

BIOMOLECULAR PROBES AND METHODS OF DETECTING GENE AND PROTEIN EXPRESSION

BACKGROUND OF THE INVENTION

[0001] Standard immunohistochemical and *in situ* hybridization methods allow for simultaneous detection of, at most, six to ten protein or nucleic acid targets, with three to four targets being typical. There exists a need for probes, compositions, methods, and kits for simultaneous, multiplexed detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell. Furthermore, there is a need for such systems to be adaptable for use with existing sequencing technologies that are already available to a large number of end users.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 11, 2018, is named NATE-037/P01US_SeqList.txt and is 49,564 bytes in size.

SUMMARY OF THE INVENTION

[0003] The present disclosure relates to probes, compositions, methods, and kits for simultaneous, multiplexed, spatial detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell.

[0004] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released

identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the ligation product produced in step (4); and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0005] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises: a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias, and wherein at least one of the first or second amplification primer binding sites identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; (5) amplifying the ligation product produced in step (4); and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0006] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released

identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises: a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the extension product produced in step (4) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0007] The nucleic acid adapter of step (4) can be a partially double-stranded nucleic acid molecule. A partially double-stranded nucleic acid adapter can comprise a double-stranded annealed region, a first single-stranded mismatched region and a second single-stranded mismatched region. The first single-stranded mismatched region and the second single stranded mismatched region can be present on opposing sides of the double-stranded annealed region.

[0008] The nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter.

[0009] The constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter.

[0010] A unique molecular identifier can be present in at least one of the first or second single-stranded mismatched regions of a partially double-stranded nucleic acid adapter.

[0011] The first amplification primer binding site can be present in the first single-stranded mismatched region of a partially double-stranded nucleic acid adapter and the second amplification primer binding site can be present in the second single-stranded mismatched region of the same partially double-stranded nucleic acid adapter.

[0012] The methods of the present disclosure described in the preceding can further comprise prior to step (4), performing an end repair reaction. The methods can also further comprise prior to step (4), performing a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide. The methods can further comprise, prior to step (4), performing

an end repair reaction and a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide. The tailing reaction and the end repair reaction can be performed sequentially or concurrently.

[0013] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first amplification primer binding site, and a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the ligation product produced in step (4); and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0014] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises: a first amplification primer binding site and a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises a nucleic acid sequence comprising a unique molecular identifier, a second amplification primer binding site, and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the extension product produced in step (4) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification

primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0015] The nucleic acid adapter of step (4) can be a partially double-stranded nucleic acid molecule. A partially double-stranded nucleic acid adapter can comprise a double-stranded annealed region and a single-stranded mismatched region.

[0016] The nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter.

[0017] The constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter. The constant nucleic acid sequence can also comprise a cleavable moiety. The cleavable moiety can be an enzymatically cleavable moiety. The enzymatically cleavable moiety can be a USER sequence.

[0018] A unique molecular identifier can be present in the single-stranded mismatched region of a partially double-stranded nucleic acid adapter.

[0019] The second amplification primer binding site can be present in the single-stranded mismatched region of a partially double-stranded nucleic acid adapter.

[0020] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first amplification primer binding site, and a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a single stranded nucleic acid template, wherein the nucleic acid template comprises a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier, a nucleic acid sequence complementary to a second amplification primer binding site and optionally, an affinity molecule; (5) extending the

identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the unique molecular identifier, and the second amplification primer binding site; (6) amplifying the extension product produced in step (5) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0021] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first amplification primer binding site and a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a single stranded nucleic acid template, wherein the nucleic acid template comprises a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier, a nucleic acid sequence complementary to a second amplification primer binding site and optionally, an affinity molecule; (5) extending the identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, the nucleic acid sequence complementary to the unique molecular identifier and the second amplification primer binding site; (6) amplifying the extension product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the

amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0022] The single stranded nucleic acid template can further comprise an affinity molecule. In aspects in which the single stranded nucleic acid template comprises an affinity molecule, the methods of the present disclosure described in the preceding can further comprise an affinity purification step between steps (4) and (5).

[0023] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0024] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2)

providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a second amplification primer binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0025] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding site, and wherein the

first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0026] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a second amplification primer binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0027] In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is located on the first

nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be located 5' to the first amplification primer binding site.

[0028] In aspects in which a unique molecular identifier is located on the second nucleic acid probe, the unique molecular identifier can be located 3' to the second amplification primer binding site.

[0029] In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a unique molecular identifier are present in the first nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a unique molecular identifier can be located 5' to the first amplification primer binding site.

[0030] In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a unique molecular identifier are present in the second nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the unique molecular identifier can be located 3' to the second amplification primer binding site.

[0031] In aspects in which a unique molecular identifier is present in the first nucleic acid probe and the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is present in the second nucleic acid probe, the unique molecular identifier can be located 5' to the first amplification primer binding site and the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be located 3' to the second amplification binding site.

[0032] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe,

wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier, a nucleic acid sequence comprising a third unique molecular identifier and a second flow cell binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0033] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier and a second flow cell binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a third unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was

released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0034] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier, a nucleic acid sequence comprising a third unique molecular identifier and a second flow cell binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0035] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target

analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier and a second flow cell binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a third unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0036] In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the first unique molecular identifier are present in the first nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the first unique molecular identifier can be located 5' to the first flow cell binding site.

[0037] In aspects in which the second and the third unique molecular identifiers are present in the second nucleic acid probe, the second and the third unique molecular identifiers can be located 3' to the second flow cell binding site.

[0038] In some aspects, the first unique molecular identifier can be present in the first nucleic acid probe and can be located 5' to the first flow cell binding site. In other aspects, the second unique molecular identifier can be present in the second nucleic acid probe and can be located 3' to the second flow cell binding site.

[0039] In some aspects, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the third unique molecular identifier can be present in the first nucleic acid probe and can be located 5' to the first flow cell binding site.

[0040] In some aspects, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the third unique molecular identifier can be present in the second nucleic acid probe and can be located 3' to the second flow cell binding site.

[0041] In some aspect, the third unique molecular identifier can be present in the first nucleic acid probe and can be located 5' to the first flow cell binding site. In this same aspect, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be present in the second nucleic acid probe and can be located 3' to the second flow cell binding site.

[0042] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, and a second amplification primer binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; (5) Identifying the released identifier oligonucleotide by sequencing the amplified

products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0043] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain, a first amplification primer binding site and a second amplification primer binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein at least one of the amplification primers comprises a nucleic acid sequence comprising a unique molecular identifier; and (5) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0044] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain and a capture probe binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a capture probe, wherein the capture probe comprises an affinity molecule and a region complementary to the capture probe binding site; and (5) Identifying the released identifier oligonucleotide by sequencing the amplified hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0045] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain, a capture probe binding site and a multiplexing probe binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a capture probe and a multiplexing probe, wherein the capture probe comprises an affinity molecule and a region complementary to the capture probe binding site, and wherein the multiplexing probe comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a region complementary to the multiplexing probe binding site; and (5) Identifying the released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0046] In all methods of the present disclosure, the sequencing can be an enzyme free sequencing method.

[0047] In all methods of the present disclosure, the identifier oligonucleotide can be double-stranded. In aspects in which the identifier oligonucleotide is double-stranded, at least one of the two strands of the identifier oligonucleotide can comprise at least two separate nucleic acid molecules. Alternatively, at least one 3' end of an identifier oligonucleotide can comprise a single nucleotide overhang.

[0048] In all methods of the present disclosure, the unique nucleic acid sequence which identifies the target analyte bound to a target domain can comprise between about 5 nucleotides and about 40 nucleotides preferably about 35 nucleotides, preferably still about 10 nucleotides.

[0049] In all methods of the present disclosure, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can comprise between about 6 nucleotides and about 15 nucleotides, preferably about 12 nucleotides, preferably still about 10 nucleotides.

[0050] In all methods of the present disclosure, an amplification primer binding site can comprise between about 18 nucleotides and about 40 nucleotides, preferably about 32

nucleotides, preferably still about 25 nucleotides. An amplification primer binding site can comprise an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1. An amplification primer binding site can comprise an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.

[0051] In all methods of the present disclosure, an amplification primer can comprise a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing. An amplification primer can comprise a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3. An amplification primer can comprise a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

[0052] In all methods of the present disclosure, a flow cell binding site can comprise a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing. A flow cell binding site can comprise a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3. A flow cell binding site can comprise a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

[0053] Any of the above aspects can be combined with any other aspect.

[0054] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. In the Specification, the singular forms also include the plural unless the context clearly dictates otherwise; as examples, the terms “a,” “an,” and “the” are understood to be singular or plural and the term “or” is understood to be inclusive. By way of example, “an element” means one or more element. Throughout the specification the word “comprising,” or variations such as “comprises” or “comprising,” will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from the context, all numerical values provided herein are modified by the term “about.”

[0055] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are

described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The references cited herein are not admitted to be prior art to the claimed invention. In the case of conflict, the present Specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting. Other features and advantages of the disclosure will be apparent from the following detailed description and claim.

BREIF DESCRIPTION OF THE DRAWINGS

[0056] The above and further features will be more clearly appreciated from the following detailed description when taken in conjunction with the accompanying drawings.

[0057] Figure 1 is a schematic of a two-ended adapter ligation method of the present disclosure.

[0058] Figure 2 is a schematic of a one-ended adapter ligation method of the present disclosure.

[0059] Figure 3 is a schematic of a templated-primer extension method of the present disclosure.

[0060] Figure 4 is a schematic of a template-extended identifier oligonucleotide of the present disclosure.

[0061] Figure 5 is a schematic of a short probe hybridization method of the present disclosure.

[0062] Figure 6 is a schematic of a short probe hybridization method of the present disclosure.

[0063] Figure 7 is a schematic of a short probe hybridization method of the present disclosure.

[0064] Figure 8 is a schematic of a long probe hybridization method of the present disclosure.

[0065] Figure 9 is a schematic of a long probe hybridization method of the present disclosure.

[0066] Figure 10 is a schematic of a long probe hybridization method of the present disclosure.

[0067] Figure 11 is a schematic of a direct-PCR method of the present disclosure.

[0068] Figure 12 is a schematic of an enzyme free method of the present disclosure.

[0069] Figure 13 is a schematic of a multiplexed enzyme free method of the present disclosure.

[0070] Figure 14 is a schematic of a probe of the present disclosure indirectly binding to a target nucleic acid.

DETAILED DESCRIPTION OF THE INVENTION

[0071] The present disclosure is based in part on probes, compositions, methods, and kits for simultaneous, multiplexed spatial detection and quantification of protein and/or nucleic acid

expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell using existing sequencing methods.

[0072] The present disclosure provides a comparison of the identity and abundance of target proteins and/or target nucleic acids present in a first region of interest (*e.g.*, tissue type, a cell (including normal and abnormal cells), and a subcellular structure within a cell) and the identity and abundance of target proteins and/or target nucleic acids present in a second region of interest. There is no pre-defined upper limit to the number of regions of interest and comparisons that can be made; the upper limit relates to the size of the region of interest relative the size of the sample. As examples, when a single cell represent a region of interest, then a section may have hundreds to thousands of regions of interest; however, if a tissue section includes only two cell types, then the section may have only two regions of interest (each including only one cell type).

[0073] The present disclosure provides a higher degree of multiplexing than is possible with standard immunohistochemical or *in situ* hybridization methods. Standard immunohistochemical methods allow for maximal simultaneous detection of six to ten protein targets, with three to four protein targets being more typical. Similarly, *in situ* hybridization methods are limited to simultaneous detection of fewer than ten nucleic acid targets. The present disclosure provides detection of large combinations of nucleic acid targets and/or protein targets from a defined region of a sample. The present disclosure provides an increase in objective measurements by digital quantification and increased reliability and consistency, thereby enabling comparison of results among multiple centers.

[0074] Various compositions and methods of the present disclosure are describe in full detail herein.

[0075] In one aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a “two-ended adapter ligation method”.

[0076] A two ended-adapter ligation method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The probes and samples of the present disclosure are described in further detail herein. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is

a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain.

[0077] Following contacting the at least one target analyte with the at least one probe, a two-ended adapter ligation method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[0078] Following release of the identifier oligonucleotide, a two-ended adapter ligation method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location, thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[0079] Following collection of the released identifier oligonucleotide, a two-ended adapter ligation method can further comprise: (4) ligating to the released identifier oligonucleotide collected in step (3) at least one nucleic acid adapter.

[0080] The nucleic acid adapter can comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated “ROI #1”, the nucleic acid adapter would comprise a nucleic acid sequence that corresponds to “ROI #1”.

[0081] The nucleic acid adapter can also comprise a unique molecular identifier.

[0082] The nucleic acid adapter can also comprise a first amplification primer binding site. In other aspects, the nucleic acid adapter can also comprise a second amplification primer binding site.

[0083] In some aspects, the nucleic acid adapter can also comprise a constant nucleic acid sequence to minimize ligation bias caused by differences in sequences of particular identifier oligonucleotides.

[0084] The nucleic acid adapter can be a partially double-stranded nucleic acid molecule. In aspects in which the nucleic acid adapter is partially double-stranded, the nucleic acid adapter comprises a double-stranded annealed region, a first single-stranded mismatched region, and a second single-stranded mismatched region. The first single-stranded mismatched region and the second single stranded mismatched region can be present on opposing sides of the double-stranded annealed region.

[0085] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of the nucleic acid adapter.

[0086] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a constant nucleic acid sequence to minimize ligation bias, the constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of the nucleic acid adapter.

[0087] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a unique molecular identifier, the unique molecular identifier can be present in at least one of the first or second single-stranded mismatched regions of the nucleic acid adapter.

[0088] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a first and a second amplification primer binding site, the first amplification primer binding site can be present in the first single-stranded mismatched region of the nucleic acid adapter and the second amplification primer binding site can be present in the second single-stranded mismatched region of the nucleic acid adapter.

[0089] After ligation of the at least one nucleic acid adapter, a two-ended adapter ligation method can further comprise: (5) amplifying the ligation product produced in step (4) using amplification primers that bind to the first and second amplification primer binding sites; and (6) identifying the released oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the sample.

[0090] A two-ended adapter ligation method of the present disclosure can further comprise, prior to step (4), performing an end repair reaction using methods known in the art. The method can also further comprise, prior to step (4), performing a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide using methods known in the art. In aspects, the end repair reaction and the tailing reaction can be performed sequentially or concurrently.

[0091] In preferred aspects of a two-ended adapter ligation method, a nucleic acid adapter is ligated to both ends of the released and collected identifier oligonucleotide.

[0092] In other aspects of a two-ended adapter ligation method, at least one of the amplification primers used in step (5) to amplify the ligation product produced in step (4) comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", at least one of the amplification primers would comprise a nucleic acid sequence that corresponds to "ROI #1".

[0093] Figure 1 shows a schematic of a preferred aspect of a two-ended adapter ligation method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising an antibody that binds to a target protein. In the upper left panel, the probe binds to the target protein. In the upper right panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target protein bound to the target domain. In the bottom panel, a nucleic acid adapter is ligated to both ends of the identifier oligonucleotide. In this non-limiting example, the nucleic acid adapter is partially double-stranded and comprises a double-stranded annealed region, a first single-stranded mismatched region, and a second single-stranded mismatched region. Present in the double-stranded annealed region is a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. Present in the first single-stranded mismatched region is a first amplification primer binding site. Present in the second single-stranded mismatched region is a unique molecular identifier and a second amplification primer binding site. Following ligation of the nucleic acid adapters to the identifier oligonucleotide, the product is amplified

using amplification primers that bind the first and the second amplification primer binding sites and sequenced to identify the target protein bound by the probe.

[0094] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide dually ligated to two nucleic acid adapters for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide dually ligated to two nucleic acid adapters comprises an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which is capable of identifying a target analyte in a sample. Each end of the identifier oligonucleotide is attached to a nucleic acid adapter molecule, wherein the nucleic acid adapter molecule is partially double-stranded and comprises a double-stranded annealed region, a first single-stranded mismatched region and a second single-stranded mismatched region. The first single-stranded mismatched region and the second single stranded mismatched region are present on opposing sides of the double-stranded annealed region. The double-stranded mismatch region comprises a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence which is capable of identifying a specific location of a sample. The first single-stranded mismatched region comprises a first amplification primer binding site. The second single-stranded mismatched region comprises a second amplification primer binding site and a nucleic acid sequence comprising a unique molecular identifier. A schematic of an identifier oligonucleotide dually ligated to two nucleic acid adapters is shown in the bottom panel of Figure 1.

[0095] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a “one-ended adapter ligation method”.

[0096] A one-ended adapter ligation method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte

bound to the target domain. The identifier oligonucleotide can also comprise a first amplification primer binding site. In some aspects, the identifier oligonucleotide also comprises at least one 3' end with a single nucleotide overhang.

[0097] Following contacting the at least one target analyte with the at least one probe, a one-ended adapter ligation method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[0098] Following release of the identifier oligonucleotide, a one-ended adapter ligation method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[0099] Following collection of the released identifier oligonucleotide, a one-ended adapter ligation method can further comprise: (4) ligating to the released oligonucleotide collected in step (3) at least one nucleic acid adapter;

[00100] The nucleic acid adapter can comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", the nucleic acid adapter would comprise a nucleic acid sequence that corresponds to "ROI #1". The nucleic acid adapter can also comprise a unique molecular identifier. The nucleic acid adapter can also comprise a second amplification primer binding site.

[00101] In some aspects, the nucleic acid adapter can also comprise a constant nucleic acid sequence to minimize ligation bias caused by differences in sequences of particular identifier oligonucleotides. The constant nucleic acid sequence can comprise a cleavable moiety. The cleavable moiety can be enzymatically cleavable. In a non-limiting example, the enzymatically cleavable moiety can be a USER sequence, wherein the USER sequence comprises the sequence GUGUATUG.

[00102] The nucleic acid adapter can comprise any combination of the features described above.

[00103] The nucleic acid adapter can be a partially double-stranded nucleic acid molecule. In aspects in which the nucleic acid adapter is partially double-stranded, the nucleic acid adapter comprises a double-stranded annealed region and a single-stranded mismatched region.

[00104] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of the nucleic acid adapter.

[00105] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a constant nucleic acid sequence to minimize ligation bias, the constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of the nucleic acid adapter.

[00106] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a unique molecular identifier, the unique molecular identifier can be present in the single-stranded mismatched region.

[00107] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a second amplification primer binding site, the second amplification primer binding site can be present in the single-stranded mismatched region of the nucleic acid adapter.

[00108] After ligation of the at least one nucleic acid adapter, a two-ended adapter ligation method can further comprise: (5) amplifying the ligation product produced in step (4); and (6) identifying the released oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the sample.

[00109] In other aspects of a one-ended adapter ligation method of the present disclosure, at least one of the amplification primers used in step (5) to amplify the ligation product produced in step (4) comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated “ROI #1”, at least one of the amplification primers would comprise a nucleic acid sequence that corresponds to “ROI #1”.

[00110] Figure 2 shows a schematic of a preferred aspect of a one-ended adapter ligation method of the present disclosure. In this aspect, the probe comprises a target binding domain that is an

antibody that binds to a target protein. In the upper left panel, the probe binds to the target protein. In upper right panel, the UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target protein and a first amplification primer binding site. In this non-limiting example, the identifier oligonucleotide is double-stranded with one strand that comprises three separate nucleic acid molecules. The identifier oligonucleotide also comprises one 3' end with a single nucleotide overhang.

[00111] In the bottom panel of Figure 2, a nucleic acid adapter is ligated to the end of the identifier oligonucleotide that comprises the 3' single nucleotide overhang. In this non-limiting example, the nucleic acid adapter is partially double-stranded and comprises a double-stranded annealed region and a single-stranded mismatched region. Present in the double-stranded annealed region is a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. Present in the single-stranded mismatched region is a unique molecular identifier and a second amplification primer binding site. Following ligation of the nucleic acid adapter to the identifier oligonucleotide, the product is amplified using amplification primers that bind to the first and the second amplification primer binding sites and sequenced to identify the target protein bound by the probe.

