Summary

Trivalent, bispecific antibody:

Herein is reported a recombinant mammalian cell expressing a trivalent, bispecific antibody, especially a bivalent, monospecific antibody which comprises an additional scFv or Fab fragment at one C-terminus of one of its heavy chains. A trivalent, bispecific antibody is a heteromultimeric polypeptide not naturally expressed by said mammalian cell. More specifically, trivalent, bispecific antibody is a heteromultimeric protein consisting of four polypeptides: one light chain, which is a full length light chain; a further light chain, which is a domain exchanged light chain; one heavy chain, which is a full length heavy chain; and a further heavy chain, which is an extended heavy chain comprising an addition domain exchanged heavy or light chain Fab fragment at its C-terminus. To achieve expression of a trivalent, bispecific antibody a recombinant nucleic acid comprising multiple different expression cassettes in a specific and defined sequence has been integrated into the genome of a mammalian cell.

Especially, the method according to the current disclosure can be used for the generation of brain-shuttle antibodies. These can have a format as described, e.g. in WO 2014/033074. Those molecules can simultaneously bind to human transferrin receptor (first specificity) on a cell of the blood-brain-barrier and to a target therapeutic antigen (second specificity) and thereby induce transport and therapeutic effects.

Herein is also reported a method for generating a recombinant mammalian cell expressing trivalent, bispecific antibody and a method for producing trivalent, bispecific antibody using said recombinant mammalian cell.

In one preferred example the trivalent, bispecific antibody comprises

- a first heavy chain comprising from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain, a peptidic linker, a second heavy chain variable domain and a CL domain,

- a second heavy chain comprising from N- to C-terminus the first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,

- a first light chain comprising from N- to C-terminus a first light chain variable domain and a CH1 domain, and
- a second light chain comprising from N- to C- terminus a second light chain variable domain and a CL domain,

wherein the second heavy chain variable domain and the first light chain variable domain form a first binding site and the first heavy chain variable domain and the second light chain variable domain form a second binding site,

wherein the first binding site specifically binds to human transferrin receptor.

In one preferred example the trivalent, bispecific antibody comprises

- a first heavy chain comprising from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain, a peptidic linker, a first light chain variable domain and a CH1 domain,
- a second heavy chain comprising from N- to C-terminus the first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- a first light chain comprising from N- to C-terminus a second light chain variable domain and a CH1 domain, and
- a second light chain comprising from N- to C- terminus a second heavy chain variable domain and a CL domain,

wherein the second heavy chain variable domain and the first light chain variable domain form a first binding site and the first heavy chain variable domain and the second light chain variable domain form a second binding site,

wherein the first binding site specifically binds to human transferrin receptor.

In one preferred example none of the first light chain and the second light chain of the trivalent, bispecific antibody is a common light chain or a universal light chain.

The current disclosure is based, at least in part, on the finding that the sequence of the different expression cassettes required for the expression of the heteromultimeric trivalent, bispecific antibody, i.e. the expression cassette organization, as integrated

into the genome of a mammalian cell influences the expression yield of trivalent, bispecific antibody (e.g. a brain-shuttle antibody).

The current disclosure is based, at least in part, on the finding that by integrating a nucleic acid encoding the heteromultimeric, trivalent, bispecific antibody that has a specific expression cassette organization into the genome of a mammalian cell efficient recombinant expression and production of the trivalent, bispecific antibody can be achieved (e.g. of a brain-shuttle antibody).

It has been found that the defined expression cassette sequence can advantageously be integrated into the genome of a mammalian cell by a double recombinase mediated cassette exchange reaction.

The present disclosure provides a method for producing trivalent, bispecific antibody comprising the steps of

a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding trivalent, bispecific antibody optionally under conditions suitable for the expression of trivalent, bispecific antibody, and

b) recovering trivalent, bispecific antibody from the cell or the cultivation medium,

wherein the deoxyribonucleic acid encoding trivalent, bispecific antibody is stably integrated into the genome of the mammalian cell and comprises in 5’- to 3’-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the second light chain, and
- an eighth expression cassette encoding the second light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,

- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second heavy chain,
- a seventh expression cassette encoding the second light chain, and
- an eighth expression cassette encoding the second light chain,

or (3)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain, and
- a seventh expression cassette encoding the first light chain.

In one aspect, the present disclosure provides a method for producing a trivalent, bispecific antibody comprising the steps of

a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding the trivalent, bispecific antibody, and

b) recovering the trivalent, bispecific antibody from the cell or the cultivation medium,

wherein the deoxyribonucleic acid encoding the trivalent, bispecific antibody is stably integrated into the genome of the mammalian cell and comprises in 5’- to 3’-direction

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain, and
- a seventh expression cassette encoding the second light chain.

In one example exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

The present disclosure provides a deoxyribonucleic acid encoding trivalent, bispecific antibody comprising in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the second light chain, and
- an eighth expression cassette encoding the second light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second heavy chain,
- a seventh expression cassette encoding the second light chain, and
- an eighth expression cassette encoding the second light chain,

or (3)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain, and
- a seventh expression cassette encoding the first light chain.

In another aspect, the present disclosure provides a deoxyribonucleic acid encoding a trivalent, bispecific antibody comprising in 5'- to 3'-direction

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain, and
- a seventh expression cassette encoding the second light chain.

The present disclosure provides the use of a deoxyribonucleic acid comprising in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the second light chain, and
- an eighth expression cassette encoding the second light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,

- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second heavy chain,
- a seventh expression cassette encoding the second light chain, and
- an eighth expression cassette encoding the second light chain,

or (3)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain, and
- a seventh expression cassette encoding the first light chain.

for the expression of trivalent, bispecific antibody in a mammalian cell.

In another aspect, the present disclosure provides the use of a deoxyribonucleic acid comprising in 5'- to 3'-direction

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain, and
- a seventh expression cassette encoding the second light chain,

for the expression of the trivalent, bispecific antibody in a mammalian cell.

In one example of the use the deoxyribonucleic acid is integrated into the genome of the mammalian cell.

