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(71) Demandeur/Applicant:
FLUIDIGM CORPORATION, US
(72) Inventeurs/Inventors:
LIVAK, KENNETH J., US;
FEKETE, RICHARD A., US
(74) Agent: ROBIC

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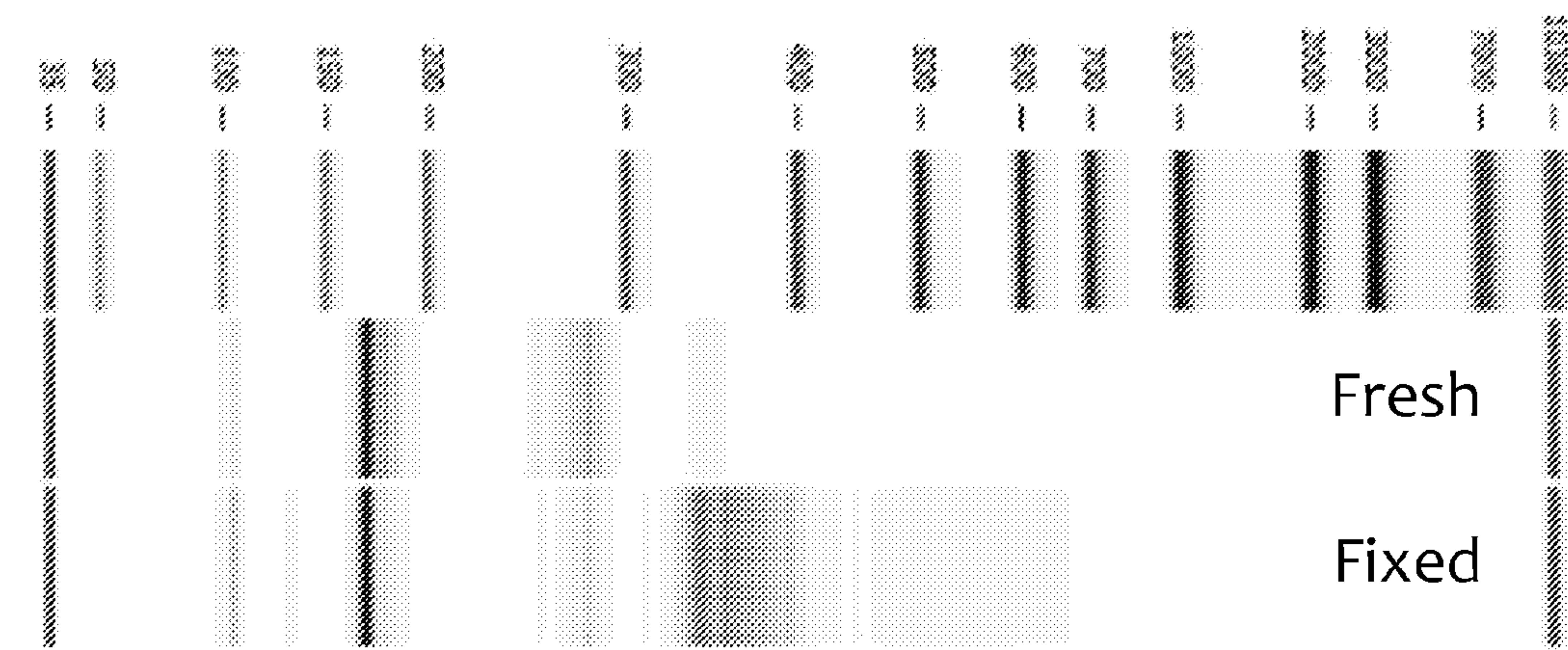


Figure 10

(57) **Abrégé/Abstract:**

Described herein are methods for preparing DNA templates for single-cell transcript sequencing of RNA from a population of cells. The methods entail distributing cells from the population into separate reaction volumes so that a plurality of separate reaction volumes each contain a single, isolated cell, wherein the cells have been treated with a fixative prior to distribution. The isolated cells are then permeabilized or disrupted, and cDNA is prepared by reverse transcript, followed by amplification. Also provided is a novel chemistry for efficient production of DNA templates from T-cell receptors or immunoglobulins in single cells.

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(71) Applicant: **FLUIDIGM CORPORATION** [US/US];
7000 Shoreline Court, Suite 100, South San Francisco, California 94080 (US).

(72) Inventors: **LIVAK, Kenneth J.**; 7000 Shoreline Court, Suite 100, South San Francisco, California 94080 (US).
FEKETE, Richard A.; 7000 Shoreline Court, Suite 100, South San Francisco, California 94080 (US).

(74) Agent: **HALIDAY, Emily M.** et al.; Weaver Austin Villeneuve & Sampson LLP, P.O. Box 70250, Oakland, California 94612-0250 (US).

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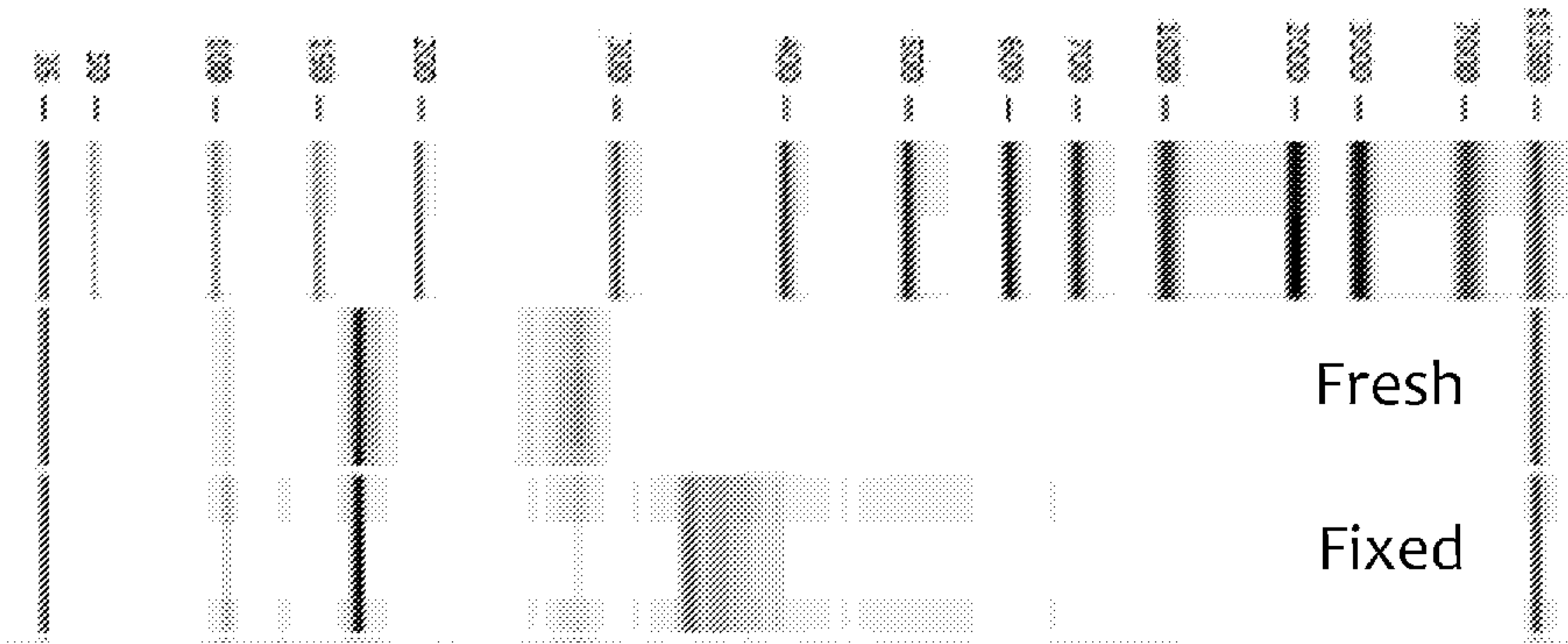


Figure 10

(57) Abstract: Described herein are methods for preparing DNA templates for single-cell transcript sequencing of RNA from a population of cells. The methods entail distributing cells from the population into separate reaction volumes so that a plurality of separate reaction volumes each contain a single, isolated cell, wherein the cells have been treated with a fixative prior to distribution. The isolated cells are then permeabilized or disrupted, and cDNA is prepared by reverse transcript, followed by amplification. Also provided is a novel chemistry for efficient production of DNA templates from T-cell receptors or immunoglobulins in single cells.

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SINGLE-CELL TRANSCRIPT SEQUENCING

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional
5 Patent Application Serial Numbers 62/362,425, filed July 14, 2016, and 62/463,487, filed February 24, 2017, both of which are incorporated herein by reference in their entireties for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

10 [0002] Not applicable.

FIELD

[0003] The subject matter disclosed herein relates to generally to the area of analysis of single cells. In particular, the subject matter relates to methods and compositions for performing single-cell transcript sequencing.

15 BACKGROUND

[0004] The ability to sequence mRNA from single eukaryotic cells is providing new information about the scope of cellular heterogeneity and the nature of cell identity. Single cells can be isolated, e.g., by micromanipulation, laser microdissection, fluorescence-activated cell sorting, and in microfluidic devices. Methods for preparing
20 sequencing libraries from single-cell mRNA have been developed. The two main classes of methods are whole transcript and end-tagging. In the whole transcript approach, each library contains fragments obtained from the full length of the transcript; in the end-tagging approach, each library contains fragments from just one end of the transcript. Whole transcript libraries provide more information, but end-tagging offers advantages in
25 terms of workflow and quantification. Primary processing of the sequencing data is generally similar to the processing used in bulk mRNA sequencing.

[0005] Single-cell transcript of sequencing is of particular interest in the context of determining RNA sequences encoding the T-cell receptor (TCR) or immunoglobulin

produced by a particular cell. For example, each T lymphocyte expresses TCR that binds antigen. Antigen binding is a key event in the cascade of the immune response. Each TCR is a heterodimer consisting of α - and β -chains encoded by the TRA and TRB genes, respectively. Diversity in antigen recognition is generated by somatic V(D)J

5 recombination occurring at the TRA and TRB loci. At single-cell resolution, determining the sequence of an appropriate portion of TRA and TRB serves as a unique identifier of a T cell's ancestry because it is likely that any two T cells expressing the same TCR $\alpha\beta$ pair arose from a common T-cell clone. Because immunoglobulin genes are organized similarly to TCR genes and also generate diversity by somatic V(D)J recombination,
10 immunoglobulin RNA sequences can likewise be used to identify a B cell's ancestry.

SUMMARY

[0006] Various embodiments contemplated herein may include, but need not be limited to, one or more of the following:

[0007] Embodiment 1: A method for preparing DNA templates for single-cell
15 transcript sequencing of RNA from a population of cells, the method including:
distributing cells from the population into separate reaction volumes so that a plurality of separate reaction volumes each comprise a single cell, wherein the cells have been treated with a fixative prior to distribution, wherein the fixative includes a cell-permeant fixative that enables the production of DNA templates from transcripts derived from single cells
20 with greater efficiency than in the absence of said cell-permeant fixative; permeabilizing or disrupting each cell in each separate reaction volume; reverse transcribing cDNA from RNA in each separate reaction volume; and amplifying the cDNA to produce DNA templates, wherein the amplification incorporates one or more nucleotide sequences that facilitate DNA sequencing of the DNA templates.

25 [0008] Embodiment 2: The method of embodiment 1, wherein the fixative stabilizes the cell nucleus and/or stabilizes RNA.

[0009] Embodiment 3: The method of embodiment 1, wherein the method includes the prior treatment of the cells with the fixative.

[0010] Embodiment 4: The method of embodiments 1 or 3, wherein the fixative
30 includes biomarker and histology preservative (BHP).

[0011] Embodiment 5: The method of embodiments 1 or 3, wherein the fixative includes dithiobis(succinimylal proprionate) (DSP).

[0012] Embodiment 6: The method of any of embodiments 1-5, wherein the DNA templates are recovered from the separate reaction volumes in one or more pools of DNA
5 templates.

[0013] Embodiment 7: The method of any of embodiments 1-6, wherein the DNA templates are further amplified after recovery.

[0014] Embodiment 8: The method of any of embodiments 1-7, wherein the method additionally includes subjecting the DNA templates to DNA sequencing.

10 [0015] Embodiment 9: The method of any of embodiments 1-8, wherein the method includes preparing DNA templates for single-cell transcript sequencing of T-cell receptor or immunoglobulin RNA from the population, wherein: the cells comprise T cells or B cells; and the DNA templates are generated from T-cell receptor or immunoglobulin RNA, respectively.

15 [0016] Embodiment 10: The method of embodiment 9, wherein the cells are T cells.

[0017] Embodiment 11: The method of embodiment 9, wherein the cells are B cells.

[0018] Embodiment 12: The method of embodiments 10 or 11, wherein the cells
20 are activated.

[0019] Embodiment 13: The method of any of embodiments 1-9, wherein the separate reaction volumes comprise separate capture sites in a microfluidic device.

[0020] Embodiment 14: The method of embodiment 13, including providing reverse transcription reagents to each capture site, wherein the reverse transcription
25 reagents comprise: a first reverse transcription (RT) primer having a portion specific for the Constant segment (CS) of an RNA encoding a first chain of a T-cell receptor or immunoglobulin, the T-cell receptor or immunoglobulin also including a second chain; a second reverse transcription (RT) primer having a portion specific for the Constant segment (CS) of an RNA encoding the second chain of the T-cell receptor or
30 immunoglobulin, respectively; wherein the first and second RT primers each additionally comprise a first nucleotide tag including a first primer binding site for a first DNA

sequencing primer, the first nucleotide tag being 5' of the CS-specific portion; and a first barcode primer that includes, from 3' to 5', a portion specific for the first primer binding site, a first barcode nucleotide sequence, and a first sequencing adaptor.

[0021] Embodiment 15: The method of embodiment 14, wherein the first barcode primer additionally includes nucleotide sequences that form a stem-duplex, whereby the first barcode primer has a hairpin configuration during reverse transcription and a linear configuration during amplification.

[0022] Embodiment 16: The method of embodiment 15, wherein the first barcode primer is provided to each capture site before permeabilizing or disrupting each cell in each separate reaction volume.

[0023] Embodiment 17: The method of embodiments 15 or 16, wherein the reverse transcription reagents additionally comprise a second barcode primer that includes, from 3' to 5', a portion specific for a second primer binding site for a second DNA sequencing primer, a second barcode nucleotide sequence, and a second sequencing adaptor.

[0024] Embodiment 18: The method of embodiment 17, wherein the second barcode primer is provided to each capture site with one or more reagents that permeabilize or disrupt each cell in each separate reaction volume.

[0025] Embodiment 19: The method of embodiments 17 or 18, wherein the second barcode primer additionally includes nucleotide sequences that form a stem-duplex, whereby the second barcode primer has a hairpin configuration during reverse transcription and a linear configuration during amplification.

[0026] Embodiement 20: The method of any of embodiments 15-19, including providing amplification reagents to each capture site, wherein the amplification reagents are provided separately from the reverse transcription reagents, and the amplification reagents comprise: a plurality of first amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the first chain of the T-cell receptor or immunoglobulin; a plurality of second amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the second chain of the T-cell receptor or immunoglobulin; wherein the first and second amplification primers each additionally comprise a second nucleotide tag including the second primer binding site, the second nucleotide tag being 5' of the VS-specific portion;

wherein the first and second amplification primers each comprise nucleotide sequences that form a stem-duplex, and wherein the first and second amplification primers each has a linear configuration during amplification.

[0027] Embodiment 21: The method of any of embodiments 1-20, wherein said
5 amplifying the cDNA to produce DNA templates includes polymerase chain reaction (PCR), which includes multiple cycles of denaturation, annealing, and elongation, wherein the PCR includes reducing the reaction temperature to rehydrate the reaction volume after two or more cycles of PCR.

[0028] Embodiment 22: The method of embodiment 21, wherein each of the first
10 and second amplification primers has a hairpin configuration during rehydration and a linear configuration during denaturation, annealing, and extension.

[0029] Embodiment 23: The method of any of embodiments 14-22, wherein the reverse transcription reagents additionally comprise a third reverse transcription (RT) primer having an oligo-dT sequence and a third nucleotide tag including the first primer
15 binding site, the third nucleotide tag being 5' of the oligo-dT sequence.

[0030] Embodiment 24: The method of embodiment 23, wherein the third RT primer additionally includes a unique molecular identifier (UMI) between the oligo-dT sequence and the first primer binding site.

[0031] Embodiment 25: The method of any of embodiments 20-24, wherein the
20 amplification reagents comprise a third amplification primer including a portion specific for a particular target RNA and the second nucleotide tag including the second primer binding site, the second nucleotide tag being 5' of the target-specific portion, wherein the third amplification primer includes nucleotide sequences that form a stem-duplex, wherein the third amplification primer has a linear configuration during amplification.

[0032] Embodiment 26: The method of embodiment 14, including providing
25 amplification reagents to each capture site, wherein the amplification reagents are provided separately from the reverse transcription reagents, and the amplification reagents comprise: a plurality of first amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the first chain of the T-cell receptor
30 or immunoglobulin; a plurality of second amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the second chain of the T-cell receptor or immunoglobulin, respectively; wherein the first and second

amplification primers each additionally comprise a second nucleotide tag including a second primer binding site for a second DNA sequencing primer, the second nucleotide tag being 5' of the VS-specific portion; and a second barcode primer that includes, from 3' to 5', a portion specific for the second primer binding site, a second barcode nucleotide sequence, and a second sequencing adaptor.

[0033] Embodiment 27: The method of embodiment 26, wherein the reverse transcription reagents additionally comprise a third reverse transcription (RT) primer having an oligo-dT sequence and a third nucleotide tag including the first primer binding site, the third nucleotide tag being 5' of the oligo-dT sequence.

10 **[0034]** Embodiment 28: The method of embodiment 27, wherein the third RT primer additionally includes a unique molecular identifier (UMI) between the oligo-dT sequence and the first primer binding site.

[0035] Embodiment 29: The method of any of embodiments 26-28, wherein the amplification reagents comprise a third amplification primer including a portion specific for a particular target RNA and the second nucleotide tag including the second primer binding site, the second nucleotide tag being 5' of the target-specific portion.

[0036] Embodiment 30: The method of embodiment 29, wherein the target-specific portion of the third amplification primer includes an insert that does not anneal to the target RNA, wherein the insert is flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the target RNA.

[0037] Embodiment 31: The method of embodiment 14, wherein the reverse transcription reagents additionally comprise a 5' oligonucleotide including, from 5' to 3', the second primer binding site, and an oligo-riboG sequence.

[0038] Embodiment 32: The method of embodiment 31, wherein the 5' oligonucleotide additionally includes a unique molecular identifier (UMI) between the second primer binding site and the oligo-riboG sequence.

[0039] Embodiment 33: The method of embodiment 32, including providing amplification reagents to each capture site, wherein the amplification reagents are provided separately from the reverse transcription reagents, and the amplification reagents comprise a second barcode primer that includes, from 3' to 5', a portion specific for the second primer binding site, a second barcode nucleotide sequence, and a second sequencing adaptor.

[0040] Embodiment 34: The method of any of embodiments 14-33, wherein the method produces DNA templates for T-cell receptor chains.

[0041] Embodiment 35: The method of any of embodiments 14-33, wherein the method produces DNA templates for immunoglobulin chains.

5 **[0042]** Embodiment 36: The method of any of embodiments 13-26, wherein the microfluidic device is a matrix-type microfluidic device including: capture sites arranged in a matrix of R rows and C columns, wherein R and C are integers greater than 1, and wherein the capture sites can be fluidically isolated from one another after distribution of cells to the capture sites; a set of R first input lines configured to deliver the first reagent(s)
10 to capture sites in a particular row; a set of C second input lines configured to deliver second reagent(s) to capture sites in a particular column, wherein said delivery is separate from the delivery first reagent(s), wherein, after a reaction, reaction products can be recovered from the microfluidic device in pools of reaction products from individual rows or columns.

15 **[0043]** Embodiment 37: The method of embodiment 36, wherein either: the reverse transcription reagents are provided to each capture site via the first input lines, and the amplification reagents are provided to each capture site via the second input lines; or the reverse transcription reagents are provided to each capture site via the second input lines, and the amplification reagents are provided to each capture site via the first input
20 lines.

[0044] Embodiment 38: The method of embodiment 37, wherein: each barcode primer provided to a first input line includes a barcode nucleotide sequence that is different from that in the other barcode primers provided to all other first input lines; each barcode primer provided to a second input line includes a barcode nucleotide sequence
25 that is different from that in the other barcode primers provided to all other second input lines; and each DNA template produced at each capture site includes the structure: 5'-(second sequencing adaptor)-(second barcode nucleotide sequence)-(second primer binding site)-(VS)-(complementarity determining region)-(CS)-(reverse complement first primer binding site)-(reverse complement first barcode nucleotide sequence)-(reverse
30 complement first sequencing adaptor)-3', wherein the first and second barcode nucleotide sequences, together, uniquely identify the capture site at which the DNA template was produced.

[0045] Embodiment 39: The method of any of embodiments 14-38, wherein: the CS-specific portion of the first RT primer includes an insert that does not anneal to the CS of the RNA encoding the first chain of the T-cell receptor or immunoglobulin; and/or the CS-specific portion of the second RT primer includes an insert that does not anneal to the CS of the RNA encoding the second chain of the T-cell receptor or immunoglobulin; wherein the insert is flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the CS.

[0046] Embodiment 40: The method of any of embodiments 26-39, wherein: the VS-specific portion of the plurality of first amplification primers includes an insert that does not anneal to the VS of the RNA encoding the first chain of the T-cell receptor or immunoglobulin; and/or the VS-specific portion of the plurality of second amplification primers includes an insert that does not anneal to the VS of the RNA encoding the second chain of the T-cell receptor or immunoglobulin; wherein the insert is flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the VS.

[0047] Embodiment 41: The method of embodiment 40, wherein the first and second RT primers do not comprise an insert that does not anneal to the CS of the RNA.

[0048] Embodiment 42: The method of any of embodiments 1-40, wherein the method includes preparing DNA templates for single-cell transcriptome sequencing.

[0049] Embodiment 43: The method of any of embodiments 1-42, wherein the method includes preparing DNA templates for single-cell transcript sequencing of more than one specific target RNA.

[0050] Embodiment 44: The method of embodiment 43, wherein the reverse transcription reagents additionally comprise a third reverse transcription (RT) primer having a portion specific for a particular target RNA and a third nucleotide tag including the first primer binding site for the first DNA sequencing primer, the third nucleotide tag being 5' of the target-specific portion.

[0051] Embodiment 45: The method of embodiment 44, wherein the target-specific portion of the third RT primer includes an insert that does not anneal to the particular target RNA, wherein the insert is flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the target RNA.

[0052] Embodiment 46: The method of any of embodiments 1-26 and 36-45, wherein the reverse transcription reagents additionally comprise a 5' oligonucleotide

including, from 5' to 3', the second primer binding site for the second DNA sequencing primer and an oligo-riboG sequence.

[0053] Embodiment 47: The method of embodiment 46, wherein the 5' oligonucleotide additionally includes a unique molecular identifier (UMI) between the second primer binding site and the oligo-riboG sequence.