[00112] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide ligated to one nucleic acid adapter for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide ligated to one nucleic acid adapter comprises an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which is capable of identifying a target analyte in a sample and a first amplification primer binding site. One end of the identifier oligonucleotide is attached to a nucleic acid adapter molecule, wherein the nucleic acid adapter molecule is partially double-stranded and comprises a double-stranded annealed region and a single-stranded mismatched region and a second single-stranded mismatched region. The double-stranded mismatch region comprises a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence nucleic acid sequence which is capable of identifying a specific location of a sample. The single-stranded mismatched region

comprises a second amplification primer binding site. A schematic of an identifier oligonucleotide ligated to one nucleic acid adapter is shown in the bottom panel of Figure 2.

[00113] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a “templated-primer extension method”.

[00114] A templated-primer extension method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain. The identifier oligonucleotide can also comprise a first amplification primer binding site.

[00115] Following contacting the at least one target analyte with the at least one probe, a templated-primer extension method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00116] Following release of the identifier oligonucleotide, a templated-primer extension method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00117] Following collection of the released identifier oligonucleotide, a templated-primer extension method can further comprise: (4) hybridizing to the released identifier oligonucleotide collected in step (3) a single stranded nucleic acid template.

[00118] The single stranded nucleic acid template can comprise a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, thereby allowing for the hybridization of the single stranded nucleic acid template and the collected identifier oligonucleotide.

[00119] The single stranded nucleic acid template can also comprise a nucleic acid sequence comprising a unique molecular identifier.

[00120] The single stranded nucleic acid template can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released.

[00121] The single stranded nucleic acid template can also comprise a nucleic acid sequence that is complementary to a second amplification primer binding site.

[00122] The single stranded nucleic acid template can comprise any combination of the features described above.

[00123] Following hybridization of the identifier oligonucleotide to the single stranded nucleic acid template, a templated-primer extension method can further comprise: (5) extending the identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide and the sequence complementary to the single stranded nucleic acid template; (6) amplifying the extension product of step (5) using amplification primers that hybridize to the first and second amplification primer binding sites; and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the sample.

[00124] In some aspects, the single stranded nucleic acid template can comprise an affinity molecule. In aspects in which the single stranded nucleic acid template comprises an affinity molecule, a templated-primer extension method can further comprise an affinity purification step between steps (4) and (5).

[00125] Figure 3 shows a schematic of a preferred aspect of a templated-primer extension method of the present disclosure. In this aspect, the probe comprises a target binding domain that is an

antibody that binds to a target protein. In the upper left panel, the probe binds to the target protein. In upper right panel, the UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target protein and a first amplification primer binding site. In the lower right panel, the identifier oligonucleotide is hybridized to a single stranded nucleic acid template. In this non-limiting example, the single-stranded nucleic acid template comprises an affinity molecule, a nucleic acid sequence complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a first unique molecular identifier, and a sequence complementary to a second amplification primer binding site. The identifier oligonucleotide is extended to form an extension product complementary to the single stranded nucleic acid template. As shown in the lower left panel, the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the first unique molecular identifier, and the second amplification primer binding site. Following the extension reaction, the primer extension product is amplified using amplification primers that bind to the first and the second amplification primer binding sites. In this non-limiting example, one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. The amplified product is then sequenced to identify the target protein bound by the probe.

[00126] In one aspect, the present disclosure provides a composition of a template-extended identifier oligonucleotide for spatially detecting at least one target analyte in a sample. The template-extended identifier oligonucleotide comprises a first flow cell adapter sequence suitable for sequencing, followed by a first unique molecular identifier, followed by an identifier oligonucleotide, followed by a second unique molecular identifier, followed by a second amplification primer binding site, followed by a third unique molecular identifier, followed by a second flow cell adapter sequence suitable for sequencing. The identifier oligonucleotide comprises a first amplification primer binding site and a unique nucleic acid sequence which is capable of identifying a target analyte in a sample. A schematic of a template-extended identifier oligonucleotide is shown in the bottom panel of Figure 4.

[00127] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a “short probe hybridization method”.

[00128] A short probe hybridization method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain.

[00129] Following contacting the at least one target analyte with the at least one probe, a short probe hybridization method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00130] Following release of the identifier oligonucleotide, a short probe hybridization method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00131] Following collection of the released identifier oligonucleotide, a short probe hybridization method can further comprise: (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe.

[00132] The first or the second nucleic acid probe can comprise a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The first or the second nucleic acid

probe can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. The first or the second nucleic acid probe can also comprise a nucleic acid sequence comprising unique molecular identifier. The first nucleic acid probe can comprise a first amplification primer binding site. The second nucleic acid probe can comprise a second amplification primer binding site.

[00133] The first or the second nucleic acid probe can comprise any combination of the features described above. In a preferred aspect depicted in Figure 5, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released is located 5' to the first amplification primer binding site and the unique molecular identifier is located 3' to the second amplification primer binding site.

[00134] In another preferred aspect depicted in Figure 6, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and the unique molecular identifier are located 5' to the first amplification primer binding site.

[00135] In another preferred aspect depicted Figure 7, the first nucleic acid probe comprises a first amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, a

nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and the unique molecular identifier are located 3' to the second amplification primer binding site.

[00136] The first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Alternatively, the first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping.

[00137] Following hybridization of the first and the second nucleic acid probe to the identifier oligonucleotide, a short probe hybridization method can further comprise: (5) in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping, performing a nick repair reaction such that the first and the second nucleic acid probes are ligated together. Alternatively, in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping, the method comprises performing a gap extension reaction and a nick repair reaction, such that the first and the second nucleic acid probes are ligated together.

[00138] Following ligation of the first and the second nucleic acid probe, a short probe hybridization method can further comprise: (6) amplifying the ligation product produced in step (5) using amplification primers that hybridize to the first and second amplification primer binding sites; and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the sample.

[00139] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-short nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-short nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first amplification primer binding

site, followed by a unique nucleic acid sequence capable of identifying a specific location in a sample, followed by a region complementary to the identifier oligonucleotide. The second nucleic acid probe comprises a second amplification primer binding site, followed by a nucleic acid sequence comprising a unique molecular identifier, followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in Figure 5.

[00140] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a “long probe hybridization method”.

[00141] A long probe hybridization method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain.

[00142] Following contacting the at least one target analyte with the at least one probe, a long probe hybridization method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00143] Following release of the identifier oligonucleotide, a long probe hybridization method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in

step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00144] Following collection of the released identifier oligonucleotide(s), a long probe hybridization method can further comprise: (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe.

[00145] The first or the second nucleic acid probe can comprise a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The first or the second nucleic acid probe can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released.

[00146] The first or the second nucleic acid probe can also comprise a first unique molecular identifier. The first or the second nucleic acid probe can also comprise a second unique molecular identifier. The first or the second nucleic acid probe can also comprise a third unique molecular identifier.

[00147] The first nucleic acid probe can comprise a first amplification primer binding site.

[00148] The first nucleic acid probe can also comprise a first flow cell binding site. The second nucleic acid probe can comprise a second flow cell binding site.

[00149] The first and the second nucleic acid probes can comprise any combination of the features described above. In a preferred aspect depicted in Figure 8, the first nucleic acid probe comprises a first flow cell binding site, a first unique molecular identifier, a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second flow cell binding site, a second unique molecular identifier, a third unique molecular identifier and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and the first unique molecular identifier are located 5' to the first flow cell binding site and the second and the third unique molecular identifiers are located 3' to the second flow cell binding site.

[00150] In another preferred aspect depicted in Figure 9, the first nucleic acid probe comprises a first flow cell binding site, a first unique molecular identifier, a second unique molecular identifier, a first amplification primer binding site, a nucleic acid sequence which identifies the

specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second flow cell binding site, a third unique molecular identifier and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the first unique molecular identifier, the second unique molecular identifier and the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released are located 5' to the first flow cell binding site and the third unique molecular identifier is located 3' to the second flow cell binding site.

[00151] In another preferred aspect depicted in Figure 10, the first nucleic acid probe comprises a first flow cell binding site, a first unique molecular identifier, a first amplification primer binding site and a nucleic acid sequence complementary to the identifier oligonucleotide. In this same preferred aspect, the second nucleic acid probe comprises a second flow cell binding site, a second unique molecular identifier, a third unique molecular identifier, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the first unique molecular identifier is located 5' to the first flow cell binding site and the second unique molecular identifier, the third unique molecular identifier, and the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released are located 3' to the second amplification primer binding site.

[00152] The first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Alternatively, the first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping.

[00153] Following hybridization of the first and the second nucleic acid probe to the identifier oligonucleotide, a long probe hybridization method can further comprise: (5) in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping, performing a nick repair reaction such that the first and the second nucleic acid probes are ligated together. Alternatively, in the aspect in which the first and the second nucleic acid probe hybridize to the

identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping, the method comprises performing a gap extension and a nick repair reaction such that the first and the second nucleic acid probes are ligated together.

[00154] The name method can further comprise: (6) amplifying the ligation product produced in step (5) using amplification primers that hybridize to the first and second amplification primer binding sites; and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the sample.

[00155] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-long nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-long nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first flow cell binding site suitable for sequencing, followed by a first unique molecular identifier, followed by a first amplification primer binding site, followed by a unique nucleic acid sequence capable of identifying a specific location in a sample, followed by a region complementary to the identifier oligonucleotide. The second nucleic acid probe comprises a second flow cell binding site, followed by a second unique molecular identifier, followed by a third unique molecular identifier, followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in Figure 8.

[00156] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a “direct PCR method”.

[00157] A direct PCR method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest.

The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain. The identifier oligonucleotide can also comprise a first amplification primer binding site, a second amplification primer binding site, or a unique molecular identifier. The identifier oligonucleotide can comprise any combination of these features. Any of these features can also be flanked by regions comprising constant nucleic acid sequences of about 1 nucleotide to about 10 nucleotides.

[00158] Following contacting the at least one analyte with the at least one probe, a direct PCR method further comprises: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00159] A direct PCR method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00160] Following release of the identifier oligonucleotide, a direct PCR method can further comprise: (4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site. In some aspects, at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated “ROI #1”, at least one of the amplification primers would comprise a nucleic acid sequence that corresponds to “ROI #1”. In still other aspects, at least one of the amplification primers comprises a unique molecular identifier.

[00161] Following amplification, a direct PCR method of the present disclosure can further comprise: (5) identifying the released oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the sample.

[00162] Figure 11 shows a schematic of a preferred aspect of a direct PCR method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising a nucleic acid sequence that is complementary to a target nucleic acid. In the upper panel, the probe hybridizes to the target nucleic acid. In the lower panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a first amplification primer binding site, a second amplification primer binding site, a unique molecular identifier, and a unique nucleic acid sequence which identifies the target analyte bound to the target domain. Located between these four features are constant spacer regions that are 3 nucleotides in length. The identifier oligonucleotide is double-stranded and comprises a strand that comprises 3 separate nucleic acid molecules. After release, the identifier oligonucleotide is amplified using a first amplification primer that hybridizes to the first amplification primer binding site and comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a second amplification primer that hybridizes to the second amplification primer binding site. The amplified product is then sequenced to identify the target nucleic acid bound by the probe.

[00163] In one aspect, the present disclosure provides a composition of a direct-PCR compatible identifier oligonucleotide for spatially detecting at least one target analyte in a sample. A direct-PCR compatible identifier oligonucleotide comprises a first amplification primer binding site, followed a unique nucleic acid sequence which is capable of identifying a target analyte in a sample, followed by a unique molecular identifier, followed by a second amplification primer binding site. A schematic of a direct-PCR compatible identifier oligonucleotide is depicted in the lower panel of Figure 11.

[00164] The present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as an “enzyme free method”.

[00165] An enzyme free method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one

target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. An identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain. An identifier oligonucleotide can also comprise a capture probe binding site.

[00166] Following contacting at least one target analyte with at least one probe, an enzyme free method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00167] Following release of an identifier oligonucleotide, an enzyme free method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00168] Following collection of a released identifier oligonucleotide, an enzyme free method can further comprise: (4) hybridizing to a released identifier oligonucleotide a capture probe.

[00169] A capture probe can comprise a region complementary to the capture probe binding site. A capture probe can also comprise an affinity molecule.

[00170] Following hybridization of a capture probe, an enzyme free method can further comprise: (5) Identifying a released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[00171] A hybridized product produced in step (4) can be sequenced using an enzyme free method of sequencing. Enzyme-free methods of sequencing have been described in, e.g.,

US2014946386 and US15/819,151, each of which is incorporated herein by reference in its entirety.

[00172] Figure 12 shows a schematic of a preferred aspect of an enzyme free method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising a nucleic acid sequence that is complementary to a target nucleic acid. In the top panel, the probe hybridizes to the target nucleic acid. In the middle panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target nucleic acid bound to the target domain and a capture probe binding site. After release, the identifier oligonucleotide is hybridized to a capture probe, as depicted in the bottom panel. The capture probe comprises a nucleic acid sequence complementary to the capture probe binding site and an affinity molecule. The hybridized product is then sequenced using enzyme free sequencing methods to identify the target nucleic acid bound by the probe.

[00173] In one aspect, the present disclosure provides a composition of a hybridized identifier oligonucleotide-capture probe complex for spatially detecting at least one target analyte in a sample. A hybridized identifier oligonucleotide-capture probe complex comprises an identifier oligonucleotide hybridized to a capture probe. An identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a specific target analyte in a sample and a capture probe binding site. A capture probe comprises an affinity molecule and a region complementary to the capture probe binding site. A schematic of a hybridized identifier oligonucleotide-capture probe complex is depicted in the bottom of panel Figure 12.

[00174] The present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a “multiplexed enzyme free method”.

[00175] A multiplexed enzyme free method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target

nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain. The identifier oligonucleotide can also comprise a capture probe binding site. The identifier oligonucleotide can also comprise a multiplexing probe binding site.

[00176] Following contacting the at least one target analyte with the at least one probe, a multiplexed enzyme free method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00177] Following release of the identifier oligonucleotide, a multiplexed enzyme free method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00178] Following collection of the released identifier oligonucleotide, a multiplexed enzyme free method can further comprise: (4) hybridizing to the released identifier oligonucleotide a capture probe and a multiplexing probe.

[00179] A capture probe can comprise a region complementary to the capture probe binding site. A capture probe can also comprise an affinity molecule.

[00180] A multiplexing probe can comprise a region complementary to the multiplexing probe binding site. A multiplexing probe can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a region complementary to the multiplexing probe binding site.

[00181] Following hybridization of a capture probe and a multiplexing probe, a multiplexed enzyme free method can further comprise: (5) Identifying the released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[00182] A hybridized product produced in step (4) can be sequenced using an enzyme free method of sequencing. Enzyme-free methods of sequencing have been described in, e.g., US2014946386 and US15/819,151, each of which is incorporated herein by reference in its entirety.

[00183] Figure 13 shows a schematic of a preferred aspect of a multiplexed enzyme free method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising a nucleic acid sequence that is complementary to a target nucleic acid. In the top panel, the probe hybridizes to the target nucleic acid. In the middle panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain, a capture probe binding site, and a multiplexing probe binding site, as shown in the middle panel. After release, the identifier oligonucleotide is hybridized to a capture probe and a multiplexing probe as shown in the lower panel. The capture probe comprises a nucleic acid sequence complementary to the capture probe binding site and an affinity molecule. The multiplexing probe comprises a nucleic acid sequence complementary to the multiplexing probe binding site and a nucleic acid sequence which identifies the specific location of a sample from which the identifier oligonucleotide was released. The hybridized product is then sequenced using enzyme free sequencing methods to identify the target nucleic acid bound by the probe.

[00184] In one aspect, the present disclosure provides a composition of a hybridized identifier oligonucleotide-capture probe-multiplex probe complex for spatially detecting at least one target analyte in a sample. A hybridized identifier oligonucleotide-capture probe-multiplex probe complex comprises an identifier oligonucleotide hybridized to a capture probe and a multiplexing probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a specific target analyte in a sample, a capture probe binding site and a multiplexing probe binding site. The capture probe comprises an affinity molecule and a region complementary to the capture probe binding site. The multiplexing probe comprises a nucleic acid sequence which identifies the specific location of a sample from which the identifier oligonucleotide was released and a region complementary to the multiplexing probe binding site. A schematic of a hybridized identifier oligonucleotide-capture probe-multiplex probe complex is depicted in the bottom panel of Figure 13.

[00185] As described in the preceding, the present disclosure provides probes for the compositions and methods of spatially detecting at least one target analyte in a sample. The present disclosure provides probes comprising a target binding domain and an identifier oligonucleotide. The target binding domain is a region of the probe that specifically binds to at least one target analyte in a sample.

[00186] Probes of the present disclosure can be used for spatially detecting a target nucleic acid. In this aspect, the target binding domain can be a target nucleic acid-binding region. The target nucleic acid-binding region is preferably at least 15 nucleotides in length, and more preferably is at least 20 nucleotides in length. In specific aspects, the target nucleic acid-binding region is approximately 10 to 500, 20 to 400, 25, 30 to 300, 35, 40 to 200, or 50 to 100 nucleotides in length. Probes and methods for binding and identifying a target nucleic acid have been described in, *e.g.*, US2003/0013091, US2007/0166708, US2010/0015607, US2010/0261026, US2010/0262374, US2010/0112710, US2010/0047924, and US2014/0371088, each of which is incorporated herein by reference in its entirety.

[00187] The target nucleic acid-binding region can directly hybridize to a target nucleic acid present in a sample. Alternatively, the probes of the present disclosure can indirectly hybridize to a target nucleic acid present in a sample (via an intermediary oligonucleotide). Figure 14 illustrates a probe (or composition) of this aspect. The probe includes a target nucleic-acid binding domain which binds to a synthetic oligonucleotide (the intermediary oligonucleotide) that in turn binds to a target nucleic acid in a biological sample. It could be said that the intermediary oligonucleotide is a probe, as defined herein, since it comprises a nucleic acid backbone and is capable of binding a target nucleic acid. In these aspects, a probe's target nucleic acid-binding region hybridizes to a region of an intermediary oligonucleotide (*i.e.*, a synthetic oligonucleotide) which is different from the target nucleic acid present in a sample. Thus, the probe's target binding region is independent of the ultimate target nucleic acid in the sample. This allows economical and rapid flexibility in an assay design, as the target (present in a sample)-specific components of the assay are included in inexpensive and widely-available synthetic DNA oligonucleotides rather than the more expensive probes. Such synthetic oligonucleotides are simply designed by including a region that hybridizes to the target nucleic acid present in a sample and a region that hybridizes to a probe. Therefore, a single set of indirectly-binding probes can be used to detect an infinite variety of target nucleic acids (present

in a sample) in different experiments simply by replacing the target-specific (synthetic) oligonucleotide portion of the assay.

[00188] A target nucleic acid may be DNA or RNA and preferably messenger RNA (mRNA) or miRNA.

[00189] Probes of the present disclosure can be used for detecting a target protein. In this aspect, the target binding domain can be a target protein-binding region. A target protein-binding region includes molecules or assemblies that are designed to bind to at least one target protein, at least one target protein surrogate, or both and can, under appropriate conditions, form a molecular complex comprising the probe and the target protein. The target-protein binding region can include an antibody, a peptide, an aptamer, or a peptoid. The antibody can be obtained from a variety of sources, including but not limited to polyclonal antibody, monoclonal antibody, monospecific antibody, recombinantly expressed antibody, humanized antibody, plantibodies, and the like. The terms protein, polypeptide, peptide, and amino acid sequence are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids or synthetic amino acids. The terms also encompass an amino acid polymer that has been modified, for example, by disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term amino acid refers to either natural and/or unnatural or synthetic amino acids, including but not limited to glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. Probes and methods for binding and identifying a target protein have been described, *e.g.*, in US2011/0086774, the contents of which is incorporated herein by reference in its entirety.

[00190] An identifier oligonucleotide is a nucleic acid molecule that identifies the target analyte bound to the target domain. The identifier oligonucleotide comprises a unique nucleic acid sequence that identifies the target analyte bound to the target domain of the probe. In a non-limiting example, a probe with a target binding domain that binds to the protein P53 comprises an identifier oligonucleotide with a unique nucleic acid sequence that corresponds to P53, while a probe with a target binding domain that binds to the protein P97 comprises an identifier oligonucleotide with a unique nucleic acid sequence that corresponds to P97.

[00191] An identifier oligonucleotide can be DNA, RNA, or a combination of DNA and RNA.