In one example of the use exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

The present disclosure provides a recombinant mammalian cell comprising a deoxyribonucleic acid encoding trivalent, bispecific antibody integrated in the genome of the cell,

wherein the deoxyribonucleic acid encoding trivalent, bispecific antibody comprises in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the second light chain, and
- an eighth expression cassette encoding the second light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second heavy chain,
- a seventh expression cassette encoding the second light chain, and
- an eighth expression cassette encoding the second light chain,

or (3)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,

- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain, and
- a seventh expression cassette encoding the first light chain.

In another aspect, the present disclosure provides a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a trivalent, bispecific antibody integrated in the genome of the cell, wherein the deoxyribonucleic acid encoding the trivalent, bispecific antibody comprises in 5'- to 3'-direction

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain, and
- a seventh expression cassette encoding the second light chain.

In one example exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

In one example of the above the deoxyribonucleic acid encoding trivalent, bispecific antibody further comprises

- a first recombination recognition sequence located 5’ to the first (most 5’) expression cassette,
- a second recombination recognition sequence located 3’ to the seventh or eighth (most 3’) expression cassette, and
- a third recombination recognition sequence located
    - between the first and the second recombination recognition sequence, and
    - between two of the expression cassettes,

and

wherein all recombination recognition sequences are different.

In one example the third recombination recognition sequence is located between the fourth and the fifth expression cassette.

In one example the deoxyribonucleic acid encoding trivalent, bispecific antibody comprises a further expression cassette encoding for a selection marker and the expression cassette encoding for the selection marker is located partly 5’ and partly 3’ to the third recombination recognition sequence, wherein the 5’-located part of said expression cassette comprises the promoter and the start-codon and the 3’-located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal, wherein the start-codon is operably linked to the coding sequence.

The present disclosure provides a composition comprising two deoxyribonucleic acids, which comprise in turn three different recombination recognition sequences and seven or eight expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5’- to 3’-direction,
    - a first recombination recognition sequence,

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction

either (1)

- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the second light chain,
- an eighth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (2)

- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second heavy chain,
- a seventh expression cassette encoding the second light chain,
- an eighth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (3)

- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- a second recombination recognition sequence.

In another aspect, the present disclosure provides a composition comprising two deoxyribonucleic acids, which comprise in turn three different recombination recognition sequences and four expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5’- to 3’-direction,
- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5’- to 3’-direction
- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain,
- a seventh expression cassette encoding the second light chain, and
- a second recombination recognition sequence.

In one example of the above the deoxyribonucleic acid encoding trivalent, bispecific antibody further comprises a further expression cassette encoding for a selection marker.

In one example the expression cassette encoding for a selection marker is located either

i) 5’, or

ii) 3’, or

iii) partly 5’ and partly 3’

to the third recombination recognition sequence.

In one example the expression cassette encoding for a selection marker is located partly 5’ and partly 3’ to the third recombination recognition sequences, wherein the 5’-located part of said expression cassette comprises the promoter and a start-codon and the 3’-located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal.

In one example the 5’-located part of the expression cassette encoding the selection marker comprises a promoter sequence operably linked to a start-codon, whereby the promoter sequence is flanked upstream by (i.e. is positioned downstream to) the fourth expression cassette and the start-codon is flanked downstream by (i.e. is positioned upstream of) the third recombination recognition sequence; and the 3’-located part of the expression cassette encoding the selection marker comprises a nucleic acid encoding the selection marker lacking a start-codon and is flanked upstream by the third recombination recognition sequence and downstream by the fifth expression cassette.

In one example the start-codon is a translation start-codon. In one example the start-codon is ATG.

The present disclosure provides a recombinant mammalian cell comprising a deoxyribonucleic acid encoding trivalent, bispecific antibody integrated in the genome of the cell,

wherein the deoxyribonucleic acid encoding trivalent, bispecific antibody comprises the following elements:
a first, a second and a third recombination recognition sequence,
a first and a second selection marker, and
a first to eighth expression cassette,

wherein the sequences of said elements in 5’-to-3’ direction is

RRS1-1st EC-2nd EC-3rd EC-4th EC-RRS3-SM1-5th EC-6th EC-7th EC-8th EC-RRS2

or

RRS1-1st EC-2nd EC-3rd EC-4th EC-RRS3-SM1-5th EC-6th EC-7th EC-RRS2

with

RRS = recombination recognition sequence,

EC = expression cassette,

SM = selection marker.

The present disclosure provides a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding trivalent, bispecific antibody and secreting trivalent, bispecific antibody comprising the following steps:

a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and seven or eight expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5’- to 3’-direction,
  - a first recombination recognition sequence,
  - a first expression cassette encoding the first heavy chain,
  - a second expression cassette encoding the first heavy chain,
  - a third expression cassette encoding the first light chain,
  - a fourth expression cassette encoding the first light chain, and
  - a first copy of a third recombination recognition sequence,

  and

- the second deoxyribonucleic acid comprises in 5’- to 3’-direction either (1)
  - a second copy of the third recombination recognition sequence,
  - a fifth expression cassette encoding the second heavy chain,
  - a sixth expression cassette encoding the first light chain,
  - a seventh expression cassette encoding the second light chain,
  - an eighth expression cassette encoding the second light chain, and

thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding trivalent, bispecific antibody and secreting trivalent, bispecific antibody.

In another aspect, the present disclosure provides a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a trivalent, bispecific antibody and secreting the trivalent, bispecific antibody, comprising the following steps:

a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and at least seven expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5’- to 3’-direction,
- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5’- to 3’-direction
- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,

- a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain,
- a seventh expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

wherein the first to third recombination recognition sequences of the first and second deoxyribonucleic acids are matching the first to third recombination recognition sequence on the integrated exogenous nucleotide sequence,

wherein the 5'-terminal part and the 3'-terminal part of the expression cassette encoding the one second selection marker when taken together form a functional expression cassette of the one second selection marker;

c) introducing
i) either simultaneously with the first and second deoxyribonucleic acid of b);
or
ii) sequentially thereafter
one or more recombinases,

wherein the one or more recombinases recognize the recombination recognition sequences of the first and the second deoxyribonucleic acid; (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)

and

d) selecting for cells expressing the second selection marker and secreting the trivalent, bispecific antibody,

thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the trivalent, bispecific antibody and secreting the trivalent, bispecific antibody.