[0054] Embodiment 48: The method of any of embodiments 9-47, wherein the method includes determining that a T-cell receptor or immunoglobulin sequence is present in a cell having a specific phenotype.

[0055] Embodiment 49: A primer combination for producing DNA templates from RNA encoding T-cell receptor or immunoglobulin chains, the primer combination including: a first reverse transcription (RT) primer having a portion specific for the Constant segment (CS) of an RNA encoding a first chain of a T-cell receptor or immunoglobulin, the T-cell receptor or immunoglobulin also including a second chain; a second reverse transcription (RT) primer having a portion specific for the Constant segment (CS) of an RNA encoding the second chain of the T-cell receptor or immunoglobulin, respectively; wherein the first and second RT primers each additionally comprise a first nucleotide tag including a first primer binding site for a first DNA sequencing primer, the first nucleotide tag being 5' of the CS-specific portion; and a first barcode primer that includes, from 3' to 5', a portion specific for the first primer binding site, a first barcode nucleotide sequence, and a first sequencing adaptor; a plurality of first amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the first chain of the T-cell receptor or immunoglobulin; a plurality of second amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the second chain of the T-cell receptor or immunoglobulin, respectively; wherein the first and second amplification primers each additionally comprise a second nucleotide tag including a second primer binding site for a second DNA sequencing primer, the second nucleotide tag being 5' of the VS-specific portion; and a second barcode primer that includes, from 3' to 5', a portion specific for the second primer binding site, a second barcode nucleotide sequence, and a second sequencing adaptor.

[0056] Embodiment 50: The primer combination of embodiment 49, wherein the first barcode primer includes nucleotide sequences that form a stem-duplex, whereby the

first barcode primer has a hairpin configuration when used for reverse transcription and a linear configuration when used for amplification.

[0057] Embodiment 51: The primer combination of embodiments 49 or 50, wherein the second barcode primer includes nucleotide sequences that form a stem-duplex, whereby the second barcode primer has a hairpin configuration when used for reverse transcription and a linear configuration when used for amplification.

[0058] Embodiment 52: The primer combination any of embodiments 49-51, wherein the first and second amplification primers each comprise nucleotide sequences that form a stem-duplex, whereby the first and second amplification primers each has a linear configuration when used for amplification.

[0059] Embodiment 53: The primer combination of any of embodiments 49-52, additionally including a third reverse transcription (RT) primer having an oligo-dT sequence and a third nucleotide tag including the first primer binding site, the third nucleotide tag being 5' of the oligo-dT sequence.

[0060] Embodiment 54: The primer combination of embodiment 53, wherein the third RT primer additionally includes a unique molecular identifier (UMI) between the oligo-dT sequence and the first binding site.

[0061] Embodiment 55: The primer combination of any of embodiments 49-54, additionally including a third amplification primer including a portion specific for a particular target RNA and the second nucleotide tag including the second primer binding site, the second nucleotide tag being 5' of the target-specific portion, wherein the third amplification primer includes nucleotide sequences that form a stem-duplex, whereby the third amplification primer has a linear configuration during amplification.

[0062] Embodiment 56: The primer combination of embodiment 49, wherein: the CS-specific portion of the first RT primer includes an insert that does not anneal to the CS of the RNA encoding the first chain of the T-cell receptor or immunoglobulin; and/or the CS-specific portion of the second RT primer includes an insert that does not anneal to the CS of the RNA encoding the second chain of the T-cell receptor or immunoglobulin; wherein the insert is flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the CS.

[0063] Embodiment 57: The primer combination of embodiments 49 or 56, wherein: the VS-specific portion of the plurality of first amplification primers includes an

insert that does not anneal to the VS of the RNA encoding the first chain of the T-cell receptor or immunoglobulin; and/or the VS-specific portion of the plurality of second amplification primers includes an insert that does not anneal to the VS of the RNA encoding the second chain of the T-cell receptor or immunoglobulin; wherein the insert is
 5 flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the VS.

[0064] Embodiment 58: The primer combination of embodiment 57, wherein the first and second RT primers do not comprise an insert that does not anneal to the CS of the RNA.

[0065] Embodiment 59: The primer combination of any of embodiments 56-58,
 10 additionally including a third reverse transcription (RT) primer having an oligo-dT sequence and a third nucleotide tag including the first primer binding site, the third nucleotide tag being 5' of the oligo-dT sequence.

[0066] Embodiment 60: The primer combination of embodiment 59, wherein the third RT primer additionally includes a unique molecular identifier (UMI) between the
 15 oligo-dT sequence and the binding site for the first DNA sequencing primer.

[0067] Embodiment 61: The primer combination of embodiments 59 or 60, additionally including a third amplification primer including a portion specific for a particular target RNA a second nucleotide tag including a second primer binding site, the second nucleotide tag being 5' of the target-specific portion.

20 **[0068]** Embodiment 62: The method of embodiment 61, wherein the target-specific portion of the third amplification primer includes an insert that does not anneal to the target RNA, wherein the insert is flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the target RNA.

[0069] Embodiment 63: The primer combination of any of embodiments 49-58,
 25 wherein the primer combination additionally includes a third reverse transcription (RT) primer having a portion specific for a particular target RNA and a third nucleotide tag including the first primer binding site, the third nucleotide tag being 5' of the target-specific portion.

[0070] Embodiment 64: The primer combination of embodiment 63, wherein the
 30 target-specific portion of the third RT primer includes an insert that does not anneal to the particular target RNA, wherein the insert is flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the target RNA.

[0071] Embodiment 65: The primer combination of embodiments 63 or 64, wherein the primer combination additionally includes a 5' oligonucleotide including, from 5' to 3', the second primer binding site and an oligo-riboG sequence.

[0072] Embodiment 66: The primer combination of embodiment 65, wherein the 5' oligonucleotide additionally includes a unique molecular identifier (UMI) between the binding site for the second DNA sequencing primer and the oligo-riboG sequence.

[0073] Embodiment 67: A primer combination for producing DNA templates from RNA encoding T-cell receptor or immunoglobulin chains, the primer combination including: a first reverse transcription (RT) primer having a portion specific for the Constant segment (CS) of an RNA encoding a first chain of a T-cell receptor or immunoglobulin, the T-cell receptor or immunoglobulin also including a second chain; a second reverse transcription (RT) primer having a portion specific for the Constant segment (CS) of an RNA encoding the second chain of the T-cell receptor or immunoglobulin, respectively; wherein the first and second RT primers each additionally comprise a first nucleotide tag including a first primer binding site for a first DNA sequencing primer, the first nucleotide tag being 5' of the CS-specific portion; and a first barcode primer that includes, from 3' to 5', a portion specific for the first primer binding site, a first barcode nucleotide sequence, and a first sequencing adaptor; a 5' oligonucleotide including, from 5' to 3', the second primer binding site for the second DNA sequencing primer, a unique molecular identifier (UMI), and an oligo-riboG sequence; and a second barcode primer that includes, from 3' to 5', a portion specific for the second primer binding site, a second barcode nucleotide sequence, and a second sequencing adaptor.

[0074] Embodiment 68: The primer combination of embodiment 67, wherein: the CS-specific portion of the first RT primer includes an insert that does not anneal to the CS of the RNA encoding the first chain of the T-cell receptor or immunoglobulin; and/or the CS-specific portion of the second RT primer includes an insert that does not anneal to the CS of the RNA encoding the second chain of the T-cell receptor or immunoglobulin; wherein the insert is flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the CS.

[0075] Embodiment 69: The primer combination of embodiments 67 or 68, wherein the combination additionally includes a third reverse transcription (RT) primer

having a portion specific for a particular target RNA and a third nucleotide tag including the first primer binding site, the third nucleotide tag being 5' of the target-specific portion.

[0076] Embodiment 70: The primer combination of embodiment 69, wherein the target-specific portion of the third RT primer includes an insert that does not anneal to the particular target RNA, wherein the insert is flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the target RNA.

[0077] Embodiment 71: The primer combination of any of embodiments 49-70, wherein the primer combination is adapted to produce DNA templates for T-cell receptor chains.

10 [0078] Embodiment 72: The primer combination of any of embodiments 49-70, wherein the primer combination is adapted to produce DNA templates for immunoglobulin chains.

[0079] Embodiment 73: A kit including the primer combination of any of embodiments 49-72 and a matrix-type microfluidic device including: capture sites
15 arranged in a matrix of R rows and C columns, wherein R and C are integers greater than 1, and wherein the capture sites can be fluidically isolated from one another after distribution of cells to the capture sites; a set of R first input lines configured to deliver the first reagent(s) to capture sites in a particular row; a set of C second input lines configured to deliver second reagent(s) to capture sites in a particular column, wherein said delivery is
20 separate from the delivery first reagent(s), wherein, after a reaction, reaction products can be recovered from the microfluidic device in pools of reaction products from individual rows or columns.

BRIEF DESCRIPTION OF THE DRAWINGS

[0080] The patent or application file contains at least one drawing executed in
25 color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0081] Figure 1A-D: An illustrative matrix-type microfluidic device is shown schematically in (1A). (1B) illustrates the delivery of R different barcodes through the R different first input lines to the capture sites. (1C) illustrates the delivery of C different
30 barcodes through C different input lines to the capture sites. (1D) illustrates that, after the reaction has been carried out, reaction products can be harvested for each column as a

pool, for example, by applying a harvesting fluid to the C second input lines to push the reaction products out of outlets at one end of the input lines.

[0082] Figure 2: A photograph of the illustrative matrix-type microfluidic device shown schematically in Figure 1.

5 **[0083]** Figure 3A-3D: Schematic representation of the structure of the genes encoding the α - and β -chains of the T-cell receptor (*TRA* and *TRB*, respectively). (3A) Somatic recombination to produce a gene encoding a TCR α -chain. (3B) Somatic recombination to produce a gene encoding a TCR β -chain. (3C) Detail of the complementarity determining regions (CDRs) in the TCR α -chain gene after somatic recombination; N denotes non-templated nucleotides inserted into CDR3. (3D) Detail of the CDRs in the TCR β -chain gene after somatic recombination.

[0084] Figure 4A-4B: (4A) Schematic representation of the primers added to a column (or row) of a matrix-type microfluidic device in an embodiment of a method of single-cell TCR transcript sequencing. The TRAC and TRBC sequences are
15 complementary to the TCR α and β mRNAs and serve as specific primers during the reverse transcriptase reaction. In the first barcode primer, illustrated in Figure 4A, Rd2 is the Illumina TruSeq Read 2 sequence, BC1-20 refers to 20 different barcodes, and P7 is one of the Illumina sequencing adaptors. The P7.BC.Rd2 primer does not participate in the RT reaction but is there so that barcodes are added to this end of the amplified
20 segments during the amplification step. (4B) Schematic representation of the primers added to a row (or column) of a matrix-type microfluidic device in this embodiment of a method of single-cell TCR transcript sequencing.

[0085] Figure 5: A diagrammatic representation of a Super Selective primer. The Anchor segment keeps the primer stably bound to the template. Because of the unpaired
25 Insert/Loop region, the Bait segment (typically 5-10 nucleotides) hybridizes to the template only transiently.

[0086] Figure 6A-6B: Schematic representations of the use of Super Selective primers in the method illustrated in Figure 4A-4B, above. (6A) Reverse transcription. (6B) amplification.

30 **[0087]** Figure 7: Schematic representation of the use of template switching for generating a DNA template for a particular target transcript (targeted transcript sequencing). In the template switching oligonucleotide, Rd1 is the Illumina TruSeq Read

1 sequence, UMI is a unique molecular identifier, and GGG is an oligo-riboG sequence. In the RT primer, Rd2 is the Illumina TruSeq Read 2 sequence. The P7.BC.Rd2 primer discussed with respect to Figures 4 and 6 can prime subsequent amplification of the cDNA resulting from template switching.

5 [0088] Figure 8A-B: Schematic representation of the structure of genes encoding particular heavy and light chains that make up immunoglobulins. (8A) Somatic recombination to produce genes encoding μ or δ heavy chains. (8B) Somatic recombination to produce a gene encoding a κ light chain.

[0089] Figure 9: Gel showing results of Example 1. Desired DNA fragments
10 derived from a TCR transcript are in the size range 250 to 350 nucleotides. Undesired, nonspecific fragments are in the size range 100 to 220 nucleotides. The reaction products in lane 1 were generated with No Loop RT primers and No Loop V primers; lane 2, Loop RT primers and No Loop V primers; lane 3, No Loop RT primers and Loop V primers; lane 4, Loop RT primers and Loop V primers. The best ratio of desired fragments to
15 undesired fragments is observed for combination 3, which is No Loop RT primers and Loop V primers.

[0090] Figure 10: Gel showing results of Example 2. Because of the addition of P5, P7, and other sequences, the desired DNA fragments derived from a TCR transcript are in the size range 320 to 400 nucleotides. Results show that the fixed cells gave a much
20 better yield of desired fragments than fresh cells.

[0091] Figure 11A-11B: Schematic representation of an illustrative method of single-cell T-cell receptor template production designed for tubes, which is described in detail in Example 5.

[0092] Figure 12A-12B: Schematic representation of a method of single-cell T-
25 cell receptor template production similar to that shown in Figure 11A-11B, but modified for use with Fluidigm's C₁TM High-Throughput IFC (described in detail in Example 5).

[0093] Figure 13: Gel showing results of Example 5. This method produces TCR libraries predominantly in the expected 350-450 bp size range.

DETAILED DESCRIPTION

30 [0094] Described herein are methods for preparing DNA templates for single-cell transcript sequencing of RNA from a population of cells. The methods entail distributing

cells from the population into separate reaction volumes so that a plurality of separate reaction volumes each contain a single, isolated cell, wherein the cells have been treated with a fixative prior to distribution. In certain embodiments, the fixative can be a cell-permeant fixative. In particular embodiments, the method includes the step of pre-treating
5 the cells with the fixative. The method is carried out by permeabilizing or disrupting (e.g., lysing) each cell in its separate reaction volume and reverse transcribing cDNA from RNA in each separate reaction volume (i.e., wherein each reverse transcription of RNA from an isolated cell is carried out separately from the reverse transcription of RNA from every other isolated cell). The cDNA is then amplified to produce DNA, with the amplification
10 also being carried out to produce DNA templates from each isolated cell in separate reaction volumes. The amplification incorporates one or more nucleotide sequences that facilitate DNA sequencing of the DNA templates. After amplification, the DNA templates can be recovered, e.g., for further amplification and/or DNA sequencing. In some embodiments, the DNA templates are recovered from the separate reaction volumes in one
15 or more pools of DNA templates, which can, optionally, be further pooled to produce a single pool of DNA templates for further amplification and/or DNA sequencing.

[0095] These methods described herein enable the production of DNA templates from transcripts derived from single cells with greater efficiency than was previously possible (e.g., without the fixative). In certain embodiments, aimed at single-cell
20 sequencing of T-cell receptors or immunoglobulins, a particular chemistry is described herein which allow the production of DNA templates using a simpler protocol than any previously available, especially when implemented on a matrix-type microfluidic device.

Definitions

[0096] Terms used in the claims and specification are defined as set forth below
25 unless otherwise specified. These terms are defined specifically for clarity, but all of the definitions are consistent with how a skilled artisan would understand these terms.

[0097] As used herein, the term “microfluidic device” refers to any device that includes chambers and/or fluid channels wherein at least one dimension is less than 1 millimeter. In certain embodiments, a microfluidic device includes fluid flow channels (or
30 lines) and separate control channels (or lines) that function to control or regulate flow through the fluid channels.

[0098] The term nucleic acid includes any form of DNA or RNA, including, for example, genomic DNA; complementary DNA (cDNA), which is a DNA representation of mRNA, usually obtained by reverse transcription of messenger RNA (mRNA) or by amplification; DNA molecules produced synthetically or by amplification; and mRNA.

5 [0099] The term nucleic acid encompasses double- or triple-stranded nucleic acids, as well as single-stranded molecules. In double- or triple-stranded nucleic acids, the nucleic acid strands need not be coextensive (i.e., a double-stranded nucleic acid need not be double-stranded along the entire length of both strands).

[0100] The term nucleic acid also encompasses any chemical modification thereof,
10 such as by methylation and/or by capping. Nucleic acid modifications can include addition of chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and/or functionality to the individual nucleic acid bases or to the nucleic acid as a whole. Such modifications may include base modifications such as 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine
15 modifications, modifications at cytosine exocyclic amines, substitutions of 5-bromo-uracil, backbone modifications, unusual base pairing combinations such as the isobases isocytidine and isoguanidine, and the like.

[0101] More particularly, in certain embodiments, nucleic acids, can include polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing
20 D-ribose), and any other type of nucleic acid that is an N- or C-glycoside of a purine or pyrimidine base, as well as other polymers containing nonnucleotidic backbones, for example, polyamide (e.g., peptide nucleic acids (PNAs)) and polymorpholino (commercially available from the Anti-Virals, Inc., Corvallis, Oregon, as Neugene) polymers, and other synthetic sequence-specific nucleic acid polymers providing that the
25 polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. The term nucleic acid also encompasses linked nucleic acids (LNAs), which are described in U.S. Patent Nos. 6,794,499, 6,670,461, 6,262,490, and 6,770,748, which are incorporated herein by reference in their entirety for their disclosure of LNAs.

30 [0102] The nucleic acid(s) can be derived from a completely chemical synthesis process, such as a solid phase-mediated chemical synthesis, from a biological source, such as through isolation from any species that produces nucleic acid, or from processes that

involve the manipulation of nucleic acids by molecular biology tools, such as DNA replication, amplification (e.g., PCR), reverse transcription, or from a combination of those processes.

5 [0103] The term “template” is used herein to refer to a nucleic acid molecule that serves as a template for a polymerase to synthesize a complementary nucleic acid molecule.

[0104] A “DNA template,” for example, can be used in DNA sequencing, typically after adding one or more nucleotide sequences that facilitate DNA sequencing (e.g., barcodes, unique molecular identifiers, binding sites for DNA sequencing primers, and/or
10 sequencing adaptors, such as, e.g., a flow cell sequence useful in cluster generation in bridge sequencing).

[0105] As used herein the terms “nucleotide barcode” and “barcode” refer to a specific nucleotide sequence that encodes information about cDNA produced when a barcoded primer or oligonucleotide is employed in reverse transcription or the amplicon
15 produced when one or more barcoded primer(s) is/are employed in an amplification reaction.

[0106] In some embodiments, the barcode encodes an item of capture site information. For example, for reactions carried out on a matrix-type microfluidic device, a barcode can encode the row or column of a capture site. Two barcodes, one encoding
20 the row in which the barcode is introduced and the other encoding the column in which that barcode is introduced can define the specific capture site residing at the intersection of the row and column identified by the barcodes.

[0107] As used herein, “UMI” is an acronym for “unique molecular identifier,” also referred to as “molecular identifier.” A UMI is one in a group of identifiers in which
25 each identifier is distinguishable from any of the other identifiers in the group. One way to achieve this “uniqueness” is to use a string of nucleotides. For example, if the length of this string is 10 bases, there are more than 1 million unique sequences; if it is 20 bases long, there will be 10^{12} unique sequences. See Hug and Schuler, “Measurement of the Number of Molecules of a Single mRNA Species in a Complex mRNA Preparation,” J.
30 Theor. Biol. (2003) 221, 615–624 and Hollas and Schuler, “A Stochastic Approach to Count RNA Molecules Using DNA Sequencing Methods” in Algorithms in Bioinformatics (2003): Third International Workshop, WABI 2003, Budapest, Hungary,

September 15-20, 2003, Series title: Lecture Notes in Computer Science Volume 2812, pp 55-62 (eds. Benson and Page), which is incorporated by reference herein for its description of the UMI concept.

[0108] The term “target nucleic acids” or “target RNA” is used herein to refer to particular nucleic acids to be detected in the methods described herein. Accordingly, amplification of a particular RNA transcript, for example, is an example of target-specific amplification, whereas “Whole Transcriptome Amplification” is an example of an amplification that aims to amplify all transcripts present in a given cell. When the amplification products are sequenced, the sequencing of a particular transcript is termed “target-specific” or “targeted” sequencing, whereas “transcriptome sequencing” aims to sequence all transcripts present in a cell.

[0109] As used herein, the term “target nucleotide sequence” refers to a molecule that includes the nucleotide sequence of a target nucleic acid, such as, for example, the amplification product obtained by amplifying a target nucleic acid or the cDNA produced upon reverse transcription of an RNA target nucleic acid.

[0110] As used herein, the term “complementary” refers to the capacity for precise pairing between two nucleotides. I.e, if a nucleotide at a given position of a nucleic acid is capable of hydrogen bonding with a nucleotide of another nucleic acid, then the two nucleic acids are considered to be complementary to one another at that position.

Complementarity between two single-stranded nucleic acid molecules may be “partial,” in which only some of the nucleotides bind, or it may be complete when total complementarity exists between the single-stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. A first nucleotide sequence is said to be the “complement” of a second sequence if the first nucleotide sequence is complementary to the second nucleotide sequence. A first nucleotide sequence is said to be the “reverse complement” of a second sequence, if the first nucleotide sequence is complementary to a sequence that is the reverse (i.e., the order of the nucleotides is reversed) of the second sequence.

[0111] “Specific hybridization” refers to the binding of a nucleic acid to a target nucleotide sequence in the absence of substantial binding to other nucleotide sequences present in the hybridization mixture under defined stringency conditions. Those of skill in

the art recognize that relaxing the stringency of the hybridization conditions allows sequence mismatches to be tolerated.

[0112] In particular embodiments, hybridizations are carried out under stringent hybridization conditions. The phrase “stringent hybridization conditions” generally refers to a temperature in a range from about 5°C to about 20°C or 25°C below than the melting temperature (T_m) for a specific sequence at a defined ionic strength and pH. As used herein, the T_m is the temperature at which a population of double-stranded nucleic acid molecules becomes half-dissociated into single strands. Methods for calculating the T_m of nucleic acids are well known in the art (see, e.g., Berger and Kimmel (1987) METHODS IN ENZYMOLOGY, VOL.152: GUIDE TO MOLECULAR CLONING TECHNIQUES, San Diego: Academic Press, Inc. and Sambrook et al. (1989) MOLECULAR CLONING: A LABORATORY MANUAL, 2ND ED., VOLS. 1-3, Cold Spring Harbor Laboratory), both incorporated herein by reference). As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G+C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (see, e.g., Anderson and Young, Quantitative Filter Hybridization in NUCLEIC ACID HYBRIDIZATION (1985)). The melting temperature of a hybrid (and thus the conditions for stringent hybridization) is affected by various factors such as the length and nature (DNA, RNA, base composition) of the primer or probe and nature of the target nucleic acid (DNA, RNA, base composition, present in solution or immobilized, and the like), as well as the concentration of salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol). The effects of these factors are well known and are discussed in standard references in the art. Illustrative stringent conditions suitable for achieving specific hybridization of most sequences are: a temperature of at least about 60°C and a salt concentration of about 0.2 molar at pH7.

