

Multiplex immunohistochemistry (mIHC) with barcoded-antibodies

Technical Field

The present invention relates to the field of detection and quantification of antigens.

- 5 In particular the present invention relates to a multiplex immunohistochemistry (mIHC) method that uses a barcoded-antibody, a specifically-designed template circle, a fluorescent probe and/or a mild denaturing buffer for signal amplification and detection.

Background

- 10 Immunohistochemistry refers to the identification of antigens in a tissue section by utilizing antibodies that bind specifically to the antigens of interest. The antibody-antigen binding is most commonly visualized by color or fluorescence, with the use of color-producing enzyme reactions or fluorophores respectively. IHC is widely used in many areas including research and medical applications. In particular, histopathological evaluation of
15 biomarkers in tissue samples is not only used as a diagnostic tool, it also enables prognostic and risk stratification assessments for precision therapy.

- However with the discovery of increasing numbers of clinically relevant biomarkers, especially in complex diseases such as cancer, the ability to simultaneously identify multiple
20 biomarkers on a single tissue section has become crucial. mIHC is limited by the compatibility and number of host species in which antibodies are produced and, in the case of fluorescence microscopy, the spectral overlap between fluorophores. Therefore there is a need for new and improved mIHC methods that allow sensitive detection of multiple antigens on a single tissue section.

25

- The invention described here exploits amplification of oligonucleotides conjugated to antibodies as a means for signal amplification. Besides signal amplification, the antibody-specific oligonucleotides also serve as identification barcodes. Furthermore the invention attains multiplexity by incorporating multiple rounds of fluorescent probe hybridization,
30 imaging and probe removal and the compositions and conditions for achieving multiplexity is described in the method.

Summary

The invention uses a combination of oligo-conjugated antibodies, nucleic acid amplification and fluorescent probe hybridization to achieve signal amplification and detection.

5

The invention makes use of antibodies (Abs) that are highly specific for their target antigens and each conjugated with its specific oligonucleotide. This specific oligonucleotide serves two main functions. It acts as an identification barcode for its conjugated antibody (Ab) and it also acts as a primer for nucleic acid amplification.

10

After any necessary pretreatment of tissue sections for IHC, all the barcoded-Abs are first incubated with the sample for antigen binding in a single step. The oligonucleotides are then simultaneously amplified by rolling circle amplification (RCA) with specifically-designed template circles. There are 2 main features of each template circle. It will include
15 a complementary sequence for binding to its specific oligonucleotide barcode as well as a unique probe sequence. After polymerization and amplification of the oligonucleotides with its specific template circle, each oligonucleotide should form a long single-stranded amplicon containing several copies of the unique probe complementary sequence. The first round of detection is then performed with the addition of a mixture of fluorescent probes
20 that are specific for the first set of antigens (and hence amplicons) to be detected. The fluorescent probes will hybridize to their specific targets (amplicons) via the unique probe complementary sequences contained in the amplicons and signal amplification is achieved through the multiple copies of probe complementary sequences found in each amplicon. After excess probes are washed off and the sample is imaged, the first set of probes will be
25 removed with a mild denaturing buffer. The second round of detection is next performed with the addition of another mixture of fluorescent probes (second set) that are specific for the second set of antigens to be detected. Subsequent imaging of the sample and probe removal will take place, which then prepares the tissue sample for the next round of probe hybridization. Thus multiplexity is achieved with multiple rounds of probe hybridization,
30 sample imaging and probe removal.

Besides RCA, other nucleic acid amplification reactions can also be utilized such as hybridization chain reaction (HCR). The mild denaturing buffer may comprise of any

chemicals or reagents that disrupt or denature double-stranded DNA hybridization, but do not affect the structure and integrity of the tissue sample, proteins or Abs.

5 Thus, the present invention first relates to a template circle comprising
a complementary sequence for binding to a specific oligonucleotide barcode; and
a unique probe sequence.

10 According to one embodiment of the present invention, the sequence of the template
circle has less than 95% identity to any human sequences or bacterial sequences.

According to one embodiment of the present invention, the sequence of the template
circle is selected from plant-related genes and Tobacco mosaic virus.