[00192] In some aspects, an identifier oligonucleotide can comprise at least one amplification primer binding site. An amplification primer binding site is a nucleic acid sequence capable of binding to an amplification primer. An amplification primer can be used to amplify the nucleic molecule to which it is bound using methods known in the art, including, but not limited to, polymerase chain reaction (PCR).

[00193] In some aspects, an identifier oligonucleotide can comprise at least one unique molecular identifier.

[00194] An identifier oligonucleotide can be a single-stranded, a double-stranded, or a partially double-stranded nucleic acid molecule. In the aspects in which an identifier oligonucleotide is double-stranded or partially double-stranded, at least one of the two strands can comprise at least two separate nucleic acid molecules which, without being bound by theory, allows for denaturing of the identifier oligonucleotide at lower temperatures.

[00195] An identifier oligonucleotide can also comprise at least one 3' end that comprises a single nucleotide overhang.

[00196] An identifier oligonucleotide can also comprise a capture probe binding site. A capture probe binding site is a nucleic acid sequence to which a capture probe can bind.

[00197] A capture probe of the present disclosure can comprise a nucleic acid sequence complementary to a capture probe binding site. A capture probe can also comprise an affinity molecule.

[00198] An identifier oligonucleotide can also comprise a multiplexing probe binding site. A multiplexing probe binding site is a nucleic acid sequence to which a multiplexing probe can bind.

[00199] A multiplexing probe of the present disclosure can comprise a nucleic acid sequence complementary to a multiplexing probe binding site. A multiplexing probe can also comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released.

[00200] A probe of the present disclosure can include a region which permits the release of an identifier oligonucleotide following the application of a suitable force. In one non-limited example, the region is a cleavable motif (*e.g.*, a restriction enzyme site or cleavable linker). The cleavable motif allows release of an identifier oligonucleotide from a bound target nucleic acid or protein and the identifier oligonucleotide can then be collected and detected. The region

which permits the release of an identifier oligonucleotide can be positioned between the target-binding domain and the identifier oligonucleotide, allowing for the release of the identifier oligonucleotide from the target binding domain. An identifier oligonucleotide is said to be releasable when it can be separated (*i.e.*, cleaved and released) from the remainder of the probe. Examples of cleavable motives include but are not limited to photo-cleavable linkers. Photo-cleavable linkers can be cleaved by light provided by a suitable coherent light source (*e.g.*, a laser and a UV light source) or a suitable incoherent light source (*e.g.*, an arc-lamp and a light-emitting diode (LED)).

[00201] In aspects where the target-binding domain of a probe is an antibody, the probe can be prepared using a cysteine bioconjugation method that is stable, site-specific to, preferably, the antibody's hinge-region heavy-chain. This preparation method provides relatively controllable identifier oligonucleotides to antibody stoichiometric ratios. A probe can comprise a plurality (*i.e.*, more than one, *e.g.*, 2, 3, 4, 5, or more) identifier oligonucleotides per antibody. Generally, "heavier" probes, which comprise 3 or 4 identifier oligonucleotides per antibody, are significantly less sensitive than antibodies lacking an identifier oligonucleotide or "lighter" probes, which comprise 1 or 2 identifier oligonucleotides per antibody.

[00202] In aspects, probes are provided to a sample at concentrations typically less than that used for immunohistochemistry (IHC) or for in situ hybridization (ISH). Alternately, the concentration may be significantly less than that used for IHC or ISH. For example, the probe concentration may be 2 fold less, 5 fold less, 10 fold less, 20 fold less, 25 fold less, 30 fold less, 50 fold less, 60 fold less, 70 fold less, 80 fold less, 90 fold less, 100 fold less, 200 fold less, 300 fold less, 400 fold less, 500 fold less, 600 fold less, 700 fold less, 800 fold less, 900 fold less, 1000 fold less, 2000 fold less, or less and any number in between. In aspects, probes are provided at a concentration of 100 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 9 nM, 8 nM, 7 nM, 6 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM, 0.9 nM, 0.8 nM, 0.7 nM, 0.6 nM, 0.5 nM, 0.4 nM, 0.3 nM, 0.2 nM, 0.1 nM, 0.09 nM, 0.08 nM, 0.07 nM, 0.06 nM, 0.05 nM, 0.04 nM, 0.03 nM, 0.02 nM, 0.01 nM, and less and any concentration in between.

[00203] Background noise, during protein detection, can be reduced by performing a negative purification of the intact probe molecule. This can be done by conducting an affinity purification of the antibody or photo-cleavable linker after collection of eluate from a region of interest. Normally, released signal oligonucleotides will not be pulled out of solution. A protein-G or -O

mechanism in a pipet tip, tube, or plate can be employed for this step. Such devices and reagents commercially available.

[00204] Background noise, during nucleic acid detection, can be reduced by performing a negative purification of the intact probe molecule. This can be done by conducting an affinity purification of the target binding domain or photo-cleavable linker after collection of eluate from a region of interest. Normally, released signal oligonucleotides will not be pulled out of solution. To assist in the negative purification, a universal purification sequence may be included in a probe, e.g., in the target binding domain.

[00205] Protein-targeting probes and nucleic acid-targeting probes may be applied simultaneously as long as conditions allow for binding of both a protein target and a nucleic acid target. Alternately, protein-targeting probes and nucleic acid-targeting probes may be applied sequentially when conditions allowing for binding of both a protein target and a nucleic acid target are not possible.

[00206] A set of probes is synonymous with a composition of probes. A set of probes includes at least one species of probes, *i.e.*, directed to one target. A set of probes preferably includes at least two, e.g., 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or more species of probes. A probe set may include one or multiple copies of each species of probe.

[00207] A first set of probes only may be applied to a sample. Alternately, a second set (or higher number) of probes may be later applied to the sample. The first set and second (or higher number) may target only nucleic acids, only proteins, or a combination thereof.

[00208] In the present disclosure, two or more targets (*i.e.*, proteins, nucleic acids, or a combination thereof) are detected; 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or more targets, and any number there between, are detected.

[00209] A set of probes may be pre-defined based upon the cell type or tissue type to be targeted. For example, if the tissue is a breast cancer, then the set of probes will include probes directed to proteins relevant to breast cancer cells (e.g., Her2, EGFR, and PR) and/or probes directed to proteins relevant to normal breast tissues. Additionally, the set of probes may be pre-defined based upon developmental status of a cell or tissue to be targeted. Alternately, the set of probes may be pre-defined based upon subcellular localizations of interest, e.g., nucleus, cytoplasm, and

membrane. For example, antibodies directed to Foxp3, Histone H3, or P-S6 label the nucleus, antibodies directed to CD3, CD4, PD-1, or CD45RO label the cytoplasm, and antibodies directed to PD-L1 label membranes.

[00210] A probe may be chemically synthesized or may be produced biologically using a vector into which a nucleic acid encoding the probe has been cloned.

[00211] Any probe or set of probes described herein may be used in methods and kits of the present disclosure.

[00212] For the herein-described probes, association of a unique nucleic acid sequence to a specific target nucleic acid or target protein is not fixed.

[00213] As described in the preceding, probes of the present disclosure can be used to detect a target nucleic acid or target protein present in any sample, *e.g.*, a biological sample. As will be appreciated by those in the art, the sample may comprise any number of things, including, but not limited to: cells (including both primary cells and cultured cell lines) and tissues (including cultured or explanted). In aspects, a tissue sample (fixed or unfixed) is embedded, serially sectioned, and immobilized onto a microscope slide. As is well known, a pair of serial sections will include at least one cell that is present in both serial sections. Structures and cell types, located on a first serial section will have a similar location on an adjacent serial section. The sample can be cultured cells or dissociated cells (fixed or unfixed) that have been immobilized onto a slide.

[00214] In aspects, a tissue sample is a biopsied tumor or a portion thereof, *i.e.*, a clinically-relevant tissue sample. For example, the tumor may be from a breast cancer. The sample may be an excised lymph node.

[00215] The sample can be obtained from virtually any organism including multicellular organisms, *e.g.*, of the plant, fungus, and animal kingdoms; preferably, the sample is obtained from an animal, *e.g.*, a mammal. Human samples are particularly preferred.

[00216] In some aspects, the probes, compositions, methods, and kits described herein are used in the diagnosis of a condition. As used herein the term diagnose or diagnosis of a condition includes predicting or diagnosing the condition, determining predisposition to the condition, monitoring treatment of the condition, diagnosing a therapeutic response of the disease, and prognosis of the condition, condition progression, and response to particular treatment of the condition. For example, a tissue sample can be assayed according to any of the probes, methods, or kits described herein to determine the presence and/or quantity of markers of a disease or

malignant cell type in the sample (relative to the non-diseased condition), thereby diagnosing or staging a disease or a cancer.

[00217] In general, samples attached to a slide can be first imaged using fluorescence (e.g., fluorescent antibodies or fluorescent stains (e.g., DAPI)) to identify morphology, regions of interest, cell types of interest, and single cells and then expression of proteins and/or nucleic acids can be digitally counted from the sample on the same slide.

[00218] Compositions and kits of the present disclosure can include probes and other reagents, for example, buffers and other reagents known in the art to facilitate binding of a protein and/or a nucleic acid in a sample, *i.e.*, for performing hybridization reactions.

[00219] A kit also will include instructions for using the components of the kit, including, but not limited to, information necessary to hybridize labeled oligonucleotides to a probe, to hybridize a probe to a target-specific oligonucleotide, to hybridize a target-specific oligonucleotide to a target nucleic acid and/or to hybridize a probe to target protein.

[00220] A region of interest may be a tissue type present in a sample, a cell type, a cell, or a subcellular structure within a cell.

[00221] Together, a comparison of the identity and abundance of the target proteins and/or target nucleic acids present in a first region of interest (e.g., tissue type, a cell type (including normal and abnormal cells), and a subcellular structure within a cell) and the identity and abundance of the target proteins and/or target nucleic acids present in second region of interest or more regions of interest can be made using the methods of the present disclosure.

[00222] As described in the preceding, the products produced by the methods of the present disclosure can be used for nucleic acid amplification. In a preferred aspect, the nucleic acid amplification can be solid-phase nucleic acid amplification. Thus, in further aspects the invention provides a method of solid-phase nucleic acid amplification of template polynucleotide molecules which comprises: preparing a library of template polynucleotide molecules which have common sequences at their 5' and 3' ends using the methods of the present disclosure and carrying out a solid-phase nucleic acid amplification reaction wherein said template polynucleotide molecules are amplified. Compositions and methods for nucleic acid amplification and sequencing have been described in, e.g., US9376678, which is incorporated herein by reference in its entirety.

[00223] The term "solid-phase amplification" as used herein refers to any nucleic acid amplification reaction carried out on or in association with a solid support such that all or a portion of the amplified products are immobilized on the solid support as they are formed. In particular, the term encompasses solid-phase polymerase chain reaction (solid-phase PCR), which is a reaction analogous to standard solution phase PCR, except that one or both of the forward and reverse amplification primers is/are immobilized on the solid support.

[00224] Although the invention encompasses "solid-phase" amplification methods in which only one amplification primer is immobilized (the other primer usually being present in free solution), it is preferred for the solid support to be provided with both the forward and the reverse primers immobilized. In practice, there will be a "plurality" of identical forward primers and/or a "plurality" of identical reverse primers immobilized on the solid support, since the PCR process requires an excess of primers to sustain amplification. References herein to forward and reverse primers are to be interpreted accordingly as encompassing a "plurality" of such primers unless the context indicates otherwise.

[00225] As will be appreciated by the skilled reader, any given PCR reaction requires at least one type of forward primer and at least one type of reverse primer specific for the template to be amplified. However, in certain aspects the forward and reverse primers may comprise template-specific portions of identical sequence, and may have entirely identical nucleotide sequence and structure (including any non-nucleotide modifications). In other words, it is possible to carry out solid-phase amplification using only one type of primer, and such single-primer methods are encompassed within the scope of the invention. Other aspects may use forward and reverse primers which contain identical template-specific sequences but which differ in some other structural features. For example one type of primer may contain a non-nucleotide modification which is not present in the other.

[00226] In other aspects of the invention the forward and reverse primers may contain template-specific portions of different sequence.

[00227] Amplification primers for solid-phase PCR are preferably immobilized by covalent attachment to the solid support at or near the 5' end of the primer, leaving the template-specific portion of the primer free for annealing to its cognate template and the 3' hydroxyl group free for primer extension. Any suitable covalent attachment means known in the art may be used for this purpose. The chosen attachment chemistry will depend on the nature of the solid support, and

any derivatization or functionalization applied to it. The primer itself may include a moiety, which may be a non-nucleotide chemical modification, to facilitate attachment. In one particularly preferred aspect the primer may include a sulphur-containing nucleophile, such as phosphorothioate or thiophosphate, at the 5' end. In the case of solid-supported polyacrylamide hydrogels (as described below), this nucleophile will bind to a "C" group present in the hydrogel. The most preferred means of attaching primers and templates to a solid support is via 5' phosphorothioate attachment to a hydrogel comprised of polymerised acrylamide and N-(5-bromoacetamidylpentyl)acrylamide (BRAPA).

[00228] The terms "cluster" and "colony" are used interchangeably herein to refer to a discrete site on a solid support comprised of a plurality of identical immobilized nucleic acid strands and a plurality of identical immobilized complementary nucleic acid strands. The term "clustered array" refers to an array formed from such clusters or colonies. In this context the term "array" is not to be understood as requiring an ordered arrangement of clusters.

[00229] The invention also encompasses methods of sequencing the amplified nucleic acids generated by solid-phase amplification. Thus, the invention provides a method of nucleic acid sequencing comprising amplifying a library of nucleic acid templates by the methods of the present disclosure described above, using solid-phase amplification as described above to amplify this library on a solid support, and carrying out a nucleic acid sequencing reaction to determine the sequence of the whole or a part of at least one amplified nucleic acid strand produced in the solid-phase amplification reaction.

[00230] Sequencing, as referred to herein, can be carried out using any suitable "sequencing-by-synthesis" technique, wherein nucleotides are added successively to a free 3' hydroxyl group, resulting in synthesis of a polynucleotide chain in the 5' to 3' direction. The nature of the nucleotide added is preferably determined after each nucleotide addition.

[00231] The initiation point for the sequencing reaction may be provided by annealing of a sequencing primer to a product of the whole genome or solid-phase amplification reaction. In this connection, one or both of the adapters added during formation of the template library may include a nucleotide sequence which permits annealing of a sequencing primer to amplified products derived by whole genome or solid-phase amplification of the template library.

[00232] The products of solid-phase amplification reactions wherein both forward and reverse amplification primers are covalently immobilized on the solid surface are so-called "bridged"

structures formed by annealing of pairs of immobilized polynucleotide strands and immobilized complementary strands, both strands being attached to the solid support at the 5' end. Arrays comprised of such bridged structures provide inefficient templates for nucleic acid sequencing, since hybridization of a conventional sequencing primer to one of the immobilized strands is not favored compared to annealing of this strand to its immobilized complementary strand under standard conditions for hybridization.

[00233] In order to provide more suitable templates for nucleic acid sequencing it is preferred to remove substantially all or at least a portion of one of the immobilized strands in the "bridged" structure in order to generate a template which is at least partially single-stranded. The portion of the template which is single-stranded will thus be available for hybridization to a sequencing primer. The process of removing all or a portion of one immobilized strand in a "bridged" double-stranded nucleic acid structure may be referred to herein as "linearization".

[00234] Bridged template structures may be linearized by cleavage of one or both strands with a restriction endonuclease or by cleavage of one strand with a nicking endonuclease. Other methods of cleavage can be used as an alternative to restriction enzymes or nicking enzymes, including inter alia chemical cleavage (e.g. cleavage of a diol linkage with periodate), cleavage of abasic sites by cleavage with endonuclease, or by exposure to heat or alkali, cleavage of ribonucleotides incorporated into amplification products otherwise comprised of deoxyribonucleotides, photochemical cleavage or cleavage of a peptide linker.

[00235] It will be appreciated that a linearization step may not be essential if the solid-phase amplification reaction is performed with only one primer covalently immobilized and the other in free solution.

[00236] In order to generate a linearized template suitable for sequencing it is necessary to remove "unequal" amounts of the complementary strands in the bridged structure formed by amplification so as to leave behind a linearized template for sequencing which is fully or partially single stranded. Most preferably one strand of the bridged structure is substantially or completely removed.

[00237] Following the cleavage step, regardless of the method used for cleavage, the product of the cleavage reaction may be subjected to denaturing conditions in order to remove the portion(s) of the cleaved strand(s) that are not attached to the solid support. Suitable denaturing conditions will be apparent to the skilled reader with reference to standard molecular biology protocols.

[00238] Denaturation (and subsequent re-annealing of the cleaved strands) results in the production of a sequencing template which is partially or substantially single-stranded. A sequencing reaction may then be initiated by hybridization of a sequencing primer to the single-stranded portion of the template.

[00239] Thus, the nucleic acid sequencing reaction may comprise hybridizing a sequencing primer to a single-stranded region of a linearized amplification product, sequentially incorporating one or more nucleotides into a polynucleotide strand complementary to the region of amplified template strand to be sequenced, identifying the base present in one or more of the incorporated nucleotide(s) and thereby determining the sequence of a region of the template strand.

[00240] One preferred sequencing method which can be used in accordance with the invention relies on the use of modified nucleotides that can act as chain terminators. Once the modified nucleotide has been incorporated into the growing polynucleotide chain complementary to the region of the template being sequenced there is no free 3'-OH group available to direct further sequence extension and therefore the polymerase cannot add further nucleotides. Once the nature of the base incorporated into the growing chain has been determined, the 3' block may be removed to allow addition of the next successive nucleotide. By ordering the products derived using these modified nucleotides it is possible to deduce the DNA sequence of the DNA template. Such reactions can be done in a single experiment if each of the modified nucleotides has attached a different label, known to correspond to the particular base, to facilitate discrimination between the bases added at each incorporation step. Alternatively, a separate reaction may be carried out containing each of the modified nucleotides separately.

[00241] The modified nucleotides may carry a label to facilitate their detection. Preferably this is a fluorescent label. Each nucleotide type may carry a different fluorescent label. However the detectable label need not be a fluorescent label. Any label can be used which allows the detection of an incorporated nucleotide.

[00242] One method for detecting fluorescently labelled nucleotides comprises using laser light of a wavelength specific for the labelled nucleotides, or the use of other suitable sources of illumination. The fluorescence from the label on the nucleotide may be detected by a CCD camera or other suitable detection means.

[00243] The invention is not intended to be limited to use of the sequencing method outlined above, as essentially any sequencing methodology which relies on successive incorporation of nucleotides into a polynucleotide chain can be used. Suitable alternative techniques include, for example, Pyrosequencing, FISSEQ (fluorescent in situ sequencing), MPSS (massively parallel signature sequencing) and sequencing by ligation-based methods.

[00244] In methods of the present disclosure, the unique nucleic acid sequence present in the identifier oligonucleotide of a probe which identifies the target analyte bound to the target binding domain of the probe can comprise between about 5 nucleotides and about 50 nucleotides. Preferably, the sequence comprises between about 20 nucleotides and about 40 nucleotides. Even more preferably, the sequence comprises about 35 nucleotides. In some preferred aspects, the sequence comprises 10 nucleotides.

[00245] In methods of the present disclosure, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises between about 6 nucleotides and about 15 nucleotides. Preferably, the sequence comprises about 12 nucleotides.

[00246] In methods of the present disclosure, an amplification primer binding sites comprises between about 18 nucleotides and about 40 nucleotides. Preferably, an amplification primer binding sites comprises about 32 nucleotides.

[00247] In some aspects of the methods of the present disclosure, an amplification primer binding site can comprise an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1.

[00248] In some aspects of the methods of the present disclosure, an amplification primer binding site can comprise an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.

[00249] In some aspects of the methods of the present disclosure, an amplification primer can comprise a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing. Preferably, at least one amplification primer used in the methods of the present disclosure comprises a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3. Preferably still, at least one amplification primer used in the methods of the present disclosure comprises a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

[00250] In methods of the present disclosure, a unique molecular identifier can comprise between about 6 nucleotides and about 30 nucleotides. Preferably, a unique molecular identifier can comprise about 15 nucleotides. The terms unique molecular identifier and random molecular tags are used interchangeably herein. Using methods known in that art, unique molecular identifiers are random sequences that can be used to correct for biases in amplification prior to sequencing.

[00251] In methods of the present disclosure, a constant nucleic acid sequence to minimize ligation bias comprises between about 1 nucleotide and about 15 nucleotides. Preferably, the constant sequence comprises about 8 nucleotides.

