

BINDING PROTEINS AND METHODS AND USES THEREOF**FIELD OF THE INVENTION**

5 The present invention relates to binding proteins which bind the alanine aminotransferase (ALT) enzyme. The present invention also relates to bivalent, trivalent, quadrivalent or multivalent binding proteins that bind ALT. The present invention also relates to nucleic acids, vectors and host cells for producing such binding proteins, methods for producing such binding proteins, kits comprising such binding proteins and methods and uses of such binding proteins.

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BACKGROUND OF THE INVENTION

Within the liver, ALT enzymes play a major role in the intermediary catabolism of glucose and amino acids. Enzymatic levels of ALT are measured as part of routine management of patients where suspected liver injury has occurred. Serum enzymatic activity levels of ALT, considered the sum of ALT1 and ALT2 in the blood, is used as a biomarker of liver injury. The enzymatic activity of ALT1 and ALT2 isoform 1 are similar for alanine and pyruvate with ALT2 isoform 2 lacking enzymatic activity (Glinghammar et al., 2009). A study by Rafter et al (2012) measured the percentage contribution of ALT1 and ALT2 enzymatic activity in healthy subjects and people with liver disease. In healthy people, on average 92% of circulating activity was ALT1, while 8% was ALT2. In people with non-alcoholic fatty liver disease, 94% of activity was ALT1, and 6% ALT2. In people with hepatitis C, 96% of ALT activity was ALT1, while the healthy control group's ALT1 contribution was 93%; ALT2 activity only increased 2.5-fold in people with hepatitis C. In people who underwent liver surgery, ALT levels changed from 31 IU/L to 357 IU/L. The contribution of ALT1 to this activity changed from 91% (before surgery) to 97% (after surgery) while the contribution of ALT2 activity changed from 8% to 3%. By contrast, after heavy exercise, the contribution of ALT1 in overall ALT activity decreased from 96% to 93% and ALT2 increased from 4% to 7%. Together this evidence suggests that the measurement of the amount of ALT1 protein in blood samples will reflect the enzymatic level detected in traditional enzyme-based assays.

30 ALT levels in the blood become elevated when the liver is damaged, and measurement of these levels is critical for diagnosis and patient management. ALT can be elevated for a number of reasons, including for example, as a result of drug toxicity (for example due to paracetamol overdose or prescribed pharmaceuticals), infection with liver-tropic viruses such as hepatitis A, B, C, D or E, SARS-CoV-2 infection, excessive or frequent alcohol consumption, fatty liver disease, preeclampsia, inflammatory bowel disease, and through the use of antibody-based therapeutics for the treatment of various human health conditions. Routine tests to

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measure ALT levels require collection of blood and measurement in pathology laboratories leading to potential delays in treatment and loss to follow up, and in resource-constrained settings, pathology services may be limited or unavailable.

5 The ability to measure ALT levels at point-of-care represents an advance in patient management with clinicians able to make immediate decisions on further diagnostic tests and treatment pathways. A previous point-of-care test developed by Anderson et al., (PCT/IB2017/055943) employed a rabbit polyclonal immune serum raised against ALT1 to detect the amount of ALT protein present in blood samples. The invention describes that the rabbit polyclonal antibody overcomes limitations observed with the use of mouse monoclonal
10 antibodies and the inhibition of detection of ALT in plasma samples. Specifically, that interfering substances in human plasma obscure the epitopes recognized by mouse antibodies and thereby limit detection of ALT1. In fact, this phenomenon is likely to be related to the presence of human anti-mouse antibodies (HAMA). Endogenous human heterophilic antibodies are observed, not only to mouse, but also other common animal species, in 10-40%
15 of people (Garcia-Gonzalez et al., 2016). Human anti-animal antibodies are usually directed to the Fc portion of an antibody but can also be directed to the F(ab)2' region. These antibodies can bind to antibodies used in immunodiagnostics and impact assay performance by interfering with the binding of the diagnostic antibody to its target epitope. In some cases, this results in a false positive if it bridges capture and signal antibodies or can result in a false negative if it
20 blocks binding to the antigen in a sandwich assay. Alternatively, in competitive assays the heterophilic antibodies bind to the diagnostic antibody and inhibit binding to the antigen resulting in a false negative. Why these HAMA antibodies develop is unclear but may be related to the use of monoclonal antibody therapies for treatments and imaging procedures, autoimmune disorders and through frequent contact with animals. Regardless of the cause of
25 the interference of mouse antibodies with the detection of ALT1, rabbit polyclonal immune serum was suggested as an alternative agent to develop antigenic assays to measure ALT1 in point-of-care assays such as lateral flow. However, a significant limitation to the use of such polyclonal reagents, which are made in animals through vaccination, is the ability to produce a consistent product, as well as to produce the quantities required for large-scale manufacture of
30 diagnostic tests.

Thus, there is a requirement for new reagents that bind ALT1.

SUMMARY OF THE INVENTION

35 The present inventors have developed binding proteins that bind alanine aminotransferase (ALT).

In an aspect, the present invention provides a binding protein that binds alanine aminotransferase 1 (ALT1), comprising amino acid sequences selected from the following:

- a) amino acid sequences GPAVSNVA (**SEQ ID NO: 2**) as complementarity-determining region (CDR) 1, ITWSGWT (**SEQ ID NO: 3**) as CDR2 and NLIGLRVGPENKY (**SEQ ID NO: 4**) as CDR3;
- b) amino acid sequences GRTDSFYA (**SEQ ID NO: 6**) as CDR1, ITWSAGST (**SEQ ID NO: 7**) as CDR2 and AADSLSAGYESSWLEAFGS (**SEQ ID NO: 8**) as CDR3; and
- c) amino acid sequences GRTFSSYS (**SEQ ID NO: 10**) as CDR1, ISRSGFST (**SEQ ID NO: 11**) as CDR2 and AVGRAYLPTASGTRCPREAYDY (**SEQ ID NO: 12**) as CDR3;
- and wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an embodiment, the binding protein binds an epitope of alanine aminotransferase 1 (ALT1).

In an embodiment, the binding protein binds an epitope of human ALT1.

- In an embodiment, the binding protein is a nanobody.

In an embodiment, the binding protein comprises the amino acid sequence **SEQ ID NO: 1** (C8), or a sequence at least 76% identical thereto, or a humanised, or germlined version thereof.

- In an embodiment, the binding protein comprises the amino acid sequence **SEQ ID NO: 5** (G6), or a sequence at least 76% identical thereto, or a humanised, or germlined version thereof.

In an embodiment, the binding protein comprises the amino acid sequence **SEQ ID NO: 9** (C10), or a sequence at least 76% identical thereto, or a humanised, or germlined version thereof.

- In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1), the binding protein comprising the antigen binding site of an antibody comprising amino acid sequences selected from;

- a) GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2, ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3, ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3;

- b) GFDLSSYY (**SEQ ID NO: 117**) as heavy chain CDR1, IWLGSIGNI (**SEQ ID NO: 118**) as heavy chain CDR2 and ARGWLDDSFDP (**SEQ ID NO: 119**) as heavy chain CDR3, VSVHYNKKW (**SEQ ID NO: 121**) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (**SEQ ID NO: 122**) as light chain CDR3;

c) GFSLITYS (**SEQ ID NO: 124**) as heavy chain CDR1, ISASGTA (**SEQ ID NO: 125**) as heavy chain CDR2, ARGSGPSGIESYKL (**SEQ ID NO: 126**) as heavy chain CDR3, QSIGN Y (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGY YGIHIT (**SEQ ID NO: 129**) as light chain CDR3;

5 d) GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, ARKDWTSGDSFNP (**SEQ ID NO: 133**) as heavy chain CDR3, QSISTA (**SEQ ID NO: 135**) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (**SEQ ID NO: 136**) as light chain CDR3; and

10 e) GFSLSNDA (**SEQ ID NO: 138**) as heavy chain CDR1, ISSAGRP (**SEQ ID NO: 139**) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (**SEQ ID NO: 140**) as heavy chain CDR3, QSISSSY (**SEQ ID NO: 142**) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (**SEQ ID NO: 143**) as light chain CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

15 In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1), the binding protein comprising a heavy chain variable region (V_H) and a light chain variable region (V_L), wherein the V_H and V_L comprise six complementary determining regions (CDRs) selected from:

20 a) GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2, ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3, ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3;

25 b) GFDLSSYY (**SEQ ID NO: 117**) as heavy chain CDR1, IWLGSNGI (**SEQ ID NO: 118**) as heavy chain CDR2 and ARGWLDDSFDP (**SEQ ID NO: 119**) as heavy chain CDR3, VSVHYNKW (**SEQ ID NO: 121**) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (**SEQ ID NO: 122**) as light chain CDR3;

30 c) GFSLITYS (**SEQ ID NO: 124**) as heavy chain CDR1, ISASGTA (**SEQ ID NO: 125**) as heavy chain CDR2, ARGSGPSGIESYKL (**SEQ ID NO: 126**) as heavy chain CDR3, QSIGN Y (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGY YGIHIT (**SEQ ID NO: 129**) as light chain CDR3;

d) GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, ARKDWTSGDSFNP (**SEQ ID NO: 133**) as heavy chain CDR3, QSISTA (**SEQ ID NO: 135**) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (**SEQ ID NO: 136**) as light chain CDR3; and

35 e) GFSLSNDA (**SEQ ID NO: 138**) as heavy chain CDR1, ISSAGRP (**SEQ ID NO: 139**) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (**SEQ ID NO: 140**) as heavy

chain CDR3, QSISSSY (**SEQ ID NO: 142**) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (**SEQ ID NO: 143**) as light chain CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

5 In an embodiment, the binding protein is a monoclonal antibody.

In an embodiment, the binding protein is a rabbit monoclonal antibody.

In an embodiment, the binding protein binds an epitope that comprises residues on both monomers of ALT1.

In an embodiment, the binding protein does not significantly bind heat denatured ALT1.

10 In an embodiment, the binding protein does not detectably bind heat denatured ALT1.

In an embodiment, the VL is kappa 1.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1 comprising the antigen binding site of an antibody.

15 In an aspect, the present invention provides a binding protein which binds a conformational epitope of alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1 comprising the antigen binding site of an antibody.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1) wherein the binding protein does not significantly and/or detectably
20 bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1
25 comprising the antigen binding site of an antibody and wherein the binding protein does not significantly and/or detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1
30 comprising the antigen binding site of an antibody comprising amino acid sequences selected from:

a) GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2, ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3,
35 ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3;

b) GFDLSSYY (SEQ ID NO: 117) as heavy chain CDR1, IWLGSJNI (SEQ ID NO: 118) as heavy chain CDR2, ARGWLDDSFDP (SEQ ID NO: 119) as heavy chain CDR3, VSVHYNKK (SEQ ID NO: 121) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (SEQ ID NO: 122) as light chain CDR3;

5 c) GFSLITYS (SEQ ID NO: 124) as heavy chain CDR1, ISASGTA (SEQ ID NO: 125) as heavy chain CDR2, ARGSGPSGIESYKL (SEQ ID NO: 126) as heavy chain CDR3, QSIGNNY (SEQ ID NO: 128) as light chain CDR1, RAS as light chain CDR2 and QGYGIIHT (SEQ ID NO: 129) as light chain CDR3;

d) GIDLSVNA (SEQ ID NO: 131) as heavy chain CDR1, IHTYDVT (SEQ ID NO: 132) as heavy chain CDR2, ARKDWTSGDSFNP (SEQ ID NO: 133) as heavy chain CDR3, QSISTA (SEQ ID NO: 135) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (SEQ ID NO: 136) as light chain CDR3;

e) GFSLSNDA (SEQ ID NO: 138) as heavy chain CDR1, ISSAGRP (SEQ ID NO: 139) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (SEQ ID NO: 140) as heavy chain CDR3, QSISSSY (SEQ ID NO: 142) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (SEQ ID NO: 143) as light chain CDR3; and

f) GPAVSNA (SEQ ID NO: 2) as CDR1, ITWSGWT (SEQ ID NO: 3) as CDR2 and NLIGLRVGPENKY (SEQ ID NO: 4) as CDR3;

20 wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No,

wherein when the amino acid sequences are a) the binding protein is a nanobody, and wherein when the amino acid sequences are b), c), d), e) or f) the binding protein is an antibody.

25 In an aspect, the present invention provides binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1 comprising amino acid sequences selected from the following:

a) GFSLNNYN (SEQ ID NO: 110) as heavy chain CDR1, ITAGJNI (SEQ ID NO: 111) as heavy chain CDR2, ARDLAGNVYYDFDL (SEQ ID NO: 112) as heavy chain CDR3, ENIYSG (SEQ ID NO: 114) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (SEQ ID NO: 115) as light chain CDR3;

b) GFDLSSYY (SEQ ID NO: 117) as heavy chain CDR1, IWLGSJNI (SEQ ID NO: 118) as heavy chain CDR2, ARGWLDDSFDP (SEQ ID NO: 119) as heavy chain CDR3, VSVHYNKK (SEQ ID NO: 121) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (SEQ ID NO: 122) as light chain CDR3;

35 c) GFSLITYS (SEQ ID NO: 124) as heavy chain CDR1, ISASGTA (SEQ ID NO: 125) as heavy chain CDR2, ARGSGPSGIESYKL (SEQ ID NO: 126) as heavy chain CDR3,

QSIGNY (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGYGIIHIT (**SEQ ID NO: 129**) as light chain CDR3;

d) GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, ARKDWTSGDSFNP (**SEQ ID NO: 133**) as heavy chain CDR3,
5 QSISTA (**SEQ ID NO: 135**) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (**SEQ ID NO: 136**) as light chain CDR3;

e) GFSLSNDA (**SEQ ID NO: 138**) as heavy chain CDR1, ISSAGRP (**SEQ ID NO: 139**) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (**SEQ ID NO: 140**) as heavy chain CDR3, QSISSSY (**SEQ ID NO: 142**) as light chain CDR1, RVS as light chain CDR2
10 and QGTYGSGSSSYGNA (**SEQ ID NO: 143**) as light chain CDR3; and

f) GPAVSNA (**SEQ ID NO: 2**) as CDR1, ITWSGWT (**SEQ ID NO: 3**) as CDR2 and NLIGLRVGPENKY (**SEQ ID NO: 4**) as CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No,

15 wherein when the amino acid sequences are a) the binding protein is a nanobody, and wherein when the amino acid sequences are b), c), d), e) or f) the binding protein is an antibody.

In an aspect, the present invention provides a bivalent, trivalent, quadrivalent or multivalent binding protein comprising at least one binding protein as described herein or a
20 combination thereof.

In an aspect, the present invention provides an isolated nucleic acid encoding the amino acid sequence of the isolated binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein.

In an aspect, the present invention provides a vector comprising the nucleic acid as
25 described herein.

In an aspect, the present invention provides a host cell comprising the nucleic acid as described herein, or the vector as described herein.

In an aspect, the present invention provides a method of producing a binding protein as described herein, or a bivalent, trivalent, quadrivalent or multivalent binding protein as
30 described herein, comprising culturing the host cell as described herein in cell culture medium and expressing the binding protein or the bivalent, trivalent, quadrivalent or multivalent binding protein.

In an aspect, the present invention provides a kit or panel comprising a binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as
35 described herein.

In an aspect, the present invention provides a lateral flow assay comprising: a solid support that comprises a binding protein as described herein, and/or a bivalent, trivalent, quadrivalent or multivalent binding protein as described herein immobilized on the solid support.

5 In an aspect, the present invention provides a lateral flow assay comprising: (i) a detector binding protein conjugated to a detectable label; (ii) a capture binding protein in a capture region on a solid support, wherein the binding protein in (i) and/or (ii) is a binding protein as described herein, and/or a bivalent, trivalent, quadrivalent or multivalent binding protein as described herein.

10 In an aspect, the present invention provides use of a binding protein as described herein, or a bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, or the kit or panel as described herein, or the lateral flow assay as described herein for detecting ALT1.

In an aspect, the present invention provides a method of detecting ALT1, the method comprising contacting a sample with a binding protein as described herein, or a bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, to form an antigen-binding protein complex and directly or indirectly detecting the antigen-binding protein complex.

15 In an aspect, the present invention provides use of a binding protein as described herein, or a bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, or the kit or panel as described herein, or the lateral flow assay as described herein for detecting a subject with liver damage and/or liver disease.

In an aspect, the present invention provides use of a binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, or the kit or panel as described herein, or the lateral flow assay as described herein for detecting ALT1 in a subject.

25 In an aspect, the present invention provides a method of detecting ALT1, the method comprising contacting a sample with a binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein to form an antigen-binding protein complex and directly or indirectly detecting the antigen-binding protein complex.

30 In an aspect, the present invention provides use of a binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, or the kit or panel as described herein, or the lateral flow assay as described herein for detecting a subject with liver damage and/or liver disease.

35 In an aspect, the present invention provides a method of detecting a subject with liver damage and/or liver disease the method comprising contacting a sample with a binding protein

as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, to form an antigen-binding protein complex and directly or indirectly detecting the antigen-binding protein complex.

5 In an aspect, the present invention provides a solid support or semi-solid support having immobilized thereon the binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein.

Any embodiment herein shall be taken to apply *mutatis mutandis* to any other embodiment unless specifically stated otherwise. For instance, as the skilled person would understand examples of amino acid sequences outlined above for the binding proteins of the invention equally apply to the bivalent, trivalent, quadrivalent or multivalent binding proteins of the invention.

10 The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

20 The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANING DRAWINGS

25 **Figure 1. A)** Shows the structure of a typical human IgG molecule with a heavy and light chain forming a heterodimeric 150 kDa protein. **B)** Shows a typical camelid antibody as a homodimer of two heavy chain molecules. **C)** Shows the VHH antigen-binding fragment that can be expressed independently in bacterial cells.

30 **Figure 2. A)** Shows the amino acid sequences of VHH domains resulting from screening a phage display library for nanobodies reactive to ALT1 in a panning experiment. 94 clones were screened for reactivity to ALT1-coated ELISA plates. Of these, 91 were positive to ALT1, of which 86 had full length VHH domains. **B)** Shows the results of screening a phage display library for nanobodies reactive to ALT1 in a second panning experiment. 188 clones were screened for reactivity to ALT1 coated ELISA plates. Of these, 60 had full length VHH domains.

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Figure 3. Shows the frequency of clones isolated with the same complementarity determining region 3 (CDR3) within the library isolated in panning experiment one where the two strongly reactive nanobodies were identified. These are referred to as Nb_C8 and Nb_G6 hereafter. Nb_C8 was unique within this library with only a single clone having the same CDR3 region.

5 Nb_G6 was the dominant clone isolated in this library with 82 of 91 clones possessing the same CDR3 sequence.

Figure 4. Shows the results of an enzyme linked immunosorbent assay (ELISA) on a selection of nanobodies with unique complementarity determining regions. The results show that Nb_C8 and Nb_G6 have very strong binding to ALT1 (ALT1-avi), indicated by high absorbance at 450nm, while Nb_C10 has very low binding. All other nanobodies screened show no binding to ALT1 (**Figure 2** and not shown). For comparison, the binding of the nanobodies to bovine serum albumin (BSA) is shown which represents the background of the assay. WK6 represents cells only and is the background of the assay.

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Figure 5. A) Shows the protein coding sequence and annotation showing the predicted boundaries of the framework (FR) and complementarity determining regions (CDR) for the Nb_C8. **B)** Shows the protein coding sequence for Nb_C8_FLAG. The CDR domains are in bold text, while FR regions are in grey. The FLAG-tag is underlined, and the poly-histidine sequence is boxed.

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Figure 6. A) Shows the protein coding sequence and annotation showing the predicted boundaries of the framework (FR) and complementarity determining regions (CDR) for the Nb_G6. **B)** Shows the protein coding sequence for Nb_G6_FLAG. The CDR domains are in bold text, while FR regions are in grey. The FLAG-tag is underlined and the poly-histidine sequence is boxed.

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Figure 7. Shows the protein coding sequence and annotation showing the predicted boundaries of the framework (FR) and complementarity determining regions (CDR) for the nanobody Nb_C10. The CDR domains are in bold text, while FR regions are in grey.

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Figure 8. Shows the percentage identity between the nanobodies (detailed in in **Figures 5 to 7**).

Figure 9. Shows the *Vicugna pacos* (Alpaca) V-gene, J-gene and D-gene usage of Nb_G6 and Nb_C8. RF= reading frame.

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Figure 10. Shows an alignment between nanobodies Nb_G6 and Nb_C8 highlighting differences in the protein coding sequence. Complementarity determining regions 1, 2, and 3 are shaded in grey. Conserved mutation (:), semi-conserved mutation (.), gap (-) non conserved mutation ().

Figure 11. Shows an alignment between nanobodies Nb_C8 and Nb_C10 highlighting differences in the protein coding sequence. Complementarity determining regions 1, 2, and 3 are shaded in grey. Conserved mutation (:), semi-conserved mutation (.), gap (-) non conserved mutation ().

Figure 12. Shows an alignment between nanobodies Nb_C10 and Nb_G6 highlighting differences in the protein coding sequence. Complementarity determining regions 1, 2, and 3 are shaded in grey. Conserved mutation (:), semi-conserved mutation (.), gap (-) non conserved mutation ().

Figure 13. A) Shows the Alphafold2-predicted structures of the two active nanobodies with Nb_C8 (light grey) superimposed on the predicted structure of Nb_G6 (medium grey). **B)** Shows both active Nb_C8 and Nb_G6 superimposed on the structure of the inactive Nb_C10 (black) which binds weakly to ALT1. Arrows indicate complementarity determining regions (CDR) 1, 2, and 3.

Figure 14. Shows whole cell lysates separated on an SDS-PAGE gel of cells expressing nanobodies Nb_C8 and Nb_G6, which have been modified through the addition of a His-tag, FLAG-tag or Avi-tag. Protein size in kilodaltons (kDa) is indicated on the left-hand side.

Figure 15. Shows binding of antibodies in an enzyme linked immunosorbent assay (ELISA) against ALT1, ALT2 and AST. Absorbance at an optical density (OD) of 450nm is shown versus increasing concentrations of the indicated antibody. **A)** Shows binding of Nb_G6; **B)** Shows binding of Nb_C8; **C)** Shows binding of rabbit polyclonal antibodies raised to ALT1; **D)** Shows the binding of a commercial rabbit monoclonal antibody raised to ALT1. A modified form of ALT2 was used in ELISAs that contains a deletion of 48 residues at the N-terminus to facilitate expression in *E. coli*.

Figure 16. Shows a summary of the concentration of each antibody required to achieve ten times binding over background to ALT1, ALT2 and AST.

Figure 17. Shows an alignment of the protein coding sequences of ALT1 and isoform 1 of ALT2. Conserved mutation (:), semi-conserved mutation (.), gap (-) non conserved mutation ().

Figure 18. Shows an alignment of the protein coding sequences of AST, ALT1, ALT2 isoform 1 and ALT2 isoform 2. Conserved mutation (:), semi-conserved mutation (.), gap (-) non conserved mutation ().

Figure 19. Shows modifications to the nanobodies where Nb_C8 and Nb_G6 were joined to themselves (homobivalent) or each other (heterobivalent) via different linker sequences comprising highly flexible glycine (G) and serine (S) residues. Alternative linkers used were 3 or 4 repeats of GGGGS. Hereafter, bivalent nanobodies are referred to by the code indicated in the table (e.g., C8-3-C8 is referred to as C3C).

Figure 20. Shows the expression of bivalent nanobodies from bacterial cells run on SDS-PAGE gels and confirms the increase in expected molecular mass from monovalent (~14kDa) to bivalent nanobodies (28 kDa). Different conditions (18 or 28 °C, grown in either terrific broth (TB) or autoinduction media (AI)) were explored to optimise yield of nanobody and show consistent production of ~28kDa species containing two 14 kDa Nb VHH sequences. Protein size in kilodaltons (kDa) is indicated on the left-hand side. **A)** Shows bivalent nanobodies C3C, C4C, **B)** Shows bivalent nanobodies G3G, G4G.

Figure 21. Shows relative binding affinity of bivalent nanobodies to ALT1 in an enzyme linked immunosorbent assay. Nanobodies were added at 1000ng/mL. The parental monovalent nanobodies Nb_C8 and Nb_G6 are shown for comparison.

Figure 22. A) Shows a biolayer interferometry (BLI) experiment that measures the association and dissociation of receptor-ligand interactions. ALT1 was immobilised on avidin sensors and the binding of Nb_C8, and rabbit polyclonal antibodies to the sensors was measured in real-time in seconds (s). **B)** Shows a BLI experiment in which monovalent (C8, G6) or bivalent (G4C, C4C, C4G) nanobodies were immobilised on nickel sensors and the binding of five consecutively lower concentrations of ALT1 (1000, 333, 111, 39, 12 nM) to the sensors was measured in real-time. The association and dissociation curves of the different ALT1 concentrations are shown for each nanobody. Different linkers and different combinations of nanobody alter the association and dissociation towards ALT1. Such differences can provide advantages in their applicability to different assay systems such as plate-based assays, biosensor-based assays, and lateral flow assays. Nb_C8 rapidly associates to ALT1 and

dissociates relatively fast, whereas the bivalent nanobodies show slower dissociation rates, while maintaining fast association rates. Nb_G6 does not bind to ALT1 in this orientation.

Figure 23. A) Summarises the association (k_{on} , or on-rate) and dissociation (k_{dis} , or off-rate) for the nanobodies using a 1:1 model of binding. Bivalent nanobodies showed differences in their on-rates towards ALT1, with G4C displaying faster on-rates than other bivalent nanobodies tested. Dissociation also differed for bivalent nanobodies with C4G having the fastest off-rate. K_D is the equilibrium dissociation constant and R^2 indicates the goodness of fit. **B)** Describes the fold improvement of the association, dissociation and K_D for the bivalent nanobodies compared to monovalent Nb_C8. The data shows that the association is improved for both G4C and C4C, whereas the dissociation and K_D are improved for all three bivalent nanobodies tested.

Figure 24. Shows a schematic of an ALT1 detection system in a lateral flow format. The nanobody is conjugated to a visible or fluorescent molecule such as colloidal gold, gold nanoshells, europium and other examples known to those skilled in the art. These are applied to a conjugate pad and interact with ALT present in serum, plasma or blood samples upon contact. Addition of a running buffer to the conjugate pad (shown in top panel) then allows the nanobody-conjugate-ALT complexes to flow along the nitrocellulose (indicated by the arrow in the bottom panel), where they are captured by a second nanobody striped onto the nitrocellulose membrane. If nanobody-conjugate-ALT complexes are present these will be retained on the nanobody stripe (line indicated by 'T' in top panel). Free nanobody-conjugate then flows through to a control line (line indicated by 'C' in top panel) where ALT is striped on the line and captures the free-conjugate confirming the test result is valid.

Figure 25. Shows an example of how a nanobody (Nb_G6) can be used to detect ALT1 in a lateral flow assay when ALT1 is striped onto nitrocellulose and comparison with detection with polyclonal antibody to ALT1. Antibodies were conjugated to europium and used to detect different concentrations of ALT1 protein striped directly onto nitrocellulose.

Figure 26. Shows an example of europium conjugated to rabbit polyclonal anti-ALT1. Shows an example of the utility of nanobody C8_FLAG (Nb_C8_FLAG) to capture different amounts of ALT1 applied to a lateral flow test. Here different amounts of Nb_C8_FLAG were striped onto nitrocellulose ranging from 0.1 mg/mL to 1 mg/mL. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with europium conjugated to a polyclonal antibody to ALT1 and allowed to diffuse laterally. ALT1 was captured by the

Nb_C8_FLAG stripe and residual europium-conjugated antibody was then captured by the ALT control line.

Figure 27. Shows an example of europium conjugated to rabbit polyclonal anti-ALT1. Shows an example of the utility of nanobody G6_FLAG (Nb_G6_FLAG) to capture different amounts of ALT1 applied to a lateral flow test. Here different amounts of Nb_G6_FLAG were striped onto nitrocellulose ranging from 0.1 mg/mL to 1 mg/mL. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with europium conjugated to a polyclonal antibody to ALT1 and allowed to diffuse laterally. ALT1 was captured by the Nb_G6_FLAG stripe and residual europium-conjugated antibody was then captured by the ALT control line.

Figure 28. A) Shows an example of the utility of nanobody C8 (Nb_C8_FLAG) to capture different amounts of ALT1 applied to a lateral flow test and detected with a nanobody conjugated to gold nanoshells. A constant amount of Nb_C8_FLAG was striped onto nitrocellulose. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with gold nanoshells conjugated to either G3G or G4C and allowed to diffuse laterally. ALT1 was captured by the Nb_C8_FLAG stripe and residual gold nanoshell conjugated antibody was then captured by the ALT control line. **B)** Shows the quantitation of the results using a lateral flow strip reader. Increasing peak intensity (y-axis) reflects more visible test lines on lateral flow strips.

Figure 29. A) Shows an example of a lateral flow test in which the nanobody C8 (Nb_C8_FLAG) is used as a capture antibody striped onto nitrocellulose membrane. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with europium conjugated to nanobody G6, G3G and G4C and allowed to diffuse laterally. ALT1 was captured by the Nb_C8_FLAG stripe and residual europium conjugated antibody was then captured by the ALT control line. **B)** Shows the quantitation of the results using a lateral flow strip reader. **C)** Shows the quantitation of the results using a lateral flow strip reader, corrected for the background in the assay at 0 µg/mL ALT1. This allows a comparison of the relative ability of each nanobody to detect ALT1. Bivalent nanobodies G3G or G4C have improved binding ability to ALT1 relative to monovalent nanobody G6.

Figure 30. A) Shows an example of a lateral flow test in which the heterobivalent nanobodies can be used for both capture and detection. Here G4C was striped onto nitrocellulose as a capture antibody. Different amounts of ALT1 were added to the bottom of the nitrocellulose

membrane in contact with gold nanoshells conjugated to G4C and allowed to diffuse laterally. ALT1 was captured by the G4C stripe and residual gold nanoshell conjugated antibody was then captured by the ALT control line. **B)** Shows the quantitation of the results using a lateral flow strip reader.

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Figure 31. A) – H) Shows examples of different nanobodies acting as capture reagents on the stripe with the heterobivalent G4C as a detector and their ability to detect different amounts of recombinant ALT1-his protein spiked into buffer solution.

10 **Figure 32. A) – H)** Shows examples of different nanobodies acting as capture reagents on the stripe with the heterobivalent C4G as a detector and their ability to detect different amounts of recombinant ALT1-his protein spiked into buffer solution.

15 **Figure 33. A) – H)** Shows examples of different nanobodies acting as capture reagents on the stripe with the heterobivalent C3G as a detector and their ability to detect different amounts of recombinant ALT1-his protein spiked into buffer solution.

20 **Figure 34. A) – F)** Shows examples of different nanobodies acting as capture reagents on the stripe with the homobivalent G3G as a detector and their ability to detect different amounts of recombinant ALT1-his protein spiked into buffer solution.

25 **Figure 35. A) – C)** Shows examples of different nanobodies acting as capture reagents on the stripe with the homobivalent G4G as a detector and their ability to detect different amounts of recombinant ALT1-his protein spiked into buffer solution.

Figure 36. A) – E) Shows examples of different nanobodies acting as capture reagents on the stripe with polyclonal antibody to ALT1 as a detector and their ability to detect different amounts of recombinant ALT1-his protein spiked into buffer solution.

30 **Figure 37. A)** Shows an example of the effect that the addition of excipients can have on the performance of the test capture line and control line. Here monovalent Nb_C8_FLAG (C8F) was striped onto nitrocellulose at 0.5 mg/mL for the test line, with and without the presence of BSA and/or sucrose. ALT1 was striped at 50 µg/mL onto the control line, with and without the presence of BSA and/or sucrose. **B)** Shows the quantitation of the test line results using a lateral flow strip reader. **C)** Shows the quantitation of the control line results using a lateral flow strip reader.

35

Figure 38. A) Shows an example of the effect that the addition of excipients can have on the performance of the test capture line and control line. Here bivalent G4C was striped onto nitrocellulose at 0.5 mg/mL for the test line, with and without the presence of BSA and/or sucrose. ALT1 was striped at 50 µg/mL onto the control line with and without the presence of BSA and/or sucrose B) Shows the quantitation of the test line results using a lateral flow strip reader. C) Shows the quantitation of the control line results using a lateral flow strip reader.

Figure 39. Shows the assessment of ALT1 in clinical samples with known concentrations of enzymatic ALT using the bivalent A) G4C as capture and B) C4C as capture. Capture antibodies were detected with europium particles conjugated to anti-ALT polyclonal antibody in a lateral flow test. Human plasma was obtained, and ALT levels determined by standard of care testing in a pathology laboratory, which does not distinguish between ALT1 and ALT2, as described earlier in the Background Of The Invention section. The values of enzymatic ALT in international units (IU/L) are shown in brackets after the sample identifier.

Figure 40. A) Shows the correlations between enzymatic ALT as determined by standard pathology testing and antigenic ALT1 using G4C capture, as determined by lateral flow analysis of clinical samples in Figure 39. B) Shows the correlations between enzymatic ALT as determined by standard pathology testing and lateral flow assay measured ALT1 using C4C as capture as determined by lateral flow analysis of clinical samples in Figure 39.

Figure 41. Shows a schematic of nanobodies modified to increase size, avidity and polyspecificity. A) Shows a schematic of two monovalent nanobodies. B) Shows a schematic of a bivalent nanobody, which is two monovalent nanobodies connected by a linker. C) Shows a schematic of a bivalent nanobody fused to a human IgG Fc domain, resulting in a quadrivalent molecule.

Figure 42. A) Shows an SDS-PAGE gel, run under reducing conditions, of quadrivalent Fc-fusion nanobodies expressed in mammalian cells and confirms the increase in expected molecular mass from ~28 kDa to ~53 kDa for each Fc chain fused to the bivalent nanobody. B) Shows an SDS-PAGE gel, run under non-reducing and reducing conditions, of Protein G sepharose-purified quadrivalent Fc-fusion nanobodies expressed in mammalian cells and confirms the increase in expected molecular mass from ~53 kDa to ~106 kDa as expected for a quadrivalent nanobody-Fc fusion under non-reducing conditions.

Figure 43. Shows the binding ability of the Fc-fusion nanobodies to bind to ALT1 in an enzyme linked immunosorbent assay. Nanobodies were added at 1000 ng/mL and serially diluted two-fold, and absorbance was measured at 450nm.

5 **Figure 44. A)** Shows quadrivalent nanobody-Fc proteins (C4C-Fc, C4G-Fc or G4C-Fc) or bivalent nanobody (G4C) protein in lateral flow tests, which were striped onto nitrocellulose membranes in the presence of the excipients, BSA and sucrose. Different spiked amounts of ALT1 were added to the bottom of the nitrocellulose membrane in buffer alone, or in presence
10 of human plasma, and mixed with europium particles conjugated to rabbit polyclonal anti-ALT antibody and allowed to diffuse laterally. Spiked ALT1 was captured by the striped protein and residual europium conjugated antibody was then captured by the ALT control line. **B-C)** Shows the quantitation of the results using a lateral flow strip reader. ALT1 can be specifically captured and detected in a dose-dependent manner in **B)** buffer and **C)** plasma by quadrivalent Fc-fusion nanobodies with greater sensitivity than by the bivalent G4C.

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Figure 45. Shows a diagrammatic representation, including the dimensions, of an example of a lateral flow test to be used in the assembled cassette.

20 **Figure 46.** Shows an example of an assembled lateral flow test cassette. On the left, the top view of the cassette is depicted, and on the right, the interior of the cassette is depicted, with an assembled lateral flow strip inside.

25 **Figure 47.** Shows a quantitation using a lateral flow strip reader of a lateral flow tests' ability to detect different levels of ALT1, when assembled in a lateral flow cassette such as the one shown in Figure 46. In this example quadrivalent Fc-fusion C4G-Fc was striped onto nitrocellulose membrane in the presence of the excipients BSA and sucrose. Different amounts of ALT1 were added to the sample port and three drops of buffer added and allowed to diffuse laterally. ALT1 was captured by the striped protein and detected with rabbit polyclonal antibody raised to ALT conjugated to europium and used at two different concentrations (1/1000 and
30 1/2000).

35 **Figure 48.** Shows how a lateral flow assay performed in a cassette can measure ALT1 in human plasma. Quadrivalent Fc-fusion C4G-Fc was striped onto nitrocellulose membrane in the presence of the excipients BSA and sucrose. A fixed amount of plasma was added to the sample port and three drops of buffer added to rehydrate the europium particles conjugated to rabbit polyclonal anti-ALT antibody and allowed to diffuse laterally. ALT1 in the clinical sample was

captured by the striped protein. The enzymatic ALT levels in the human plasma was determined by standard of care testing in a pathology laboratory. The values of enzymatic ALT in international units (IU/L) are shown in brackets after the sample identifier.

5 **Figure 49.** Shows a summary of the nucleotide sequences encoding binding proteins as described herein. For *E. coli* expression constructs (non-Fc) genes have an N-terminal pelB signalling peptide for periplasmic expression (indicated in bold). Fc-fusion bivalent nanobody genes were codon optimised for mammalian cell expression and the gene synthesised before being cloned into an Fc-pcDNA3 vector. These genes have a tPA leader sequence at the N-terminus (indicated in bold).
10

Figure 50. Shows the interaction of Fc domain nanobody-fusion antibodies with ALT1. Nickel sensors were loaded with 5 ug/mL ALT1 protein and exposed to various concentrations from 3.125nM to 100nM of either **A)** G6-Fc, **B)** C8-Fc, or **C)** C4G-Fc for 600 seconds to monitor association and then allowed to dissociate for 600 seconds. Sensorgrams are shown for each concentration of antibody and the association and dissociation phases are to the left and right of the vertical dotted line at 600s, respectively.
15

Figure 51. Shows the sequence of rabbit monoclonal antibody 15B5 (RmAb1) specific to ALT1. The variable domain sequences of the heavy chain (HC) and light chain (LC) are shown with CDR1, 2, 3 underlined.
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Figure 52. Shows the sequence of a rabbit monoclonal antibody 20C12 (RmAb2). The variable domain sequences of the heavy chain (HC) and light chain (LC) are shown with CDR1, 2, 3 underlined.
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Figure 53. Shows the sequence of a rabbit monoclonal antibody 22G10 (RmAb3). The variable domain sequences of the heavy chain (HC) and light chain (LC) are shown with CDR1, 2, 3 underlined.
30

Figure 54. Shows the sequence of a rabbit monoclonal antibody 36D4 (RmAb4). The variable domain sequences of the heavy chain (HC) and light chain (LC) are shown with CDR1, 2, 3 underlined.

Figure 55. Shows the sequence of a rabbit monoclonal antibody 42F12 (RmAb5). The variable domain sequences of the heavy chain (HC) and light chain (LC) are shown with CDR1, 2, 3 underlined.

5 **Figure 56.** Shows the alignment of heavy chain sequences of the rabbit monoclonal antibodies to ALT1. Sequences were aligned using Clustal 2.1 software and the CDR1, 2 and 3 regions are boxed. Conserved mutation (:), semi-conserved mutation (.), gap (-), non conserved mutation ().

10 **Figure 57.** Shows the alignment of light chain sequences of the rabbit monoclonal antibodies to ALT1. Sequences were aligned using Clustal 2.1 software and the CDR1, 2 and 3 regions are boxed. Conserved mutation (:), semi-conserved mutation (.), gap (-), non conserved mutation ().

15 **Figure 58.** Shows an SDS-PAGE of purified rabbit monoclonal antibodies. Antibodies were expressed by co-transfection of heavy and light chain encoding plasmids into Expi293 cells. Proteins were purified using protein A affinity chromatography and buffer exchanged into PBS (phosphate buffered saline). The proteins were then run on SDS-PAGE under reducing and non-reducing conditions. Purified C4G-Fc was also run for comparison. On the left-hand size,
20 molecular weight markers are indicated.

Figure 59. Shows differential scanning fluorimetry plots of ALT-specific rabbit monoclonal antibodies. Proteins were subjected to thermal denaturation in the presence of Sypro Orange.

25 **Figure 60. A)** Shows an enzyme linked immunosorbent assay (ELISA) of the binding of rabbit monoclonal antibodies to ALT1. Serial dilutions of each rabbit anti-ALT monoclonal antibody and the rabbit polyclonal antibody were applied to plates coated with ALT1. Data shown are mean $\pm$ standard deviation. **B)** Shows an enzyme linked immunosorbent assay (ELISA) of the binding of rabbit monoclonal antibodies to ALT2. Serial dilutions of each rabbit anti-ALT
30 monoclonal antibody and the rabbit polyclonal antibody were applied to plates coated with ALT2. Data shown are mean $\pm$ standard deviation. **C)** Shows a summary of the concentration of each antibody required to achieve ten times binding over background to ALT1 and ALT2.

Figure 61. Shows the association and dissociation of antibodies to ALT using biolayer interferometry. Nickel sensors were used to capture ALT1-chis protein and different
35 concentrations of antibody were applied and associated for 600 s and dissociation for 600 s.

Figure 62. Shows an example of the adaptation of the lateral flow test to detect ALT in a self-test device. The schematic shows the assembly the components of a lateral flow test and the relative positions of the sample pad containing blood capture reagent anti-glycophorin A (Anti-GPA) and europium conjugated rabbit anti-ALT1 (Conjugate area), the nitrocellulose membrane sprayed with 1mg/mL C4G-Fc (Test line) and 0.1mg/mL ALT1 (Control line), the absorbent pad and backing card. The position of the compression points, the blister well and sample port on the device are indicated.

Figure 63. Shows the quantitative results of a lateral flow test using europium conjugated rabbit-anti-ALT polyclonal antibody. Different amounts of ALT1 spiked into buffer were applied to the cassette to show quantitative detection of ALT1. After addition of running buffer, tests were run for 20 minutes before imaging on a fluorescent lateral flow reader. Data shown are mean $\pm$ standard deviation.

Figure 64. Shows the image of a lateral flow device after running. Venous blood was spiked with 0 or 2 ng ALT1 and run for 20 minutes before photography. The same schematic as shown in **Figure 62** was used.

Figure 65. Shows the images of test strips run in an AtomoRapid Pascal device after running with venous blood spiked with ALT1. The same lateral flow configuration was used as described in **Figure 62**. Three different volunteer bloods (A, B and C) were spiked with either no (0 ng) or 2 ng of ALT1 and run for 20 minutes on the lateral flow device before imaging strips and quantitation on a lateral flow reader.

Figure 66. Shows the quantitative results of the test line in **Figure 65**. Data shown are mean $\pm$ standard deviation.