15 According to one embodiment of the present invention, a folding structure of the
template circle in circular form has a free energy ≥ 2 kcal/mol.

According to one embodiment of the present invention, the template circle is less
than 90 bases in length.

20 According to one embodiment of the present invention, the template circle comprises
a nucleotide sequence as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ
ID NO: 4, or comprises a nucleotide sequence having at least or about 80%, 85%, 90%,
95%, 96%, 97%, 98%, or 99% identity to the sequence as set forth in SEQ ID NO: 1, SEQ
ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

25

The present invention also relates to a multiplexable set comprising
a template circle as described above,
a probe having the unique probe sequence comprised in the template circle, and
a barcoded-antibody comprising a specific oligonucleotide barcode which binds to
30 the template circle.

According to one embodiment of the present invention, the probe is 15 – 30 bases in
length, preferably about 20 bases in length.

According to one embodiment of the present invention, the specific oligonucleotide barcode is 20 – 40 bases in length, preferably about 30 bases in length.

5 According to one embodiment of the present invention, the probe is conjugated to an observable moiety such as a fluorophore. In some embodiments of the present invention, same observable moiety (such as the same fluorophore) can be used for different rounds of imaging so that multiplexing is not limited by spectral overlap of observable moieties in multiple rounds of imaging. Any fluorophore commonly used in the art can be applied
10 without limitation. For instance, the fluorophore can be selected from the group comprising FAM (5-or 6-carboxyfluorescein), VIC, NED, Fluorescein, Fluorescein isothiocyanate (FITC), IRD-700/800, Cyanine dyes, such as CY3, CY5, CY3.5, CY5.5, Cy7, Xanthene, 6-Carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), TET, 6-Carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE), N,N,N',N'-Tetramethyl-6-carboxyrhodamine (TAMRA), 6-
15 Carboxy-X-rhodamine (ROX), 5-Carboxyrhodamine-6G (R6G5), 6-carboxyrhodamine-6G (RG6), Rhodamine, Rhodamine Green, Rhodamine Red, Rhodamine 110, BODIPY dyes, such as BODIPY TMR, Oregon Green, Coumarines such as Umbelliferone, Benzimides, such as Hoechst 33258; Phenanthridines, such as Texas Red, Yakima Yellow, Alexa Fluor, PET, Ethidium bromide, Acridinium dyes, Carbazole dyes, Phenoxazine dyes, Porphyrin
20 dyes, Polymethine dyes, and the like.

 According to one embodiment of the present invention, the probe comprises a nucleotide sequence as set forth in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or SEQ ID NO: 8 or comprises a nucleotide sequence having at least or about 80%, 85%, 90%, 95%,
25 96%, 97%, 98%, or 99% identity to the sequence as set forth in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or SEQ ID NO: 8.

 According to one embodiment of the present invention, the oligonucleotide barcode comprises a nucleotide sequence as set forth in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID
30 NO: 11 or SEQ ID NO: 12, or comprises a nucleotide sequence having at least or about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence as set forth in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, or SEQ ID NO: 12.

The present invention also relates to a multiplex system including a plurality of multiplexable sets as described above, wherein template circles, barcodes and probes in different sets have no cross-reactivity or interference from each other.

5 The present invention also relates to a mild denaturing buffer for the gentle removal of probes, comprising:

- (1) a low ionic strength buffer;
- (2) a denaturant; and
- (3) a detergent.

10

According to one embodiment of the present invention, any low ionic strength buffer commonly used in the field can be comprised without limitations. According to one particular embodiment of the present invention, the low ionic strength buffer is 0.1x – 1x PBS. According to one embodiment of the present invention, the mild denaturing buffer
15 comprises PBS in a concentration of less than 10x, less than 5x, less than 2x, less than 1x, less than 0.5x, less than 0.2x. According to another embodiment of the present invention, the mild denaturing buffer comprises PBS in a concentration of more than 0.01x, more than 0.02x, more than 0.05x. Preferably, the mild denaturing buffer comprises PBS in a concentration of about 0.1x.