In one preferred example of the present disclosure the first binding site specifically binds to human transferrin receptor.

In one example of the present disclosure the trivalent, bispecific antibody is an anti-TfR/CD20 bispecific antibody. Such an antibody is reported in WO 2017/055542, which is incorporated herein by reference in its entirety.

In one example of the present disclosure the trivalent, bispecific antibody is an anti-TfR/Abeta bispecific antibody. Such an antibody is reported in WO 2017/055540, which is incorporated herein by reference in its entirety.

Detailed Description of Embodiments of the Invention

Bispecific, trivalent antibody:

Herein is reported a recombinant mammalian cell expressing a trivalent antibody, especially a bispecific, trivalent antibodies, such as a T-cell bispecific antibody (TCB). A trivalent antibody is a heteromultimeric polypeptide not naturally expressed by said mammalian cell. More specifically, a trivalent antibody is a heteromultimeric protein consisting of four polypeptides or polypeptide chains: one light chain, which is a full length light chain; a further light chain, which is a domain exchanged light chain; one heavy chain, which is a full length heavy chain; and a further heavy chain, which is an extended heavy chain comprising an addition domain exchanged heavy or light chain Fab fragment. To achieve expression of a trivalent antibody a recombinant nucleic acid comprising multiple different expression cassettes in a specific and defined sequence has been integrated into the genome of a mammalian cell.

Especially, the method according to the current invention can be used for the generation of T-cell bispecific antibodies (TCBs). These can have a format as described, e.g. in WO 2013/026831. Those molecules can simultaneously bind to CD3 (first specificity) on T-cells and to an antigen on a target (e.g. tumor) cell (second specificity) and thereby induce killing of target cells.

Herein is also reported a method for generating a recombinant mammalian cell expressing a trivalent antibody, especially a trivalent, bispecific antibody, more specifically a TCB and a method for producing a trivalent antibody, especially a

The current invention is based, at least in part, on the finding that for the expression of trivalent, bispecific antibody, which is a complex molecule comprising different polypeptides, i.e. which is a heteromultimer, the use of a defined and specific expression cassette organization results in efficient expression and production of the trivalent, bispecific antibody in mammalian cells, such as CHO cells.

The current invention is based, at least in part, on the finding that double recombinase mediated cassette exchange (RMCE) can be used for producing a recombinant mammalian cell, such as a recombinant CHO cell, in which a defined and specific expression cassette sequence has been integrated into the genome, which in turn results in the efficient expression and production of a trivalent, bispecific antibody. The integration is effected at a specific site in the genome of the mammalian cell by targeted integration. Thereby it is possible to control the expression ratio of the different polypeptides of the heteromultimeric, trivalent, bispecific antibody relative to each other. Thereby in turn an efficient expression, correct assembly and successful secretion in high expression yield of correctly folded and assembled trivalent, bispecific antibody is achieved.

The current invention is based, at least in part, on the finding that for the expression of a trivalent antibody (e.g. a TCB), which is a complex molecule comprising different polypeptides, i.e. which is a heteromultimer, the use of a defined and specific expression cassette organization results in efficient expression and production of the trivalent antibody (e.g. a TCB) in mammalian cells, such as CHO cells.

The current invention is based, at least in part, on the finding that double recombinase mediated cassette exchange (RMCE) can be used for producing a recombinant mammalian cell, such as a recombinant CHO cell, in which a defined and specific expression cassette sequence has been integrated into the genome, which in turn results in the efficient expression and production of a trivalent antibody (e.g. a TCB). The integration is effected at a specific site in the genome of the mammalian cell by targeted integration. Thereby it is possible to control the expression ratio of the different polypeptides of the heteromultimeric, trivalent antibody (e.g. a TCB) relative to each other. Thereby in turn an efficient expression, correct assembly and successful secretion in high expression yield of correctly folded and assembled trivalent antibody (e.g. a TCB) is achieved.

encoding a part of a heteromultimeric polypeptide and/or at least one selection marker or part thereof flanked by two heterospecific RRSs, are both integrated into a pre-determined site of the genome of a mammalian cell suitable for TI. In certain embodiments, targeted integration is achieved by multiple RMCEs, wherein DNA sequences from multiple vectors, each comprising at least one expression cassette encoding a part of a heteromultimeric polypeptide and/or at least one selection marker or part thereof flanked by two heterospecific RRSs, are all integrated into a predetermined site of the genome of a mammalian cell suitable for TI. In certain embodiments the selection marker can be partially encoded on the first the vector and partially encoded on the second vector such that only the correct integration of both by double RMCE allows for the expression of the selection marker. An example of such a system is presented in Figure 1.

In certain embodiments, targeted integration via recombinase-mediated recombination leads to selection marker and/or the different expression cassettes for the multimeric polypeptide integrated into one or more pre-determined integration sites of a host cell genome free of sequences from a prokaryotic vector.

There is provided a method for producing a trivalent antibody comprising the steps of

a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding the trivalent antibody, and

b) recovering the trivalent antibody from the cell or the cultivation medium,

wherein the deoxyribonucleic acid encoding the trivalent antibody is stably integrated into the genome of the mammalian cell and comprises in 5’- to 3’-direction either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain, and
- a fifth expression cassette encoding the second light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain,

or (3)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the second light chain,

or (4)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the first heavy chain,
- a sixth expression cassette encoding the second light chain,

or (5)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second light chain,
- a seventh expression cassette encoding the second light chain,

or (6)

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first heavy chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain.

There is provided a deoxyribonucleic acid encoding a trivalent antibody comprising in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain, and
- a fifth expression cassette encoding the second light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain,

or (3)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the second light chain,

or (4)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the first heavy chain,
- a sixth expression cassette encoding the second light chain,

or (5)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second light chain,
- a seventh expression cassette encoding the second light chain,

or (6)

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first heavy chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain.

There is provided use of a deoxyribonucleic acid comprising in 5'- to 3'-direction either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain, and
- a fifth expression cassette encoding the second light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain,

or (3)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the second light chain,

or (4)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the first heavy chain,
- a sixth expression cassette encoding the second light chain,

or (5)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second light chain,
- a seventh expression cassette encoding the second light chain,

or (6)

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first heavy chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain,

for the expression of the trivalent antibody in a mammalian cell.