Figure 67. Shows an example of a modification of the lateral flow test replacing anti-glycophorin A sprayed glass fibre pads with commercial blood retention pads. Two alternative schematics are shown where the sample pad and blood separation pad lengths size and positions are varied to modulate blood retention capacity and sensitivity of the test. The region on the sample pad containing the europium conjugated rabbit anti-ALT1 is shown as the conjugate area. The other components of the test are the same as that described **Figure 62**.

Figure 68 Shows images of lateral flow tests run using venous blood using a blood retention pad. The lateral flow tests uses the configurations described in **Figure 67** showing their ability to retain red blood cells and prevent red cells from running into the test window. The tests were assembled in the AtomoRapid™ Pascal device. Human venous blood spiked with 0, 1 or 5 ng ALT1 was applied to tests. After addition of running buffer, tests were run for 20 minutes before imaging on a fluorescent lateral flow reader. A single volunteer venous blood was used and either not spiked or spiked with different amounts of ALT1.

Figure 69. A) Shows the images of the nitrocellulose strips after running the ALT test. **B)** Shows the quantitative data for the tests strips shown in **A**). The test strips were the same as those shown in **Figure 68**.

Figure 70. Shows a schematic of a lateral flow test for the detection of ALT1 in blood using rabbit monoclonal antibodies and rabbit polyclonal antibody conjugated to europium. In this schematic a separate rehydration pad is used, a conjugate pad with europium conjugated anti-ALT antibody and a separate blood retention sample pad. Other components of the test are as described in **Figure 62**.

Figure 71. Shows an example of images taken of lateral flow tests performed using the schematic in **Figure 70**. The tests were assembled in the AtomoRapid™ Pascal device using human venous blood spiked with 0, 1 or 5 ng ALT1 applied to the tests. After addition of running buffer, tests were run for 20 minutes before imaging on a fluorescent lateral flow reader.

Figure 72. Shows the quantitation of the test line data in **Figure 71**. Data shown are mean $\pm$ standard deviation.

Figure 73. Shows quantitative data for the detection of naturally occurring ALT in human plasma samples using the rabbit monoclonal antibodies to detect C4G-Fc captured ALT1 on nitrocellulose membranes. Nitrocellulose strips striped with C4G-Fc at 1mg/mL and 0.1 mg/mL ALT1 were used in a wet system experiment. Human plasma samples were added to europium conjugated rabbit monoclonal antibody and preincubated in 96 well plates before dipping in tests strips. After 8 minutes, running buffer was added to the sample pad and after a further 8 minutes read in a fluorescent reader.

Figure 74. Shows an example of a visible version of the ALT1 lateral flow test. Nitrocellulose membranes striped with 1mg/mL C4G-Fc and 0.1 mg/mL ALT1 were used in a wet system test. Estapor blue and black intense particles were conjugated to different amounts of RmAb5 at either 50ug/mg or 150ug/mg. Various amounts of ALT1 was added to running buffer and mixed with either blue or black conjugated rRmAb5 in a 96 well plate. Nitrocellulose test strips were added to the mixture of ALT1 and blue/black conjugated RmAb5 for 8 minutes followed by addition of running buffer. After 10 min, tests were inserted and imaged and quantitated in a visible tests strip reader.

Figure 75. Shows the quantitation of the tests performed in **Figure 74.**

Figure 76. Shows a test schematic for a visible version of the ALT1 lateral flow test. In this schematic, the conjugate pad was sprayed with blue intense conjugated RmAb5 in the conjugate area. A longer version of the conjugate pad was used that acts as the sample pad as well. Two blood retention pads were overlayed to improve blood retention capacity in front of the sample port. The nitrocellulose membrane was striped with C4G-Fc at either 0.05 or 0.2 mg/mL and ALT1 at 0.1 mg/mL.

Figure 77. Shows the results of a lateral flow test performed using the schematic shown in **Figure 76.** The lateral flow devices were assembled in a AtomoRapid™ Pascal device. Various amounts of ALT1 were spiked into whole blood and added to the blood collection unit for delivery to the devices. After delivery, the blisters were popped to release running buffer and tests imaged after 20 minutes in a lateral flow strip reader.

Figure 78. Shows the quantitation of the results shown in **Figure 77.** Data shown are mean $\pm$ standard deviation.

Figure 79. Shows an example of how the test can be modified to allow detection of ALT above a cut-off level of ALT. Nitrocellulose membranes striped with 0.5 mg/mL C4G-Fc and 0.1 mg/ml ALT1 for the control line were prepared and used in wet system testing. RmAb5 was used at different concentrations to conjugate with blue-intense and black particles. Conjugate was mixed with various amounts of ALT1 in running buffer in a 96 well plate and nitrocellulose membranes added. After 8 min, additional running buffer was added and after a further 8 minutes, tests imaged using a strip reader. Images of the tests are shown using black and blue-intense particles for detection of ALT1.

Figure 80. Shows the quantitation of **Figure 79**.

Figure 81. Shows an example of how the test can be further modified to allow detection of ALT above a cut-off level of ALT. Nitrocellulose membranes striped with 0.2 and 0.05 mg/mL C4G-Fc and 0.1 mg/mL ALT1 for the control line were prepared and used in wet system testing. RmAb5 was used at different concentrations to conjugate with blue-intense and black particles. Conjugate was mixed with various amounts of ALT1 in running buffer in a 96 well plate and nitrocellulose membranes added. After 8 min, additional running buffer was added and after a further 8 minutes, tests imaged using a strip reader. Images of the tests are shown using black and blue-intense particles for detection of ALT1.

Figure 82. Shows the quantitation of **Figure 81**. Data shown are mean $\pm$ standard deviation.

Figure 83. Shows an example of how the rabbit monoclonal antibodies to ALT can be used to capture ALT1 as well as detect ALT1. RmAb1, 3, 4 and 5 and the polyclonal rabbit anti-ALT (poly) were conjugated to europium. Nitrocellulose was spotted with RmAb1,3,4 and 5 and the polyclonal rabbit anti-ALT to capture ALT1 as well as spotted with recombinant ALT1. ALT1 diluted in running buffer was applied to the test strips (+) and bound ALT1 detected with europium conjugated antibody and imaged on a fluorescent strip reader. Alternatively, no ALT was added to running buffer as a negative control (-).

Figure 84. Shows an example of an epitope binning experiment performed using biolayer interferometry (BLI). ALT-1 captured on nickel sensors via its c-terminal histidine tag was saturated with the antibodies adjacent to the traces indicated with arrows (**A and B**) and on the x-axis (**C and D**). A second competitor antibody either **A**) C8-Fc or **B**) G6-Fc was then added and the association and dissociation phases monitored. The percentage inhibition was calculated as $100 - [\text{maximum nm shift saturating antibody} / \text{maximum nm shift competing antibody}] \times 100$ for **C**) C8-Fc and **D**) G6-Fc.

Figure 85. Shows the X-ray crystal structure of ALT1 in complex with nanobody C8. ALT1, which is a homodimer, is displayed in surface mode with each monomer in different shades of grey. There are two molecules of nanobody C8 bound to the ALT1 dimer shown in cartoon representation. Rotation of the ALT1-C8 crystal structure by 90° (**Figure 85B**) shows the top view of the ALT1-C8 complex. The interface between ALT1 and nanobody C8 is shaded in the same shade as the interacting nanobody.

Figure 86. Shows an overlay of the ALT1-C8 crystal structure with that of ALT2 (PDB ID: 3IHJ) or nanobody NbALFA (PDB ID 6I2G). **A)** Shows ALT1 monomer A (black) overlayed with ALT2 monomer (light grey). ALT1 monomer adopts a similar fold to that of ALT2 as reflected by an RMSD of 0.868 Å. **B)** shows the ALT1 dimer (black) overlayed with ALT2 dimer (light grey). There is some difference in the manner in which ALT1 and ALT2 form a homodimer as reflected in a higher RMSD of 6.633 Å. **C)** Shows nanobody C8 (black) overlayed with NbALFA (light grey), a random alpaca nanobody structure found in the RCSB PDB database. Dotted lines in C8 structure denote unmodelled residues. The two nanobodies show excellent alignment with and RMSD of 0.615 Å. **D)** Shows the overlay of nanobody C8 with the predicted alphafold 2.0 structure presented in **Figure 13**.

Figure 87. Shows the residues that are involved in the ALT1 and nanobody C8 interface. Residues which are involved in salt bridge interactions are shown with a solid line, while residues involved in hydrogen bonding are shown with a dashed line. Nanobody C8 makes contacts to both monomers of the ALT1 homodimer.

Figure 88. Shows a close up of the bonds made between ALT1 and nanobody C8 in the ALT1-C8 crystal structure as determined by PISA analysis. Salt bridges are shown in dashed lines, hydrogen bonds are shown in dotted lines. Residues from ALT1 are underlined.

Figure 89. Shows a heat map of the ability of nanobody G6-Fc to bind to single alanine mutations of ALT1. Residues in ALT1 were replaced with alanine at indicated positions and protein expressed and used to coat ELISA plates. Nanobody G6-Fc was applied to each well in triplicate and bound antibody detected with an anti-species-specific antibody conjugated to horse radish peroxidase. The percentage binding was calculated relative to wild type ALT1. Data are presented as a heat map of the average of two independent experiments.

Figure 90. Shows a list of all amino acids where replacement with alanine causes a reduction in binding to nanobody G6. The data are derived from **Figure 89**, that caused either a 75-100% reduction in binding or 50-75% reduction in binding or a 25-50% decrease in binding are listed.

Figure 91. Shows a heat map of the ability of rabbit monoclonal antibodies to bind to single alanine mutations of ALT1. Residues in ALT1 were replaced with alanine at indicated positions and protein expressed and used to coat ELISA plates via anti-His capture. Each of the rabbit monoclonal antibodies was applied to each well in triplicate and bound antibody detected with an anti-rabbit antibody conjugated to horse radish peroxidase. The percentage binding was

calculated relative to wild type ALT1. Data are presented as a heat map of the average of two independent experiments.

5 **Figure 92.** Shows a list of all amino acids where replacement with alanine causes a reduction in binding to rabbit monoclonal antibody 1. The data are derived from **Figure 91**, that caused either a 75-100% reduction in binding or 50-75% reduction in binding or a 25-50% decrease in binding are listed.

10 **Figure 93.** Shows a list of all amino acids where replacement with alanine causes a reduction in binding to rabbit monoclonal antibody 3. The data are derived from **Figure 91**, that caused either a 75-100% reduction in binding or 50-75% reduction in binding or a 25-50% decrease in binding are listed.

15 **Figure 94.** Shows a list of all amino acids where replacement with alanine causes a reduction in binding to rabbit monoclonal antibody 4. The data are derived from **Figure 91**, that caused either a 75-100% reduction in binding or 50-75% reduction in binding or a 25-50% decrease in binding are listed.

20 **Figure 95.** Shows a list of all amino acids where replacement with alanine causes a reduction in binding to rabbit monoclonal antibody 5. The data are derived from **Figure 91**, that caused either a 75-100% reduction in binding or 50-75% reduction in binding or a 25-50% decrease in binding are listed.

25 **Figure 96.** Shows an epitope binning experiment performed using ALT-1 and the rabbit monoclonal antibodies. ALT1 captured on nickel sensors via its c-terminal histidine tag was saturated either buffer only (KN), or RmAb1, 3, 4 or 5 (indicated for each trace). A second competitor antibody either RmAb1, 3, 4 or 5 was then added and the association and dissociation phases monitored.

30 **Figure 97.** Shows the quantitation of epitope binning experiment performed using ALT-1 and the rabbit monoclonal antibodies. The percentage inhibition was calculated as $100 - [\text{maximum nm shift saturating antibody} / \text{maximum nm shift competing antibody}] \times 100$ for RmAb1, 3, 4 or 5.

35 **Figure 98.** Shows a dot blot of native and denatured ALT1 probed using various anti-ALT1 antibodies. Varying concentrations of ALT1, native (N) or denatured (D), was spotted onto

nitrocellulose membrane and probed with either G6-FLAG, C8-FLAG, C4G-Fc **A**), rabbit monoclonal antibodies **B**), rabbit polyclonal, commercial Abcam anti-ALT, commercial MyBioSource.com anti-ALT2 **C**), or mouse monoclonal antibodies **D**).

- 5 **Figure 99.** Shows an epitope binning experiment performed using ALT1 and the mouse monoclonal antibodies. ALT1 captured on nickel sensors via its C-terminal His-tag was saturated with various antibodies (see graph legend). A second competitor antibody either mouse monoclonal 3H12 (**A and B**), 4A9 (**C and D**), 5H2 (**E and F**) or 6B5 (**G and H**) was then added and the association and dissociation phases monitored.

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Figure 100. Shows the quantitation of the epitope binning experiment performed using ALT1 and the mouse monoclonal antibodies. The percentage inhibition was calculated as $100 - [\text{maximum nm shift saturating antibody} / \text{maximum nm shift competing antibody}] \times 100$ 3H12 **A**), 4A9 **B**), 5H2 **C**) or 6B5 **D**). **E**) shows a summary of (**A-D**) in heat map form.

15

Figure 101. Provide embodiments of the assay of the invention. Grey boxes indicate combinations of capture and detection binding proteins. Light grey boxes indicate a preferred combination.

- 20 **Figure 102.** Shows differential scanning fluorimetry plot of ALT protein. The thermal stability of ALT1 was assessed by differential scanning fluorimetry. The data shows that ALT1 has a T_m of 60.0°C as observed by the peak inflection point. The protein begins unfolding at approximately 43°C and is unfolded by about 68°C.

25 **KEY TO SEQUENCE LISTING**

SEQ ID NO: 1	Nb C8 full length	Amino acid sequence
SEQ ID NO: 2	Nb C8 CDR1	Amino acid sequence
SEQ ID NO: 3	Nb C8 CDR2	Amino acid sequence
SEQ ID NO: 4	Nb C8 CDR3	Amino acid sequence
SEQ ID NO: 5	Nb G6 full length	Amino acid sequence
SEQ ID NO: 6	Nb G6 CDR1	Amino acid sequence
SEQ ID NO: 7	Nb G6 CDR2	Amino acid sequence
SEQ ID NO: 8	Nb G6 CDR3	Amino acid sequence
SEQ ID NO: 9	Nb C10 full length	Amino acid sequence
SEQ ID NO: 10	Nb C10 CDR1	Amino acid sequence
SEQ ID NO: 11	Nb C10 CDR2	Amino acid sequence
SEQ ID NO: 12	Nb C10 CDR3	Amino acid sequence
SEQ ID NO: 13	A1	Amino acid sequence
SEQ ID NO: 14	A2, A4, A7, A10, A12, B2, B3, B4, B5, B6, B7, B8, B11, C1, C2, C4, C6, C7, C10, C11, C12, D1, D4, D5, D6, D7, D8, D10, D11, D12, E2, E3, E4, E5, E6, E7, E8, E10, E11, E12, F2, F3, F4, F5, F6, F7, F8, F11,	Amino acid sequence

	G1, G2, G4, G5, G7, G8, G10, G12, H3, H4, H5, H7, H8, H11	
SEQ ID NO: 15	A11, F1	Amino acid sequence
SEQ ID NO: 16	B1	Amino acid sequence
SEQ ID NO: 17	B9, E9, H9	Amino acid sequence
SEQ ID NO: 18	B10, F12, H6, H10	Amino acid sequence
SEQ ID NO: 19	B12	Amino acid sequence
SEQ ID NO: 20	C3, D3	Amino acid sequence
SEQ ID NO: 21	C5	Amino acid sequence
SEQ ID NO: 22	D2	Amino acid sequence
SEQ ID NO: 23	D9	Amino acid sequence
SEQ ID NO: 24	F10	Amino acid sequence
SEQ ID NO: 25	G3	Amino acid sequence
SEQ ID NO: 26	G9	Amino acid sequence
SEQ ID NO: 27	R2 B5	Amino acid sequence
SEQ ID NO: 28	R2 B6	Amino acid sequence
SEQ ID NO: 29	R2 B8	Amino acid sequence
SEQ ID NO: 30	R2 B9	Amino acid sequence
SEQ ID NO: 31	R2 B10	Amino acid sequence
SEQ ID NO: 32	R2 B11	Amino acid sequence
SEQ ID NO: 33	R2 B12	Amino acid sequence
SEQ ID NO: 34	R2 C1	Amino acid sequence
SEQ ID NO: 35	R2 C3	Amino acid sequence
SEQ ID NO: 36	R2 C5	Amino acid sequence
SEQ ID NO: 37	R2 C6	Amino acid sequence
SEQ ID NO: 38	R2 C7	Amino acid sequence
SEQ ID NO: 39	R2 C9	Amino acid sequence
SEQ ID NO: 40	R2 C11	Amino acid sequence
SEQ ID NO: 41	R2 C12	Amino acid sequence
SEQ ID NO: 42	R2 D4	Amino acid sequence
SEQ ID NO: 43	R2 D5	Amino acid sequence
SEQ ID NO: 44	R2 D6	Amino acid sequence
SEQ ID NO: 45	R2 D7	Amino acid sequence
SEQ ID NO: 46	R2 D8	Amino acid sequence
SEQ ID NO: 47	R2 D9	Amino acid sequence
SEQ ID NO: 48	R2 D11	Amino acid sequence
SEQ ID NO: 49	R2 D12	Amino acid sequence
SEQ ID NO: 50	R2 E1	Amino acid sequence
SEQ ID NO: 51	R2 E2	Amino acid sequence
SEQ ID NO: 52	R2 E4	Amino acid sequence
SEQ ID NO: 53	R2 E6	Amino acid sequence
SEQ ID NO: 54	R2 E7	Amino acid sequence
SEQ ID NO: 55	R2 E8	Amino acid sequence
SEQ ID NO: 56	R2 E9	Amino acid sequence
SEQ ID NO: 57	C3C	Amino acid sequence
SEQ ID NO: 58	C4C	Amino acid sequence
SEQ ID NO: 59	G3G	Amino acid sequence
SEQ ID NO: 60	G4G	Amino acid sequence
SEQ ID NO: 61	C3G	Amino acid sequence
SEQ ID NO: 62	C4G	Amino acid sequence
SEQ ID NO: 63	G3C	Amino acid sequence
SEQ ID NO: 64	G4C	Amino acid sequence
SEQ ID NO: 65	(Gly)2-Ala-(Gly)2	Amino acid sequence
SEQ ID NO: 66	(Gly)5	Amino acid sequence
SEQ ID NO: 67	(Gly)8	Amino acid sequence

SEQ ID NO: 68	(Gly)6	Amino acid sequence
SEQ ID NO: 69	(Gly)7	Amino acid sequence
SEQ ID NO: 70	(Gly)10	Amino acid sequence
SEQ ID NO: 71	Gly-Ser-Gly-Ser-Gly	Amino acid sequence
SEQ ID NO: 72	(Gly)4	Amino acid sequence
SEQ ID NO: 73	Gly-Ala-Gly	Amino acid sequence
SEQ ID NO: 74	Gly)2-Arg-(Gly)2-Ser	Amino acid sequence
SEQ ID NO: 75	(Gly-Gly-Gly-Gly-Ser)3	Amino acid sequence
SEQ ID NO: 76	(Gly-Gly-Gly-Gly-Ser)4	Amino acid sequence
SEQ ID NO: 77	Ser-(Gly)2-Ser-Gly	Amino acid sequence
SEQ ID NO: 78	Fc region	Amino acid sequence
SEQ ID NO: 79	C4C-Fc	Amino acid sequence
SEQ ID NO: 80	G4G-Fc	Amino acid sequence
SEQ ID NO: 81	C4G-Fc	Amino acid sequence
SEQ ID NO: 82	G4C-Fc	Amino acid sequence
SEQ ID NO: 83	AST	Amino acid sequence
SEQ ID NO: 84	ALT1	Amino acid sequence
SEQ ID NO: 85	ALT2iso1	Amino acid sequence
SEQ ID NO: 86	ALT2iso2	Amino acid sequence
SEQ ID NO: 87	Nb C8 full length	Nucleotide sequence
SEQ ID NO: 88	Nb G6 full length	Nucleotide sequence
SEQ ID NO: 89	Nb C10 full length	Nucleotide sequence
SEQ ID NO: 90	C3C	Nucleotide sequence
SEQ ID NO: 91	C4C	Nucleotide sequence
SEQ ID NO: 92	C4C codon optimized	Nucleotide sequence
SEQ ID NO: 93	G3G	Nucleotide sequence
SEQ ID NO: 94	G4G	Nucleotide sequence
SEQ ID NO: 95	G4G codon optimized	Nucleotide sequence
SEQ ID NO: 96	C3G	Nucleotide sequence
SEQ ID NO: 97	C4G	Nucleotide sequence
SEQ ID NO: 98	C4G codon optimized	Nucleotide sequence
SEQ ID NO: 99	G3C	Nucleotide sequence
SEQ ID NO: 100	G4C	Nucleotide sequence
SEQ ID NO: 101	G4C codon optimized	Nucleotide sequence
SEQ ID NO: 102	C4C-Fc	Nucleotide sequence
SEQ ID NO: 103	G4G-Fc	Nucleotide sequence
SEQ ID NO: 104	C4G-Fc	Nucleotide sequence
SEQ ID NO: 105	G4C-Fc	Nucleotide sequence
SEQ ID NO: 106	Fc region	Nucleotide sequence
SEQ ID NO: 107	N-terminal pelB signaling peptide	Nucleotide sequence
SEQ ID NO: 108	tPA leader sequence	Nucleotide sequence
SEQ ID NO: 109	RmAb1 15B5 Heavy Chain	Amino acid sequence
SEQ ID NO: 110	RmAb1 15B5 Heavy Chain CDR1	Amino acid sequence
SEQ ID NO: 111	RmAb1 15B5 Heavy Chain CDR2	Amino acid sequence
SEQ ID NO: 112	RmAb1 15B5 Heavy Chain CDR3	Amino acid sequence
SEQ ID NO: 113	RmAb1 15B5 Light Chain	Amino acid sequence
SEQ ID NO: 114	RmAb1 15B5 Light Chain CDR1	Amino acid sequence
SEQ ID NO: 115	RmAb1 15B5 Light Chain CDR3	Amino acid sequence
SEQ ID NO: 116	RmAb2 20C12 Heavy chain	Amino acid sequence
SEQ ID NO: 117	RmAb2 20C12 Heavy chain CDR1	Amino acid sequence
SEQ ID NO: 118	RmAb2 20C12 Heavy chain CDR2	Amino acid sequence
SEQ ID NO: 119	RmAb2 20C12 Heavy chain CDR3	Amino acid sequence
SEQ ID NO: 120	RmAb2 20C12 Light chain	Amino acid sequence
SEQ ID NO: 121	RmAb2 20C12 Light chain CDR1	Amino acid sequence
SEQ ID NO: 122	RmAb2 20C12 Light chain CDR3	Amino acid sequence

SEQ ID NO: 123	RmAb3 22G10 Heavy chain	Amino acid sequence
SEQ ID NO: 124	RmAb3 22G10 Heavy chain CDR1	Amino acid sequence
SEQ ID NO: 125	RmAb3 22G10 Heavy chain CDR2	Amino acid sequence
SEQ ID NO: 126	RmAb3 22G10 Heavy chain CDR3	Amino acid sequence
SEQ ID NO: 127	RmAb3 22G10 Light chain	Amino acid sequence
SEQ ID NO: 128	RmAb3 22G10 Light chain CDR1	Amino acid sequence
SEQ ID NO: 129	RmAb3 22G10 Light chain CDR3	Amino acid sequence
SEQ ID NO: 130	RmAb4 36D4 Heavy Chain	Amino acid sequence
SEQ ID NO: 131	RmAb4 36D4 Heavy Chain CDR1	Amino acid sequence
SEQ ID NO: 132	RmAb4 36D4 Heavy Chain CDR2	Amino acid sequence
SEQ ID NO: 133	RmAb4 36D4 Heavy Chain CDR3	Amino acid sequence
SEQ ID NO: 134	RmAb4 36D4 Light Chain	Amino acid sequence
SEQ ID NO: 135	RmAb4 36D4 Light Chain CDR1	Amino acid sequence
SEQ ID NO: 136	RmAb4 36D4 Light Chain CDR3	Amino acid sequence
SEQ ID NO: 137	RmAb5 42F12 Heavy chain	Amino acid sequence
SEQ ID NO: 138	RmAb5 42F12 Heavy chain CDR1	Amino acid sequence
SEQ ID NO: 139	RmAb5 42F12 Heavy chain CDR2	Amino acid sequence
SEQ ID NO: 140	RmAb5 42F12 Heavy chain CDR3	Amino acid sequence
SEQ ID NO: 141	RmAb5 42F12 Light chain	Amino acid sequence
SEQ ID NO: 142	RmAb5 42F12 Light chain CDR1	Amino acid sequence
SEQ ID NO: 143	RmAb5 42F12 Light chain CDR3	Amino acid sequence
SEQ ID NO: 144	RmAb1 15B5 Heavy Chain	Nucleotide sequence
SEQ ID NO: 145	RmAb1 15B5 Light Chain	Nucleotide sequence
SEQ ID NO: 146	RmAb2 20C12 Heavy chain	Nucleotide sequence
SEQ ID NO: 147	RmAb2 20C12 Light chain	Nucleotide sequence
SEQ ID NO: 148	RmAb3 22G10 Heavy chain	Nucleotide sequence
SEQ ID NO: 149	RmAb3 22G10 Light chain	Nucleotide sequence
SEQ ID NO: 150	RmAb4 36D4 Heavy Chain	Nucleotide sequence
SEQ ID NO: 151	RmAb4 36D4 Light Chain	Nucleotide sequence
SEQ ID NO: 152	RmAb5 42F12 Heavy chain	Nucleotide sequence
SEQ ID NO: 153	RmAb5 42F12 Light chain	Nucleotide sequence
SEQ ID NO: 154	C3C-Fc	Nucleotide sequence
SEQ ID NO: 155	C3C-Fc	Amino acid sequence
SEQ ID NO: 156	G3G-Fc	Nucleotide sequence
SEQ ID NO: 157	G3G-Fc	Amino acid sequence
SEQ ID NO: 158	C3G-Fc	Nucleotide sequence
SEQ ID NO: 159	C3G-Fc	Amino acid sequence
SEQ ID NO: 160	G3C-Fc	Nucleotide sequence
SEQ ID NO: 161	G3C-Fc	Amino acid sequence

DETAILED DESCRIPTION

General techniques and definitions

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., amino acid sequences).

The term “and/or”, e.g., “X and/or Y” shall be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or

group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

As used herein, the term “subject” refers to any animal. In one example, the animal is a vertebrate. In an embodiment, the subject is a mammal. In an embodiment, the mammal is a human. In an embodiment, the mammal is a human companion animal. In an embodiment, the animal is livestock or a research animal.

As used herein, the term “binds” or “binding” refers to the interaction of a binding protein or an antigen binding domain thereof with an antigen means that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the antigen. For example, a binding protein recognizes and binds to a specific protein structure rather than to proteins generally. If a binding protein binds to epitope “A”, the presence of a molecule containing epitope “A” (or free, unlabelled “A”), in a reaction containing labelled “A” and the binding protein, will reduce the amount of labelled “A” bound to the binding protein.

As used herein, the term “epitope” refers to a structure on the surface of an antigen that is recognized by and can bind to a specific antibody.

As used herein, the term “a conformational epitope”, also referred to as a “discontinuous epitope” or “topographic epitope”, is an epitope built from non-contiguous parts of one or more amino amino acid sequence/s that are brought together by protein folding in its native state. In an embodiment, a conformational epitope comprises residues on two protomers of a multimeric protein (e.g. a homo dimer). In an embodiment, the conformation epitope is a conformational epitope of ALT1. In an embodiment, the conformational epitope is dependent on the non-covalent bond formation in ALT.

As used herein, the term “paratope” with respect to a binding protein or an antigen binding domain thereof refers to a group of amino acid residues on the variable regions of the antibody that makes direct contact with the antigen and form the antigen binding site of the variable regions. A paratope often comprises or consists of amino acid residues in one or more CDR sequences. In some embodiments, a paratope comprises residues of the one or more CDR sequences and one of more framework regions.

As used herein, the term “specifically binds” or “specific for X” shall be taken to mean a binding protein of the disclosure reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular antigen or antigens or cell expressing same than it does with alternative antigens or cells. For example, a protein that specifically binds to an antigen binds that antigen with greater affinity (e.g., 20-fold or 40-fold or 60-fold or 80-fold to 100-fold or 150-fold or 200-fold greater affinity), avidity, more readily, and/or with greater duration than it binds to other antigens. It is also understood by reading this

definition that, for example, a protein that specifically binds to a first antigen may or may not specifically bind to a second antigen. In an embodiment, the antigen is alanine aminotransferase 1 (ALT1). In an embodiment, the antigen is alanine aminotransferase 2 (ALT2). In an embodiment, the second antigen is aspartate aminotransferase (AST). In an embodiment, the antigen is human ALT1. In an embodiment, the antigen is human ALT2. In an embodiment, the second antigen is human AST.

As used herein, “does not substantially bind” shall be understood to mean that a binding protein does not bind to an antigen at a level greater than 20% or 15% or 10% or 9% or 8% or 7% or 6% or 5% or 4% or 3% or 2% of the level of binding to an antigen to which the protein is known to bind. In one example, the binding is detected by Western blotting and/or FACS and/or ELISA and/or antibody panning (e.g., with antibody variable regions on the surface of a particle, such as a phage). For example, a protein of the present invention binds to heat denatured ALT1 at a level no greater than about 20% or 15% or 10% of the level bound to correctly folded ALT1.

As used herein, “does not detectably bind” shall be understood to mean that a binding protein does not bind to an antigen at a level significantly greater than background, e.g., binds to ALT1 at a level less than 10%, or 8% or 6% or 5% above background. In the case of an antibody, the antibody binds to the antigen at a level less than 10% or 8% or 6% or 5% greater than an isotype control antibody. In one example, the binding is detected by Western blotting and/or FACS and/or ELISA and/or antibody panning (e.g., with antibody variable regions on the surface of a particle, such as a phage).

As used herein, the term “isolated” shall be taken to mean that the binding protein or nucleic acid is substantially removed from its naturally-occurring environment, e.g., is in a heterologous environment and/or that it is substantially free of contaminating agents, e.g., at least about 70% or 75% or 80% or 85% or 90% or 95% or 96% or 97% or 98% or 99% free of contaminating agents.

As used herein, the term “recombinant” shall be taken to mean created by combining genetic material from two or more different sources.

As used herein, reference to a “sample” refers to a biological sample e.g. blood or a fraction thereof, urine, tissue, cells, saliva. In an embodiment, the sample is selected from: plasma, serum, whole blood and capillary blood. In an embodiment, the sample is plasma. In an embodiment, the sample is serum. In an embodiment, the sample is human plasma. In an embodiment, the sample is human serum. In an embodiment, the sample is whole blood. In an embodiment, the sample is capillary blood. In an embodiment, the sample is finger prick blood. In an embodiment, the whole blood is venous blood. In an embodiment, the whole blood is capillary blood.

As used herein, a “visual” or “visible assay” refers to an assay wherein the result of the test can be directly interpreted by a user upon viewing without the aid of special technical equipment to detect and read the result.

As used herein, “sensitivity” refers to the ability of a binding protein to detect an antigen.
5 A higher sensitivity means that the binding protein can detect the antigen at a lower concentration in a sample.

As used herein, “specificity” refers to the ability of a binding protein to detect an antigen in a mixture of other antigens (that could be similar and/or dissimilar).

As used herein, “protomer” refers to the basic structural unit of an oligomeric protein
10 (e.g. the monomers of a homodimer).

As used herein, the term “antigen binding site” shall be taken to mean a structure formed by a protein that is capable of binding or specifically binding to an antigen. The antigen binding site need not be a series of contiguous amino acids, or even amino acids in a single polypeptide chain. For example, in a Fv produced from two different polypeptide chains the antigen binding
15 site is made up of a series of amino acids of a V_L and a V_H that interact with the antigen and that are generally, however not always in the one or more of the CDRs in each variable region. In some examples, an antigen binding site is a V_H or a V_L or a Fv.

As used herein, the term “avidity” refers to avidity refers to the strength of the interaction between an antigen and a binding protein (the overall strength of an antibody-binding protein
20 complex. In an embodiment, addition of a Fc region or fragment thereof increases the avidity of a binding protein. In an embodiment, a multimeric form of a binding protein has a higher avidity than a monomeric form of a binding protein.

Alanine transaminase

25 As used herein “alanine transaminase”, “ALT”, “alanine aminotransferase”, “glutamate-pyruvate transaminase” or “serum glutamic-pyruvic transaminase” is an enzyme that catalyzes the reversible transamination between L-alanine and 2-oxoglutarate (α -ketoglutarate) by transferring an amino group from L-alanine to α -ketoglutarate to generate pyruvate and L-glutamate. ALT, like other transaminases, requires the coenzyme pyridoxal phosphate in the
30 first phase of the reaction. The native/naturally occurring enzyme is a homodimer (comprises two protomers). Alanine transaminases play roles in gluconeogenesis and amino acid metabolism in many tissues including skeletal muscle, kidney, and liver. In an embodiment, the ALT is ALT1. In an embodiment, a monomer of the ALT1 homodimer is encoded by the amino acid sequence set forth in **SEQ ID NO:84**. In an embodiment, ALT1 is human ALT1. In an
35 embodiment, the ALT is ALT2. In an embodiment, ALT2 is human ALT2. In an embodiment, ALT2 is ALT2 isoform 1. In an embodiment, a monomer of the ALT2 isoform 1 homodimer is

encoded by the amino acid sequence set forth in **SEQ ID NO:85**. In an embodiment, ALT2 is ALT2 isoform 2. In an embodiment, a monomer of the ALT2 isoform 2 homodimer is encoded by the amino acid sequence set forth in **SEQ ID NO:85**.

As used herein, “denatured ALT1” refers to ALT1 that has lost the folded structure of the protein in its native state. In an embodiment, ALT1 is treated to break non-covalent bonds. In an embodiment, ALT1 is denatured by heating. The melting temperature of ALT1 is shown in **Figure 102**. In an embodiment, ALT1 is denatured by heating at a temperature of about 68°C. In an embodiment, ALT1 is denatured by heating at a temperature of about 68°C or higher. In an embodiment, ALT1 is denatured by chemical treatment.

The gene encoding ALT1 is located on chromosome 8 in humans with the highest gene transcription observed in the liver, followed by colon, duodenum, fat, and kidney with lower levels detected in skin, small intestine and stomach. ALT1 is a cytosolic protein and under normal conditions has been reported to comprise more than 90% of the detectable enzymatic activity in blood (Rafter et al., 2012).

The gene encoding alanine transaminase 2 (ALT2) is located on chromosome 16 in humans. ALT2 isoform 1 represents the longer transcript and encodes the longer variant. ALT2 isoform 2 is produced when alternate exons are used in the 5' UTR and 5' coding sequence, resulting in use of a downstream start codon compared to variant 1. It has a shorter N-terminus than isoform 1. Unlike ALT1, multiple single nucleotide polymorphisms exist in ALT2 that are of clinical significance. ALT2 is a mitochondrial enzyme and has a distinct tissue distribution with RNA transcripts found predominantly in fat, followed by oesophagus, pancreas, liver, stomach, brain, skin, salivary gland, with low levels detected in gall bladder, testis and thyroid. ALT2 constitutes a relatively small proportion of the enzymatic ALT activity found in blood at <10% (Rafter et al., 2012). ALT2 dysregulation has been reported to promote cell survival and growth in cancer. Recessive mutations in ALT2 are associated with intellectual and developmental disabilities, post-natal microcephaly and spastic paraplegia (Ouyang Qing et al., 2019).

Binding proteins

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase (ALT).

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase (ALT), comprising amino acid sequences selected from the following:

a) amino acid sequences GPAVSNVA (**SEQ ID NO: 2**) as CDR1, ITWSGWT (**SEQ ID NO: 3**) as CDR2 and NLIGLRVGPENKY (**SEQ ID NO: 4**) as CDR3;

b) amino acid sequences GRTDSFYA (**SEQ ID NO: 6**) as CDR1, ITWSAGST (**SEQ ID NO: 7**) as CDR2 and AADSLSAGYESSWLEAFGS (**SEQ ID NO: 8**) as CDR3; and

c) amino acid sequences GRTFSSYS (**SEQ ID NO: 10**) as CDR1, ISRSGFST (**SEQ ID NO: 11**) as CDR2 and AVGRAYLPTASGTRCPREAYDY (**SEQ ID NO: 12**) as CDR3;

5 and

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1), comprising amino acid sequences selected from the following:

10 a) amino acid sequences GPAVSNVA (**SEQ ID NO: 2**) as CDR1, ITWSGWT (**SEQ ID NO: 3**) as CDR2 and NLI GLRVGPENKY (**SEQ ID NO: 4**) as CDR3;

b) amino acid sequences GRTDSFYA (**SEQ ID NO: 6**) as CDR1, ITWSAGST (**SEQ ID NO: 7**) as CDR2 and AADSLSAGYESSWLEAFGS (**SEQ ID NO: 8**) as CDR3; and

15 c) amino acid sequences GRTFSSYS (**SEQ ID NO: 10**) as CDR1, ISRSGFST (**SEQ ID NO: 11**) as CDR2 and AVGRAYLPTASGTRCPREAYDY (**SEQ ID NO: 12**) as CDR3;

and

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an embodiment, the present invention provides a binding protein which binds ALT1, comprising the amino acid sequences GPAVSNVA (**SEQ ID NO: 2**) as CDR1, ITWSGWT (**SEQ ID NO: 3**) as CDR2 and NLI GLRVGPENKY (**SEQ ID NO: 4**) as CDR3, wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an embodiment, the present invention provides a binding protein which binds ALT1, comprising the amino acid sequences GRTDSFYA (**SEQ ID NO: 6**) as CDR1, ITWSAGST (**SEQ ID NO: 7**) as CDR2 and AADSLSAGYESSWLEAFGS (**SEQ ID NO: 8**) as CDR3, wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an embodiment, the present invention provides a binding protein which binds ALT2, comprising the amino acid sequences GRTDSFYA (**SEQ ID NO: 6**) as CDR1, ITWSAGST (**SEQ ID NO: 7**) as CDR2 and AADSLSAGYESSWLEAFGS (**SEQ ID NO: 8**) as CDR3, wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an embodiment, the present invention provides a binding protein which binds ALT1, comprising the amino acid sequences GRTFSSYS (**SEQ ID NO: 10**) as CDR1, ISRSGFST (**SEQ ID NO: 11**) as CDR2 and AVGRAYLPTASGTRCPREAYDY (**SEQ ID NO: 12**) as

CDR3, wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an embodiment, one of the CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

5 In an embodiment, two of the CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an embodiment, three of the CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

10 In an embodiment, one of the CDR1 has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an embodiment, one of the CDR2 has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

In an embodiment, one of the CDR3 has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.

15 In an embodiment, the present invention provides a binding protein which binds ALT1, comprising the amino acid sequences GPAVSNVA (**SEQ ID NO: 2**) as CDR1, ITWSGWT (**SEQ ID NO: 3**) as CDR2 and NLIGLRVGPENKY (**SEQ ID NO: 4**) as CDR3.

In an embodiment, the present invention provides a binding protein which binds ALT1, comprising the amino acid sequences GRTDSFYA (**SEQ ID NO: 6**) as CDR1, ITWSAGST (**SEQ ID NO: 7**) as CDR2 and AADSLSAGYESSWLEAFGS (**SEQ ID NO: 8**) as CDR3.

20 In an embodiment, the present invention provides a binding protein which binds ALT2, comprising the amino acid sequences GRTDSFYA (**SEQ ID NO: 6**) as CDR1, ITWSAGST (**SEQ ID NO: 7**) as CDR2 and AADSLSAGYESSWLEAFGS (**SEQ ID NO: 8**) as CDR3.

25 In an embodiment, the present invention provides a binding protein which binds ALT1, comprising the amino acid sequences GRTFSSYS (**SEQ ID NO: 10**) as CDR1, ISRSGFST (**SEQ ID NO: 11**) as CDR2 and AVGRAYLPTASGTRCPREAYDY (**SEQ ID NO: 12**) as CDR3.

30 In an embodiment, the binding protein comprises the amino acid sequence **SEQ ID NO: 1** (C8), or a sequence at least 76% identical thereto, or a humanised, or germlined version thereof.

In an embodiment, the binding protein comprises the amino acid sequence **SEQ ID NO: 5** (G6), or a sequence at least 76% identical thereto, or a humanised, or germlined version thereof.

35 In an embodiment, the binding protein comprises the amino acid sequence **SEQ ID NO: 9** (C10), or a sequence at least 76% identical thereto, or a humanised, or germlined version thereof.

In an embodiment, the binding protein comprises, consists of or consists essentially of an amino acid sequence that is at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to **SEQ ID NO: 1** (C8).

5 In an embodiment, the binding protein comprises, consists of or consists essentially of an amino acid sequence that is at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to **SEQ ID NO: 5** (G6).

10 In an embodiment, the binding protein comprises, consists of or consists essentially of an amino acid sequence that is at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to **SEQ ID NO: 9** (C10).

In an embodiment, the binding protein comprises the amino acid sequence **SEQ ID NO: 1** (C8). In an embodiment, the binding protein comprises the amino acid sequence **SEQ ID NO: 5** (G6). In an embodiment, the binding protein comprises the amino acid sequence **SEQ ID NO: 9** (C10).

In an embodiment, the binding protein comprises an amino acid sequence selected from **SEQ ID NO: 13** to **SEQ ID NO: 56**, or a sequence at least 76% identical thereto, or a humanised, or germlined version thereof.

20 In an embodiment, the binding protein binds an epitope that comprises residues on both protomers of ALT1.

In an embodiment, the binding protein does not significantly bind denatured ALT1.

In an embodiment, the binding protein does not significantly bind heat denatured ALT1.

In an embodiment, the binding protein does not detectably bind denatured ALT1.

25 In an embodiment, the binding protein does not detectably bind heat denatured ALT1.

In an embodiment, ALT1 is denatured by heating to a temperature of about 68°C. In an embodiment, ALT1 is denatured by heating to a temperature of 68°C or higher.

In an embodiment, the binding protein binds denatured ALT1. In an embodiment, the binding protein binds heat denatured ALT1.

30 In an embodiment, the binding protein does not significantly bind an epitope of ALT2.

In an embodiment, the binding protein does not significantly bind an epitope of human ALT2.

In an embodiment, the binding protein does not significantly bind AST. In an embodiment, the binding protein does not significantly bind human AST.

35 In an embodiment, the binding protein does not detectably bind an epitope of ALT2. In an embodiment, the binding protein does not detectably bind an epitope of human ALT2.