20

According to one embodiment of the present invention, any denaturant commonly used in the field can be comprised without limitations. According to one particular embodiment of the present invention, the denaturant is selected from sodium hydroxide, dimethyl sulfoxide, ethylene carbonate, urea, formamide, or any combination thereof.
25 Preferably, the denaturant is formamide.

According to one embodiment of the present invention, the denaturant is comprised in a concentration of more than 20%, more than 30%, more than 40%, more than 50%, more than 60%, more than 70%, or more than 80%. According to another embodiment of the present invention, the denaturant is comprised in a concentration of less than 99%, less than 95%, less than 90%, less than 80%, less than 70%, or less than 60%. According to one particular embodiment of the present invention, the denaturant is comprised in a
30 concentration of 40%-80%, 50%-70%, 55%-65%, or preferably about 60%.

According to one embodiment of the present invention, any detergent commonly used in the field can be comprised without limitations. According to one particular embodiment of the present invention, the detergent is selected from Triton X-100, Tween-
5 20 or any combination thereof. Preferably, the detergent is Tween-20.

According to one embodiment of the present invention, the detergent is comprised in a concentration of more than 0.01%, more than 0.02%, more than 0.05%, more than 0.1%, more than 0.2%, more than 0.5%, or more than 1%. According to another embodiment of
10 the present invention, the detergent is comprised in a concentration of less than 2%, less than 1.5%, less than 1%, less than 0.5%, less than 0.2%, or less than 0.1%. According to one particular embodiment of the present invention, the detergent is comprised in a concentration of 0.01%-2%, 0.02%-1%, 0.05%-0.5%, or preferably about 0.1%.

15 The present invention also relates to a method for mIHC, comprising the use of a template circle as described above, a multiplexable set as described above, a multiplex system as described above, a mild denaturing buffer as described above, or any combination thereof.

20 According to one embodiment of the present invention, the method comprises incubating all the barcoded-antibodies with a sample for antigen binding in a single step, and then simultaneously amplifying the barcodes by rolling circle amplification (RCA) with template circles as described above.

25 According to one embodiment of the present invention, the method comprises multiple rounds of probe hybridization, sample imaging and probe removal, and a mild denaturing buffer as described above is used for probe removal between different rounds.

According to one embodiment of the present invention, the method comprises
30 applying heat (30°C – 50°C) in combination with the mild denaturing buffer.

The present invention also relates to a kit for mIHC, comprising a template circle as described above, a multiplexable set as described above, a multiplex system as described above, a mild denaturing buffer as described above, or any combination thereof.

5 The present invention also relates to the use of a multiplexable set as described above, a multiplex system as described above, a mild denaturing buffer as described above, or any combination thereof for mIHC.

Positive effects

10 Usage of oligonucleotides conjugated to the Abs increases specificity as the oligonucleotides serve as identification barcodes, and increases sensitivity by signal amplification via nucleic acid amplification. Furthermore the use of oligonucleotide fluorescent hybridization probes that are easily removed by mild denaturing buffer enables high multiplexity to be attained. In addition, since all the Ab addition and barcode
15 amplification are performed in single steps, much time is saved compared to traditional mIHC in which Abs have to be stripped from the sample before a new set of Abs are incubated with the tissue section.

Brief Description of Drawings

20 To clearly indicate the technical solution of the present invention, a brief introduction thereto is provided below in reference to the Figures. Apparently, these Figures are merely some embodiments recorded in this application. The present invention includes but is not limited to these Figures.

25 Figure 1: mIHC images of DAPI (A), CD8 (B), PD-L1 (C) and CD68 (D) signals on a single section of FFPE tissue sample stained with barcoded-Abs.

 Figure 2: 4-plex mIHC images of single section of FFPE tissue sample stained with barcoded-Abs. After first round of probe hybridization, CD8 (B) and PD-L1 (C) signals were imaged, followed by probe removal, and second round of probe hybridization and imaging to detect DAPI (A), CD68 (D) and PanCK (E) signals.