[0113] The term “oligonucleotide” is used to refer to a nucleic acid that is relatively short, generally shorter than 200 nucleotides, more particularly, shorter than 100 nucleotides, even more particularly, shorter than 50 nucleotides, and most particularly shorter than 40, 30, 20, 15, 10, or 5 nucleotides. Typically, oligonucleotides are single-stranded DNA molecules.

[0114] A stretch of nucleotides of the same type are referred to, e.g., as “oligo-riboG” (for a stretch of repeated guanines) or “poly-A” (for a stretch of repeated adenines).

[0115] The term “primer” refers to an oligonucleotide that is capable of hybridizing (also termed “annealing”) with a nucleic acid and serving as an initiation site for nucleotide (RNA or DNA) polymerization under appropriate conditions (i.e., in the presence of four different nucleoside triphosphates and an agent for polymerization, such as DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The term “primer site” or “primer binding site” refers to the segment of the target nucleic acid to which a primer hybridizes.

[0116] A primer is said to “anneal” to another nucleic acid if the primer, or a portion thereof, hybridizes to a nucleotide sequence within the nucleic acid. The statement that a primer hybridizes to a particular nucleotide sequence is not intended to imply that the primer hybridizes either completely or exclusively to that nucleotide sequence. For example, a portion of a primer may anneal to a particular nucleic acid, in which case, that portion is said to be “specific for” that nucleic acid.

[0117] The primer can be perfectly complementary to the target nucleic acid sequence or can be less than perfectly complementary. In certain embodiments, the primer has at least 65% identity to the complement of the target nucleic acid sequence over a sequence of at least 7 nucleotides, more typically over a sequence in the range of 10-30 nucleotides, and often over a sequence of at least 14-25 nucleotides, and more often has at least 75% identity, at least 85% identity, at least 90% identity, or at least 95%, 96%, 97%, 98%, or 99% identity. It will be understood that certain bases (e.g., the 3' base of a primer) are generally desirably perfectly complementary to corresponding bases of the target nucleic acid sequence. Primer and probes typically anneal to the target sequence under stringent hybridization conditions.

[0118] The term “nucleotide tag” is used herein to refer to a predetermined nucleotide sequence that is added to another nucleotide sequence, typically during reverse transcription or amplification. Nucleotide tags are conveniently added using a primer having a non-hybridizing portion (i.e., the “tag”). Nucleotide tags can include, e.g., primer binding sites for sequencing primers, barcodes, UMIs, sequencing adaptors, etc.

[0119] As used herein the term “barcode primer” refers to a primer that includes a specific barcode nucleotide sequence that encodes information about the amplicon produced when the barcode primer is employed in an amplification reaction.

[0120] A “linker” can, but need not, be or include a nucleic acid. Nucleotide linkers can be added to either end of a nucleotide sequence to be amplified to facilitate unbiased amplification using primers specific for the nucleotide linkers, which can be the same or different.

5 [0121] As used herein with reference to a portion of a primer, the term “target-specific” nucleotide sequence refers to a sequence that can specifically anneal to a target nucleic acid or a target nucleotide sequence under suitable annealing conditions.

[0122] As used herein, an “anchor sequence” refers to a sequence in an oligonucleotide (e.g., a primer) that keeps the oligonucleotide stably bound to a
10 complementary nucleotide sequence.

[0123] As used herein, a “bait sequence” refers to a sequence in an oligonucleotide (e.g., a primer) that binds only transiently to a complementary nucleotide sequence. When used in conjunction with an anchor sequence, the bait sequence will form a less stable hybrid than the anchor sequence.

15 [0124] As used in conjunction with anchor and bait sequences, an “insert” refers to a non-hybridizing element residing between the anchor and bait sequences, the insert is typically a nucleotide sequence, but is not limited to such.

[0125] The term “flanked by” is used herein to describe a situation in which a central nucleotide sequence has other nucleotide sequences on either side.

20 [0126] As used herein, a “stem-duplex” is formed when two self-complementary regions of nucleic acid molecule (typically an oligonucleotide) hybridize with one another.

[0127] The two self-complementary regions of a stem-duplex are linked, typically by a nucleotide loop, to form a “hairpin” configuration at temperatures below the T_m of the stem-duplex.

25 [0128] A “hairpin primer” is an oligonucleotide that is capable of priming nucleotide polymerization (e.g., amplification) and that, at lower temperatures, assumes a hairpin configuration.

[0129] A primer is said to have “a hairpin configuration during reverse transcription” if it has a stem-duplex with a T_m higher than the temperature used for
30 reverse transcription (e.g., 37-42 °C). In this configuration, the primer cannot function to prime nucleotide polymerization. In effect, the primer is “turned off.”

[0130] A primer is said to have “a linear configuration” during amplification such that it can anneal to a sufficiently complementary sequence and prime nucleotide polymerization. In effect, the primer is “turned on.” When amplification is carried out by polymerase chain reaction (PCR), the primer assumes a linear configuration on
 5 denaturation, which is maintained during annealing (e.g., at 50-65°C) and extension.

[0131] Amplification according to the present teachings encompasses any means by which at least a part of at least one target nucleic acid is reproduced, typically in a template-dependent manner, including without limitation, a broad range of techniques for amplifying nucleic acid sequences, either linearly or exponentially. Illustrative means for
 10 performing an amplifying step include ligase chain reaction (LCR), ligase detection reaction (LDR), ligation followed by Q-replicase amplification, PCR, primer extension, strand displacement amplification (SDA), hyperbranched strand displacement amplification, multiple displacement amplification (MDA), nucleic acid strand-based amplification (NASBA), two-step multiplexed amplifications, rolling circle amplification
 15 (RCA), and the like, including multiplex versions and combinations thereof, for example but not limited to, OLA/PCR, PCR/OLA, LDR/PCR, PCR/PCR/LDR, PCR/LDR, LCR/PCR, PCR/LCR (also known as combined chain reaction--CCR), and the like.

Descriptions of such techniques can be found in, among other sources, Ausbel et al.; PCR Primer: A Laboratory Manual, Diffenbach, Ed., Cold Spring Harbor Press (1995); The
 20 Electronic Protocol Book, Chang Bioscience (2002); Msuih et al., J. Clin. Micro. 34:501-07 (1996); The Nucleic Acid Protocols Handbook, R. Rapley, ed., Humana Press, Totowa, N.J. (2002); Abramson et al., Curr Opin Biotechnol. 1993 Feb.;4(1):41-7, U.S. Pat. No. 6,027,998; U.S. Pat. No. 6,605,451, Barany et al., PCT Publication No. WO 97/31256; Wenz et al., PCT Publication No. WO 01/92579; Day et al., Genomics, 29(1): 152-162
 25 (1995), Ehrlich et al., Science 252:1643-50 (1991); Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press (1990); Favis et al., Nature Biotechnology 18:561-64 (2000); and Rabenau et al., Infection 28:97-102 (2000); Belgrader, Barany, and Lubin, Development of a Multiplex Ligation Detection Reaction DNA Typing Assay, Sixth International Symposium on Human Identification, 1995 (available on the world
 30 wide web at: promega.com/geneticidproc/ussymp6proc/blegrad.html); LCR Kit Instruction Manual, Cat. #200520, Rev. #050002, Stratagene, 2002; Barany, Proc. Natl. Acad. Sci. USA 88:188-93 (1991); Bi and Sambrook, Nucl. Acids Res. 25:2924-2951 (1997); Zirvi et al., Nucl. Acid Res. 27:e40i-viii (1999); Dean et al., Proc Natl Acad Sci

USA 99:5261-66 (2002); Barany and Gelfand, Gene 109:1-11 (1991); Walker et al., Nucl. Acid Res. 20:1691-96 (1992); Polstra et al., BMC Inf. Dis. 2:18- (2002); Lage et al., Genome Res. 2003 Feb.;13(2):294-307, and Landegren et al., Science 241:1077-80 (1988), Demidov, V., Expert Rev Mol Diagn. 2002 Nov.;2(6):542-8., Cook et al., J Microbiol Methods. 2003 May;53(2):165-74, Schweitzer et al., Curr Opin Biotechnol. 2001 Feb.;12(1):21-7, U.S. Pat. No. 5,830,711, U.S. Pat. No. 6,027,889, U.S. Pat. No. 5,686,243, PCT Publication No. WO0056927A3, and PCT Publication No. WO9803673A1.

[0132] In some embodiments, amplification comprises at least one cycle of the sequential procedures of: annealing at least one primer with complementary or substantially complementary sequences in at least one target nucleic acid; synthesizing at least one strand of nucleotides in a template-dependent manner using a polymerase; and denaturing the newly-formed nucleic acid duplex to separate the strands. The cycle may or may not be repeated. Amplification can comprise thermocycling or can be performed isothermally.

[0133] “Whole transcriptome amplification” (“WTA”) refers to any amplification method that aims to produce an amplification product that is representative of a population of RNA from the cell from which it was prepared. An illustrative WTA method entails production of cDNA bearing linkers on either end that facilitate unbiased amplification. In many implementations, WTA is carried out to analyze messenger (poly-A) RNA.

[0134] The term “substantially” as used herein with reference to a parameter means that the parameter is sufficient to provide a useful result. Thus, “substantially complementary,” as applied to nucleic acid sequences generally means sufficiently complementary to work in the described context. Typically, substantially complementary means sufficiently complementary to hybridize under the conditions employed.

[0135] A “reagent” refers broadly to any agent used in a reaction, other than an analyte (e.g., nucleic acid being analyzed). Illustrative reagents for a nucleic acid amplification reaction include, but are not limited to, buffer, metal ions, polymerase, reverse transcriptase, primers, nucleotides, oligonucleotides, labels, dyes, nucleases, and the like. Reagents for enzyme reactions include, for example, substrates, cofactors, buffer, metal ions, inhibitors, and activators. The term reagent also encompasses any component

that influences cell growth or behavior, such as, e.g., buffer, culture medium or components thereof, agonists or antagonists, etc.

[0136] The term “label,” as used herein, refers to any atom or molecule that can be used to provide a detectable and/or quantifiable signal. In particular, the label can be attached, directly or indirectly, to a nucleic acid or protein. Suitable labels that can be attached to probes include, but are not limited to, radioisotopes, fluorophores, chromophores, mass labels, electron dense particles, magnetic particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, and enzyme substrates.

[0137] As used herein with respect to reactions, reaction mixtures, reaction volumes, etc., the term “separate” refers to reactions, reaction mixtures, reaction volumes, etc., where reactions are carried out in isolation from other reactions. Separate reactions, reaction mixtures, reaction volumes, etc. include those carried out in droplets (See, e.g., U.S. Patent No., 7,294,503, issued November 13, 2007 to Quake et al., entitled “Microfabricated crossflow devices and methods,” which is incorporated herein by reference in its entirety and specifically for its description of devices and methods for forming and analyzing droplets; U.S. Patent Publication No. 20100022414, published January 28, 2010, by Link et al., entitled “Droplet libraries,” which is incorporated herein by reference in its entirety and specifically for its description of devices and methods for forming and analyzing droplets; and U.S. Patent Publication No. 20110000560, published January 6, 2011, by Miller et al., entitled “Manipulation of Microfluidic Droplets,” which is incorporated herein by reference in its entirety and specifically for its description of devices and methods for forming and analyzing droplets.), which may, but need not, be in an emulsion, as well as those wherein reactions, reaction mixtures, reaction volumes, etc. are separated by mechanical barriers, e.g., separate vessels, separate wells of a microtiter plate, or separate compartments of a matrix-type microfluidic device.

[0138] The term “fluidically isolated” is used herein to refer to state in which two or more elements of a microfluidic device are not in fluid communication with one another.

[0139] The term “elastomer” has the general meaning used in the art. Thus, for example, Allcock et al. (Contemporary Polymer Chemistry, 2nd Ed.) describes elastomers in general as polymers existing at a temperature between their glass transition temperature

and liquefaction temperature. Elastomeric materials exhibit elastic properties because the polymer chains readily undergo torsional motion to permit uncoiling of the backbone chains in response to a force, with the backbone chains recoiling to assume the prior shape in the absence of the force. In general, elastomers deform when force is applied, but then
 5 return to their original shape when the force is removed.

[0140] As used herein, “fixative” is compound or mixture of compounds that stabilizes cells, cellular structures, and/or macromolecules (e.g., RNA) for subsequent analysis.

[0141] When used with reference to a T cell or B cell, the term “activated” refers
 10 to the state of the cell after exposure to an antigen that stimulates an immune response in the cell.

Single-Cell Transcript Sequencing Using Fixed Cells – In General

[0142] The methods described herein can be employed to prepare DNA templates for sequencing from single, fixed cells. The templates can be prepared from any type of
 15 RNA, e.g., from micro-RNA to messenger RNA. Templates can be prepared from specific RNA targets in a single cell or the entire complement of a type of RNA, e.g., all messenger RNA as in whole transcriptome sequencing. While the present disclosure focuses on the preparation of DNA templates for determining RNA sequences, those of skill in the art understand that RNA expression levels can be determined in the course of
 20 sequencing because expression levels are correlated with the number of sequencing reads obtained. In addition, as those of skill in the art will readily appreciate from the discussion below, these methods for generating and sequencing DNA templates can be coupled with other types of analyses of nucleic acids, proteins, or other aspects of single cells. Because multiple types of analyses can be carried out in the separate reaction volumes employed in
 25 these methods, multiple characteristics of individual cells can be identified, facilitating the linkage of particular RNA sequences, optionally at particular levels, with phenotype. An example of this type of approach is described in Darmanis S, Gallant CJ, Marinescu VD, Niklasson M, Segerman A, Flamourakis G, Fredriksson S, Assarsson E, Lundberg M, Nelander S, et al: Simultaneous Multiplexed Measurement of RNA and Proteins in Single
 30 Cells. Cell Rep 2016, 14:380-389, which is incorporated by reference herein for this description.

Cells Analyzed

[0143] The methods described herein can be used to analyze transcripts from any type of cells, e.g., any self-replicating, membrane-bounded biological entity or any non-replicating, membrane-bounded descendant thereof. Non-replicating descendants may be
 5 senescent cells, terminally differentiated cells, cell chimeras, serum-starved cells, infected cells, non-replicating mutants, anucleate cells, intact nuclei, and fixed, intact (dead) cells, etc. Cells used in the methods described herein may have any origin, genetic background, state of health, state of fixation, membrane permeability, pretreatment, and/or population purity, among other characteristics. Suitable cells may be eukaryotic, prokaryotic,
 10 archaeon, etc., and may be from animals, plants, fungi, protists, bacteria, and/or the like. In illustrative embodiments, human cells are analyzed. Cells may be from any stage of organismal development, e.g., in the case of mammalian cells (e.g., human cells), embryonic, fetal, or adult cells may be analyzed. In certain embodiments, the cells are stem cells. Cells may be wildtype; natural, chemical or viral mutants; engineered mutants
 15 (such as transgenics); and/or the like. In addition, cells may be growing, quiescent, senescent, transformed, and/or immortalized, among other states. Furthermore, cells may be a monoculture, generally derived as a clonal population from a single cell or a small set of very similar cells; may be preselected by any suitable mechanism, such as affinity binding, FACS, drug selection, etc.; and/or may be a mixed or heterogeneous population
 20 of distinct cell types.

[0144] In particular embodiments, the cells are preselected populations of T or B cells. Both (along with natural killer T or NKT cells and monocytes) are peripheral blood mononuclear cells (PMBCs), which can be isolated using standard methods. PMBCs can be enriched for desired populations also using standard methods. For example, T helper
 25 (T_H) cells can be enriched by selecting for CD4⁺ cells; cytotoxic T cells (T_C cells or CTLs) can be enriched by selecting for CD8⁺ cells; memory T cells can be enriched by selecting for CD45RO⁺ cells; etc.

[0145] One advantage of the methods described herein is that they can be used to analyze virtually any number of single cells, e.g., in a particular cell population. In
 30 various embodiments, the number of single cells analyzed can, e.g., be at least any of the following values: 10, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7,000, 8000, 9,000, or 10,000. When the cells to be analyzed are scarce, the methods described herein offer the advantage over cell sorting

that fewer cells are needed for the analysis. In various embodiments, the number of single cells analyzed can be fewer than any of the following values: 10,000, 9,000, 8,000, 7000, 6,000, 5,000, 4,000, 3,000, 2,000, or 1,000. In specific embodiments, the number of cells analyzed can fall within a range bounded by any two values listed above, such as, for
 5 example, 300-1,300, 400-1,200, 500-1,100, 600-1,000, or 700-900. An illustrative embodiment aims to analyze 800 cells.

Fixing Cells

[0146] Treatment of the cells with a cell-permeant fixative might be expected to hamper subsequent reactions, such as reverse transcription and/or amplification.

10 Surprisingly, based on the work described herein, it has now been found that fixing cells can improve the quality of the results observed upon sequencing DNA templates produced by reverse transcription, followed by amplification.

[0147] Accordingly, the methods described herein enable the production of DNA templates from transcripts derived from single cells with greater efficiency than was
 15 previously possible (e.g., without the fixative). In various embodiments, these methods enable sequencing and/or quantification of the level of any transcript of interest from more than: 50%, 60%, 70%, 80%, 85%, 90%, 95% of cells that have been isolated in separate reaction volumes, wherein the number of isolated cells ranges, e.g., from 90-10,000, 100-5,000, 200-1,500, 300-1,300, 400-1,200, 500-1,100, 600-1,000, or 700-900. For example,
 20 using the methods described herein, one can obtain the sequences (and optionally expression levels) of both TCR or Ig chains, and optionally one or more phenotypic markers, in more than 85%, more than 90%, or more than 95% of of 200-1,500, isolated cells using a simpler protocol than any previously available. In certain embodiments, suitable fixatives provide such results by stabilizing the cell nucleus, so that cell lysis is
 25 less likely to disrupt the nucleus (which increases the percentage of RNA in the released nucleic acids) and/or by stabilizing RNA.

[0148] In illustrative embodiments, the fixative is biomarker and histology preservative (BHP) and/or dithiobis(succinimydal proprionate) (DSP). BHP is described in Mueller et al., "One-Step Preservation of Phosphoproteins and Tissue Morphology at
 30 Room Temperature for Diagnostic and Research Specimens (August, 2011) PLoS ONE, 6(8) e23780, and in Espina et al., US 2013/0137094, published May 30, 2013, both of

which are incorporated by reference for this description. Table 1 of Mueller et al. provides the following regarding BHP composition:

Ingredient	Function
Kinase inhibitors	Inhibition of kinase activity
Phosphatase inhibitors	Inhibition of phosphatase activity
Permeation enhancers	Increased penetration rate into tissue/cells
Reversible crosslinkers	Easily cleavable cross-linkage of proteins beyond the lifetime of inhibitors
Water based buffer solution	Maintenance of functional osmotic balance and shape during fixation
Precipitating fixative	Inhibition of enzymatic activity
Carboxylic acid	Maintenance of tissue morphology

US 2013/0137094 describes the production of an illustrative BHP as follows:

- 5
1. Protocol for making the blood cell fixative without lactic acid:
Prepare stock solutions of 10% polyethylene glycol (MW 8000) (Fisher),
100 mM sodium orthovanadate (Sigma), and 1.0M Beta Glycerophosphate
(Calbiochem) in type 1 reagent grade water. Prepare a stock solution of
200 mM Genistein (Alexis Biochemicals) in DMSO.
- 10
- Dissolve 0.05 mg DSP (Pierce) in 500 μ L DMSO. Dissolve 0.05 mg
DTBP in 500 μ L Type 1 reagent grade water.
Add 6.0 mL of 200 proof ethanol (Sigma) to 38 mL of Hanks Balanced
Salt Solution (Hyclone, Fisher).
Add 250 μ L 10% polyethylene glycol, 1000 μ L sodium orthovanadate
- 15
- (Sigma), 3.75 mL Beta Glycerophosphate (Calbiochem), 50.0 μ L of 1.0
mM Staurosporine ready-to-use in DMSO (Sigma cat #S6942) and 2.5 μ L
Genistein (Alexis Biochemicals), 500 μ L DSP solution, and 500 μ L DTBP
solution to the alcohol/Hanks Balanced Salt Solution. Mix gently.

[0149] Cells can be fixed with BHP by adding the BHP to the cells at room
20 temperature.

[0150] The fixative DSP is described in Xiang, et al., “Using DSP, a reversible
cross-linker, to fix tissue sections for immunostaining, microdissection and expression
profiling” (December 16, 2004) Nucleic Acids Res., 32(22) e185. DSP, which is also
known as Lomant’s reagent, is a cell permeant, homobifunctional, thiol-cleavable
25 molecule, which is believed to link primary amino groups to one another in aqueous
buffers at pHs between 6.5 and 8.5. Cells can be fixed, for example, as described in
Xiang, et al. Briefly, fibroblast cells are grown to about 85% confluency, washed once

with 1X PBS buffer and then fixed with DSP (Pierce, Rockford, IL) at a final concentration of 1 mg/ml for 5 min. Stock solutions of DSP in 100% anhydrous DMSO (Sigma-Aldrich, St Louis, MO) are prepared and stored at -80°C. The stock solution is diluted to working concentration with 1X PBS immediately before use. To prevent the DSP from precipitating when the DMSO stock is added to the PBS, the latter is vortexed gently and the stock solution added dropwise.

Distributing Cells into Separate Reaction Volumes

[0151] The separate reaction volumes can be created and maintained by any available means. For example, separate reaction volumes can be made up of separate wells in a plate, separate droplets in an emulsion, separate segments of fluid in a channel, or separate reaction sites (e.g., chambers) in a microfluidic device.

[0152] At least four standard methods/systems are available for isolating cells for single-cell sequencing: micromanipulation, laser-capture microdissection (LCM), fluorescence-activated cell sorting (FACS), and microfluidic systems. The last two approaches can provide the high throughput needed for the large sample numbers required to reliably reflect a cell population's heterogeneity. See Livak, "Eukaryotic Single-Cell mRNA Sequencing," Field Guidelines for Genetic Experimental Designs in High-Throughput Sequencing, Springer, ISBN 978-3-319-31348-1 (2016), which is incorporated by reference herein for its description of single-cell mRNA sequencing.

[0153] In some embodiments, the distributing step is carried so as to increase the number of separate reaction volumes that contain only one cell. Methods for achieving this are well known and include, for example, limiting dilution. In particular embodiments, the method entails determining which reaction volumes contain a single, isolated cell.

[0154] Conveniently, the method can be carried out using a microfluidic device that facilitates capture of individual cells at a plurality of capture sites. In some embodiments, the capture sites are capable of being fluidically isolated from one another, for example, after cell distribution throughout the device. In certain embodiments, the capture sites each have a capture feature that retains the cell or group of cells in the place. In some embodiments, the capture feature resides within a chamber that can be fluidically isolated from other chambers within the capture site. The device can be configured so that one or more first reagent(s) can be provided to each capture site, and one or more second

reagent(s) can be provided to each capture site, wherein the second reagent(s) is/are different from the first reagent(s) and is/are provided separately from the first reagent(s). Each pair of reagents can, for example, be provided to a pair of fluidically isolatable chambers in the capture site that are distinct from one another and, optionally, distinct
 5 from the chamber used for cell capture.

[0155] In some embodiments, at least one surface of the microfluidic device is transparent to permit visualization of the cell and/or a signal from a label. In such embodiments, the method can optionally include imaging the cell-occupied capture sites before reverse transcription.