There is provided a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a trivalent antibody integrated in the genome of the cell, wherein the deoxyribonucleic acid encoding the trivalent antibody comprises in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain, and
- a fifth expression cassette encoding the second light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain,

or (3)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the second light chain,

or (4)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the first heavy chain,
- a sixth expression cassette encoding the second light chain,

or (5)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second light chain,
- a seventh expression cassette encoding the second light chain,

or (6)

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first heavy chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain.

There is provided a composition comprising two deoxyribonucleic acids, which comprise in turn three different recombination recognition sequences and five to seven expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5’- to 3’-direction,

either (1)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

or (2)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

or (3)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain, and
- a first copy of a third recombination recognition sequence,

or (4)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain, and
- a first copy of a third recombination recognition sequence,

or (5)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain, and
- a first copy of a third recombination recognition sequence,

or (6)

- a first recombination recognition sequence,
- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first heavy chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5’- to 3’-direction

either (1)

- a second copy of the third recombination recognition sequence,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (2)

- a second copy of the third recombination recognition sequence,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (3)

- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (4)

- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the first heavy chain,
- a sixth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (5)

- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second light chain,
- a seventh expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (6)

- a second copy of the third recombination recognition sequence,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain, and
- a second recombination recognition sequence.

There is provided a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a trivalent antibody and secreting the trivalent antibody, comprising the following steps:

a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and five to seven expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5’- to 3’-direction,

either (1)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

or (2)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

or (3)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain, and
- a first copy of a third recombination recognition sequence,

or (4)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain, and
- a first copy of a third recombination recognition sequence,

or (5)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second light chain, and
- a first copy of a third recombination recognition sequence,

or (6)

- a first recombination recognition sequence,
- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first heavy chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5’- to 3’-direction

either (1)

- a second copy of the third recombination recognition sequence,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (2)
- a second copy of the third recombination recognition sequence,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (3)
- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (4)
- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the first heavy chain,
- a sixth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (5)
- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the second light chain,
- a seventh expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

or (6)
- a second copy of the third recombination recognition sequence,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the second light chain,
- a sixth expression cassette encoding the second light chain, and
- a second recombination recognition sequence,

wherein the first to third recombination recognition sequences of the first and second deoxyribonucleic acids are matching the first to third recombination recognition sequence on the integrated exogenous nucleotide sequence,
wherein the 5’-terminal part and the 3’-terminal part of the expression cassette encoding the one second selection marker when taken together form a functional expression cassette of the one second selection marker;

c) introducing

i) either simultaneously with the first and second deoxyribonucleic acid of b);

or

ii) sequentially thereafter

one or more recombinases,

wherein the one or more recombinases recognize the recombination recognition sequences of the first and the second deoxyribonucleic acid; (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)

and

d) selecting for cells expressing the second selection marker and secreting the trivalent antibody,

thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the trivalent antibody and secreting the trivalent antibody.

In one example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat), or vice versa.

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein one of the heavy chains further comprises the mutation S354C and the respective other heavy chain comprises the mutation Y349C (numbering according to Kabat).

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the first heavy chain is an extended heavy chain comprising an additional domain exchanged Fab fragment.

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the first light chain is a domain exchanged light chain VH-VL or CH1-CL.

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein

- the first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a first light chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- the second heavy chain comprises from N- to C-terminus the first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- the first light chain comprises from N- to C-terminus a second heavy chain variable domain and a CL domain, and
- the second light chain comprises from N- to C- terminus a second light chain variable domain and a CL domain,

wherein the first heavy chain variable domain and the second light chain variable domain form a first binding site and the second heavy chain variable domain and the first light chain variable domain form a second binding site.

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the deoxyribonucleic acid encoding the trivalent antibody comprises a further expression cassette encoding for a selection marker, wherein the expression cassette encoding for the selection marker is located partly 5’ and partly 3’ to the third recombination recognition sequence, wherein the 5’-located part of said expression cassette comprises the promoter and the start-codon and the 3’-

located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal, wherein the start-codon is operably linked to the coding sequence.

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the promoter is the human CMV promoter with intron A, the polyadenylation signal sequence is the bGH polyadenylation signal sequence and the terminator is the hGT terminator except for the expression cassette of the selection marker, wherein the promoter is the SV40 promoter and the polyadenylation signal sequence is the SV40 polyadenylation signal sequence and a terminator is absent.

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the mammalian cell is a CHO cell.

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein in case the organization in 5’ to 3’-direction has as first expression cassette an expression cassette encoding the first heavy chain all cassettes are arranged unidirectional.

In another example, there is provided the method for producing a trivalent antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein in case the organization in 5’ to 3’-direction is first expression cassette encoding the first light chain, second expression cassette encoding the first light chain, third expression cassette encoding the first heavy chain, fourth expression cassette encoding the second heavy chain, fifth expression cassette encoding the second light chain, sixth expression cassette encoding the second light chain, the first to third expression cassettes are arranged unidirectional and the fourth to sixth expression cassette are arranged unidirectional whereby the

first to third expression cassettes are arranged in opposite direction of the fourth to sixth expression cassette.