30

Detailed Description

Abbreviations

Antibodies, Abs;
Bovine Serum Albumin, BSA;
5 4', 6-diamidino-2-phenylindole, DAPI;
Deoxynucleoside triphosphate, dNTP;
Ethylenediaminetetraacetic acid, EDTA;
Formalin-Fixed Paraffin-Embedded, FFPE;
hour, hr;
10 Multiplexed immunohistochemistry, mIHC;
minute, min;
Phosphate-Buffered Saline, PBS;
Rolling Circle Amplification, RCA;
Sodium Chloride-Sodium Citrate, SSC;
15 Tris-Buffered Saline, TBS.

Definitions

Unless defined otherwise, all terms of art, notations and other technical and scientific terms or terminology used herein are intended to have the same meaning as is commonly
20 understood by one of ordinary skill in the art.

As used herein, the term “about” as applied to one or more values of interest, refers to a value that is similar to a stated reference value, or within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in
25 part on how the value is measured or determined, such as the limitations of the measurement system. In one aspect, the term “about” refers to any values that are within a variation of up to $\pm 10\%$ of the value modified by the term “about.” Alternatively, “about” can mean within 3 or more standard deviations. Alternatively, such as with respect to biological systems or processes, the term “about” can mean within an order of magnitude, in some embodiments
30 within 5-fold, and in some embodiments within 2-fold, of a value.

As used herein, the term “include,” “comprise,” or “have” refers to the presence of stated features, numbers, steps, elements, or combinations thereof, but do not preclude the

presence or addition of one or more other features, numbers, steps, elements, or combinations thereof. In one aspect, the term “include,” “comprise,” or “have” encompasses “essentially consisting of”, or even “consisting of”.

5 As used herein, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. For example, “a” or “an” means “at least one” or “one or more.”

10 As used herein, the term “antibody” refers to any immunoglobulin from any species that can specifically recognize a target molecule. The term “antibody” also encompasses any engineered variation or fragment of an antibody such as Fab, Fab', F(ab')₂, single heavy chain, diabody, and the like (antigen binding fragments of antibodies). Those skilled in the art can contemplate substituting antibody in the present invention with any known binding partner of a target molecule and engineered variants of such binding partner, such as
15 aptamers.

As used herein, the term “ionic strength” is a measure of total concentration of dissolved ions in a solution. Ionic compounds (i.e., salts) will dissociate into ions after dissolving, increasing the ionic strength of a solution.

20

Design of Template Circle, Barcode and Probe

For the present invention, several sets of template circles with their respective barcodes and probes were designed. Tables 1- 3 show the sequences of template circles, probes and barcodes.

25

Table 1: Template Circle Sequences

Name	Sequence (5'→3')	SEQ ID NO
Template Circle 1 (T1)	TGCTAGACGAGGTCTACTGGGTGAGATGCTTGA CGTAGATCGTGCGAGTTGGAAGTCTGATGGTGC GTGTCGTAGCAGCACAAGTCC	SEQ ID NO: 1
Template Circle 6 (T6)	GGTCAGTCACATAAGTGCCTTATGTTGAGTGGA GGAGGTCATCGCTCGCCTGTGTCGTAGTCCATG CAGCTTCCTTGCGTAGTACAACCC	SEQ ID NO: 2

Name	Sequence (5'→3')	SEQ ID NO
Template Circle 11 (T11)	TTTTGGGTCCCCACCATTTCCCAAACCAGAAGC TGAGCATTGCTTTGTGCTTAAGTAGCCATTGTC TTGAACTGGGTTTCATTCC	SEQ ID NO: 3
Template Circle 12 (T12)	GGTATTGCTGAAAGCGGAAGGCATTCCTGTGG GAATCACTTATGTCCAGAGTGCGGTAGCCAAA GGAGCTGTTTGTGTTGGATGGA	SEQ ID NO: 4

Table 2: Probe Sequences

Name	Sequence (5'→3')	SEQ ID NO
Probe 1 (P1)	FAM-TCGTGCGAGTTGGAAGTCTGATGGT	SEQ ID NO: 5
Probe 6 (P6)	Cy5-TGAGTGGAGGAGGTCATCGCT	SEQ ID NO: 6
Probe 11 (P11)	Alexa546-CCACCATTTCCCAAACCAGA	SEQ ID NO: 7
Probe 12 (P12)	FAM-AAAGGAGCTGTTTGTGTTGGA	SEQ ID NO: 8