10 Matrix-Type Microfluidic Devices

[0156] In some embodiments, methods described herein can be carried out on a matrix-type microfluidic device, which facilitates the introduction of a barcode that identifies a particular row in the device and a barcode that identifies a particular column, whereby the combination uniquely identifies a particular capture site and therefore a
 15 particular cell or group of cells from which the reaction products were derived. The method has been tested on such a device and demonstrated to work (see Example 3).

[0157] In certain embodiments, a matrix-type microfluidic device useful in the method described above includes capture sites arranged in a matrix of R rows and C columns, wherein R and C are integers greater than 1. Each capture site can include a
 20 capture feature that is capable of capturing just one cell or, where cells are to be analyzed in groups, not more than the desired number of cells for each group of cells. The capture sites can be fluidically isolated from one another after distribution of cells to the capture sites. The device also includes a set of R first input lines configured to deliver the first reagent(s) to capture sites in a particular row, and a set of C second input lines configured
 25 to deliver second reagent(s) to capture sites in a particular column, wherein this delivery is separate from the delivery first reagent(s). An illustrative device of this type is shown schematically in Figure 1A. Figure 1B illustrates the delivery of R different barcodes through the R different first input lines to the capture sites. Figure 1C illustrates the delivery of C different barcodes through C different input lines to the capture sites. In
 30 particular embodiments, all barcodes will be unique, i.e., different from every other barcode provided to the device. Figure 1D illustrates that, after the reaction has been carried out, reaction products can be harvested from each column as a pool, for example,

by applying a harvesting fluid to the C second input lines to push the reaction products out of outlets at one end of the input lines. (Those of skill in the art readily appreciate that the designation of “rows” and “columns” is arbitrary, since, if one rotates the device 90 degrees, the rows become columns and vice versa, in which case, reaction products would be harvested from each row as a pool.) Figure 2 shows a photograph of the device shown schematically in Figure 1.

[0158] In certain embodiments, the matrix-type microfluidic device permits analysis of individual cells or groups of cells, e.g., up to (and including) 1000. The cells can be intact or partially or fully disrupted (e.g., permeabilized or lysed) after capture or isolation of one or more cells at each capture site. In the latter case, the device is configured to provide this functionality. In some embodiments, the device is transparent on at least one surface to permit imaging to visualize cell number or phenotype (e.g., where the cells or their contents have been reacted with an optically detectable label). In some embodiments, the device is configured to perform “X-Y” combinatorial barcoding, whereby reaction products may be exported in one or more pools (which may themselves be pooled) and further analyzed in multiplex (e.g., by amplification), followed by “de-multiplexing” (“demux”) to assign particular reaction products to particular capture sites. This type of barcoding is illustrated in Figure 1, which shows the same set of 3’ barcodes (“3’BC” in Figure 1B) being delivered to each column and the same set of 5’ barcodes (“5’BC” in Figure 1C) being delivered to each row.

[0159] In various embodiments, a microfluidic device having from about 90 to about 10,000 separate capture sites is employed to carry out one or more of the methods described herein, particularly from about 90 to about 5,000 capture sites, more particularly from about 90 to about 2,500 capture sites, and even more particularly from about 90 to about 1,000 capture sites. In some embodiments the microfluidic device can have greater than 100, greater than 200, greater than 300, greater than 400, greater than 500, greater than 600, greater than 700, greater than 800, greater than 900, or greater than 1000 capture sites. In illustrative embodiments, the microfluidic device has a number of capture sites in the range of 300-1300, 400-1200, 500-1100, 600-1000, 700-900, or 800.

[0160] In some embodiments, the capture sites have one or more reaction chambers ranging from about 0.2 nL to about 500 nL. The lower the reaction chamber volume, the higher the effective concentration of any target nucleic acid. In certain embodiments, the reaction chamber is from about 0.2 nL to about 50 nL, preferably 0.5 nL

to about 5 nL, more preferably from about 1 nL to about 4 nL. In some embodiments, the reaction chamber volume is 1.5 nL, 2.0 nL, 2.5 nL, 3.0 nL, or 3.5 nL, or falls within any range bounded by any of these values.

[0161] Microfluidic devices meeting the specifications described herein, and

5 systems employing them the carry out the disclosed method can be designed and fabricated based on the guidance herein and in prior co-owned patent publications, such as U.S. Patent Publication No. 2013/0323732, published May 12, 2013, Anderson et al. and U.S. Application No. 15/055,252, filed February 26, 2016, Conant et al. (both of which are incorporated by reference for their descriptions of single-cell analysis methods and

10 systems). For example, the C₁TM Single-Cell Auto Prep System available from Fluidigm Corporation (South San Francisco, CA) provides bench-top automation of the multiplexed isolation, lysis, and reactions on nucleic acids from single cells in an IFCTM. In particular, the C₁ Single-Cell Auto Prep Array IFC is a matrix-type microfluidic device that facilitates capture and highly parallel preparation of products from up to 96 individual

15 cells. Fluidigm also sells a high-throughput version of this type of device, called the C₁ mRNA Seq HT Array IFC, which facilitates capture and preparation of products from up to 800 individual cells. When used properly, each capture site within the chip captures one single cell. Sometimes, a site may capture zero, two, or more cells; however, the exact number of captured cells in each captured site of a C₁ chip is easily verified at high

20 confidence and easily documented in a microscopic picture. In certain embodiments, cells are captured and barcoding is carried out in each separate reaction volume to produce barcoded nucleic acid molecules, which are analyzed, most conveniently by DNA sequencing, be it Sanger sequencing, next-generation sequencing, or third-generation sequencing, optionally after preamplification.

25 **[0162]** The C₁ architecture enables the processing of discretely captured cells in combination with any multistep biochemical process that facilitates the analysis of intracellular macromolecules. Such process include, in addition to preparing templates for DNA sequencing, multiplexed protein proximity ligation assays or multiplexed protein proximity elongation assays to quantitate specific proteins, multiplexed microRNA

30 preamplification, target-specific amplification of RNA transcripts or DNA sequences (e.g., for the purpose of genotyping polymorphic markers, such as SNPs, or otherwise analyzing genetic variations, such as copy number variations), for example, or any combination thereof. This architecture can also be exploited to culture discretely captured cells under

any desired conditions, which can be modified on-chip by adding components such as, e.g., agonists or antagonists for particular receptors.

Producing Encoded DNA Templates

[0163] Once the cells are distributed into separate reaction volumes, the individual
 5 cells are permeabilized or disrupted to release RNA. Preferably, the cell are disrupted (e.g., lysed), and any conventional means of achieving this can be used, such as, for example, treatment with proteinase K. An illustrative lysis solution is 0.5% NP-40, 50 mM Tris-HCl, pH 8.4, 1 mM EDTA. This provides a pH that is optimal for reverse transcriptase, and the NP-40 does not inhibit reverse transcriptase. A brief incubation (1–2
 10 minutes) at 65–70 °C is sufficient to lyse many mammalian cells. If harsher lysis is required, this solution can be supplemented with 30 µg/mL proteinase K, and incubation can be carried out at 50 °C for 30 minutes, followed by 70 °C for 1 minute. If proteinase K is included in the lysis solution, the proteinase K inhibitor AAPF (Cat. No. 539470, EMD Millipore) can be included in the reverse transcriptase reaction at a concentration of
 15 0.33 mM. Commercially available lysis solutions include CelluLyser™ (TATAA Biocenter), RealTime ready™ Cell Lysis (Roche Diagnostics), and Single Cell-to-CT™ (Life Technologies).

[0164] One advantage of working with the RNA from a single cell is that the RNA need not be purified. The volume of a single cell is on the order of picoliters. Even at the
 20 nanoliter scale typically used in microfluidics, the cell lysate can be diluted enough that enzymatic reactions can be performed directly in the lysate.

[0165] In certain embodiments, reverse transcription reagents are added to the separate reaction volumes and reverse transcription is carried out. In particular
 25 embodiments, amplification reagents are subsequently added to the separate reaction volumes and amplification is carried out.

[0166] In some embodiments, the reactions carried out in each reaction volume (separately from every other reaction volume) produce DNA templates that encode one or more items of information regarding reaction volume identity and/or cell and/or target (e.g., in targeted transcript sequencing). The DNA templates can be recovered, optionally
 30 pooled, and subjected to DNA sequencing, which determines both the sequence of the DNA template and the item(s) of information encoded therein. In particular embodiments,

this allows the identification of particular DNA templates as having been derived from a single cell in a particular reaction volume.

[0167] One way that this identification can be achieved is by incorporating one or more barcode nucleotide sequences (“barcodes”) into the DNA templates. Barcodes can be of virtually any length, although shorter barcodes (e.g., 4-6 nucleotides in length) may be preferred in some embodiments. In various embodiments, suitable barcodes are 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, or 20 nucleotides in length or can fall within a range bounded by any of these values, e.g., 2-10 or 3-8.

[0168] In some embodiments, each reaction can incorporate at least one unique molecular identifier (“UMI”), which is a nucleotide sequence that uniquely identifies the molecule into which it is incorporated. Kivioja et al. (2012) describe the process of counting the absolute number of molecules using UMIs. In some embodiments, a UMI is a random sequence label (generally N4 to N10) that is attached to a molecule to be sequenced prior to any amplification step. For conventional RNA Seq, quantification is inferred from the total number of reads that map to each particular transcript. With UMIs, quantification is done by counting the number of unique UMIs observed for each particular transcript regardless of how many times each UMI is read. For 5’-end-tagging, the UMI can be incorporated into the template switch oligo. For 3’-end-tagging, the UMI can be incorporated into, e.g., an oligo dT primer. In terms of the number of random nucleotides to use for the UMI, N5 should be adequate for most mammalian cells. For larger cells, which would be expected to have a greater number of total transcripts, the use of N6 or N7 might be advisable.

[0169] In variations of such embodiments, the reaction incorporates one or more barcodes in addition to one or more UMIs (e.g., two barcodes to encode row and column of a matrix-type microfluidic device plus a UMI to encode the molecular identity of the original RNA template). UMIs can be any length, and the length required for a given analysis will increase as the number of unique molecules to be identified increases. In various embodiments, suitable UMIs are 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, or 20 nucleotides in length or can fall within a range bounded by any of these values, e.g., 2-10, 3-8, 4-7, or 5-6.

Single-Cell Sequencing of T-Cell Receptor or Immunoglobulin Transcripts

[0170] In certain embodiments the above-described methods can be carried out to sequence T-cell receptor (TCR) or immunoglobulin (Ig) transcripts in single T or B cells, respectively. In some embodiments, the T or B cells are activated in order to increase the levels of TCR or Ig transcripts available for analysis. T or B cells can be activated using any method known to those of skill in the art. For example, T helper (T_H) cells are activated when presented with peptide antigens by MHC class II molecules, which are expressed on the surface of antigen-presenting cells (APCs). Cytotoxic T cells (T_C cells or CTLs) are activated by binding to antigen associate with MHC class I molecules, which are present on the surface of all nucleated cells. B cells are activated by antigen binding to cell surface receptors.

TCR Transcript Production

[0171] Each T lymphocyte expresses TCR that binds antigen. Antigen binding is a key event in the cascade of the immune response. Most TCRs are a heterodimer consisting of α - and β -chains encoded by the *TRA* and *TRB* genes, respectively. A small percentage of TCRs are a heterodimer consisting of γ - and δ -chains encoded by the *TRG* and *TRD* genes, respectively.

[0172] Diversity in antigen recognition is generated by somatic V(D)J recombination occurring at the *TRA*, *TRB*, *TRG*, and *TRD* loci. For ease of discussion, the methods described below focus on sequencing transcripts from the *TRA* and *TRB* loci, respectively, although these methods are equally applicable to transcripts from the *TRG* and *TRD* loci. At single-cell resolution, determining the sequence of an appropriate portion of *TRA* and *TRB* serves as a unique identifier of a T cell's ancestry because it is likely that any two T cells expressing the same $TCR\alpha\beta$ pair arose from a common T-cell clone. This disclosure describes improved methods for targeted single-cell transcript sequencing to determine T-cell clonal identity. The improved methods are conveniently carried out on microfluidic devices, such as those described herein, e.g., like the C₁ Single-Cell Auto Prep Array™ IFC or the C₁ mRNA Seq HT Array IFC, both sold by Fluidigm.

[0173] *TRA* and *TRB* have a complex structure diagrammed in Figure 3. In any given T cell, V(D)J recombination has joined one of many Variable (V), Joining (J), and Diversity (D) segments to a Constant (C) segment at both *TRA* and *TRB* to create the genes that encode the α - and β -chains expressed by that cell. In this process, non-

templated nucleotides (N) are inserted in a segment denoted CDR3. The sequence of CDR3 is the best marker to use to determine clonal identity. By using primers specific for V and C, the segment containing CDR3 can be amplified and sequenced. Because there are many different V segments, many different TRAV and TRBV primers need to be included in the PCR mix. For any given cell, though, typically only one TRAV and one TRBV primer participate in the PCR.

Preparing DNA Templates from TRCs by Reverse Transcription and Amplification

[0174] The Summary above describes methods for preparing DNA templates for the two types of TCR transcripts from single T cells in general terms. These methods enable the use of just one amplification, which is much simpler than any available protocol for preparing TCR templates from single cells. In addition, these methods separate the reverse transcription step from the amplification step, which provides greater specificity. In some embodiments, specificity is further enhanced by primer design as described below.

[0175] These methods can be implemented in a variety of ways that are within the level of skill in the art. In specific embodiments, the goal of performing single-cell TCR sequencing (scTCRseq) on the C₁ mRNA Seq HT Array IFC is to generate 800 single-cell libraries with appropriate barcodes and sequencing adapters such that the material harvested from the IFC can be pooled, cleaned up with beads, and directly loaded onto an Illumina sequencer. The first step after capturing cells is to use the 20 column inlets of the C₁ to add reverse transcriptase (RT) reagents to the captured cells. Referring to Figure 4A, the RT reagents include two RT primers, a first RT having a portion specific for the Constant segment (CS) of an RNA encoding a first chain of a T-cell receptor, and a second RT primer having a portion specific for the Constant segment (CS) of an RNA encoding the second chain of the T-cell receptor. The first and second RT primers each additionally include a first nucleotide tag comprising a first primer binding site for a first DNA sequencing primer, the first nucleotide tag being 5' of the CS-specific portion.

[0176] The RT reagents include an additional type of primer, termed a "first barcode primer," which includes from 3' to 5', a portion specific for the first primer binding site, a first barcode nucleotide sequence, and a first sequencing adaptor. A different first barcode primer, bearing a different barcode is added in each of the columns, as shown in Figure 4A (so that each barcode uniquely identifies a particular column). The

TRAC and TRBC sequences are complementary to the TCR α and β mRNAs and serve as specific primers during the reverse transcriptase reaction. In the first barcode primer illustrated in Figure 4A, Rd2 is the Illumina TruSeq Read 2 sequence, BC1-20 refers to 20 different barcodes, and P7 is one of the Illumina sequencing adaptors. The P7.BC.Rd2 primer does not participate in the reverse transcription reaction but is there so that barcodes are added to this end of the amplified segments during the amplification step.

[0177] Illustrative sequences designed for the two RT primers (TRAC and TRBC) and the first barcode primers (P7.BC1-20.Rd2, i.e., 20 different first barcode primers) are as follows:

10 AC.A1: gtgactggagttcagacgtgtgctcttccgatctTGAATAGGCAGACAGACTTGTC
(SEQ ID NO:1)

BC.A1: gtgactggagttcagacgtgtgctcttccgatctCACACCAGTGTGGCCTTT
(SEQ ID NO:2)

15 P7.BC.Rd2x: CAAGCAGAAGACGGCATAACGAGAT[barcode seq]GTGACTGGAGTTCAGACGT
(SEQ ID NO:3)

[0178] After adding these RT reagents, the cells are lysed, e.g., by addition of a proteinase K/NP-40 mixture and incubation at elevated temperature (50°C). Then, additional components required for reverse transcription (e.g., buffer, dNTPs, proteinase K inhibitor, reverse transcriptase, and C₁TM Loading Reagent (Fluidigm 100-5170)) are transferred from a common reservoir to the captured cells and reverse transcription is carried out.

25 **[0179]** The next step of this illustrative method is to use the 40 row inlets of the C₁ to add the amplification reagents. Referring to Figure 4B, the amplification reagents include two sets amplification primers, a set of first amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the α chain of the T-cell receptor, and a set of second amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the β chain of the T-cell receptor. The first and second amplification primers each additionally include a second nucleotide tag comprising a second primer binding site for a second DNA sequencing primer, the second nucleotide tag being 5' of the VS-specific portion.

30

[0180] The amplification reagents include an additional type of primer, termed a "second barcode primer," which includes from 3' to 5', a portion specific for the second primer binding site, a second barcode nucleotide sequence, and a second sequencing

35

adaptor. A different second barcode primer, bearing a different barcode is added in each of the rows, as shown in Figure 4B (so that each barcode uniquely identifies a particular row). The TRAV and TRBV sequences are specific for the Variable segments that may be in the TCR α and β mRNAs. Because there are many different Variable segments, multiple TRAV and TRBV primers must be used at each capture site. Rd1 is the Illumina TruSeq Read 1 sequence, BC1-40 refers to 40 different barcodes, and P5 is one of the Illumina sequencing adaptors. Illustrative sequences designed for the two sets of amplification primers (38 TRAV and 31 TRBV) and the second barcode primers (P5.BC1-40.Rd1, i.e., 40 different second barcode primers) are as follows:

10

AV01.A1	acactctttccctacacgacgctcttccgatctAGCACCCACATTT CTKTCTTACAA	(SEQ ID NO:4)
AV02.A1	acactctttccctacacgacgctcttccgatctCAAGACTCCTTGT TAAAGGCTCAA	(SEQ ID NO:5)
AV03.A1	acactctttccctacacgacgctcttccgatctTGAAGCTGAATTT AACAAGAGCCAA	(SEQ ID NO:6)
AV04.A1	acactctttccctacacgacgctcttccgatctCTGTTTATCCCTG CCGACAGAA	(SEQ ID NO:7)
AV05.A1	acactctttccctacacgacgctcttccgatctAACAAGACCAAAG ACTCACTGTTCTA	(SEQ ID NO:8)
AV06.A1	acactctttccctacacgacgctcttccgatctACTGAAGGTCACC TTTGATACCA	(SEQ ID NO:9)
AV07.A1	acactctttccctacacgacgctcttccgatctATTCAGCTGGATA TGAGAAGCAGAA	(SEQ ID NO:10)
AV08.A11	acactctttccctacacgacgctcttccgatctMATCAACGGTTTT GAGGCTGAA	(SEQ ID NO:11)
AV08.A12	acactctttccctacacgacgctcttccgatctGATCCACTGGTTA AAGGCATCAA	(SEQ ID NO:12)
AV08.A13	acactctttccctacacgacgctcttccgatctTGAGGCTGAATTT AAGAGGAGTCAA	(SEQ ID NO:13)
AV09.A1	acactctttccctacacgacgctcttccgatctTACCGTAAGGAAA CCACTTCTTTCCA	(SEQ ID NO:14)
AV10.A1	acactctttccctacacgacgctcttccgatctCGGAAGATATACA GCAACTCTGGAT	(SEQ ID NO:15)
AV12.A1	acactctttccctacacgacgctcttccgatctWGAAGATGGAAGG TTTACAGCACA	(SEQ ID NO:16)
AV13.A11	acactctttccctacacgacgctcttccgatctCGAAAAGAAAGAC CAACGAATTGCT	(SEQ ID NO:17)
AV13.A12	acactctttccctacacgacgctcttccgatctGGCCAAAGAGTCA CCGTTTTTA	(SEQ ID NO:18)
AV14.A1	acactctttccctacacgacgctcttccgatctACAGAAGGTCGCT ACTCATTGAA	(SEQ ID NO:19)
AV16.A1	acactctttccctacacgacgctcttccgatctAGGCTTCACTGCT GACCTTAA	(SEQ ID NO:20)
AV17.A1	acactctttccctacacgacgctcttccgatctAGATTAAGAGTCA CGCTTGACACT	(SEQ ID NO:21)
AV18.A1	acactctttccctacacgacgctcttccgatctGGTTTTTCAGGCCA GTCCTATCAA	(SEQ ID NO:22)
AV19.A1	acactctttccctacacgacgctcttccgatctTCGGTATTCTTGG AACTTCCAGAA	(SEQ ID NO:23)
AV20.A1	acactctttccctacacgacgctcttccgatctGGAAAGCTTTCTG CACATCACA	(SEQ ID NO:24)

AV21.A1	acactctttccctacacgacgctcttccgatctAAGACTTAATGCC TCGCTGGATAA	(SEQ ID NO:25)
AV22.A1	acactctttccctacacgacgctcttccgatctACGACTGTCGCTA CGGAA	(SEQ ID NO:26)
AV23.A1	acactctttccctacacgacgctcttccgatctCTCCTTCAATAAA AGTGCCAAGCA	(SEQ ID NO:27)
AV24.A1	acactctttccctacacgacgctcttccgatctCTTAATACCAAGG AGGGTTACAGCTA	(SEQ ID NO:28)
AV25.A1	acactctttccctacacgacgctcttccgatctCCTGCACATCACA GCCAC	(SEQ ID NO:29)
AV26.A11	acactctttccctacacgacgctcttccgatcAAGTCCAGCACCTT GATCCT	(SEQ ID NO:30)
AV26.A12	acactctttccctacacgacgctcttccgatcCAATGTGAACAACA GAATGGCCT	(SEQ ID NO:31)
AV27.A1	acactctttccctacacgacgctcttccgatcGACAGTAGTTACGG GTGGAGAA	(SEQ ID NO:32)
AV29.A1	acactctttccctacacgacgctcttccgatcCAAAAGTGCCAAGC ACCTCT	(SEQ ID NO:33)
AV30.A1	acactctttccctacacgacgctcttccgatcGCACCCGTCTTCCT GATGATA	(SEQ ID NO:34)
AV34.A1	acactctttccctacacgacgctcttccgatcGCAGCAAAGTTCCC TGCATA	(SEQ ID NO:35)
AV35.A1	acactctttccctacacgacgctcttccgatcTCAAATGGAAGACT GACTGCTCA	(SEQ ID NO:36)
AV36.A1	acactctttccctacacgacgctcttccgatcCATCCTGAACATCA CAGCCAC	(SEQ ID NO:37)
AV38.A1	acactctttccctacacgacgctcttccgatcCGCCAAGAAGCTTA TAAGCAACA	(SEQ ID NO:38)
AV39.A1	acactctttccctacacgacgctcttccgatcTGAAGCAGGAGGGA CGATTAA	(SEQ ID NO:39)
AV40.A1	acactctttccctacacgacgctcttccgatcCAAAAACCTTCGGAG GCGGAA	(SEQ ID NO:40)
AV41.A1	acactctttccctacacgacgctcttccgatcAGCTCCCTGCACAT CACA	(SEQ ID NO:41)
BV02.A1	acactctttccctacacgacgctcttccgatcCTCTGAAGATCCGG TCCACAA	(SEQ ID NO:42)
BV03.A1	acactctttccctacacgacgctcttccgatcCGMTTCTCACCTAA ATCTCCAGACAA	(SEQ ID NO:43)
BV04.A1	acactctttccctacacgacgctcttccgatcCCAAGTCGCTTCTC ACCTGAA	(SEQ ID NO:44)
BV05.A11	acactctttccctacacgacgctcttccgatcTGAGACACAGAGAA ACAAAGGAAACT	(SEQ ID NO:45)
BV05.A12	acactctttccctacacgacgctcttccgatcGAGCTGAATGTGAA CGCCTT	(SEQ ID NO:46)
BV06.A11	acactctttccctacacgacgctcttccgatcCACTGRCAAAGGAG AAGTCCC	(SEQ ID NO:47)
BV06.A12	acactctttccctacacgacgctcttccgatcGTMCTGGTATCGAC AAGACCCA	(SEQ ID NO:48)
BV07.A11	acactctttccctacacgacgctcttccgatcCCAATTTAGGTCA TACTGCCCT	(SEQ ID NO:49)
BV07.A12	acactctttccctacacgacgctcttccgatcGGATGTAGCTCTCA GGTGTGAT	(SEQ ID NO:50)
BV07.A13	acactctttccctacacgacgctcttccgatcYCAGTGATCGGTTC TCTGCA	(SEQ ID NO:51)
BV07.A14	acactctttccctacacgacgctcttccgatcCTCAGTGATCGGAT CTCTGCA	(SEQ ID NO:52)
BV09.A1	acactctttccctacacgacgctcttccgatcCTTGAACGATTCTC CGCACAA	(SEQ ID NO:53)
BV10.A11	acactctttccctacacgacgctcttccgatcGGGCTGAGGCTGAT CCA	(SEQ ID NO:54)