[00252] As used in this Specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise.

[00253] Unless specifically stated or obvious from context, as used herein, the term “or” is understood to be inclusive and covers both “or” and “and”.

[00254] Unless specifically stated or obvious from context, as used herein, the term “about” is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from the context, all numerical values provided herein are modified by the term “about.”

[00255] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although other probes, compositions, methods, and kits similar, or equivalent, to those described herein can be used in the practice of the present disclosure, the preferred materials and methods are described herein. It is to be understood that the terminology used herein is for the purpose of describing particular aspects only, and is not intended to be limiting.

EXAMPLES

[00256] Example 1—two-ended adapter ligation method for 96 multiplexed samples

[00257] In this example, a two-ended adapter ligation method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. The nucleic acid adapters used in this experiment were partially double-stranded. The nucleic acid adapters comprised a first strand and a second strand. The first strand comprised a 5' phosphate moiety for ligation. The first strand also comprised a constant nucleic acid sequence to minimize ligation

bias (GCGTAGTG), a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a first amplification primer binding site (SEQ ID NO: 2). The second strand comprised a single overhanging thymine nucleotide at the 3' end, a sequence complementary to the constant nucleic acid sequence to minimize ligation bias present in the first strand, a sequence complementary to the unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released present in the first strand and a second amplification primer binding site (SEQ ID NO: 1).

[00258] To form the partially double stranded nucleic acid adapters, first strand oligonucleotides and second strand oligonucleotides were combined in equimolar proportion for a final total oligonucleotide concentration of 28 μ M in buffer comprising 50mM NaCl. The oligonucleotide mixture was heated at 95°C for 2 minutes and cooled at ambient temperature for 30 minutes, thereby annealing the first stand and second strand oligonucleotides together to form the partially double-stranded nucleic acid adapters. Annealed nucleic acid adaptors were diluted to final concentration ranging between 0.02 μ M to 0.002 μ M in a solution of 10mM Tris pH 8 and 0.05% Tween20.

[00259] Collected identifier oligonucleotides were end repaired and A-tailed using NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) with a modified protocol. End repair/A-tail master mix was prepared by combining the following: 627.8 μ L of PCR-grade H₂O, 143.9 μ L of NEBNext Ultra II End Prep Reaction Buffer, and 61.7 μ L of NEBNext Ultra II End Prep Enzyme Mix. 8.3 μ L of end repair/A-tail master mix was added to 4 μ L of each sample of identifier oligonucleotides. The reaction was incubated for 30 minutes at 20°C with a heated lid of >75°C, followed by a second incubation for 30 minutes at 65°C. The repaired/A-tailed identifier oligonucleotide mixtures were then stored at 4°C.

[00260] Following end repair and A-tailing, the nucleic acid adaptors were ligated to the repaired/A-tailed identifier oligonucleotides by adding 6.4 μ L of NEBNext Ultra II Ligation Master Mix, 0.2 μ L of NEBNext Ligation Enhancer, and 1 μ L of the nucleic acid adapter dilution to each repaired/A-tailed identifier oligonucleotide mixture. These reactions were incubated for 15 minutes at 20°C with the heated lid off and subsequently quenched with 1 μ L 0.5M EDTA.

All of the reactions were then pooled into a single 15mL conical tube to form a pooled adapter-ligated sample.

[00261] The pooled adaptor-ligated sample was purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350µL of AMPure XP beads and 3.15mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). AMPure XP bead cleanup was performed with 3.5mL of diluted AMPure XP beads and eluted in 200µL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20. AMPure XP bead cleanup was then repeated with 400µL of AMPure XP beads and eluted in 20µL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20 to obtain purified adapter-ligated samples.

[00262] Following AMPure XP cleanup, PCR reactions with purified adaptor-ligated sample were prepared to amplify the adapter-ligated identifier oligonucleotides. To 6µL of the purified adapter-ligated sample, 10µL of NEBNext Ultra II Q5 Master Mix, 0.2µL of 100µM forward and reverse primers, and 3.6µL of PCR-grade H2O was added. The forward primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the first strand of the nucleic acid adapter. Table 1 provides the sequences of the forward primers used.

Table 1. Forward primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACGCTCAGATATAGCCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTACACGCTCAGAATAGAGGCACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACACGCTCAGACCTATCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGGCTCTGAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACACGCTCAGAAGGCGAAGACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACACGCTCAGATAATCTTAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACACGCTCAGACAGGACGTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGTACTGACACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	12

[00263] The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located on the second strand of the nucleic acid adapter. Table 2 provides the sequences of the reverse primers used.

Table 2. Reverse primers for two-ended adapter ligation

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	13
CAAGCAGAAGACGGCATACGAGATTCTCCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	14

CAAGCAGAAGACGGCATAACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	15
CAAGCAGAAGACGGCATAACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	16
CAAGCAGAAGACGGCATAACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	17
CAAGCAGAAGACGGCATAACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	18
CAAGCAGAAGACGGCATAACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	19
CAAGCAGAAGACGGCATAACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	20
CAAGCAGAAGACGGCATAACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	21
CAAGCAGAAGACGGCATAACGAGATTTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	22
CAAGCAGAAGACGGCATAACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	23
CAAGCAGAAGACGGCATAACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	24

[00264] The optimal number of PCR cycles was determined empirically with triplicate PCR reactions. Alternately, the optimal number of PCR cycles could have been determined using real-time/qPCR. The PCR program used comprised the following steps:

- (1) 30 seconds at 98°C
- (2) 10 seconds at 98°C
- (3) 1 minute at 65°C
- (4) Repeating steps (2) and (3) nine times
- (5) 5 minutes at 65°C

[00265] The amplified products were purified using 18µL of AMPure XP beads and eluting with 20µL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20.

[00266] The amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The amplified products were also diluted to 15pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer comprising the nucleotide sequence
ACACTCTTTAAGACGACGTCGCTATGGCCTCTCC (SEQ ID NO: 25).

[00267] Example 2—one-ended adapter ligation method for 96 multiplexed samples

[00268] In this example, a one-ended adapter ligation method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. The nucleic acid adapters used in this experiment were partially double-stranded. The nucleic acid adapters comprised a first strand and a second strand. The first strand comprised a 5' phosphate moiety

for ligation. The first strand also comprised a constant nucleic acid sequence to minimize ligation bias (CACTACGC), a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a first amplification primer binding site (SEQ ID NO: 1). The second strand comprised a single overhanging thymine nucleotide at the 3' end, a sequence complementary to the constant nucleic acid sequence to minimize ligation bias present in the first strand and a sequence complementary to the unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released present in the first strand.

[00269] To form the partially double stranded nucleic acid adapters, first strand oligonucleotides and second strand oligonucleotides were combined in equimolar proportion for a final total oligonucleotide concentration of 28 μ M in 50mM NaCl. The oligonucleotide mixture was heated to 95°C for 2 minutes and cooled at ambient temperature for 30 minutes, thereby annealing the first stand and second strand oligonucleotides together to form the partially double-stranded nucleic acid adapters. Annealed nucleic acid adapters were diluted to a final concentration ranging between 0.02 μ M to 0.002 μ M in a solution of 10mM Tris pH 8 and 0.05% Tween20.

[00270] Nucleic acid adapters were ligated to the collected identifier oligonucleotides by addition of 10 μ L of 2X rapid ligation buffer (Enzymatics), 1 μ L of T4 DNA Rapid Ligase (Enzymatics), and 1 μ L of annealed nucleic acid adapter dilutions to each sample of collected identifier oligonucleotides. Samples were incubated for 15 minutes at 20°C with the heated lid off and subsequently quenched with 1 μ L 0.5M EDTA. All of the reactions were then pooled into a single 15mL conical tube to form a pooled adapter-ligated sample.

[00271] The pooled adaptor-ligated sample was purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350 μ L of AMPure XP beads and 3.15mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). AMPure XP bead cleanup was performed with 3.5mL of diluted AMPure XP beads and eluted in 200 μ L of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20. AMPure XP bead cleanup was then repeated with 400 μ L of AMPure XP beads and eluted in 20 μ L of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20 to obtain purified adapter-ligated samples.

[00272] Following AMPure XP cleanup, PCR reactions with purified adaptor-ligated sample were prepared to amplify the adapter-ligated identifier oligonucleotides. To 6 μ L of the purified

adapter-ligated sample, 10µL of NEBNext Ultra II Q5 Master Mix, 0.2µL of 100µM forward and reverse primers, and 3.6µL of PCR-grade H₂O was added. The forward primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the first strand of the nucleic acid adapter. Table 3 provides the sequences of the forward primers used.

Table 3. Forward primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACGCTCAGATATAGCCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTACACGCTCAGAATAGAGGCACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACACGCTCAGACCTATCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGGCTCTGAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACACGCTCAGAAGGCGAAGACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACACGCTCAGATAATCTTAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACACGCTCAGACAGGACGTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGTACTGACACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	12

[00273] The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located on the second strand of the nucleic acid adapter. Table 4 provides the sequences of the reverse primers used.

Table 4. Reverse primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATAACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	13
CAAGCAGAAGACGGCATAACGAGATTCTCCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	14
CAAGCAGAAGACGGCATAACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	15
CAAGCAGAAGACGGCATAACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	16
CAAGCAGAAGACGGCATAACGAGATTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	17
CAAGCAGAAGACGGCATAACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	18
CAAGCAGAAGACGGCATAACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	19
CAAGCAGAAGACGGCATAACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	20
CAAGCAGAAGACGGCATAACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	21
CAAGCAGAAGACGGCATAACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	22
CAAGCAGAAGACGGCATAACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	23
CAAGCAGAAGACGGCATAACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	24

[00274] The optimal number of PCR cycles was determined empirically with triplicate PCR reactions. Alternately, the optimal number of PCR cycles could have been determined using real-time/qPCR. The PCR program used comprised the following steps:

- (1) 30 seconds at 98°C
- (2) 10 seconds at 98°C
- (3) 1 minute at 65°C
- (4) Repeating steps (2) and (3) nine times
- (5) 5 minutes at 65°C

[00275] The amplified products were purified using 18µL of AMPure XP beads and eluting with 20µL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20.

[00276] The amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The amplified products were also diluted to 15pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer comprising the nucleotide sequence

ACACTCTTTAAGACGACGTCGCTATGGCCTCTCC (SEQ ID NO: 25).

[00277] Example 3—templated-primer extension method for 96 multiplexed samples

[00278] In this example, a templated-primer extension method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. The single stranded nucleic acid templates used in this example comprised a 3' biotin moiety, a region complementary to the unique nucleic acid sequences present in the collected identifier oligonucleotides, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding sequence

(GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT, SEQ ID NO: 26). Table 5 provides the sequences of the single-stranded nucleic acid templates used in this example.

Table 5. Single stranded nucleic acid templates for templated-primer extension method

Single-stranded nucleic acid templates	SEQ ID NO
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNTGAAGCACACCGTTTTTCTTCTCTTTCACGG	27
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNACCCACAGGTTATACGGGATTATCCGGTTATCCA	28
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCGACACCGAGTTCGACCGTTATGTTGGTAGGATC	29
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCGGTGTGTAAGCGTAACGATGTTGGTGTGCTCT	30
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCAGACACTGCGACAACCTACGATCATGACACAGA	31
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNATATTCTGTAAGTGCCTATCCACCTAATAGG	32
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNTTCAGTTATAATGTGTCCAGCAGAAGCAGGAATT	33
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNGTCCTTTGTTGGGCGGACCGTAATGAGGAATTTG	34
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNGATGAGACTTCTACATGTCCGATGTTTGTGCT	35
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNACTCACATAGTACTGACACGTAAGATAGGATG	36

GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNTTACCCTATCTCGTCTATGTACGTCAGGCTGAAT	37
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNATCAACGTAGGGTAAGGTCATATTTTACCTTAC	38
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNTCCCTCTTCTCCGCTTATGGATGAAAGGACAG	39
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCCTGCACAGTGAGTTTCTTCACTCTAACTCTCT	40
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNTGTCGCTCTAGTGTGACTTTTCCACCTCGCATCT	41
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNATATCTTCTCGGGTAAAGATTAGGCGTCCGATA	42
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCGATTAGCCGTAGACGCAACTCATTGCCGAAGAT	43
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNTGTGAGCAATTTCAGTACGAGTGATGCAGATAAAC	44
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNTATAGTTACCAAGTACTATGGGTTGGTGGAAGCC	45
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCCAATTACTGTCTGTTATGTTCTCGGATAAGC	46
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNTCAGGTGCTTGTAGGCTCATGATAGGGGTAATGC	47
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCTCTGTGTAATCTCAGCTCCACTTGTTCCTAAG	48
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNGTGCATATTGCAGCTGAGCCAGCTCAATTTGAAG	49
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCCGGTTGATTTACGCAACAGCGGCTTATATAGCTC	50
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCATCATCGACAGTTCGCAGCCCTATAACATGATA	51
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNATCGCAGGATGGTACAGCATCATACATGATGAGC	52
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCTGATAAGTCGTAGGAATGTCGCTTAATACGGAT	53
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNATGGCGGTTTCGGGTCCTGCACTATTCTAATAA	54
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNCCAGTACGGGTACTAATAAGTGCATATCTATTG	55
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNTGTTGGAGAGGTTAGAGGTGAGGAGGCGAAGATA	56

[00279] Single stranded nucleic acid templates were ordered from Integrated DNA Technologies, Inc. and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

Individual single stranded nucleic acid templates were normalized to a standard concentration and then pooled to be equimolar. The pool of single stranded nucleic acid templates was diluted to 0.83nM in a buffer comprising 10mM Tris pH 8 and 0.05% Tween20.

[00280] The collected identifier oligonucleotides were hybridized to the single stranded nucleic acid templates and extended by addition of 10µL of NEBNext Ultra II Q5 Master Mix (New England Biolabs), 4µL of the diluted single stranded nucleic acid template pool and 4µL of H₂O to 2µL of each sample of identifier oligonucleotides. The following PCR program was used to extend the identifier oligonucleotides:

- (1) 30 seconds at 98°C, 10×
- (2) 1 minutes at 98°C,
- (3) 1 minutes at 68°C
- (4) 1 minutes at 72°C
- (5) Repeating steps (2)-(4) ten times
- (6) 2 minutes at 72°C

[00281] The extension products were stored at 4°C. Magnetic streptavidin beads (MyOne Streptavidin C1 beads, Thermo Fisher Scientific) were washed in 1X Binding and Washing Buffer (5mM Tris-HCl, 0.5mM EDTA, 1M NaCl), and 5µL of streptavidin beads were added to each extension product sample. The extension product samples were incubated with the beads on an orbital mixer for a minimum of 15 minutes. Following incubation, the samples were heated to 95°C for 3 minutes and transferred to a magnetic plate. Supernatant was extracted immediately after sufficient bead pelleting to yield the purified extension product samples.

[00282] The purified extension product samples were amplified by adding to 7.5 µL of each purified extension product sample, 12.5µL of NEBNext Ultra II Q5 Master Mix, 0.25µL of 100µM forward primer, 1µL of 25µM reverse primer and 3.8µL of PCR-grade H₂O. The forward primer comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the identifier oligonucleotide. Table 6 provides the sequences of the forward primers used in this example.

Table 6. Forward primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACGCTCAGATATAGCCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTACACGCTCAGAATAGAGGCACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACACGCTCAGACCTATCCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGGCTCTGAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACACGCTCAGAAGGCGAAGACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACACGCTCAGATAATCTTAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACACGCTCAGACAGGACGTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGTACTGACACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	12

[00283] The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located single-stranded nucleic acid template. Table 7 provides the sequences of the reverse primers used.

Table 7. Reverse primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATGTCGGTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	57
CAAGCAGAAGACGGCATACGAGATAGGTCACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	58
CAAGCAGAAGACGGCATACGAGATGAATCCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	59
CAAGCAGAAGACGGCATACGAGATGTACCTTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	60

CAAGCAGAAGACGGCATAACGAGATCATGAGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	61
CAAGCAGAAGACGGCATAACGAGATTGACTGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	62
CAAGCAGAAGACGGCATAACGAGATCGTATTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	63
CAAGCAGAAGACGGCATAACGAGATCTCCTAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	64
CAAGCAGAAGACGGCATAACGAGATTAGTTGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	65
CAAGCAGAAGACGGCATAACGAGATGAGATACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	66
CAAGCAGAAGACGGCATAACGAGATAGGTGTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	67
CAAGCAGAAGACGGCATAACGAGATTAATGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	68
CAAGCAGAAGACGGCATAACGAGATTCAGACGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	69
CAAGCAGAAGACGGCATAACGAGATGATAGGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	70
CAAGCAGAAGACGGCATAACGAGATTGGTACAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	71
CAAGCAGAAGACGGCATAACGAGATCAAGGTCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	72
CAAGCAGAAGACGGCATAACGAGATGCTATCCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	73
CAAGCAGAAGACGGCATAACGAGATATGGAAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	74
CAAGCAGAAGACGGCATAACGAGATTCAAGGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	75
CAAGCAGAAGACGGCATAACGAGATGTTACGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	76
CAAGCAGAAGACGGCATAACGAGATAGTCTGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	77
CAAGCAGAAGACGGCATAACGAGATGCACGTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	78
CAAGCAGAAGACGGCATAACGAGATAACCTTGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	79
CAAGCAGAAGACGGCATAACGAGATATTGCGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	80
CAAGCAGAAGACGGCATAACGAGATACCTGGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	81
CAAGCAGAAGACGGCATAACGAGATGGAGATGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	82
CAAGCAGAAGACGGCATAACGAGATGTACTCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	83
CAAGCAGAAGACGGCATAACGAGATGTAACGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	84
CAAGCAGAAGACGGCATAACGAGATATTCCTCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	85
CAAGCAGAAGACGGCATAACGAGATGTGTTCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	86
CAAGCAGAAGACGGCATAACGAGATAAGCACTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	87
CAAGCAGAAGACGGCATAACGAGATCTAGCAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	88
CAAGCAGAAGACGGCATAACGAGATTGCTTCCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	89
CAAGCAGAAGACGGCATAACGAGATGCTTAGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	90
CAAGCAGAAGACGGCATAACGAGATAACCGTTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	91
CAAGCAGAAGACGGCATAACGAGATGACATTCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	92
CAAGCAGAAGACGGCATAACGAGATAGACCGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	93
CAAGCAGAAGACGGCATAACGAGATGATACTGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	94
CAAGCAGAAGACGGCATAACGAGATTGCGTAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	95
CAAGCAGAAGACGGCATAACGAGATTCCGTTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	96
CAAGCAGAAGACGGCATAACGAGATATGACGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	97
CAAGCAGAAGACGGCATAACGAGATGCTGTAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	98
CAAGCAGAAGACGGCATAACGAGATGCAATGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	99
CAAGCAGAAGACGGCATAACGAGATATCTCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	100
CAAGCAGAAGACGGCATAACGAGATGGCTATTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	101
CAAGCAGAAGACGGCATAACGAGATGGTGTCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	102
CAAGCAGAAGACGGCATAACGAGATTCAACTGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	103
CAAGCAGAAGACGGCATAACGAGATCTTACCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	104
CAAGCAGAAGACGGCATAACGAGATACGGTCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	105
CAAGCAGAAGACGGCATAACGAGATTCTCGCAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	106
CAAGCAGAAGACGGCATAACGAGATGGAATTGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	107
CAAGCAGAAGACGGCATAACGAGATACGGATTCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	108
CAAGCAGAAGACGGCATAACGAGATTAAAGCGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	109

CAAGCAGAAGACGGCATAACGAGATTGCAGGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	110
CAAGCAGAAGACGGCATAACGAGATCAATCGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	111
CAAGCAGAAGACGGCATAACGAGATGTGCCATAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	112
CAAGCAGAAGACGGCATAACGAGATTGTTTCGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	113
CAAGCAGAAGACGGCATAACGAGATTGGAGTTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	114
CAAGCAGAAGACGGCATAACGAGATACGATGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	115
CAAGCAGAAGACGGCATAACGAGATTGATGTCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	116
CAAGCAGAAGACGGCATAACGAGATTGAACCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	117
CAAGCAGAAGACGGCATAACGAGATCTTCGTTTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	118
CAAGCAGAAGACGGCATAACGAGATCTTCTGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	119
CAAGCAGAAGACGGCATAACGAGATTGCTCATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	120
CAAGCAGAAGACGGCATAACGAGATAGTTCGTCTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	121
CAAGCAGAAGACGGCATAACGAGATTAGCGTCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	122
CAAGCAGAAGACGGCATAACGAGATGGCGTTATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	123
CAAGCAGAAGACGGCATAACGAGATGGTGATTTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	124
CAAGCAGAAGACGGCATAACGAGATAACTTGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	125
CAAGCAGAAGACGGCATAACGAGATGCAAGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	126
CAAGCAGAAGACGGCATAACGAGATTTCGATTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	127
CAAGCAGAAGACGGCATAACGAGATTGTACACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	128
CAAGCAGAAGACGGCATAACGAGATAGCTCCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	129
CAAGCAGAAGACGGCATAACGAGATGCAATTTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	130
CAAGCAGAAGACGGCATAACGAGATCTTAGGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	131
CAAGCAGAAGACGGCATAACGAGATGTCTTAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	132
CAAGCAGAAGACGGCATAACGAGATAACGTGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	133
CAAGCAGAAGACGGCATAACGAGATCTGTGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	134
CAAGCAGAAGACGGCATAACGAGATGTTAAGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	135
CAAGCAGAAGACGGCATAACGAGATCACCTTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	136
CAAGCAGAAGACGGCATAACGAGATTGGTAGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	137
CAAGCAGAAGACGGCATAACGAGATCAGTGAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	138
CAAGCAGAAGACGGCATAACGAGATGTTCAACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	139
CAAGCAGAAGACGGCATAACGAGATTGGCTATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	140
CAAGCAGAAGACGGCATAACGAGATCTGGAGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	141
CAAGCAGAAGACGGCATAACGAGATTCTCTTCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	142
CAAGCAGAAGACGGCATAACGAGATTCTAACGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	143
CAAGCAGAAGACGGCATAACGAGATGGTCAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	144
CAAGCAGAAGACGGCATAACGAGATCTCTGGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	145
CAAGCAGAAGACGGCATAACGAGATTGTGGTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	146
CAAGCAGAAGACGGCATAACGAGATCCTATACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	147
CAAGCAGAAGACGGCATAACGAGATTTCTCTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	148
CAAGCAGAAGACGGCATAACGAGATGTATGCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	149
CAAGCAGAAGACGGCATAACGAGATAAGTCGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	150
CAAGCAGAAGACGGCATAACGAGATAACCGAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	151
CAAGCAGAAGACGGCATAACGAGATTGTTGTGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	152

[00284] The PCR program used to amplify the purified extension products comprised the following steps:

(1) 30 seconds at 98°C

- (2) 10 seconds at 98°C
- (3) 30 seconds at 65°C
- (4) 30 seconds at 72°C
- (5) Repeat steps (2)-(4) eighteen times
- (6) 2 minutes at 72°C

[00285] The amplified extension products were stored at 4°C. 4µL of each PCR reaction were combined into four pools, for 24 samples per pool.