Table 3: Barcode Sequences

Name	Sequence (5'→3')	SEQ ID NO
Barcode 1	GGACTTGTGCTGCTACGACACGCACCATCA	SEQ ID NO: 9
Barcode 6	GGGTTGTACTACGCAAGGAAGCTGCATGGA	SEQ ID NO: 10
Barcode 11	TGGCTACTTAAGCACAAAGCAATGCTCAGC	SEQ ID NO: 11
Barcode 12	TGGCTACCGCACTCTGGACATAAGTGATTC	SEQ ID NO: 12

5

The essential features of the template circle include a complementary sequence for binding to its specific oligonucleotide barcode and a unique probe sequence. It is preferred to be less than 90 bases in length and it should not be aligned to any human sequences and other common environmental contaminants (such as bacteria). Template circle sequences were selected from plant-related genes and Tobacco mosaic virus, and a BLAST against NCBI nucleotide databases was performed to ensure that there are no similar sequences found in human and bacteria. Specifically, a BLAST hit is defined as (1) matching length $\geq$ 80 (out of 85 bp for query length); (2) identity $\geq$ 95%; and (3) E-value $\leq$ 0.01. In addition, the template circle should not fold into stable structures which will hinder the amplification

10

process. The sequences were evaluated for folding structures in circular form as predicted by mFold (version v3.6) and only those with a free energy ≥ 2 kcal/mol were selected.

For the barcode sequence, it is preferred to be about 30 bases in length, but can be 20 – 40 bases. The sequences were evaluated with primer3 and selected based on the following criteria: (1) GC content of 40 - 60%, (2) Tm about 70°C, (3) no stable hairpin formation and (4) no stable self-dimer formation.

For the probe sequence, it is preferred to be about 20 bases in length, but can be 15 – 30 bases. The sequences were evaluated with primer3 and selected based on the following criteria: (1) GC content of 40 - 60%, (2) Tm about 60°C, (3) no stable hairpin formation and (4) no stable self-dimer formation. Furthermore, to allow multiplexing and ensure specificity, in-silico testing was performed to check that the different sets of template circles, barcodes and probes would not bind non-specifically with each other.

15

Mild Denaturing Buffer Composition

A mild denaturing buffer was developed for the gentle removal of probes. The buffer comprises of the following: -

- (1) a low ionic strength buffer, such as 0.1x – 1x PBS (Phosphate-Buffered Saline);
- (2) a denaturant such as sodium hydroxide, dimethyl sulfoxide, ethylene carbonate, urea and formamide;
- (3) a detergent such as Triton X-100 and Tween-20.

For more efficient probe removal, heat (30°C – 50°C) can also be applied in combination with the mild denaturing buffer.

The preferred composition is 60% formamide, 0.1% Tween 20 in 0.1x PBS.

Preparation of Template Circles

The template circles were generated by ligation of 5'-phosphorylated linear oligonucleotides with T4 DNA Ligase [1]. The ligation reaction setup is as shown in the table below. The splint is a 30 bases-oligonucleotide complementary to both ends of the linear template oligonucleotide and designed to hold both ends of the linear template in close

proximity to facilitate ligation. All oligonucleotides were purchased from Integrated DNA Technologies and T4 DNA Ligase kit was purchased from New England Biolabs.