BV10.A12	acactctttccctacacgacgctcttccgatcCATGGGCTGAGGCT GATCTA	(SEQ ID NO:55)
BV10.A13	acactctttccctacacgacgctcttccgatcTGGGCTGAGGCTAA TCCA	(SEQ ID NO:56)
BV11.A1	acactctttccctacacgacgctcttccgatcTGCCTAAGGATCGA TTTTCTGCA	(SEQ ID NO:57)
BV12.A11	acactctttccctacacgacgctcttccgatcCGTTCCGATAGATG ATTCAGGGAT	(SEQ ID NO:58)
BV12.A12	acactctttccctacacgacgctcttccgatcATTCTCAGCAGAGA TGCCTGAT	(SEQ ID NO:59)
BV13.A1	acactctttccctacacgacgctcttccgatcAGCGATAAAGGAAG CATCCCT	(SEQ ID NO:60)
BV14.A1	acactctttccctacacgacgctcttccgatcCGGTATGCCCAACA ATCGATTCTTA	(SEQ ID NO:61)
BV15.A1	acactctttccctacacgacgctcttccgatcCAGACACCCCTGAT AACTTCCAA	(SEQ ID NO:62)
BV16.A1	acactctttccctacacgacgctcttccgatcTGAAACAGGTATGC CCAAGGAA	(SEQ ID NO:63)
BV18.A1	acactctttccctacacgacgctcttccgatcCAGGAATGCCAAAG GAACGAT	(SEQ ID NO:64)
BV19.A1	acactctttccctacacgacgctcttccgatcGCTGAAGGGTACAG CGTCT	(SEQ ID NO:65)
BV20.A1	acactctttccctacacgacgctcttccgatcAAGGCGTCGAGAAG GACAA	(SEQ ID NO:66)
BV24.A1	acactctttccctacacgacgctcttccgatcGATGGATACAGTGT CTCTCGACA	(SEQ ID NO:67)
BV25.A1	acactctttccctacacgacgctcttccgatcCTCTGAGTCAACAG TCTCCAGAA	(SEQ ID NO:68)
BV27.A1	acactctttccctacacgacgctcttccgatcCTGAAGGGTACAAA GTCTCTCGAA	(SEQ ID NO:69)
BV28.A1	acactctttccctacacgacgctcttccgatcGCTACGGCTGATCT ATTTCTCATATGAT	(SEQ ID NO:70)
BV29.A1	acactctttccctacacgacgctcttccgatcGCCACATATGAGAG TGGATTTGTCAT	(SEQ ID NO:71)
BV30.A1	acactctttccctacacgacgctcttccgatcGGTGCCCCAGAATC TCTCA	(SEQ ID NO:72)

P5.BC.Rd1x:AATGATACGGCGACCAACGAGATCT[barcode seq]ACACTCTTTCCTACACGA
(SEQ ID NO:73)

5 **[0181]** The amplification reagents include, in addition to the above-described primers, other components for performing an amplification reaction (e.g., PreAmp Master Mix (Fluidigm 100-5581) and C₁TM Loading Reagent (Fluidigm 100-5170)).

10 **[0182]** The RT primers (TRAC and TRBC) and first barcode (P7.BC.Rd2) primers are still present from the RT step and are utilized during the amplification step. The combinatorial arrangement of 40 barcodes on this end of the amplified segment and 20 barcodes on the other end provides each of the 800 cells with a unique barcode identity.

[0183] The amplicons harvested in the 20 harvest ports should have the structure:

P5-BC-Rd1-V(α or β)-CDR3(α or β)-C(α or β)-Rd2-BC-P7

15

[0184] Because each cell has its own combinatorial barcode, the 20 harvests can be pooled, purified using beads or some other method, and loaded on an Illumina sequencer to determine CDR3 sequences for each of the cells. By using different sets of 20 barcodes in the P7.BC.Rd2 primers for additional IFCs, the libraries from multiple IFCs can be
5 pooled together before sequencing.

[0185] In particular embodiments, the amplification reaction is PCR, preferably carried out under hot-start conditions to improve amplification specificity.

Super Selective Primers

[0186] In some embodiments of the methods described herein, amplification
10 specificity can be further enhanced using Super Selective (also termed “Loop”) primers (Marras S, Vargas-Gold D, Tyagi S, Kramer FR, PCT/US2014/015351, which is incorporated by reference for its description of Super Selective primers). An illustrative Super Selective primer is shown diagrammatically in Figure 5. The Anchor segment (typically 18-35 nucleotides) keeps the primer stably bound to the template. Because of
15 the unpaired Insert/Loop region, the Bait segment (typically 5-10 nucleotides) hybridizes to the template only transiently. Occasionally, during this hybridization time, the primer is extended by polymerase. Any mismatch in the Bait segment drastically reduces the occupancy time for any hybridization event and thus drastically reduces the probability that the primer will be extended. Therefore, the only templates that should participate in
20 primer extension are those that have the Anchor sequence and a close-by, perfectly-matched Bait sequence. An illustrative Super Selective primer can have a 24-nucleotide Anchor segment, a 14-nucleotide Insert, and a Bait sequence of 5-7 nucleotides. The Loop formed by unpaired template nucleotides need not be the same length as the Insert in the primer. For example, the Loop opposite a 14-nucleotide Insert might only contain 10
25 nucleotides.

[0187] For TCR sequencing, the same procedure outlined above can be used, with the modification that the TCR-specific primers include Inserts that do not hybridize to the TCR transcripts. Figure 6A illustrates the use of Super Selective primers in reverse transcription, whereas Figure 6B illustrates their use in amplification.

30 [0188] Referring to Figure 6A, the CS-specific portion of the first RT primer includes an insert that does not anneal to the CS of the RNA encoding the α chain of the TCR, and/or the CS-specific portion of the second RT primer includes an insert that does

not anneal to the CS of the RNA encoding the β chain of the TCR. The inserts are flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the CS.

Preferably, both first and second RT primers include inserts; in some embodiments, the inserts are similar or the same with respect to sequence and/or length.

- 5 **[0189]** Referring to Figure 6B, the VS-specific portion of the plurality of first amplification primers includes an insert that does not anneal to the VS of the RNA encoding the α chain of the TCR, and/or the VS-specific portion of the plurality of second amplification primers includes an insert that does not anneal to the VS of the RNA encoding the β chain of the TCR. The inserts are flanked by a 5' anchor sequence and a 3' bait sequence, both of which anneal to the VS. Preferably, both first and second amplification primers include inserts; in some embodiments, the inserts are similar or the same with respect to sequence and/or length.
- 10

- [0190]** In various embodiments, the first and second RT primers can include inserts, with standard primers (i.e., without inserts) used for amplification; standard primers can be used for reverse transcription in conjunction with amplification primers that include inserts; or both sets of primer can include inserts. As an example of the latter embodiment, the sequences of illustrative Super Selective forms of RT primers (1 TRAC and 1 TRBC) and amplification primers (41 TRAV and 31 TRBV) are given below:
- 15

AV01.L1	acactctttccctacacgacgctcttccgatctGCGRAGCACCC ACATTTCTKTCTTACAAatcaacatcaacGGATGGT	(SEQ ID NO:74)
AV02.L1	acactctttccctacacgacgctcttccgatctCACCAAGACTC CTTGTTAAAGGCTCAAtcaaccaacaaTCAGCAG	(SEQ ID NO:75)
AV03.L1	acactctttccctacacgacgctcttccgatctGGCTTTGAAGC TGAATTTAACAAGAGCCAAtaacaatCCTGAAG	(SEQ ID NO:76)
AV04.L1	acactctttccctacacgacgctcttccgatctCCCTGTTTATC CCTGCCGACAGAAacaacttCTCTGAG	(SEQ ID NO:77)
AV05.L1	acactctttccctacacgacgctcttccgatctACATGAAACAA GACCAAAGACTCACTGTTCTAcaccttttAGGATAAAC	(SEQ ID NO:78)
AV06.L1	acactctttccctacacgacgctcttccgatctAGAAAGACTGA AGGTCACCTTTGATACCAaaacacctttCAGAGTTT	(SEQ ID NO:79)
AV07.L2	acactctttccctacacgacgctcttccgatctAGCAGAAAGGA AGACTAAATGCTACATTACTGAtetcatGCTTGTAC	(SEQ ID NO:80)
AV08.L11	acactctttccctacacgacgctcttccgatctARGMATCAACG GTTTTGAGGCTGAAccctttctGTGAAACC	(SEQ ID NO:81)
AV08.L12	acactctttccctacacgacgctcttccgatctGGGATCCACTG GTTAAAGGCATCAAttcataccctAGGCTGA	(SEQ ID NO:82)
AV08.L13	acactctttccctacacgacgctcttccgatctGCTTTGAGGCT GAATTTAAGAGGAGTCAACaactaCTGAGGA	(SEQ ID NO:83)
AV08.L14	acactctttccctacacgacgctcttccgatctARGMATCAACG GTTTTGAGGCTGAAacattttcaacattCAAGAGTC	(SEQ ID NO:84)
AV09.L11	acactctttccctacacgacgctcttccgatctACCGTAAAGAA ACCACTTCTTTCCACTTGctcttttttctCTCAGTTC	(SEQ ID NO:85)
AV09.L12	acactctttccctacacgacgctcttccgatctCCACATACCGT AAGGAAACCACTTCTTTCCAacctttCTCAGTTC	(SEQ ID NO:86)
AV10.L1	acactctttccctacacgacgctcttccgatctCGAACGGAAGA TATACAGCAACTCTGGATcatattttGCAAAGC	(SEQ ID NO:87)
AV12.L11	acactctttccctacacgacgctcttccgatctTGGTAATGAAG ATGGAAGGTTTACAGCACAAacattcttAGCCAGC	(SEQ ID NO:88)
AV12.L12	acactctttccctacacgacgctcttccgatctRACAAAGAAGA TGGAAGGTTTACAGCACAAacattcttAGCCAGC	(SEQ ID NO:89)
AV12.L13	acactctttccctacacgacgctcttccgatctRACAAAGAAGA TGGAAGGTTTACAGCACAAacattcttATCCAGCA	(SEQ ID NO:90)
AV13.L11	acactctttccctacacgacgctcttccgatctGGCGAAAAGAA AGACCAACGAATTGCTTcacttttCAAGACAG	(SEQ ID NO:91)
AV13.L12	acactctttccctacacgacgctcttccgatctGCAAGGCCAAA GAGTCACCGTTTTTAaccttttcttGACAGTG	(SEQ ID NO:92)
AV14.L1	acactctttccctacacgacgctcttccgatctTGCAACAGAAG GTCGCTACTCATTGAAaacaatcttGGCAAGA	(SEQ ID NO:93)
AV16.L1	acactctttccctacacgacgctcttccgatctCATCAAAGGCT TCACTGCTGACCTTAAatateCTTTCCAC	(SEQ ID NO:94)
AV17.L1	acactctttccctacacgacgctcttccgatctTGGAAGATTAA GAGTCACGCTTGACACTcaattttttGACAGTTC	(SEQ ID NO:95)
AV18.L1	acactctttccctacacgacgctcttccgatctAGAGGTTTTCA GGCCAGTCCTATCAAActtccaaCCTTCCA	(SEQ ID NO:96)
AV19.L1	acactctttccctacacgacgctcttccgatctTGGTCGGTATT CTTGGAACCTCCAGAAatcatccaCCTTCAAC	(SEQ ID NO:97)
AV20.L1	acactctttccctacacgacgctcttccgatctAAGAAGGAAAG CTTTCTGCACATCACAAAAactttCCTGAAG	(SEQ ID NO:98)
AV21.L1	acactctttccctacacgacgctcttccgatctTGGAAGACTTA ATGCCTCGCTGGATAAtcattttctCGTAGTAC	(SEQ ID NO:99)
AV22.L1	acactctttccctacacgacgctcttccgatctGCCACGACTGT CGCTACGGAAactacGTACATTTT	(SEQ ID NO:100)
AV23.L1	acactctttccctacacgacgctcttccgatctACAATCTCCTT CAATAAAAGTGCCAAGCAccataATCATGGA	(SEQ ID NO:101)
AV24.L1	acactctttccctacacgacgctcttccgatctCACTCTTAATA CCAAGGAGGGTTACAGCTAacaattAGGATCC	(SEQ ID NO:102)

AV25.L1	acactctttccctacacgacgctcttccgatctTCCCTGCACAT CACAGCCACaatctaaatctaTACAGATG	(SEQ ID NO:103)
AV26.L11	acactctttccctacacgacgctcttccgatcACAGAAAGTCCA GCACCTTGATCCTcaatacaaACGCTGA	(SEQ ID NO:104)
AV26.L12	acactctttccctacacgacgctcttccgatcAGCAATGTGAAC AACAGAATGGCCTacaatcattTCGCTGA	(SEQ ID NO:105)
AV27.L1	acactctttccctacacgacgctcttccgatcGGTGACAGTAGT TACGGGTGGAGAAcacttttcttGCTGAAG	(SEQ ID NO:106)
AV29.L1	acactctttccctacacgacgctcttccgatcTCTTAAACAAAA GTGCCAAGCACCTCTacacaatatTTGTGCC	(SEQ ID NO:107)
AV30.L1	acactctttccctacacgacgctcttccgatcTGAAGCACCCGT CTTCCTGATGATAcctaaaGGAGAAC	(SEQ ID NO:108)
AV34.L1	acactctttccctacacgacgctcttccgatcAGAAAAAGCAGC AAAGTTCCCTGCATAcatacaaCAGCCCA	(SEQ ID NO:109)
AV35.L1	acactctttccctacacgacgctcttccgatcACCTCAAATGGA AGACTGACTGCTCAcaccttCCAGAAAG	(SEQ ID NO:110)
AV36.L1	acactctttccctacacgacgctcttccgatcAGCATCCTGAAC ATCACAGCCACaatttataacGAGACTC	(SEQ ID NO:111)
AV38.L1	acactctttccctacacgacgctcttccgatcCGTTATTCGCCA AGAAGCTTATAAGCAACActtattatGAGAATCG	(SEQ ID NO:112)
AV39.L1	acactctttccctacacgacgctcttccgatcGCAGTGAAGCAG GAGGGACGATTAAactaaaacatCTTGATACC	(SEQ ID NO:113)
AV40.L1	acactctttccctacacgacgctcttccgatcAGCAAAAAC TTC GGAGGCGGAAtctcacctttGACAAAAC TC	(SEQ ID NO:114)
AV41.L1	acactctttccctacacgacgctcttccgatcCACAGCTCCCTG CACATCACACaacaataatTCCCAGA	(SEQ ID NO:115)
BV02.L1	acactctttccctacacgacgctcttccgatcTCACTCTGAAGA TCCGGTCCACAAtcaatcttcACTCAGC	(SEQ ID NO:116)
BV03.L1	acactctttccctacacgacgctcttccgatcCCAAATCGMTTC TCACCTAAATCTCCAGACAAAtcttaCTTCACATC	(SEQ ID NO:117)
BV04.L1	acactctttccctacacgacgctcttccgatcGCCAAGTCGCTT CTCACCTGAAacaattatGCTCTC	(SEQ ID NO:118)
BV05.L11	acactctttccctacacgacgctcttccgatcTCAGTGAGACAC AGAGAAACAAAGGAACTcaactaTCTCAGG	(SEQ ID NO:119)
BV05.L12	acactctttccctacacgacgctcttccgatcCTCTGAGCTGAA TGTGAACGCCTTcctttctaGACTCG	(SEQ ID NO:120)
BV06.L11	acactctttccctacacgacgctcttccgatcGTACCACTGRCA AAGGAGAAGTCCCattattTGTCTCC	(SEQ ID NO:121)
BV06.L12	acactctttccctacacgacgctcttccgatcACATGTMCTGGT ATCGACAAGACCCAtcatactcatacGGGCTGA	(SEQ ID NO:122)
BV07.L11	acactctttccctacacgacgctcttccgatcTGATCCAATTTC AGGTCATACTGCCCTcatacacctaACCGACA	(SEQ ID NO:123)
BV07.L12	acactctttccctacacgacgctcttccgatcGACRGGATGTAG CTCTCAGGTGTGATAaattcacatGGTCATG	(SEQ ID NO:124)
BV07.L13	acactctttccctacacgacgctcttccgatcGCYCAGTGATCG GTTCTCTGCActctaactAGGGATC	(SEQ ID NO:125)
BV07.L14	acactctttccctacacgacgctcttccgatcTGCTCAGTGATC GGATCTCTGCActctaactAGGGATC	(SEQ ID NO:126)
BV09.L1	acactctttccctacacgacgctcttccgatcACATTCTTGAAC GATTCTCCGCACAAatcacaaacGACTTGC	(SEQ ID NO:127)
BV10.L11	acactctttccctacacgacgctcttccgatcCATGGGCTGAGG CTGATCCAcatacacatcATGGTGTT	(SEQ ID NO:128)
BV10.L12	acactctttccctacacgacgctcttccgatcGGACATGGGCTG AGGCTGATCTAactacatcaAGCTGCT	(SEQ ID NO:129)
BV10.L13	acactctttccctacacgacgctcttccgatcGCATGGGCTGAG GCTAATCCAcatacacatcATGGTGTT	(SEQ ID NO:130)
BV11.L1	acactctttccctacacgacgctcttccgatcCAGTTGCCTAAG GATCGATTTTCTGCAtcttttGGAGTAG	(SEQ ID NO:131)
BV12.L11	acactctttccctacacgacgctcttccgatcACAACGTTCCGA TAGATGATTCAGGGATcaaactcttTCGATTC	(SEQ ID NO:132)

BV12.L12	acactctttccctacacgacgctcttccgatcTCGATTCTCAGC AGAGATGCCTGATcattataacaAGCCACT	(SEQ ID NO:133)
BV13.L1	acactctttccctacacgacgctcttccgatcGCAGAGCGATAA AGGAAGCATCCCTttcactcaaTCAGCTC	(SEQ ID NO:134)
BV14.L1	acactctttccctacacgacgctcttccgatcTCCGGTATGCCC AACAAATCGATTCTTAcaactttctACTGGAG	(SEQ ID NO:135)
BV15.L1	acactctttccctacacgacgctcttccgatcAGCAGACACCCC TGATAACTTCCAAaaattccaCGAACAC	(SEQ ID NO:136)
BV16.L1	acactctttccctacacgacgctcttccgatcCTTTGATGAAAC AGGTATGCCCAAGGAAtctacacatGCTAAGTG	(SEQ ID NO:137)
BV18.L1	acactctttccctacacgacgctcttccgatcGAGTCAGGAATG CCAAAGGAACGATcaaacaacaGAATTTCCC	(SEQ ID NO:138)
BV19.L1	acactctttccctacacgacgctcttccgatcGAGATATAGCTG AAGGGTACAGCGTCTacactcttGAAGGAATC	(SEQ ID NO:139)
BV20.L1	acactctttccctacacgacgctcttccgatcGCAAGGCGTCGA GAAGGACAAtacaacatcCAACCATG	(SEQ ID NO:140)
BV24.L1	acactctttccctacacgacgctcttccgatcTCTCTGATGGAT ACAGTGTCTCTCGACActatatctaCTAAATTCTC	(SEQ ID NO:141)
BV25.L1	acactctttccctacacgacgctcttccgatcCTTTCCTCTGAG TCAACAGTCTCCAGAActtcacttGCATTTTC	(SEQ ID NO:142)
BV27.L1	acactctttccctacacgacgctcttccgatcTTCCTGAAGGGT ACAAAGTCTCTCGAAAtcttcttGAATTTCC	(SEQ ID NO:143)
BV28.L1	acactctttccctacacgacgctcttccgatcGGGCTACGGCTG ATCTATTTCTCATATGATtcctttGAAAAAGGA	(SEQ ID NO:144)
BV29.L1	acactctttccctacacgacgctcttccgatcAGGCCACATATG AGAGTGGATTTGTCATcttatcaaCCCATCA	(SEQ ID NO:145)
BV30.L1	acactctttccctacacgacgctcttccgatcTGAGGTGCCCCA GAATCTCTCAtaactaaCCAGGAC	(SEQ ID NO:146)

AC.L1:gtgactggagttcagacgtgtgctcttccgatctCGGTGAATAGGCAGACAGACTTGTCAactctaa
ctGAGTCT (SEQ ID NO:147)

5 BC.L1:gtgactggagttcagacgtgtgctcttccgatctGGCACACCAGTGTGGCCTTTatctccatctGGA
GATC (SEQ ID NO:148)

[0191] Using these Super Selective primers, the resulting amplicons still have the
same general structure (P5-BC-Rd1-V(α or β)-CDR3(α or β)-C(α or β)-Rd2-BC-P7) and
10 can be pooled and sequenced as outlined above.

[0192] In certain, preferred embodiments of the above-described methods, Super
Selective primers are used for amplification, but not for reverse transcription, as this
primer combination, rather surprisingly, enhances production of the desired products.

[0193] In some embodiments, the concentration of the “outer” barcode primers
15 exceeds that of the “inner” target-specific primers. For example, the outer, barcode
primers can be used in a 100-fold, 75-fold, 50-fold, 40-fold, 30-fold, 20-fold, 10-fold, or
5-fold excess over the inner, target-specific primers or in a degree of excess falling within
any range bounded by any of these values, e.g., 30-fold to 50-fold. In implementations on
the C₁ chip, for example, good results are obtained using inner, target-specific primers at
20 50 nM and outer, barcode primers at 2 μ M, which is a 40-fold excess.