There is provided a method for producing a bivalent, bispecific antibody comprising the steps of

a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding the bivalent, bispecific antibody, and

b) recovering the bivalent, bispecific antibody from the cell or the cultivation medium,

wherein the deoxyribonucleic acid encoding the bivalent, bispecific antibody is stably integrated into the genome of the mammalian cell and comprises in 5'- to 3'-direction

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the second heavy chain,

wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

There is provided a deoxyribonucleic acid encoding a bivalent, bispecific antibody comprising in 5'- to 3'-direction

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the second heavy chain,

wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

There is provided use of a deoxyribonucleic acid comprising in 5'- to 3'-direction

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the second heavy chain,

for the expression of the bivalent, bispecific antibody in a mammalian cell,

wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

There is provided a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a bivalent, bispecific antibody integrated in the genome of the cell, wherein the deoxyribonucleic acid encoding the bivalent, bispecific antibody comprises in 5'- to 3'-direction

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the second heavy chain,

wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

There is provided a composition comprising two deoxyribonucleic acids, which comprise in turn three different recombination recognition sequences and four expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction,
- a first recombination recognition sequence,
- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction
- a second copy of the third recombination recognition sequence,
- a third expression cassette encoding the second light chain,
- a fourth expression cassette encoding the second heavy chain, and
- a second recombination recognition sequence,

wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

There is provided a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a bivalent, bispecific antibody and secreting the bivalent, bispecific antibody, comprising the following steps:

a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and four expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction,
- a first recombination recognition sequence,
- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction
- a second copy of the third recombination recognition sequence,
- a third expression cassette encoding the second light chain,
- a fourth expression cassette encoding the second heavy chain, and
- a second recombination recognition sequence,

wherein the first to third recombination recognition sequences of the first and second deoxyribonucleic acids are matching the first to third recombination recognition sequence on the integrated exogenous nucleotide sequence,

wherein the 5'-terminal part and the 3'-terminal part of the expression cassette encoding the one second selection marker when taken together form a functional expression cassette of the one second selection marker;

wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat);

c) introducing

i) either simultaneously with the first and second deoxyribonucleic acid of b);

or

ii) sequentially thereafter

one or more recombinases,

wherein the one or more recombinases recognize the recombination recognition sequences of the first and the second deoxyribonucleic acid; (and optionally wherein

the one or more recombinases perform two recombinase mediated cassette exchanges;)
and
d) selecting for cells expressing the second selection marker and secreting the bivalent, bispecific antibody;
thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the bivalent, bispecific antibody and secreting the bivalent, bispecific antibody.

In another example, there is provided the method for producing a bivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein one of the heavy chains further comprises the mutation S354C and the respective other heavy chain comprises the mutation Y349C (numbering according to Kabat).

In another example, there is provided the method for producing a bivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the second light chain is a domain exchanged light chain VH-CH1 after VH-VL exchange or a domain exchanged light chain VL-CH1 after CH1-CL exchange.

In another example, there is provided the method for producing a bivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein
- the first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- the second heavy chain comprises from N- to C-terminus the first light chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- the first light chain comprises from N- to C-terminus a second heavy chain variable domain and a CL domain, and
- the second light chain comprises from N- to C- terminus a second light chain variable domain and a CL domain,

wherein the first heavy chain variable domain and the second light chain variable domain form a first binding site and the second heavy chain variable domain and the first light chain variable domain form a second binding site.

In another example, there is provided the method for producing a bivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

In another example, there is provided the method for producing a bivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the deoxyribonucleic acid encoding the bivalent, bispecific antibody comprises a further expression cassette encoding for a selection marker, wherein the expression cassette encoding for the selection marker is located partly 5’ and partly 3’ to the third recombination recognition sequence, wherein the 5’-located part of said expression cassette comprises the promoter and the start-codon and the 3’-located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal, wherein the start-codon is operably linked to the coding sequence, wherein the 5’-located part of the expression cassette encoding the selection marker comprises a promoter sequence operably linked to a start-codon, whereby the promoter sequence is flanked upstream by the second expression cassette and the start-codon is flanked downstream by the third recombination recognition sequence; and the 3’-located part of the expression cassette encoding the selection marker comprises a nucleic acid encoding the selection marker lacking a start-codon and is flanked upstream by the third recombination recognition sequence and downstream by the third expression cassette, wherein the start-codon is operably linked to the coding sequence.

In another example, there is provided the method for producing a bivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein

each expression cassette for an antibody chain comprises in 5’-to-3’ direction a promoter, a nucleic acid encoding an antibody chain, and a polyadenylation signal sequence and optionally a terminator sequence

and

each expression cassette encoding the selection marker comprises in 5'-to-3' direction a promoter, a nucleic acid encoding the selection marker, and a polyadenylation signal sequence and optionally a terminator sequence,

wherein the promoter is the human CMV promoter with intron A, the polyadenylation signal sequence is the bGH polyadenylation signal sequence and the terminator is the hGT terminator except for the expression cassette of the selection marker, wherein the promoter is the SV40 promoter and the polyadenylation signal sequence is the SV40 polyadenylation signal sequence and a terminator is absent.

In another example, there is provided the method for producing a bivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the mammalian cell is a CHO cell.

In another example, there is provided the method for producing a bivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein all cassettes are arranged unidirectional.

There is provided a method for producing a multivalent, bispecific antibody comprising the steps of

a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding the multivalent, bispecific antibody, and

b) recovering the multivalent, bispecific antibody from the cell or the cultivation medium,

wherein the deoxyribonucleic acid encoding the multivalent, bispecific antibody is stably integrated into the genome of the mammalian cell and comprises in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and
- a sixth expression cassette encoding the first light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- an eighth expression cassette encoding the first light chain.

There is provided a deoxyribonucleic acid encoding a multivalent, bispecific antibody comprising in 5'- to 3'-direction
either (1)
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and
- a sixth expression cassette encoding the first light chain,

or (2)
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- an eighth expression cassette encoding the first light chain.

There is provided use of a deoxyribonucleic acid comprising in 5'- to 3'-direction
either (1)
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and
- a sixth expression cassette encoding the first light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- an eighth expression cassette encoding the first light chain,

for the expression of the multivalent, bispecific antibody in a mammalian cell.

There is provided a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a multivalent, bispecific antibody integrated in the genome of the cell, wherein the deoxyribonucleic acid encoding the multivalent, bispecific antibody comprises in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and
- a sixth expression cassette encoding the first light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- an eighth expression cassette encoding the first light chain.

There is provided a composition comprising two deoxyribonucleic acids, which comprise in turn three different recombination recognition sequences and six or eight expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction,

either (1)

- a first recombination recognition sequence,

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

or (2)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction

either (1)

- a second copy of the third recombination recognition sequence,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain,
- a sixth expression cassette encoding the first light chain, and
- a second recombination recognition sequence,

or (2)

- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain,
- an eighth expression cassette encoding the first light chain, and
- a second recombination recognition sequence.