[00286] The pooled PCR reactions were purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 100µL of AMPure XP beads and 400µL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). Purification was performed with 76.8µL of diluted AMPure XP beads and eluted in 20µL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20. Beads were retained and cleanup process was repeated with 24µL of AMPure XP buffer and eluted in 20µL of buffer comprising 10mM Tris pH 8 and 0.05% Tween20.

[00287] The purified PCR products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The purified PCR products were also diluted to 15pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer comprising the nucleotide sequence
ACACTCTTTAAGACGACGTCGCTATGGCCTCTCC (SEQ ID NO: 25).

[00288] Example 4—long probe hybridization method for 96 multiplexed samples

[00289] In this example, a long probe hybridization method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. In this example, the first nucleic acid probe comprises a 5' phosphate moiety, a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a first amplification primer binding site comprising an i7 sequence (SEQ ID NO: 1), unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a P7 flow cell adapter sequence (SEQ ID NO: 4). The second nucleic acid probe comprises a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier, a second amplification primer binding site comprising and i5 sequence (SEQ ID NO: 2) and a P5 flow cell adapter sequence (SEQ ID NO: 3).

[00290] The first and second nucleic acid probes were ordered from Integrated DNA Technologies, Inc. and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Individual nucleic acid probes were normalized to a standard concentration, pooled to be equimolar, and diluted to 0.83nM in a buffer comprising 10mM Tris pH 8 and 0.05% Tween20. The nucleic acid probes and the identifier oligonucleotides were hybridized by combining 0.5µL of diluted nucleic acid probe pool with 2µL of a mixture of identifier oligonucleotides collected from a sample solution in a buffer comprising 50mM NaCl. This mixture was heated for 2 minutes at 95°C and cooled for 30 minutes at ambient temperature to yield an annealed identifier oligonucleotide-nucleic acid probe mixture.

[00291] In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were not adjacent and were not overlapping, a gap extension reaction was performed. To 2.5µL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 3.8µL of NEBNext Ultra II Q5 Master mix and 1.3µL of PCR-grade H₂O was added. The mixture was then subjected to the following Gap extension temperature cycle:

- (1) 30 seconds at 98°C
- (2) 1 minute at 98°C
- (3) 1 minute at 68°C
- (4) 1 minute at 72°C,
- (5) Repeat steps (2)-(4) ten times
- (6) 2 minutes at 72°C

[00292] The gap extension products were then stored at 4°C. The first and second nucleic acid probes were then ligated together by adding to 1µL of the gap extension product, 10µL of 2X rapid ligation buffer (Enzymatics), 1µL of T4 DNA Rapid Ligase (Enzymatics), and 8µL of PCR-grade H₂O. These ligation reactions were incubated for 15 minutes 20°C, subsequently quenched with 1µL 0.5M EDTA, and pooled into a single 15mL conical tube.

[00293] In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were adjacent and were not overlapping, nick repair ligation reaction was performed. To 2.5µL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 10µL of 2X rapid ligation buffer (Enzymatics), 1µL of T4 DNA Rapid Ligase (Enzymatics), and 1µL of PCR-grade H₂O was

added. These ligation reactions were incubated for 15 minutes at 20°C, subsequently quenched with 1μL of 0.5M EDTA, and pooled into a single 15mL conical tube.

[00294] The pools of quenched ligation reactions were then purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350μL of AMPure XP beads and 3.15mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). The purification was performed with 3.5mL of diluted AMPure XP beads and eluted in 200μL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20. The AMPure XP bead cleanup was then repeated with 400μL of AMPure XP beads and eluted in 20μL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20.

[00295] To amplify the purified ligation products, PCR reactions with purified ligation products and primers were prepared. To 6μL of purified ligation product, 10μL of NEBNext Ultra II Q5 Master Mix, 0.2μL of 100μM forward primer (CAAGCAGAAGACGGCATACGA, SEQ ID NO: 153) and reverse primer (AATGATACGGCGACCACCGA, SEQ ID NO: 154) and 3.6μL of PCR-grade H₂O was added. The PCR program used to amplify the purified extension products comprised the following steps:

- (1) 30 seconds at 98°C
- (2) 10 seconds at 98°C
- (3) 30 seconds at 65°C
- (4) 30 seconds at 72°C
- (5) Repeat steps (2)-(4) eighteen times
- (6) 2 minutes at 72°C

[00296] The amplified products were stored at 4°C. 4μL of each PCR reaction were combined into six pools, for 16 samples per pool. The amplified products were further purified using an AMPure XP bead cleanup with 64μL of AMPure XP beads and eluting with 20μL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20.

[00297] The purified amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The purified amplified products were also diluted to 15pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with either standard sequencing primers or a custom spike-in Read1 primer (SEQ ID NO: 25).

[00298] Example 5—short probe hybridization method for 96 multiplexed samples

[00299] In this example, a short probe hybridization method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. In this example, the first nucleic acid probe comprises a 5' phosphate moiety, a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a first amplification primer binding site comprising an i7 sequence (SEQ ID NO: 1) and a unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. The second nucleic acid probe comprises a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding site comprising an i5 sequence (SEQ ID NO: 2).

[00300] The first and second nucleic acid probes were ordered from Integrated DNA Technologies, Inc. and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Individual nucleic acid probes were normalized to a standard concentration, pooled to be equimolar, and diluted to 0.83nM in a buffer comprising 10mM Tris pH 8 and 0.05% Tween20. The nucleic acid probes and the identifier oligonucleotides were hybridized by combining 0.5μL of diluted nucleic acid probe pool with 2μL of a mixture of identifier oligonucleotides collected from a sample solution in a buffer comprising 50mM NaCl. This mixture was heated for 2 minutes at 95°C and cooled for 30 minutes at ambient temperature to yield an annealed identifier oligonucleotide-nucleic acid probe mixture.

[00301] In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were not adjacent and were not overlapping, a gap extension reaction was performed. To 2.5μL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 3.8μL of NEBNext Ultra II Q5 Master mix and 1.3μL of PCR-grade H₂O was added. The mixture was then subjected to the following Gap extension temperature cycle:

- (1) 30 seconds at 98°C
- (2) 1 minute at 98°C
- (3) 1 minute at 68°C
- (4) 1 minute at 72°C,
- (5) Repeat steps (2)-(4) ten times
- (6) 2 minutes at 72°C

[00302] The gap extension products were then stored at 4°C. The first and second nucleic acid probes were then ligated together by adding to 1µL of the gap extension product, 10µL of 2X rapid ligation buffer (Enzymatics), 1µL of T4 DNA Rapid Ligase (Enzymatics), and 8µL of PCR-grade H₂O. These ligation reactions were incubated 15 min 20°C, quenched with 1µL 0.5M EDTA, and pooled into a single 15mL conical tube.

[00303] In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were adjacent and were not overlapping, nick repair ligation reaction was performed. To 2.5µL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 10µL of 2X rapid ligation buffer (Enzymatics), 1µL of T4 DNA Rapid Ligase (Enzymatics), and 1µL of PCR-grade H₂O was added. These ligation reactions were incubated for 15 minutes at 20°C, subsequently quenched with 1µL of 0.5M EDTA, and pooled into a single 15mL conical tube.

[00304] The pools of quenched ligation reactions were then purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350µL of AMPure XP beads and 3.15mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). The purification was performed with 3.5mL of diluted AMPure XP beads and eluted in 200µL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20. The AMPure XP bead cleanup was then repeated with 400µL of AMPure XP beads and eluted in 20µL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20.

[00305] To amplify the purified ligation products, PCR reactions with purified ligation products and primers were prepared. To 6µL of purified ligation product, 10µL of NEBNext Ultra II Q5 Master Mix, 0.2µL of 100µM forward primer and reverse primer and 3.6µL of PCR-grade H₂O was added. . The forward primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the first strand of the nucleic acid adapter. Table 8 provides the sequences of the forward primers used.

Table 8. Forward primers for short probe hybridization method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATAACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	13
CAAGCAGAAGACGGCATAACGAGATTCTCCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	14
CAAGCAGAAGACGGCATAACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	15
CAAGCAGAAGACGGCATAACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	16
CAAGCAGAAGACGGCATAACGAGATTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	17

CAAGCAGAAGACGGCATAACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	18
CAAGCAGAAGACGGCATAACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	19
CAAGCAGAAGACGGCATAACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	20
CAAGCAGAAGACGGCATAACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	21
CAAGCAGAAGACGGCATAACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	22
CAAGCAGAAGACGGCATAACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	23
CAAGCAGAAGACGGCATAACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	24

[00306] The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located on the second strand of the nucleic acid adapter. Table 9 provides the sequences of the reverse primers used.

Table 9. Reverse primers for short probe hybridization method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	155
AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCT	156
AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	157
AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACGACGCTCTTCCGATCT	158
AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGACGCTCTTCCGATCT	159
AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGACGCTCTTCCGATCT	160
AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACACGACGCTCTTCCGATCT	161
AATGATACGGCGACCACCGAGATCTACACGTAAGTACACACTCTTTCCCTACACGACGCTCTTCCGATCT	162

[00307] The PCR program used to amplify the purified extension products comprised the following steps:

- (1) 30 seconds at 98°C
- (2) 10 seconds at 98°C
- (3) 30 seconds at 65°C
- (4) 30 seconds at 72°C
- (5) Repeat steps (2)-(4) eighteen times
- (6) 2 minutes at 72°C

[00308] The amplified products were stored at 4°C. 4µL of each PCR reaction were combined into six pools, for 16 samples per pool. The amplified products were further purified using an AMPure XP bead cleanup with 64µL of AMPure XP beads and eluting with 20µL of a buffer comprising 10mM Tris pH 8 and 0.05% Tween20.

[00309] The purified amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The purified amplified products were also diluted to 15pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with either standard sequencing primers or a custom spike-in Read1 primer (SEQ ID NO: 25).

[00310] Example 6—direct PCR method for 96 multiplexed samples

[00311] In this example, a direct PCR method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. In this example, 8 species of forward amplification primers and 12 species of reverse amplification primers were used. The forward primers comprised a P5 flow cell adapter (SEQ ID NO: 3), a nucleic acid sequence comprising a unique molecular identifier and a region complementary to a first amplification primer binding site present on the identifier oligonucleotide. Table 10 provides the sequences of the forward amplification primers used.

Table 10. Forward amplification primers for direct PCR method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACGCTCAGATATAGCCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTACACGCTCAGAATAGAGGCACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACACGCTCAGACCTATCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGGCTCTGAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACACGCTCAGAAGGCGAAGACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACACGCTCAGATAATCTTAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACACGCTCAGACAGGACGTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGTACTGACACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	12

[00312] The reverse primers comprised a P7 flow cell adapter (SEQ ID NO: 4), a nucleic acid sequence comprising a unique molecular identifier and a region complementary to a second amplification primer binding site present on the identifier oligonucleotide. Table 11 provides the sequences of the reverse amplification primers used.

Table 11. Reverse amplification primers for direct PCR method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	163
CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	164
CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	165

CAAGCAGAAGACGGCATAACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	166
CAAGCAGAAGACGGCATAACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	167
CAAGCAGAAGACGGCATAACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	168
CAAGCAGAAGACGGCATAACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	169
CAAGCAGAAGACGGCATAACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	170
CAAGCAGAAGACGGCATAACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	171
CAAGCAGAAGACGGCATAACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	172
CAAGCAGAAGACGGCATAACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	173
CAAGCAGAAGACGGCATAACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNAACGGACA GGATGCAGCAAAAT	174

[00313] In the case of 8 forward amplification primers and 12 reverse amplification primers, when the unique molecular identifiers from a pair of forward and reverse primers are combined, a total of 96 unique combinations can be obtained, allowing for the multiplexing of 96 samples.

[00314] To amplify the collected identifier oligonucleotides for sequencing, PCR reactions with collected identifier oligonucleotides and forward and reverse amplification primers were prepared on a 96-well plate with 2µL of each identifier oligonucleotide sample, 10µL of NEBNext Ultra II Q5 Master Mix, 2µL of 10µM forward amplification primer, 2µL of reverse amplification primer, and 4µL of PCR-grade H₂O. Each well in the 96-well plate contained an identifier oligonucleotide sample and a unique combination of forward and reverse amplification primers. The PCR program used comprised the following steps:

- (1) 30 seconds at 98°C
- (2) 10 seconds at 98°C
- (3) 30 seconds at 65°C
- (4) 30 seconds at 72°C
- (5) Repeat steps (2)-(4) six to ten times
- (6) 2 minutes at 72°C

[00315] The amplified products were stored at 4°C. 10µL of each PCR reaction was combined into a single 15mL conical tube.

[00316] The pooled PCR reactions were purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 115.2µL of AMPure XP beads and 1036.8µL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). Purification was performed with 1152µL of diluted AMPure XP beads and eluted in 60µL of a

buffer comprising 10mM Tris pH 8. The purification process was repeated with 60µL of AMPure XP beads and eluted in 70µL of a buffer comprising 10mM Tris pH 8.

[00317] Following AMPure XP cleanup, PCR reactions with universal primers were prepared with 9µL of pooled direct PCR product, 15µL of NEBNext Ultra II Q5 Master Mix, 3µL of 10µM universal P7 primer (SEQ ID NO: 153) and 2µL of 10µM universal P5 primer (SEQ ID NO: 154). The PCR program used was:

- (1) 30 seconds at 98°C,
- (2) 10 seconds at 98°C
- (3) 30 seconds at 65°C
- (4) 30 seconds at 72°C
- (5) Repeat steps (2)-(4) 15 to 24 times
- (6) 2 minutes at 72°C

[00318] Two rounds of AMPure XP bead cleanup was performed. The first round was performed with 30µL of beads and eluted with 20µL of a buffer comprising 10mM Tris pH 8 and the second round was performed with 20µL beads and eluted with 11µL of a buffer comprising 10mM Tris pH 8.

[00319] These purified PCR products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics). The purified PCR products were also diluted for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer (SEQ ID NO: 25).

What is claimed is:

1. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises:

a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released,

a nucleic acid sequence comprising a unique molecular identifier,

a first amplification primer binding site,

a second amplification primer binding site, and

optionally, a constant nucleic acid sequence to minimize ligation bias;

(5) amplifying the ligation product produced in step (4); and

(6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

2. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises:

a nucleic acid sequence comprising a unique molecular identifier,

a first amplification primer binding site,

a second amplification primer binding site, and

optionally, a constant nucleic acid sequence to minimize ligation bias, and

wherein at least one of the first or second amplification primer binding sites identifies the specific location of the tissue sample from which the identifier oligonucleotide was released;

(5) amplifying the ligation product produced in step (4); and

(6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

3. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises:

a nucleic acid sequence comprising a unique molecular identifier,

a first amplification primer binding site,

a second amplification primer binding site, and

optionally, a constant nucleic acid sequence to minimize ligation bias;

(5) amplifying the extension product produced in step (4) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; and

(6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

4. The method of claim 1, wherein the nucleic acid adapter of step (4) is a partially double-stranded nucleic acid molecule.

5. The method of claim 4, wherein the nucleic acid adapter comprises:

- a double-stranded annealed region,
- a first single-stranded mismatched region, and
- a second single-stranded mismatched region.

6. The method of claim 5, wherein the first single-stranded mismatched region and the second single stranded mismatched region are present on opposing sides of the double-stranded annealed region.

7. The method of claim 5, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is present in the double-stranded annealed region of the nucleic acid adapter.

8. The method of claim 2 or claim 3, wherein the nucleic acid adapter of step (4) is a partially double-stranded nucleic acid molecule.

9. The method of claim 8, wherein the nucleic acid adapter comprises:

- a double-stranded annealed region,
- a first single-stranded mismatched region, and

a second single-stranded mismatched region.

10. The method of claim 9, wherein the first single-stranded mismatched region and the second single stranded mismatched region are present on opposing sides of the double-stranded annealed region.
11. The method of claim 5 or claim 9, wherein the nucleic acid adapter comprises a constant nucleic acid sequence to minimize ligation bias, and wherein the constant nucleic acid sequence is present in the double-stranded annealed region of the nucleic acid adapter.
12. The method of claim 5 or claim 9, wherein the unique molecular identifier is present in at least one of the first or second single-stranded mismatched regions.
13. The method of claim 6 or claim 10, wherein the first amplification primer binding site is present in the first single-stranded mismatched region of the nucleic acid adapter and the second amplification primer binding site is present in the second single-stranded mismatched region of the nucleic acid adapter.
14. The method of any of claims 1 to 3, wherein the identifier oligonucleotide is double-stranded.
15. The method of claim 14, wherein at least one of the two strands of the identifier oligonucleotide comprises at least two separate nucleic acid molecules.
16. The method of any of claims 1-3, the method further comprising, prior to step (4), performing an end repair reaction.
17. The method of any of claims 1-3, the method further comprising, prior to step (4), performing a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide.

18. The method of any of claims 1-3, the method further comprising, prior to step (4), performing an end repair reaction followed by a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide
19. The method of any of claims 1-3, the method further comprising, prior to step (4), concurrently performing an end repair reaction and a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide
20. The method of any of claims 1-3, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain comprises between about 25 nucleotides and about 40 nucleotides
21. The method of claim 20, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain comprises about 35 nucleotides.
22. The method of any of claims 1-3, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises between about 6 nucleotides and about 15 nucleotides.
23. The method of claim 22, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises about 12 nucleotides.
24. The method of any of claims 1-3, wherein the unique molecular identifier comprises between about 6 nucleotides and about 30 nucleotides.
25. The method of claim 24, wherein the unique molecular identifier comprises about 15 nucleotides.
26. The method of any of claims 1-3, wherein the first or the second amplification primer binding sites comprise between about 18 nucleotides and about 40 nucleotides.