Preparing DNA Templates by Reverse Transcription, Template Switching, and Amplification

[0194] In some embodiments, DNA templates can be prepared from single cells using template switching. This well-known technique is outlined in Zhu YY, Machleder EM, Chenchik A et al (2001) Reverse transcriptase template switching: a SMART approach for full-length cDNA library construction. Biotechniques 30:892–897, which is incorporated herein by reference for this description. The Summary above discusses methods for preparing the DNA templates described herein using template switching in general terms. Figure 7 illustrates the application of an embodiment of this technique to generate a DNA template for a particular target transcript (targeted transcript sequencing). The number and identity of the target-specific primers is determined by the mRNAs to be sequenced. These target-specific primers are typically positioned close to the 5' end of their respective mRNAs. In the Figure 7, the RT primer is shown with an insert, but primers without inserts could be used as well. Reverse transcriptase extends the target-specific primers then, taking advantage of the template-switch mechanism, copies the information in the template-switch oligonucleotide (Rd1-UMI-GGG). By incorporating the UMI, more accurate quantitative information for the targeted mRNAs can be obtained. “GGG” denotes an oligo-riboG sequence. Most typically, three guanines are used in template switch oligonucleotides, but any number that works can be used. One or more of the guanines can be a locked nucleic acid (LNA).

[0195] This approach can be adapted for whole transcriptome sequencing by using an RT primer in which the target-specific portion is replaced with a portion specific for the poly-A tail of mRNA molecules (e.g., poly-dT). In either case, templates can be amplified and barcodes and sequencing adaptors added using barcode primers such as those described above (e.g., P5-BC-Rd1 and Rd2-BC-P7), followed by pooling and sequencing as described above. If whole transcriptome sequencing is performed, TCR and Ig sequences can be determined from the resulting data set using standard bioinformatic methods, thus avoiding the need for the specialized single-cell TCR or Ig transcript sequencing methods described above. Illustrative bioinformatics methods are found in Nat Methods. 2016 Apr;13(4):329-32. doi: 10.1038/nmeth.3800. Epub 2016 Mar 7. T cell fate and clonality inference from single-cell transcriptomes. Stubbington MJ, Lönnberg T, Proserpio V, Clare S, Speak AO, Dougan G, Teichmann SA, which is incorporated by reference herein for this description. In an intermediate approach, template switching can

be carried out to obtain templates corresponding to the whole transcriptome, followed by TCR- or Ig-transcript specific amplification.

[0196] Alternatively, either or both of targeted transcript sequencing or transcriptome sequencing can be combined with the single-cell TCR or Ig transcript sequencing methods described above.

[0197] In some embodiments, using the template-switch oligonucleotide provides another mechanism for obtaining TCR/Ig sequence information. In this case, the reverse transcription step would be performed with TRAC primer, TRBC primer, optionally target-or poly-A-specific primers for additional mRNAs, P7.BC.Rd2 primer, and a template-switch oligonucleotide. The TRAC and TRBC primers would be extended to the 5' ends of the TCR mRNAs, followed by template switching. For the amplification step, all that needs to be added is amplification reagents including the P5.BC.Rd1 primer. There would be no need to add all the different TRAV and TRBV primers. If necessary, the number of sequencing cycles could be increased to be certain of obtaining CDR3 sequence.

[0198] In particular embodiments, the template switching method is carried out in a microfluidic device, as described above. In this case, the RT primer can be delivered to capture sites via one set of the input lines, and the 5' oligonucleotide can be delivered to the capture sites via the other set of input lines.

Ig Transcript Production

[0199] Natural antibodies typically have two identical Ig heavy chains. Each of the Ig heavy chain genes is assembled from multiple possible V, D, J, and C segments by somatic recombination in a manner similar to that described above for TCRs. An example of heavy chain rearrangement is shown in Figure 8A. There are multiple different C segments (encoding constant regions) that give rise to different isotypes. Natural antibodies also typically have two identical Ig light chains, which can either be κ light chains or λ light chains. Each of the Ig light chain genes is assembled from multiple possible V, J, and C segments by somatic recombination. An example of κ light chain rearrangement is shown in Figure 8B. As those of skill in the art readily appreciate, the strategies described herein with respect to sequencing TCR transcripts can also be carried out to sequence Ig transcripts simply by designing primers that will anneal to the V and C regions of Igs, rather than the V and C regions of TCRs.

DNA Sequencing

[0200] In some embodiments, the methods described herein include subjecting the DNA templates produced to DNA sequencing, e.g., Sanger sequencing, next-generation sequencing (e.g., bridge sequencing), or third-generation sequencing. In variations of such
5 embodiments, the sequences obtained from DNA sequencing can be identified as having been derived from a particular capture site based on one or two barcodes.

[0201] As discussed above, reaction products from a particular row or column of a matrix-type microfluidic device can be exported as a pool. Any subsequent characterization of reaction products, such as DNA sequencing, can be carried out on
10 individual exported pools. However, it is also contemplated that the pools themselves can be pooled prior to further characterization. In this case, the DNA template(s) from each separate capture site in the microfluidic device is typically distinct, which is readily achieved, e.g., by using two barcode sequences to encode the row and column location of the capture site in the microfluidic device.

[0202] In some embodiments, it is advantages to further amplify reaction products before carrying out DNA sequencing. Where all reaction products bear common end sequences, all reaction products to be sequenced can be pooled and amplified together, using primers specific for the end sequences (i.e., in the illustrative embodiment of preparing TCR templates described above, P5 and P7 primers would be used).

Primer Combinations

[0203] Any of the primers or oligonucleotides described above may be combined to form primer combinations. Typically, primer combinations include 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more primers or oligonucleotides that are used together
25 in a method such as those described herein. Individual primers can be packaged individually or packaged together, when used as a set in a reaction. For example, when the primers are to be used for sequencing TCRs or Igs, the set of all possible Variable segment-specific primers are conveniently packaged together.

Kits

[0204] Kits according to the invention can include one or more reagents useful for
30 practicing one or more methods described herein. A kit generally includes a package with one or more containers holding the reagent(s), as one or more separate compositions or,

optionally, as admixture where the compatibility of the reagents will allow. The kit can also include other material(s) that may be desirable from a user standpoint, such as a buffer(s), a diluent(s), a standard(s), and/or any other material useful in sample processing, washing, or conducting any other step of the assay. In specific embodiments, the kit
 5 includes one or more matrix-type microfluidic devices and/or primers/oligonucleotides discussed above or combinations thereof.

[0205] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and
 10 purview of this application and scope of the appended claims.

[0206] In addition, all other publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

Example 1: Use of Super Selective Primers for T-Cell Receptor Template

Production

[0207] RT primer set 1 (RT-No Loop) is a mix of AC.A1 and BC.A1. RT primer set 2 (RT-Loop) is a mix of AC.L1 and BC.L1. V primer set 1 (V-No Loop) is a mix of AV01.A1, AV02.A1, AV03.A1, AV04.A1, AV05.A1, AV06.A1, AV07.A1, AV08.A11, AV08.A12, AV08.A13, AV09.A1, AV10.A1, AV12.A1, AV13.A11, AV13.A12,
 20 AV14.A1, AV16.A1, AV17.A1, AV18.A1, AV19.A1, AV20.A1, AV21.A1, AV22.A1, AV23.A1, AV24.A1, AV25.A1, AV26.A11, AV26.A12, AV27.A1, AV29.A1, AV30.A1, AV34.A1, AV35.A1, AV36.A1, AV38.A1, AV39.A1, AV40.A1, AV41.A1, BV02.A1, BV03.A1, BV04.A1, BV05.A11, BV05.A12, BV06.A11, BV06.A12, BV07.A11, BV07.A12, BV07.A13, BV07.A14, BV09.A1, BV10.A11, BV10.A12, BV10.A13,
 25 BV11.A1, BV12.A11, BV12.A12, BV13.A1, BV14.A1, BV15.A1, BV16.A1, BV18.A1, BV19.A1, BV20.A1, BV24.A1, BV25.A1, BV27.A1, BV28.A1, BV29.A1, and BV30.A1. V primer set 2 (V-Loop) is a mix of AV01.L1, AV02.L1, AV03.L1, AV04.L1, AV05.L1, AV06.L1, AV07.L2, AV08.L11, AV08.L12, AV08.L13, AV08.L14, AV9.L11, AV9.L12, AV10.L1, AV12.L11, AV12.L12, AV12.L13, AV13.L11, AV13.L12, AV14.L1,
 30 AV16.L1, AV17.L1, AV18.L1, AV19.L1, AV20.L1, AV21.L1, AV22.L1, AV23.L1, AV24.L1, AV25.L1, AV26.L11, AV26.L12, AV27.L1, AV29.L1, AV30.L1, AV34.L1,

AV35.L1, AV36.L1, AV38.L1, AV39.L1, AV40.L1, AV41.L1, BV02.L1, BV03.L1, BV04.L1, BV05.L11, BV05.L12, BV06.L11, BV06.L12, BV07.L11, BV07.L12, BV07.L13, BV07.L14, BV09.L1, BV10.L11, BV10.L12, BV10.L13, BV11.L1, BV12.L11, BV12.L12, BV13.L1, BV14.L1, BV15.L1, BV16.L1, BV18.L1, BV19.L1, BV20.L1, BV24.L1, BV25.L1, BV27.L1, BV28.L1, BV29.L1, and BV30.L1. In the experiment described below, these primers were used in the following combinations:

1: No Loop RT primers with No Loop V primers

2: Loop RT primers with No Loop V primers

3: No Loop RT primers with Loop V primers

10 4: Loop RT primers with Loop V primers

[0208] Reverse transcriptase reactions contained 100 ng total RNA from human peripheral blood mononuclear cells (PBMCs), 220 nM each RT primer (RT-No Loop or RT-Loop), 15 mM Tris-HCl, pH 8.4, 75 mM KCl, 10 mM MgCl₂, 5% (v/v) glycerol, 10 mM dithiothreitol, 0.8 units/ μ L RNaseOUT™ (Thermo Fisher Scientific), 500 μ M each dNTP, and 8 units/ μ L SuperScript® II reverse transcriptase (Thermo Fisher Scientific) in a volume of 11.5 μ L. These reactions were incubated at 25°C for 10 minutes, 42°C for 60 minutes, then placed on ice. To each reaction, 14 μ L of a preamp/primer mix was added so that the final concentrations were 1 $\times$ PreAmp Master Mix (Fluidigm) and 50 nM each V primer (V-No Loop or V-Loop). The conditions for PCR were 95°C for 5 minutes, 20 cycles of 96°C for 5 seconds and 60 °C for 6 minutes, followed by 4°C hold. Ten microliters of a mix consisting of 2 μ L Exonuclease I (New England BioLabs), 1 μ L 10 $\times$ Exonuclease I reaction buffer, 7 μ L water were added to each reaction. These reactions were incubated at 37°C for 30 minutes, 80°C for 15 minutes, then placed on ice. Ninety microliters 10 mM Tris-HCl, pH 8.0, 1 mM EDTA were added to each reaction. For purification, 50 μ L of each sample was mixed with 50 μ L AMPure XP Beads (Beckman Coulter), mixed well, and kept at room temperature for 5 minutes. Each sample tube was put on a magnet plate for 5 minutes, then the supernatant was discarded. The beads were washed with newly made 70% ethanol twice. The beads were dried at room temperature for at least 7 minutes, then each was resuspended in 30 μ L 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. After sitting at room temperature for 5 minutes, each sample tube was put on a magnet plate for 2 minutes and each supernatant was transferred to a fresh tube. A 1- μ L

aliquot of each sample was analyzed on the 2100 Bioanalyzer system using the High Sensitivity DNA kit (Agilent).

[0209] Results are shown in Figure 9. Desired DNA fragments derived from the TCR transcript are in the size range 250 to 350 nucleotides. Undesired, nonspecific fragments are in the size range 100 to 220 nucleotides. The best ratio of desired fragments to undesired fragments is observed for combination 3, which is No Loop RT primers and Loop V primers.

Example 2: Fixation Enhances the Recovery of DNA Templates Produced from RNA Transcripts

[0210] Human peripheral blood mononuclear cells (PBMCs) were enriched for CD4⁺ cells using magnetic bead purification. Pelleted cells, resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing IL-2 and IL-7, were activated by addition of 2 µg/mL phytohemagglutinin (PHA). After five days of activation, cells were washed and enriched again for CD4⁺ cells using magnetic bead purification. Two aliquots of these cells were pelleted. The cells designated fresh were resuspended in phosphate-buffered saline (PBS). The cells designated fixed were resuspended in PBS containing 1 mg/mL 3,3'-dithiodipropionic acid di(N-hydroxysuccinimide ester) (DSP, Sigma-Aldrich). Eight hundred fresh or fixed cells were pelleted and resuspended in 5.5 µL Lysis Mix consisting of 50 mM Tris-HCl, pH 8.4, 40 mM dithiothreitol, 0.5% NP-40 detergent, 8 units/mL proteinase K (New England BioLabs), 230 nM each RT-No Loop primer, 9.2 µM P7.rcCBC001.Rd2x primer (CAAGCAGAAGACGGCATACTGAGATcgcgactgaaGTGACTGGAGTTCAGACGT) (SEQ ID NO:149), and 1× C1 Loading Reagent (Fluidigm). These reactions were incubated at 50°C for 30 minutes, 72°C for 1 minute, then placed on ice. To each reaction, 6 µL of a RT mix was added so that the final concentrations were 15 mM Tris-HCl, pH 8.4, 75 mM KCl, 10 mM MgCl₂, 5% (v/v) glycerol, 360 µM AAPF proteinase K inhibitor (EMD Millipore), 500 µM each dNTP, 8 units/µL SuperScript[®] II reverse transcriptase, and 1× C1 Loading Reagent. These reactions were incubated at 42°C for 60 minutes, 85°C for 5 minutes, then placed on ice. To each reaction, 14 µL of a preamp/primer mix was added so that the final concentrations were 1× PreAmp Master Mix (Fluidigm) and 50 nM each V-Loop primer, and 2 µM P5.Rd1x primer (AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA) (SEQ ID

NO:150), and 1× C1 Loading Reagent. The conditions for PCR were 95°C for 5 minutes, 20 cycles of 96°C for 20 seconds and 60°C for 6 minutes, followed by 4°C hold. Twenty-five microliters 10 mM Tris-HCl, pH 8.0, 1 mM EDTA were added to each reaction. For purification, each sample was mixed with 35 µL AMPure XP Beads (Beckman Coulter), mixed well, and kept at room temperature for 5 minutes. Each sample tube was put on a magnet plate for 5 minutes, then the supernatant was discarded. The beads were washed with newly made 70% ethanol twice. The beads were dried at room temperature for at least 7 minutes, then each was resuspended in 20 µL 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. After sitting at room temperature for 5 minutes, each sample tube was put on a magnet plate for 2 minutes and each supernatant was transferred to a fresh tube. The following was added to each sample: 2.5 µL 10 µM P5 primer (AATGATACGGCGACCACCGAGATCTACAC) (SEQ ID NO:151), 2.5 µL 10 µM P7 primer (CAAGCAGAAGACGGCATACGAGAT) (SEQ ID NO:152), and 25 µL 2× Q5[®] Hot Start HiFi PCR Master Mix (New England BioLabs). The conditions for PCR were 98°C for 30 seconds, 18 cycles of 98°C for 10 seconds and 62 °C for 2 minutes, 75°C for 2 minutes, followed by 4°C hold. For purification, each sample was mixed with 35 µL AMPure XP Beads (Beckman Coulter), mixed well, and kept at room temperature for 5 minutes. Each sample tube was put on a magnet plate for 5 minutes, then the supernatant was discarded. The beads were washed with newly made 70% ethanol twice. The beads were dried at room temperature for at least 7 minutes, then each was resuspended in 20 µL 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. After sitting at room temperature for 5 minutes, each sample tube was put on a magnet plate for 2 minutes and each supernatant was transferred to a fresh tube.

[0211] A 1-µL aliquot of each sample was analyzed on the 2100 Bioanalyzer system using the High Sensitivity DNA kit. Results are shown in Figure 10. Because of the addition of P5, P7, and other sequences, the desired DNA fragments derived from the TCR transcript are in the size range 320 to 400 nucleotides. Results show that the fixed cells gave a much better yield of desired fragments than fresh cells.

Example 3: Single-Cell T-Cell Receptor Template Production Using C1 mRNA Seq HT Array IFC

[0212] In the procedure that follows, any reference to standard protocol refers to “Using the C1 High-Throughput IFC to Generate Single-Cell cDNA Libraries for mRNA

Sequencing” (Fluidigm PN 100-9886). This document also provides the details for instrument operation.

Stocks

5× RT Buffer

- 5 75 mM Tris-HCl, pH 8.4
 375 mM KCl
 50 mM MgCl₂
 25% glycerol (50%, Teknova G1796, G1797, G1798, G1799)
- 10 18 mM AAPF Proteinase K inhibitor
 Dissolve 10 mg AAPF Proteinase K inhibitor in 1 mL DMSO
 Store at -20°C

C1 Mixes

15 20× Column Mixes

Mix:

- 187.5 μL Cell Rinsing Reagent (Fluidigm)
 12.5 μL C1 Loading Reagent (Fluidigm 100-5170)

Dispense 8 μL to each of 20 wells

- 20 Add 2 μL 100 μM P7.rcCBC001-020.Rd2x, with a different barcode added to each well
 During the set up for the Prime step, transfer 8 μL of each to one of the 20 Inlets labeled as “Column inlets.” Addition of the barcode mix will actually happen after cell staining.

Lysis Mix (163.5 μL)

- 25 15 μL 1 M Tris-HCl, pH8.4
 12 μL 1M dithiothreitol
 15 μL 10% NP-40 (0.5% in final rxn)
 6.9 μL 10 μM each RT-No Loop primer
 3 μL 800 units/mL (20 mg/mL) proteinase K (8 units/mL in final rxn)
- 30 8.2 μL C1 Loading Reagent (Fluidigm 100-5170)
 103.4 μL H₂O
- Transfer 130 μL Lysis Mix to Inlet L2 (big reservoir) during the set up for the Final Reaction.

RT Mix (26 μL)

- 35 10 μL 5× RT Buffer
 1 μL 25 mM each dNTPs
 1 μL 18 mM AAPF Proteinase K inhibitor (360 μM in final rxn)
 1 μL 200 units/μL SuperScript[®] II Reverse Transcriptase
 1.3 μL C1 Loading Reagent (Fluidigm 100-5170)
- 40 11.7 μL H₂O
- Transfer 20 μL RT Mix to Inlet RT during the set up for the Final Reaction.

40× Row Mixes

PCR Mix (225 μL)

- 45 130 μL 5× PreAmp Master Mix (Fluidigm 100-5581) (1× in final rxn)
 65 μL 500 nM each V-Loop primer (50 nM each primer in final rxn)
 18 μL C1 Loading Reagent (Fluidigm 100-5170)
 12 μL H₂O
- Dispense 5 μL PCR Mix to each of 40 wells
- 50 Add 2.9 μL 10 μM P5.rcRBC001-040.Rd1x, with a different barcode added to each well
 Transfer 5 μL of each to Inlets labeled as “Row Barcode” during the set up for the Final Reaction.

Harvest Mix (200 μL)

- 55 194 μL C1 Harvest Reagent (Fluidigm 100-7081)
 6 μL 10 mg/mL yeast tRNA (Thermo Fisher Scientific AM7119)

180 µL to each of the Harvest Inlet during the set up for the Final Reaction.

C1 Steps

- 5 **1.** Prime:
 - a. Pipette mixes following the standard prime loading map. For Valve Fluid, add Tween 20 to a final concentration of 0.05% and load 250 µL each into Acc1 and Acc2.
 - b. Pipette 8 µL each column mix to 20 column inlets
 - c. Run prime script: "mRNA Seq Prime R&C barcode". ~51 min
- 10 **2.** Cell Loading:
 - a. Replace liquid following the standard protocol
 - b. Keep the column mixes in the 20 column inlets
 - c. Using activated, DSP-fixed CD4+ T cells prepared as described in Example 2, dispense 20 µL cell suspension to each of the two cell inlets
 - 15 d. If you want cell load and stain: Run script "mRNA Seq HT: Cell Load &Stain with Column barcode" ~39min
 - e. If you only want cell load and wash: Run script "mRNA Seq HT: Cell load with column barcode" ~9 min
- 20 **3.** Final Reaction:
 - a. Replace cell-related liquid following the standard protocol.
 - b. Pipette Lysis Mix into L2 zone (up right corner), ~130 µL
 - c. Pipette RT Mix into RT inlet, ~20 µL
 - d. Pipette PCR Mixes containing barcode primers into 40 row inlets, ~5 µL each
 - 25 e. Check the entire reagent, such as harvest reagent, before inserting IFC into machine.
 - f. Run script "scTCR Seq HT" ~6 hr. Set time delay as usual.

Final Reaction consists of:

- 30 1. Lysis Mix to chambers 0+1
50°C, 30 min (1800 sec)
72°C, 1 min (60 sec)
10°C, 1 min (60 sec)
- 35 2. RT Mix to chambers 0+1+2
42°C, 60 min (3600 sec)
85°C, 5 min (300 sec)
- 40 3. PCR Mix to chambers 0+1+2+3
70°C, 30 min (1800 sec)
95°C, 5 min (300 sec)
20 cycles: 96°C, 20 sec
60°C, 6 min (360 sec)
4. Harvest

Transfer the 20 Harvest samples to a 96-well plate.

Pool 5 µL of each sample.