There is provided a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a multivalent, bispecific antibody and secreting the multivalent, bispecific antibody, comprising the following steps:

a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and six or eight expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction,

either (1)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

or (2)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction

either (1)

- a second copy of the third recombination recognition sequence,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain,
- a sixth expression cassette encoding the first light chain, and
- a second recombination recognition sequence,

or (2)

- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain,
- an eighth expression cassette encoding the first light chain, and
- a second recombination recognition sequence,

wherein the first to third recombination recognition sequences of the first and second deoxyribonucleic acids are matching the first to third recombination recognition sequence on the integrated exogenous nucleotide sequence,

wherein the 5'-terminal part and the 3'-terminal part of the expression cassette encoding the one second selection marker when taken together form a functional expression cassette of the one second selection marker;

c) introducing

i) either simultaneously with the first and second deoxyribonucleic acid of b);

or

ii) sequentially thereafter

one or more recombinases,

wherein the one or more recombinases recognize the recombination recognition sequences of the first and the second deoxyribonucleic acid; (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)

and

d) selecting for cells expressing the second selection marker and secreting the multivalent, bispecific antibody,

thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the multivalent, bispecific antibody and secreting the multivalent, bispecific antibody.

In another example, there is provided the method for producing a multivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat), or vice versa.

In another example, there is provided the method for producing a multivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein one of the heavy chains further comprises the mutation S354C and the respective other heavy chain comprises the mutation Y349C (numbering according to Kabat).

In another example, there is provided the method for producing a multivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant

mammalian cell as described herein, wherein the first light chain is a domain exchanged light chain VH-VL or CH1-CL.

In another example, there is provided the method for producing a multivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein

- the first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain and a first light chain variable domain,
- the second heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain and a second heavy chain variable domain, and
- the first light chain comprises from N- to C-terminus a second light chain variable domain and a CL domain,

wherein the first heavy chain variable domain and the second light chain variable domain form a first binding site and the second heavy chain variable domain and the first light chain variable domain form a second binding site.

In another example, there is provided the method for producing a multivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

In another example, there is provided the method for producing a multivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the deoxyribonucleic acid encoding the multivalent, bispecific antibody comprises a further expression cassette encoding for a selection marker, wherein the expression cassette encoding for the selection marker is located partly 5’ and partly 3’ to the third recombination recognition sequence, wherein the 5’-located part of said expression cassette comprises the promoter and the start-codon and the 3’-located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal, wherein the start-codon

is operably linked to the coding sequence, wherein the 5'-located part of the expression cassette encoding the selection marker comprises a promoter sequence operably linked to a start-codon, whereby the promoter sequence is flanked upstream by the third or fourth expression cassette, respectively, and the start-codon is flanked downstream by the third recombination recognition sequence; and the 3'-located part of the expression cassette encoding the selection marker comprises a nucleic acid encoding the selection marker lacking a start-codon and is flanked upstream by the third recombination recognition sequence and downstream by the fourth or fifth expression cassette, respectively, wherein the start-codon is operably linked to the coding sequence.

In another example, there is provided the method for producing a multivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein
each expression cassette for an antibody chain comprises in 5'-to-3' direction a promoter, a nucleic acid encoding an antibody chain, and a polyadenylation signal sequence and optionally a terminator sequence
and
each expression cassette encoding the selection marker comprises in 5'-to-3' direction a promoter, a nucleic acid encoding the selection marker, and a polyadenylation signal sequence and optionally a terminator sequence,
wherein the promoter is the human CMV promoter with intron A, the polyadenylation signal sequence is the bGH polyadenylation signal sequence and the terminator is the hGT terminator except for the expression cassette of the selection marker, wherein the promoter is the SV40 promoter and the polyadenylation signal sequence is the SV40 polyadenylation signal sequence and a terminator is absent.

In another example, there is provided the method for producing a multivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein the mammalian cell is a CHO cell.

In another example, there is provided the method for producing a multivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell as described herein, wherein all expression cassettes are arranged unidirectional.

There is provided a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a polypeptide and secreting the polypeptide comprising the following steps:

a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different, wherein the mammalian cell is free of Cre-recombinase encoding DNA;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and one to eight expression cassettes, wherein

the first deoxyribonucleic acid comprises in 5’- to 3’-direction,

- a first recombination recognition sequence,
- one or more expression cassette(s),
- a 5’-terminal part of an expression cassette encoding one second selection marker, and
- a first copy of a third recombination recognition sequence,

and

the second deoxyribonucleic acid comprises in 5’- to 3’-direction

- a second copy of the third recombination recognition sequence,
- a 3’-terminal part of an expression cassette encoding the one second selection marker,
- one or more expression cassette(s), and
- a second recombination recognition sequence,

wherein the first to third recombination recognition sequences of the first and second deoxyribonucleic acids are matching the first to third recombination recognition sequence on the integrated exogenous nucleotide sequence,

wherein the 5'-terminal part and the 3'-terminal part of the expression cassette encoding the one second selection marker when taken together form a functional expression cassette of the one second selection marker,
wherein the deoxyribonucleic acids are free of Cre-recombinase encoding DNA;
c) introducing
i) either simultaneously with the first and second deoxyribonucleic acid of b); or
ii) sequentially thereafter
Cre-recombinase mRNA as sole source of Cre-recombinase,
wherein the Cre-recombinases recognize the recombination recognition sequences of the first and the second deoxyribonucleic acid; (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)
and
d) selecting for cells expressing the second selection marker and secreting the polypeptide,
thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the polypeptide and secreting the polypeptide.

In another example, there is provided the method as described herein, wherein the Cre mRNA encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 20.

In another example, there is provided the method as described herein, wherein the Cre mRNA comprises the nucleotide sequence of SEQ ID NO: 21 or a codon usage optimized variant thereof.

In another example, there is provided the method as described herein, wherein exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

In another example, there is provided the method as described herein, wherein the deoxyribonucleic acid encoding the polypeptide comprises at least 4 expression cassettes wherein
- a first recombination recognition sequence is located 5' to the most 5' (i.e. first) expression cassette,
- a second recombination recognition sequence is located 3' to the most 3' expression cassette, and
- a third recombination recognition sequence is located

- between the first and the second recombination recognition sequence, and
- between two of the expression cassettes,

and

wherein all recombination recognition sequences are different.