27. The method of claim 26, wherein the first or the second amplification primer binding sites comprise about 32 nucleotides.
28. The method of any of claims 1 to 3, wherein the at least one nucleic acid adapter comprises a constant nucleic acid sequence to minimize ligation bias, wherein the constant nucleic acid sequence comprises between about 1 nucleotide and about 15 nucleotides.
29. The method of claim 28, wherein the constant nucleic acid sequence comprises about 8 nucleotides.
30. The method of any of claims 1 to 3, wherein at least one of the amplification primer binding sites comprises an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1.
31. The method of any of claims 1 to 3, wherein at least one of the amplification primer binding sites comprises an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.
32. The method of any of claims 1 to 3, wherein at least one of the amplification primers comprises a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing.
33. The method of claim 1 to 3, wherein at least one of the amplification primers comprises a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3.
34. The method of any of claim 1 to 3, wherein at least one of the amplification primers comprises a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

35. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises:

a first amplification primer binding site, and

a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises:

a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released,

a nucleic acid sequence comprising a unique molecular identifier,

a second amplification primer binding site, and

optionally, a constant nucleic acid sequence to minimize ligation bias;

(5) amplifying the ligation product produced in step (4); and

(6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

36. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises:

a first amplification primer binding site, and

a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises:

a nucleic acid sequence comprising a unique molecular identifier,

a second amplification primer binding site, and

optionally, a constant nucleic acid sequence to minimize ligation bias;

(5) amplifying the extension product produced in step (4) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; and

(6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

37. The method of claim 35, wherein the nucleic acid adapter of step (4) is a partially double-stranded nucleic acid molecule.

38. The method of claim 37, wherein the nucleic acid adapter comprises:

a double-stranded annealed region,

a single-stranded mismatched region.

39. The method of claim 38, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is present in the double-stranded annealed region of the nucleic acid adapter.

40. The method of claim 36, wherein the nucleic acid adapter of step (4) is a partially double-stranded nucleic acid molecule.
41. The method of claim 40, wherein the nucleic acid adapter comprises:
 - a double-stranded annealed region,
 - a single-stranded mismatched region.
42. The method of claim 38 or claim 41, wherein the nucleic acid adapter comprises a constant nucleic acid sequence to minimize ligation bias, and wherein the constant nucleic acid sequence is present in the double-stranded annealed region of the nucleic acid adapter.
43. The method of claim 42, wherein the constant nucleic acid sequence comprises a cleavable moiety.
44. The method of claim 43, wherein the cleavable moiety is an enzymatically cleavable moiety.
45. The method of claim 44, wherein the enzymatically cleavable moiety is a USER sequence, wherein the USER sequence comprises the sequence GUGUATUG.
46. The method of claim 38 or claim 41, wherein the unique molecular identifier is present in the single-stranded mismatched region.
47. The method of claim 38 or claim 41, wherein the second amplification primer binding site is present in the single-stranded mismatched region of the nucleic acid adapter.
48. The method of claim 35 or claim 36, wherein the identifier oligonucleotide is double-stranded.
49. The method of claim 48, wherein at least one of the two strands of the identifier oligonucleotide comprises at least two separate nucleic acid molecules.

50. The method of claim 35 or claim 36, wherein at least one 3' end of the identifier oligonucleotide comprises a single nucleotide overhang.
51. The method of claim 35 or claim 36, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain comprises between about 25 nucleotides and about 40 nucleotides
52. The method of claim 51, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain comprises about 35 nucleotides.
53. The method of claim 35 or claim 36, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises between about 6 nucleotides and about 15 nucleotides.
54. The method of claim 53, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises about 12 nucleotides.
55. The method of claim 35 or claim 36, wherein the unique molecular identifier comprises between about 6 nucleotides and about 30 nucleotides.
56. The method of claim 55, wherein the unique molecular identifier comprises about 15 nucleotides.
57. The method of claim 35 or claim 36, wherein the first or the second amplification primer binding sites comprise between about 18 nucleotides and about 40 nucleotides.
58. The method of claim 57, wherein the first or the second amplification primer binding sites comprise about 32 nucleotides.

59. The method of claim 35 or claim 36, wherein the at least one nucleic acid adapter comprises a constant nucleic acid sequence to minimize ligation bias, wherein the constant nucleic acid sequence comprises between about 1 nucleotide and about 15 nucleotides.
60. The method of claim 59, wherein the constant nucleic acid sequence comprises about 8 nucleotides.
61. The method of claim 35 or claim 36, wherein at least one of the amplification primer binding sites comprises an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1.
62. The method of claim 35 or claim 36, wherein at least one of the amplification primer binding sites comprises an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.
63. The method of claim 35 or claim 36, wherein at least one of the amplification primers comprises a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing.
64. The method of claim 35 or claim 36, wherein at least one of the amplification primers comprises a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3.
65. The method of claim 35 or claim 36, wherein at least one of the amplification primers comprises a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.
66. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises:

- a first amplification primer binding site, and
- a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a single stranded nucleic acid template, wherein the nucleic acid template comprises:

- a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide,
- a nucleic acid sequence comprising a unique molecular identifier, and
- a nucleic acid sequence complementary to a second amplification primer binding site, and

optionally, an affinity molecule;

(5) extending the identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises:

- the identifier oligonucleotide,
- the nucleic acid sequence complementary to the unique molecular identifier, and
- the second amplification primer binding site;

(6) amplifying the extension product produced in step (5) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released;

(7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

67. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises:

a first amplification primer binding site,
a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a single stranded nucleic acid template, wherein the nucleic acid template comprises:

a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide,
a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released,
a nucleic acid sequence comprising a unique molecular identifier, and
a nucleic acid sequence complementary to a second amplification primer binding site, and
optionally, an affinity molecule;

(5) extending the identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises:

the identifier oligonucleotide,
the nucleic acid sequence complementary to the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released
the nucleic acid sequence complementary to the unique molecular identifier, and
the second amplification primer binding site;

(6) amplifying the extension product produced in step (5); and
(7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

68. The methods of claim 65 or claim 67, wherein the single stranded nucleic acid template of step (4) further comprises an affinity molecule.

69. The method of claim 68, wherein the method further comprises an affinity purification step between steps (4) and (5).

70. The method of claim 65 or claim 67, wherein the identifier oligonucleotide is double-stranded.

71. The method of claim 70, wherein at least one of the two strands of the identifier oligonucleotide comprises at least two separate nucleic acid molecules.

72. The method of claim 65 or claim 67, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain comprises between about 25 nucleotides and about 40 nucleotides

73. The method of claim 72, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain comprises about 35 nucleotides.

74. The method of claim 65 or claim 67, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises between about 6 nucleotides and about 15 nucleotides.

75. The method of claim 74, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises about 12 nucleotides.

76. The method of claim 65 or claim 67, wherein the unique molecular identifier comprises between about 6 nucleotides and about 30 nucleotides.

77. The method of claim 76, wherein the unique molecular identifier comprises about 15 nucleotides.

78. The method of claim 65 or claim 67, wherein the first or the second amplification primer binding sites comprise between about 18 nucleotides and about 40 nucleotides.

79. The method of claim 78, wherein the first or the second amplification primer binding sites comprise about 32 nucleotides.

80. The method of claim 65 or claim 67, wherein the at least one nucleic acid adapter comprises a constant nucleic acid sequence to minimize ligation bias, wherein the constant nucleic acid sequence comprises between about 1 nucleotide and about 15 nucleotides.

81. The method of claim 80, wherein the constant nucleic acid sequence comprises about 8 nucleotides.

82. The method of claim 65 or claim 67, wherein at least one of the amplification primer binding sites comprises an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1.

83. The method of claim 65 or claim 67, wherein at least one of the amplification primer binding sites comprises an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.

84. The method of claim 65 or claim 67, wherein at least one of the amplification primers comprises a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for next-generation sequencing applications.

85. The method of claim 65 or claim 67, wherein at least one of the amplification primers comprises a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3.

86. The method of claim 65 or claim 67, wherein at least one of the amplification primers comprises a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

87. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe,

wherein the first nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide,

a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and

a first amplification primer binding site, and

wherein the second nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide

a nucleic acid sequence comprising a unique molecular identifier, and

a second amplification primer binding site, and

wherein the first the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping;

(5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together;

(6) amplifying the ligation product produced in step (5); and

(7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

88. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe,

wherein the first nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide, and

a first amplification primer binding site, and

wherein the second nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide, and

a second amplification primer binding site, and

wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a unique molecular identifier, and

wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and

wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping;

(5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together;

(6) amplifying the ligation product produced in step (5); and

(7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

89. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe,

wherein the first nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide,
a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and
a first amplification primer binding site, and

wherein the second nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide,
a nucleic acid sequence comprising a unique molecular identifier, and
a second amplification primer binding site, and

wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping;

(5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together;

(6) amplifying the ligation product produced in step (5); and

(7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

90. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe,

wherein the first nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide, and

a first amplification primer binding site, and

wherein the second nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide, and

a second amplification primer binding site, and

wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a unique molecular identifier, and

wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and

wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping;

(5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together;

(6) amplifying the ligation product produced in step (5); and

(7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

91. The method of claim 87 or claim 89, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is located 5' to the first amplification primer binding site, and

wherein the unique molecular identifier is located 3' to the second amplification primer binding site.

92. The method of claim 88 or claim 90, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the unique molecular identifier are present in the first nucleic acid probe of step (4), and

wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the unique molecular identifier are located 5' to the first amplification site.

93. The method of claim 88 or claim 90, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the unique molecular identifier are present in the second nucleic acid probe of step (4), and

wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the unique molecular identifier are located 3' to the second amplification site.

94. The method of claim 88 or claim 90, wherein the unique molecular identifier is present in the first nucleic acid probe of step (4) and is located 5' to the first amplification binding site, and wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is present in the second nucleic acid probe of step (4) and is located 3' to the second amplification binding site.

95. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe,

wherein the first nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide,
a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and
a first amplification primer binding site,
a nucleic acid sequence comprising a first unique molecular identifier, an
a first flow cell binding site, and

wherein the second nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide
a nucleic acid sequence comprising a second unique molecular identifier,

a nucleic acid sequence comprising a third unique molecular identifier, and
a second flow cell binding site, and

wherein the first and the second nucleic acid probes hybridize to the identifier
oligonucleotide such that the first and the second nucleic acid probes are adjacent but not
overlapping;

(5) performing nick repair such that the hybridized first and second nucleic acid probes
are ligated together;

(6) amplifying the ligation product produced in step (5); and

(7) Identifying the released identifier oligonucleotide by sequencing the amplified
products produced in step (6), thereby spatially detecting the at least one target analyte in the at
least one cell in a tissue sample.

96. A method for spatially detecting at least one target analyte in at least one cell from a
tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least
one probe comprising a target binding domain and an identifier oligonucleotide, wherein the
identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target
analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier
oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a
second nucleic acid probe,

wherein the first nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide,
a first amplification primer binding site,
a nucleic acid sequence comprising a first unique molecular identifier, and
a first flow cell binding site, and

wherein the second nucleic acid probe comprises:

a nucleic acid complementary to a portion of the identifier oligonucleotide
a nucleic acid sequence comprising a second unique molecular identifier, and

a second flow cell binding site, and

wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a third unique molecular identifier, and

wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and

wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping;

(5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together;

(6) amplifying the ligation product produced in step (5); and

(7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

97. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe,

wherein the first nucleic acid probe comprises:

a nucleic acid complementary to the identifier oligonucleotide,

a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and

a first amplification primer binding site,
a nucleic acid sequence comprising a first unique molecular identifier, and
a first flow cell binding site, and

wherein the second nucleic acid probe comprises:

a nucleic acid complementary to the identifier oligonucleotide
a nucleic acid sequence comprising a second unique molecular identifier,
a nucleic acid sequence comprising a third unique molecular identifier, and
a second flow cell binding site, and

wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping;

(5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together;

(6) amplifying the ligation product produced in step (5); and

(7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

98. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target domain;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe,

wherein the first nucleic acid probe comprises:

a nucleic acid complementary to the identifier oligonucleotide,

a first amplification primer binding site,
a nucleic acid sequence comprising a first unique molecular identifier, and
a first flow cell binding site, and

wherein the second nucleic acid probe comprises:

a nucleic acid complementary to the identifier oligonucleotide
a nucleic acid sequence comprising a second unique molecular identifier, and
a second flow cell binding site, and

wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a third unique molecular identifier, and

wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and

wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping;

(5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together;

(6) amplifying the ligation product produced in step (5); and

(7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

99. The method of claim 95 or claim 97, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the first unique molecular identifier are located 5' to the first flow cell binding site, and

wherein the second and the third unique molecular identifiers are located 3' to the second flow cell binding site.

100. The method of claim 96 or claim 98, wherein the first unique molecular identifier is located 5' to the first flow cell binding site and the second unique molecular identifier is located 3' to the second flow cell binding site.

101. The method of claim 100, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the third unique molecular identifier are present in the first nucleic acid probe of step (4) and are located 5' to the first flow cell binding site.

102. The method of claim 100, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the third unique molecular identifier are present in the second nucleic acid probe of step (4) and are located 3' to the second flow cell binding site.

103. The method of claim 100, wherein the third unique molecular identifier is present in the first nucleic acid probe of step (4) and is located 5' to the first flow cell binding site, and
wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is present in the second nucleic acid probe of step (4) and is located 3' to the second flow cell binding site.

104. The method of any of claims 87 to 90 or claims 95 to 98, wherein the identifier oligonucleotide is double-stranded.

105. The method of claim 104, wherein at least one of the two strands of the identifier oligonucleotide comprises at least two separate nucleic acid molecules.

106. The method of any of claims 87 to 90 or claims 95 to 98, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain comprises between about 25 nucleotides and about 40 nucleotides

107. The method of claim 106, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain comprises about 35 nucleotides.

108. The method of any of claims 87 to 90 or claims 95 to 98, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises between about 6 nucleotides and about 15 nucleotides.

109. The method of claim 108, wherein the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises about 12 nucleotides.

110. The method of any of claims 87 to 90 or claims 95 to 98, wherein a unique molecular identifier comprises between about 6 nucleotides and about 30 nucleotides.

111. The method of claim 110, wherein a unique molecular identifier comprises about 15 nucleotides.

112. The method of any of claims 87 to 90 or claims 95 to 98, wherein the first amplification primer binding site comprises between about 18 nucleotides and about 40 nucleotides.

113. The method of claim 112, wherein the first amplification primer binding site comprises about 32 nucleotides.

114. The method of any of claims 87 to 90 or claims 95 to 98 wherein at least one of the amplification primer binding sites comprises an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1.

115. The method of any of claims 87 to 90 or claims 95 to 98, wherein at least one of the amplification primer binding sites comprises an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.

116. The method of any of claims 87 to 90 or claims 95 to 98, wherein at least one of the amplification primers comprises a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing.

117. The method of any of claims 87 to 90 or claims 95 to 98, wherein at least one of the amplification primers comprises a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3.

118. The method of any of claims 87 to 90 or claims 95 to 98, wherein at least one of the amplification primers comprises a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

119. The method of any of claims 95 to 98, wherein at least one of the flow cell binding sites comprises a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing.

120. The method of any of claims 95 to 98, wherein at least one of the flow cell binding sites comprises a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3.

121. The method of any of claims 95 to 98, wherein at least one of the flow cell binding sites comprises a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

122. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises:

- a unique nucleic acid sequence which identifies the target analyte bound to the target domain,
- a nucleic acid sequence comprising a unique molecular identifier,
- a first amplification primer binding site, and
- a second amplification primer binding site;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released;

(5) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

123. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises:

a unique nucleic acid sequence which identifies the target analyte bound to the target domain,

a first amplification primer binding site, and

a second amplification primer binding site;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site,

wherein at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and

wherein at least one of the amplification primers comprises a nucleic acid sequence comprising a unique molecular identifier; and

(5) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

124. The method of claim 122 or claim 123, wherein the identifier oligonucleotide is double-stranded.

125. The method of claim 124, wherein at least one of the two strands of the identifier oligonucleotide comprises at least two separate nucleic acid molecules.

126. The method of claim 122 or claim 123, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain comprises about 10 nucleotides.

127. The method of any of claims 126, wherein the unique molecular identifier comprises about 10 nucleotides.

128. The method of claim 122 or claim 123, wherein the first amplification primer binding site comprises about 25 nucleotides.

129. The method of claim 122 or claim 123, wherein the second amplification primer binding sites comprises about 32 nucleotides.

130. The method of any of claim 122 or claim 123, wherein the unique nucleic acid sequence which identifies the target analyte bound to the target domain is flanked on at least one side by about 3 nucleotides.

131. The method of claim 122, wherein the unique molecular identifier is flanked on at least one side by about 3 nucleotides.

132. The method of claim 122 or claim 123, wherein the first amplification primer binding site is flanked on at least one side by about 3 nucleotides.

133. The method of claim 122 or claim 123, wherein the second amplification primer binding site is flanked on at least one side by about 3 nucleotides.

134. The method of claim 122 or claim 123, wherein at least one of the amplification primer binding sites comprises an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1.

135. The method of claim 122 or claim 123, wherein at least one of the amplification primer binding sites comprises an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.

136. The method of claim 122 or claim 123, wherein at least one of the amplification primers comprises a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing.

137. The method of claim 122 or claim 123, wherein at least one of the amplification primers comprises a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3.

138. The method of claim 122 or claim 123, wherein at least one of the amplification primers comprises a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

139. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises:

a unique nucleic acid sequence which identifies the target analyte bound to the target domain,

a capture probe binding site;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a capture probe ,

wherein the capture probe comprises:

an affinity molecule, and

a region complementary to the capture probe binding site; and

(5) Identifying the released identifier oligonucleotide by sequencing the amplified hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

140. A method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising:

(1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises:

a unique nucleic acid sequence which identifies the target analyte bound to the target domain,

a capture probe binding site,

a multiplexing probe binding site;

(2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide;

(3) collecting the released identifier oligonucleotide;

(4) hybridizing to the released identifier oligonucleotide a capture probe and a multiplexing probe,

wherein the capture probe comprises:

an affinity molecule, and

a region complementary to the capture probe binding site, and

wherein the multiplexing probe comprises:

a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and

a region complementary to the multiplexing probe binding site; and

(5) Identifying the released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

141. The method of claim 139 or claim 140, wherein the sequencing of step (5) is an enzyme free sequencing method.

**BIOMOLECULAR PROBES AND METHODS OF DETECTING GENE AND PROTEIN
EXPRESSION**

ABSTRACT OF THE INVENTION

The present invention relates to, among other things, probes, compositions, methods, and kits for simultaneous, multiplexed detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell that are adaptable for use with existing sequencing technologies.