Table 4: Ligation Reaction

Component	Volume (μL)	Final Concentration/Amount
10x T4 DNA Ligase Buffer	10.00	1x
5'-phosphorylated linear DNA (100 μM)	1.00	100 pmol
Splint (100 μM)	3.00	300 pmol
Nuclease-free water	85.00	--
T4 DNA Ligase (400,000 U/mL)	1.00	400 U
Total	100.00	--

5

The ligation reaction was carried out at 16°C overnight and the ligase was then heat inactivated at 65°C for 10 min. Non-ligated linear DNA was removed by exonuclease treatment by adding 1 μL of *E. coli* Exonuclease I (New England Biolabs) directly to the ligation reaction and incubating at 37°C for 1 hr, followed by Exonuclease I heat inactivation at 80°C for 15 min. Next, the template circle was purified from the ligation reaction by ethanol precipitation as follows: Add 1/10 of total reaction volume of 3 M sodium acetate, pH 5.2 (Sigma-Aldrich), to the ligation reaction. Mix and spin down briefly. Add 3 times of total reaction volume of 100% ethanol (Sigma-Aldrich). Mix well and incubate at -80°C for at least 2 hours. Centrifuge the reaction at 14,000 rpm for 30 min at 4°C to pellet out the template circle DNA and discard the supernatant. Rinse the DNA pellet with 70% ethanol and repeat the centrifugation. The supernatant is discarded and DNA pellet is allowed to air dry. Lastly the pellet is resuspended in TE, pH 8.0 (Integrated DNA Technologies).

20 Preparation of Barcoded-Abs and Probes

All barcodes were ordered from Integrated DNA Technologies with terminal amine group and HPLC purification. CD8, PD-L1, CD68 and PanCK antibodies were purchased from Cell Signalling Technology. Conjugation of barcodes to their respective antibodies was performed with Oligonucleotide Conjugation Kit (abcam) according to manufacturer's

instructions. All probes were ordered from Integrated DNA Technologies with their respective fluorophores and HPLC purification.

Examples

5 To further understand the present invention, preferable solutions of the present invention are described in details below in reference to examples. However, these examples are merely used to illustrate the characteristics and merits of the present invention, but not to limit the protection scope of the present invention.

10 **Example 1**

 To show that the barcoded-Abs (and their respective template circles and probes) can be multiplexed and used for mIHC, a panel of 3 barcoded-Abs (PD-L1, CD8 and CD68 antibodies) was used to perform mIHC on 4 μ m human FFPE (Formalin-Fixed Paraffin-Embedded) tissue sample.

15 The FFPE tissue slides were first dehydrated at 60°C overnight, and then deparaffinized and rehydrated with xylene and ethanol, respectively. Next, heat-induced antigen retrieval was performed on the slides by placing them in Tris-EDTA buffer, pH 9.0 (abcam) and boiling them in a microwave oven. Slides are allowed to cool to room
20 temperature before transferring them to 1x TBST Buffer (0.05% Tween 20, pH 7.5) for 5 min, followed by blocking buffer (10% normal goat serum, 1% bovine serum albumin (BSA), 0.2 mg/mL salmon sperm DNA, 0.1% Tween 20, 0.05% ProClin 300 in 1x PBS) for 10 min.

25 The slides are then incubated with all the barcoded-Abs (diluted in blocking buffer) for an hour at room temperature, wherein PD-L1 antibody was conjugated with Barcode 1, CD8 antibody was conjugated with Barcode 6, and CD68 antibody was conjugated with Barcode 11. After 1x TBST Buffer washes (2 min, 3 times), RCA reaction mix (1x ϕ 29 DNA Polymerase Reaction Buffer, 0.1 mg/mL recombinant albumin, 10 mM dNTP, 10 U
30 ϕ 29 DNA Polymerase and 8 pmol of each template circle) was added to the slides and incubated at 30°C for an hr. This is followed by 1x TBST Buffer washes (2 min, 3 times), and then probe hybridization with all probes (0.1 μ M each) at room temperature for an hour. Probe hybridization is carried out in Probe Hybridization Buffer (5x Sodium Chloride-

Sodium Citrate (SSC) Buffer, 0.5% BSA and 0.1% Tween 20). After probe hybridization, the slides are washed several times in Probe Wash Buffer (0.5x SSC and 0.1% Tween 20), before DAPI (4', 6-diamidino-2-phenylindole) staining for nuclei visualization. Lastly the slides were mounted with mounting medium (Vector Labs) and coverslips for imaging.