- 45 Clean up with 80 µL AMPure XP Beads. Elute with 50 µL 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

Clean up with 40 µL AMPure XP Beads. Elute with 20 µL 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

- 50 Add:
 - 2.5 µL 10 µM P5 Primer
 - 2.5 µL 10 µM P7 Primer
 - 25 µL Q5[®] Hot Start HiFi PCR Master Mix
- 55 98°C, 30 sec

20 cycles: 98°C, 10 sec
62°C, 2 min
75°C, 2 min
4°C, hold

- 5 Clean up with 35 µL AMPure XP Beads. Elute with 20 µL 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

[0213] The sequences of primers not previously given are below:

P7.rcCBC001 .Rd2x	CAAGCAGAAGACGGCATAACGAGATcgcgactgaaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:149)
P7.rcCBC002 .Rd2x	CAAGCAGAAGACGGCATAACGAGATagcatcgataGTGACTGGA GTTTCAGACGT	(SEQ ID NO:153)
P7.rcCBC003 .Rd2x	CAAGCAGAAGACGGCATAACGAGATcgacacatggGTGACTGGA GTTTCAGACGT	(SEQ ID NO:154)
P7.rcCBC004 .Rd2x	CAAGCAGAAGACGGCATAACGAGATcgactacgcaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:155)
P7.rcCBC005 .Rd2x	CAAGCAGAAGACGGCATAACGAGATcactgctgagGTGACTGGA GTTTCAGACGT	(SEQ ID NO:156)
P7.rcCBC006 .Rd2x	CAAGCAGAAGACGGCATAACGAGATtcgctgtacaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:157)
P7.rcCBC007 .Rd2x	CAAGCAGAAGACGGCATAACGAGATcgctgcagtaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:158)
P7.rcCBC008 .Rd2x	CAAGCAGAAGACGGCATAACGAGATagacttgcagGTGACTGGA GTTTCAGACGT	(SEQ ID NO:159)
P7.rcCBC009 .Rd2x	CAAGCAGAAGACGGCATAACGAGATgcgaaactaagGTGACTGGA GTTTCAGACGT	(SEQ ID NO:160)
P7.rcCBC010 .Rd2x	CAAGCAGAAGACGGCATAACGAGATgctagaacagGTGACTGGA GTTTCAGACGT	(SEQ ID NO:161)
P7.rcCBC011 .Rd2x	CAAGCAGAAGACGGCATAACGAGATtacgctaacgGTGACTGGA GTTTCAGACGT	(SEQ ID NO:162)
P7.rcCBC012 .Rd2x	CAAGCAGAAGACGGCATAACGAGATcagctatccgGTGACTGGA GTTTCAGACGT	(SEQ ID NO:163)
P7.rcCBC013 .Rd2x	CAAGCAGAAGACGGCATAACGAGATgtgtcctacaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:164)
P7.rcCBC014 .Rd2x	CAAGCAGAAGACGGCATAACGAGATatgatgcatgGTGACTGGA GTTTCAGACGT	(SEQ ID NO:165)
P7.rcCBC015 .Rd2x	CAAGCAGAAGACGGCATAACGAGATgaatgcatcaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:166)
P7.rcCBC016 .Rd2x	CAAGCAGAAGACGGCATAACGAGATgtctcgacaaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:167)
P7.rcCBC017 .Rd2x	CAAGCAGAAGACGGCATAACGAGATagcacgctcaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:168)
P7.rcCBC018 .Rd2x	CAAGCAGAAGACGGCATAACGAGATctatcgaacaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:169)
P7.rcCBC019 .Rd2x	CAAGCAGAAGACGGCATAACGAGATaagtcacagGTGACTGGA GTTTCAGACGT	(SEQ ID NO:170)
P7.rcCBC020 .Rd2x	CAAGCAGAAGACGGCATAACGAGATttcgtcgtgaGTGACTGGA GTTTCAGACGT	(SEQ ID NO:171)
P5.rcRBC001 .Rd1x	AATGATACGGCGACCACCGAGATCTACACgcaacgtaagACAC TCTTTCCCTACACGA	(SEQ ID NO:172)
P5.rcRBC002 .Rd1x	AATGATACGGCGACCACCGAGATCTACACgctgctcgaaACAC TCTTTCCCTACACGA	(SEQ ID NO:173)
P5.rcRBC003 .Rd1x	AATGATACGGCGACCACCGAGATCTACACtagaacaacgACAC TCTTTCCCTACACGA	(SEQ ID NO:174)
P5.rcRBC004 .Rd1x	AATGATACGGCGACCACCGAGATCTACACacgtcggataACAC TCTTTCCCTACACGA	(SEQ ID NO:175)
P5.rcRBC005	AATGATACGGCGACCACCGAGATCTACACtcagagaatgACAC	(SEQ ID NO:176)

.Rd1x	TCTTTCCCTACACGA	
P5.rcRBC006 .Rd1x	AATGATACGGCGACCACCGAGATCTACACatagctcgaaACAC TCTTTCCCTACACGA	(SEQ ID NO:177)
P5.rcRBC007 .Rd1x	AATGATACGGCGACCACCGAGATCTACACgacacggataACAC TCTTTCCCTACACGA	(SEQ ID NO:178)
P5.rcRBC008 .Rd1x	AATGATACGGCGACCACCGAGATCTACACattgttggaACAC TCTTTCCCTACACGA	(SEQ ID NO:179)
P5.rcRBC009 .Rd1x	AATGATACGGCGACCACCGAGATCTACACc gatagaagaACAC TCTTTCCCTACACGA	(SEQ ID NO:180)
P5.rcRBC010 .Rd1x	AATGATACGGCGACCACCGAGATCTACACtaccgtatgaACAC TCTTTCCCTACACGA	(SEQ ID NO:181)
P5.rcRBC011 .Rd1x	AATGATACGGCGACCACCGAGATCTACACatggagtctaACAC TCTTTCCCTACACGA	(SEQ ID NO:182)
P5.rcRBC012 .Rd1x	AATGATACGGCGACCACCGAGATCTACACagctccgtagACAC TCTTTCCCTACACGA	(SEQ ID NO:183)
P5.rcRBC013 .Rd1x	AATGATACGGCGACCACCGAGATCTACACgaatctacagACAC TCTTTCCCTACACGA	(SEQ ID NO:184)
P5.rcRBC014 .Rd1x	AATGATACGGCGACCACCGAGATCTACACcaatacgtctaACAC TCTTTCCCTACACGA	(SEQ ID NO:185)
P5.rcRBC015 .Rd1x	AATGATACGGCGACCACCGAGATCTACACcaacatgagaACAC TCTTTCCCTACACGA	(SEQ ID NO:186)
P5.rcRBC016 .Rd1x	AATGATACGGCGACCACCGAGATCTACACggcaacagaaACAC TCTTTCCCTACACGA	(SEQ ID NO:187)
P5.rcRBC017 .Rd1x	AATGATACGGCGACCACCGAGATCTACACagagtgcataACAC TCTTTCCCTACACGA	(SEQ ID NO:188)
P5.rcRBC018 .Rd1x	AATGATACGGCGACCACCGAGATCTACACcactgtcgggaACAC TCTTTCCCTACACGA	(SEQ ID NO:189)
P5.rcRBC019 .Rd1x	AATGATACGGCGACCACCGAGATCTACACgatcgtaacaACAC TCTTTCCCTACACGA	(SEQ ID NO:190)
P5.rcRBC020 .Rd1x	AATGATACGGCGACCACCGAGATCTACACacatgctccaACAC TCTTTCCCTACACGA	(SEQ ID NO:191)
P5.rcRBC021 .Rd1x	AATGATACGGCGACCACCGAGATCTACACgcacattcgaACAC TCTTTCCCTACACGA	(SEQ ID NO:192)
P5.rcRBC022 .Rd1x	AATGATACGGCGACCACCGAGATCTACACggcgatactcACAC TCTTTCCCTACACGA	(SEQ ID NO:193)
P5.rcRBC023 .Rd1x	AATGATACGGCGACCACCGAGATCTACACacgctacatcACAC TCTTTCCCTACACGA	(SEQ ID NO:194)
P5.rcRBC024 .Rd1x	AATGATACGGCGACCACCGAGATCTACACacaatgctcgACAC TCTTTCCCTACACGA	(SEQ ID NO:195)
P5.rcRBC025 .Rd1x	AATGATACGGCGACCACCGAGATCTACActacacgacgcACAC TCTTTCCCTACACGA	(SEQ ID NO:196)
P5.rcRBC026 .Rd1x	AATGATACGGCGACCACCGAGATCTACACagatgcactcACAC TCTTTCCCTACACGA	(SEQ ID NO:197)
P5.rcRBC027 .Rd1x	AATGATACGGCGACCACCGAGATCTACActcatctaggaACAC TCTTTCCCTACACGA	(SEQ ID NO:198)
P5.rcRBC028 .Rd1x	AATGATACGGCGACCACCGAGATCTACActggtatacagACAC TCTTTCCCTACACGA	(SEQ ID NO:199)
P5.rcRBC029 .Rd1x	AATGATACGGCGACCACCGAGATCTACACgcactcaacgACAC TCTTTCCCTACACGA	(SEQ ID NO:200)
P5.rcRBC030 .Rd1x	AATGATACGGCGACCACCGAGATCTACACactgtatgtgACAC TCTTTCCCTACACGA	(SEQ ID NO:201)
P5.rcRBC031 .Rd1x	AATGATACGGCGACCACCGAGATCTACACattcgtgcacACAC TCTTTCCCTACACGA	(SEQ ID NO:202)
P5.rcRBC032 .Rd1x	AATGATACGGCGACCACCGAGATCTACACcataatgcgcACAC TCTTTCCCTACACGA	(SEQ ID NO:203)
P5.rcRBC033 .Rd1x	AATGATACGGCGACCACCGAGATCTACActagattgtcgACAC TCTTTCCCTACACGA	(SEQ ID NO:204)
P5.rcRBC034 .Rd1x	AATGATACGGCGACCACCGAGATCTACActaagaagacgACAC TCTTTCCCTACACGA	(SEQ ID NO:205)

P5.rcRBC035 .Rd1x	AATGATACGGCGACCACCGAGATCTACACacattgagacACAC TCTTTCCTACACGA	(SEQ ID NO:206)
P5.rcRBC036 .Rd1x	AATGATACGGCGACCACCGAGATCTACACagtcggactcACAC TCTTTCCTACACGA	(SEQ ID NO:207)
P5.rcRBC037 .Rd1x	AATGATACGGCGACCACCGAGATCTACACgcaatcaatcACAC TCTTTCCTACACGA	(SEQ ID NO:208)
P5.rcRBC038 .Rd1x	AATGATACGGCGACCACCGAGATCTACActacgattgagACAC TCTTTCCTACACGA	(SEQ ID NO:209)
P5.rcRBC039 .Rd1x	AATGATACGGCGACCACCGAGATCTACACatatacgctgaACAC TCTTTCCTACACGA	(SEQ ID NO:210)
P5.rcRBC040 .Rd1x	AATGATACGGCGACCACCGAGATCTACACaccacgtgacACAC TCTTTCCTACACGA	(SEQ ID NO:211)

[0214] Below is an example of an alpha CDR3 sequence detected in a single stimulated CD4+ T cell analyzed using this protocol:

5 TGTGCTCCTACTAGCAACACAGGCAAATAATCTTT (SEQ ID NO:212)
C A P T S N T G K L I F (SEQ ID NO:213)

The top line is the DNA sequence determined by sequencing. The bottom line is the inferred amino acid sequence. The same CDR3 sequence was detected when single cells from the same population were analyzed by a method similar to that described in Nat Biotechnol. 2014 Jul;32(7):684-92, “Linking T-cell receptor sequence to functional phenotype at the single-cell level,” Han A, Glanville J, Hansmann L, Davis MM.

Example 4: Single-Cell T-Cell Receptor Plus Target Transcript Template Production Using C1 mRNA Seq HT Array IFC

[0215] The protocol is the same as in Example 3 except Target RT primers are added to the Lysis Mix and a template switch oligo (TSO) is added to the RT Mix. The modified recipes are shown below:

Lysis Mix (163.5 µL)

15 µL 1 M Tris-HCl, pH8.4
12 µL 1M dithiothreitol
15 µL 10% NP-40 (0.5% in final rxn)
6.9 µL 10 µM each RT-No Loop primer
6.9 µL 10 µM each Target RT primer
3 µL 800 units/mL (20 mg/mL) proteinase K (8 units/mL in final rxn)
25 8.2 µL C1 Loading Reagent (Fluidigm 100-5170)
96.5 µL H₂O
Transfer 130 µL Lysis Mix to Inlet L2 (big reservoir) during the set up for the Final Reaction.

RT Mix (26 µL)

10 µL 5× RT Buffer
5 µL 20 µM TSO.Rd1.1
1 µL 25 mM each dNTPs

- 1 μL 18 mM AAPF Proteinase K inhibitor (360 μM in final rxn)
 1 μL 200 units/μL SuperScript II[®] Reverse Transcriptase
 1.3 μL C1 Loading Reagent (Fluidigm 100-5170)
 6.7 μL H₂O
 5 Transfer 20 μL RT Mix to Inlet RT during the set up for the Final Reaction.

[0216] The sequence of the template switch oligo TSO.Rd1.1 is /5'-

Biotin/rArCrArCrUrCrUrUrUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCr

UrNrNrNrHrHrHrHrGrGrG/3'-Amino Modifier/, r indicates a ribonucleotide, rN is rA,

- 10 rC, rG, or rU, and rH is rA, rC, or rU (SEQ ID NO:214). The sequences for Target RT primers for 96 target transcripts are:

ACTB.RT1	gtgactggagttcagacgtgtgctcttccgatctTCGTGCC CACATAGGAA	(SEQ ID NO:215)
B2M.RT1	gtgactggagttcagacgtgtgctcttccgatctCAACTTCA ATGTCGGATGGATGAA	(SEQ ID NO:216)
BCL6.RT1	gtgactggagttcagacgtgtgctcttccgatctTCAACAGA GCTCAATTCTCGGAA	(SEQ ID NO:217)
BTLA.RT1	gtgactggagttcagacgtgtgctcttccgatctCCAAGCAT GGCAGGCAA	(SEQ ID NO:218)
CBLB.RT1	gtgactggagttcagacgtgtgctcttccgatctGGAGTGGG ATCGCTGAGAA	(SEQ ID NO:219)
CCL3.RT1	gtgactggagttcagacgtgtgctcttccgatctCTCGTCTC AAAGTAGTCAGCTATGAA	(SEQ ID NO:220)
CCL4.RT1	gtgactggagttcagacgtgtgctcttccgatctGAGGCTGC TGGTCTCATAGTAA	(SEQ ID NO:221)
CCL5.RT1	gtgactggagttcagacgtgtgctcttccgatctCACTTGCC ACTGGTGTAGAAA	(SEQ ID NO:222)
CCR1.RT1	gtgactggagttcagacgtgtgctcttccgatctGACCACCA GGATGTTTCCAA	(SEQ ID NO:223)
CCR10.RT1	gtgactggagttcagacgtgtgctcttccgatctAGAGTCAG GGCCAGCAA	(SEQ ID NO:224)
CCR2.RT1	gtgactggagttcagacgtgtgctcttccgatctTTGGGTTG AGGTCTCCAGAA	(SEQ ID NO:225)
CCR3.RT1	gtgactggagttcagacgtgtgctcttccgatctTCTCCAAT ACAACCTCAGCAGTGAA	(SEQ ID NO:226)
CCR4.RT1	gtgactggagttcagacgtgtgctcttccgatctCTTGATGC CTTCTTTGGTGCAA	(SEQ ID NO:227)
CCR5.RT1	gtgactggagttcagacgtgtgctcttccgatctGCCAGATG AGCTGTGCAA	(SEQ ID NO:228)
CCR6.RT1	gtgactggagttcagacgtgtgctcttccgatctGCAGCGGT AGCAGGAAA	(SEQ ID NO:229)
CCR7.RT1	gtgactggagttcagacgtgtgctcttccgatctACGTCCTT CTTGAGCACAA	(SEQ ID NO:230)
CCR8.RT1	gtgactggagttcagacgtgtgctcttccgatctGAGCAACT TGCCATTTGTCTGAA	(SEQ ID NO:231)
CCR9.RT1	gtgactggagttcagacgtgtgctcttccgatctTGAAGTTA ACGTAGTCTTCCATGGAA	(SEQ ID NO:232)
CD160.RT1	gtgactggagttcagacgtgtgctcttccgatctCCTTGGCT TCAGTCTCCTGAA	(SEQ ID NO:233)
CD27.RT1	gtgactggagttcagacgtgtgctcttccgatctAGAACGCA CAGCCACCA	(SEQ ID NO:234)
CD274.RT1	gtgactggagttcagacgtgtgctcttccgatctACAATTAG TGCAGCCAGGTCTAA	(SEQ ID NO:235)

CD276.RT1	gtgactggagttcagacgtgtgctcttccgatctCTGTGAGG CGGCTGACA	(SEQ ID NO:236)
CD28.RT1	gtgactggagttcagacgtgtgctcttccgatctTGAAGGGA ATAAGTTGAGAGCCAA	(SEQ ID NO:237)
CD3E.RT1	gtgactggagttcagacgtgtgctcttccgatctCCCGACTG CATCTTTGTTTCAT	(SEQ ID NO:238)
CD4.RT1	gtgactggagttcagacgtgtgctcttccgatctGGACCTGA GCCCCACAGAA	(SEQ ID NO:239)
CD40LG.RT1	gtgactggagttcagacgtgtgctcttccgatctCCAACCTT CTATGAAGATACACAGCA	(SEQ ID NO:240)
CD44.RT1	gtgactggagttcagacgtgtgctcttccgatctAGCGAAGG ACACACCCAA	(SEQ ID NO:241)
CD5.RT1	gtgactggagttcagacgtgtgctcttccgatctCTGGCACT TCGAGTTGGAA	(SEQ ID NO:242)
CD69.RT1	gtgactggagttcagacgtgtgctcttccgatctTGGCCAC TGATAAGGCAA	(SEQ ID NO:243)
CD80.RT1	gtgactggagttcagacgtgtgctcttccgatctGGTGTTCC TGGGTCTCAA	(SEQ ID NO:244)
CD8A.RT1	gtgactggagttcagacgtgtgctcttccgatctGCGCTGCT GACCTCATT	(SEQ ID NO:245)
CSF2.RT1	gtgactggagttcagacgtgtgctcttccgatctCGGCTCCT GGAGGTCAA	(SEQ ID NO:246)
CTLA4.RT1	gtgactggagttcagacgtgtgctcttccgatctAGGGCCAG GTCCTGGTA	(SEQ ID NO:247)
CXCL8.RT1	gtgactggagttcagacgtgtgctcttccgatctGTTCTTTA GCACTCCTTGCAA	(SEQ ID NO:248)
CXCR3.RT1	gtgactggagttcagacgtgtgctcttccgatctCGGTGCAA GTTCAGGCTGAA	(SEQ ID NO:249)
CXCR4.RT1	gtgactggagttcagacgtgtgctcttccgatctGCCACAA TGCCAGTTAAGAA	(SEQ ID NO:250)
CXCR5.RT1	gtgactggagttcagacgtgtgctcttccgatctCCAATCTG TCCAGTTCCCAGAA	(SEQ ID NO:251)
CXCR6.RT1	gtgactggagttcagacgtgtgctcttccgatctAGGCTCTG CAACTTATGGTAGAA	(SEQ ID NO:252)
EBI3.RT1	gtgactggagttcagacgtgtgctcttccgatctGAGCCTGT ACGTGGCAA	(SEQ ID NO:253)
EGR2.RT1	gtgactggagttcagacgtgtgctcttccgatctTCCTTTTG CCCTCCACACTTAA	(SEQ ID NO:254)
EGR3.RT1	gtgactggagttcagacgtgtgctcttccgatctGAAAAGCA TGCGAGAGGGAAA	(SEQ ID NO:255)
EOMES.RT1	gtgactggagttcagacgtgtgctcttccgatctCGTCCTTT CCGGAAGGAAA	(SEQ ID NO:256)
FAS.RT1	gtgactggagttcagacgtgtgctcttccgatctAGCTTCCC CAACTCCGTA	(SEQ ID NO:257)
FOXP3.RT1	gtgactggagttcagacgtgtgctcttccgatctCTGAGGCT TTGGGTGCA	(SEQ ID NO:258)
GATA3.RT1	gtgactggagttcagacgtgtgctcttccgatctCTGCAATT CTGCGAGCCA	(SEQ ID NO:259)
GGT1.RT1	gtgactggagttcagacgtgtgctcttccgatctTGTTACCT CCCTCTGCCTCTA	(SEQ ID NO:260)
GZMA.RT1	gtgactggagttcagacgtgtgctcttccgatctAGTTACAG TGAGCTGCAGTCAA	(SEQ ID NO:261)
GZMB.RT1	gtgactggagttcagacgtgtgctcttccgatctGCAGCTGT CAGCACGAA	(SEQ ID NO:262)
HAVCR2.RT1	gtgactggagttcagacgtgtgctcttccgatctGGACACCT CTGTTAGGCACA	(SEQ ID NO:263)
HLA-DRA.RT1	gtgactggagttcagacgtgtgctcttccgatctCCTGATTG GTCAGGATTCAGATAGAA	(SEQ ID NO:264)
ICOS.RT1	gtgactggagttcagacgtgtgctcttccgatctTCCTTTTG TCTTAGTGAGATCGCA	(SEQ ID NO:265)

IFNG.RT1	gtgactggagttcagacgtgtgctcttccgatctACATATGG GTCCTGGCAGTAA	(SEQ ID NO:266)
IL10.RT1	gtgactggagttcagacgtgtgctcttccgatctCCTCCAGC AAGGACTCCTTTAA	(SEQ ID NO:267)
IL12A.RT1	gtgactggagttcagacgtgtgctcttccgatctGGCACTGC AGGGACACA	(SEQ ID NO:268)
IL12B.RT1	gtgactggagttcagacgtgtgctcttccgatctCCTCACTG CTCTGGTCCAA	(SEQ ID NO:269)
IL13.RT1	gtgactggagttcagacgtgtgctcttccgatctGCTGTCAG GTTGATGCTCCATA	(SEQ ID NO:270)
IL15.RT1	gtgactggagttcagacgtgtgctcttccgatctTGGGCTGG CTATCTGCTAA	(SEQ ID NO:271)
IL16.RT1	gtgactggagttcagacgtgtgctcttccgatctTGCAGTCA GAAATCCTAACCCAA	(SEQ ID NO:272)
IL17A.RT1	gtgactggagttcagacgtgtgctcttccgatctCATTGCGG TGGAGATTCCAA	(SEQ ID NO:273)
IL2.RT1	gtgactggagttcagacgtgtgctcttccgatctTGGCCTTC TTGGGCATGTAA	(SEQ ID NO:274)
IL2RA.RT1	gtgactggagttcagacgtgtgctcttccgatctGCCAGGCA CCATGATGAA	(SEQ ID NO:275)
IL2RB.RT1	gtgactggagttcagacgtgtgctcttccgatctTGTTGGCT CTCGAGTTGTAGAA	(SEQ ID NO:276)
IL4.RT1	gtgactggagttcagacgtgtgctcttccgatctAGCAAAGA TGTCTGTTACGGTCAA	(SEQ ID NO:277)
IL5.RT1	gtgactggagttcagacgtgtgctcttccgatctCCAGTGTG CCTATTCCTGAA	(SEQ ID NO:278)
IL6.RT1	gtgactggagttcagacgtgtgctcttccgatctGGCGGCTA CATCTTTGGAA	(SEQ ID NO:279)
IL7.RT1	gtgactggagttcagacgtgtgctcttccgatctCTGGATGA GGACCAGAGGAA	(SEQ ID NO:280)
IL7R.RT1	gtgactggagttcagacgtgtgctcttccgatctTCCAACTG GCTATAGCATGAGAA	(SEQ ID NO:281)
IL9.RT1	gtgactggagttcagacgtgtgctcttccgatctCATCTGAG ACAGTCTCTCACTGAA	(SEQ ID NO:282)
ITCH.RT1	gtgactggagttcagacgtgtgctcttccgatctTGGTGAGG CTACCCATTGAA	(SEQ ID NO:283)
ITGA2.RT1	gtgactggagttcagacgtgtgctcttccgatctGAGACCAA CATTGTAGGCCAAA	(SEQ ID NO:284)
ITGAE.RT1	gtgactggagttcagacgtgtgctcttccgatctCTGGTGAC CAGGAGCCA	(SEQ ID NO:285)
ITGAL.RT1	gtgactggagttcagacgtgtgctcttccgatctCCATCACA GTGATGCAGGAA	(SEQ ID NO:286)
ITGAX.RT1	gtgactggagttcagacgtgtgctcttccgatctGGACCACG CTGTCTCCAA	(SEQ ID NO:287)
KLRG1.RT1	gtgactggagttcagacgtgtgctcttccgatctGCTAGGAC AGCTGGCACAA	(SEQ ID NO:288)
NEDD4.RT1	gtgactggagttcagacgtgtgctcttccgatctCCGGCTAT AACTCTTACTCTCACAA	(SEQ ID NO:289)
PECAM1.RT1	gtgactggagttcagacgtgtgctcttccgatctGGGTCAGC AGGACTCCAA	(SEQ ID NO:290)
PPIA.RT1	gtgactggagttcagacgtgtgctcttccgatctTGAAAGCA GGAACCCTTATAACCAA	(SEQ ID NO:291)
PRDM1.RT1	gtgactggagttcagacgtgtgctcttccgatctCGTACCCA CACGTTTTTCCAA	(SEQ ID NO:292)
PRF1.RT1	gtgactggagttcagacgtgtgctcttccgatctCTGCGCTT GCACTCTGA	(SEQ ID NO:293)
PTPRC.RT1	gtgactggagttcagacgtgtgctcttccgatctCAAATGCC AAGAGTTTAAGCCACAA	(SEQ ID NO:294)
RNF128.RT1	gtgactggagttcagacgtgtgctcttccgatctACAGTCAC ATAGGCACTCATTGAA	(SEQ ID NO:295)