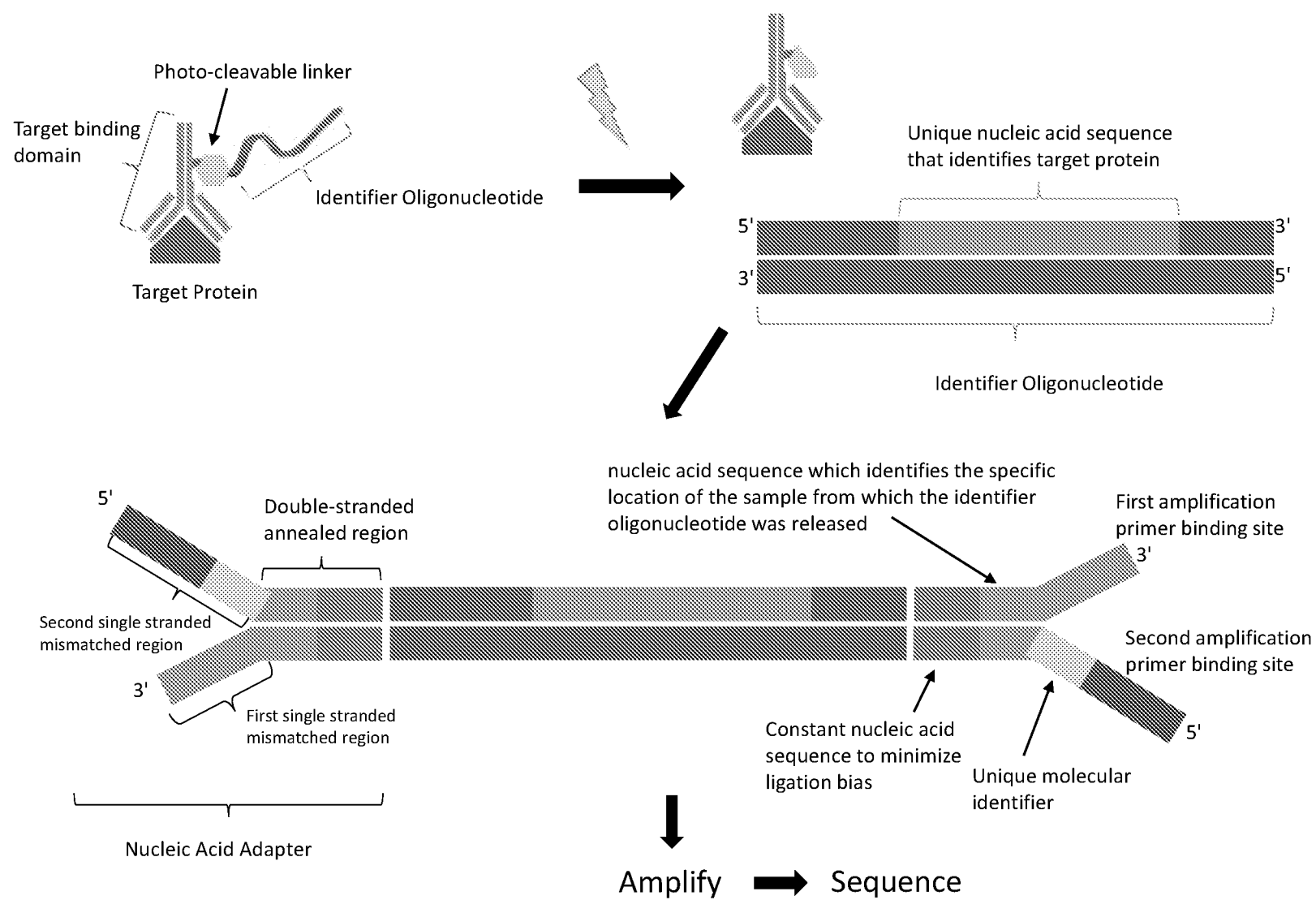


Figure 1

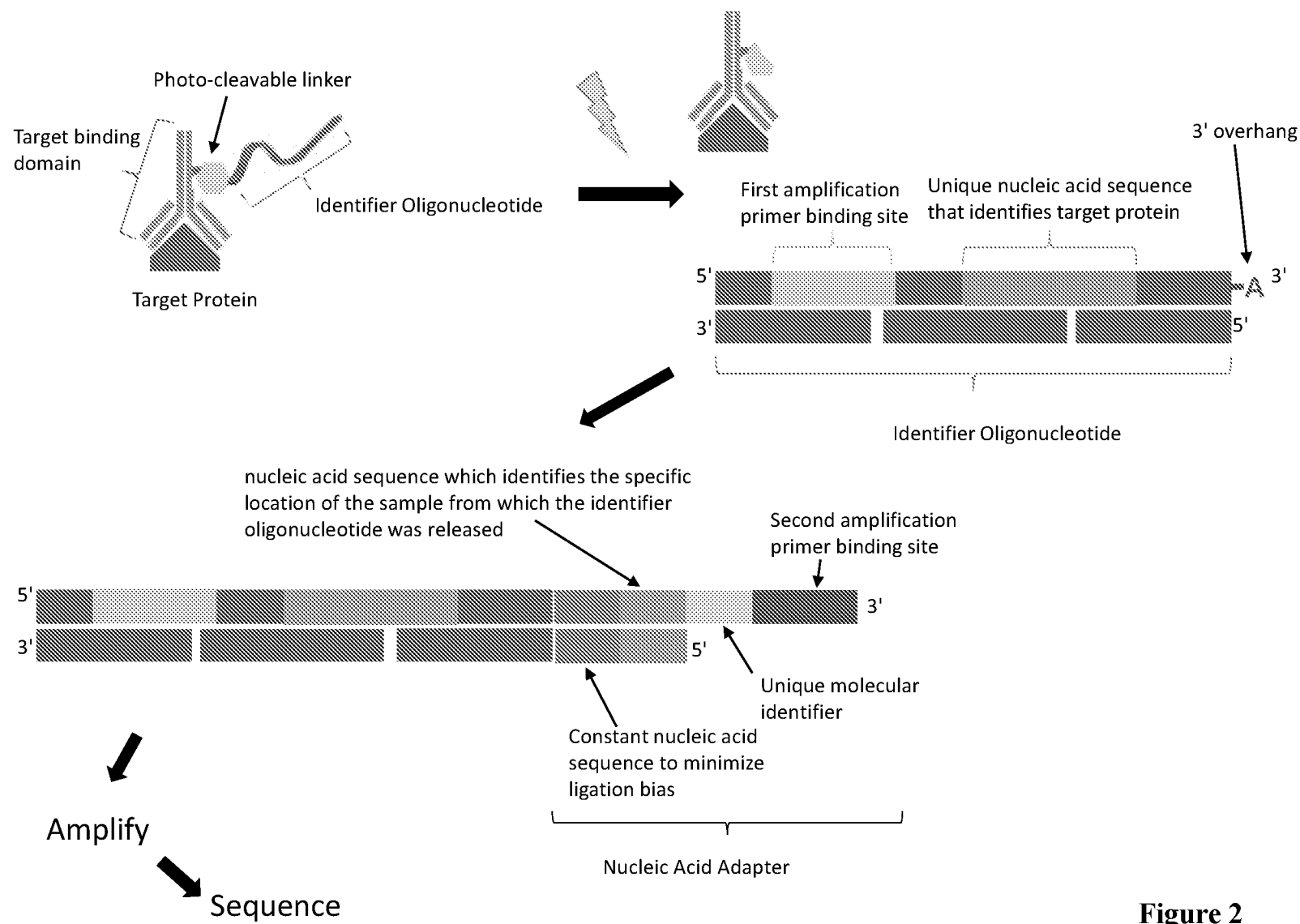


Figure 2

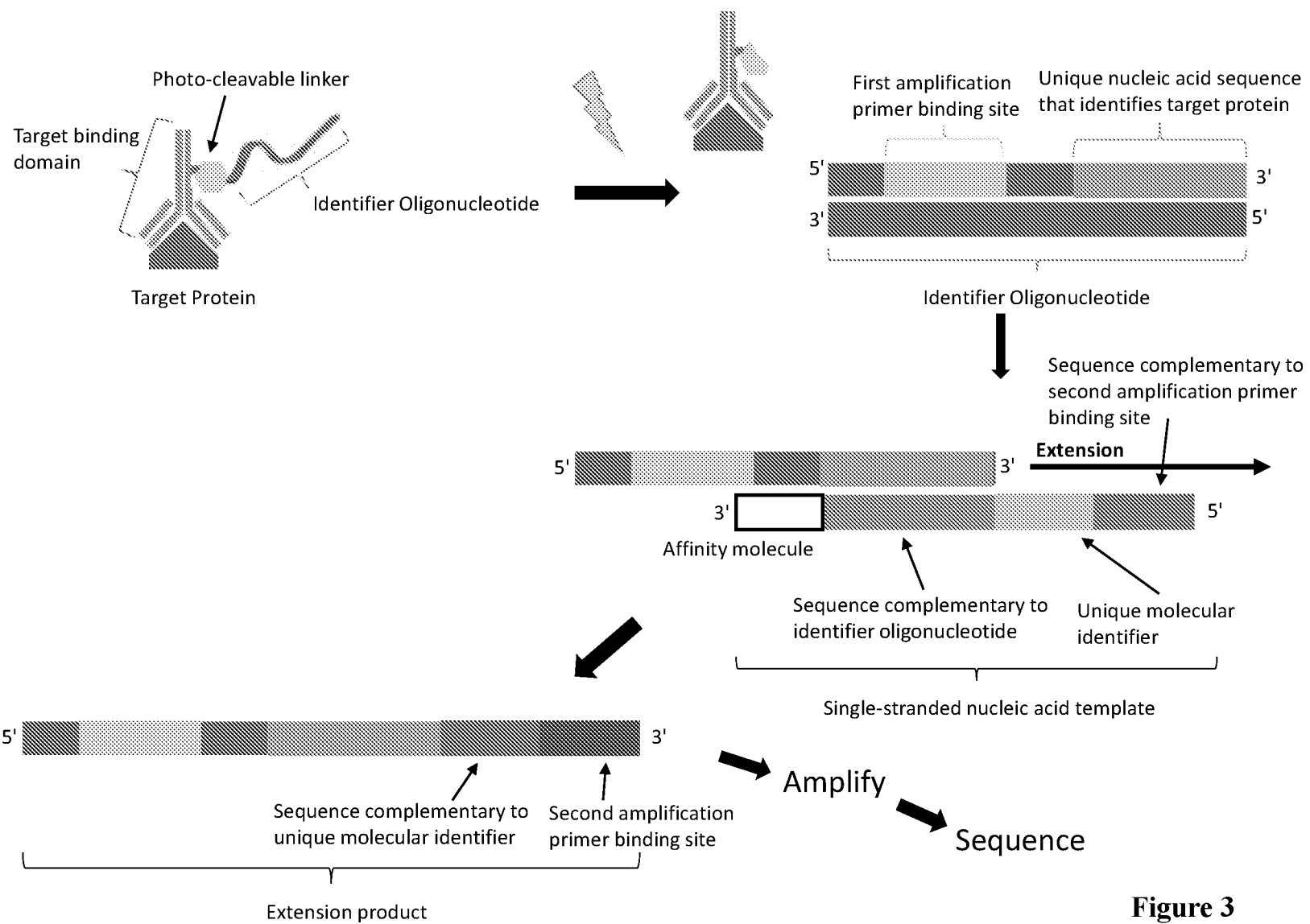


Figure 3

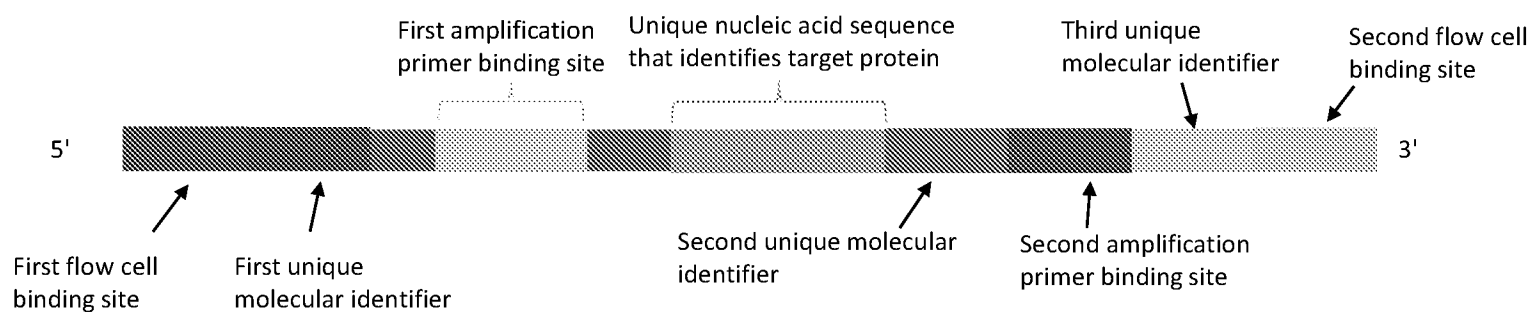


Figure 4

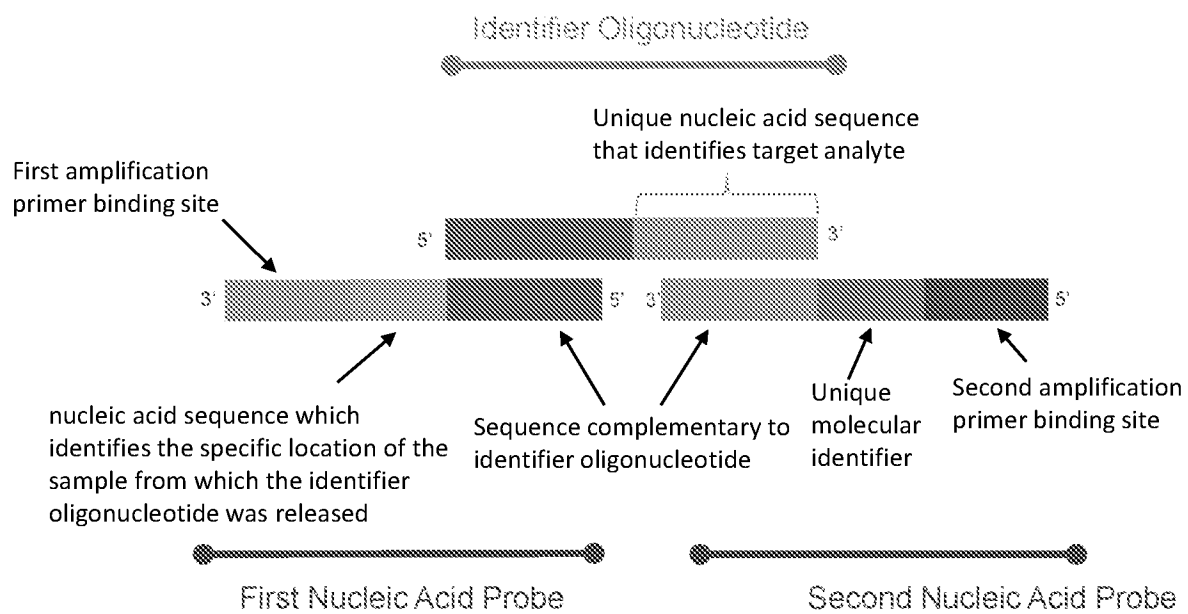


Figure 5

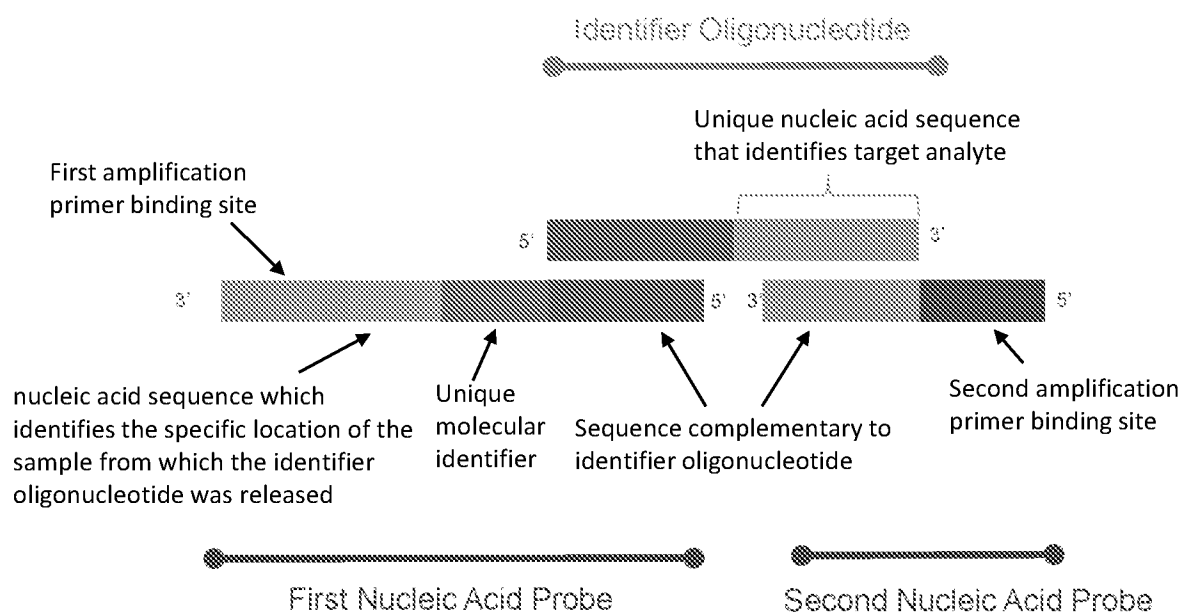


Figure 6

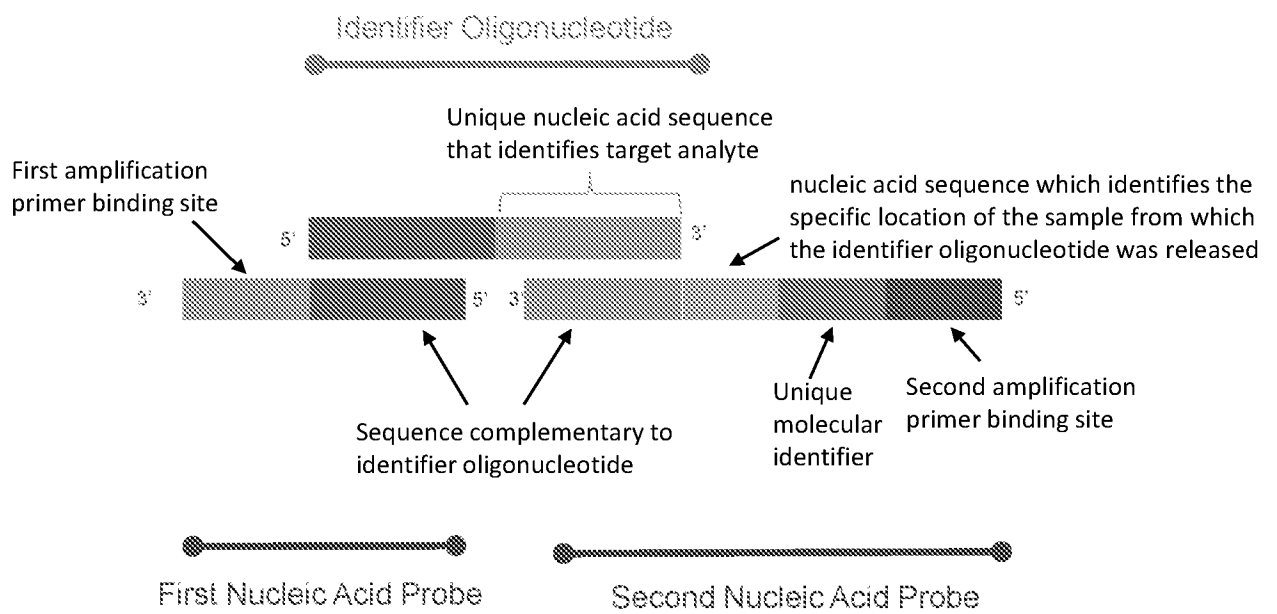


Figure 7