5 Imaging was performed on Olympus VS200 Slide Scanner and analysed with OlyVIA software. TBST Buffer, SSC Buffer and PBS Buffer were obtained from 1st BASE. Xylene, normal goat serum, BSA, Tween 20, ProClin 300, DAPI were purchased from Sigma-Aldrich. Salmon sperm DNA and dNTP were bought from Thermo Fisher Scientific. ϕ 29 DNA Polymerase (with reaction buffer and recombinant albumin) was purchased from New

10 England Biolabs.

As shown in Figure 1, robust and specific PD-L1, CD8 and CD68 signals were detected simultaneously on a single section of FFPE tissue sample. This shows that our specially designed template circles, barcodes and probes work well together in a multiplex

15 system without any cross-reactivity or interference from each other. Therefore both multiplexity and specificity were achieved with our template circle designs. Furthermore since antibody incubation, RCA amplification and probe hybridization for the different sets of barcoded-Ab, template circle and probe were all performed together in single steps, much time is saved compared to conventional mIHC technology such as tyramine signal

20 amplification (TSA), in which antigen retrieval, blocking, primary and secondary antibody incubation and TSA steps are performed and repeated for each antibody, until all antibodies were stained.

Example 2

25 Besides the use of multiplexable sets of template circles, the level of multiplexing can be further increased by removing the probe signal after the first round of imaging, and proceeding with more rounds of probe hybridization and imaging for other antigens/targets. A mild denaturing buffer (Probe Removal Buffer) was developed to facilitate this. In a similar procedure that was described in Example 1, mIHC for a panel of 4 barcoded-Abs

30 (PD-L1 antibody conjugated with Barcode 1, CD8 antibody conjugated with Barcode 6, CD68 antibody conjugated with Barcode 11 and PanCK antibody conjugated with Barcode 12) was performed on 4 μ m human FFPE tissue sample. After RCA and 1x TBST Buffer washes were performed, only probes specific for PD-L1 and CD8 were used for the first

round of probe hybridization. The slides are then washed several times in Probe Wash Buffer before being mounted for imaging. Figures 2B and 2C show strong CD8 and PD-L1 signals detected simultaneously on the same section respectively.

5 Following this, the slides are washed in 1x TBST Buffer (2 min, 3 times) prior to being incubated for 15 min in Probe Removal Buffer (60% formamide (Sigma-Aldrich), 0.1% Tween 20 and 0.1x PBS). The slides are then washed several times in Probe Wash Buffer. Next the slides were washed first in 1x TBST Buffer (2 min, 3 times), and then Probe Hybridization Buffer (2 min, 3 times), before the second round of probe hybridization for
10 probes specific for PanCK and CD68 was performed. After probe hybridization, the slides are washed several times in Probe Wash Buffer before DAPI staining for nuclei visualization was performed. Lastly the slides were mounted with mounting medium and coverslips for imaging of both CD68 and PanCK (Figures 2D and 2E).

15 Thus with the combined use of the optimized mild denaturing buffer and multiplexable sets of barcoded-Abs, template circles and probes, high level multiplexity can be achieved with several rounds of probe hybridization, sample imaging and probe removal. Different sets of probes modified with the same set of fluorophores can be used for different rounds of imaging so multiplexing is not limited by spectral overlap of fluorophores. Below
20 is a table of the comparison between the present invention and TSA (commonly used method for mIHC).

Table 5: Comparison between mIHC performed with TSA and barcoded-Ab

	TSA	Barcoded-Ab	Advantage
1	Sequential labelling of Abs for multiplexing	One-step labelling of Abs for multiplexing	Present invention takes much less time
2	Antibody stripping and antigen retrieval is repeated for each Ab to be stained and imaged	Mild denaturing buffer to remove probe for next round of probe hybridization and imaging	Mild denaturing buffer do not damage sample, whereas in TSA, the repeated Ab stripping may

			destroy sample and reduce imaging sensitivity.
3	Different fluorophores used for multiplexing	Same set of fluorophores can be used repeatedly for multiplexing	Multiplexing is not limited by spectral overlap of fluorophores