In an embodiment, the binding protein does not detectably bind AST. In an embodiment, the binding protein does not detectably bind human AST.

In an embodiment, the binding protein has a higher affinity for ALT1 than ALT2.

In an embodiment, the binding protein binds native ALT1 in solution.

5 In an embodiment, the binding protein binds conformationally intact ALT1.

In an embodiment, the binding protein binds a conformational epitope of ALT1.

In an embodiment, the binding protein binds native ALT1 striped to a solid surface/ conjugated to a solid surface.

10 In an embodiment, the paratope of the binding protein comprises residues on both protomers of ALT (the active enzyme is a homodimer of two ALT1 protomers). In an embodiment, the paratope of the binding protein binds residues on both promoters of ALT.

In an embodiment, the binding protein binds Asn 99 and Gln 374 of one protomer of ALT1 and 93 Asp, 96 Ser and 109 Glu on a second protomer of ALT1.

15 In an embodiment, the paratope of the binding protein comprises residues on both protomers of ALT (the active enzyme is a homodimer of two ALT1 monomers).

In an embodiment, the paratope of the binding protein comprises residues in one or more of: CDR2, FR1 and FR2. In an embodiment, the paratope of the binding protein comprises residues in CDR2. In an embodiment, the paratope of the binding protein comprises residues in FR1. In an embodiment, the paratope of the binding protein comprises residues in FR2.

20 In an embodiment, the paratope of the binding protein comprises residues in CDR2 and not CDR1 or CDR3.

In an embodiment, the binding protein residues that mediate contact with ALT1 comprise one or more of the residues corresponding to C8: 18 Leu, 19 Arg, 58 Ser, 68 Phe, 90 Thr, 70 Ile, 71 Ser, 76 Lys, 82 Gln and 84 Asn.

25 In an embodiment, the binding protein residues that mediate contact with ALT1 comprise or consist of the residues corresponding to 18 Leu, 19 Arg, 58 Ser, 68 Phe, 90 Thr, 70 Ile, 71 Ser, 76 Lys, 82 Gln and 84 Asn.

30 In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu.

35 In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 53

Arg, 61 Thr, 154 Val, 175 Glu, 178 Thr, 183 Leu, 196 Leu, 202 Val, 205 Asp, 212 Arg, 225 Leu, 239 Val, 260 Arg, 266 Arg, 304 Gln, 327 Val, 358 Leu, 369 Asp, 430 Glu, 431 Leu and 433 Leu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 53 Arg, 61 Thr, 154 Val, 175 Glu, 178 Thr, 183 Leu, 196 Leu, 202 Val, 205 Asp, 212 Arg, 225 Leu, 239 Val, 260 Arg, 266 Arg, 304 Gln, 327 Val, 358 Leu, 369 Asp, 430 Glu, 431 Leu and 433 Leu.

In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 48 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 53 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 61 Thr. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 154 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 175 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 178 Thr. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 196 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 202 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 205 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 212 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 225 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 327 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not significantly bind to ALT1

comprising an alanine substitution at position 369 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 431 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 433 Leu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 48 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 53 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 61 Thr. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 154 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 175 Glu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 178 Thr. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 196 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 202 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 205 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 212 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 225 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 327 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 369 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the

binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 431 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 433 Leu.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1), the binding protein comprising a heavy chain variable region (V_H) and a light chain variable region (V_L), wherein the V_H and V_L comprise six complementary determining regions (CDRs) selected from:

a) GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2, ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3, ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3;

b) GFDLSSYY (**SEQ ID NO: 117**) as heavy chain CDR1, IWLGSJNI (**SEQ ID NO: 118**) as heavy chain CDR2 and ARGWLDDSFDP (**SEQ ID NO: 119**) as heavy chain CDR3, VSVHYNKW (**SEQ ID NO: 121**) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (**SEQ ID NO: 122**) as light chain CDR3;

c) GFSLITYS (**SEQ ID NO: 124**) as heavy chain CDR1, ISASGTA (**SEQ ID NO: 125**) as heavy chain CDR2, ARGSGPSGIESYKL (**SEQ ID NO: 126**) as heavy chain CDR3, QSIGNY (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGYYGIHIT (**SEQ ID NO: 129**) as light chain CDR3;

d) GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, ARKDWTSGDSFNP (**SEQ ID NO: 133**) as heavy chain CDR3, QSISTA (**SEQ ID NO: 135**) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (**SEQ ID NO: 136**) as light chain CDR3; and

e) GFSLSNDA (**SEQ ID NO: 138**) as heavy chain CDR1, ISSAGRP (**SEQ ID NO: 139**) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (**SEQ ID NO: 140**) as heavy chain CDR3, QSISSSY (**SEQ ID NO: 142**) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (**SEQ ID NO: 143**) as light chain CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

In an embodiment, the binding protein binds an epitope that comprises residues on both monomers of ALT1.

In an embodiment, the binding protein does not bind significantly bind human ALT2. In an embodiment, the binding protein does not bind significantly bind aspartate aminotransferase (AST).

In an embodiment, the binding protein does not bind detectably bind human ALT2. In an embodiment, the binding protein does not bind detectably bind aspartate aminotransferase (AST).

5 In an embodiment, the binding protein does not significantly bind denatured ALT1. In an embodiment, the binding protein does not significantly bind heat denatured ALT1.

In an embodiment, the binding protein does not detectably bind denatured ALT1. In an embodiment, the binding protein does not detectably bind heat denatured ALT1.

In an embodiment, the binding protein binds native ALT1 in solution.

In an embodiment, the binding protein binds conformationally intact ALT1.

10 In an embodiment, the binding protein binds a conformational epitope of ALT1.

In an embodiment, the paratope of the binding protein comprises residues on both protomers of ALT.

In an embodiment, the binding protein binds native ALT1 striped to a solid surface/ conjugated to a solid surface.

15 In an embodiment, the binding protein melting temperature is about 74 °C to about 76 °C. In an embodiment, the binding protein melting temperature is about 74.8 °C to about 75.8 °C.

In an embodiment, the binding protein has an on rate that is at least about 5-fold higher than a rabbit polyclonal ALT-1 antibody. In an embodiment, the binding protein has an on rate that is at least about 5.47-fold higher than a rabbit polyclonal ALT-1 antibody. In an embodiment, the binding protein has an on rate that is about 5.47-fold to about 16.08-fold higher than a rabbit polyclonal ALT-1 antibody.

20 In an embodiment, the binding protein has an affinity for ALT1 that is at least about 10-fold higher than a rabbit polyclonal anti-ALT antibody. In an embodiment, the binding protein has an affinity for ALT1 that is at least about 10.56-fold higher than a rabbit polyclonal anti-ALT antibody. In an embodiment, the binding protein has an affinity for ALT1 that is at least about 10.56-fold higher to about 45.16-fold than a rabbit polyclonal anti-ALT antibody. In an embodiment, the binding protein has an affinity for ALT1 that is at least about 28.38-fold higher to about 2160-fold than a rabbit polyclonal anti-ALT antibody. In an embodiment, the binding protein has an affinity for ALT1 that is at least about 10.56-fold higher to about 2160-fold than a rabbit polyclonal anti-ALT antibody.

25 In an embodiment, the binding protein can be isolated at least about 90% purity. In an embodiment, the binding protein can be isolated at least about 91% purity. In an embodiment, the binding protein can be isolated at least about 92% purity. In an embodiment, the binding protein can be isolated at least about 93% purity. In an embodiment, the binding protein can be

isolated at least about 94% purity. In an embodiment, the binding protein can be isolated at least about 95% purity.

In an embodiment, the binding protein has at least a 2-fold higher sensitivity for detecting human ALT1 compared to a rabbit polyclonal anti-ALT antibody.

5 In an embodiment, the binding protein has at least a 3-fold higher sensitivity for detecting human ALT1 compared to a rabbit polyclonal anti-ALT antibody.

In an embodiment, the binding protein has about a 2-fold to about a 3-fold higher sensitivity for detecting human ALT1 compared to a rabbit polyclonal anti-ALT antibody.

10 In an embodiment, the binding protein comprises a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 109** and a V_L comprising the amino acid sequence as set forth in **SEQ ID NO: 113** (RmAb1) or a sequence at least 70% identical thereto.

In an embodiment, the binding protein comprises a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 116** and a V_L comprising the amino acid sequence as set forth in **SEQ ID NO: 120** (RmAb2) or a sequence at least 70% identical thereto.

15 In an embodiment, the binding protein comprises a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 123** and a V_L comprising the amino acid sequence as set forth in **SEQ ID NO: 127** (RmAb3) or a sequence at least 70% identical thereto.

In an embodiment, the binding protein comprises a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 130** and a V_L comprising the amino acid sequence as set forth in **SEQ ID NO: 120** (RmAb4) or a sequence at least 70% identical thereto.

20 In an embodiment, the binding protein comprises a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 137** and a V_L comprising the amino acid sequence as set forth in **SEQ ID NO: 141** (RmAb5) or a sequence at least 70% identical thereto.

25 In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 129 Ile, 131 Leu, 154 Val, 177 His, 30 178 Thr, 183 Leu, 202 Val, 205 Asp, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 340 Met, 358 Leu, 408 Val, 430 Glu and 433 Leu.

In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 61 Thr, 129 Ile, 131 Leu, 154 Val, 183 Leu, 196 Leu, 202 Val, 212 Arg, 35 222 His, 225 Leu, 229 Arg, 239 Val, 260 Arg, 266 Arg, 284 Ser, 304 Gln, 333 Asp, 340 Met, 358 Leu, 408 Val, 430 Glu and 433 Leu.

In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 77 Arg, 109 Glu, 129 Ile, 131 Leu, 138 Arg, 141 Glu, 154 Val, 157 Ser, 175 Glu, 177 His, 178 Thr, 183 Leu, 196 Leu, 202 Val, 205 Asp, 212 Arg, 225 Leu, 239 Val, 260 Arg, 266 Arg, 276 Gln, 304 Gln, 327 Val, 333 Asp, 340 Met, 358 Leu, 376 Gln, 408 Val, 430 Glu, 431 Leu and 433 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 31 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 43 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 48 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 56 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 77 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 109 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 129 Ile. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 131 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 138 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 141 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 154 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 157 Ser. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 175 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 177 His. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 178 Thr. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 196 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 202 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 205 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at

position 212 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 225 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 276 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 327 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 333 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 340 Met. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 376 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 408 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 431 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 433 Leu.

In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 61 Thr, 129 Ile, 131 Leu, 154 Val, 183 Leu, 196 Leu, 202 Val, 212 Arg, 222 His, 225 Leu, 229 Arg, 239 Val, 260 Arg, 266 Arg, 284 Ser, 304 Gln, 333 Asp, 340 Met, 358 Leu, 376 Gln, 408 Val, 418 Val, 430 Glu or 433 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 31 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 43 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 48 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 56 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine

substitution at position 61 Thr. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 129 Ile. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 131 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 154 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 196 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 202 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 212 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 222 His. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 225 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 229 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 284 Ser. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 333 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 340 Met. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 376 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 408 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 418 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 433 Leu.

In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of the following positions: 4 Ser, 7 Asp, 8 Arg, 12 Val, 16 Leu, 19 Lys, 20 Val, 24 Asp, 31 Arg, 32 Arg, 43 Gln, 44 Arg, 48 Leu, 53 Arg, 54 Gln, 56

Val, 61 Thr, 77 Arg, 102 Asp, 129 Ile, 130 Gln, 131 Leu, 154 Val, 157 Ser, 183 Leu, 196 Leu, 202 Val, 205 Asp, 212 Arg, 222 His, 225 Leu, 229 Arg, 239 Val, 251 Thr, 260 Arg, 266 Arg, 276 Gln, 284 Ser, 285 Gln, 303 Gln, 304 Gln, 327 Val, 333 Asp, 340 Met, 358 Leu, 408 Val, 430 Glu, 433 Leu, 461 Asp, 485 Ser, 493 Leu or 494 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 4 Ser. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 7 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 8 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 12 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 16 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 20 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 31 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 32 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 43 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 44 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 48 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 53 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 54 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 56 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 61 Thr. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 77 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 102 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 129 Ile. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 130 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 131 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 154 Val. In an embodiment, the

binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 157 Ser. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 196 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 202 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 205 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 212 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 222 His. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 225 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 229 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 251 Thr. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 276 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 284 Ser. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 285 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 303 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 327 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 333 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 340 Met. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 408 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 433 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at

position 461 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 485 Ser. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 493 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 494 Glu.

In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of the following positions: 4 Ser, 12 Val, 13 Arg, 19 Lys, 24 Asp, 31 Arg, 43 Gln, 44 Arg, 48 Leu, 53 Arg, 56 Val, 61 Thr, 129 Ile, 131 Leu, 154 Val, 177 His, 178 Thr, 183 Leu, 202 Val, 205 Asp, 212 Arg, 239 Val, 260 Arg, 266 Arg, 284 Ser, 304 Gln, 340 Met, 358 Leu, 408 Val, 430 Glu, 433 Leu, 461 Asp or 485 Ser. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 4 Ser. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 12 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 13 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 31 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 43 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 44 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 48 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 53 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 56 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 61 Thr. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 129 Ile. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 131 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 154 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 177 His. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 178 Thr. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine

substitution at position 202 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 205 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 212 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 284 Ser. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 340 Met. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 408 Val. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 433 Leu. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 461 Asp. In an embodiment, the binding protein does not significantly bind to ALT1 comprising an alanine substitution at position 485 Ser.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at one or more of an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 129 Ile, 131 Leu, 154 Val, 177 His, 178 Thr, 183 Leu, 202 Val, 205 Asp, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 340 Met, 358 Leu, 408 Val, 430 Glu and 433 Leu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 61 Thr, 129 Ile, 131 Leu, 154 Val, 183 Leu, 196 Leu, 202 Val, 212 Arg, 222 His, 225 Leu, 229 Arg, 239 Val, 260 Arg, 266 Arg, 284 Ser, 304 Gln, 333 Asp, 340 Met, 358 Leu, 408 Val, 430 Glu and 433 Leu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 77 Arg, 109 Glu, 129 Ile, 131 Leu, 138 Arg, 141 Glu, 154 Val, 157 Ser, 175

Glu, 177 His, 178 Thr, 183 Leu, 196 Leu, 202 Val, 205 Asp, 212 Arg, 225 Leu, 239 Val, 260 Arg, 266 Arg, 276 Gln, 304 Gln, 327 Val, 333 Asp, 340 Met, 358 Leu, 376 Gln, 408 Val, 430 Glu, 431 Leu and 433 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 31 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 43 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 48 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 56 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 77 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 109 Glu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 129 Ile. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 131 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 138 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 141 Glu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 154 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 157 Ser. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 175 Glu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 177 His. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 178 Thr. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 196 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 202 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 205 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 212 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 225 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding protein does not detectably bind

to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 276 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 327 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 333 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 340 Met. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 376 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 408 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 431 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 433 Leu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 61 Thr, 129 Ile, 131 Leu, 154 Val, 183 Leu, 196 Leu, 202 Val, 212 Arg, 222 His, 225 Leu, 229 Arg, 239 Val, 260 Arg, 266 Arg, 284 Ser, 304 Gln, 333 Asp, 340 Met, 358 Leu, 376 Gln, 408 Val, 418 Val, 430 Glu or 433 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 31 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 43 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 48 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 56 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 61 Thr. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 129 Ile. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 131 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 154 Val.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 196 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 202 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 212 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 222 His. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 225 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 229 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 284 Ser. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 333 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 340 Met. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 376 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 408 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 418 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 433 Leu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 4 Ser, 7 Asp, 8 Arg, 12 Val, 16 Leu, 19 Lys, 20 Val, 24 Asp, 31 Arg, 32 Arg, 43 Gln, 44 Arg, 48 Leu, 53 Arg, 54 Gln, 56 Val, 61 Thr, 77 Arg, 102 Asp, 129 Ile, 130 Gln, 131 Leu, 154 Val, 157 Ser, 183 Leu, 196 Leu, 202 Val, 205 Asp, 212 Arg, 222 His, 225 Leu, 229 Arg, 239 Val, 251 Thr, 260 Arg, 266 Arg, 276 Gln, 284 Ser, 285 Gln, 303 Gln, 304 Gln, 327 Val, 333 Asp, 340 Met, 358 Leu, 408 Val, 430 Glu, 433 Leu, 461 Asp, 485 Ser, 493 Leu or 494 Glu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 4 Ser. In an

embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 7 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 8 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 12 Val.

5 In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 16 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 20 Val.

10 In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 31 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 32 Arg.

15 In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 43 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 44 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 48 Leu.

20 In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 53 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 54 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 56 Val.

25 In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 61 Thr. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 77 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 102 Asp.

30 In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 129 Ile. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 130 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 131 Leu.

35 In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 154 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 157 Ser. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 196 Leu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 202 Val. In an embodiment, the binding protein does not detectably bind

to ALT1 comprising an alanine substitution at position 205 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 212 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 222 His. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 225 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 229 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 251 Thr. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 276 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 284 Ser. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 285 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 303 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 327 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 333 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 340 Met. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 408 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 433 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 461 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 485 Ser. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 493 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 494 Glu.

In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 4 Ser, 12 Val, 13 Arg, 19 Lys, 24

Asp, 31 Arg, 43 Gln, 44 Arg, 48 Leu, 53 Arg, 56 Val, 61 Thr, 129 Ile, 131 Leu, 154 Val, 177 His, 178 Thr, 183 Leu, 202 Val, 205 Asp, 212 Arg, 239 Val, 260 Arg, 266 Arg, 284 Ser, 304 Gln, 340 Met, 358 Leu, 408 Val, 430 Glu, 433 Leu, 461 Asp or 485 Ser. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position

5 4 Ser. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 12 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 13 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 19 Lys. In an embodiment, the binding protein does not detectably bind

10 to ALT1 comprising an alanine substitution at position 24 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 31 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 43 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 44 Arg. In an embodiment, the binding

15 protein does not detectably bind to ALT1 comprising an alanine substitution at position 48 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 53 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 56 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 61 Thr.

20 In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 129 Ile. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 131 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 154 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 177 His. In an embodiment, the binding protein does not detectably bind

25 to ALT1 comprising an alanine substitution at position 178 Thr. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 183 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 202 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 205 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 212 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 239 Val. In an embodiment, the binding

30 protein does not detectably bind to ALT1 comprising an alanine substitution at position 260 Arg. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 266 Arg. In an embodiment, the binding protein does not

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detectably bind to ALT1 comprising an alanine substitution at position 284 Ser. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 304 Gln. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 340 Met. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 358 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 408 Val. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 430 Glu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 433 Leu. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 461 Asp. In an embodiment, the binding protein does not detectably bind to ALT1 comprising an alanine substitution at position 485 Ser.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1), the binding protein comprising the antigen binding site of an antibody comprising amino acid sequences selected from;

a) GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2, ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3, ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3;

b) GFDLSSYY (**SEQ ID NO: 117**) as heavy chain CDR1, IWLGSJNI (**SEQ ID NO: 118**) as heavy chain CDR2 and ARGWLDDSFDP (**SEQ ID NO: 119**) as heavy chain CDR3, VSVHYNKK (**SEQ ID NO: 121**) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (**SEQ ID NO: 122**) as light chain CDR3;

c) GFSLITYS (**SEQ ID NO: 124**) as heavy chain CDR1, ISASGTA (**SEQ ID NO: 125**) as heavy chain CDR2, ARGSGPSGIESYKL (**SEQ ID NO: 126**) as heavy chain CDR3, QSIGNNY (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGYGIIHT (**SEQ ID NO: 129**) as light chain CDR3;

d) GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, ARKDWTSGDSFNP (**SEQ ID NO: 133**) as heavy chain CDR3, QSISTA (**SEQ ID NO: 135**) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (**SEQ ID NO: 136**) as light chain CDR3; and

e) GFSLSNDA (**SEQ ID NO: 138**) as heavy chain CDR1, ISSAGRP (**SEQ ID NO: 139**) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (**SEQ ID NO: 140**) as heavy chain CDR3, QSISSSY (**SEQ ID NO: 142**) as light chain CDR1, RVS as light chain CDR2 and QGTGSGSSSYGNA (**SEQ ID NO: 143**) as light chain CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1), the binding protein comprising a heavy chain variable region (V_H) wherein the V_H comprises complementary determining regions (CDRs) selected from:

a) GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2, and ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3;

b) GFDLSSYY (**SEQ ID NO: 117**) as heavy chain CDR1, IWLGSJNI (**SEQ ID NO: 118**) as heavy chain CDR2 and ARGWLDDSFDP (**SEQ ID NO: 119**) as heavy chain CDR3;

c) GFSLITYS (**SEQ ID NO: 124**) as heavy chain CDR1, ISASGTA (**SEQ ID NO: 125**) as heavy chain CDR2, and ARGSGPSGIESYKL (**SEQ ID NO: 126**) as heavy chain CDR3;

d) GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, and ARKDWTSGDSFNP (**SEQ ID NO: 133**) as heavy chain CDR3; and

e) GFSLSNDA (**SEQ ID NO: 138**) as heavy chain CDR1, ISSAGRP (**SEQ ID NO: 139**) as heavy chain CDR2, and ARDKGYYSYHYAYDTRLDL (**SEQ ID NO: 140**) as heavy chain CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1), the binding protein comprising a light chain variable region (V_L), wherein V_L comprises complementary determining regions (CDRs) selected from:

a) amino acid sequences ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3;

b) amino acid sequences VSVHYNKW (**SEQ ID NO: 121**) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (**SEQ ID NO: 122**) as light chain CDR3;

c) amino acid sequences QSIGNNY (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGYGGIHIT (**SEQ ID NO: 129**) as light chain CDR3;

d) amino acid sequences QSISTA (**SEQ ID NO: 135**) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (**SEQ ID NO: 136**) as light chain CDR3; and

e) amino acid sequences QSISSSY (**SEQ ID NO: 142**) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (**SEQ ID NO: 143**) as light chain CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1 comprising the antigen binding site of an antibody.

5 In an aspect, the present invention provides a binding protein which binds a conformational epitope of alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1 comprising the antigen binding site of an antibody.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1) wherein the binding protein does not significantly and/or detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19
10 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1 comprising the antigen binding site of an antibody and wherein the binding protein does not
15 significantly and/or detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1
20 comprising the antigen binding site of an antibody comprising amino acid sequences selected from:

a) GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2, ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3, ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and
25 QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3;

b) GFDLSSYY (**SEQ ID NO: 117**) as heavy chain CDR1, IWLGSNGI (**SEQ ID NO: 118**) as heavy chain CDR2, ARGWLDDSFDP (**SEQ ID NO: 119**) as heavy chain CDR3, VSVHYNKW (**SEQ ID NO: 121**) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (**SEQ ID NO: 122**) as light chain CDR3;

30 c) GFSLITYS (**SEQ ID NO: 124**) as heavy chain CDR1, ISASGTA (**SEQ ID NO: 125**) as heavy chain CDR2, ARGSGPSGIESYKL (**SEQ ID NO: 126**) as heavy chain CDR3, QSIGNY (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGYGIIHIT (**SEQ ID NO: 129**) as light chain CDR3;

d) GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, ARKDWTSKGSDFNP (**SEQ ID NO: 133**) as heavy chain CDR3,
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QSISTA (SEQ ID NO: 135) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (SEQ ID NO: 136) as light chain CDR3;

e) GFSLSNDA (SEQ ID NO: 138) as heavy chain CDR1, ISSAGRP (SEQ ID NO: 139) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (SEQ ID NO: 140) as heavy chain CDR3, QSISSSY (SEQ ID NO: 142) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (SEQ ID NO: 143) as light chain CDR3; and

f) GPAVSNVA (SEQ ID NO: 2) as CDR1, ITWSGWT (SEQ ID NO: 3) as CDR2 and NLIGLRVGPENKY (SEQ ID NO: 4) as CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No,

wherein when the amino acid sequences are a) the binding protein is a nanobody, and wherein when the amino acid sequences are b), c), d), e) or f) the binding protein is an antibody.

In an aspect, the present invention provides a binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1 comprising amino acid sequences selected from the following:

a) GFSLNNYN (SEQ ID NO: 110) as heavy chain CDR1, ITAGGNI (SEQ ID NO: 111) as heavy chain CDR2, ARDLAGNVYYDFDL (SEQ ID NO: 112) as heavy chain CDR3, ENIYSG (SEQ ID NO: 114) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (SEQ ID NO: 115) as light chain CDR3;

b) GFDLSSYY (SEQ ID NO: 117) as heavy chain CDR1, IWLGSGNI (SEQ ID NO: 118) as heavy chain CDR2 and ARGWLDDSFDP (SEQ ID NO: 119) as heavy chain CDR3, VSVHYNKW (SEQ ID NO: 121) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (SEQ ID NO: 122) as light chain CDR3;

c) GFSLITYS (SEQ ID NO: 124) as heavy chain CDR1, ISASGTA (SEQ ID NO: 125) as heavy chain CDR2, ARGSGPSGIESYKL (SEQ ID NO: 126) as heavy chain CDR3, QSIGNY (SEQ ID NO: 128) as light chain CDR1, RAS as light chain CDR2 and QGYYGIIHIT (SEQ ID NO: 129) as light chain CDR3;

d) GIDLSVNA (SEQ ID NO: 131) as heavy chain CDR1, IHTYDVT (SEQ ID NO: 132) as heavy chain CDR2, ARKDWTSGDSFNP (SEQ ID NO: 133) as heavy chain CDR3, QSISTA (SEQ ID NO: 135) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (SEQ ID NO: 136) as light chain CDR3;

e) GFSLSNDA (SEQ ID NO: 138) as heavy chain CDR1, ISSAGRP (SEQ ID NO: 139) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (SEQ ID NO: 140) as heavy chain CDR3, QSISSSY (SEQ ID NO: 142) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (SEQ ID NO: 143) as light chain CDR3; and

f) GPAVSNVA (SEQ ID NO: 2) as CDR1, ITWSGWT (SEQ ID NO: 3) as CDR2 and NLIGLRVGPENKY (SEQ ID NO: 4) as CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

5

Antibodies and fragments thereof

In an embodiment, the binding protein is an antibody or fragment thereof. The term "antibody" as used herein includes monoclonal antibodies, bispecific antibodies, fusion diabodies, triabodies, heteroconjugate antibodies, chimeric antibodies including intact molecules as well as fragments thereof (antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab').sub.2), single-chain antibody molecules (e.g. scFv) nanobodies and other antibody-like molecules. Antibody fragments include antigen binding fragments either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA methodologies. In an embodiment, the antibody is a monoclonal antibody.

10 In embodiment, the antibody is a rabbit polyclonal antibody. In an embodiment, the antibody is not a polyclonal antibody. In an embodiment, the antibody is not a rabbit polyclonal antibody.

Antibodies include modifications in a variety of forms including, for example, but not limited to, domain antibodies including either the VH or VL domain, a dimer of the heavy chain variable region (VHH, as described for a camelid), a dimer of the light chain variable region (VLL), Fv fragments containing only the light (VL) and heavy chain (VH) variable regions which may be joined directly or through a linker, or Fd fragments containing the heavy chain variable region and the CH1 domain.

20

Antibodies can consist of VHH regions, in isolation or multiple VHH domains joined directly with linkers, and then connected to the Fc region comprising CH2 and CH3 domains of an immunoglobulin.

25

A scFv consisting of the variable regions of the heavy and light chains linked together to form a single-chain antibody (Bird et al., 1988; Huston et al., 1988) and oligomers of scFvs such as diabodies and triabodies are also encompassed by the term "antibody". Also encompassed are fragments of antibodies such as Fab, (Fab')₂ and FabFc₂ fragments which contain the variable regions and parts of the constant regions. Complementarity determining region (CDR)-grafted antibody fragments and oligomers of antibody fragments are also encompassed. The heavy and light chain components of an Fv may be derived from the same antibody or different antibodies thereby producing a chimeric Fv region. The antibody may be of animal (for example mouse, rabbit or rat) or may be chimeric (Morrison et al., 1984). The antibody may be produced by any method known in the art.

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The antibodies may be Fv regions comprising a variable light (V_L) and a variable heavy (V_H) chain in which the light and heavy chains may be joined directly or through a linker. In an embodiment, the V_L is a kappa. In an embodiment, the V_L is a kappa 1. In an embodiment the light chain J gene germline usage comprises J1-2 (IGKJ1_2).

5 As used herein, “variable region” refers to the portions of the light and/or heavy chains of a binding protein as defined herein that specifically binds to an antigen and, for example, includes amino acid sequences of CDRs; i.e., CDR1, CDR2, and CDR3, and framework regions (FRs). For example, the variable region comprises three or four FRs (e.g., FR1, FR2, FR3 and optionally FR4) together with three CDRs. As used herein, the term “complementarity
10 determining regions” (i.e., CDR1, CDR2, and CDR3) refers to the amino acid residues of a binding protein variable region (e.g., a VHH chain) the presence of which are major contributors to specific antigen binding. Each VHH chain of e.g. a camelid-derived binding protein typically has three CDR regions identified as CDR1, CDR2 and CDR3. “Framework regions” are those variable domain residues other than the CDR residues.

15 There are multiple conventions to define, annotate and describe the CDRs (and by extension FRs) of a binding protein, such as a VHH chain or single domain binding protein. To this end, the length and sequence of specific CDRs of a binding protein can vary depending upon the specific nomenclature, algorithm or the like used to define them. Exemplary conventions to define CDRs include the Kabat definition (which is based on sequence
20 variability and is the most commonly used; See, e.g., Sequences of Proteins of Immunological Interest, Kabat, et al.; National Institutes of Health, Bethesda, Md.; 5th ed.; NIH Publ. No. 91-3242 (1991)), the Chothia definition (which is based on the location of the structural loop regions; See, e.g., Chothia, et al., (1987) J Mol. Biol. 196:901-917), the AbM definition (which is a compromise between the Kabat and Chothia definitions and is based on Oxford Molecular's
25 AbM antibody modelling software), the IMGT definition (see, e.g., <https://www.imgt.org/IMGTindex/CDR.php>) and the method described by Kontermann and Diibel (Eds., Antibody Engineering, vol 2, Springer Verlag Heidelberg Berlin, Martin, Chapter 3, pp. 33-51, 2010).

30 In some embodiments, the amino acid sequences of the CDR1, CDR2 and CDR3 of the binding proteins of the present disclosure are determined or defined by the Lefranc unique numbering definition (Lefranc, 1997, Lefranc, 1999, Lefranc, 2003).

Monoclonal antibodies are preferred. The term “monoclonal antibody” or “MAb” refers to a homogeneous antibody population capable of binding to the same antigen(s) and, preferably, to the same epitope within the antigen. This term is not intended to be limited as
35 regards to the source of the antibody or the manner in which it is made. For the production of

Mabs any one of a number of known techniques may be used, such as, for example, the procedure exemplified in US4,196,265 or Harlow and Lane (1988) or Zola (1987).

Nanobodies

5 In an embodiment, the binding protein is a nanobody. As used herein, the term “nanobody” or “nanobodies” or “Nb” or “single domain binding protein” refers to the single antigen-binding domain (VHH) binding protein derived from camelid-based heavy chain antibodies (HCAs) which are naturally devoid of light chains (Muyltermans, 2013).

10 It is noted that the terms “nanobody” and “nanobodies” are registered trademarks of Ablynx N.V. and thus may also be referred to as Nanobody® and/or Nanobodies®. Nanobodies generally each have three CDRs, denoted CDR1, CDR2 and CDR3, respectively. Additionally, nanobodies typically include three or four framework regions (FRs; FR1, FR2, FR3 and optionally FR4). The nanobodies as described herein can be derived from camel, dromedary, llama or alpaca HCAs. In particular examples, the nanobodies according to the present
15 disclosure are derived from alpaca HCAs.

In an embodiment, the binding protein as described herein, comprises a framework region derived from an alpaca. In an embodiment, the binding protein as described herein, comprises a framework region derived from a camel. In an embodiment, the binding protein as described herein, comprises a framework region derived from a llama. In an embodiment, the
20 binding protein as described herein, comprises a framework region derived from a shark. In an embodiment, the binding protein as described herein, comprises a modified version of the framework region of the binding protein derived from an alpaca. In an embodiment, the binding protein as described herein, comprises a modified version of the framework region of the binding protein derived from a camel. In an embodiment, the binding protein as described
25 herein, comprises a modified version of the framework region of the binding protein derived from a llama. In an embodiment, the binding protein as described herein, comprises a modified version of the framework region of the binding protein derived from a shark. In an embodiment, the binding protein as described herein, comprises a humanised version of the framework region of the binding protein derived from an alpaca. In an embodiment, the binding protein as
30 described herein, comprises a humanised version of the framework region of the binding protein derived from a camel. In an embodiment, the binding protein as described herein, comprises a humanised version of the framework region of the binding protein derived from a llama. In an embodiment, the binding protein as described herein, comprises a humanised version of the framework region of the binding protein derived from a shark.

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Humanised binding proteins

The term "humanised binding protein" or "humanised" as used herein, shall be understood to refer to a binding protein comprising a human-like variable region, which includes CDRs from a binding protein from a non-human species (e.g., camelids) grafted onto or inserted into FRs from a human binding protein (this type of binding protein is also referred to a "CDR-grafted binding protein"). Humanised binding proteins also include binding proteins in which one or more residues of the human protein are modified by one or more amino acid substitutions and/or one or more FR residues of the human binding protein are replaced by corresponding non-human residues. Humanised binding proteins may also comprise residues which are found in neither the human binding protein or in the non-human binding protein. Additional regions of the binding protein (e.g., Fc region) are generally human. Humanisation can be performed using a method known in the art, e.g., US5225539, US6054297, US7566771 or US5585089. The term "humanised binding protein" also encompasses a super-humanised binding protein, e.g., as described in US7732578. In an embodiment, the binding protein is a humanised nanobody e.g. as described in Vincke et al. 2009.

15

Deimmunized binding proteins

As used herein the term "deimmunized binding protein", shall be understood to refer to a binding protein having one or more epitopes, e.g., B cell epitopes or T cell epitopes removed (i.e., mutated) to thereby reduce the likelihood that a subject will raise an immune response against the antibody or protein (e.g. as described in WO2000/34317 and WO2004/108158). A person skilled in the art will appreciate that nanobodies as described herein are not "deimmunized" as they naturally lack B cell epitopes and T cell epitopes.

20

Germlined binding proteins

As discussed above a "germlined binding protein" is derived from or corresponds to sequences from a human or camelid e.g. germ line or somatic cells which can include amino acids residues not encoded by the host species e.g. in some instances a mutation may be introduced for example by affinity maturation or as a result of a use of a synthetic library that is not encoded by the species from which the sequence was originally derived. In some embodiments, a "germlined binding protein" is a camelid binding protein in which one or more amino acids in a chain which are not encoded by the camelid species are replaced or substituted with an amino acid from the germline species.

30

Modified binding proteins

Binding protein sequences can be modified through the addition of sequences and moieties for a number of reasons, including for example, to aid purification and/or processing during manufacture, direct detection, indirect detection, and modifying stability among others.

In an embodiment, the binding protein is modified to comprise one or more of, a tag,
5 linker, radionucleotide, toxin, another protein (e.g. Fc region).

In an embodiment, the binding protein as described herein comprises a tag. The skilled person will appreciate that binding proteins are routinely fused to certain tags for detection and/or purification purposes, for example, as described in Muyldermans (2013). In an embodiment, the tag is selected from one or more of: poly-histidine, FLAG, antibody epitope
10 tags, c-myc, haemagglutinin, headlock, C-tag, ALFA tag, Avi, GST, maltose-binding protein (MBP). In an embodiment, the binding protein is fused to a poly-histidine tag. In an embodiment, the binding protein is fused to a FLAG tag. In an embodiment, the binding protein is fused to an antibody epitope tag. In an embodiment, the binding protein is fused to a c-myc tag. In an embodiment, the binding protein is fused to a haemagglutinin tag. In an embodiment,
15 the binding protein is fused to a headlock tag. In an embodiment, the binding protein is fused to a C-tag. In an embodiment, the binding protein is fused to an ALFA tag. In an embodiment, the binding protein is fused to an Avi tag. In an embodiment, the binding protein is fused to a GST tag. In an embodiment, the binding protein is fused to a MBP tag. In an embodiment, the tag is cleavable from the binding protein.

In an embodiment, the binding protein comprises a detectable label, for example, as
20 described in Muyldermans (2013). In an embodiment, the detectable label is directly detectable. In an embodiment, the detectable label is indirectly detected. Examples of detectable labels include metal labels, magnetic labels, beads, fluorescent labels, chemical labels, radionucleotides, coloured particles, quantum dots, fluorescent latex particles, carbon
25 nanoparticles, chemiluminescence based label, liposome based probes, raman-active tags and protein labels (Song et al., 2008; Nuntawong et al., 2022; Muyldermans, 2013). In an embodiment, the detectable label is detectable via a smartphone (Zangheri et al., 2015).

In an embodiment, the detectable lab is an Estapor® Coloured Microsphere.

In an embodiment, the detectable label is selected from one or more of: red intense
30 microspheres (Merck e.g. catalogue numbers FR180380637 and FR180380638), cellulose nanobeads, latex beads, alkaline phosphatase, horseradish peroxidase, colloidal gold, gold nanoshells, europium, fluorescent label and a luminescent label. In an embodiment, the detectable label is red intense microspheres. In an embodiment, the detectable label is cellulose nanobeads. In an embodiment, the detectable label is latex beads. In an embodiment, the
35 detectable label is alkaline phosphatase. In an embodiment, the detectable label is horseradish peroxidase. In an embodiment, the detectable label is colloidal gold. In an embodiment, the

detectable label is gold nanoshells. In an embodiment, the detectable label is europium. In an embodiment, the detectable label is red fluorescent protein. In an embodiment, the detectable label is green fluorescent protein. In an embodiment, the detectable label is estapor-blue. In an embodiment, the detectable label is estapor-blue conjugate.

5 In an embodiment, the fluorescent label is selected from, but not limited to, Green fluorescent protein (GFP), enhanced green fluorescent protein (EGFP), fluorescein (FITC), alexa fluor, 5,6-carboxymethyl fluorescein, texas red, nitrobenz-2-oxa-1,3- diazol-4-yl (NBD), coumarin, dansyl chloride, rhodamine, 4'-6-diamidino-2- phenylindole (DAPI), and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7, fluorescein (5-carboxyfluorescein-N-
10 hydroxysuccinimide ester), and rhodamine (5,6- tetramethyl rhodamine), Emerald, Superfolder GFP, Azami Green, mWasabi, TagGFP, TurboGFP, mNeonGreen, mUKG, AcGFP, ZsGreen, Cloverm Sapphire, T-Sapphire, Enhanced blue fluorescent protein (EBFP), EBFP2, Azurite, TagBFP, mTagBFP, mKalama1, Cyan fluorescent protein (CFP), mCFP, Enhanced cyan fluorescent protein (ECFP), mECFP, Cerulean, SCFP3A, mTurquoise, mTurquoise2, CyPet,
15 AmCyan1, Midori-Ishi Cyan, TagCFP, mTFP1 (Teal), Yellow fluorescent protein (YFP), Enhanced yellow fluorescent protein (EYFP), Super yellow fluorescent protein (SYFP), Topaz, Venus, Citrine, mCitrine, YPet, TagYFP, TurboYFP, PhiYFP, ZsYellow1, mBanana, Kusabira Orange, Kusabira Orange2, mOrange, mOrange2, dTomato, dTomato-Tandem, Red fluorescent protein (RFP), TurboRFP, TurboFP602, TurboFP635, Tag ref fluorescent protein
20 (RFP), TagRFP-T, DsRed, DsRed2, DsRed-Express (T1), DsRed-Monomer, mTangerine, mKeima-Red, mRuby, mRuby2, mApple, mStrawberry, AsRed2, mRFP1, JRed, mCherry, mKate2, mKate (TagFP635), HcRed1, mRaspberry, dKeima-Tandem, HcRed-Tandem, mPlum, mNeptune, NirFP, Sirius, TagRFP657, AQ143, Kaede, KikGR1, PX-CFP2, mEos2, IrisFP, mEOS3.2, PSmOrange, PAGFP, Dronpa, Allophycocyanin, GFPuv, R-phycoerythrin
25 (RPE), Peridinin Chlorophyll (PerCP), P3, Katusha, B-phycoerythrin (BPE), mKO, and J-Red. In an embodiment, the fluorescent protein is RFP. In an embodiment, the fluorescent protein is GFP. The absorption and emission maxima, respectively, for these fluors are: FITC (490 nm; 520 nm), Cy3 (554 nm; 568 nm), Cy3.5 (581 nm; 588 nm), Cy5 (652 nm; 672 nm), Cy5.5 (682 nm; 703 nm) and Cy7 (755 nm; 778 nm).

30 In an embodiment, the luminescent label is selected from: aequorin, firefly luciferase, renilla luciferase, gaussia luciferase, bacterial luciferase and nanoluc.

In an embodiment, the magnetic label is a magnetic or paramagnetic compound, such as, iron, steel, nickel, cobalt, rare earth materials, neodymium-iron-boron, ferrous-chromium-cobalt, nickel-ferrous, cobalt- platinum, or strontium ferrite.

35 In an embodiment, the binding protein comprises one or more linkers. Examples of such linker sequences are described in Beirnaert et al. 2017, Sabourin et al. 2007, Yang and Gruebele,

2006, Dipti et al. 2006, Anandarao et al. 2006, Wyatt et al. 1995, Bellamy-McIntyre et al. 2007, Arai et al., 2004 and Bahrami et al., 2007. The linker may connect the binding protein to a further binding protein. The linker may connect the binding protein to a further binding protein which detects the same antigen. The linker may connect the binding protein to a different protein. In an embodiment, the linker comprises a sequence selected from one or more of **SEQ ID NO: 65** to **SEQ ID NO: 77**.

In an embodiment, the binding protein is modified to comprise a radionuclide. A variety of radionuclides are available. Examples include, but are not limited to, low energy radioactive nuclei (e.g., suitable for diagnostic purposes), such as ^{13}C , ^{15}N , ^2H , ^{125}I , ^{123}I , ^{99}Tc , ^{43}K , ^{52}Fe , ^{67}Ga , ^{68}Ga , ^{111}In and the like. For example, the radionuclide is a gamma, photon, or positron-emitting radionuclide with a half-life suitable to permit activity or detection after the elapsed time between administration and localization to the imaging site. The present disclosure also encompasses high energy radioactive nuclei (e.g., for therapeutic purposes), such as ^{125}I , ^{131}I , ^{123}I , ^{111}In , ^{105}Rh , ^{153}Sm , ^{67}Cu , ^{67}Ga , ^{166}Ho , ^{177}Lu , ^{186}Re and ^{188}Re . These isotopes typically produce high energy α - or β -particles which have a short path length. Such radionuclides kill cells to which they are in close proximity, for example neoplastic cells to which the conjugate has attached or has entered. They have little or no effect on non-localized cells and are essentially non-immunogenic. Alternatively, high-energy isotopes may be generated by thermal irradiation of an otherwise stable isotope, for example as in boron neutron-capture therapy (Guan *et al.*, 1998).