In another example, there is provided the method as described herein, wherein the deoxyribonucleic acid encoding the polypeptide comprises a further expression cassette encoding for a selection marker.

In another example, there is provided the method as described herein, wherein the deoxyribonucleic acid encoding the polypeptide comprises a further expression cassette encoding for a selection marker and the expression cassette encoding for the selection marker is located partly 5' and partly 3' to the third recombination recognition sequence, wherein the 5'-located part of said expression cassette comprises the promoter and the start-codon and the 3'-located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal, wherein the start-codon is operably linked to the coding sequence.

In another example, there is provided the method as described herein, wherein the ratio by weight between Cre mRNA and mixture of first and second vector is in the range of from 1:3 to 2:1.

In another example, there is provided the method as described herein, wherein the ratio by weight between Cre mRNA and mixture of first and second vector is about 1:5.

In another example, there is provided the method as described herein, wherein each of the expression cassettes comprise in 5'-to-3' direction a promoter, a coding sequence and a polyadenylation signal sequence optionally followed by a terminator sequence, wherein the promoter is the human CMV promoter with intron A, the polyadenylation signal sequence is the bGH polyadenylation signal sequence and the terminator is the hGT terminator except for the expression cassette of the selection marker, wherein the promoter is the SV40 promoter and the polyadenylation signal sequence is the SV40 polyadenylation signal sequence and a terminator is absent.

In another example, there is provided the method as described herein, wherein the mammalian cell is a CHO cell.

In another example, there is provided the method as described herein, wherein the polypeptide is a heterotetramer comprising a first antibody heavy chain, a second antibody heavy chain, a first antibody light chain and a second antibody light chain, the deoxyribonucleic acid comprises four expression cassettes and

- the first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a first light chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- the second heavy chain comprises from N- to C-terminus s first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- the first light chain comprises from N- to C-terminus a second heavy chain variable domain and a CL domain, and
- the second light chain comprises from N- to C- terminus a second light chain variable domain and a CL domain,

wherein the first heavy chain variable domain and the second light chain variable domain form a first binding site and the second heavy chain variable domain and the first light chain variable domain form a second binding site.

In another example, there is provided the method as described herein, wherein the polypeptide is a heterotetramer comprising a first antibody heavy chain, a second antibody heavy chain, a first antibody light chain and a second antibody light chain, the deoxyribonucleic acid comprises four expression cassettes and

- the first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- the second heavy chain comprises from N- to C-terminus a first light chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- the first light chain comprises from N- to C-terminus a second heavy chain variable domain and a CL domain, and
- the second light chain comprises from N- to C- terminus a second light chain variable domain and a CL domain,

wherein the first heavy chain variable domain and the second light chain variable domain form a first binding site and the second heavy chain variable domain and the first light chain variable domain form a second binding site.

In another example, there is provided the method as described herein, wherein the polypeptide is a heterotetramer comprising a first antibody heavy chain, a second

antibody heavy chain, a first antibody light chain and a second antibody light chain, the deoxyribonucleic acid comprises four expression cassettes and

- the first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain, a peptidic linker, a second heavy chain variable domain and a CL domain,
- the second heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- the first light chain comprises from N- to C-terminus a first light chain variable domain and a CH1 domain, and
- the second light chain comprises from N- to C- terminus a second light chain variable domain and a CL domain,

wherein the second heavy chain variable domain and the first light chain variable domain form a first binding site and the first heavy chain variable domain and the second light chain variable domain form a second binding site.

In another example, there is provided the method as described herein, wherein the first recombinase recognition sequence is L3, the second recombinase recognition sequence is 2L and the third recombinase recognition sequence is LoxFas.

There is provided use of Cre-recombinase mRNA for increasing the number of recombinant mammalian cells comprising a deoxyribonucleic acid encoding a polypeptide or protein of interest stably integrated at a single site in the genome of said cell by targeted integration.

In another example, there is provided the use as described herein, wherein the recombinant cell further secrets the polypeptide of interest into the cultivation medium upon cultivation therein.

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In addition to the various embodiments depicted and claimed, the disclosed subject matter is also directed to other embodiments having other combinations of the features disclosed and claimed herein. As such, the particular features presented herein can be combined with each other in other manners within the scope of the disclosed subject matter such that the disclosed subject matter includes any suitable combination of the features disclosed herein. The foregoing description of specific embodiments of the disclosed subject matter has been presented for purposes of

illustration and description. It is not intended to be exhaustive or to limit the disclosed subject matter to those embodiments disclosed.

It will be apparent to those skilled in the art that various modifications and variations can be made in the compositions and methods of the disclosed subject matter without departing from the spirit or scope of the disclosed subject matter. Thus, it is intended that the disclosed subject matter include modifications and variations that are within the scope of the appended claims and their equivalents.

Various publications, patents and patent applications are cited herein, the contents of which are hereby incorporated by reference in their entireties.

Patent Claims

1. A method for producing a trivalent, bispecific antibody comprising the steps of

   a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding the trivalent, bispecific antibody, and

   b) recovering the trivalent, bispecific antibody from the cell or the cultivation medium,

   wherein the deoxyribonucleic acid encoding the trivalent, bispecific antibody is stably integrated into the genome of the mammalian cell and comprises in 5'- to 3'-direction
   - a first expression cassette encoding the first heavy chain,
   - a second expression cassette encoding the first heavy chain,
   - a third expression cassette encoding the first light chain,
   - a fourth expression cassette encoding the first light chain,
   - a fifth expression cassette encoding the second heavy chain,
   - a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain, and
   - a seventh expression cassette encoding the second light chain.

2. A deoxyribonucleic acid encoding a trivalent, bispecific antibody comprising in 5'- to 3'-direction
   - a first expression cassette encoding the first heavy chain,
   - a second expression cassette encoding the first heavy chain,
   - a third expression cassette encoding the first light chain,
   - a fourth expression cassette encoding the first light chain,
   - a fifth expression cassette encoding the second heavy chain,
   - a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain, and
   - a seventh expression cassette encoding the second light chain.