CLAIMS

1. A template circle comprising
a complementary sequence for binding to a specific oligonucleotide barcode; and
5 a unique probe sequence.
2. The template circle according to claim 1, wherein the sequence of the template circle has less than 95% identity to any human sequences or bacterial sequences.
- 10 3. The template circle according to claim 1 or 2, wherein the sequence of the template circle is selected from plant-related genes and Tobacco mosaic virus.
4. The template circle according to any one of claims 1-3, wherein a folding structure of the template circle in circular form has a free energy ≥ 2 kcal/mol.
- 15 5. The template circle according to any one of claims 1-4, wherein the template circle is less than 90 bases in length.
6. The template circle according to any one of claims 1-5, wherein the template circle
20 comprises a nucleotide sequence as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4, or comprises a nucleotide sequence having at least or about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.
- 25 7. A multiplexable set comprising
a template circle according to any one of claims 1-6,
a probe having the unique probe sequence comprised in the template circle, and
a barcoded-antibody comprising a specific oligonucleotide barcode which binds to
the template circle.
- 30 8. The multiplexable set according to claim 7, wherein the probe is 15 – 30 bases in length and the specific oligonucleotide barcode is 20 – 40 bases in length.

9. The multiplexable set according to claim 7 or 8, wherein the probe is conjugated to an observable moiety such as a fluorophore.
10. The multiplexable set according to any one of claims 7-9, wherein the probe comprises
5 a nucleotide sequence as set forth in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or SEQ ID NO: 8, or comprises a nucleotide sequence having at least or about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence as set forth in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or SEQ ID NO: 8.
- 10 11. The multiplexable set according to any one of claims 7-10, wherein the oligonucleotide barcode comprises a nucleotide sequence as set forth in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 or SEQ ID NO: 12, or comprises a nucleotide sequence having at least or about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence as set forth in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, or SEQ ID NO:
15 12.
12. A multiplex system including a plurality of multiplexable sets according to any one of claims 7-11, wherein template circles, barcodes and probes in different sets have no cross-reactivity or interference from each other.
- 20 13. A mild denaturing buffer for the gentle removal of probes, comprising:
(1) a low ionic strength buffer;
(2) a denaturant; and
(3) a detergent.
- 25 14. The mild denaturing buffer according to claim 13, wherein the low ionic strength buffer is 0.1x – 1x PBS.
15. The mild denaturing buffer according to claim 13 or 14, wherein the denaturant is
30 selected from sodium hydroxide, dimethyl sulfoxide, ethylene carbonate, urea, formamide, or any combination thereof, preferably in a concentration of 40%-80%, 50%-70%, 55%-65%, or about 60%.

16. The mild denaturing buffer according to any one of claims 13-15, wherein the detergent is selected from Triton X-100, Tween-20 or any combination thereof, preferably in a concentration of 0.01%-2%, 0.02%-1%, 0.05%-0.5%, or about 0.1%.
- 5 17. A method for multiplex immunohistochemistry (mIHC), comprising the use of a template circle according to any one of claims 1-6, a multiplexable set according to any one of claims 7-11, a multiplex system according to claim 12, a mild denaturing buffer according to any one of claims 13-16, or any combination thereof.
- 10 18. The method according to claim 17, wherein the method comprises incubating all the barcoded-antibodies with a sample for antigen binding in a single step, and then simultaneously amplifying the barcodes by rolling circle amplification (RCA) with template circles according to any one of claims 1-6.
- 15 19. The method according to claim 17 or 18, wherein the method comprises multiple rounds of probe hybridization, sample imaging and probe removal, and a mild denaturing buffer according to any one of claims 13-16 is used for probe removal between different rounds.
- 20 20. The method according to claim 19, wherein heat (30°C – 50°C) is applied in combination with the mild denaturing buffer.
- 25 21. A kit for mIHC, comprising a template circle according to any one of claims 1-6, a multiplexable set according to any one of claims 7-11, a multiplex system according to claim 12, a mild denaturing buffer according to any one of claims 13-16, or any combination thereof.
- 30 22. Use of a template circle according to any one of claims 1-6, a multiplexable set according to any one of claims 7-11, a multiplex system according to claim 12, a mild denaturing buffer according to any one of claims 13-16, or any combination thereof for mIHC.