In an embodiment, the binding protein is modified to comprise or react with a chemiluminescent label (e.g. as described in Cabello et al., 2023). In an embodiment, the label is horseradish peroxidase agent. In an embodiment, the chemiluminescent label is a label detectable with a smart phone (Zangheri et al (2015).

In an embodiment, the binding protein of the present disclosure is modified to comprise a quantum dot e.g. as described in Mousavi et al., 2023. In an embodiment, the quantum dot is selected from: highly bright multi-quantum dots embedded in silica-encapsulated nanoparticles (M-QD-SNs), Cde/ZNS, CdSe/ZnS QDs, Qdot, CdSe/ZnS, Cu:Zu-In-S/ZnS, CdTe QDs and CdTe. In an embodiment, the quantum dot is a M-QD-SNs e.g. as described in Kim et al., 2021.

In an embodiment, the binding protein of the present invention is fused or conjugated to a protein. Such proteins are used for the purpose of one or more of: enhancing expression, facilitating purification, increasing the size, solubility, enhancing the binding to or retention on a solid support and performance characteristics of the binding protein (e.g. antigen binding).

In an embodiment, the protein is selected from: Fc region or a fragment thereof, ferritin, maltose-binding protein (MBP), leucine zipper, glutathione S-transferase (GST), keyhole

limpet hemocyanin (KLH), albumin, cyclophilin, FKBP, calcineurin, CyPFAS, GyrB, neutravidin, avidin and streptavidin. In an embodiment, the protein is an Fc region. In an embodiment, the protein is a fragment of an Fc region. In an embodiment, the protein is a maltose-binding protein (MBP). In an embodiment, the protein is a leucine zipper. In an embodiment, the protein is a glutathione S-transferase (GST). In an embodiment, the protein is a keyhole limpet hemocyanin (KLH). In an embodiment, the protein is albumin. In an embodiment, the protein is cyclophilin. In an embodiment, the protein is FKBP. In an embodiment, the protein is calcineurin. In an embodiment, the protein is CyPFAS. In an embodiment, the protein is GyrB. In an embodiment, the protein is neutravidin. In an embodiment, the protein is avidin. In an embodiment, the protein is streptavidin.

In an embodiment, the Fc region or a fragment thereof increases the association rate of the binding protein with ALT1.

In an embodiment, the Fc region or a fragment thereof decreases the association rate of the binding protein with ALT1.

In an embodiment, the Fc region or a fragment thereof increases the avidity of ALT1 and the binding protein.

In an embodiment, the binding protein is a nanobody fused to an Fc region or a fragment thereof.

In an embodiment, the protein forms a dimer when expressed (e.g. a dimer is formed between two Fc regions (Fc-region-Fc-region). In an embodiment, the protein forms a trimer when expressed. In an embodiment, the protein forms a tetramer when expressed (e.g. streptavidin).

Examples of dimers are described in Dang et al (2022). In an embodiment, the protein forms a homodimer when expressed. In an embodiment, the protein forms a heterodimer when expressed. In an embodiment, the protein is selected from: a Fc region, ferritin, maltose-binding protein (MBP), leucine zipper, glutathione S-transferase (GST), keyhole limpet hemocyanin (KLH), albumin, cyclophilin, FKBP, calcineurin, CyPFAS, GyrB, neutravidin, avidin or other such proteins. In an embodiment, the dimer is selected from: Fc region-Fc region, ferritin-ferritin, MBP-MBP, leucine zipper-leucine zipper, GST-GST, KLH-KLH, albumin-albumin, cyclophilin-cyclophilin, cyclophilin-calcineurin, FKBP-calcineurin, FKBP-CyPFas, FKBP-FKBP, and GyrB-GyrB. In an embodiment, the dimer is Fc region-Fc region.

In an embodiment, the binding protein is fused to a protein which can form a dimer wherein upon expression the protein forms a dimer thereby forming a bivalent, quadrivalent or multivalent binding protein. An example of a bivalent binding protein fused to a protein that forms a dimer is provided in **Figure 41C**. When the dimer is formed a quadrivalent binding protein is produced.

The binding proteins of the present disclosure can be modified to comprise additional non-proteinaceous moieties that are known in the art and readily available. For example, the moieties suitable for derivatization of the protein are physiologically acceptable polymer, e.g., a water soluble polymer. Such polymers are useful for increasing stability. Non-limiting
5 examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), polyvinyl alcohol (PVA), or propylene glycol (PPG).

Bivalent, trivalent, quadrivalent or multivalent binding proteins

In an aspect, the present invention provides a bivalent, trivalent, quadrivalent or
10 multivalent binding protein comprising at least one binding protein as described herein or a combination thereof. Such formats can be desirable to enhance or otherwise modify the effectiveness of the binding proteins of the present disclosure. The advantages of such formats are described, for example in Conrath et al., 2001 and Sparkles et al., 2018.

In some embodiments, the binding protein is a bivalent binding protein. As used herein,
15 a bivalent binding protein comprises two antigen-binding domains. In an embodiment, the bivalent binding protein comprises at least one binding protein as described herein. In an embodiment, the bivalent binding protein comprises two binding proteins as described herein. In an embodiment, the bivalent binding protein is homobivalent. In an embodiment, the bivalent binding protein is heterobivalent.

20 In some embodiments, the binding protein is a trivalent binding protein. As used herein, a trivalent binding protein comprises three antigen-binding domains. In an embodiment, the trivalent binding protein comprises at least one binding protein as described herein. In an embodiment, the trivalent binding protein comprises two binding proteins as described herein. In an embodiment, the trivalent binding protein comprises three binding proteins as described
25 herein. In an embodiment, the trivalent binding protein is homotrivalent. In an embodiment, the trivalent binding protein is heterotrivalent (comprising two different binding proteins or three different binding proteins).

In some embodiments, the binding protein is a quadrivalent binding protein. As used
30 herein, a quadrivalent binding protein comprises four antigen-binding domains. In an embodiment, the quadrivalent binding protein comprises at least one binding protein as described herein. In an embodiment, the quadrivalent binding protein comprises two binding proteins as described herein. In an embodiment, the quadrivalent binding protein comprises three binding proteins as described herein. In an embodiment, the quadrivalent binding protein comprises four binding proteins as described herein. In an embodiment, the quadrivalent
35 binding protein is homoquadrivalent. In an embodiment, the quadrivalent binding protein is heteroquadrivalent (comprising two different binding proteins or three different binding

proteins or four different binding proteins). In an embodiment, the quadrivalent binding protein comprises an Fc region (e.g. as shown in **Figure 41C**). As used herein, an “Fc region” refers to monomer of the tail region of an antibody that interacts with cell surface receptors called Fc receptors. In an embodiment, the Fc region comprises the sequence set forth in **SEQ ID NO: 78** or a sequence at least 70% identical thereto or a fragment thereof. In an embodiment, the Fc region comprises the sequence set forth in **SEQ ID NO: 78**. In an embodiment, the Fc region comprises the nucleotide sequence set forth in **SEQ ID NO: 106** or a sequence at least 70% identical thereto or a fragment thereof. In an embodiment, the Fc region comprises the nucleotide sequence set forth in **SEQ ID NO: 106**. In an embodiment, the Fc region is a human Fc region. In an embodiment, the Fc region is a human IgG Fc region.

In some embodiments, the binding protein is a multivalent binding protein. As used herein a multivalent binding protein comprises five or more antigen-binding domains. In an embodiment, the multivalent binding protein comprises at least one binding protein as described herein. In an embodiment, the multivalent binding protein comprises two binding proteins as described herein. In an embodiment, the multivalent binding protein comprises three binding proteins as described herein. In an embodiment, the multivalent binding protein comprises four binding proteins as described herein. In an embodiment, the multivalent binding protein is a homomultivalent binding protein. In an embodiment, the multivalent binding protein is heteromultivalent binding protein (comprising two different binding proteins or three different binding proteins or four different binding proteins).

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, has the ability to bind to two epitopes on the same molecule (biparatopic) (Oliveira et al., 2013).

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, has the ability to bind to multiple epitopes on the same molecule (multiparatopic) (Palomo et al., 2016).

In an embodiment, a bivalent form of a binding protein has a higher avidity than a monomeric form of a binding protein.

In an embodiment, a trivalent form of a binding protein has a higher avidity than a monomeric form of a binding protein.

In an embodiment, a quadrivalent form of a binding protein has a higher avidity than a monomeric form of a binding protein.

In an embodiment, a multivalent form of a binding protein has a higher avidity than a monomeric form of a binding protein.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises a binding protein comprising the amino acid sequence as set forth in **SEQ ID NO:**

1 (C8), or a sequence at least 71% identical thereto, or a humanised, deimmunized or germlined version thereof.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises a binding protein comprising the amino acid sequence as set forth in **SEQ ID NO: 5** (G6), or a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version thereof.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises (i) a binding protein comprising the amino acid sequence as set forth in **SEQ ID NO: 1** (C8), or a sequence at least 71% identical thereto, or a humanised, deimmunized or germlined version thereof; and (ii) a binding protein comprising the amino acid sequence as set forth in **SEQ ID NO: 5** (G6), or a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version thereof.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises a binding protein comprising an amino acid sequence selected from **SEQ ID NO: 13** to **SEQ ID NO: 56**, or a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version thereof.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises a sequence selected from: C3C (**SEQ ID NO: 57**), C4C (**SEQ ID NO: 58**), G3G (**SEQ ID NO: 59**), G4G (**SEQ ID NO: 60**), C3G (**SEQ ID NO: 61**), C4G (**SEQ ID NO: 62**), G3C (**SEQ ID NO: 63**) and G4C (**SEQ ID NO: 634**) or a sequence at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the sequence C3C (**SEQ ID NO: 57**) or a sequence at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the sequence C4C (**SEQ ID NO: 58**) or a sequence at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the sequence G3G (**SEQ ID NO: 59**) or a sequence at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the sequence G4G (**SEQ ID NO: 60**) or a sequence at least 71%, 76%, 77%, 78%,

79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the sequence C3G (**SEQ ID NO: 61**) or a sequence at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the sequence C4G (**SEQ ID NO: 62**) or a sequence at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the sequence G3C (**SEQ ID NO: 63**) or a sequence at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the sequence G4C (**SEQ ID NO: 64**) or a sequence at least 71%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein is C3C (**SEQ ID NO: 57**). In an embodiment, the bivalent, trivalent or multivalent binding protein is C4C (**SEQ ID NO: 58**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein is G3G (**SEQ ID NO: 59**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein is G4G (**SEQ ID NO: 60**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein is C3G (**SEQ ID NO: 61**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein is C4G (**SEQ ID NO: 62**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein is G3C (**SEQ ID NO: 63**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein is G4C (**SEQ ID NO: 64**).

To generate bivalent, trivalent, quadrivalent or multivalent binding proteins as described herein, binding proteins as described herein may be connected by any method known to a person skilled in the art and for example as described in Conrath et al., 2001 and Sparkles et al., 2018). In an embodiment, the binding proteins are connected by a linker, such as a polypeptide linker, or more than one linker to form a bivalent, trivalent, quadrivalent or multivalent binding protein.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein provided herein comprise at least two binding proteins as described herein linked by a linker sequence of amino acids of varying lengths. In an embodiment, the two binding proteins are

different binding proteins. In an embodiment, the at least two binding proteins are the same binding protein. Examples of such linkers are described above.

In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises a linker selected from one or more of **SEQ ID NO: 65** to **SEQ ID NO: 77**.

- 5 In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GGAGG (**SEQ ID NO: 65**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GGGGG (**SEQ ID NO: 66**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GGGGGGGG (**SEQ ID NO: 67**). In an
- 10 In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GGGGGG (**SEQ ID NO: 68**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GGGGGGG (**SEQ ID NO: 69**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GGGGGGGGG (**SEQ ID NO: 70**). In an embodiment,
- 15 the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence of GSGSG (**SEQ ID NO: 71**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GGGG (**SEQ ID NO: 72**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GAG (**SEQ ID NO: 73**). In an embodiment, the bivalent, trivalent, quadrivalent
- 20 or multivalent binding protein comprises the linker sequence GGAGGS (**SEQ ID NO: 74**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GGGGSGGGSGGGGS (**SEQ ID NO: 75**). In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the linker sequence GGGGSGGGSGGGSGGGGS (**SEQ ID NO: 76**). In an embodiment, the bivalent,
- 25 trivalent, quadrivalent or multivalent binding protein comprises the linker sequence SGGSG (**SEQ ID NO: 77**).

- In an embodiment, the quadrivalent binding protein comprises an amino acid sequence selected from: C4C-Fc (**SEQ ID NO: 79**), G4G-Fc (**SEQ ID NO: 80**), C4G-Fc (**SEQ ID NO: 81**) G4C-Fc (**SEQ ID NO: 82**), C3G-Fc (**SEQ ID NO: 159**), G3C-Fc (**SEQ ID NO: 161**),
- 30 C3C-Fc (**SEQ ID NO: 155**) and G3G-Fc (**SEQ ID NO: 157**) or a sequence at least 76% identical thereto. In an embodiment, the quadrivalent binding protein comprises the amino acid sequence C4C-Fc (**SEQ ID NO: 79**) or a sequence at least 76% identical thereto. In an embodiment, the quadrivalent binding protein comprises the amino acid sequence G4G-Fc (**SEQ ID NO: 80**) or a sequence at least 76% identical thereto. In an embodiment, the
- 35 quadrivalent binding protein comprises the amino acid sequence C4G-Fc (**SEQ ID NO: 81**) or a sequence at least 76% identical thereto. In an embodiment, the quadrivalent binding protein

comprises the amino acid sequence G4C-Fc (**SEQ ID NO: 82**) or a sequence at least 76% identical thereto. In an embodiment, the quadrivalent binding protein comprises the amino acid sequence C3G-Fc (**SEQ ID NO: 159**) or a sequence at least 76% identical thereto. In an embodiment, the quadrivalent binding protein comprises the amino acid sequence G3C-Fc (**SEQ ID NO: 161**) or a sequence at least 76% identical thereto. In an embodiment, the quadrivalent binding protein comprises the amino acid sequence C3C-Fc (**SEQ ID NO: 155**) or a sequence at least 76% identical thereto. In an embodiment, the quadrivalent binding protein comprises the amino acid sequence G3G-Fc (**SEQ ID NO: 157**) or a sequence at least 76% identical thereto.

10 In an embodiment, In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises CDR sequences selected from:

a) GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2, ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3, ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and
15 QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3;

b) GFDLSSYY (**SEQ ID NO: 117**) as heavy chain CDR1, IWLGSJNI (**SEQ ID NO: 118**) as heavy chain CDR2 and ARGWLDDSFDP (**SEQ ID NO: 119**) as heavy chain CDR3, VSVHYNWK (**SEQ ID NO: 121**) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (**SEQ ID NO: 122**) as light chain CDR3;

20 c) GFSLITYS (**SEQ ID NO: 124**) as heavy chain CDR1, ISASGTA (**SEQ ID NO: 125**) as heavy chain CDR2, ARGSGPSGIESYKL (**SEQ ID NO: 126**) as heavy chain CDR3, QSIGNY (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGYGIIHT (**SEQ ID NO: 129**) as light chain CDR3;

d) GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, ARKDWTSGDSFNP (**SEQ ID NO: 133**) as heavy chain CDR3, QSISTA (**SEQ ID NO: 135**) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (**SEQ ID NO: 136**) as light chain CDR3; and

e) GFSLSNDA (**SEQ ID NO: 138**) as heavy chain CDR1, ISSAGRP (**SEQ ID NO: 139**) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (**SEQ ID NO: 140**) as heavy chain CDR3, QSISSSY (**SEQ ID NO: 142**) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (**SEQ ID NO: 143**) as light chain CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

35 In an embodiment, In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the following amino acid sequences: GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2,

ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3, ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3; and wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

5 In an embodiment, In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the following amino acid sequences: GFDLSSYY (**SEQ ID NO: 117**) as heavy chain CDR1, IWLGSGNI (**SEQ ID NO: 118**) as heavy chain CDR2 and ARGWLDDSFDP (**SEQ ID NO: 119**) as heavy chain CDR3, VSVHYNKW (**SEQ ID NO: 121**) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (**SEQ ID NO: 122**) as light chain CDR3; and wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

In an embodiment, In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the following amino acid sequences: GFSLITYS (**SEQ ID NO: 124**) as heavy chain CDR1, ISASGTA (**SEQ ID NO: 125**) as heavy chain CDR2, 15 ARGSGPSGIESYKL (**SEQ ID NO: 126**) as heavy chain CDR3, QSIGNNY (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGYGIIHIT (**SEQ ID NO: 129**) as light chain CDR3; and wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

In an embodiment, In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the following amino acid sequences: GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, 20 ARKDWTSBGDSFNP (**SEQ ID NO: 133**) as heavy chain CDR3, QSISTA (**SEQ ID NO: 135**) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (**SEQ ID NO: 136**) as light chain CDR3; and wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

In an embodiment, In an embodiment, the bivalent, trivalent, quadrivalent or multivalent binding protein comprises the following amino acid sequences: GFSLSNDA (**SEQ ID NO: 138**) as heavy chain CDR1, ISSAGRP (**SEQ ID NO: 139**) as heavy chain CDR2, 30 ARDKGYYSYHYAYDTRLDL (**SEQ ID NO: 140**) as heavy chain CDR3, QSISSSY (**SEQ ID NO: 142**) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (**SEQ ID NO: 143**) as light chain CDR3; and wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

Production of binding proteins

35 A person skilled in the art will appreciate that the binding proteins as described herein can be produced by a variety of methods, including cell based and cell-free expression systems.

In one example, a binding protein of the disclosure is produced by culturing a cell under conditions sufficient to produce the binding protein as described herein.

A person skilled in the art will appreciate that an antibody as described herein can be produced by recombinant genetic technology (recombinant protein production).

5 In some embodiments, a nucleic acid encoding a binding protein as described herein is placed into one or more expression construct/s, e.g., expression vector(s), which is/are then transfected into a host cell, such as a bacterial cell, a yeast cell, an insect cell, or a mammalian cell.

Exemplary bacterial cells include *E. coli*. Exemplary mammalian cells include simian
10 COS cells, Human Embryonic Kidney (HEK) cells and their derivatives, Chinese Hamster Ovary (CHO) cells, HeLa, Human embryonic kidney 293 cells (HEK293), human osteosarcoma U2OS, A549, HT1080, Cath. -a-differentiated cells (CAD), P19, NIH 3T3, L929, N2a, Hep G2 or myeloma cells that do not otherwise produce immunoglobulin protein. Molecular cloning techniques to achieve these ends are known in the art and described, for example in Ausubel or
15 Sambrook. A wide variety of cloning and *in vitro* amplification methods are suitable for the construction of recombinant nucleic acids. Methods of producing recombinant antibodies are also known in the art. See US4816567; US7923221 and US7022500.

In some embodiments, the nucleic acid is operably linked to a promoter. As used herein, the term “promoter” is to be taken in its broadest context and includes the transcriptional
20 regulatory sequences of a genomic gene, including the TATA box or initiator element, which is required for accurate transcription initiation, with or without additional regulatory elements (e.g., upstream activating sequences, transcription factor binding sites, enhancers and silencers) that alter expression of a nucleic acid, e.g., in response to a developmental and/or external stimulus, or in a tissue specific manner. In the present context, the term “promoter” is also used
25 to describe a recombinant, synthetic or fusion nucleic acid, or derivative which confers, activates or enhances the expression of a nucleic acid to which it is operably linked. Exemplary promoters can contain additional copies of one or more specific regulatory elements to further enhance expression and/or alter the spatial expression and/or temporal expression of said nucleic acid. As used herein, the term “operably linked to” means positioning a promoter
30 relative to a nucleic acid such that expression of the nucleic acid is controlled by the promoter.

It will be understood that it is possible to improve the expression of a nucleic acid in a host organism or host cell by replacing the nucleotide sequences coding for a particular amino acid (i.e., a codon) with another codon which is better expressed in the host organism (i.e., codon optimization). One reason that this effect arises is due to the fact that different organisms
35 show preferences for different codons. In some embodiments, a nucleic acid as disclosed herein is modified or optimized such that the nucleotide sequence reflects the codon preference for the

particular host cell, preferably mammalian or bacterial cell. In an embodiment, the nucleic acid is codon optimized for mammalian cell culture. In an embodiment, the nucleic acid is codon optimized for bacterial cell culture. Method of codon optimization will be apparent to the skilled person. For example, tools for codon optimization include, for example, GeneArt
5 GeneOptimizer (Thermofisher®) or GenSmart® (GeneScript®).

In some embodiments, the nucleic acid will comprise an N-terminal sequence to aid expression in a host cell. For example, for expression in a bacteria, such as *E. coli*, the nucleic acid may comprises an N-terminal pelB signaling peptide for periplasmic expression (e.g. as shown in **SEQ ID NO: 107**). For example, for expression in a mammalian cell, the nucleic acid
10 comprises an N-terminal tPA leader sequence (e.g. as shown in **SEQ ID NO: 108**).

In an embodiment, the nucleic acid comprises a nucleotide sequence selected from: **SEQ ID NO: 87** to **SEQ ID NO: 106** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 87** or a codon optimized version thereof of or a sequence at least 70% identical
15 thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 88** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 89** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 90** or a codon optimized
20 version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 91** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence: **SEQ ID NO: 92** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide
25 sequence **SEQ ID NO: 93** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 94** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 95** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an
30 embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 96** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 97** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 98** or a codon optimized version thereof
35 of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 99** or a codon optimized version thereof of or a sequence

at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 100** or a codon optimized version thereof or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 101** or a codon optimized version thereof or a sequence at least 70% identical thereto.

[illegible]

acid comprises the nucleotide sequence **SEQ ID NO: 158** or a codon optimized version thereof of or a sequence at least 70% identical thereto. In an embodiment, the nucleic acid comprises the nucleotide sequence **SEQ ID NO: 160** or a codon optimized version thereof of or a sequence at least 70% identical thereto.

5

Isolation of binding proteins

A binding protein of the present disclosure can be isolated or purified using any method known to a person skilled in the art.

When using recombinant techniques, the binding protein of the disclosure can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the protein is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Where the protein is secreted into the medium, supernatants from such expression systems can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or
10 Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants. Supernatants can also be used directly for purification.

The binding protein prepared from the cells or supernatant can be purified using, for example, ion exchange, hydroxyapatite chromatography, hydrophobic interaction
20 chromatography, gel electrophoresis, dialysis, affinity chromatography (e.g., protein A affinity chromatography or protein G chromatography), or any combination of the foregoing. These methods are known in the art and described, for example in WO99/57134 or Zola (1987).

The skilled artisan will also be aware that a binding protein of the disclosure can be modified to include a tag to facilitate purification or detection (examples of such are described
25 above). The resulting protein is then purified using methods known in the art, such as affinity purification. For example, a protein comprising a hexa-his tag is purified by contacting a sample comprising the protein with nickel-nitrilotriacetic acid (Ni-NTA) that specifically binds a hexa-His-tag immobilized on a solid or semi-solid support, washing the sample to remove unbound protein, and subsequently eluting the bound protein. Alternatively, or in addition a ligand or
30 antibody that binds to a tag is used in an affinity purification method.

Kits

In an aspect, the present invention provides a kit or panel comprising the binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as
35 described herein.

In an embodiment, the kit or panel comprises a strip, chip or cartridge for use on point-of care device.

In an embodiment, the kit or panel selected from a: lateral flow assay kit, ELISA kit and an Luminex® assay kit.

In an embodiment, the kit or panel is designed for use by a health care practitioner.

In an embodiment, the kit or panel is a self-test kit designed for home use.

- 5 In an embodiment, the kit or panel is contained within an all-in-one device for home use or health care practitioner use. In an embodiment, the kit or panel comprises a cassette with a built in lance and a blister pack of running buffer. No extra consumables are provided with the test. The device is designed for at home use. For some types of treatments (drugs, antibody therapies) where an indication of liver toxicity has been described, monitoring of ALT levels
- 10 may be required daily, multiple times a week or weekly.

Assays

- The methods and uses as described herein are suitable for *in vitro* use and may be performed in an assay format known to a person skilled in the art, including as an immunoassay, chromatographic assay or a homogenous assay.
- 15

- As used herein, "immunoassay" refers to assays using immunoglobulins or parts thereof that are capable of detecting and quantifying a desired biomarker such as ALT. The immunoassay may be one of a range of immune assay formats known to the skilled addressee. A wide range of immunoassay techniques are available, such as those described in Wild D. "The Immunoassay Handbook" Nature Publishing Group, 4th Edition, 2013 and subsequent innovations.
- 20

- In an embodiment, the immunoassay detects total ALT (ALT1 and ALT2). In an embodiment, the immunoassay detects ALT1. In an embodiment, the immunoassay detects total ALT at level comparable to that of the current gold standard ALT measurement (enzymatic activity of ALT (ALT1 and 2) in serum/plasma (L-alanine + 2-oxoglutarate is converted to pyruvate + L-glutamate by ALT). The enzymatic assay is a coupled assay. That is the product of the ALT reaction, pyruvate, becomes the substrate for the 2nd reaction catalysed by lactate dehydrogenase (LDH). This second reaction uses cofactors NADH which is converted to NAD⁺. This cofactor can be monitored spectrophotometrically.
- 25

- In an embodiment, the immunoassay is selected from: electrochemiluminescence (ELICA), enzyme-linked immunosorbent assay (ELISA), chemiluminescent ELISA, fluorescent immunosorbent assay (FIA), biosensor based assay, a bead-type immunoassay and a particle based immunoassay (e.g. mesoscale delivery platform (MSD)). Examples of detectable-groups include, for example and without limitation: fluorochromes, enzymes, epitopes for binding a second binding reagent (for example, when the second binding reagent/antibody is a mouse antibody, which is detected by a fluorescently-labelled anti-mouse
- 30
- 35

antibody), for example an antigen or a member of a binding pair, such as biotin. The surface may be a planar surface, such as in the case of a typical grid-type array (for example, but without limitation, 96-well plates and planar microarrays) or a non-planar surface, as with coated bead array technologies, where each "species" of bead is labelled with, for example, a fluorochrome
5 (such as the Luminex technology described in U. S. Patent Nos. 6,599,331, 6, 592,822 and 6,268,222), or quantum dot technology (for example, as described in U. S. Patent No. 6,306,610).

In an embodiment, the immunoassay is performed on an automated platform e.g. a Cobas Immunology Analyzer (Roche) or an Architect immunoassay analyzer (Abbott).

10 In an embodiment, the immunoassay is a supplementary/monitoring assay for a clinical trial.-

Lateral flow assays and more recently non-lateral flow and microfluidics provide a useful set up for biological assays. Such assays can be qualitative, quantitative or semi quantitative. In microfluidic devices, small volumes of liquid are moved through microchannels
15 generated in, for example, a chip or cartridge. A wide range of detection reagents are available including metal nanoparticles, coloured or luminescent materials. Resonance enhanced adsorption (REA) of bioconjugated metal nanoparticles offers rapid processing times and other advantages. These devices have been combined with barcode technologies to identify the patient and the analyte being tested. Computer software and hardware for assessing input data
20 are encompassed by the present disclosure. Point-of-care devices and arrays and high throughput screening methods are also contemplated. In an embodiment, the assay is a point-of-care device. In an embodiment, the point-of-care device comprises or is accompanied with a lance for obtaining a sample as described herein.

Qualitative assays providing an intermediate or definitive diagnosis require integrated
25 thresholds, gates or windows that permit scoring of samples as likely or not to have a condition. Instrument readers and software are often employed to collate data and process it through a diagnostic algorithm or decision tree.

In an embodiment, the bead-type immunoassay is selected from a Luminex LabMAP assay, Bio-Plex Multiplex immunoassay (Bio-Rad). In the bead-type immunoassays, the
30 Luminex LabMAP system can be utilized. The LabMAP system incorporates polystyrene microspheres that are dyed internally with two spectrally distinct fluorochromes. Using precise ratios of these fluorochromes, an array is created consisting of different microsphere sets with specific spectral addresses. Each microsphere set can possess a different reactant on its surface. Because microsphere sets can be distinguished by their spectral addresses, they can be
35 combined, allowing up to 100 different analytes to be measured simultaneously in a single reaction vessel. A third fluorochrome coupled to a reporter molecule quantifies the

biomolecular interaction that has occurred at the microsphere surface. Microspheres are interrogated individually in a rapidly flowing fluid stream as they pass by two separate lasers in the Luminex analyzer. High-speed digital signal processing classifies the microsphere based on its spectral address and quantifies the reaction on the surface in a few seconds per sample.

5 In one embodiment, the assay is a homogenous assay, meaning an assay format allowing the make an assay-measurement by a simple mix and read procedure without the necessity to process samples by separating or washing. Such assays do not include an immunosorbent solid phase step. In one embodiment, the homogenous assay is time-resolved Förster resonance energy transfer (FRET).

10 In one embodiment, the assay is a flow cytometry-, bead array-, lateral flow-, cartridge-, microfluidic- or immunochromatographic-based method or the like. In one embodiment, the assay is a point-of-care assay. In one embodiment, the point-of-care assay reader is an Axxin AX-2X-type reader, or equivalent or modified device. For example, the device may be modified to include LEDs and filters of the appropriate wavelength for the subject assays.

15 In an embodiment, the assay is a biosensor assay. In an embodiment, the assay is a plasmon resonance assay or a biolayer interferometry (BLI) assay for example as described in Capelli et al (2013) and Tokel et al (2014). In some embodiments, the assay allows label free detection of ALT1.

20 *Capture and detection*

 A person skilled in the art will appreciate that the assays as described herein may use one binding protein as described herein or may use more than one binding protein as described here. In an embodiment, an assay as described herein may comprise the use of two different binding proteins as described herein. In an embodiment, an assay as described herein may
25 comprise the use of a binding agent as described herein with a rabbit polyclonal ALT-1 antibody.

 In an aspect, the present invention provides an assay comprising: a solid support that comprises a binding protein as described herein, and/or a bivalent, trivalent, quadrivalent or multivalent binding protein as described herein immobilized on the solid support.

30 In an aspect, the present invention provides an immunoassay comprising: a solid support that comprises a binding protein immobilized on the solid support.

 In an aspect, the present invention provides an immunoassay comprising a bivalent, trivalent, quadrivalent or multivalent binding protein as described herein immobilized on the solid support.

35 In embodiment, the assay as described herein comprises a binding protein for detecting ALT (a detection or detector binding protein) and a binding protein for capturing ALT (a

capture binding protein). As used, herein the “detection binding protein” comprises a detectable label as described herein which can be used to directly or indirectly measure the level of ALT in a sample. As used herein, a “capture binding protein” is used to capture ALT in the sample. In an embodiment, the capture binding protein binds ALT before it binds the detection binding protein. In an embodiment, the capture binding protein binds ALT after it has bound the detection binding protein. In an embodiment, the capture binding protein is bound/immobilised to a solid support. The term “immobilised” “immobilisation” is to be understood to involve various methods and techniques to fix proteins onto specific matrices, e.g. as described in WO99/56126 or WO02/26292. For example, immobilization can serve to stabilize the proteins so that its activity is not reduced or adversely modified by biological, chemical or physical exposure, especially during storage or in single-batch use. In an embodiment, the capture binding protein is bound to the solid support in the presence of one or more excipients. In an embodiment, the excipient is selected from one or more of: sucrose, bovine serum albumin (BSA), an immunoglobulin, trehalose, casein, lactose, galactose and a detergent (e.g. Tween). In an embodiment, the excipient is sucrose. In an embodiment, the excipient is BSA. In an embodiment, the excipient is sucrose and BSA. In an embodiment, the detection and capture binding proteins are nanobodies as described herein. In an embodiment, the detection and capture binding proteins are bivalent binding proteins as described herein. In an embodiment, the detection and capture binding proteins are trivalent binding protein as described herein. In an embodiment, the detection and capture binding proteins are quadrivalent binding proteins as described herein. In an embodiment, the detection and capture binding proteins are multivalent binding proteins as described herein. In an embodiment, the detection and capture binding proteins are monoclonal antibodies as described herein. In an embodiment, the detection and capture binding proteins are rabbit monoclonal antibodies as described herein.

25 In an embodiment, the detection binding protein is a nanobody as described herein. In an embodiment, the detection binding protein is a bivalent binding protein as described herein. In an embodiment, the detection binding protein is a trivalent binding protein as described herein. In an embodiment, the detection binding protein is a quadrivalent binding protein as described herein. In an embodiment, the detection binding protein is a multivalent binding protein as described herein. In an embodiment, the detection binding protein is a monoclonal antibody as described herein. In an embodiment, the detection binding protein is a rabbit monoclonal antibody as described herein.

30 In an embodiment, the capture binding protein is a nanobody as described herein. In an embodiment, the capture binding protein is a bivalent binding protein as described herein. In an embodiment, the capture binding protein is a trivalent binding protein as described herein. In an embodiment, the capture binding protein is a quadrivalent binding protein as

described herein. In an embodiment, the capture binding is a multivalent binding protein as described herein. In an embodiment, the capture binding protein is a monoclonal antibody as described herein. In an embodiment, the capture binding protein is a rabbit monoclonal antibody as described herein.

- 5 In an embodiment, the detection binding proteins is an rabbit polyclonal ALT antibody. In an embodiment, the capture binding protein is an rabbit polyclonal ALT antibody.

Examples of binding proteins that can be used combinations for capture and detection are shown in **Figure 101**.

- 10 In an embodiment, the capture binding protein is a nanobody as described herein and the detector binding protein is a rabbit polyclonal ALT-1 antibody. In an embodiment, the capture binding protein is a nanobody as described herein and the detector binding protein is nanobody as described herein. In an embodiment, the capture binding protein is a nanobody as described herein and the detector binding protein is a rabbit monoclonal antibody as described herein. In an embodiment, the capture binding protein is a rabbit monoclonal antibody as described herein and the detector binding protein is a rabbit monoclonal antibody as described herein. In an embodiment, the capture binding protein is a rabbit monoclonal antibody as described herein and the detector binding protein is a rabbit polyclonal ALT-1 antibody. In an embodiment, the capture binding protein is a rabbit monoclonal antibody as described herein and the detector binding protein is a nanobody as described herein. In an embodiment, the capture binding protein is a rabbit polyclonal ALT-1 antibody and the detector binding protein is a nanobody as described herein.

- 20 In an embodiment, the capture binding protein is C4G-Fc and the detector binding protein is selected from one or more of: RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5. In an embodiment, the capture binding protein is C4G-Fc and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is C4G-Fc and the detector binding protein is RmAb2. In an embodiment, the capture binding protein is C4G-Fc and the detector binding protein is RmAb3. In an embodiment, the capture binding protein is C4G-Fc and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is C4G-Fc and the detector binding protein is RmAb5.

- 30 In an embodiment, the capture binding protein is RmAb1 and the detector binding protein is selected from one or more of: RmAb3, RmAb4, RmAb5 and C4G-Fc. In an embodiment, the capture binding protein is RmAb1 and the detector binding protein is RmAb3. In an embodiment, the capture binding protein is RmAb1 and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is RmAb1 and the detector binding protein is RmAb5. In an embodiment, the capture binding protein is RmAb1 and the detector binding protein is C4G-Fc.

In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is selected from one or more of: RmAb1, RmAb3, RmAb4, RmAb5 and C4G-Fc.

In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is RmAb3. In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is RmAb5. In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is C4G-Fc.

In an embodiment, the capture binding protein is RmAb4 and the detector binding protein is selected RmAb5 and C4G-Fc. In an embodiment, the capture binding protein is RmAb4 and the detector binding protein is RmAb5. In an embodiment, the capture binding protein is RmAb4 and the detector binding protein is C4G-Fc.

In an embodiment, the capture binding protein is RmAb5 and the detector binding protein is selected from one or more of: RmAb3, RmAb4, RmAb5 and C4G-Fc. In an embodiment, the capture binding protein is RmAb5 and the detector binding protein is RmAb3. In an embodiment, the capture binding protein is RmAb5 and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is RmAb5 and the detector binding protein is RmAb5. In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is C4G-Fc.

In an embodiment, the capture binding protein is C4C-Fc and the detector binding protein is selected from one or more of: RmAb1, RmAb4 and RmAb5. In an embodiment, the capture binding protein is C4C-Fc and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is C4C-Fc and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is C4C-Fc and the detector binding protein is RmAb5.

In an embodiment, the capture binding protein is C3G-Fc and the detector binding protein is selected from one or more of: RmAb1, RmAb4 and RmAb5. In an embodiment, the capture binding protein is C3G-Fc and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is C3G-Fc and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is C3G-Fc and the detector binding protein is RmAb5.

In an embodiment, the capture binding protein is G3C-Fc and the detector binding protein is selected from one or more of: RmAb1, RmAb4 and RmAb5. In an embodiment, the capture binding protein is G3C-Fc and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is G3C-Fc and the detector binding protein is RmAb4.

In an embodiment, the capture binding protein is G3C-Fc and the detector binding protein is RmAb5.

In an embodiment, the capture binding protein is G4C-Fc and the detector binding protein is selected from one or more of: RmAb1, RmAb4 and RmAb5. In an embodiment, the
5 capture binding protein is G4C-Fc and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is G4C-Fc and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is G4C-Fc and the detector binding protein is RmAb5.

In an embodiment, the capture binding protein is C8 and the detector binding protein is
10 G6. In an embodiment, the capture binding protein is C8 and the detector binding protein is a rabbit polyclonal ALT-1 antibody. In an embodiment, the capture binding protein is C8 and the detector binding protein is G4C. In an embodiment, the capture binding protein is C8 and the detector binding protein is C3G. In an embodiment, the capture binding protein is C8 and the detector binding protein is C4G. In an embodiment, the capture binding protein is C8 and the
15 detector binding protein is G3G. In an embodiment, the capture binding protein is C8 and the detector binding protein is G4G. In an embodiment, the capture binding protein is G6 and the detector binding protein is a rabbit polyclonal ALT-1 antibody.

In an embodiment, the capture binding protein is a rabbit polyclonal ALT-1 antibody and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is a
20 rabbit polyclonal ALT-1 antibody and the detector binding protein is RmAb3. In an embodiment, the capture binding protein is a rabbit polyclonal ALT-1 antibody and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is a rabbit polyclonal ALT-1 antibody and the detector binding protein is RmAb5.

In an embodiment, the capture binding protein is C4G-Fc and the detector binding
25 protein is a rabbit polyclonal ALT-1 antibody. In an embodiment, the capture binding protein is C4G-Fc antibody and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is C4G-Fc antibody and the detector binding protein is RmAb3. In an embodiment, the capture binding protein is C4G-Fc antibody and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is C4G-Fc antibody and the detector
30 binding protein is RmAb5. In an embodiment, the capture binding protein is RmAb1 and the detector binding protein is a rabbit polyclonal ALT-1 antibody.

In an embodiment, the capture binding protein is RmAb1 and the detector binding protein is RmAb1.

In an embodiment, the capture binding protein is RmAb3 and the detector binding
35 protein is a rabbit polyclonal ALT-1 antibody. In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is RmAb1. In an embodiment, the capture binding

protein is RmAb3 and the detector binding protein is RmAb3. In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is RmAb3 and the detector binding protein is RmAb5.

5 In an embodiment, the capture binding protein is RmAb4 and the detector binding protein is a rabbit polyclonal ALT-1 antibody. In an embodiment, the capture binding protein is RmAb4 and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is RmAb4 and the detector binding protein is RmAb3. In an embodiment, the capture binding protein is RmAb4 and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is RmAb4 and the detector binding protein is RmAb5.

10 In an embodiment, the capture binding protein is RmAb5 and the detector binding protein is a rabbit polyclonal ALT-1 antibody. In an embodiment, the capture binding protein is RmAb5 and the detector binding protein is RmAb1. In an embodiment, the capture binding protein is RmAb5 and the detector binding protein is RmAb3. In an embodiment, the capture binding protein is RmAb5 and the detector binding protein is RmAb4. In an embodiment, the capture binding protein is RmAb5 and the detector binding protein is RmAb5. In an embodiment, the capture binding protein is G4C and the detector binding protein is a RmAb1.

15 In an embodiment, the capture binding protein is G4C and the detector binding protein is G4C. In an embodiment, the capture binding protein is G4C and the detector binding protein is C3G. In an embodiment, the capture binding protein is G4C and the detector binding protein is C4G. In an embodiment, the capture binding protein is G4C and the detector binding protein is G3G.

20 In an embodiment, the capture binding protein is C3G and the detector binding protein is a rabbit polyclonal ALT-1 antibody. In an embodiment, the capture binding protein is C3G and the detector binding protein is G4C. In an embodiment, the capture binding protein is C3G and the detector binding protein is C3G. In an embodiment, the capture binding protein is C3G and the detector binding protein is C4G. In an embodiment, the capture binding protein is C3G and the detector binding protein is G3G. In an embodiment, the capture binding protein is C3G and the detector binding protein is G4G.

25 In an embodiment, the capture binding protein is C4G and the detector binding protein is a rabbit polyclonal ALT-1 antibody. In an embodiment, the capture binding protein is C4G and the detector binding protein is G4C. In an embodiment, the capture binding protein is C4G and the detector binding protein is C3G. In an embodiment, the capture binding protein is C4G and the detector binding protein is C4G. In an embodiment, the capture binding protein is C4G and the detector binding protein is G3G. In an embodiment, the capture binding protein is C4G and the detector binding protein is G4G. In an embodiment, the capture binding protein is C3C and the detector binding protein is G4C. In an embodiment, the capture binding protein is C3C

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and the detector binding protein is C3G. In an embodiment, the capture binding protein is C3C and the detector binding protein is C4G. In an embodiment, the capture binding protein is C3C and the detector binding protein is G3G. In an embodiment, the capture binding protein is C4C and the detector binding protein is a rabbit polyclonal ALT-1 antibody.

5 In an embodiment, the capture binding protein is C4C and the detector binding protein is G4C. In an embodiment, the capture binding protein is C4C and the detector binding protein is C3G. In an embodiment, the capture binding protein is C4C and the detector binding protein is C4G. In an embodiment, the capture binding protein is C4C and the detector binding protein is G3G. In an embodiment, the capture binding protein is G3G and the detector binding protein is a rabbit polyclonal ALT-1 antibody.