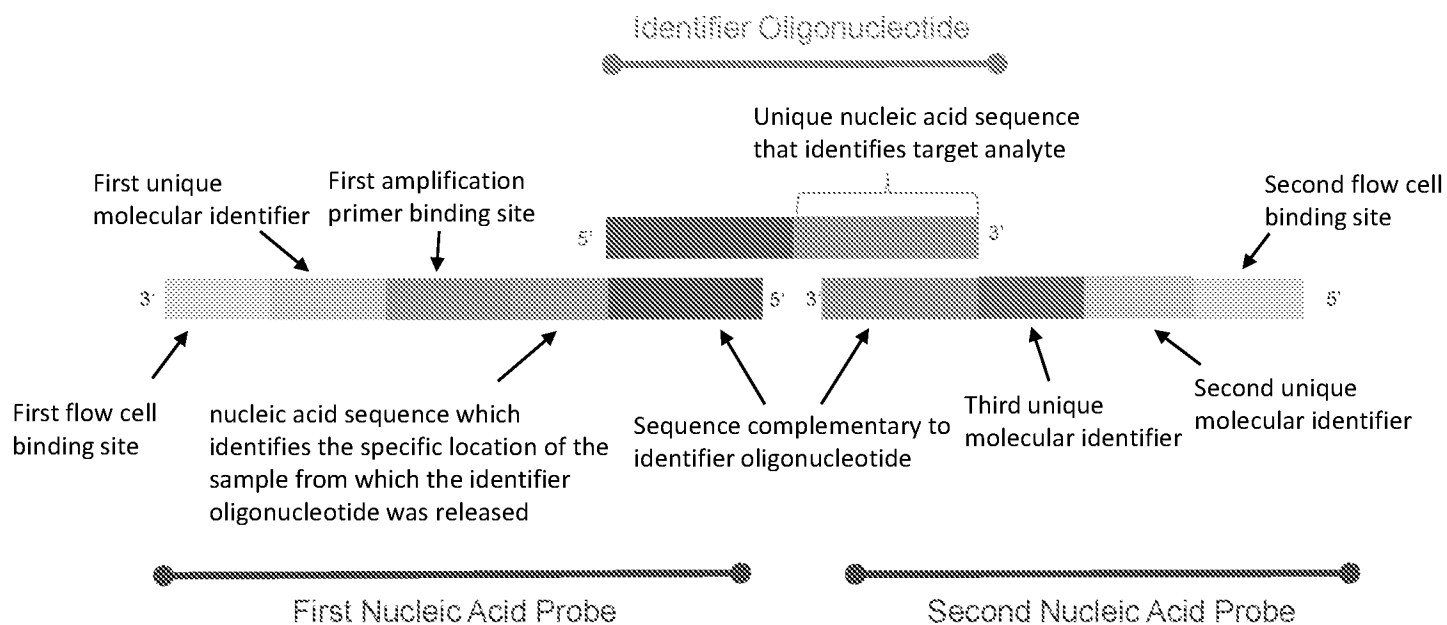


Figure 8

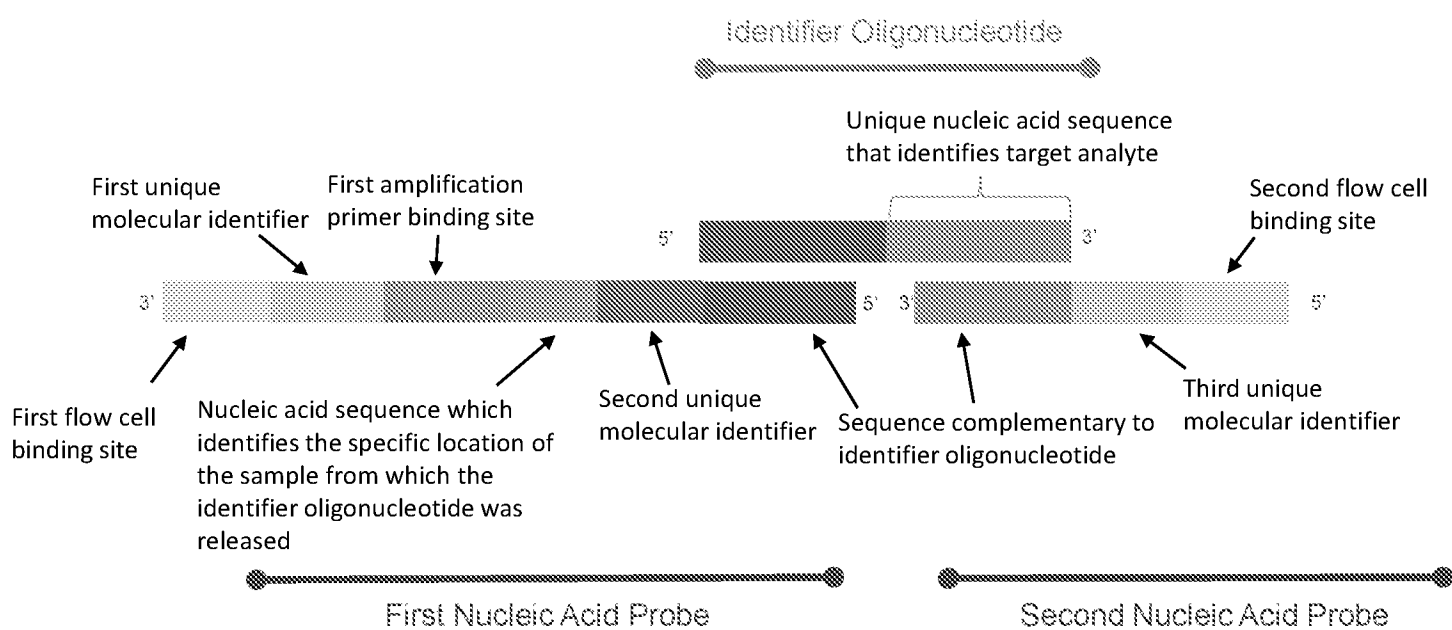


Figure 9

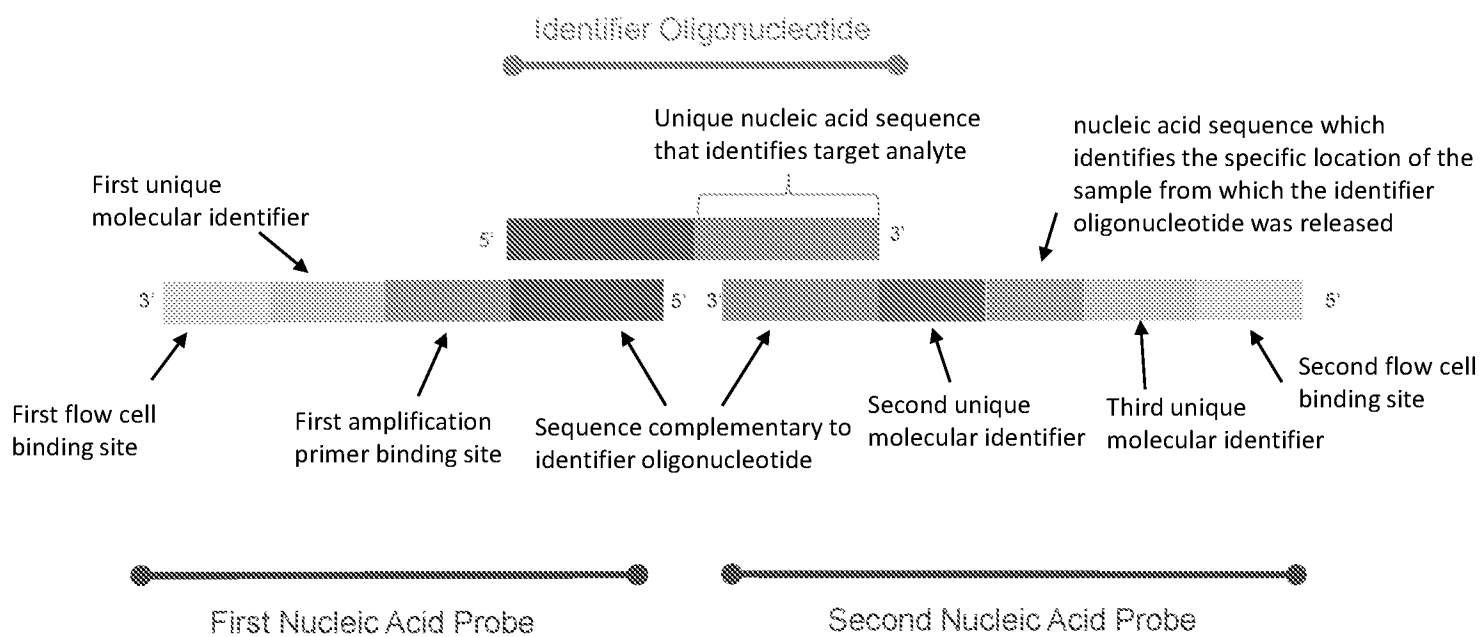


Figure 10

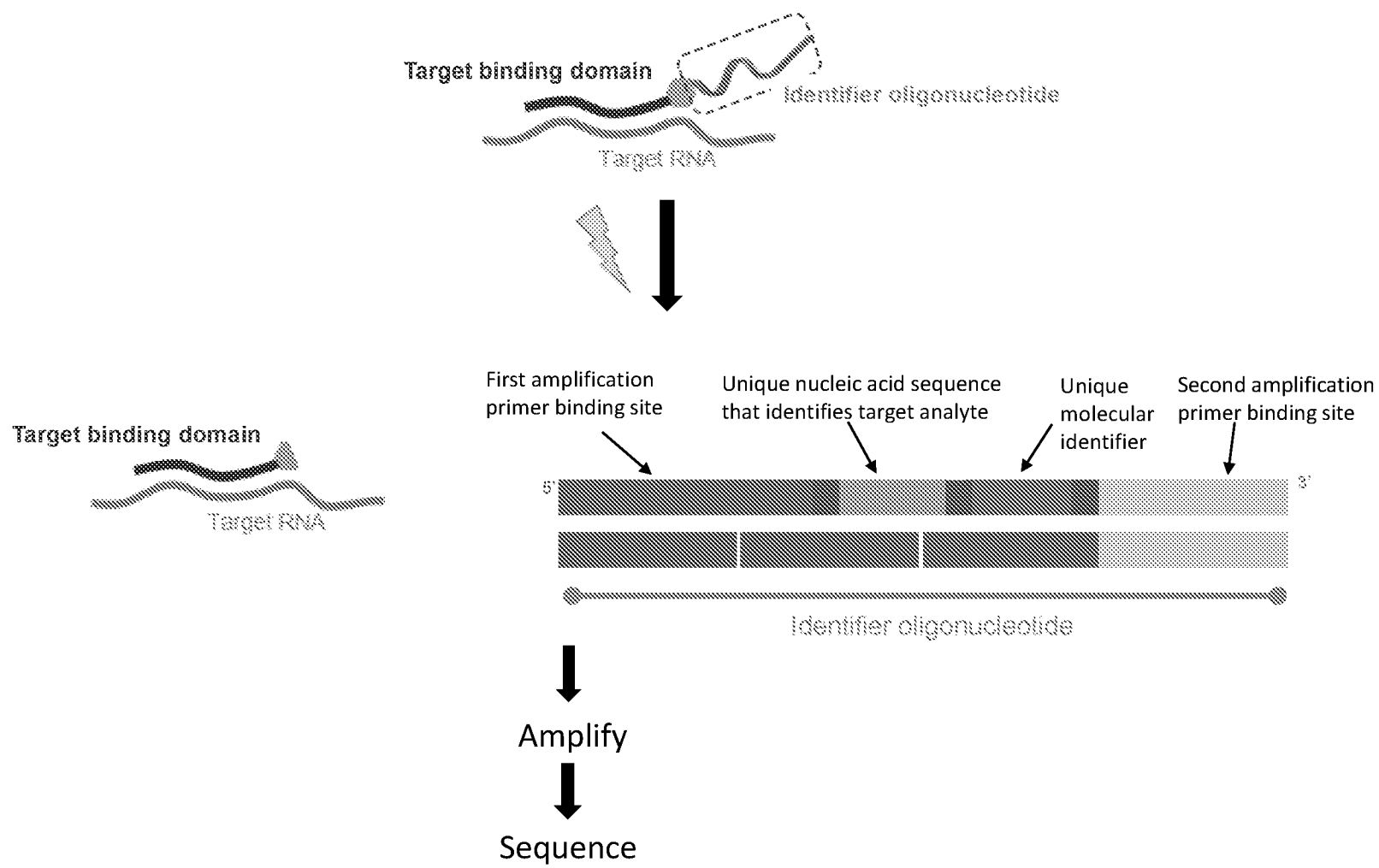


Figure 11

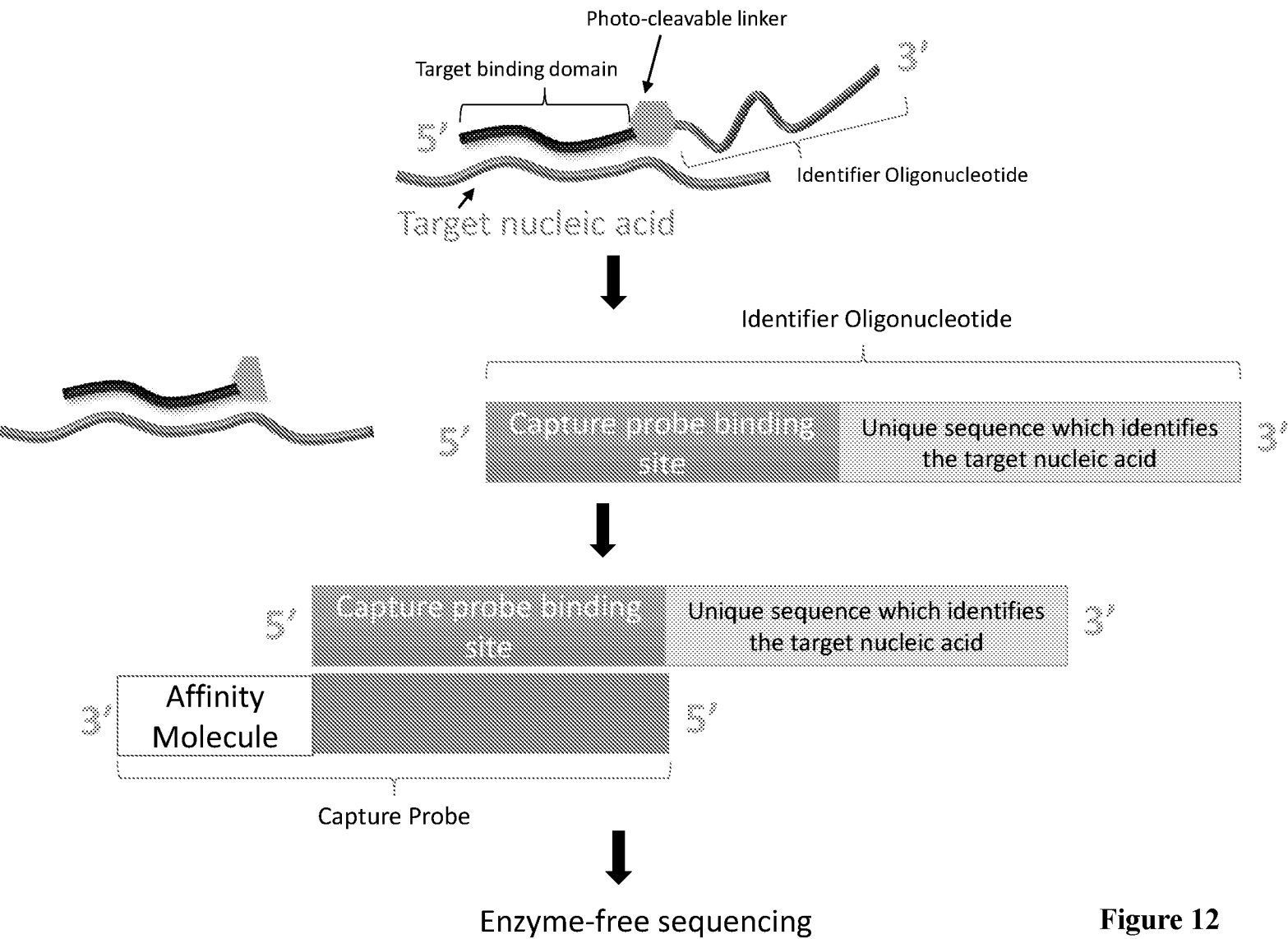


Figure 12

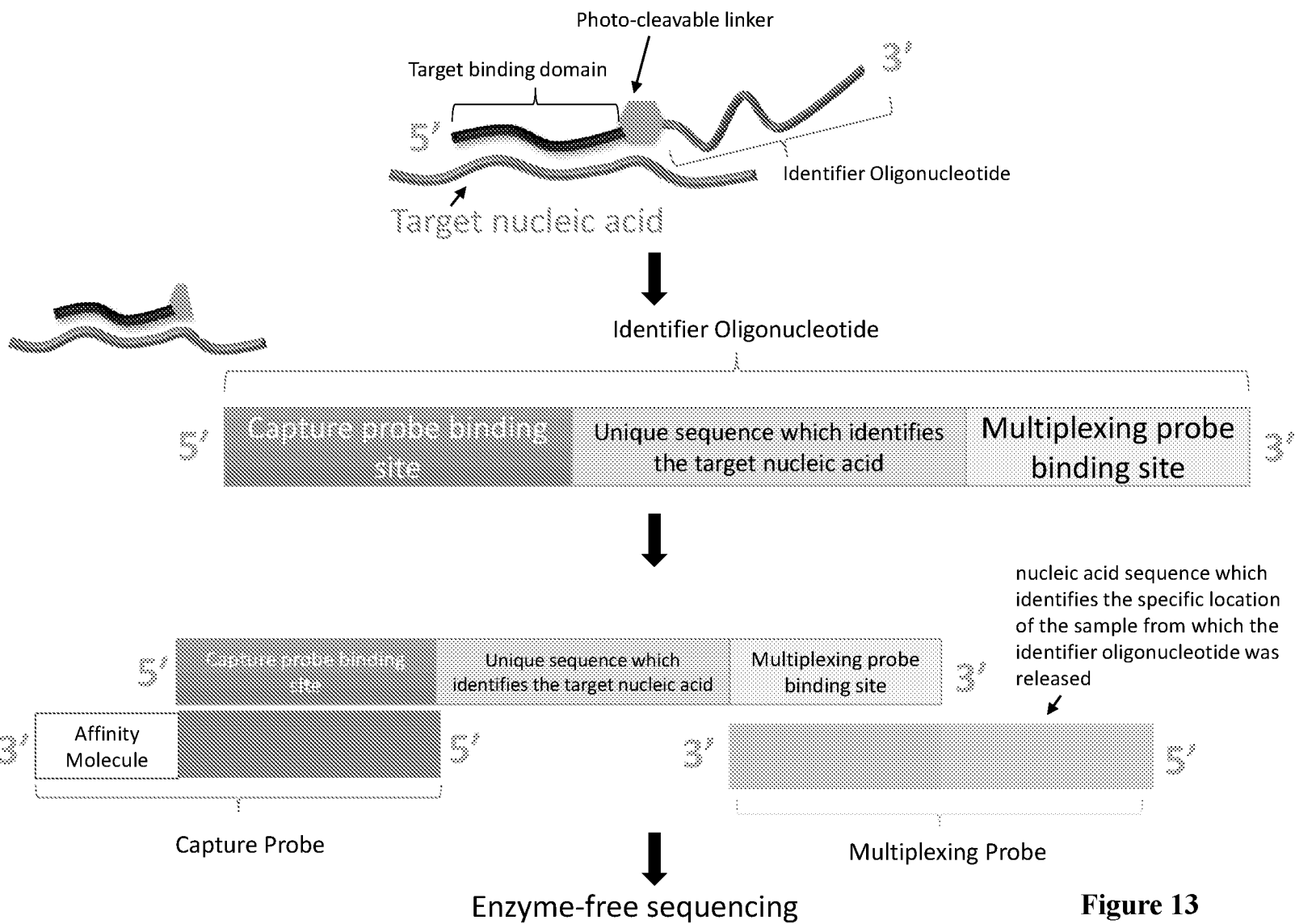


Figure 13

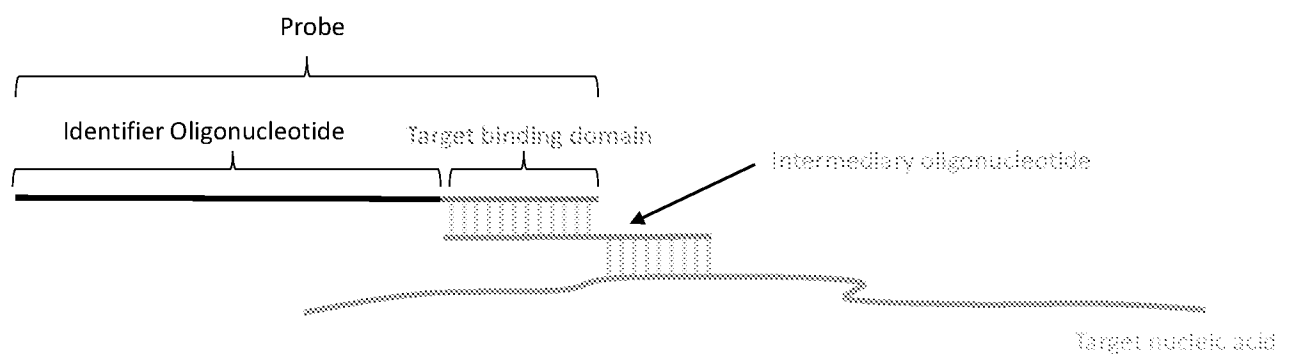


Figure 14