3. Use of a deoxyribonucleic acid comprising in 5'- to 3'-direction
   - a first expression cassette encoding the first heavy chain,
   - a second expression cassette encoding the first heavy chain,
   - a third expression cassette encoding the first light chain,
   - a fourth expression cassette encoding the first light chain,

- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain, and
- a seventh expression cassette encoding the second light chain,

for the expression of the trivalent, bispecific antibody in a mammalian cell.

4. A recombinant mammalian cell comprising a deoxyribonucleic acid encoding a trivalent, bispecific antibody integrated in the genome of the cell, wherein the deoxyribonucleic acid encoding the trivalent, bispecific antibody comprises in 5'- to 3'-direction
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain, and
- a seventh expression cassette encoding the second light chain.

5. A composition comprising two deoxyribonucleic acids, which comprise in turn three different recombination recognition sequences and four expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction,
  - a first recombination recognition sequence,
  - a first expression cassette encoding the first heavy chain,
  - a second expression cassette encoding the first heavy chain,
  - a third expression cassette encoding the first light chain,
  - a fourth expression cassette encoding the first light chain, and
  - a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction
  - a second copy of the third recombination recognition sequence,
  - a fifth expression cassette encoding the second heavy chain,
  - a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain,
  - a seventh expression cassette encoding the second light chain, and

- a second recombination recognition sequence.

6. A method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a trivalent, bispecific antibody and secreting the trivalent, bispecific antibody, comprising the following steps:

a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and at least seven expression cassettes, wherein
   - the first deoxyribonucleic acid comprises in 5'- to 3'-direction,
     - a first recombination recognition sequence,
     - a first expression cassette encoding the first heavy chain,
     - a second expression cassette encoding the first heavy chain,
     - a third expression cassette encoding the first light chain,
     - a fourth expression cassette encoding the first light chain, and
     - a first copy of a third recombination recognition sequence,

   and

   - the second deoxyribonucleic acid comprises in 5'- to 3'-direction
     - a second copy of the third recombination recognition sequence,
     - a fifth expression cassette encoding the second heavy chain,
     - a sixth expression cassette encoding either the first light chain or the second heavy chain or the second light chain,
     - a seventh expression cassette encoding the second light chain, and
     - a second recombination recognition sequence,

wherein the first to third recombination recognition sequences of the first and second deoxyribonucleic acids are matching the first to third recombination recognition sequence on the integrated exogenous nucleotide sequence,

wherein the 5’-terminal part and the 3’-terminal part of the expression cassette encoding the one second selection marker when taken together form a functional expression cassette of the one second selection marker;

c) introducing
   i) either simultaneously with the first and second deoxyribonucleic acid of b);
   or
   ii) sequentially thereafter
   one or more recombinases,

   wherein the one or more recombinases recognize the recombination recognition sequences of the first and the second deoxyribonucleic acid; (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)

and

d) selecting for cells expressing the second selection marker and secreting the trivalent, bispecific antibody,

thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the trivalent, bispecific antibody and secreting the trivalent, bispecific antibody.

7. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1 to 6, wherein the deoxyribonucleic acid comprises after the seven expression cassette an eighth expression cassette encoding the second light chain

8. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1 to 7, wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

9. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to claim 8, wherein one of the heavy chains further comprises the mutation S354C and the respective other heavy chain comprises the mutation Y349C (numbering according to Kabat).

10. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1 to 9, wherein the first heavy chain is an extended heavy chain comprising an additional domain exchanged Fab fragment VH-VL or CH1-CL.

11. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1 to 10, wherein the first light chain is a domain exchanged light chain VH-VL or CH1-CL.

12. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1 to 11, wherein

- the first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain, a peptidic linker, a second heavy chain variable domain and a CL domain,

- the second heavy chain comprises from N- to C-terminus the first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,

- the first light chain comprises from N- to C-terminus a first light chain variable domain and a CH1 domain, and

- the second light chain comprises from N- to C- terminus a second light chain variable domain and a CL domain,

wherein the second heavy chain variable domain and the first light chain variable domain form a first binding site and the first heavy chain variable domain and the second light chain variable domain form a second binding site.

13. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1, 3, 4 and 6 to 12, wherein exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

14. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1 to 5 and 7 to 13, wherein the deoxyribonucleic acid encoding the trivalent, bispecific antibody comprises a further expression cassette encoding for a selection marker, ´

wherein the expression cassette encoding for the selection marker is located partly 5’ and partly 3’ to the third recombination recognition sequence, wherein the 5’-located part of said expression cassette comprises the promoter and the start-codon and the 3’-located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal, wherein the start-codon is operably linked to the coding sequence,

wherein the 5’-located part of the expression cassette encoding the selection marker comprises a promoter sequence operably linked to a start-codon, whereby the promoter sequence is flanked upstream by the fourth expression cassette and the start-codon is flanked downstream by the third recombination recognition sequence; and the 3’-located part of the expression cassette encoding the selection marker comprises a nucleic acid encoding the selection marker lacking a start-codon and is flanked upstream by the third recombination recognition sequence and downstream by the fifth expression cassette, wherein the start-codon is operably linked to the coding sequence.

15. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1 to 14, wherein

each expression cassette for an antibody chain comprises in 5’-to-3’ direction a promoter, a nucleic acid encoding an antibody chain, and a polyadenylation signal sequence and optionally a terminator sequence

and

each expression cassette encoding the selection marker comprises in 5’-to-3’ direction a promoter, a nucleic acid encoding the selection marker, and a polyadenylation signal sequence and optionally a terminator sequence,

wherein the promoter is the human CMV promoter with intron A, the polyadenylation signal sequence is the bGH polyadenylation signal sequence and the terminator is the hGT terminator except for the expression cassette of the selection marker, wherein the promoter is the SV40 promoter and the polyadenylation signal sequence is the SV40 polyadenylation signal sequence and a terminator is absent.

16. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1, 3, 4 and 6 to 15, wherein the mammalian cell is a CHO cell.

17. The method for producing a trivalent, bispecific antibody or the deoxyribonucleic acid or the use or the recombinant mammalian cell or the composition or the method for producing a recombinant mammalian cell according to any one of claims 1 to 16, wherein all cassettes are arranged unidirectional.