10 In an embodiment, the capture binding protein is G3G and the detector binding protein is G4C. In an embodiment, the capture binding protein is G3G and the detector binding protein is C3G. In an embodiment, the capture binding protein is G3G and the detector binding protein is C4G. In an embodiment, the capture binding protein is G3G and the detector binding protein is G3G. In an embodiment, the capture binding protein is G4G and the detector binding protein is a rabbit polyclonal ALT-1 antibody.

In an embodiment, the capture binding protein is G4G and the detector binding protein is G4C. In an embodiment, the capture binding protein is G4G and the detector binding protein is C3G. In an embodiment, the capture binding protein is G4G and the detector binding protein is C4G. In an embodiment, the capture binding protein is G4G and the detector binding protein is G3G.

Lateral flow assays

In an aspect, the present invention provides a lateral flow assay comprising: a solid support that comprises a binding protein as described herein, and/or a bivalent, trivalent, quadrivalent or multivalent binding protein as described herein immobilized on the solid support.

In an aspect, the present invention provides a lateral flow assay comprising: a solid support that comprises a binding protein immobilized on the solid support.

30 In an aspect, the present invention provides a lateral flow assay comprising a bivalent, trivalent, quadrivalent or multivalent binding protein as described herein immobilized on the solid support.

In an embodiment, the lateral flow assay provides for the detection of ALT1. =

In an aspect, the present invention provides a lateral flow assay comprising: (i) a detector binding protein conjugated to a detectable label; (ii) a capture binding protein in a capture region on a solid support, wherein the binding protein in (i) and/or (ii) is a binding protein as

described herein, and/or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein.

In an embodiment, the lateral flow assay, further comprises a red blood cell capture portion. In an embodiment, the red blood cell capture portion is selected from a red blood cell binding protein and a blood retention pad. In an embodiment, the red blood cell binding protein is selected from binding protein targeting: glycophorin B, lewis, CD238 and CD234. In an embodiment, the red blood cell binding protein is glycophorin B. In an embodiment, the red blood cell binding protein is lewis. In an embodiment, the red blood cell binding protein is CD238. In an embodiment, the red blood cell binding protein is CD234. In an embodiment, the blood retention pad is selected from one or more of: FR1, MDI, Vivid GX, CytoSep1663, CytSep 1660, CytoSep1668, Vivid GR, Vivid GX, Vivid GR, and Vivid GF. In an embodiment, the blood retention pad is FR1. In an embodiment, the blood retention pad is MDI. In an embodiment, the blood retention pad is Vivid GX. In an embodiment, the blood retention pad is CytoSep1663. In an embodiment, the blood retention pad is CytSep 1660. In an embodiment, the blood retention pad is CytoSep1668. In an embodiment, the blood retention pad is Vivid GR. In an embodiment, the blood retention pad is Vivid GX. In an embodiment, the blood retention pad is Vivid GR. In an embodiment, the blood retention pad is Vivid GF. In an embodiment, the lateral flow assay comprises one or more of a lancet, blood collection unit and blister pack containing buffer. In an embodiment, the lateral flow assay comprises a lancet. In an embodiment, the lateral flow assay comprises a blood collection unit. In an embodiment, the lateral flow assay comprises a blister pack containing buffer.

In an embodiment, the lateral flow device further comprises a control line on the solid support comprising an ALT or an ALT epitope.

In an embodiment, the binding protein in i) and/or ii) is a binding protein as described herein. In an embodiment, the binding protein in i) and/or ii) is a nanobody as described herein. In an embodiment, the binding protein in i) and/or ii) is a nanobody fusion protein as described herein. In an embodiment, the binding protein in i) and/or ii) is a nanobody fusion protein as described herein. In an embodiment, the binding protein in i) and/or ii) is a bivalent binding protein as described herein. In an embodiment, the binding protein in i) and/or ii) is a trivalent binding protein as described herein. In an embodiment, the binding protein in i) and/or ii) is a quadrivalent binding protein as described herein. In an embodiment, the binding protein in i) and/or ii) is a multivalent binding protein as described herein.

In an embodiment, the binding protein in i) is a binding protein as described herein. In an embodiment, the binding protein in i) is a nanobody as described herein. In an embodiment, the binding protein in i) is a bivalent binding protein as described herein. In an embodiment,

the binding protein in i) is a trivalent binding protein as described herein. In an embodiment, the binding protein in i) is a quadrivalent binding protein as described herein. In an embodiment, the binding protein in i) is a multivalent binding protein as described herein.

In an embodiment, the binding protein in ii) is a binding protein as described herein. In an embodiment, the binding protein in ii) is a nanobody as described herein. In an embodiment, the binding protein in ii) is a bivalent binding protein as described herein. In an embodiment, the binding protein in ii) is a trivalent binding protein as described herein. In an embodiment, the binding protein in ii) is a quadrivalent binding protein as described herein. In an embodiment, the binding protein in ii) is a multivalent binding protein as described herein.

In an embodiment, the binding protein in i) and/or ii) is selected from: C8, C6, G4C, C3G, C4G, G4G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.

In an embodiment, the binding protein in i) and/or ii) is selected from: C8, G6, G4C, C3G, C4G, G4G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc and ALT rabbit polyclonal antibody. In an embodiment, the binding protein in i) and/or ii) is selected from: C4C-Fc, G4G-Fc, C4G-Fc and G4C-Fc.

In an embodiment, the binding protein in i) and/or ii) is C8. In an embodiment, the binding protein in i) and/or ii) is G6. In an embodiment, the binding protein in i) and/or ii) is G4C. In an embodiment, the binding protein in i) and/or ii) is C3G. In an embodiment, the binding protein in i) and/or ii) is G4G. In an embodiment, the binding protein in i) and/or ii) is C4C-Fc. In an embodiment, the binding protein in i) and/or ii) is G4G-Fc. In an embodiment, the binding protein in i) and/or ii) is G4G-Fc. In an embodiment, the binding protein in i) and/or ii) is G4C-Fc. In an embodiment, the binding protein in i) and/or ii) is C4G-Fc. In an embodiment, the binding protein in i) and/or ii) is an ALT rabbit polyclonal antibody. In an embodiment, the ALT rabbit polyclonal antibody is an antibody described in PCT/IB2017/055943.

In an embodiment, the binding protein in i) is C8. In an embodiment, the binding protein in i) is G6. In an embodiment, the binding protein in i) is G4C. In an embodiment, the binding protein in i) is C3C. In an embodiment, the binding protein in i) is G4G. In an embodiment, the binding protein in i) is C4C-Fc. In an embodiment, the binding protein in i) is G4G-Fc. In an embodiment, the binding protein in i) is G4G-Fc. In an embodiment, the binding protein in i) is G4C-Fc. In an embodiment, the binding protein in i) is C4C-Fc. In an embodiment, the binding protein in i) is an ALT rabbit polyclonal antibody.

In an embodiment, the binding protein in ii) is C8. In an embodiment, the binding protein in ii) is G6. In an embodiment, the binding protein in ii) is G4C. In an embodiment, the binding protein in ii) is C36. In an embodiment, the binding protein in ii) is G4G. In an embodiment,

the binding protein in ii) is C4C-Fc. In an embodiment, the binding protein in ii) is G4G-Fc. In an embodiment, the binding protein in ii) is G4G-Fc. In an embodiment, the binding protein in ii) is G4C-Fc. In an embodiment, the binding protein in ii) is G4G-Fc. In an embodiment, the binding protein in ii) is an ALT rabbit polyclonal antibody.

5 In an embodiment, the binding protein in i) is G4C the binding protein in ii) is selected from: C4G, C4C, C3C, G4C, C8, G4G, G3G and C3G. In an embodiment, the binding protein in i) is C3G the binding protein in ii) is selected from: G4G, G3G, C8, G4C, C3C, C4C, C3G and C4G. In an embodiment, the binding protein in i) is G4G the binding protein in ii) is selected from: C8, C3G and C4G. In an embodiment, the binding protein in i) is anti-ALT polyclonal
10 antibody the binding protein in ii) is selected from G4G, G3G, G4C, C3G and C4G.

In an embodiment, the lateral flow assay can distinguish between different levels of high (>120 IU/L) and low (<120 IU/L) enzymatic ALT1 in samples as described herein.

In an embodiment, when the binding protein in i) is G4C and the binding protein in ii) is selected from: C4G, C4C, C3C, G4C, C8, G4G, G3G, C3G, RmAb1, RmAb2, RmAb3,
15 RmAb4 and RmAb5.

In an embodiment, when the binding protein in i) is C3G and the binding protein in ii) is selected from: G4G, G3G, C8, G4C, C3C, C4C, C3G, C4G, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.

In an embodiment, when the binding protein in i) is G4G and the binding protein in ii) is selected from: C8, C3G, C4G, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.
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In an embodiment, when the binding protein in i) is an anti-ALT polyclonal antibody and the binding protein in ii) is selected from G4G, G3G, G4C, C3G, C4G, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.

In an embodiment, when the binding protein in i) is RmAb1 and the binding protein in ii) is selected from: G4G, G3G, C8, G4C, C3C, C4C, C3G, C4G, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.
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In an embodiment, when the binding protein in i) is RmAb2 and the binding protein in ii) is selected from: G4G, G3G, C8, G4C, C3C, C4C, C3G, C4G, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.

In an embodiment, when the binding protein in i) is RmAb3 and the binding protein in ii) is selected from: G4G, G3G, C8, G4C, C3C, C4C, C3G, C4G, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.
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In an embodiment, when the binding protein in i) is RmAb4 and the binding protein in ii) is selected from: G4G, G3G, C8, G4C, C3C, C4C, C3G, C4G, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.
35

In an embodiment, when the binding protein in i) is RmAb5 and the binding protein in ii) is selected from: G4G, G3G, C8, G4C, C3C, C4C, C3G, C4G, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.

In an embodiment, the binding protein in i) and/or ii) is selected from: C4C-Fc, G4G-Fc, C4G-Fc and G4C-Fc.

In an embodiment, the binding protein in ii) is striped at a concentration of 0.05 mg/mL to about 2.5 mg/mL. In an embodiment, the binding protein in ii) is striped at a concentration of 0.02 mg/mL to about 0.5 mg/mL.

10 Methods

It will be apparent from the description herein that the present disclosure provides various methods for detecting ALT. It will be apparent from the description herein that the present disclosure provides various methods/ uses for diagnosing/prognosing and/or monitoring conditions/treatments associated with ALT expression. It will be apparent from the description herein that the present disclosure provides methods for monitoring drug induced treatment.

In an aspect, the present invention provides use of a binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, or the kit as described herein, or the lateral flow assay as described herein for detecting ALT1.

In an aspect, the present invention provides a method of detecting ALT1, the method comprising contacting a sample with a binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein of as described herein to form an antigen-binding protein complex and directly or indirectly detecting the antigen-binding protein complex.

In an aspect, the present invention provides use of a binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, or the kit as described herein, or the lateral flow assay as described herein for detecting a subject with liver damage and/or liver disease.

In an aspect, the present invention provides method of detecting a subject with liver damage and/or liver disease the method comprising containing a sample with a binding protein as described herein, or the bivalent, trivalent, quadrivalent or multivalent binding protein as described herein, to form an antigen-binding protein complex and directly or indirectly detecting the antigen-binding protein complex.

In an embodiment, the use or method as described herein is used to detect liver damage and/or liver disease in a subject.

In an embodiment the use or method as described herein is used to detect a condition or treatment in a subject associated with liver damage and or to monitor a condition or treatment

in a subject associated with liver damage. Examples as such conditions and treatments are described in Schaefer and John, 2023 and provided in **Table 1**.

In an embodiment, the condition is a pregnancy related condition, infection with a pathogen, non-alcoholic fatty liver disease, fatty liver disease or another liver damage and/or
5 liver disease related condition.

In an embodiment, the condition is selected from a/an: hepatotropic virus, non-hepatotropic virus, bacteria, fungi, parasite, toxin or substance related cause, inflammatory condition, metabolic or hereditary condition, pregnancy related condition, ischemic or vascular condition.

10 In an embodiment, the condition is a chronic condition.

In an embodiment the condition is an acute condition.

In an embodiment, the condition is selected from: hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, Epstein-Barr virus, cytomegalovirus, herpes simplex virus, coxsackievirus, mononucleosis, adenovirus, dengue virus, coronavirus-19, fatty liver disease,
15 acute alcoholic hepatitis, or alcoholic cirrhosis, sea anemone sting, autoimmune hepatitis, biliary disease such as primary biliary cholangitis or primary sclerosing cholangitis. non-alcoholic fatty liver disease, hemochromatosis, Wilson's disease, preeclampsia, acute fatty liver of pregnancy, HELLP syndrome, cardiogenic/distributive shock, hypotension, heatstroke, cocaine, methamphetamine, ephedrine, acute Budd-Chiari syndrome, sinusoidal obstruction
20 syndrome, malignancy, eclampsia, Reye's syndrome, primary graft non-function after liver transplantation.

In an embodiment, the hepatotropic virus is selected from: hepatitis A, hepatitis B, hepatitis C, hepatitis D and hepatitis E.

In an embodiment, the non-hepatotropic virus is a virus as described in Spengler et al
25 (2019), Jafari (2023), and Gupta et al (2021).

In an embodiment, the non-hepatotropic virus is selected from viruses that cause viral hemorrhagic fevers affecting the liver within the families of Flaviviridae, Arenaviridae, Filoviridae, Bunyaviridae and Togaviridae. including but not limited to yellow fever, lassa fever, Argentinian hemorrhagic fever, ebola fever and Marburg fever, rift valley fever, kongo-
30 krim hemorrhagic fever and chikungunya virus.

In an embodiment, the non-hepatotropic virus is selected from herpesviruses including but not limited to human herpesviruses 1-8, Influenza A and B viruses, , adenoviruses, and coronaviruses including but not limited to severe acute respiratory syndrome viruses 1 and 2, parvovirus including B19, enteroviruses including coxsackie B virus and echovirus,
35 paramyxovirus including measles, togaviruses including rubella.

In an embodiment, the toxin or substance related cause is selected from: fatty liver disease, acute alcoholic hepatitis, or alcoholic cirrhosis, paracetamol, antibiotic, anticonvulsants, statins, NSAIDs, herbal/nutritional supplements, mushroom (e.g. amanita phalloides), herbal and dietary supplement, carbon tetrachloride and sea anemone sting.

5 In an embodiment, the inflammatory condition is selected from: autoimmune hepatitis, biliary disease such as primary biliary cholangitis and primary sclerosing cholangitis.

In an embodiment, the metabolic or hereditary condition is selected from: non-alcoholic fatty liver disease, hemochromatosis and Wilson's disease.

10 In an embodiment, the pregnancy related condition is selected from: pregnancy related condition, eclampsia, preeclampsia, high ALT1 levels in pregnancy in a subject, acute fatty liver of pregnancy and HELLP syndrome.

In an embodiment, the ischemic or vascular condition is selected from: cardiogenic/distributive shock, hypotension, heatstroke, cocaine, methamphetamine, ephedrine, acute Budd-Chiari syndrome, sinusoidal obstruction syndrome.

15 In an embodiment, the condition is selected from: malignancy, eclampsia, Reye's syndrome and primary graft non-function after liver transplantation.

In an embodiment, the method or use is used monitor a condition as described herein in a subject.

20 In an embodiment, the method or use is used to monitor treatment in a subject. A person skilled in the art will appreciate that the treatment can be any treatment wherein liver toxicity is a known concern or a potential concern. The method or use can be used as an early warning of rising ALT levels (e.g. rising over time, approaching the upper limit of normal or above the upper limit of normal). The method or use can be used to determine when a treatment should be adjusted, altered or terminated.

25 In an embodiment, the treatment is selected from: a tuberculosis treatment, neurological condition treatment, a cancer treatment and an inflammatory bowel disease treatment. In an embodiment, the treatment is a tuberculosis treatment. In an embodiment, the treatment is a neurological condition treatment. In an embodiment, the treatment is a cancer treatment. In an embodiment, the treatment is an inflammatory bowel disease treatment.

30 In an embodiment, the treatment is selected from: an anti-TNFalpha antibody, infliximab, adalimumab, Bruton tyrosine kinase inhibitors, 2MHRZ/2MHR, 2EMRZ/2MR, paracetamol, antibiotic, anticonvulsants, statins, NSAIDs and a herbal/nutritional supplement. In an embodiment, the treatment is an anti-TNFalpha antibody. In an embodiment, the treatment is infliximab. In an embodiment, the treatment is adalimumab. In an embodiment, the treatment is Bruton tyrosine kinase inhibitors. In an embodiment, the treatment is 2MHRZ/2MHR. In an embodiment, the treatment is 2EMRZ/2MR. In an embodiment, the treatment is paracetamol.

35

In an embodiment, the method or use comprises re-testing at a later time point or testing at multiple time points to determine progression of a condition and/or response to treatment. Many therapeutic treatments can negatively impact the liver and cause damage. In an embodiment, the methods and uses as described herein can be used to monitor a subject's response to a treatment with an indication of being above upper limit normal ALT1 levels as an indication of when treatment should be adjusted, altered or ceased. In an embodiment, testing is daily. In an embodiment, testing is weekly. In an embodiment, testing is twice weekly. In an embodiment, testing is thrice weekly. In an embodiment, testing is monthly. In an embodiment, testing is bi-monthly. In an embodiment, testing is thrice monthly.

In an embodiment, testing occurs for at least one week. In an embodiment, testing is for at least two weeks. In an embodiment, testing is for at least 3 weeks. In an embodiment, testing is for at least 4 weeks. In an embodiment, testing is for at least 5 weeks. In an embodiment, testing is for at least 6 weeks. In an embodiment, testing is for at least 7 weeks. In an embodiment, testing is for at least 8 weeks. In an embodiment, testing is for at least 9 weeks. In an embodiment, testing is for at least 10 weeks. In an embodiment, testing is for at least 11 weeks. In an embodiment, testing is for at least 12 weeks. In an embodiment, testing is for at least 13 weeks. In an embodiment, testing is for at least 14 weeks. In an embodiment, testing is for at least 15 weeks. In an embodiment, testing is for at least 16 weeks. In an embodiment, testing is for at least 17 weeks. In an embodiment, the present invention provides a companion assay to monitor liver toxicity in pharmaceutical treatment.

In an embodiment, detection of the antigen binding protein complex above a threshold level indicates liver damage and/or liver disease in a subject. In an embodiment, the threshold is the upper limit normal (ULN). Although the ULN for ALT1 or ALT1+ALT2 can vary between jurisdictions, gender, time of day, health condition and ethnicity it is most commonly 40 IU/L. In some jurisdictions the, ULN for ALT1 or ALT1+ALT2 is 70 IU/L.

Table 1: Example methods and uses.

Method/use	Therapeutic drug	Example time points	ALT threshold	ALT1 link
Companion diagnostic				
Drug toxicity associated with TB treatment	2MHRZ/2MHR or 2EMRZ/2MR	0,2,4, 8,12, 17 weeks	5ULN alone or 3ULN + 2ULN bilirubin	Monitoring of ALT levels while under treatment to detect liver toxicity flares that may require treatment interruption/halt.
anti-TNFα treatment for IBD	anti-TNFα MAb generally	see below		Monitoring of ALT levels while under treatment to detect liver toxicity flares that may require treatment interruption/halt.

Inflammatory bowel disease	Infliximab	every 4 months when on maintenance treatment	3ULN	Monitoring of ALT levels while under treatment to detect liver toxicity flares that may require treatment interruption/halt.
Inflammatory bowel disease	adalimumab		3 ULN	Monitoring of ALT levels while under treatment to detect liver toxicity flares that may require treatment interruption/halt.
Health condition	Any agent that causes liver toxicity	Daily, weekly, monthly or as required	>2ULN or set limit as required	Monitoring of ALT levels while under treatment to detect liver toxicity flares that may require treatment interruption/halt.
Diagnostic screening tool				
Preeclampsia	None	3-9 months gestation	>1ULN	Monitoring of ALT levels during pregnancy to detect development of pre-eclampsia.
Pregnancy	None	0-9 months gestation	>1ULN	More regular testing of ALT levels during pregnancy might be required based on the results of this study that showed 10% of women had very high levels of ALT.
Paracetamol poisoning		Emergency department, ambulance, PoC	>2ULN	Most frequent cause for presentation to ED due to OD is paracetamol poisoning. Can cause acute and permanent liver damage, and in some cases a transplant may be required. Use case is for use in ambulance at PoC for patient triage, at ED for immediate diagnosis at treatment. Half of all acute liver toxicity is due to paracetamol in USA.
Infectious causes				
Hepatitis viruses A,B,C,D,E		Ad hoc	>1 ULN	Ability to monitor liver damage at PoC, esp in low resource settings.
SARS-CoV-2		Severe disease management		Cases of liver damage reported. Due to inflammation, but could be due to infection of bile duct which express ACE2.
Mononucleosis and CMV				Reported to cause liver flares.

ULN = upper limit of normal 3ULN = 3 times the upper limit of normal etc.

EXAMPLES

Example 1 – Methods

Generation of nanobodies and polyclonal antibodies

Alpacas were immunised six times with ALT1 over 42 days. The alpacas were bled on day 45 post-immunisation. Biopanning for ALT1 reactive nanobodies using phage display was performed as previously described with the following modifications (Pardon et al., 2014). Phages displaying ALT-specific nanobodies were enriched after two rounds of biopanning on 5 ng/mL and then 10ng/mL biotinylated ALT protein. After the second round of panning, individual clones were selected for further analyses by ELISA for the presence of ALT reactive nanobodies, on microtitre plates coated with 125nM non-biotinylated ALT1. Positive clones were sequenced and annotated using the International ImMunoGeneTics database and aligned in Geneious Prime.

To generate polyclonal antibodies to ALT, rabbits were immunized multiple times with purified ALT1. Immunoglobulins were purified from serum using protein G Sepharose. Further purification was performed using affinity purification with ALT1 coupled to Sepharose. Polyclonal rabbit anti-ALT1 antibodies were buffer exchanged into PBS and stored at -80°C.

Expression and purification of nanobodies

Nanobodies were expressed in *E. coli* WK6 in Terrific broth supplemented with 0.1% glucose and 100 µg/mL ampicillin at 37°C. Expression of nanobodies were induced by addition of 1 mM IPTG at 28°C overnight. Cells were harvested and resuspended in 200 mM Tris, 0.5 mM EDTA, 20% sucrose, pH 8.0 and incubated on ice for at least 1 h. Ice cold Milli-Q water was added and the cells incubated for at least a further 1 h on ice. The periplasmic extracts were isolated by centrifugation. Magnesium sulfate or magnesium chloride (usually 2-10 mM) was then added to prevent nickel ion-EDTA chelation. Nanobodies were purified by affinity chromatography using HisTrap FF column (Cytiva) with 20 mM Tris, 300 mM NaCl, 25 mM imidazole, pH 8.0 and elution with 20–500 mM imidazole gradient. Recombinant nanobody was further purified by gel-filtration chromatography using a Superose 12 10/30 (Amersham) pre-equilibrated with PBS if required. Otherwise, the appropriate fractions were concentrated and buffer exchanged into DPBS.

Expression and purification of ALT1 and ALT2

The amino acid sequence of human ALT1 and ALT2 was synthesised by GeneArt and subcloned into pET expression vectors using Gibson assembly (NEB). ALT1 with a C-terminal His-tag was subcloned into pET28a before the N-terminal His-tag and thrombin cleavage site. ALT2 (residues 49-523) was subcloned in to pET151 after the TEV cleavage site.

ALT1 was expressed in *E. coli* BL21 Star(DE3) cells (Invitrogen) in LB supplemented with 100 µg/mL ampicillin at 37 °C. Expression of ALT1 was induced by addition of 0.1 mM IPTG at 18 °C overnight. Cells were harvested, resuspended in 20 mM Tris, pH 8.0 containing 100 µM PLP, 1 mM phenylmethylsulfonyl (PMSF) and 0.2 mg/mL lysozyme and lysed by sonication (Branson). ALT1 was purified from clarified cell lysate by affinity chromatography using HisTrap FF (Cytiva) with 20 mM Tris, 300 mM NaCl, 25 mM imidazole, pH 8.0 and elution with 20–500 mM imidazole gradient. Recombinant ALT1 was further purified by gel-filtration chromatography using a Superose 12 10/30 (Amersham) pre-equilibrated with 25 mM HEPES, 150 mM NaCl, pH 8.0 or PBS.

ALT2-del was expressed in *E. coli* Rosetta 2(DE3) cells (Merck Millipore) in LB supplemented with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol at 37°C. Expression of ALT2-del was induced by addition of 0.1 mM IPTG at 18°C overnight. Cells were harvested, resuspended in 20 mM Tris, pH 8.0 containing 40 µM PLP, 1 mM phenylmethylsulfonyl (PMSF) and 0.2 mg/mL lysozyme and lysed by sonication (Branson). ALT1 was purified from clarified cell lysate by affinity chromatography using HisTrap FF (Cytiva) with 20 mM Tris, 300 mM NaCl, 25 mM imidazole, pH 8.0 and elution with 20–500 mM imidazole gradient. Recombinant ALT1 was further purified by gel-filtration chromatography using a Superose 12 10/30 (Amersham) pre-equilibrated with 25 mM HEPES, 150 mM NaCl, pH 8.0 or PBS.

Cloning, expression and purification of FLAG-tagged nanobodies

Nanobodies, including pelB signal peptide and C-terminal His-tag, were subcloned into pET28a before the N-terminal His-tag and thrombin cleavage site. FLAG-tag was introduced into pET28a constructs using the Q5 Site-Directed Mutagenesis Kit (NEB, E0554S) at the C-terminus of the nanobody before the His-tag. Successful incorporation of the tag was verified by dideoxynucleotide sequencing (Micromon, Monash University). FLAG-tagged nanobodies were expressed in BL21 Star(DE3) in autoinduction media at 28°C overnight and purified as described above for the untagged versions.

Cloning, expression and purification of bivalent nanobodies

Bivalent nanobodies were cloned by inserting a second nanobody gene between the pelB sequence and the original nanobody gene. GGGGS linkers with three or four repeats were engineered between the two nanobodies using PCR overhangs before the construct was assembled using Gibson Assembly (NEB). Bivalent nanobodies were expressed in C41(DE3) in autoinduction media at 20°C overnight and purified as described above for the monomeric nanobodies.

Cloning, expression and purification of bivalent nanobody-Fc fusion proteins

Bivalent nanobody genes were optimised for mammalian cell expression using GenSmart™ Codon Optimization tool (Genscript) and synthesised by GenScript. Bivalent nanobodies were cloned into the pcDNA3 expression vector (Invitrogen) between a tissue plasminogen activator leader sequence and the Fc region of human Fc IgG1 using NEBuilder HiFi DNA Assembly (NEB).

Quadrivalent nanobody-Fc fusion proteins were expressed in Expi293 cells (the expressed nucleic acid encodes a bivalent monomer that when expressed produces a quadrivalent nanobody Fc fusion protein) (Gibco) maintained in suspension at 37°C and 8 % CO₂. The cells (3 x 10⁶/mL) were transfected with 1 µg DNA/mL of culture with ExpiFectamine™293 Reagent following manufacturer's instructions (Gibco). The day after transfection, ExpiFectamine™293 transfection Enhancer 1 and 2 were added. The culture supernatant was collected by centrifugation and filtered five days post-transfection and diluted with 10x PBS (Gibco). Culture supernatant was incubated with Protein G sepharose (GenScript), the resin washed with PBS and recombinant protein eluted using 0.1 M glycine, pH 2.5 and immediately neutralised with 2 M Tris, pH 8.3.

Avidin-capture ELISA

Maxisorp plates (96 well, Nunc) were coated overnight at 4°C or 3-4 h at ambient temperature with 5 µg/mL avidin in carbonate-bicarbonate buffer (Sigma-Aldrich, C3041). The wells were blocked for 1 h with 1.5% (w/v) BSA in PBS-T (PBS with 0.05% Tween-20) before incubation with biotinylated proteins (ALT1, ALT2, AST) at 1 µg/mL in ELISA buffer (0.5% BSA (w/v) in PBS-T) for 1 h. The wells were subsequently incubated with serial dilutions of anti-ALT1 nanobodies, anti-ALT1 quadrivalent nanobody-Fc, anti-ALT1 rabbit-polyclonal or anti-ALT1 rabbit monoclonal antibody in ELISA buffer for 1 h. Wells containing anti-ALT1 nanobodies were further incubated with mouse anti-FLAG antibody (Sigma-Aldrich, F1804, 1/1000) for 1 h, before incubation with HRP-conjugated anti-mouse secondary antibody (Sigma-Aldrich, A3415, 1:1500) in ELISA Buffer for a further 1 h. Wells containing quadrivalent nanobody-Fc were further incubated with HRP-conjugated anti-human IgG antibody (DAKO, P0214, 1:10000). Wells containing rabbit anti-ALT1 antibodies were incubated with HRP-conjugated anti-rabbit secondary antibody (Sigma-Aldrich, A1949, 1/50000). Bound protein was quantified using KPL Sure Blue Reserve TMB Microwell Peroxidase Substrate (Seracare) and the reaction stopped by addition of 0.5 M sulfuric Acid. The optical densities at 620 nm and 450 nm were measured using a Multiskan SkyHigh reader (ThermoFisher) and the OD (450 nm -620 nm) calculated. All incubations were conducted at room temperature unless specified. Wells were washed three times with PBS-T after each step.

For initial screening of monoclonal nanobody reactivity to ALT1, the ELISA was performed as outlined above, however, ALT1 was added to avidin-coated plates at a dilution of 1/200 for 1 h. Nanobodies were subsequently added at a single dilution of 15 µg/mL for 1 h. Screening of bivalent nanobodies to ALT1 was also performed using avidin capture assay as
5 outlined above, with ALT1 at a dilution of 1/1000 and bivalent nanobodies added at a single dilution of 1 µg/mL. In both assays, bound nanobodies were detected by incubation with anti-mouse anti-His (1/1000) and subsequent species-specific HRP-conjugated antibody.

Direct ELISA

10 Maxisorp plates (96 well, Nunc) were coated overnight at 4 °C with 1 µg/mL ALT1, ALT2 or AST in carbonate-bicarbonate buffer (Sigma-Aldrich, C3041). The wells were blocked for 1 h with 1.5% (w/v) BSA in PBS-T and subsequently incubated with serial dilutions of anti-ALT1 nanobody for 1h. Bound anti-ALT1 nanobody was detected using HRP-conjugated anti-llama secondary antibody (Invitrogen, 1/2000). Plates were developed and
15 measured as outlined above.

Nanobody modelling

Structures of nanobodies were modelled using AlphaFold2 (Jumper et al., 2021) via ChimeraX (Pettersen et al., 2021). Figures were generated using The PyMOL Molecular
20 Graphics System, Version 2.5.3 Schrödinger, LLC.

SDS-PAGE gels and western blot

SDS-PAGE analysis was conducted using Bolt™ Bis-Tris Plus Mini Protein Gels, 4-12% (Invitrogen) with Bolt MES SDS Running Buffer (Invitrogen). Western blot transfer to
25 nitrocellulose membrane was carried out with iBlot 2 Western Blot Transfer Device (Invitrogen). The membrane was blocked in 3% skim milk in TBS-T (TBS + 0.05% Tween-20) for 1 h before it was incubated in primary antibody, anti-His (Rockland, 600-401-382, 1:1000) in blocking buffer for 1 h. The membrane was washed with TBS-T and incubated with anti-rabbit/HRP (Sigma, A1949, 1:15000) in blocking buffer for 1 h. The membrane was washed
30 before addition of ECL Prime (Amersham) and visualisation on a ChemiDoc Imaging System (Bio-Rad).

Generation of monoclonal antibodies

To generate rabbit monoclonal antibodies, peripheral blood mononuclear cells from
35 ALT1 immunized rabbits were isolated and positive B cells enriched, cultured, cloned and sequenced (Genscript). The resulting supernatants were screened and the top binders sequenced

and cloned into pcDNA3.4 for recombinant protein production. Sequence analysis of heavy and light chain variable domains was performed using the IMGT/V QUEST server (Brochet et al., 2008).

5 *Expression and purification of monoclonal antibodies*

Rabbit monoclonal antibodies were expressed in Expi293 cells (Gibco) and purified using Protein G Sepharose in the same manner as described for nanobody-Fc fusion proteins.

Cloning and expression of ALT1 mutants for epitope mapping

10 ALT1 alanine mutants were generated by Genscript using the ALT1-cHis-pET28 backbone. ALT1 mutants were expressed in *E. coli* BL21 Star(DE3) (Invitrogen) in Luria-Bertani broth (LB) supplemented with 100 µg/mL ampicillin at 37 °C. Expression of ALT1 was induced by addition of 0.1 mM IPTG at 18 °C overnight. Cells were harvested and resuspended in 1/25 culture volume of BugBuster Master Mix (Merck, 71456-4). After 10 min, the lysed
15 cells were centrifuged at 20000g for 10 min at 4 °C and the supernatant removed. This clarified lysate was used in epitope mapping experiments.

X-ray crystallography

ALT1-C8 complexes were crystallised using the hanging drop vapour diffusion method
20 with drops containing 1 µL of protein solution (10 mg/mL in 25 mM HEPES, 150 mM NaCl, pH 7.5) and 1 µL of reservoir solution (19% PEG 1.5 K v/v, 20% glycerol v/v) at 20 °C as described in Brochet et al., 2008 and Giudicelli et al., 2011. Crystals appeared within 7 days. For X-ray data collection, the crystals were soaked in cryoprotectant solution containing reservoir solution with PEG 1.5 K increased to 24% v/v and directly flash cooled in liquid
25 nitrogen.

Data were collected for 200° using 0.954 Å at 100 K with an EIGER x 16 M detector with 0.1 degree per frame with 0.02 s exposure at the Australian Synchrotron on the MX2 Beamline (Aragao et al., 2018). The data was indexed and integrated using XDS (Kabsch, 2010) and scaled using AIMLESS (Evans & Murshudov, 2013) to 2.7 Å resolution, belonging to space
30 group P4₁2₁2 with cell dimensions of a = 146.846 Å, b = 146.846 Å, c = 157.369 Å and α = 90.00°, β = 90.00° and γ = 90.00°. Matthews coefficient predicted two ALT1-C8 complexes per asymmetric unit.

The structure of the ALT1-C8 complex was solved using molecular replacement using AlphaFold2 (Jumper et al., 2021) models of ALT1 and the C8 nanobody as the search model.
35 Structural refinement of the resulting complex model performed using REFMAC5 (Murshudov et al., 2011) and Phenix (Liebschner et al., 2019) with iterative model building with COOT

(Emsley et al., 2010). Structure quality was assessed by MolProbity (Williams et al., 2018). Analysis of ALT1-C8 interface was conducted using PISA (Krissinel & Henrick, 2007). Molecular figures were generated using PyMOL (DeLano, 2002).

5 *Biolayer interferometry (BLI)*

Affinities of the nanobodies to ALT1 were measured using an Octet RED96 instrument (ForteBio). Assays were performed using Streptavidin (SA) or Ni-NTA capture sensors (Sartorius) with kinetics buffer (20mM HEPES, 150 mM sodium chloride pH 7.5, supplemented with 0.1 % BSA and 0.05% Tween 20). All experiments were run at 25 °C in solid black 96-well plates (Greiner). To measure binding to ALT1, following a 180 s baseline step ALT1 (5 µg/mL) was loaded onto the SA sensor until a response signal of 1 nm was achieved for each sensor. Sensors were washed in kinetics buffer for 180 s and association of nanobodies or polyclonal anti-ALT1 antibody was performed using a 1/3 dilution series starting at 1000 nM by submerging sensors for 600 s. Following association, dissociation was measured in kinetics buffer for 600 s.

To measure the interaction of ALT1 with nanobodies, following a 180 s baseline step, respective nanobodies (5 µg/mL) were loaded on to the Ni-NTA sensor until a response signal of 0.5 nm was achieved for each sensor. Sensors were washed in kinetics buffer for 180 s and association of ALT1 was performed using a 1/3 dilution series starting at 1000 nM by submerging sensors for 600 s. Following association, dissociation was measured in kinetics buffer for 600s. Sensors were regenerated using a cycle of 10s in either 10 mM Glycine, pH 1.7 or 300 mM imidazole, followed by 10 s in kinetics buffer, repeated 5 times. Data was fitted using the Octet Analysis Software (Forte Bio) using a global fit 1:1 model to determine kinetics and KD values.

In-tandem binning was performed using Ni-NTA biosensors (Sartorius). ALT1-cHis were diluted in kinetic buffer to 5µg/ml and immobilised onto Ni-NTA for 600s. Saturating antibodies were associated at 300nM for 600s. With complete self-blocking ensured, competing antibodies at 150nM were associated for 600s and dissociated for 600s. Epitope binning analysis was constructed using Octet Analysis Studio 13.0.3.52 software.

Quantitation of expressed protein in ALT1 mutant lysate was performed via BLI using an Octet RED96 instrument (ForteBio). Experiments were run at 25 °C in solid black 96-well plates (Greiner) with Ni-NTA capture sensors (Sartorius) activated in kinetics buffer (1X Dulbecco's Phosphate-Buffered Saline (DPBS) supplemented with 0.1 mg/mL BSA and 0.002 % Tween 20). Varying concentrations (0 to 80 µg/mL) of purified recombinant His-tagged ALT1 were immobilised to the sensors and a standard curve was generated using the binding rate. Next, His-tagged ALT1 mutants in lysate diluted at 1:10 in kinetics buffer were

immobilised and the resulting maximum binding rate was then used to determine the concentration of ALT1 mutant in the lysate. Results were analysed with Octet Analysis Studio 13.0.3.52 software (Sartorius).

Additionally, to evaluate whether C4G-Fc, C8-Fc, G6-Fc and the rabbit monoclonal antibodies (RmAb1, RmAb3, RmAb4, RmAb5) have varied or overlapping epitopes, BLI was also used to perform epitope binning. 5 µg/mL of purified recombinant His-tagged ALT1 was loaded onto Ni-NTA sensors for 300s and a saturating concentration (300 nM) of Antibody 1 (saturating antibody) was allowed to associate for 600 s. This was then competed off with Antibody 2 (competitor antibody) for 600 s at the following concentrations [C4G-Fc (90 nM); C8-Fc (90 nM); G6-Fc (200 nM); RmAb1, RmAb3, RmAb4, RmAb5 (66.7 nM); 3H12, 4A9, 5H2, 6B5 (150 nM)]. Epitope binning analysis was constructed using the Octet Analysis Studio 13.0.3.52 software (Sartorius).

Differential scanning fluorimetry (DSF)

Differential scanning fluorimetry was used to assess protein thermostability (Niesen et al., 2007). Protein (10 µg) was diluted into 25 µL 1x PBS with 2.5 µL of 5x concentration SYPRO Orange Protein Gel Stain (Sigma Aldrich) in duplicate. The samples were then heated in an QuantStudio 7 Real-time qPCR System (Thermo Fisher) in 0.5°C increments from 25°C to 95°C for 1 minute per increment. Measurements of fluorescence were taken at the end of each increment. Excitation was at 492 nm, and emission at 610 nm. The T_m was determined to be the minimum of the negative first derivative of the melting curve.

Direct ELISA with ALT1 mutant lysate

High binding plates (96 well half area, Nunc, Corning 3690) were coated overnight at 4°C with ALT1 mutants lysate at 1.25 µg/mL in carbonate-bicarbonate buffer (Sigma-Aldrich, C3041) with. The wells were blocked for 1 h with 1.5% (w/v) BSA in PBS and subsequently incubated with G6-Fc at 0.1 µg/mL for 1 h. Bound anti-ALT1 nanobody fusion was detected using HRP-conjugated anti-human IgG (Dako, 1/10000). Plates were developed and measured as outlined in ELISA above.

Anti-His capture ELISA with ALT lysate.

High binding plates (96 well half area, Nunc, Corning 3690) were coated overnight at 4-8°C or 3-4 h at ambient temperature with mouse anti-Histidine Tag (Bio-RAD, MCA1396) at 1 µg/mL in carbonate-bicarbonate buffer (Sigma-Aldrich, C3041) with. The wells were blocked for 1 h with 1.5% (w/v) BSA in PBS and subsequently incubated with ALT1 mutant lysate at 1.25 µg/mL in ELISA diluent buffer. The wells were subsequently incubated with different concentrations of anti-ALT1 nanobody fusion proteins or anti-ALT1 rabbit

monoclonal antibody in ELISA diluent buffer for 1 h. Wells containing nanobody-Fc were further incubated with HRP-conjugated anti-human IgG antibody at 1:10000 or 1:20000 dilution (DAKO, P0214 or Millipore, AP504P 1:20000). Wells containing rabbit anti-ALT1 antibodies were incubated with HRP-conjugated anti-rabbit secondary antibody (Sigma-
5 Aldrich, A1949, 1/10000). Plates were developed and measured as outlined in ELISA above.

Conjugation of detectors for lateral flow

Nanobodies, nanobody-Fc fusions, anti-ALT rabbit monoclonal and anti-ALT1 rabbit polyclonal were conjugated to Europium Fluorescent Functionalized Microspheres (Merck, F1-
10 EU 030) using AnteoBind Nano Kit (AnteoTech, A-PCKS) according to the manufacturers' instructions. Nanobodies were conjugated at 75 µg/mg particles while anti-ALT1 polyclonal or monoclonal were conjugated at 30, 50 or 100 µg/mg particles.

Nanobodies, nanobody-Fc fusions, anti-ALT rabbit monoclonal and anti-ALT1 rabbit polyclonal were conjugated to gold nanoshells using the BioReady High Sensitivity Gold
15 Conjugation Kit for Lateral Flow (NanoComposix, GSZR150-10M) according to the manufacturers' instructions. 30 µg of antibody were conjugated per 1 mL of nanoparticles.

Rabbit monoclonal anti-ALT antibodies were conjugated to Estapor Blue Intense and Black particles using AnteoBind Nano Kit (AnteoTech, A-PCKS) or Anteobind NXT Kit (Anteobind, A-LNXTK-5) according to the manufacturers' instructions. 2, 5, 20, 50 µg or 150
20 µg were conjugated per 1 mg of nanoparticles.

Preparing nitrocellulose membranes for lateral flow assays

Lateral flow strips were prepared by striping two lines across a nitrocellulose membrane (NCM) (Vivid 90, Pall Corporation) using an IsoFlow dispenser (Imagene Technology). The
25 first line, test line, was stiped with monovalent, quadrivalent or Fc-fusion nanobody (Burnet Institute) at 0.05, 0.1, 0.2, 0.5 or 1 mg/mL. The second line, control line, was stiped with 50 or 100 µg/mL recombinant ALT1 protein (Burnet Institute).

Assembling and running lateral flow assays in wet system

30 For wet system testing, the striped NCM was laminated together with a 10 mm sample pad (1285 or 1281, Ahlstrom) and absorbent pad (CF6 or CF5 pad, GE Healthcare) on an adhesive backing card and cut into 4 or 5 mm test strips using a guillotine cutter (Kinbio Shanghai Kinbio Tech).

To run an assay, samples are prepared in wells of a 96-well plate to a total volume of 30
35 µL. Recombinant ALT1 was diluted to 0, 0.1, 0.25, 1 or 10 µg/mL or at a final total amount of 0 to 10 ng and combined with 6 µL Gold nanoshell detector (Burnet Institute), Europium

detector to a final concentration of 2.5 or 1.25 µg/mL, or 15 µL Estapor Blue Intense or Black detector at various dilution in the sample well. Plasma samples tested were 5 µL in volume. Running buffer (20 mM HEPES, 150 mM NaCl, pH 7.5, 0.5% Tween-20, 0.1% BSA) was added to make up the final volume of 30 µL. Test strips were placed into each sample well of the 96-well plate with the sample pad at the bottom. After 8 or 10 minutes, 30 µL of running buffer are added to the wells of the 96-well plate to reduce non-specific binding and the assay run for a further 8 or 10 minutes. Depending on the conjugate used for detection, the intensity of the test line can be assessed visually (Gold conjugate, Estapor Blue Intense or Black) or preferably using an automated reader such as the Axxin reader (AX-2X, Axxin Ltd Melbourne) or Lumos Leelu reader (Lumos Diagnostics, Melbourne). This method is also used for detection of europium-conjugates which are not visible by eye. The reader gives a numerical readout for each test line, as well as a photograph of the test strip.

The test results were interpreted through visual assessment of the photographed test strips, as well as by plotting the numerical readout of the test line intensities against the spiked ALT1 concentration.

Lateral flow in cassettes - Assembly of anti-GPA schematics

Europium detector was diluted 1/250 in BD2 conjugate drying buffer (10 mM borate, 2 mM EDTA, 0.25% Tween-20, 20% sucrose, 5% trehalose, 1% BSA, 0.35% PEG8000) or 20 mM Tris, pH 8.0, 1% BSA, 0.1% Tween 20, 10% sucrose, 5% trehalose and sprayed (0.8 µL/mm) onto a glass fibre pad (8951, Ahlstrom) using an IsoFlow dispenser (Imagene Technology). Anti-glycophorin A was diluted in BD2 conjugate drying buffer to 0.185 mg/mL and sprayed as described for the detector at 3 µL/mm twice onto the same glass fibre pad and dried at 37°C overnight.

The NCM, striped as outlined above, was then laminated together with the glass fibre pad containing blood capture reagent anti-glycophorin A (anti-GPA) and europium conjugated rabbit anti-ALT1 at the bottom of the NCM and an absorbent pad (CF5 absorbent pad, GE Healthcare) at the top of the NCM. All components were laminated together on an adhesive backing card and cut into 4 mm test strips using a guillotine cutter (Kinbio Shanghai, Kinbio Tech). The strips were assembled into a disposable plastic housing (AtomoRapid™ Diagnostics).

Lateral flow in cassettes - Assembly of blood retention pad schematics

Europium detector (europium conjugated polyclonal rabbit anti-ALT1 or europium conjugated monoclonal rabbit anti-ALT1) was diluted in BD2 conjugate drying buffer or 20

mM Tris, pH 8.0, 1% BSA, 0.1% Tween 20, 10% sucrose, 5% trehalose (1/250, 1/500) and dispensed (0.8 μ L/mm) onto a glass fibre pad (8951, Ahlstrom) and dried at 37°C overnight.

The NCM, striped as outlined above, was then laminated together with the glass fibre pad containing europium detector and a blood retention pad (FR1 (0.35 mm, mdi Membrane Technologies INC) at the bottom of the NCM and an absorbent pad (CF5 absorbent pad, GE Healthcare) at the top of the NCM. Alternatively, the NCM was laminated together with a rehydration pad (8951 glass fibre, Ahlstrom), followed by the glass fibre pad containing europium detector and a blood retention pad (FR1 0.35 mm, mdi Membrane Technologies, INC) at the bottom of the NCM and an absorbent pad (CF5 absorbent pad, GE Healthcare) at the top of the NCM.

For test strips containing Estapor Blue Intense detector, the detector was diluted in 20 mM Tris, pH 8.0, 1% BSA, 0.1% Tween 20, 10% sucrose, 5% trehalose (1/10, 1/15 or 1/20) and sprayed (0.8 μ L/mm) onto a glass fibre pad (8951, Ahlstrom) and dried at 37 °C overnight. The NCM, striped as outlined above, was laminated together with the glass fibre pad containing Estapor Blue Intense detector, two overlaid blood retention pads (FR1 (0.35 mm, mdi Membrane Technologies INC) at the bottom of the NCM and an absorbent pad (CF5 absorbent pad, GE Healthcare) at the top of the NCM.

In each schematic assembly all components were laminated together on an adhesive backing card and cut into 4 mm test strips using a guillotine cutter (Kinbio Shanghai Kinbio Tech). The strips were assembled into a disposable plastic housing (AtomoRapid™ Pascal, Atomo Diagnostics).

Running lateral flow assays in AtomoRapid™ Pascal cassettes

To run an assay using venous blood spiked with ALT, healthy human volunteer venous blood is spiked with different concentrations of ALT (0, 1, 2, 5 ng per 10 μ L). Using a pipette, 10 μ L or 11.5 μ L of spiked venous blood is delivered to the blood collection unit (BCU) of the cassette and the BCU moved to the sample area to deliver the sample. Approximately 3-10 seconds later the blister is burst to start the buffer flow across the test strip. The sample and buffer are allowed to diffuse laterally for 20 minutes. After 20 minutes the test is visualized and quantitated using an automated reader, such as the Lumos Leelu reader or Cerberus reader (Lumos Diagnostics, Melbourne).

To run assays without the buffer blister pack, the assay is run as described above, however, 85 μ L of running buffer (20 mM HEPES, 150 mM NaCl, pH 7.5, 0.5% Tween-20, 0.1% BSA, with or without ProClin 300) is added through a hole drilled in the top casing where the blister pack well is located rather than bursting the blister.

Dot blot

12.5 ng to 100 ng of ALT1 in 1 μ L was spotted on to nitrocellulose membrane (Amersham, RPN303E) and allowed to dry. The blot was blocked with 3 % skim milk for 3 h at RT or overnight at 4 °C and incubated with antibody (1 μ g/mL in blocking buffer) for 2 h at RT or overnight at 4 °C. The blots were incubated with either anti-rabbit/HRP (1:5000, Sigma, A1949), anti-human/HRP (1:1000, DAKO, P0214) or anti-mouse/HRP (1:5000, Sigma, A2554) for 1 h at RT. For FLAG-tagged nanobodies the blots were first incubated with anti-FLAG (1:5000, Sigma, F1804) for 1 h before incubation in anti-mouse/HRP. Blots were washed with PBS + 0.05% Tween-20 three times for 5 min between steps. The blots were visualised with ECL (Amersham, RPN2232) using a Bio-Rad ChemiDoc MP. Commercial rabbit anti-ALT monoclonal antibody used was Abcam EPR19616.

Example 2 – Isolation of nanobodies reactive to ALT1

The phage display libraries were screened for nanobodies reactive to ALT1 in ELISA in two panning experiments. In experiment one, 94 clones were screened for reactivity to ALT1 coated ELISA plates. Of these, 91 were positive to ALT1 of which 86 had full length VHH domains. Of these, 13 had unique variable domains, of which 2 nanobodies had unique complementarity determining region (CDR) 3 domains (**Figure 2A**). These two nanobodies were Nb_C8 and Nb_G6. Nb_C8 was unique with only one clone identified that had this exact CDR3 sequence. The CDR3 domain of Nb_G6 was the dominant nanobody in the library. A second experiment was performed, and 188 clones were selected of which 60 had full length VHH sequences (**Figure 2B**). When the second round nanobodies were purified, none retained significant activity against ALT1.

Nb_C8 was unique within this library with only a single clone having the same CDR3 region. Nb_G6 was the dominant clone isolated in this library with 82 of 91 clones possessing the same CDR3 sequence (**Figure 3**).

An ELISA was performed on a selection of nanobodies with unique CDRs. The results show that Nb_C8 and Nb_G6 have very strong binding to ALT1, while Nb_C10 has very low binding. All other nanobodies screened show no binding to ALT1 (**Figure 4**). For comparison, the binding of the nanobodies to bovine serum albumin (BSA) is shown which represents the background of the assay.

The protein coding sequence was annotated showing the predicted boundaries of the framework (FR) and complementarity determining regions (CDR) for the Nb_C8 and Nb_G6, which show strong binding to ALT1, and nanobody Nb_C10, which shows weak binding to ALT1 (**Figure 5A, 5B, 6A, 6B and 7**).

The sequences were analysed for the percent identity between the nanobodies. Analysis of nanobodies that bind strongly to ALT1 versus a nanobody that does not bind strongly to ALT1 shows that the Nb_C8 (strong binder) and Nb_C10 (which shows no/low binding to ALT1) are only 71% identical and Nb_G6 and Nb_C10 are 77.0% identical. Strong ALT1 binders, Nb_C8 and Nb_G6 are 76% identical. Results are shown in **Figure 8**.

Usage of the *Vicugna pacos* (Alpaca) V-gene, J-gene and D-gene was determined for Nb_G6 and Nb_C8. For Nb_G6, the V-gene used is IGHV3-3*01-F with 95.83% identity to the germline sequence, while Nb_C8 uses the same gene with 87.15% identity. This suggests the degree of hypermutation for Nb_C8 is higher than Nb_G6. This hypermutation created a large deletion in CDR3 present in Nb_C8. Nb_G6 uses J-gene J6*01 while Nb_C8 uses J4*01, both with 89.36% identity to the germline sequence (**Figure 9**).

An alignment between Nb_G6 and Nb_C8 was performed, highlighting differences in the protein coding sequence. The largest difference evident between these nanobodies is in the CDR3, where Nb_C8 has a six amino acid deletion relative to Nb_G6. The CDR1 and 2 regions are also very different between these nanobodies which may also contribute to differences in binding properties. Point mutations are also present in FR1, 2 and 3 which may also alter their binding properties (**Figure 10**).

An alignment between Nb_C8 and Nb_C10 was performed, demonstrating that the sequences are 68.2% identical and 74.4% similar showing that most differences are present in CDR1, 2 and 3. The CDR3 of Nb_C8 has an eight amino acid deletion relative to Nb_C10 (**Figure 11**).

An alignment between Nb_C10 and Nb_G6 was performed, demonstrating that the sequences are 76.0% identical and 80.6% similar showing that most differences are present in CDR1, 2 and 3. The CDR3 region of Nb_G6 has a three amino acid deletion relative to Nb_C10 (**Figure 12**).

Two major differences exist in the Alphafold2 predicted three-dimensional structures. One difference is located within CDR1 where Nb_C10 and Nb_C8 have predicted helical content, while the CDR1 of Nb_G6 is predicted to be a loop. The second major difference in predicted structure is located in CDR3. The predicted orientation of the CDR3 loop for Nb_G6 and Nb_C8 differs significantly to the predicted orientation observed for Nb_C10. The deletion in CDR3 observed in Nb_C8 is reflected in a shorter, more compact loop structure with a short helical segment at residues LRV that is unique to this Nb. In addition, the C-terminal segment of CDR3 in Nb_C8 does not contain a helix; Nb_G6 and Nb_C10 both contain a short beta-strand at the end of CDR3 although they are in different orientations (**Figure 13A and 13B**).

Example 3 – Activity of nanobodies

Nanobody sequences can be modified through the addition of sequences to facilitate site specific changes such as biotinylation, chemical cross-linking, conjugation to a detection reagent or addition of Fc domains. In this example, Nb_C8 and Nb_G6 have been modified through the addition of a His tag, FLAG-tag or Avi-tag. The expression of these modified nanobodies was assessed by SDS-PAGE (**Figure 14**).

Binding activity of Nb_G6 was assessed in an ELISA against ALT1, ALT2 and AST. Nb_G6 containing a C-terminal FLAG-tag binds strongly to ALT1, with the amount of Nb required to achieve 10-times the background signal being 0.008 µg/mL towards ALT1, and 1.15 µg/mL towards ALT2. The binding of Nb_G6 was ~140 times higher towards ALT1 than ALT2. No binding was detected to AST (**Figure 15A**).

Binding activity of Nb_C8 was assessed in an ELISA against ALT1, ALT2 and AST. Nb_C8 containing a C-terminal FLAG-tag binds strongly to ALT1, with the amount of Nb required to achieve 10-times the background signal being 0.003 µg/mL towards ALT1, and 6.5 µg/mL towards ALT2 and AST. The binding of Nb_C8 was ~2,200 times higher towards ALT1 than ALT2 and AST (**Figure 15B**).

Binding activity of rabbit polyclonal antibodies raised to ALT1 was assessed in an ELISA against ALT1, ALT2 and AST. Rabbit polyclonal antibodies are an example of polyclonal reagents raised to ALT1 in the prior art and such reagents which contain a mixture of antibody specificities do not show high selectivity in binding to ALT1 versus ALT2. The amount of rabbit polyclonal antibody required to achieve 10-times background binding to ALT1 was 0.011 µg/mL while 0.084 µg/mL was required for 10 times background binding to ALT2 and 0.569 µg/mL was required to bind AST. The relative amount of rabbit polyclonal antibody required to achieve 10x background binding was only ~8-times higher for ALT2 and 51 times higher for AST compared to ALT1 (**Figure 15C**).

Binding activity of a commercial rabbit monoclonal antibody raised to ALT1 was assessed in an ELISA against ALT1, ALT2 and AST. Rabbit monoclonal antibodies are commercially available reagents raised to ALT1 and do not show high selectivity in binding to ALT1 versus ALT2. The amount of rabbit monoclonal antibody required to achieve 10-times background binding to ALT1 was 3.8 µg/mL while 0.477 µg/mL was required for 10 times background binding to ALT2 and no binding was observed to AST. This reagent appears to preferentially bind ALT2 with 8 times higher binding than ALT1 (**Figure 15D**).

Nanobodies raised to ALT1 are highly selective for binding to ALT1 and show greatly reduced activity towards ALT2 (**Figure 16**). These properties enable a greater degree of specific detection of ALT1 which is the predominant ALT species produced in liver damage and constitutes more than 90% of ALT species in blood.

The protein coding sequences of human ALT1 and ALT2 isoform 1 were aligned using a multiple sequence alignment tool (Clustal O). The two forms of ALT exhibit 67% amino acid identity (**Figure 17**). Polyclonal antibodies which contain multiple different antibody specificities bind to both ALT1 and ALT2 while Nb_C8 and Nb_G6 show a greater than 100-fold preference in binding to ALT1 suggesting their epitopes are not within highly conserved regions of the ALT protein sequence.

The protein coding sequences of ALT1, ALT2 isoform 1, ALT2 isoform 2 and AST were aligned using a multiple sequence alignment tool (Clustal O). AST shows 20% sequence homology to ALT1 and ALT2 and does not show any binding to nanobodies directed towards ALT1. ALT1 and ALT2 share 68% amino acid identity. There is a 27 amino acid deletion at the N-terminus of ALT1 relative to ALT2 isoform 1. ALT2 isoform 2 has a larger N-terminal deletion of 100 amino acids relative to ALT2 isoform 1. AST has N and C terminal truncations relative to ALT1 and ALT2 (**Figure 18**).

Example 4 – Multivalent nanobodies

Nanobodies to ALT1 can be modified to enhance their ability to capture and detect ALT in human samples. Such modifications include joining of nanobody sequences together and conjugating nanobodies to carrier proteins to enhance binding properties such as avidity and polyspecificity. Such modifications can enhance the sensitivity of detection of ALT in human samples and enable the development of tests to monitor ALT levels in human clinical samples.

In order to increase avidity and polyspecificity to ALT1, Nb_C8 and Nb_G6 were joined to themselves or each other via different linker sequences comprising highly flexible glycine and serine residues. Alternative lengths of linkers (3 repeats of GGGGS or 4 repeats of GGGGS) provide a greater distance between paratopes of the nanobodies and flexibility between VHH regions allowing bispecific binding (**Figure 19**). Alternative linkers known to those skilled in the art can also be used to join Nb_C8 and Nb_G6 for desired properties.

The expression of bivalent nanobodies from bacterial cells was assessed by SDS-PAGE/Western Blot which confirmed the increase in expected molecular mass from ~14 to 28 kDa. Different conditions were explored to optimise yield of nanobody and show consistent production of 28 kDa species containing two 14 kDa Nb VHH sequences (**Figure 20**).

The relative binding ability of bivalent nanobodies to ALT1 was measured at 1000 ng/mL in ELISA. The parental nanobodies, Nb_C8 and Nb_G6 are shown for comparison. Examples where bivalent nanobodies show increased binding towards ALT1 are G4G, C4G, G3C, G4C, C3C and C4C (**Figure 21**).

A biolayer interferometry (BLI) experiment that measures the association, dissociation and affinity constants of receptor-ligand interactions was performed. ALT1 was immobilised

on avidin sensors and the binding of each nanobody to the sensors was measured in real-time. The association and dissociation curves are shown for Nb_C8, showing rapid association to ALT1 and relatively fast dissociation compared to a polyclonal immune serum raised to ALT1. Nb_G6 does not bind to ALT1 in this orientation. Interestingly, Nb_C8 had a faster on-rate compared with the polyclonal antibody suggesting it may access its epitope faster than rabbit immunoglobulins (**Figure 22A**). This is a feature of nanobodies known in the literature.

A kinetic biolayer interferometry experiment for bivalent nanobodies G4C, C4C, C4G, and monovalent nanobodies Nb_C8 and Nb_G6 was also performed. Here it can be seen that different linkers and different combinations of nanobody alter the association and dissociation towards ALT1 (**Figure 22B**). Such differences can influence their applicability to different assay systems such as plate-based assays, biosensor-based assays, and lateral flow assays.

The association (k_{on}) and dissociation (k_{dis}) for the nanobodies using a 1:1 model of binding was assessed using the results of the biolayer interferometry experiments (**Figure 22B**). Bivalent nanobodies showed improved (slower) off-rates towards ALT1 relative to Nb_C8, with G4C and C4C displaying faster on rates than monovalent nanobody Nb_C8. Together, these improvements to on and off rates of bivalent nanobodies results in a net increase in the affinity constant (**Figure 23**).

Example 5 – Translation into practice

The nanobodies as described in the previous examples can be used in a lateral flow format for the detection of ALT1. For example, the nanobody is conjugated to a visible or fluorescent molecule such as colloidal gold, gold nanoshells, europium and other examples known to those skilled in the art. Alternatively an immunoglobulin, monoclonal or polyclonal, can be conjugated to a visible or fluorescent molecule such as colloidal gold, gold nanoshells, europium and other examples known to those skilled in the art. These are applied to a conjugate pad and interact with ALT present in serum, plasma or blood samples upon contact. Addition of a running buffer then allows the nanobody-conjugate-ALT complexes to flow along the nitrocellulose where they are then captured by a second nanobody striped onto the nitrocellulose membrane. If nanobody-conjugate-ALT complexes are present these will be retained on the nanobody stripe (line indicated by 'T'). Free nanobody-conjugate then flows through to a control line (line indicated by 'C') where ALT is striped on the line and captures the free-conjugate confirming the test result is valid (**Figure 24**).

A nanobody (Nb_G6) to ALT1 can be used to detect ALT1 when striped on nitrocellulose and compared with detection with polyclonal antibody to ALT1. Antibodies were conjugated to europium and used to detect different concentrations of ALT1 protein striped directly onto nitrocellulose (**Figure 25**).

Nb_C8_FLAG was used to capture different amounts of ALT1 applied to a lateral flow test. Here different amounts of Nb_C8_FLAG were striped onto nitrocellulose ranging from 0.1 mg/mL to 1 mg/mL. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with europium conjugated to a polyclonal antibody to ALT1 and allowed to diffuse laterally. ALT1 was captured by the Nb_C8_FLAG stripe and residual europium conjugated antibody was then captured by the ALT control line. The results show that at 0.5 and 1 mg/mL of Nb_C8_FLAG, ALT1 can be specifically captured and there is a dose dependent depletion of free europium conjugated to a polyclonal antibody to ALT1 binding to the control line (**Figure 26**).

Nb_G6_FLAG was used to capture different amounts of ALT1 applied to a lateral flow test. Here different amounts of Nb_G6_FLAG were striped onto nitrocellulose ranging from 0.1 mg/mL to 1 mg/mL. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with europium conjugated to a polyclonal antibody to ALT1 and allowed to diffuse laterally. ALT1 was captured by the Nb_G6_FLAG stripe and residual europium conjugated antibody was then captured by the ALT control line. The results show that at 0.5 and 1 mg/ml of Nb_G6_FLAG, ALT1 can be specifically captured and there is a dose dependent depletion of free europium conjugated to a polyclonal antibody to ALT1 binding to the control line (**Figure 27**).

Nb_C8_FLAG was used to capture different amounts of ALT1 applied to a lateral flow test and detected with a nanobody conjugated to gold nanoshells. A constant amount of Nb_C8_FLAG was striped onto nitrocellulose. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with gold nanoshells conjugated to either, G3G or G4C and allowed to diffuse laterally. ALT1 was captured by the Nb_C8 stripe and residual gold nanoshell conjugated antibody was then captured by the ALT control line (**Figure 28A**).

Quantitation of the results was performed using a lateral flow strip reader showing that ALT1 can be specifically captured and detected in a dose dependent manner (**Figure 28B**).

In another lateral flow experiment Nb_C8_FLAG was used as a capture striped onto nitrocellulose membrane. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with europium conjugated to nanobody G6, G3G and G4C and allowed to diffuse laterally. ALT1 was captured by the Nb_C8_FLAG stripe and residual europium conjugated antibody was then captured by the ALT control line. In this example, bivalent nanobody G3G and hetero-bivalent nanobody G4C can be used as a detector and show improvements to sensitivity above the use of the monovalent detector (**Figure 29A**).

Quantitation of the results was performed using a lateral flow strip reader showing the increased signal strength when using G3G or G4C nanobody detectors conjugated to europium (**Figure 29B**).

Quantitation of the data shown in **Figure 29A**, corrected for the background in the assay at 0 µg/mL ALT1 was assessed. This allows a comparison of the relative ability of each nanobody to detect ALT and shows that G3G and G4C can detect ALT1 at 0.1 µg/mL with slightly enhanced detection with G3G at higher concentrations of ALT1 (**Figure 29C**).

A lateral flow experiment was performed in which the heterobivalent nanobodies can be used for both capture and detection. Here G4C was striped onto nitrocellulose. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with gold nanoshells conjugated to nanobody G4C and sample allowed to diffuse laterally. ALT1 was captured by the G4C stripe and residual gold nanoshell conjugated antibody was then captured by the ALT control line. In this example, heterobivalent nanobody G4C can be used as both capture and detector (**Figure 30A**).

Quantitation of the results was performed using a lateral flow strip reader. This approach greatly simplifies the development of technology to detect ALT in blood samples as only a single protein reagent is required as both capture and detector (**Figure 30B**).

Various nanobodies can be used in lateral flow assays with different detectors with dose dependent detection of ALT in spiked buffer.

Using G4C conjugated to europium as a detector, nitrocellulose membrane striped with C4G, C4C, C3C, G4C, C8, G4G, G3G and C3G showed dose dependent detection of ALT (**Figure 31**).

Using C4G conjugated to europium as a detector, nitrocellulose striped with G4G, G3G, C8, G4C, C3C, C4C, C3G and C4G showed dose dependent detection of ALT (**Figure 32**).

Using C3G conjugated to europium as a detector, nitrocellulose striped with G4G, G3G, C8, G4C, C3C, C4C, C3G and C4G showed dose dependent detection of ALT (**Figure 33**).

Using G3G conjugated to europium as a detector, nitrocellulose striped with C8, C4C, C3C, G4C, C3G and C4G showed dose dependent detection of ALT (**Figure 34**).

Using G4G conjugated to europium as a detector, nitrocellulose striped with C8, C3G and C4G showed dose dependent detection of ALT (**Figure 35**).

Using rabbit anti-ALT polyclonal antibody conjugated to europium as a detector, nitrocellulose striped with G4G, G3G, G4C, C3G, C4G showed dose dependent detection of ALT (**Figure 36**).

To improve the performance of lateral flow assays by reducing background and thereby improved signal to noise ratios, excipients may be added to the striping solution and may improve the stability and performance of the capture and control line. In this example,

Nb_C8_FLAG was striped onto nitrocellulose in the presence of the excipient bovine serum albumin (BSA) alone or in combination with the excipient sucrose. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with europium conjugated to anti-ALT polyclonal antibody and allowed to diffuse laterally. ALT1 was captured by the Nb_C8_FLAG stripe and residual europium conjugated antibody was then captured by the ALT control line. In this example the addition of BSA alone does not show a change in signal of the Nb_C8_FLAG capture line, whereas the addition of both BSA and sucrose leads to improved signal (**Figure 37**). Quantitation of **Figure 37A** shows that ALT1 can be specifically captured and detected in a dose dependent manner in buffer and this detection is improved by the addition of the excipients BSA and sucrose to the Nb_C8_FLAG capture line. In addition, the amount of non-specific binding present in the ALT1 test line in the absence of exogenous ALT1 addition (0 lane) is reduced in the presence of BSA and sucrose (**Figure 37B**). In addition, excipients improve the consistency of the control line across different levels of ALT present in the sample when C8-FLAG is used in the test line. Such features are desirable for end users and manufacturers for consistent performance (**Figure 37C**).

Similarly, G4C striped onto nitrocellulose in the presence of the excipient bovine serum albumin (BSA) alone or in combination with the excipient sucrose improved specific binding and reduced non-specific background. Different amounts of ALT1 were added to the bottom of the nitrocellulose membrane in contact with europium conjugated to anti-ALT polyclonal antibody and allowed to diffuse laterally. ALT1 was captured by the G4C stripe and residual europium conjugated antibody was then captured by the ALT control line. In this example the addition of BSA alone does not show a change in signal of the G4C capture line, whereas the addition of both BSA and sucrose leads to improved signal (**Figure 38A**). Quantitation of stripe intensity showed that ALT1 can be specifically captured and detected in a dose dependent manner in buffer and this detection is improved by the addition of the excipients BSA and sucrose to the G4C capture line (**Figure 38B**). The control line performance here was improved using BSA alone in the case of G4C striped test lines (**Figure 38C**).

The use of a heterobivalent nanobodies G4C (**Figure 39A**) and C4C (**Figure 39B**) striped on a nitrocellulose membrane in the presence of the excipients BSA and sucrose, can be used to detect ALT in clinical samples. Five microlitres of plasma was applied to the bottom of the strip in conjunction with a drop of running buffer mixed with europium particles conjugated to anti-ALT polyclonal antibody and allowed to diffuse laterally. ALT1 in the clinical sample was captured by the striped protein. In this example it is shown that this lateral flow test can distinguish between different levels of high (>120 IU/L) and low (<120 IU/L) enzymatic ALT1 in clinical samples.

A Pearson correlation analysis performed on data generated in **Figure 39A** and **B** is shown in **Figure 40 A and B**, respectively. The correlation of enzymatic and antigenic ALT using nanobody G4C captured ALT in plasma was 0.91 ($p=0.01$) while an even stronger correlation was found using nanobody C4C of 0.99 ($p<0.001$). These data show that enzymatic and antigenic ALT is correlated consistent with >90% of the ALT present in blood being ALT1 (**Figure 40**).

Further modifications of the nanobodies to improve their performance can be made by joining the nanobody VHH domain, singly or multiply, to the fragment crystallizable (Fc) region of immunoglobulin G which contains the 2 and 3 constant domains (CH2 and CH3 sub-domains) that are a disulfide-linked dimer. The addition of protein domains, such as the human IgG1 Fc domain exemplified here, has been shown to aid protein expression and performance in assays in the literature. This modification improves avidity as two or more VHH domains on the one molecule are available to interact with the antigen. As an example, a bivalent nanobody connected to a human Fc domain results in a quadrivalent binding protein. Therefore, as one VHH comes off the antigen, another VHH domain is in close proximity to bind antigen immediately (**Figure 41**).

Evidence that bivalent nanobodies connected to Fc are quadrivalent is presented in **Figure 42** where C4C-Fc, G4G-Fc, C4G-Fc and G4C-Fc were expressed in mammalian cells and examined in reducing (**Figure 42A**) and non-reducing SDS-PAGE (**Figure 42B**) conditions. As expected under reducing conditions, these bivalent nanobodies are 53 kDa consistent with a single chain. Under non reducing conditions, dimerization is maintained, and proteins are detected at 106 kDa and possess a heterobivalent binding capacity (**Figure 42**).

Examination of the ability of quadrivalent Fc-fusion nanobodies to bind ALT1 was performed in ELISA. The quadrivalent Fc-fusion nanobodies, C4C-Fc, C4G-Fc and C4G-Fc show strong binding to ALT1 with lower levels for binding detected for G4G-Fc (**Figure 43**).

Quadrivalent nanobody-Fc proteins (C4C-Fc, C4G-Fc or G4C-Fc) or bivalent nanobody (G4C) striped onto nitrocellulose membranes can be used to detect ALT in plasma and running buffer (**Figure 44A**). The quadrivalent nanobody-Fc proteins (C4C-Fc, C4G-Fc or G4C-Fc) show similar intensity of the striped band in ALT spiked buffer (**Figure 44B**). In spiked human plasma, quadrivalent Fc-fusion nanobodies C4C-Fc, C4G-Fc or G4C-Fc retain the ability to detect ALT, while the bivalent nanobody (G4C) shows reduced ability to detect ALT in spiked plasma (**Figure 44C**).

In practice, lateral flow assays are performed within a cassette in a dry system. The assembly of such a “dry system” cassette is shown but other formats and configurations are possible to those skilled in the art (**Figure 45**).

An example of such a cassette is shown with the sample and running buffer port at the bottom of the cassette and the test line and control line towards the top of the cassette. An optional reference line can also be included if required. The inside of the cassette contains the different components listed in **Figure 45 (Figure 46)**.

5 Lateral flow assays in this cassette format can quantitatively detect ALT. In this example C4G-Fc was striped onto nitrocellulose membrane in the presence of the excipients BSA and sucrose. Different amounts of ALT1 were added to the sample port and detected with rabbit polyclonal antibody raised to ALT conjugated to Europium and used at two different concentrations (1/1000 and 1/2000). This example shows that an assembled lateral flow test as
10 described in **Figure 46** is able to detect ALT1 levels in a dose response dependent manner in the assembled cassette (**Figure 47**).

Using a lateral flow assay dried down in a cassette, ALT in clinical samples can be detected. Quadrivalent Fc-fusion nanobody C4G-Fc was used to capture ALT1 in plasma and detected with europium conjugated rabbit anti-ALT polyclonal antibody. This example shows
15 that an assembled lateral flow test, as described in **Figure 46** is able to detect different ALT1 levels in clinical samples.

Example 6 – Discussion

Up to 80% of the antibodies expressed by camelids are comprised of only a heavy chain
20 and are proposed to contribute significantly to immunity. The variable domains of such single chain antibodies (VHH) are referred to as nanobodies and they possess unique properties such as improved solubility, stability and the ability to be expressed in large quantities at relatively low cost from bacterial expression systems. Because they lack a light chain and associated variable domains, the diversity of single chain antibodies is lower. However, their very long
25 CDR3 loops (typically 18 versus 13 amino acids) increases the paratope size and contact area with antigens. *Vicugna pacos* encodes 88 functional V genes, 8 D genes and 7 J genes. The size range of CDR3 can range from 4 to 34 amino acids (Tu et al., 2020). Active nanobodies Nb_C8 and Nb_G6 have CDR3 lengths of 13 and 19, respectively smaller than average for the V3-3*01 germline gene from which they are derived. By contrast, inactive Nb_C10 has a CDR3 of
30 22 amino acids. It is possible that a shorter CDR3 is favourable for binding to ALT1. Active Nb_C8 uses J gene J4*01 while Nb_G6 J gene J6*01, while inactive Nb_C10 also uses J4*01. Tu et al. 2020 observed that J4*01 is frequently observed in nanobodies of the alpaca comprising 15% of sequences while J6*01 is only observed in 2.5% of sequences. Active Nb_G6 and Nb_C8 use D-gene D6*01 and D5*01 while inactive Nb_C10 uses D3. Both D6*01
35 and D5*01 are less frequently observed than D3*01. Modelling of the structures of the nanobodies reveals that the CDR3 loop is displaced in active Nb_C8 and Nb_G6 relative to

inactive Nb_C10, related to their shorter length but specific amino acid substitutions in both CDR3 and FR2 and FR3. Together the data indicate that activity towards ALT1 in nanobodies may be related to specific structural features and gene preferences.

5 Nanobody C8 was unique with only one clone identified that had this exact CDR3 sequence. By contrast, the CDR3 domain of Nb_G6 was the dominant nanobody in the library. Despite additional attempts to isolate other nanobody sequences with activity to ALT, this was not successful. This may suggest it is very difficult to isolate nanobodies with activity to ALT, possibly because ALT may be recognised as self, and/or not be very immunogenic in the alpaca. The relatively high frequency of isolation of nanobodies with the same CDR3 sequence as
10 Nb_G6 in the first panning suggests this was strongly enriched, possibly because of its high affinity binding to ALT1.

Nanobodies containing only the VHH domain provide many benefits over older technologies employing antibodies contain complete heavy and light chain subunits. The first of these advantages is the reduction of any possible human anti-mouse effects because of the
15 absence of a murine Fc domain. The presence of a recombined highly diverse V-D-J domain limits the amount of sequence conservation with IgG class antibodies thereby limiting the amount of cross-reactivity with antibodies directed at mouse immunoglobulins or other animal immunoglobulins. Examination of sequence identity with mouse variable domain suggests that alpaca nanobodies share less than 50% identity. This property is advantageous as it reduces
20 possible interference in diagnostic assays as has been previously observed for assays measuring levels of ALT in human blood.

Nanobodies can be modified further through the addition of specific tags for purification or labelling such as poly-histidine, FLAG, antibody epitope tags, GST, MBP, and human Fc sequences. Such tags can be left on the nanobody where they do not interfere with downstream
25 applications. Alternatively, these tags can be cleaved from the nanobody following purification to remove these sequences from the nanobody so they do not interfere with downstream applications. Other modifications include the addition of sequences allowing site-specific labelling such as an Avi-tag for the addition of biotin.

The activity of nanobodies towards ALT1 was demonstrated in ELISA where ALT was
30 either bound directly to the solid surface or captured via avidin-biotin interaction. Because nanobodies are monoclonal, their specificity towards antigen is higher than polyclonal antibodies. Both Nb_G6 and Nb_C8 preferentially bind ALT1 over ALT2 and do not cross react with AST. By contrast, polyclonal antibody raised in rabbits to ALT1 retained more significant cross reactivity to ALT2 and residual reactivity to AST. The degree of identity
35 shared between ALT1 and ALT2 is 67% while ALT1 and AST share 20% identity. Polyclonal reagents are therefore likely to produce highly cross-reactive antibodies that cannot distinguish

ALT1 and ALT2. In applications such as ELISA and lateral flow assays, nanobodies with specificity towards ALT1 would provide a more accurate estimate of the ALT1 activity in human blood than polyclonal reagents. A rabbit monoclonal antibody purchased commercially showed very low activity towards ALT1 and preferred binding to ALT2. It has been shown that the enzymatic activity of ALT1 and ALT2 are similar, and that the ALT1 constitutes more than 90% of the ALT produced by the liver during liver damage. The ability to specifically measure ALT1 levels using a nanobody brings greater precision to measuring levels of specific-liver damage, and this precision would allow elevation of ALT from other causes such as strenuous exercise, to be distinguished. Current enzymatic assays measure the total enzymatic levels of ALT and cannot distinguish between ALT1 and ALT2.

A further benefit to the development of antigenic assays to measure ALT1 is the ability to join nanobody sequences together via linkers incorporating glycine and serine residues known to retain flexibility and inert, or via specific cross linkers such as BS3, DTSSP or similar reagents, or coupling to other inert proteins such as BSA. Joining nanobody sequences together can have two effects. The first is to increase the avidity of binding where the dissociation constant is reduced, thereby increasing overall affinity. This is particularly important for lateral flow applications where a solvent front moves rapidly over a striped line of protein. Capture of antibodies or antigens on the striped protein line requires fast on rates while detecting captured antigens or antibodies required slow dissociation rates to retain the detector (colloidal gold particle, gold nanoshell, europium, CNB, or similar). Conjoining nanobodies, either the same nanobody sequence or different nanobody sequences, can increase both the affinity and avidity of the interaction with antigen. Major improvements to the on rate (k_{on}) and off rate (k_{dis}) of all conjoined antibodies was observed compared with a single nanobody sequence using either a 15 or 20 amino acid linker comprising glycine and serine residues. Alternative linkers would also be expected to work similarly.

In addition to ELISA, the utility of nanobodies to detect ALT was explored in lateral flow assays. Nanobodies can be used to stripe nitrocellulose to capture ALT in blood samples and used with polyclonal reagents conjugated to a detector. The ability to use a nanobody for striping, significantly reduces costs for manufacturing tests, reduces the volume of polyclonal reagents required to make conjugate detectors and overall reduces the cost of goods.

Nanobodies can be used for both striping nitrocellulose to capture ALT as well as detecting bound ALT when couple to a detector. Nb_C8 can capture ALT1 and Nb_G6 coupled to europium as a detector was able to detect bound ALT1. Also, bivalent and heterobivalent nanobodies, G3G and G4C, respectively were able to detect captured ALT1 and improved the sensitivity of detection over the monovalent Nb_G6.

Bivalent nanobodies can overcome the need for different capture and detectors. For example, G4C can be used to both capture ALT and detect ALT. In practice, this means that only a single preparation of protein is required to provide material to stripe the ALT1 capture line and then conjugate to a detector. This simplifies manufacturing and reduces cost of goods further.

Further modifications including joining nanobodies to Fc domains (Fc region) can provide additional benefits including increasing binding to nitrocellulose, increasing avidity and alternative expression and purification methods. Bivalent nanobodies joined to Fc region of human IgG antibodies create a quadrivalent molecule which can be monovalent or heterovalent. The binding of these molecules to antigen has been shown to increase sensitivity of the lateral flow and appear unaffected when ALT levels are measured in clinical samples such as plasma. This is unlike the monomeric and bivalent nanobodies which show reduced binding in the presence of plasma. This makes bivalent nanobody-Fc fusion proteins ideal in applications such as lateral flow assays where the sample type is plasma or whole blood. This improvement is likely to be a result of increased avidity as reflected by the increase in affinity constants as well as improved adhesion to nitrocellulose membranes, preventing the release of protein when a sample is applied to the lateral flow device.

Further improvements are observed when sucrose and BSA are used as excipients in both the test line stripe as well as the control line. This evidenced in the reduced background binding when no ALT is applied to the test device, and improved signal-to-noise ratio.

The measurement of antigenic ALT in human blood is shown to correlate with enzymatic levels measured in the standard pathology assays. This is important to consider when applying this technology to clinical studies for decision making.

Example 7 – Characterization of Fc tagged versions of nanobodies

Conversion of nanobodies into Fc fusions potentially changes their interaction with ALT1 through increased avidity as there are now at least two antigen binding domains on every molecule as opposed to one on unfused nanobodies. This was examined by capturing Fc-nanobody fusion proteins on anti-human Fc sensors and measured on and off rates of binding to different concentrations of purified ALT1 protein using biolayer interferometry. The sensorgrams (**Figure 50**) show that G6-Fc slowly associates with ALT1 but has an extremely slow dissociation rate, C8-Fc associates rapidly and has a slow dissociation rate, and C4G-Fc associates rapidly and dissociates slowly over time.

Calculation of the association, dissociation and affinity was performed using BLI software and is shown in **Table 2**. G6-Fc has an affinity of 2.4 nanomolar related to its slow off-rate. The affinity of C8-Fc for ALT1 is 0.7 nanomolar and C4G-Fc has an affinity for ALT1

of 0.84 nanomolar. The R^2 was greater than 0.99 suggesting high quality data fitting using this model.

Table 2: Shows the calculated on-rate (k_a), off-rate (k_{dis}) and affinity (KD) for Fc-nanobody fusion proteins towards ALT1. Data from Figure 49 were analyzed using the Octet Analysis Studio version 13.0.3.52 software and the kinetics curve fitted using a 1:2 binding model.

Sample ID	KD (M)	KD Error	k_a (1/Ms)	k_a Error	k_{dis} (1/s)	k_{dis} Error	Full R^2
C8-Fc	6.878E-10	4.807E-12	2.814E05	6.154E02	1.936E-04	1.285E-06	0.9941
G6-Fc	2.391E-09	8.698E-11	6.906E03	1.948E01	1.651E-05	5.988E-07	0.9992
C4G-Fc	8.413E-10	3.655E-12	2.917E05	4.966E02	2.454E-04	9.807E-07	0.9936

Table 3 shows the fold increase in affinity constant for Fc fused nanobodies compared to unfused forms. In comparison to non-Fc fused forms of nanobodies, C8-Fc showed a 195-fold improvement in affinity to ALT1 and C4G-Fc showed a 37-fold improvement in affinity of binding to ALT1. These data suggest that conversion of nanobodies from monovalent into bivalent (e.g. C8-Fc and G6-Fc) and bivalent (e.g. C4G) into quadrialent (C4G-Fc) forms improves the ability of the antibodies to interact with their ligand ALT1 through the creation of multiple binding sites to ALT1 and higher avidity of binding.

Table 3: Shows the fold-increase in binding for Fc versions of the nanobodies compared to non-Fc versions. The affinity constant for C8 and C4G were divided by the affinity constant of C8-Fc and C4G-Fc, respectively.

	Fold improvement in affinity of binding compared to non-Fc fused form
C8-Fc	195
C4G-Fc	37

Example 8 – Development of rabbit monoclonal antibodies specific to ALT1

An alternative to polyclonal immune serum to ALT1, rabbit monoclonal antibodies were isolated from New Zealand White rabbits immunized with recombinant ALT1 protein. The individual B cells were sequenced and the heavy and light chains cloned into DNA plasmid expression vectors. Purified plasmid DNA encoding the corresponding heavy and light chain genes were co-transfected into Expi 293 cells. Analysis of the heavy and light chain variable domain sequences was performed using the IMGT/V-QUEST database (Brochet et al., 2008) against *Oryctolagus cuniculus* (rabbit) immunoglobulin genes. The sequence of the heavy and light chain variable domains of 15B5 (RmAb1) is shown in **Figure 51**. The data show that 15B5

is 74% identical to heavy chain germline gene V1S69, J gene is 73% identical to J6*01 and the D gene is D2-1 in reading frame 2 with CDR3 length of 14. The light chain of 15B5 is 73% identical to kappa light chain gene V1S17, 75% identical to J gene J1-2 and its CDR3 is 12 amino acids (**Table 4**).

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Table 4: 15B5 (RmAb1). The most likely germline usage for the V, J and D genes is indicated with the percentage identity and likely reading frame indicated, as well as the amino acid junction for CDR3. Sequences were analysed using IMGT / V-QUEST (Brochet et al., 2008) and aligned to *Oryctolagus cuniculus* (rabbit) immunoglobulin sequences.

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	V gene	J gene	D gene	AA junction
15B5_HC	IGHV1S69*01 F	IGHJ6*01 F (b)	IGHD2-1*01 F	CARDLAGNVYYDFDLW CDR3 length=14
Identity (%) /frame	74.64% (206/276 nt)	73.58% (39/53 nt)	Reading frame 2	
15B5_LC	GKV1S17*01 F	IGKJ1-2*03 F		CQGGTYSSGADISF CDR3 length=12
Identity (%)	73.48% (205/279 nt)	75.68% (28/37 nt)		

The sequence of the heavy and light chain variable domains of 20C12 (RmAb2) is shown in **Figure 52**. The data show that 20C12 is 72% identical to heavy chain germline gene V1S33, J gene is 71% identical to J2*01 and the D gene is D4-1 in reading frame 2 with CDR3 length of 11. The light chain of 20C12 is 72% identical to kappa light chain gene V1S64, 82% identical to J gene J1-2 and its CDR3 is 12 amino acids (**Table 5**).

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Table 5: 20C12 (RmAb2). The most likely germline usage for the V, J and D genes is indicated with the percentage identity and likely reading frame indicated, as well as the amino acid junction for CDR3. Sequences were analysed using IMGT / V-QUEST (Brochet et al., 2008) and aligned to *Oryctolagus cuniculus* (rabbit) immunoglobulin sequences.

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	V gene	J gene	D gene	AA junction
20C12_HC	IGHV1S33*01 F	IGHJ2*01 F	IGHD4-1*01 F	CARGWLDDSFDPW CDR3=11
Identity (%) /frame	71.99% (203/282 nt)	71.43% (35/49 nt)	Reading frame 2	
20C12_LC	IGKV1S64*01 [F]	IGKJ1-2*01 F		CAGGYSSGSDKFAF CDR3=12
Identity (%)	72.28% (206/285 nt)	82.05% (32/39 nt)		

The sequence of the heavy and light chain variable domains of 22G10 (RmAb3) is shown in **Figure 53**. The data show that 22G10 is 73% identical to heavy chain germline gene V1S69, J

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gene is 70% identical to J4*01 and the D gene is D3-1 in reading frame 2 with CDR3 length of 14. The light chain of 22G10 is 73% identical to kappa light chain gene V1S10, 82% identical to J gene J1-2 and its CDR3 is 9 amino acids (**Table 6**).

- 5 **Table 6: 22G10 (RmAb3).** The most likely germline usage for the V, J and D genes is indicated with the percentage identity and likely reading frame indicated, as well as the amino acid junction for CDR3. Sequences were analysed using IMGT / V-QUEST (Brochet et al., 2008) and aligned to *Oryctolagus cuniculus* (rabbit) immunoglobulin sequences.

	V gene	J gene	D gene	Junction
22G10_HC	IGHV1S69*01 F	IGHJ4*01 F (b)	IGHD3-1*01	CARGSGPSGIESYKLW CDR3 length=14
Identity (%) /frame	73.24% (156/213 nt) - (V-REGION partial in 5', missing nt nb= 0)	70.21% (33/47 nt)	Reading frame 2	
22G10_LC	GKVI1S10*01 F, or IGKV1S42*01 [F]	IGKJ1-2*01 F		CQGYGIIHITF CDR3 length=9
Identity (%)	73.48% (205/279 nt)	79.49% (31/39 nt)		

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The sequence of the heavy and light chain variable domains of 36D4 (RmAb4) is shown in **Figure 54**. The data show that 36D4 is 69% identical to heavy chain germline gene V1S69, J gene is 78% identical to J4*01 or J4*02 and the D gene is D2-1 in reading frame 2 with CDR3 length of 13. The light chain of 36D4 is 72% identical to kappa light chain gene V1S36, 76% identical to J gene J1-2 and its CDR3 is 12 amino acids (**Table 7**).

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- 20 **Table 7: 36D4 (RmAb4).** The most likely germline usage for the V, J and D genes is indicated with the percentage identity and likely reading frame indicated, as well as the amino acid junction for CDR3. Sequences were analysed using IMGT / V-QUEST (Brochet et al., 2008) and aligned to *Oryctolagus cuniculus* (rabbit) immunoglobulin sequences.

	V gene	J gene	D gene	Junction
36D4_HC	IGHV1S69*01 F	IGHJ4*01 F, or IGHJ4*02 F	IGHD2-1*01 F	CARKDWTSGDSFNPW CDR3 length=13
Identity (%) /frame	69.01% (147/213 nt) - (V-REGION partial in 5', missing nt nb= 0)	78.72% (37/47 nt)	Reading frame 2	
36D4_LC	IGKV1S36*01 [F]	IGKJ1-2*01 F		CQCTYHSSSTGYAF CDR3 length=12
Identity (%)	72.04% (201/279 nt)	76.92% (30/39 nt)		

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The sequence of the heavy and light chain variable domains of 42F12 (RmAb5) is shown in **Figure 55**. The data show that 42F12 is 74% identical to heavy chain germline gene V1S51, J gene is 81% identical to J3*01 and the D gene is D7-1 in reading frame 2 with CDR3 length of 19. The light chain of 42F12 is 72% identical to kappa light chain gene V1S42, 79% identical to J gene J1-2 and its CDR3 is 14 amino acids (**Table 8**).

Table 8: 42F12 (RmAb5). The most likely germline usage for the V, J and D genes is indicated with the percentage identity and likely reading frame indicated, as well as the amino acid junction for CDR3. Sequences were analysed using IMGT / V-QUEST (Brochet et al., 2008) and aligned to *Oryctolagus cuniculus* (rabbit) immunoglobulin sequences.

	V gene	J gene	D gene	Junction
42F12_HC	IGHV1S51*01 F	IGHJ3*01 F	IGHD7- 1*01 F	CARDKGYYSYHYAYDTRLDLW CDR3 length=19
Identity (%) /frame	74.11% (209/282 nt)	80.77% (42/52 nt)	Reading frame 2	
42F12_LC	IGKV1S42*01 [F]	IGKJ1-2*01 F		CQGTYGSGSSSYGNAF CDR3 length=14
Identity (%)	72.40% (202/279 nt)	79.49% (31/39 nt)		

An alignment of all the heavy chain variable domains is shown in **Figure 56** with CDR1, CDR2 and CDR3 boxed. RmAb5 has a significantly longer CDR3 domain than the other rabbit monoclonal antibodies. **Table 9** shows the summary data of the heavy chain gene usage of the characterized rabbit monoclonal antibodies and reveals that the most common IGH gene employed is V1S69 while J and D gene usage is diverse. The range of heavy chain CDR3 lengths is 11-19 amino acids, typical for rabbit immunoglobulins (Lavinder et al., 2014).

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Table 9: Shows summary data for heavy chain gene usage of the characterized RmAbs. Sequences were analysed using IMGT / V-QUEST (Brochet et al., 2008) and aligned to *Oryctolagus cuniculus* (rabbit) immunoglobulin sequences and the genes with highest similarity to the respective RmAbs are shown in the table.

Antibody clone	IGH	IGHJ	IGHD	CDR3 length
15B5	V1S69	J6	D2-1	14
20C12	V1S33	J2	D4-1	11
22G10	V1S69	J4	D3-1	14
36D4	V1S69	J4	D2-1	13
42F12	V1S69	J3	D7-1	19

An alignment of all the light chain variable domains is shown in **Figure 57** with CDR1, CDR2 and CDR3 boxed. **Table 10** shows the summary data of the gene usage of the characterized rabbit monoclonal antibodies light chains and reveals that they are all kappa chain, commonly used in rabbits, and the IGKV gene employed is diverse while J gene usage is always J1-2. The range of light chain CDR3 lengths is 9-14 amino acids, typical for rabbit immunoglobulins (Lavinder et al., 2014).

Table 10: Shows summary data for light chain gene usage of the characterized RmAbs. Sequences were analysed using IMGT / V-QUEST (Brochet et al., 2008) and aligned to *Oryctolagus cuniculus* (rabbit) immunoglobulin sequences and the genes with highest similarity to the respective RmAbs are shown in the table.

Antibody clone	IGKV	IGKJ	CDR3 length
15B5	KV1S17	J1-2	12
20C12	KV1S64	J1-2	12
22G10	KV1S10	J1-2	9
36D4	KV1S36	J1-2	12
42F12	KV1S42	J1-2	14

Immunoglobulins secreted into the supernatant were purified on protein A Sepharose and buffer exchanged into PBS. Purified proteins were run under reducing and non-reducing conditions on a 4-12% Bis-Tris gel (Lavinder et al., 2014). Examination of rabbit monoclonal antibodies on SDS-PAGE revealed the presence of heavy chain (~60 kDa) and light chain (20-25 kDa) bands under reducing conditions (**Figure 58**). This contrasts to the single band (~65kDa) observed for C4G-Fc corresponding to the nanobody VHH domains fused to the Fc portion of the heavy chain. Under non-reducing conditions the homodimeric quadrivalent C4G-Fc ran at approximately 150kDa, and RmAb1,3,4,5 showed the presence of a 150kDa heterodimer as expected. RmAb4 was more susceptible to denaturation even under non reducing conditions possibly due to boiling and the presence of detergents. The purity of the RmAbs was >95%.

Rabbit monoclonal antibodies were assessed for their thermal stability using differential scanning fluorimetry (**Figure 59**). All rabbit monoclonal antibodies show a shift towards the right of graph compared to rabbit polyclonal antibodies raised to ALT indicating they are stable at higher temperatures than the rabbit polyclonal immunoglobulin. This is reflected in the peak inflection points (melting temperature or T_M) of 74.8-75.8°C for rabbit monoclonal antibodies compared to 73.3°C observed for polyclonal rabbit antibody (**Table 11**). The 1-2°C higher T_M observed for the rabbit monoclonal antibodies may indicate they are more stable at higher

temperatures compared to polyclonal antibodies to ALT. This may improve their stability in storage and when used in lateral flow as either capture reagents or detector reagents compared to the polyclonal reagent.

- 5 **Table 11: Shows the melting temperatures calculated from the peak inflection point for the rabbit monoclonal antibodies and polyclonal antibody.**

Protein	Inflection point (T _m , °C)
RmAb1	74.8
RmAb3	74.8
RmAb4	74.8
RmAb5	75.8
Rabbit polyclonal antibody	73.3

- The ability of the rabbit monoclonal antibodies to bind ALT1 versus ALT2 was assessed by ELISA. Serial dilutions of each of the rabbit monoclonal antibodies and the rabbit polyclonal anti-ALT antibody was added to plates coated with either recombinant ALT1 or ALT2. Bound immunoglobulin was detected with a goat anti-rabbit horse radish peroxidase conjugated antibody. The results show that all 4 characterised rabbit monoclonal antibodies and the rabbit polyclonal anti-ALT antibody bound strongly to ALT1 (**Figure 60A**) but only the rabbit polyclonal anti-ALT antibody showed binding to ALT2 (**Figure 60B**). Comparison of the amount of antibody required to achieve 10 times above background binding shows that the polyclonal rabbit anti-ALT antibody is at least 60 times more cross reactive to ALT2 than the rabbit monoclonal antibodies (**Figure 60C**). These data indicate that the rabbit monoclonal antibodies are highly specific to ALT1 which is desirable for the detection of the liver-derived form of ALT, ALT1.

- 20 The association and dissociation of the rabbit monoclonal antibodies to ALT1 was examined using biolayer interferometry. The sensorgrams (**Figure 61**) show that each of the rabbit monoclonal antibodies tested reacted strongly to ALT and displayed faster association (on) rates compared to the polyclonal antibody (**Table 12**). The four RmAbs tested in **Figure 61** have a faster on rate of binding to ALT compared to the polyclonal antibody ranging from 5 to 16-fold (**Table 13**). The RmAbs also have a higher affinity of binding to ALT1 than the polyclonal ranging from ~10 to >2000 fold which is unexpected as it is typical that polyclonal antibodies generally have higher affinity than monoclonal antibodies (**Table 13**). Monoclonal antibodies only recognise a single epitope while polyclonal antibodies are a mixture of immunoglobulins recognising multiple epitopes on the same antigen.

Table 12: Shows the association constants (k_a), dissociation constants (k_{dis}) and affinity constants (KD) for each of the antibodies tested in Figure 61 fitted to a 1:1 binding equation.

Sample ID	KD (M)	KD Error	k_a (1/Ms)	k_a Error	k_{dis} (1/s)	k_{dis} Error	Full R^2
15B5 (RmAb1)	2.05E-10	9.33E-12	2.06E+05	7.34E+02	4.22E-05	1.92E-06	0.99
36D4 (RmAb4)	7.61E-11	4.75E-12	3.74E+05	1.17E+03	2.85E-05	1.77E-06	0.99
22G10 (RmAb3)	<1.0E-12	<1.0E-12	6.06E+05	1.77E+03	<1.0E-07	<1.0E-07	0.98
42F12 (RmAb5)	4.78E-11	5.32E-12	4.02E+05	1.51E+03	1.92E-05	2.14E-06	0.98
Rabbit anti-ALT polyclonal	2.16E-09	7.75E-12	3.77E+04	2.91E+01	8.15E-05	2.86E-07	1.00

- 5 **Table 13:** Shows the comparative on rates and affinity of the rabbit monoclonal antibodies. The on rates and affinity of the rabbit polyclonal antibody derived from Table 12 was divided by the respective on rates and affinity of each rabbit monoclonal antibody.

Antibody	Fold-improvement of k_a versus polyclonal	Fold-improvement of KD versus polyclonal
15B5 (RmAb1)	5.47	10.56
36D4 (RmAb4)	9.92	28.38
22G10 (RmAb3)	16.08	>2160
42F12 (RmAb5)	10.66	45.16
Rabbit anti-ALT polyclonal	1.00	1.00

Example 9 – Development of lateral flow devices to detect ALT1 for self-testing using anti-glycophorin A

- 10 The ability to detect ALT1 in lateral flow devices was explored initially using the C4G-Fc nanobody as the capture reagent. To enable potential users to perform the test themselves, the lateral flow schematic was adapted to fit into the commercially available AtomoRapid™ Pascal device which contains an integrated lancet, blood collection unit and integrated blister pack containing buffer. An example of how such a lateral flow test can be assembled is shown
- 15 in **Figure 62**, and where the positions of the sample pad containing anti-glycophorin A relative

to the sample port and blister pack release area, the nitrocellulose membrane containing a stripe of 1 mg/mL C4G-Fc and 0.1 mg/mL of ALT1, the absorbent pad, the backing card and the compression points on the device are. In this example (**Figure 62**), anti-glycophorin A was sprayed onto glass fibre filters for the purpose of capturing red blood cells from blood. C4G-Fc nanobody at 1 mg/mL was sprayed onto nitrocellulose membranes as well as 100 µg/mL of ALT1. To detect bound ALT1, rabbit-anti ALT polyclonal antibody was conjugated to europium and sprayed onto glass fibre pads. Tests were assembled into an AtomoRapid™ Pascal device. In the example shown, different amounts of ALT1 were spiked into running buffer and applied to the cassettes and run for 20 minutes before reading in a fluorescent lateral flow strip reader. The control line (**Figure 63**) shows the test has been correctly performed with similar amounts of conjugate detected and bound to the ALT1 stripe. The test line (**Figure 63**) shows an increasing fluorescent signal on the y-axis as the concentration of ALT applied to the device is increased.

Demonstration of the utility of an ALT1 self-test device employing blood is shown in **Figure 64** using the same schematic as shown in **Figure 62**. Venous blood spiked with either no or 2 ng ALT1 were applied to the blood collection unit and rotated onto the sample pad to deliver 10 µl blood. The blisters were then popped by depressing the button and tests run for 20 minutes. The images of a test (**Figure 64**) show the effectiveness of anti-glycophorin A to retain blood in the sample pad. The images of the test strips (**Figure 65**) show that different healthy volunteers have different levels of endogenous ALT as without addition of exogenous ALT a test line can be observed in Volunteer A and more weakly for volunteer B and C. Addition of 2 ng ALT1 results in a steep increase in signal intensity on the test line. The results are presented quantitatively in **Figure 66** showing a 2-3 fold increase in signal with addition of 2 ng ALT1 to the blood. This represents an example of how the ALT1 test can be adapted to an integrated device for self-testing or health care practitioner supervised testing using blood as the sample matrix.

Example 10 – Development of lateral flow devices to detect ALT1 for self-testing using a blood retention pad

Anti-glycophorin A adds significantly to cost of goods and manufacturing complexity. An alternative is to use a blood retention pad which acts like a molecular sieve to retain red blood cells and allow the running buffer to release plasma into the device. Many different blood retention pads are available commercially. The assembly of devices and the positions of the sample pad and blood separation pad can be altered to improve retention of red blood cells and sensitivity of the assay. Two different schematics of an ALT lateral flow strip are shown in

Figure 67 wherein the sample pad length and blood retention pads are altered while keeping other components of the strip the same. This alters the available area for the blood to interact with the blood retention pad and the length of this interaction, and the available area for the conjugate to interact with the plasma containing ALT1. Both schematics adequately retained blood (**Figure 68**) as there were no significant amounts of blood consistently entering the test window. Images of the test strips (**Figure 69A**) show the appearance of a control line after running all tests consistent with the ability of the conjugate to bind to the striped ALT1 line on the nitrocellulose membrane. In the absence of ALT1 (0 ng), there is a very weak test line consistent with a low amount of endogenous ALT1 in the venous blood sample. When the blood was spiked with 1 or 2 ng ALT1, there is an increase in the intensity of the test line using either the B6 or B10 schematic. Quantitation of these tests (**Figure 69B**) shows that schematic B10 was significantly more sensitive for the detection of ALT1 in spiked venous blood having almost 3 times more signal than schematic B6. This represents an example of how the ALT1 test can be adapted to an integrated device for self-testing or health care practitioner supervised testing and modified to use a blood retention pad. Other schematics and blood retention pads are also possible and known to those in the art.

Example 11 – Rabbit monoclonal antibodies as detector reagents for lateral flow based detection of ALT1

Development of rabbit monoclonal antibodies was performed to overcome the limitation of polyclonal antibodies being difficult to manufacture consistently, difficult to affinity purify the rabbit polyclonal immunoglobulins specific to ALT1 and the relatively high cost of goods. The ability of the rabbit monoclonal antibodies to act as detectors was assessed by conjugating each of RmAb1, 3, 4 and 5 to europium and preparing lateral flow tests using the schematic shown in **Figure 70** wherein C4G-Fc was used on the test line to capture ALT1 in blood. Different amounts of ALT1 were spiked into venous blood and applied to the cassettes, running buffer was applied and the test run for 20 minutes. Imaging of the test strips (**Figure 71**) shows that a visible control line is present indicating the tests were run properly and the conjugate was able to bind ALT1. A test line was visible at 1 and 5 ng using all detectors and showed a steep upward inflection with increasing amounts of ALT1. Quantitation of the data (**Figure 72**) show that compared to the rabbit polyclonal antibody, the rabbit monoclonal antibodies have a far superior and unexpectedly better ability to detect ALT1 in human blood with a 2-4-fold increase in sensitivity. This is consistent with the improved affinity of binding observed for the rabbit monoclonal antibodies compared to the polyclonal anti-ALT1 antibody and suggests they are far superior reagents for lateral flow-based detection of ALT1. Further validation of the ability of rabbit monoclonal antibodies to detect C4G-Fc captured ALT1 was performed in wet system

testing using four different volunteers with differing amounts of ALT quantitated in an enzymatic assay. The data (**Figure 73**) shows that with increasing amounts of enzymatically active ALT in the blood, there is a corresponding increase in the amount of ALT1 detected using each of the rabbit monoclonal antibodies.

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Example 12 – Development of a visible ALT1 lateral flow self-test device

The ALT lateral flow test can be adapted to a visible test overcoming the need for a reader to interpret results. In this example, RmAb5 was conjugated to either Estapor Blue Intense or Estapor Black conjugate and used to detect different amounts of ALT1 diluted in running buffer. Testing was performed outside a cassette in a wet system. Two different dilutions of particles were tested either 50 µg/mg or 150 µg/mg. The results (**Figure 74**) show the presence of a test line visible at 0.2 ng of ALT with a sharp upward inflection at 1 ng ALT1 using either the Estapor Blue Intense or Black conjugated RmAb5. Quantitation of the control and test line shows that the control line is relatively stable at 0, 0.2 and 1.0 ng of ALT1 while the test line increases steeply from 0 to 0.2 and 1 ng ALT1 (**Figure 75**). The amount of RmAb5 conjugated to the Blue Intense and Black particles alters the signal intensity, especially for the control line. This suggests that there is more excess RmAb5 conjugate present in the test after detection of C4G-Fc captured ALT1 on the test line for 150 µg/mL compared to 50 µg/mL preparations.

The visible version of the ALT1 lateral flow test was adapted to the AtomoRapid™ Pascal self-test device. Further adjustments to the schematic were made to improve the retention of red blood cells (**Figure 76**). The sensitivity of the test can be varied by adjusting the concentration of C4G-Fc used to capture ALT1 in blood. Here cards were striped with 0.05 and 0.2 mg/mL C4G-Fc to capture ALT1 in spiked blood, with Estapor Blue Intense labelled RmAb5 to detect ALT1. The results (**Figure 77**) show that increasing the amount of C4G-Fc used to stripe the test line increases visibility of the test line. The control line appears relatively consistent at all concentrations of ALT1 used. Quantitation of the test lines (**Figure 78**) shows a steep upward inflection using C4G-Fc striped at 0.2 mg/mL with a smaller increase in signal with increasing ALT1 using C4G-Fc striped at 0.05 mg/mL. These data show how a visible lateral flow test can be developed using C4G-Fc to capture ALT in blood, and a rabbit monoclonal antibody conjugated to visible particle in a self-test device.

Example 13 – Adaptation to a visible cut-off test

Further modifications to the ALT visible test can be made to create a visible cut-off version of the test. This has several advantages, including no longer requiring a reader to quantitate the amount of ALT present, dramatically reducing cost of goods, and increasing

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simplicity and usability. This can be achieved by modulating the dilution of conjugate, modification of the amount of protein conjugated to particles, the concentration of protein striped onto the test line and conjugate type, this way the test can be tuned so that a visible line only becomes present at a desired level of ALT present in blood. The final product is a test that only indicates a positive result visible to the user at for example, x2, x3, x4, x5 etc times the upper limit of normal levels of ALT, typically in the order of 40-70IU/L (0.04-0.07ng equivalent).

An example of how the concentration of the C4G-Fc test line can affect sensitivity has been described in **Figures 76-77**. Additional modifications were made by adjusting the amount of rabbit monoclonal antibody conjugated to blue-intense and black particles. C4G-Fc was striped onto nitrocellulose membrane at 0.5 mg/mL. Different concentrations of ALT1 were diluted in running buffer and applied to the test strips. Here it can be seen that changing the amount of RmAb5 used in the conjugation and the colour of the particle, can alter where a visible line can be seen (**Figure 79**). Quantitation of the test line and control line (**Figure 80**) further demonstrates how adjusting the amount of rabbit monoclonal antibody used to prepare the conjugate alters where a visible line can be observed. The naked eye can observe a visible line at about 0.02 units. For example, if a cut-off version of the test that measures ten times the upper limit of normal was desired, a test would comprise nitrocellulose membranes striped with 0.5 mg/mL C4G-Fc for the test line, and 5 µg/mL rabbit monoclonal would be used to conjugate to Blue Intense particles. Such a combination would generate a visible test line at ten times the upper limit of normal.

A further example is shown in **Figure 81** where both the concentration of C4G-Fc used to prepare the test line and the concentration of rabbit monoclonal antibody to prepare the conjugate were varied. Quantitation of the data (**Figure 82**) show that decreasing the amount of C4G-Fc can modulate the sensitivity of the test to allow development of a cut-off version of the test to be developed when combined with the appropriate amount of rabbit monoclonal conjugated to visible particle.

Example 14 – Use of rabbit monoclonal antibodies as captures and detectors of ALT1 for lateral flow testing

While C4G-Fc performs excellently as a capture reagent, it was also explored whether it was possible to use the rabbit monoclonal antibodies to both capture and detect ALT1. This approach provides an alternative to the use of C4G-Fc in case stability or manufacturing issues are experienced in future development of the lateral flow test. To examine this, a dot-blot system was used where the rabbit monoclonal antibodies were dotted onto nitrocellulose, exposed to ALT1(+) or not (-). Antibodies were conjugated to europium for detection of bound

ALT1. The results show that a dot is observed when ALT1 is applied to test strips using any of the rabbit monoclonal antibodies and can be detected with any of the rabbit monoclonal antibodies. Some antibody combinations were less favorable including RmAb5 as a detection agent as a higher level of non-specific binding was observed in the no ALT1 test strips (**Figure 83**).

Example 15 – Mapping of the epitopes recognized by nanobodies and rabbit anti-ALT monoclonal antibodies

To determine whether the epitopes recognized by the nanobodies or rabbit monoclonal antibodies overlapped, epitope binning was performed using biolayer interferometry. ALT1 was captured on nickel sensors and then saturated with either one of the nanobodies C8-Fc, G6-Fc or C4G-Fc or the rabbit monoclonal antibodies RmAb1, RmAb3, RmAb4 or RmAb5. A competing antibody was then added. If the saturating antibody occupies or is proximal to the site recognized by the competing antibody, it will fail to bind resulting in low or no response. If the saturating antibody is distal to the site recognized by the competing antibody, the competing antibody will bind resulting in a response. The percentage inhibition afforded by the competing antibody can then be calculated. The response curves are presented for G6-Fc (**Figure 84A**) and C8-Fc (**Figure 84B**) when added as competing antibodies and the percentage inhibition shown in **Figure 89C** and **D**, respectively. As expected, when C8-Fc or C4G-Fc are bound at saturating levels, C8-Fc cannot bind as the competing antibody (**Figure 84 A** and **C**). However, when G6-Fc is saturating, C8-Fc can bind ALT1 (**Figure 84 A** and **C**). In addition, the four RmAbs tested only partially blocked binding by C8-Fc suggesting their epitopes are distinct or partially overlap that of C8-Fc (**Figure 84 A** and **C**). When C4G-Fc and C8-Fc were saturating and G6-Fc were added, there was a detectable drop in response suggesting that addition of G6-Fc and binding to ALT1 caused a conformational change resulting in the displacement of C4G-Fc and C8-Fc from ALT1 (**Figure 84 B** and **D**). Similar to C8-Fc, the four RmAbs tested could not completely block binding by G6-Fc as the competing antibody suggesting their epitopes are distinct or partially overlap that of G6-Fc (**Figure 84 B** and **D**).

Example 16 – Crystal structure of C8 and ALT1 to define contact residues

To determine the binding site of nanobody C8 on ALT1, ALT1 and C8 were combined and the ALT1-C8 complex purified by size exclusion chromatography. The complex was crystallised and the three-dimensional structure determined by X-ray crystallography using molecular replacement to a resolution of 2.7 Å. Two molecules of ALT1 and two molecules of nanobody C8 are present in the asymmetric unit (**Figure 85A**). Interestingly, the contact surface of one molecule of C8 spans the ALT1 dimer interface with contact residues on each unit of

ALT1 (**Figure 85B**). Each C8 nanobody binds at the interface of the two ALT1 monomers covering an area of 856 Å² (**Figure 85B**).

ALT1 adopts a fold similar to its homologue alanine transaminase 2 (ALT2) forming a homodimer. Superimposition of ALT1 and the ALT2 (PDB ID: 3IHJ) monomer structures gave an RMSD of 0.868 Å indicating excellent alignment between the two molecules (**Figure 86A**). Both ALT1 and ALT2's biological unit is a homodimer (**Figure 86B**). Alignment of the ALT1 dimer and ALT2 dimer showed an increased RMSD of 6.633 Å demonstrating that the manner in which the two monomers come together to form a dimer is not identical in the two homologues.

Nanobody C8 adopts an immunoglobulin-like fold. Overlaying nanobody C8 with NbALFA (PDB ID: 6I2G), a random alpaca nanobody structure found in the RCSB PDB database, shows excellent alignment with an RMSD of 0.615 Å (**Figure 86C**). In the ALT1-C8 complex the CDR1 and CDR3 loops show poor electron density and are therefore unmodelled suggesting flexibility in those regions. Neither CDR1 or CDR3 make contact with residues on ALT1. Instead, the majority of contacts are mediated via FR1, FR2 and CDR2 residues. Superimposition of the predicted structure shown in **Figure 13** using Alphafold 2.0 and the observed structure of C8 shows high homology with an RMSD of 0.419 (**Figure 86D**) despite the unexpected binding mechanism of C8 with ALT1 and demonstrating excellent structural prediction by the program.

Analysis by PISA reveals that the ALT1-C8 interface is composed of nine hydrogen bonds and six salt bridges (**Figure 87**). Interestingly, of the three CDR loops in C8 only CDR2 is involved in binding of ALT1. The remainder of the residues that make up the interaction are located in FR1 and FR2 regions of the nanobody. The residues that make up the ALT1 epitope and C8 paratope are shown in **Figure 87**. The composite binding surface on ALT1 includes major contacts mediated by Asn99, Gln374 on monomer A and Asp93, Ser96 and Glu109 on monomer B. Residues on C8 involved in binding to ALT1 include Leu18, Arg19, Ser58, Phe68, Thr90, Ile70, Ser71, Lys76, Gln82 and Asn84. The ALT1-C8 interface is shown in **Figure 88** displaying the salt bridge and hydrogen bond network formed between ALT1 and C8. In conclusion, the epitope on ALT1 recognized by C8 is highly conformation dependent.

Example 17 – Determining the binding site of G6 with ALT1

To determine the binding sites of antibodies on ALT1 for G6, we constructed a panel of single alanine mutants across ALT1 predicted to be surface exposed. Each mutant was expressed in bacterial cell cultures and used in ELISA to detect binding to antibodies. The percentage binding of G6-Fc for each mutation relative to unmutated ALT1 is shown as a heat map in **Figure 89**. Mutation of N-(4-19) and C-terminal (441-496) regions of ALT1 did not

affect binding to G6-Fc. Amino acid mutations that caused more than a 25% reduction in binding were considered involved in binding to G6-Fc and are listed in **Figure 90**. At certain positions, amino acid substitution appeared to affect all antibody reactivity for example Phe263, Asp217, Met296, Pro299, Leu420, Phe439 and Leu441. It is possible that mutation of these residues causes a global conformational change in ALT1 disrupting antibody reactivity. The amino acids involved in binding ALT1 formed four distinct clusters: 18-61, 154-225, 239-266 and 304-433. Mutations at Asn99, Asp93, Ser96 and Glu109, all involved in binding C8-Fc, did not affect binding by G6-Fc confirming that the epitopes recognized by C8 and G6 are distinct. Mutations at 154Val and 433Leu completely ablated binding by G6-Fc. 154Val is an internal residue, therefore mutation is likely to disrupt the structure of the epitope recognized by G6-Fc. 433Leu is surface exposed and on the opposite side of ALT1 to C8-Fc binding surface. Proximal to 433Leu are 430Glu 431Leu also whose mutation to alanine also substantially reduce binding to G6-Fc.

15 **Example 18 – Determining the binding site in ALT1 for rabbit monoclonal antibodies**

To determine the binding sites of antibodies on ALT1 for RmAbs1, 3, 4 and 5, we used the panel of single alanine mutants across ALT1 in a capture ELISA. The percentage binding of RmAbs1, 3, 4 and 5 for each mutation relative to unmutated ALT1 is shown as a heat map in **Figure 91** and show distinct binding patterns for each antibody. Amino acid mutations that caused more than a 25% reduction in binding were considered involved in binding to the rabbit monoclonal antibodies are listed in **Figure 92-95**.

Alanine substitutions at positions in ALT1 affecting binding by RmAb1 are in four clusters: 19-109, 129-157, 175-276 and 304-431. Mutations of 202Val and 358Leu completely ablated binding by RmAb1 (**Figure 92**).

25 Mutations that affected binding by RmAb3 (**Figure 93**) were located between residues 19-61, 129-154, 183 and 239 and 260-433. Mutation at 48Leu, 217Asp, 222His, 260Arg, 358Leu and 408Val completely ablated binding by RmAb3.

A greater number of mutations affected binding by RmAb4 suggesting it is highly sensitive to mutation of ALT1 (**Figure 94**). Mutations that affected binding by RmAb4 were located in 7 clusters; 4-32, 43-77, 102-157, 183-239, 251-285, 303-408 and 430-494 (**Figure 94**). Mutations that completely ablated binding to ALT1 were 31Arg, 48Leu, 129Ile, 217Asp, 260Arg, 266Arg, 340Met, 359Leu and 408Val.

35 Mutations that affected binding by RmAb5 (**Figure 95**) were located in 5 clusters between residues 4-61, 129-183, 202-284, 304-358 and 408-485. Mutation at 31Arg, 48Leu, 202Val, 205Asp, 340Met, 358Leu and 408Val completely ablated binding by RmAb5.

To further define similarity and differences between the epitopes recognized by the rabbit monoclonals, epitope binning was performed using biolayer interferometry. The results (**Figures 96-97**) show that RmAb1 and 5 are likely to recognize overlapping epitopes as binding either RmAb1 or 5 to ALT1 first results in complete inhibition of binding of the other antibody. Similarly, RmAbs 3 and 4 are also likely to be overlapping epitopes as binding of either RmAb3 or 4 to ALT1 first also results in almost complete inhibition of binding of the other antibody.

Example 19 – Assessment of binding native ALT and denatured ALT1

A dot blot with native and denatured ALT1 was probed with various anti-ALT1 antibodies to determine which forms of ALT1 could be detected by the antibodies. The results (**Figure 98**) show that all anti-ALT1 antibodies, with the exception of the rabbit monoclonal antibodies, are able to detect both natively folded ALT1 as well as denatured ALT1. This suggests that G6-FLAG, the G6 component of C4G-Fc and other G6 containing multivalent antibodies, (**Figure 98A**), anti-ALT1 rabbit polyclonal, commercial anti-ALT rabbit monoclonal (**Figure 98C**), and mouse monoclonals (**Figure 98D**) are likely to recognize a linear epitope rather than a conformational one. Conversely, the in-house rabbit monoclonals, RmAb1, RmAb3, RmAb4 and RmAb5 (**Figure 98B**), appear to bind exclusively to native ALT1 and not denatured ALT1. These results strongly suggest that the in-house rabbit monoclonal antibodies bind to conformational epitopes on ALT1 which are destroyed upon heat denaturing ALT1 and thus binding of the rabbit monoclonal antibodies to their epitope is abolished.

C8-FLAG nanobody (**Figure 98A**) could not detect either form of ALT1. The C8-FLAG result was consistent with ELISA experiments which show that C8 nanobody prefers to bind to ALT1 in solution, for example, an avidin captured biotinylated ALT1 rather than ALT1 coated directly on the ELISA plate. Together with results of X-ray crystallography this suggests that C8 and the C8 component of C4G binds a conformationally intact form of the ALT1 heterodimer.

BLI epitope binning experiments with anti-ALT1 rabbit monoclonal antibodies and anti-ALT1 mouse monoclonal antibodies (**Figure 99 and 100**) indicate that rabbit monoclonal antibodies, RmAb1, RmAb3, RmAb4 and RmAb5 are able to block binding of the mouse monoclonal antibodies, 3H12, 4A9, 5H2 and 6B5 by at least 50% except for RmAb1 with 4A9. This suggests that the anti-ALT1 rabbit monoclonal antibodies and anti-ALT1 mouse monoclonal antibodies may recognise regions of ALT1 that partially overlap or are adjacent to one another.

Example 20 – Discussion

Nanobodies contain a single heavy variable domain and are generated in camelid species such as *Vicugna pacos* possess unique properties such as their smaller size, high stability, high solubility in water, simple manufacturing processes ensuring consistent batch-to-batch variation, and high affinity binding. For use in lateral flow assays, it was found that the conversion of the single variable domain to a bivalent variable domain improved affinity of binding which translated to improved performance in lateral flow assays, further improvements were made by joining the nanobodies to the Fc domain of a human IgG such as IgG1. Further characterization of these was performed using biolayer interferometry (**Figure 50**) and compared to monovalent forms fused to the Fc domain. Comparison of C8-Fc to C4G-Fc showed 1.22-fold improvement in the affinity constant (KD) while C8-Fc had a slightly higher affinity to C4G-Fc (**Table 2**). By contrast, comparison of C8-Fc and C4G-Fc to their non-Fc counterparts revealed a 195-fold and 37-fold improvement in affinity constant (**Table 3**). This major change in affinity and size is beneficial to achieving high sensitivity in lateral flow assays for the detection of ALT1 as not only does it enable excellent capture of ALT1 in blood but also retains the protein on the test line as the fluid flows over the nitrocellulose membrane. Small proteins are relatively easier to leach off the nitrocellulose membrane resulting in a loss of signal and reduced sensitivity. Fc fused nanobodies with their larger size are stickier and remain bound to the nitrocellulose membrane. The generation of bivalent nanobodies fused to the Fc domain was instrumental in achieving a manufacturable and highly sensitive version of a lateral flow assay to detect ALT1 in blood using wither europium or a visible conjugate.

To overcome potential manufacturing issues using rabbit polyclonal immunoglobulins raised to ALT1, a panel of rabbit monoclonal antibodies specific to ALT1 was generated. Rabbits have many potential benefits for the generation of monoclonal reagents including their ability to generate very high affinity antibodies, longer CDR3, and their differences in generation of antibody diversity compared to humans and mice. B cell cloning was performed from PBMCs isolated from New Zealand White rabbits immunized with ALT1. The heavy and light chain genes from five highly reactive B cells were sequenced and their sequences shown in **Figures 51-55**. The alignment of the heavy chain sequences (**Figure 56**) shows high diversity in sequence, length of CDRH2 and 3. The average length of CDR in rabbits is 15 ± 4 amino acids; the average length of CDRH3 here was 14.2. The most commonly used germline used was IGHV1S69 (4/5) and one clone used V1S33 (**Table 9**). VH1 has been shown to be used in 80-90% of all heavy chain gene rearrangements (Weber et al., 2017). The heavy chain D and J germline usage was highly diverse for all five monoclonal antibodies generated.

Sequence alignment of the light chains of the rabbit anti-ALT1 monoclonal antibodies revealed high diversity of sequence and length in CDR1 and 3 (**Figure 57**). All 5 light chains were kappa 1 (K1) consistent with its common usage in rabbit immunoglobulins of 70-90%

(Table 10). All kappa chains contained the conserved Cys80 which forms a disulfide bridge with Cys 171 in the constant domain and may contribute to the stability of rabbit immunoglobulins. The kappa V germline usage was highly diverse however J1-2 was used by all antibodies. The CDRV3 length ranged from 9-14 amino acids with an average of 11.8, consistent with the average observed in rabbits of 12 ± 2 amino acids. suggesting there are multiple mechanisms to generate specificity to ALT1 in the rabbit. This contrasts strongly to what was observed in Alpacas where a very limited diversity was found that was reactive to ALT1.

Four of the five monoclonal antibodies were successfully expressed in human Expi293 cell cultures by co-transfection of plasmids encoding the heavy and light chain genes. The antibodies were successfully purified and over 95% purity achieved (Figure 58). The monoclonal antibodies possessed a slightly higher thermal melting temperature compared to the rabbit anti-ALT polyclonal reagent as ascertained by differential scanning fluorimetry (Figure 59). This 1-2°C shift in thermal stability may be significant in manufacturing as it suggests the antibodies will be more stable, a key requirement for the manufacture of any diagnostic assay to ensure adequate shelf life and consistent performance. Comparison of the binding properties in ELISA showed all four rabbit monoclonal antibodies had similar ability to bind ALT1 compared to the rabbit polyclonal antibody (Figure 60). Additionally, the monoclonal antibodies were highly specific for ALT1 compared to ALT2. Conversely, the rabbit polyclonal antibody showed cross-reactivity, binding to both ALT1 and ALT2.

Comparison of the ability of the monoclonal antibodies to interact with ALT1 was explored in biolayer interferometry (Figure 61 and Table 12). All four monoclonal antibodies to ALT1 had higher affinity constants compared to the polyclonal anti-ALT antibodies ranging from 10->2000 fold improvements. This is likely to be attributable to the fast on-rate of binding ranging from 5-16 fold compared to the polyclonal anti-ALT antibody (Table 13). This data reaffirms that the rabbit monoclonal antibodies have unexpectedly improved properties compared to the polyclonal immunoglobulins to ALT1.

A potential use case for lateral flow-based assays for detection of ALT1 is for at-home based monitoring of liver function either performed as a self-administered test or by a visiting health care professional or at a health care clinic. It was examined whether the test could be modified to a self-test using the AtomoRapid™ Pascal device as an exemplar of such a device designed for self-testing. A similar device, the AtomoRapid™ Galileo device is currently approved by the TGA in Australia for self-testing for HIV-1 status. The Pascal device contains an integrated lancet, blister pack and blood collection unit, and has been developed with the intention for home based self-testing. Alternatively, they can be used for health care practitioner supervised testing.

The europium-based test was adapted to the Pascal device using the schematic shown in **Figure 62**. To enable separation of red blood cells, the sample pad was sprayed with an antibody specific to a red blood cell surface protein glycophorin A (GPA, MNS). Other anti-red blood cell antibodies can also be employed including anti-glycophorin B, Lewis, ABO anti-CD238 (Kell), anti-CD234 (Duffy) etc. The sample pad was also sprayed with the europium conjugate coupled to anti-ALT1 polyclonal antibody in a distinct area of the pad. The pad was overlaid onto nitrocellulose membrane striped with C4G-Fc (test line) and ALT1 (Control line) and an absorbent pad was overlaid at the end of the membrane to wick running buffer. An example of the quantitative and dose dependent detection of ALT1 is shown in **Figure 63**. The control line is consistent across all concentrations of ALT1 applied to the test indicating sufficient residual conjugate was present in the device to facilitate detection of ALT1. Further exemplification of the self-test device is shown in **Figure 64** where the sample applied was venous blood spiked with ALT1 using the same schematic as **Figure 65**. The images of the device show that the red blood cells were retained in the sample window, confirming that the anti-glycophorin A captured the red blood cells and allowed the plasma to run through the device, without leakage into the test window which could interfere with reading the result. The images of the strips performed in **Figure 64** are shown in **Figure 65** with quantitation in **Figure 66** and reveal detection of ALT1 when 2 ng is spiked into venous blood. Three different volunteer bloods were used and show that in healthy individuals even normal endogenous levels of ALT can be detected (0 ng). These results show that a lateral flow test for self-testing can be developed using C4G-Fc to capture ALT1, anti-glycophorin A to capture red blood cells and europium conjugated anti-ALT polyclonal rabbit immunoglobulins to detect ALT1 in a self-test device.

To overcome the additional complexity in manufacturing and high cost of goods associated with the use of an antibody reagent to capture red blood cells, an alternative version of the test was developed using a commercially available blood retention pad. Blood separation pads contain highly pure natural and synthetic fibres, sometimes with the inclusion of chemicals, that separate red blood cells from plasma while limiting hemolysis. Many different varieties are available. An example of two different schematics is shown in **Figure 67** with resulting tests using whole blood spiked with ALT1 shown in **Figure 68**. Both schematics retained red blood cells adequately in the sample window. Quantitation of the data (**Figure 69**) show that Schematic B10 out-performed schematic B6 as it was able to achieve higher sensitivity. This is an example of how blood separation pads can be used together with C4G-Fc to capture ALT1 and rabbit-anti-ALT1 conjugated to europium to detect ALT1, and how the schematics can be altered to vary sensitivity. A self-test for detection of ALT1 could therefore

use C4G-Fc to capture ALT1, rabbit-anti-ALT1 conjugated to europium to detect ALT1 and a blood retention pad to capture red blood cells.

While rabbit polyclonal antibodies have high sensitivity for detecting ALT1, they are complex to make, batch-to-batch variation will be a manufacturing issue and there is a high cost associated with their production. To overcome these limitations, the use of rabbit monoclonal antibodies to ALT1 as alternative detector reagents was explored. **Figure 70** shows an example of a lateral flow schematic for a self-test device that was used to test whether any or all of the rabbit monoclonal antibodies could be used as detectors when conjugated to europium. The results using venous blood spiked with ALT1 (**Figure 71**) show that all 4 of the rabbit anti-ALT monoclonal antibodies could detect C4G-Fc captured ALT1 in lateral flow testing and displayed improved sensitivity compared to the rabbit anti-ALT polyclonal antibody detector as indicated by the improved signal strength at 1ng. Quantitatively (**Figure 72**) this translated to a 2-3 fold improvement in sensitivity at 1 and 5 ng ALT1. This unexpected improvement in sensitivity using the rabbit anti-ALT monoclonal antibodies is beneficial to the development of lateral flow tests as it allows highly sensitive detection of even low levels of ALT1 in blood. In later examples (**Figure 78**), much lower concentrations of C4G-Fc could be used to achieve equivalent detection of ALT1 using the rabbit anti-ALT monoclonal antibodies as detectors. In addition, the rabbit anti-ALT monoclonal antibodies were validated for their ability to detect naturally occurring ALT in plasma (**Figure 73**) meaning they have practical utility for a detection and monitoring of ALT levels in human blood.

Europium based detection has several benefits including the inability of the user to “see” the result of a test. This may be important when a clinician has prescribed or recommended regular monitoring of a person who is undergoing a treatment with a drug that induces liver injury. It may not be in the persons best interest to see the result of the ALT test so as not to influence their decision to continue taking the medication. In this case, the result would be sent directly to the clinician through for example an app be coupled to the lateral flow reader that sends the result to a clinician and the clinician can then discuss the result with the patient and provide instructions for how to continue treatment. Europium also allows for highly sensitive detection of ALT1 and can overcome limitations to the sensitivity able to be achieved with the available materials for the lateral flow test.

However, there are disadvantages to the use of europium including the requirement for a reader to measure the result. This can add hundreds to thousands of dollars in cost to the lateral flow assay and may be unaffordable or too expensive to warrant its use, reducing the potential application of the ALT test and the value of the technology for commercial development and licensing.

An alternative is the development of a visible version of the ALT1 test. Using the rabbit anti-ALT monoclonal antibodies conjugated to either Estapor Blue Intense or Black particles, ALT captured with C4G-Fc on the test line was successfully detected (**Figures 74 and 75**) and could be further adapted to a self-test device (**Figures 76-78**). Modulating the concentration of C4G-Fc on the test line can alter the sensitivity of the test which is useful when considering what range of ALT is to be monitored. For example, 0.2 mg/mL C4G-Fc gives highly sensitive detection at 0-2 ng (0-2000 IU/L) but plateaued at 5 ng while 0.05 mg/mL C4G-Fc gave a much shallower increase and could be useful when developing a test that measure very high amounts of ALT1 (>2000 IU/L).

A visible version of a lateral flow-based ALT test can be directly interpreted by the user. To perform semi-quantitatively a printed card with lines corresponding to ranges of ALT can be used to determine if a person has for example 0-40 IU/L, 40-150 IU/L, or 150-300 IU/L. Alternatively, a reader can be used for quantitative measurement of ALT levels and while not desirable, the cost of goods for a colorimetric reader is cheaper than a fluorescent reader due to the different optics required. Alternatively, a phone-based app can be used to photograph the result and provide an estimation of the amount of ALT present.

An alternative is a visible cut-off version of the test. The test only indicates a positive result visible to the user at for example, x2, x3, x4, x5 etc times the upper limit of normal levels of ALT, typically in the order of 40-70 IU/L (0.04-0.07 ng equivalent). This was shown to be possible by modulating the dilution of conjugate, modification of the amount of protein conjugated to particles, the concentration of protein striped onto the test line and conjugate type, the test can be tuned so that a visible line only becomes present at a desired level of ALT present in blood in **Figures 79-82**. The major benefit here is that the user only sees a result at the pre-defined level of ALT, for example, five-times the upper limit of normal. This provides simplicity in manufacture and usability by the end user.

The ability to use the rabbit monoclonal anti-ALT or rabbit polyclonal anti ALT antibodies to act as capture and detector reagents was explored (**Figure 83**) and showed that all could be used as either captures and detectors.

A version of the test may therefore have C4G-Fc captured ALT1 or rabbit monoclonal anti-ALT or rabbit polyclonal anti ALT antibody detected with a rabbit monoclonal anti-ALT or rabbit polyclonal anti ALT or Fc conjugated nanobody in a simple standard lateral flow cassette or a self-test device that provides a visible signal at a predefined level of ALT, or a semi quantitative result to show the range of ALT present with or without the use of aids such as a printed card or app, or a quantitative result interpreted by a reader.

To determine the binding region of nanobody C8 on ALT1, the crystal structure of the ALT1-C8 complex was solved by X-ray crystallography to a resolution of 2.7 Å. The structure

revealed the ALT1 dimer bound to two molecules of nanobody C8 (**Figure 85**). The C8 nanobodies were observed to bind to ALT1 in a unique manner (Majerle et al., 2021). Strikingly, majority of the interface between C8 and ALT1 consists of residues in the non-CDR region of the nanobody, namely the FR1 and FR2 regions, with only CDR2 of C8 involved with binding of ALT1 (**Figure 87**). Unusually, CDR1 and CDR3 do not make any contact to ALT1. Each C8 nanobody lies at the interface of the ALT1 dimer making contacts to both ALT1 monomers (**Figure 85, 87 and 88**). The binding across the two ALT1 monomers strongly suggests that C8 will bind only dimeric ALT1, the biologically active form of the enzyme. This makes it an ideal reagent for the detection of the biological form of ALT1 in immunoassays such as lateral flow assays.

Given that nanobodies C8 and G6 can be used as a pair in lateral flow assays (**Figure 29**), this implies that nanobody G6 binds in a different area on ALT1 to that of nanobody C8. To determine the epitopes recognized by the nanobody G6 on ALT1, we constructed 122 single alanine mutations in ALT1. An ELISA was used to measure binding to the mutants and normalized to wild type binding levels. The results (**Figures 89**) show that the nanobody G6 recognizes a distinct epitope to that recognized by C8 on ALT1. This is consistent with the ability to use these antibodies as capture and detector reagents and epitope binning experiments (**Figure 84**) suggesting they bind different epitopes. For G6, an alanine substitution at positions map into four distinct clusters. It is possible that similar to C8, nanobody G6 may bind some or all of these amino acids in context of a composite binding site on the ALT1 dimer, explaining the large number of amino acids that contribute to recognition.

Table 14: ALT1 residues involved in binding to rabbit monoclonal antibodies as determined by Ala scanning mutagenesis and ELISA.

RmAb1	RmAb3	RmAb4	RmAb5
31 Arg	31 Arg	4 Ser	19 Lys
48 Leu	48 Leu	5 Thr	24 Asp
129 Ile	129 Ile	7 Asp	43 Gln
131 Leu	131 Leu	8 Arg	48 Leu
155 Phe	155 Phe	13 Arg	56 Val
157 Ser	217 Asp	14 His	131 Leu
175 Glu	222 His	19 Lys	155 Phe
177 His	260 Arg	20 Val	202 Val
178 Thr	263 Phe	24 Asp	205 Asp
202 Val	284 Ser	29 Arg	263 Phe
205 Asp	296 Met	31 Arg	284 Ser
217 Asp	299 Pro	32 Arg	296 Met
263 Phe	340 met	43 Gln	299 Pro
296 Met	344 met	44 Arg	340 Met
299 Pro	358 Leu	48 Leu	344 Met
304 Gln	408 Val	50 Gln	358 Leu

327 Val	420 Leu	53 Arg	408 Val
340 Met	433 Leu	54 Gln	420 Leu
344 Met	439 Phe	56 Val	430 Glu
357 Leu		129 Ile	433 Leu
358 Leu		131 Leu	439 Phe
408 Val		155 Phe	
420 Leu		183 Leu	
430 Glu		202 Val	
433 Leu		217 Asp	
439 Phe		260 Arg	
		263 Phe	
		266 Arg	
		284 Ser	
		296 Met	
		299 Pro	
		340 Met	
		344 Met	
		358 Leu	
		408 Val	
		420 Leu	
		433 Leu	
		439 Phe	

The ALT1 mutants and epitope binning were further used to define the epitopes recognized by the rabbit monoclonal antibodies (RmAbs) (**Figure 91-95**). The RmAbs had distinct binding patterns suggesting they have different epitopes. Epitope binning suggests that RmAb1 and 5 and RmAbs 3 and 4 recognise similar epitopes (**Figure 97**). This is consistent with mutational analysis of ALT1 showing similar clusters involved in binding to RmAbs 1 and 5 (**Figure 86 and 95 and Table 14**). For RmAbs 3 and 4, binning suggests overlapping regions are involved in binding these mAbs to ALT1 (**Figure 97**). Mutational analysis shows that overlapping regions are found between these two mAbs but RmAb4 has a much more extensive area of binding, especially towards the N and C -termini of ALT1, or has higher sensitivity to mutational interference than RmAb3 (**Figures 93-94**).

One of the major features of both the nanobody C8 and engineered modifications containing C8, and the rabbit monoclonal antibodies, is their requirement for a conformationally intact form of ALT1. Denaturation of ALT1 abolishes antibody recognition by C8, RmAb1, RmAb3, RmAb4 and RmAb5. Consistent with this observation, structural analysis of C8 in complex with ALT1 shows it binds a composite epitope comprised of amino acids from both protomers of the ALT1 homodimer. Further, we consistently found that C8 and RmAb1, RmAb3, RmAb4 and RmAb5 could only bind ALT1 in ELISA when ALT1 was captured either via its C-terminal His-tag or via an added biotin tag, both effectively presenting ALT1 for recognition in its native state, in solution phase. Direct binding to plastic is known to partially denature the proteins and is sufficient to disrupt the epitope recognised by these

antibodies. This differs to previously described polyclonal immune serum and mouse monoclonal antibodies raised to ALT1 which can directly bind ALT coated ELISA plates and therefore likely recognise non-conformation sensitive epitopes. This is also evident in dot blot experiments with native and denatured ALT1 (**Figure 98**). The rabbit monoclonal antibodies, 5 RmAb1, RmAb3, RmAb4 and RmAb5, bind only to natively folded ALT1 and not denatured ALT1 while rabbit polyclonal, commercial rabbit monoclonal and mouse monoclonal antibodies, can bind to both forms of ALT1. The ability of C8, RmAb1, RmAb3, RmAb4 and RmAb5 to bind native ALT is likely to provide a distinct benefit for use as diagnostic agents as they are likely to recognise native and enzymatically active forms of ALT1 and should provide 10 a better correlation with the standard of care enzymatic measurement of ALT.

Further comparison of C8, G6, RmAb1, RmAb3, RmAb4 and RmAb5 with previously characterised but ineffective mouse monoclonal antibodies also reveals important differences. Unexpectedly, the epitopes recognised by C8, RmAb1,3,4 and 5 at least partially overlap with (completely in the case of RmAb5) mouse monoclonal antibodies. Our data shows that C8, 15 RmAb1, RmAb3, RmAb4 and RmAb5 containing antibodies have very high affinity for binding ALT1, higher than what was observed for mouse monoclonal antibodies shown in **Figure 99**. Further, our data shows that C8, and other engineered nanobodies containing C8, RmAb1, RmAb3, RmAb4 and RmAb5 recognise highly conformationally sensitive epitopes on ALT1 as the number of amino acids across ALT1 that affect binding is very high suggesting 20 the epitopes they recognize are extremely sensitive to slight changes in conformation at near and distal sites. The reduction in sensitivity observed in plasma for ALT1 using mouse monoclonal antibodies may be due to their lower affinity and the presence of human anti-mouse antibodies that block binding to ALT1.

It will be appreciated by persons skilled in the art that numerous variations and/or 25 modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

This application claims priority from Australian Provisional Application No. 2023903973 entitled "Binding proteins and methods and uses thereof" filed on 8 December 30 2023, the entire contents of which are hereby incorporated by reference. All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part 35 of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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CLAIMS

1. A binding protein which binds alanine aminotransferase 1 (ALT1), comprising amino acid sequences selected from the following:
- 5 a) amino acid sequences GPAVSNVA (**SEQ ID NO: 2**) as CDR1, ITWSGWT (**SEQ ID NO: 3**) as CDR2 and NLIGLRVGPENKY (**SEQ ID NO: 4**) as CDR3;
- b) amino acid sequences GRTDSFYA (**SEQ ID NO: 6**) as CDR1, ITWSAGST (**SEQ ID NO: 7**) as CDR2 and AADSLSAGYESSWLEAFGS (**SEQ ID NO: 8**) as CDR3; and
- 10 c) amino acid sequences GRTFSSYS (**SEQ ID NO: 10**) as CDR1, ISRSGFST (**SEQ ID NO: 11**) as CDR2 and AVGRAYLPTASGTRCPREAYDY (**SEQ ID NO: 12**) as CDR3; and
- wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID NO.
- 15 2. The binding protein of claim 1, wherein the binding protein is a nanobody.
3. The binding protein of claim 1 or claim 2, wherein the binding protein comprises the amino acid sequences GPAVSNVA (**SEQ ID NO: 2**) as CDR1, ITWSGWT (**SEQ ID NO: 3**) as CDR2 and NLIGLRVGPENKY (**SEQ ID NO: 4**) as CDR3.
- 20 4. The binding protein of claim 1 or claim 2, wherein the binding protein comprises the amino acid sequences GRTDSFYA (**SEQ ID NO: 6**) as CDR1, ITWSAGST (**SEQ ID NO: 7**) as CDR2 and AADSLSAGYESSWLEAFGS (**SEQ ID NO: 8**) as CDR3.
- 25 5. The binding protein of claim 1 or claim 2, wherein the binding protein comprises the amino acid sequences GRTFSSYS (**SEQ ID NO: 10**) as CDR1, ISRSGFST (**SEQ ID NO: 11**) as CDR2 and AVGRAYLPTASGTRCPREAYDY (**SEQ ID NO: 12**) as CDR3.
6. The binding protein of claim 1, 2 or claim 3, wherein the binding protein comprises the amino acid **SEQ ID NO: 1** (C8), or a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version thereof.
- 30 7. The binding protein of claim 1, 2 or claim 4, wherein the binding protein comprises the amino acid **SEQ ID NO: 5** (G6), or a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version thereof.
- 35

8. The binding protein of claim 1, 2 or claim 5, wherein the binding protein comprises the amino acid **SEQ ID NO: 9** (C10), or a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version thereof.
- 5 9. The binding protein of any one of claims 1 to 8, wherein the binding protein binds an epitope that comprises residues on both protomers of ALT1.
- 10 10. The binding protein of claim 1, 3 or 6 wherein the binding protein does not significantly or detectibly bind a single monomer of ALT1.
- 11 11. The binding protein of any one of claims 1 to 10, wherein the binding protein does not significantly or detectibly bind heat denatured ALT1.
- 15 12. The binding protein of any one of claims 1 to 11, wherein the binding protein binds an epitope of human ALT1.
- 20 13. The binding protein of any one of claims 1 to 12, wherein the binding protein does not significantly or detectibly bind human ALT2 and/or does not significantly or detectibly bind aspartate aminotransferase (AST).
- 25 14. The binding protein of any one of claims 1 to 13, wherein the binding protein comprises a framework region derived from an: alpaca, camel, llama or shark or is a modified or humanised version thereof.
- 30 15. The binding protein of claim 14, wherein the framework region of the binding protein is derived from an alpaca or is a modified or humanised version thereof.
16. The binding protein of any one of claims 1 to 15, wherein the binding protein comprises a tag.
17. The binding protein of claim 16, wherein the tag is selected from: poly-histidine, Flag, antibody epitope tags, c-myc, haemagglutinin, headlock, C-tag, ALFA tag, Avi, GST, MBP, Fc sequences.
- 35 18. The binding protein of claim 16 or claim 17, wherein the tag is cleavable.

19. The binding protein of any one of claims 1 to 18, wherein the binding protein comprises a detectable label.
20. The binding protein of claim 19, wherein the detectable label is selected from a: metal label, magnetic label, bead, colorimetric label, radioactive label, enzymatic label, luminescent label, fluorescent label, quantum dot, fluorescent latex particle, chemiluminescence based label, liposome based probe and a raman-active tag.
21. The binding protein of any one of claims 1 to 20, wherein the binding protein additionally comprises a linker.
22. The binding protein of claim 21, wherein the linker is selected from **SEQ ID NO: 65** to **SEQ ID NO: 77**.
23. The binding protein of any one of claims 1 to 22, wherein the binding protein is fused or conjugated to a protein.
24. The binding protein of claim 23, wherein the protein is selected from: Fc region or a fragment thereof, ferritin, maltose-binding protein (MBP), leucine zipper, glutathione S-transferase (GST), keyhole limpet hemocyanin (KLH), albumin, cyclophilin, FKBP, calcineurin, CyPFAS, GyrB, neutravidin, avidin and streptavidin.
25. The binding protein of claim 24, wherein the protein is an Fc region or a fragment thereof.
26. The binding protein of claim 24 or claim 25, wherein the Fc region or a fragment thereof increases the association rate of the binding protein with ALT1.
27. The binding protein of any one of claims 24 to 26, wherein the Fc region or a fragment thereof decreases the association rate of the binding protein with ALT1.
28. The binding protein of any one of claims 1 to 27, wherein the binding protein binds ALT1 in a sample selected from: human plasma, human serum, whole blood and capillary blood.
29. The binding protein of claim 28, wherein the whole blood is venous blood or capillary blood.

30. The binding protein of any one of claims 1 to 29, wherein the binding protein binds Asn 99 and Gln 374 of one protomer of ALT1 and Asp 93, Ser 96 and Glu 109 on a second protomer of ALT1.
- 5
31. The binding protein of any one of claims 1 to 30, wherein the paratope of the binding protein comprises residues in one or more of: CDR2, FR1 and FR2.
32. The binding protein of any one of claims 1 to 31, wherein the paratope of the binding protein comprises residues in CDR2 and not CDR1 or CDR3.
- 10
33. The binding protein of any one of claims 1 to 32, wherein the C8 residues that mediate contact with ALT1 comprise one or more of: Leu 18, Arg 19, Ser 58, Phe 68, Thr 90, Ile 70, Ser 71, Lys 76, Gln 82 and Asn 84.
- 15
34. The binding protein of any one of claims 1 to 33, wherein the binding protein does not significantly and/or or detectably bind to ALT1 comprising one or more of the following mutations: 19 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu or does not significantly and/or detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 53 Arg, 61 Thr, 154 Val, 155 Phe, 175 Glu, 178 Thr, 183 Leu, 196 Leu, 202 Val, 205 Asp, 212 Arg, 217 Asp, 225 Leu, 239 Val, 260 Arg, 263 Phe, 266 Arg, 296 Met, 299 Pro, 304 Gln, 327 Val, 330 Val, 357 Leu, 358 Leu, 369 Asp, 420 Leu, 430 Glu, 431 Leu, 433 Leu, 439 Phe and 441 Leu.
- 20
- 25
35. A binding protein which binds alanine aminotransferase 1 (ALT1), the binding protein comprising the antigen binding site of an antibody comprising amino acid sequences selected from::
- a) GFSLNNYN (**SEQ ID NO: 110**) as heavy chain CDR1, ITAGGNI (**SEQ ID NO: 111**) as heavy chain CDR2, ARDLAGNVYYDFDL (**SEQ ID NO: 112**) as heavy chain CDR3, ENIYSG (**SEQ ID NO: 114**) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (**SEQ ID NO: 115**) as light chain CDR3;
- 30
- b) GFDLSSYY (**SEQ ID NO: 117**) as heavy chain CDR1, IWLGSJNI (**SEQ ID NO: 118**) as heavy chain CDR2 and ARGWLDDSFDP (**SEQ ID NO: 119**) as heavy chain CDR3, VSVHYNKW (**SEQ ID NO: 121**) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (**SEQ ID NO: 122**) as light chain CDR3;
- 35

c) GFSLITYS (**SEQ ID NO: 124**) as heavy chain CDR1, ISASGTA (**SEQ ID NO: 125**) as heavy chain CDR2, ARGSGPSGIESYKL (**SEQ ID NO: 126**) as heavy chain CDR3, QSIGN Y (**SEQ ID NO: 128**) as light chain CDR1, RAS as light chain CDR2 and QGY YGIHIT (**SEQ ID NO: 129**) as light chain CDR3;

5 d) GIDLSVNA (**SEQ ID NO: 131**) as heavy chain CDR1, IHTYDVT (**SEQ ID NO: 132**) as heavy chain CDR2, ARKDWTSGDSFNP (**SEQ ID NO: 133**) as heavy chain CDR3, QSISTA (**SEQ ID NO: 135**) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (**SEQ ID NO: 136**) as light chain CDR3; and

10 e) GFSLSNDA (**SEQ ID NO: 138**) as heavy chain CDR1, ISSAGRP (**SEQ ID NO: 139**) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (**SEQ ID NO: 140**) as heavy chain CDR3, QSISSSY (**SEQ ID NO: 142**) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (**SEQ ID NO: 143**) as light chain CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No.

15

36. The binding protein of claim 35, wherein the binding protein is a monoclonal antibody.

37. The binding protein of claim 36, wherein the binding protein is a rabbit monoclonal antibody.

20

38. The binding protein of any one of claims 35 to 37, wherein the binding protein binds an epitope that comprises residues on both protomers of ALT1.

39. The binding protein of any one of claims 35 to 38, wherein the binding protein does not significantly and/or detectably bind heat denatured ALT1.

25

40. The binding protein of any one of claims 35 to 39, wherein the V_L is kappa 1.

41. The binding protein of 40, wherein the kappa 1 comprises Cys80 which forms a disulfide bond with Cys 171.

30

42. The binding protein of claim 40 or claim 41, wherein the kappa 1 variable region uses J germline J1-2.

35 43. The binding protein of anyone of claims 35 to 42, wherein the binding protein melting temperature is about 74.8°C to about 75.8°C.

44. The binding protein of any one of claims 35 to 43, wherein the binding protein has an on rate that is at least about 5-fold higher than a rabbit polyclonal ALT-1 antibody.
- 5 45. The binding protein of any one of claims 35 to 44, wherein the binding protein has an affinity for ALT1 that is at least about 10-fold higher than a rabbit polyclonal anti-ALT antibody.
46. The binding protein of any one of claims 35 to 45, wherein the binding protein can be
10 isolated at at least about 95% purity.
47. The binding protein of any one of claims 35 to 46, wherein the binding protein has at least a 2-fold higher sensitivity for detecting human ALT1 compared to a rabbit polyclonal anti-ALT antibody.
15
48. The binding protein of any one of claims 35 to 47, wherein the binding does not significantly and/or detectably bind ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 129 Ile, 131 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 340 Met, 358 Leu, 408 Val, 430 Glu and 433 Leu.
20
49. The binding protein of any one of claims 35 to 48, wherein the binding does not significantly and/or detectably bind ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 129 Ile, 131 Leu, 154 Val, 177 His, 178 Thr, 183 Leu, 202 Val, 205 Asp, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 340 Met, 358 Leu, 408 Val, 430 Glu and 433 Leu.
25
50. The binding protein of any one of claims 35 to 49, wherein the binding does not significantly or detectable bind ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 31 Arg, 43 Gln, 48 Leu, 56 Val, 61 Thr, 129 Ile, 131 Leu, 154 Val, 183 Leu, 196 Leu, 202 Val, 212 Arg, 222 His, 225 Leu, 229 Arg, 239 Val, 260 Arg, 266 Arg, 284 Ser, 304 Gln, 333 Asp, 340 Met, 358 Leu, 408 Val, 430 Glu and 433 Leu.
30
51. The binding protein of anyone of claims 35 to 49, wherein the binding protein comprises a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 109** and a V_L comprising
35

the amino acid sequence as set forth in **SEQ ID NO: 113** (RmAb1) or a sequence at least 70% identical thereto.

52. The binding protein of anyone of claims 35 to 47, wherein the binding protein comprises
5 a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 116** and a V_L comprising the amino acid sequence as set forth in **SEQ ID NO: 120** (RmAb2) or a sequence at least 70% identical thereto.

53. The binding protein of anyone of claims 35 to 48 or 50, wherein the binding protein
10 comprises a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 123** and a V_L comprising the amino acid sequence as set forth in **SEQ ID NO: 127** (RmAb3) or a sequence at least 70% identical thereto.

54. The binding protein of anyone of claims 35 to 48 or 50, wherein the binding protein
15 comprises a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 130** and a V_L comprising the amino acid sequence as set forth in **SEQ ID NO: 134** (RmAb4) or a sequence at least 70% identical thereto.

55. The binding protein of anyone of claims 35 to 49, wherein the binding protein comprises
20 a V_H comprising the amino acid sequence as set forth in **SEQ ID NO: 137** and a V_L comprising the amino acid sequence as set forth in **SEQ ID NO: 141** (RmAb5) or a sequence at least 70% identical thereto.

56. A bivalent, trivalent, quadrivalent or multivalent binding protein comprising at least one
25 binding protein of any one of claims 1 to 55 or a combination thereof.

57. The bivalent, trivalent, quadrivalent or multivalent binding protein of claim 56,
comprising a binding protein comprising the amino acid as set forth in **SEQ ID NO: 1** (C8), or
a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version
30 thereof.

58. The bivalent, trivalent, quadrivalent or multivalent binding protein of claim 56 or claim
57, comprising a binding protein comprising the amino acid as set forth in **SEQ ID NO: 5** (G6),
or a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version
35 thereof.

59. The bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 58, comprising:
- (i) a binding protein comprising the amino acid as set forth in **SEQ ID NO: 1** (C8), or a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version thereof; and
 - (ii) a binding protein comprising the amino acid as set forth in **SEQ ID NO: 5** (G6), or a sequence at least 76% identical thereto, or a humanised, deimmunized or germlined version thereof.
60. The bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 59, wherein the binding protein is biparatopic.
61. The bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 59, wherein the binding protein is multiparatopic.
62. The bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 61, wherein the binding protein comprises a linker selected from one or more of **SEQ ID NO: 65** to **SEQ ID NO: 77**.
63. The bivalent binding protein of any one of claims 56 to 61, wherein the binding protein comprises a sequence selected from: C3C (**SEQ ID NO: 57**), C4C (**SEQ ID NO: 58**), G3G (**SEQ ID NO: 59**), G4G (**SEQ ID NO: 60**), C3G (**SEQ ID NO: 61**), C4G (**SEQ ID NO: 62**), G3C (**SEQ ID NO: 63**) and G4C (**SEQ ID NO: 64**) or a sequence at least 76% identical thereto.
64. The quadrivalent binding protein of any one of claims 56 to 61, wherein the binding protein comprises a sequence selected from: C4C-Fc (**SEQ ID NO: 79**), G4G-Fc (**SEQ ID NO: 80**), C4G-Fc (**SEQ ID NO: 81**), G4C-Fc (**SEQ ID NO: 82**), C3G-Fc (**SEQ ID NO: 159**), G3C-Fc (**SEQ ID NO: 161**), C3C-Fc (**SEQ ID NO: 155**) and G3G-Fc (**SEQ ID NO: 157**) or a sequence at least 76% identical thereto.
65. The quadrivalent binding protein of claim 64, wherein the binding protein comprises the sequence C4G-Fc (**SEQ ID NO: 81**).
66. An isolated nucleic acid encoding the amino acid sequence of the isolated binding protein of any one of claims 1 to 55, or a bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65.

67. A vector comprising the nucleic acid of claim 66.
68. A host cell comprising the nucleic acid of claim 66, or the vector of claim 67.
- 5 69. The host cell of claim 68, wherein the host cell is selected from a: bacterial cell, mammalian cell or insect cell.
70. A method of producing a binding protein of any one of claims 1 to 55 or a bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65, comprising
10 culturing the host cell of claim 34 or claim 35 in cell culture medium and expressing the binding protein or the bivalent, trivalent, quadrivalent or multivalent binding protein.
71. A kit or panel comprising a binding protein of any one of claims 1 to 55 or a bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65.
- 15 72. The kit or panel of claim 71, wherein the kit or panel comprises a strip, chip or cartridge for use on point-of-care device.
73. The kit or panel of claim 71 or claim 72, wherein the kit or panel selected from a: lateral
20 flow assay, ELISA, biosensor based assay and a bead-type assay.
74. A lateral flow assay comprising: a solid support that comprises a binding protein of any one of claims 1 to 55, and/or a bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65 immobilized on the solid support.
- 25 75. A lateral flow assay comprising:
(i) a detector binding protein conjugated to a detectable label;
(ii) a capture binding protein in a capture region on a solid support,
wherein the binding protein in (i) and/or (ii) is a binding protein of any one of claims 1 to 55,
30 or the bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65.
76. The lateral flow assay of claim 74 or claim 75, further comprising a red blood cell capture portion.
- 35 77. The lateral flow assay of any one of claims 74 to 76, wherein the red blood cell capture portion is selected from a red blood cell binding protein and a blood retention pad.

78. The lateral flow assay of claim 77, wherein the blood retention pad is selected from one or more of: FR1, MDI, Vivid GX, CytoSep1663, CytSep 1660, CytoSep1668, Vivid GR, Vivid GX, Vivid GR, and Vivid GF.
- 5 79. The lateral flow assay of claim 77, wherein the red blood cell binding protein is selected from binding protein targeting: glycophorin B, lewis, CD238 and CD234.
80. The lateral flow assay of any one of claims 74 to 79, wherein the lateral flow assay
10 comprises one or more of a lancet, blood collection unit and blister pack containing buffer.
81. The lateral flow assay of any one of claims 74 to 80, wherein the binding protein is bound to capture region of the solid support in the presence of one or more excipient/s.
- 15 82. The lateral flow assay of claim 81, wherein the excipient is selected from: one or more of: sucrose, bovine serum albumin (BSA), an immunoglobulin, trehalose, casein, lactose, galactose and a detergent (e.g. tween).
83. The lateral flow assay of any one of claims 74 to 82, further comprising a control line
20 on the solid support comprising an ALT or an ALT epitope.
84. The lateral flow assay of claim 74 or 83, wherein ii) is a quadrivalent binding protein.
85. The lateral flow assay of any one of claims 74 to 84, wherein the binding protein in i)
25 and/or ii) is a nanobody or a rabbit monoclonal antibody.
86. The lateral flow assay of any one of claims 74 to 85, wherein the binding protein in i) and/or ii) is a bivalent, trivalent, quadrivalent or multivalent binding protein.
- 30 87. The lateral flow assay of any one of claims 74 to 85, wherein the binding protein in i) is a nanobody or a rabbit monoclonal antibody and wherein the binding protein in ii) is a rabbit polyclonal antibody; or wherein the binding protein in ii) is a nanobody or a rabbit monoclonal antibody and wherein the binding protein in i) is a rabbit polyclonal antibody.
- 35 88. The lateral flow assay of any one of claims 74 to 87, wherein the assay can detect about 0.1 ng to about 5 ng of ALT1 in a sample.

89. The lateral flow assay of any one of claims 74 to 88, wherein the binding protein in i) is selected from: C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.

5 90. The lateral flow assay of any one of claims 74 to 89, wherein the binding protein in ii) is selected from: C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5.

91. The lateral flow assay of claim 89, wherein the binding protein in ii) is a rabbit polyclonal ALT-1 antibody.
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92. The lateral flow assay of claim 90, wherein the binding protein in i) is a rabbit polyclonal ALT-1 antibody.

15 93. The lateral flow assay of claim 87, wherein the binding protein in i) is selected from: G4C, C3G and G4G.

94. The lateral flow assay of claim 87, wherein the binding protein in i) and/or ii) is selected from: C4C-Fc, G4G-Fc, C4G-Fc and G4C-Fc.

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95. The lateral flow assay of any one of claims 74 to 94, wherein:

a) when the binding protein in i) is G4C and the binding protein in ii) C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4, RmAb5 and a rabbit polyclonal ALT-1 antibody;

25 b) when the binding protein in i) is C3G and the binding protein in ii) C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4, RmAb5 and a rabbit polyclonal ALT-1 antibody;

c) when the binding protein in i) is G4G and the binding protein in ii) C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1,

30 RmAb2, RmAb3, RmAb4, RmAb5 and a rabbit polyclonal ALT-1 antibody;

d) when the binding protein in i) is a rabbit polyclonal ALT-1 antibody and the binding protein in ii) C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4 and RmAb5;

35 e) when the binding protein in i) is RmAb1 and the binding protein in ii) is selected from: C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4, RmAb5 and a rabbit polyclonal ALT-1 antibody;

f) when the binding protein in i) is RmAb2 and the binding protein in ii) is selected from: C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4, RmAb5 and a rabbit polyclonal ALT-1 antibody;

5 g) when the binding protein in i) is RmAb3 and the binding protein in ii) is selected from: C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4, RmAb5 and a rabbit polyclonal ALT-1 antibody;

h) when the binding protein in i) is RmAb4 and the binding protein in ii) is selected from: C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4, RmAb5 and a rabbit polyclonal ALT-1 antibody; and

10 i) when the binding protein in i) is RmAb5 and the binding protein in ii) is selected from: C8, G6, G4C, C3G, G4G, G3C, C4G, C3C, C4C, G3G, C4C-Fc, G4G-Fc, C4G-Fc, G4C-Fc, RmAb1, RmAb2, RmAb3, RmAb4, RmAb5 and a rabbit polyclonal ALT-1 antibody.

96. The lateral flow assay of any one of claims 74 to 95, wherein the binding protein in ii) is striped at a concentration of 0.05 mg/mL to about 2.5 mg/mL.

97. The kit or panel or lateral flow assay of any one of claims 74 to 96, wherein the kit or panel or lateral flow assay is for detecting liver damage and/or liver disease.

20 98. Use of a binding protein of any one of claims 1 to 55, or the bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65, or the kit or panel of any one of claims 71 to 73, or the lateral flow assay of any one of claims 74 to 96 for detecting ALT1 in a subject.

25 99. A method of detecting ALT1, the method comprising contacting a sample with a binding protein of any one of claims 1 to 55, or the bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65 to form an antigen-binding protein complex and directly or indirectly detecting the antigen-binding protein complex.

30 100. Use of a binding protein of any one of claims 1 to 55, or the bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65, or the kit or panel of any one of claims 71 to 73, or the lateral flow assay of any one of claims 74 to 96 for detecting a subject with liver damage and/or liver disease.

35 101. A method of detecting a subject with liver damage and/or liver disease the method comprising contacting a sample with a binding protein of any one of claims 1 to 55, or the bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65, to

form an antigen-binding protein complex and directly or indirectly detecting the antigen-binding protein complex.

102. The use or method of any one of claims 98 to 101, wherein detection of the antigen binding protein complex above a threshold level indicates liver damage and/or liver disease in a subject.

103. The use or method of any one of claims 98 to 102, wherein the use or method is used to detect a condition selected from: pregnancy related condition, eclampsia, preeclampsia, high ALT1 levels in pregnancy in a subject, acute fatty liver of pregnancy and HELLP syndrome.

104. The use or method of any one of claims 98 to 103, wherein the use or method is used to monitor a condition in a subject.

105. The use or method of claim 104, wherein the condition is selected from a/an: hepatotropic virus, non-hepatotropic virus, bacteria, fungi, parasite, toxin or substance (e.g. a therapeutic treatment), inflammatory condition, metabolic or hereditary condition, pregnancy related condition, and ischemic or vascular condition.

106. The use or method of claim 104 of claim 105, wherein the condition is selected from: hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, epstein-barr virus, cytomegalovirus, herpes simplex virus, coxsackievirus, mononucleosis, adenovirus, dengue virus, coronavirus-19, fatty liver disease, acute alcoholic hepatitis, or alcoholic cirrhosis, sea anemone sting, autoimmune hepatitis, biliary disease such as primary biliary cholangitis or primary sclerosing cholangitis, non-alcoholic fatty liver disease, hemochromatosis, wilson's disease, preeclampsia, acute fatty liver of pregnancy, HELLP syndrome, cardiogenic/distributive shock, hypotension, heatstroke, cocaine, methamphetamine, ephedrine, acute Budd-Chiari syndrome, sinusoidal obstruction syndrome, malignancy, eclampsia, reye' syndrome, primary graft non-function after liver transplantation.

107. The use or method of any one of claims 104 to 106, wherein the method or use is used monitor treatment in a subject.

108. The use or method of claim 107, wherein the treatment is selected from: a tuberculosis treatment, neurological condition treatment, a cancer treatment and an inflammatory bowel disease treatment.

109. The use or method of claim 107, wherein the treatment is selected from: an anti-TNF α antibody, infliximab, adalimumab, Bruton tyrosine kinase inhibitors, 2MHRZ/2MHR, 2EMRZ/2MR, paracetamol, antibiotic, anticonvulsants, statins, NSAIDs and a herbal/nutritional supplement.

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110. A binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1 comprising the antigen binding site of an antibody comprising amino acid sequences selected from:

a) GFSLNNYN (SEQ ID NO: 110) as heavy chain CDR1, ITAGGNI (SEQ ID NO: 111) as heavy chain CDR2, ARDLAGNVYYDFDL (SEQ ID NO: 112) as heavy chain CDR3, ENIYSG (SEQ ID NO: 114) as light chain CDR1, KAS as light chain CDR2 and QGGTYSSGADIS (SEQ ID NO: 115) as light chain CDR3;

b) GFDLSSYY (SEQ ID NO: 117) as heavy chain CDR1, IWLGSNGI (SEQ ID NO: 118) as heavy chain CDR2, ARGWLDDSFDP (SEQ ID NO: 119) as heavy chain CDR3, VSVHYNKKW (SEQ ID NO: 121) as light chain CDR1, GAS as light chain CDR2 and AGGYSSGSDKFA (SEQ ID NO: 122) as light chain CDR3;

c) GFSLITYS (SEQ ID NO: 124) as heavy chain CDR1, ISASGTA (SEQ ID NO: 125) as heavy chain CDR2, ARGSGPSGIESYKL (SEQ ID NO: 126) as heavy chain CDR3, QSIGNNY (SEQ ID NO: 128) as light chain CDR1, RAS as light chain CDR2 and QGYGIIHIT (SEQ ID NO: 129) as light chain CDR3;

d) GIDLSVNA (SEQ ID NO: 131) as heavy chain CDR1, IHTYDVT (SEQ ID NO: 132) as heavy chain CDR2, ARKDWTSGDSFNP (SEQ ID NO: 133) as heavy chain CDR3, QSISTA (SEQ ID NO: 135) as light chain CDR1, SAS as light chain CDR2 and QCTYHSSSTGYA (SEQ ID NO: 136) as light chain CDR3;

e) GFSLSNDA (SEQ ID NO: 138) as heavy chain CDR1, ISSAGRP (SEQ ID NO: 139) as heavy chain CDR2, ARDKGYYSYHYAYDTRLDL (SEQ ID NO: 140) as heavy chain CDR3, QSISSSY (SEQ ID NO: 142) as light chain CDR1, RVS as light chain CDR2 and QGTYGSGSSSYGNA (SEQ ID NO: 143) as light chain CDR3; and

f) GPAVSNVA (SEQ ID NO: 2) as CDR1, ITWSGWT (SEQ ID NO: 3) as CDR2 and NLIGLRVGPENKY (SEQ ID NO: 4) as CDR3;

wherein any one of more CDRs has 0, 1, 2 or 3 amino acid changes compared to that set forth in the corresponding SEQ ID No,

wherein when the amino acid sequences are a) the binding protein is a nanobody, and wherein when the amino acid sequences are b), c), d), e) or f) the binding protein is an

antibody.

111. The binding protein of claim 110, wherein the binding protein does not significantly bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19

Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu.

5 112. A binding protein which binds alanine aminotransferase 1 (ALT1) and does not significantly and/or detectably bind denatured ALT1 comprising the antigen binding site of an antibody, and wherein the binding protein does not significantly and/or detectably bind to ALT1 comprising an alanine substitution at one or more of the following positions: 19 Lys, 24 Asp, 48 Leu, 154 Val, 183 Leu, 202 Val, 212 Arg, 239 Val, 260 Arg, 266 Arg, 304 Gln, 358 Leu, 430 Glu and 433 Leu.

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113. A solid support or semi-solid support having immobilized thereon the binding protein of any one of claims 1 to 55, or the bivalent, trivalent, quadrivalent or multivalent binding protein of any one of claims 56 to 65.