RORC.RT1	gtgactggagttcagacgtgtgctcttccgatctGCACCCCT CACAGGTGATAA	(SEQ ID NO:296)
RPS3.RT1	gtgactggagttcagacgtgtgctcttccgatctACATTCTG TGTTCTGGTGGCTAA	(SEQ ID NO:297)
RUNX1.RT1	gtgactggagttcagacgtgtgctcttccgatctGCATCGTG GACGTCTCTAGAA	(SEQ ID NO:298)
RUNX3.RT1	gtgactggagttcagacgtgtgctcttccgatctGAGAAGCG GGAAAGCAGAA	(SEQ ID NO:299)
SELL.RT1	gtgactggagttcagacgtgtgctcttccgatctGTGCTCTG ACATTTCATGGAAA	(SEQ ID NO:300)
SNCA.RT1	gtgactggagttcagacgtgtgctcttccgatctCTCCCAGT TCTCCGCTCA	(SEQ ID NO:301)
TBX21.RT1	gtgactggagttcagacgtgtgctcttccgatctGTCAGCAT GTCTCCGCAA	(SEQ ID NO:302)
TGFB1.RT1	gtgactggagttcagacgtgtgctcttccgatctCGGCAACG GAAAAGTCTCAA	(SEQ ID NO:303)
TIGIT.RT1	gtgactggagttcagacgtgtgctcttccgatctGTGGAGGA GAGGTGACATTGTAA	(SEQ ID NO:304)
TNF.RT1	gtgactggagttcagacgtgtgctcttccgatctCTCCACGT CCCGGATCAT	(SEQ ID NO:305)
TNFRSF18.RT1	gtgactggagttcagacgtgtgctcttccgatctGTCCGTTC CCGTCCCAA	(SEQ ID NO:306)
TNFRSF9.RT1	gtgactggagttcagacgtgtgctcttccgatctTGGCACAG GTATGATACTAGCAAA	(SEQ ID NO:307)
UBB.RT1	gtgactggagttcagacgtgtgctcttccgatctGTCACCTA TCACCCCTCACGTA	(SEQ ID NO:308)
VEGFA.RT1	gtgactggagttcagacgtgtgctcttccgatctGGAAGTAG AGCAATCTCCCCAA	(SEQ ID NO:309)
VTCN1.RT1	gtgactggagttcagacgtgtgctcttccgatctCCAGCTGA GGCGACAGTA	(SEQ ID NO:310)

Example 5: Single-Cell T-Cell Receptor Template Production Using Hairpin Primers

[0217] This Example describes the adaptation of an illustrative method of single-cell T-cell receptor template production designed for tubes to Fluidigm’s C₁[™] High-Throughput IFC (“HT-IFC”).

[0218] The method, as designed for tubes, is shown schematically in Figure 11A-11B. During the reverse transcriptase (RT) step (Figure 11A), the primers Rd2.TRAC and Rd2.TRBC hybridize to the TCR α and β mRNAs, respectively, and are extended to generate TCR-specific cDNA. The primer Rd2.N7.T20VN (Rd2-UMI-oligo-dT, in Figure 11A) generates cDNA from all the polyA⁺ mRNA in the sample. The primer P7.rcCBC001.Rd2x (P7-CBC1-20-Rd2, in Figure 11A) is present in the reaction, but it is not used until the PCR step. During the initial cycle of PCR (Figure 11B), the primers Rd1.TRAV.L1 (42 different primers; Rd1-TRAV, in Figure 11B), Rd1.TRBV.L1 (31 different primers; Rd1-TRBV, in Figure 11B), and Rd1.Target.L1 (96 different primers; Rd1-Target, in Figure 11) generate primer extension products on TCR- α cDNA, TCR- β

cDNA and 96 target cDNAs, respectively. In order to enhance specificity, these primers are designed as Super Selective primers (Marras S, Vargas-Gold D, Tyagi S, Kramer FR, PCT/US2014/015351; Vargas DY, Kramer FR, Tyagi S, Marras SA, “Multiplex Real-Time PCR Assays that Measure the Abundance of Extremely Rare Mutations Associated with Cancer,” PLoS One 11:e0156546, 2016). At this point, the DNA strands have either an Rd1 or an Rd2 5' tail and can be amplified with the outer primers P5.rcRBC001.Rd1x (P5-RBC11-40-Rd1, in Figure 11B) and P7.rcCBC001.Rd2x (P7-CBC11-20-Rd2, in Figure 11B). This procedure produces libraries predominantly 350-450 bp in size. When sequenced, these libraries generate the desired data, namely TCR sequences and sequences from the 96 target transcripts.

[0219] When this procedure is used with HT IFCs, though, the libraries include a significant percentage of non-specific fragments generally smaller than 350 bp. This seems to be due to sub-optimal mixing of the PCR reagents with the RT reaction. In the PCR mix, the primer P5.rcRBC001.Rd1x (or one of its barcode counterparts) is present at high concentration with the DNA polymerase and all the other components required for PCR. When the PCR mix is loaded into the IFC, mixing with the RT reaction occurs largely by diffusion. Because the primers in the RT reaction are smaller than the cDNA, these primers diffuse faster and are the first to encounter P5.rcRBC001.Rd1x and the other primers in the PCR mix. Thus, spurious products involving the various primers have a chance to form before any specific products involving the cDNA are made. This problem can be partially overcome by moving the primer P5.rcRBC001.Rd1x to the RT reaction. When this is done, libraries generated in HT IFCs do have the desired products in the 350-450 bp size range, but there still are a lot of non-specific products 250 bp and smaller.

[0220] The residual non-specific products being generated seem to be due to rehydration steps used during PCR. In the standard mRNA seq protocol developed for the HT IFC, rehydration is performed every 2nd cycle of PCR. In the TCR protocol, by changing the rehydration from every 2nd cycle to every 4th cycle, the ratio of specific products (350 to 450 bp) to non-specific products (smaller than 250 bp) improves. Increasing to rehydration every 8th cycle improves the ratio more, but the effect is marginal. One explanation is that, when the reaction is lowered to 25°C, the primers have the opportunity to form non-specific products. What was needed is a way to turn the primers “off” at lower temperatures. This was done by using hairpin primers that each consist of a blunt-ended, self-complementary stem (a “stem-duplex”) connected by a loop

(Lao KQ, Straus NA, Livak KJ, "Hot start reverse transcription by primer design," U. S. Patent 8,993,240). The use of hairpin primers addresses another issue. Having too many primers in the RT reaction can also lead to formation of non-specific products. The hairpins used here are designed to be closed at the 42°C of the RT reaction, but open at the 5 62°C annealing temperature used in the PCR. Thus, these primers are not functional during the RT reaction or during the rehydration steps in PCR when the temperature of the HT-IFC is lowered to 25°C.

[0221] The following primers were replaced with hairpin versions (denoted .HP): P5.rcRBC001-040.Rd1x (40 primers), P7.rcCBC001-020.Rd2x (20 primers), 10 Rd1.TRAV.L1 (42 primers), Rd1.TRBV.L1 (31 primers), and Rd1.Target.L1 (96 primers). Using the .HP versions of these primers, specific TCR libraries in the size range 350-450 bp are generated in the HT-IFC from single cells captured in the HT IFC with only minimal amounts of non-specific fragments. This strategy is shown schematically in Figure 12A-12B. A TCR library was prepared from a mixed pool of stimulated CD4+ or 15 CD8+ T cells. Approximately 400 DSP-fixed single cells were captured and processed on a HT IFC according to the protocol given below. As shown in Figure 13, this method produces libraries predominantly 350-450 bp in size.

Protocol:

Purchases

20

Thermo Fisher Scientific

SuperScript® II Reverse Transcriptase

18064-014

25

10% NP-40

PI-28324

dNTP Mix, 25mM each

FERR1121

30

Yeast tRNA (10 mg/mL)

AM7119

WO 2018/013723

PCT/US2017/041770

New England BioLabs

Proteinase K

P8107S

- 5 NEBNext® Q5® Hot Start HiFi PCR Master Mix
M0543S

EMD Millipore

AAPF Proteinase K inhibitor

- 10 539470

Teknova

1 M Tris-HCl, pH8.4

T1084

- 15 1M DTT (Dithiothreitol)
D9750

DNA Suspension Buffer

- 20 T0221

Beckman Coulter

AMPure XP Beads

A63880

- 25

Stocks5× RT Buffer

- 30 75 mM Tris-HCl, pH 8.4
375 mM KCl
50 mM MgCl₂
25% glycerol (50%, Teknova G1796, G1797, G1798, G1799)

- 35 18 mM AAPF Proteinase K inhibitor

Dissolve 10 mg AAPF Proteinase K inhibitor in 1 mL DMSO

Store at -20°C

C1 Mixes**20× Column Mixes**

Mix:

187.5 µL Cell Rinsing Reagent (Fluidigm)

5 12.5 µL C1™ Loading Reagent (Fluidigm 100-5170)

Dispense 8 µL to each of 20 wells

Add 2 µL 50 µM P7.rcCBC001-020.Rd2x.HP (1 µM in PCR), with a different barcode added to each well

10 Before Cell Loading and Staining, transfer 9 µL of each to one of the 20 Inlets labeled as “Column inlets.” The barcode mix will happen after cell staining.

2× Lysis Mix (245.4 µL)

45 µL 1 M Tris-HCl, pH8.4

36 µL 1 M DTT

15 45 µL 10% NP-40 (0.5% in final rxn)

20.7 µL 10 µM each AC.A1/BC.A1 primers (110 nM in RT rxn)

18.9 µL 10 µM Rd2.N7.T20VN (100 nM in RT rxn)

9 µL 800 units/mL (20 mg/mL) proteinase K (8 units/mL in final rxn)

33.8 µL C1™ Loading Reagent (Fluidigm 100-5170)

20 37 µL H₂O**40× Row Mixes**

Dispense 5 µL 2× Lysis Mix to each of 40 wells

25 Add 5 µL 17 µM P5.rcRBC001-040.Rd1x.HP (1 µM in PCR), with a different barcode added to each well

Transfer 9 µL of each to Inlets labeled as “Row Barcode”

RT Mix (26 µL)

10 µL 5× RT Buffer

30 1 µL 25 mM each dNTPs

1 µL 18 mM AAPF Proteinase K inhibitor (360 µM in final rxn)

1 µL 200 units/µL SuperScript II Reverse Transcriptase

1.3 µL C1™ Loading Reagent (Fluidigm 100-5170)

11.7 µL H₂O

35 Transfer 20 µL RT Mix to Inlet RT

PCR Mix (150 µL)

- 54.6 µL 5× PreAmp Master Mix (Fluidigm 100-5581)
- 2.7 µL 5 µM each TRAV.L1.HP/TRBV.L1.HP primers (50 nM each primer in final rxn)
- 2.7 µL 5 µM each Target.L1.HP primers (50 nM each primer in final rxn)
- 5 7.5 µL C1™ Loading Reagent (Fluidigm 100-5170)
- 82.5 µL H₂O

Transfer 130 µL PCR Mix to Inlet L2 (big reservoir).

Harvest Mix (200 µL)

- 10 194 µL C1 Harvest Reagent (Fluidigm 100-7081)
- 6 µL 10 mg/mL yeast tRNA
- 180 µL to the Harvest Inlet

15 **C1 Steps**

1. Prime:
 - a. Pipette mixes following the scTCR small_v1xx v5 prime loading map. For Valve Fluid, add Tween 20 to a final concentration of 0.05%, or use Valve Fluid v2.
 - 20 b. Pipette 9 µL each column mix to 20 column inlets
 - c. Run prime script: "scTCR small: 26min Prime with Column Barcode (1911x)"
2. Cell loading:
 - 25 a. Replace liquid following the scTCR small_v1xx v5.
 - b. Keep the column mixes in the 20 column inlets
 - c. Dispense 20 µL cell suspension to each of the two cell inlets
 - d. Dispense 20 µL cell rinse reagent to each of the two cell stain inlets
 - 30 e. Run script "scTCR small: 36min, Cell Load with column barcode (1911x)"
3. Final reaction:
 - a. Replace cell related liquid following the scTCR small_v1xx v5.
 - b. Pipette lysis mixes into 40 row inlets, ~9 µL each
 - c. Pipette RT mix into RT inlet, ~20 µL
 - 35 d. Pipette PCR mix into L2 zone (up right corner), ~130 µL
 - e. Check the entire reagent, such as harvest reagent, before inserting IFC into machine.
 - f. Run script "scTCR small:7hr50min Chemistry v5D (1911x)". Set time delay as usual.
 - 40

Final Reaction consists of:

1. Lysis Mix to chambers 0+1
 - 50°C, 30 min (1800 sec)
 - 72°C, 1 min (60 sec)
 - 45 10°C, 1 min (60 sec)

2. RT Mix to chambers 0+1+2
42°C, 90 min (5400 sec)
85°C, 5 min (300 sec)

- 5 3. PCR Mix to chambers 0+1+2+3
95°C, 2 min (120 sec)
22 cycles: 95°C, 5 sec
62°C, 5 min (360 sec)

4. Harvest

10

Post-C1 Steps

Transfer the 20 Harvest samples to a 96-well plate.

Combine 2.5 µL of each sample.

15

Clean up with 40 µL AMPure XP Beads. Elute with 50 µL DNA Suspension Buffer.

Clean up with 40 µL AMPure XP Beads. Elute with 20 µL DNA Suspension Buffer.

Add:

- 20 2.5 µL 10 µM P5 Primer
2.5 µL 10 µM P7 Primer
25 µL Q5 Hot Start HiFi PCR Master Mix

- 25 98°C, 30 sec
21 cycles: 98°C, 10 sec
62°C, 2 min
75°C, 2 min
4°C, hold

- 30 Clean up with 35 µL AMPure XP Beads. Elute with 30 µL DNA Suspension Buffer.
Clean up with 21 µL AMPure XP Beads. Elute with 20 µL DNA Suspension Buffer.....
Check 1 µL on Bioanalyzer.

35

Primers:

Name	Sequence	SEQ ID NO
Rd2 . TRAC	gtgactggagttcagacgctgtgctcttccgatctT GAATAGGCAGACAGACTTGTCA	(SEQ ID NO:311)
Rd2 . TRBC	gtgactggagttcagacgctgtgctcttccgatctC ACACCAGTGTGGCCTTT	(SEQ ID NO:312)
Rd2 . N7 . T20VN	gtgactggagttcagacgctgtgctcttccgatctN HNNNHNTTTTTTTTTTTTTTTTTTTTTTVN	(SEQ ID NO:313)
TRAV01 . L1 . HP	ACCATCCGacactctttccctacacgacgctcttc cgatctGCGRAGCACCCACATTTCTKTCTTACAAa tcaacatcaacGGATGGT	(SEQ ID NO:314)
TRAV02 . L1 . HP	CTGCTGATTGacactctttccctacacgacgctct tccgatctACCAAGACTCCTTGTTAAAGGCTCAA tcaaccaacaatTCAGCAG	(SEQ ID NO:315)
TRAV03 . L1 . HP	CTTCAGGATTGTacactctttccctacacgacgct cttccgatctGGCTTTGAAGCTGAATTTAACAAGA GCCAAtaacaatCCTGAAG	(SEQ ID NO:316)
TRAV04 . L1 . HP	CTCAGAGAAGacactctttccctacacgacgctct tccgatctCCCTGTTTATCCCTGCCGACAGAAaca acttCTCTGAG	(SEQ ID NO:317)
TRAV05 . L1 . HP	GTTTATCCTAactctttccctacacgacgctcttc cgatctACATGAAACAAGACCAAAGACTCACTGTT CTAcaccttttAGGATAAAC	(SEQ ID NO:318)
TRAV06 . L1 . HP	AAACTCTGGGacactctttccctacacgacgctct tccgatctAGAAAGACTGAAGGTCACCTTTGATAC CAaaacacctccCAGAGTTT	(SEQ ID NO:319)
TRAV07 . L2 . HP	GTACAAGCATacactctttccctacacgacgctct tccgatctAGCAGAAAGGAAGACTAAATGCTACAT TACTGAtctcatGCTTGTAC	(SEQ ID NO:320)
TRAV08 . L11 . HP	GGTTTCACAGacactctttccctacacgacgctct tccgatctARGMATCAACGGTTTTGAGGCTGAAcc ctttctGTGAAACC	(SEQ ID NO:321)
TRAV08 . L12 . HP	TCAGCCTAGacactctttccctacacgacgctctt ccgatctGGGATCCACTGGTTAAAGGCATCAAttc ataccctAGGCTGA	(SEQ ID NO:322)
TRAV08 . L13 . HP	TCCTCAGTAGTTGacactctttccctacacgacgc tcttccgatctGCTTTGAGGCTGAATTTAAGAGGA GTCAAcactaCTGAGGA	(SEQ ID NO:323)
TRAV08 . L14 . HP	GACTCTTGAAacactctttccctacacgacgctct tccgatctARGMATCAACGGTTTTGAGGCTGAAac	(SEQ ID NO:324)

	atttcaacattCAAGAGTC	
TRAV9.L11.HP	GAACTGAGAGacactctttccctacacgacgctct tccgatctACCGTAAAGAAACCACTTCTTTCCACT TGctctttttctCTCAGTTC	(SEQ ID NO:325)
TRAV9.L12.HP	GAACTGAGAAacactctttccctacacgacgctct tccgatctCCACATACCGTAAGGAAACCACTTCTT TCCAacctttCTCAGTTC	(SEQ ID NO:326)
TRAV10.L1.HP	GCTTTGCAAATacactctttccctacacgacgctc ttccgatctCGAACGGAAGATATACAGCAACTCTG GATcatatttGCAAAGC	(SEQ ID NO:327)
TRAV12.L11.HP	GCTGGCTGGGacactctttccctacacgacgctct tccgatctTGGTAATGAAGATGGAAGGTTTACAGC ACAaacattcccAGCCAGC	(SEQ ID NO:328)
TRAV12.L12.HP	GCTGGCTAAGacactctttccctacacgacgctct tccgatctRACAAAGAAGATGGAAGGTTTACAGCA CAaacattcttAGCCAGC	(SEQ ID NO:329)
TRAV12.L13.HP	TGCTGGATAAGacactctttccctacacgacgctc ttccgatctRACAAAGAAGATGGAAGGTTTACAGC ACAaacattcttATCCAGCA	(SEQ ID NO:330)
TRAV13.L11.HP	CTGTCTTGGAAGTacactctttccctacacgacgc tcttccgatctGGCGAAAAGAAAGACCAACGAATT GCTtcacttcCAAGACAG	(SEQ ID NO:331)
TRAV13.L12.HP	CACTGTCGGGacactctttccctacacgacgctct tccgatctGCAAGGCCAAAGAGTCACCGTTTTAAc cttttcccGACAGTG	(SEQ ID NO:332)
TRAV14.L1.HP	TCTTGCCAAacactctttccctacacgacgctctt ccgatctTGCAACAGAAGGTCGCTACTCATTGAAa acaatcttGGCAAGA	(SEQ ID NO:333)
TRAV16.L1.HP	GTGGAAAGGAacactctttccctacacgacgctct tccgatctCATCAAAGGCTTCACTGCTGACCTTAA atatcCTTTCCAC	(SEQ ID NO:334)
TRAV17.L1.HP	GAACTGCAGGAacactctttccctacacgacgctc ttccgatctTGGAAGATTAAGAGTCACGCTTGACA CTcaattcctGCAGTTC	(SEQ ID NO:335)
TRAV18.L1.HP	TGGAAGGTTGacactctttccctacacgacgctct tccgatctAGAGGTTTTTCAGGCCAGTCCTATCAAc ttccaaCCTTCCA	(SEQ ID NO:336)
TRAV19.L1.HP	GTTGAAGGTGGacactctttccctacacgacgctc ttccgatctTGGTCGGTATTCTTGGAACCTCCAGA AtcatccaCCTTCAAC	(SEQ ID NO:337)

TRAV20.L1.HP	CTTCAGGAGAAcactctttccctacacgacgctct tccgatctAAGAAGGAAAGCTTTCTGCACATCACA caaaactctCCTGAAG	(SEQ ID NO:338)
TRAV21.L1.HP	GTACTACGAGAAcactctttccctacacgacgctc ttccgatctTGGAAGACTTAATGCCTCGCTGGATA AtcatttctCGTAGTAC	(SEQ ID NO:339)
TRAV22.L1.HP	GAAATGTACGTAGacactctttccctacacgacgc tcttccgatctGCCACGACTGTCGCTACGGAAact acGTACATTTC	(SEQ ID NO:340)
TRAV23.L1.HP	TCCATGATTAacactctttccctacacgacgctct tccgatctACAATCTCCTTCAATAAAAGTGCCAAG CAccataATCATGGA	(SEQ ID NO:341)
TRAV24.L1.HP	GGATCCTAATacactctttccctacacgacgctct tccgatctCACTCTTAATACCAAGGAGGGTTACAG CTAacaattAGGATCC	(SEQ ID NO:342)
TRAV25.L1.HP	CATCTGTATAGacactctttccctacacgacgctc ttccgatctTCCCTGCACATCACAGCCACaatcta aatctaTACAGATG	(SEQ ID NO:343)
TRAV26.L11.HP	TCAGCGTGTGacactctttccctacacgacgctct tccgatctACAGAAAGTCCAGCACCTTGATCCTca atacacACGCTGA	(SEQ ID NO:344)
TRAV26.L12.HP	TCAGCGAAacactctttccctacacgacgctcttc cgatctAGCAATGTGAACAACAGAATGGCCTacaa tcattTCGCTGA	(SEQ ID NO:345)
TRAV27.L1.HP	CTTCAGCAAGAAacactctttccctacacgacgct cttccgatctGGTGACAGTAGTTACGGGTGGAGAA cacttttcttGCTGAAG	(SEQ ID NO:346)
TRAV29.L1.HP	GGCACAATAacactctttccctacacgacgctct tccgatctTCTTAAACAAAAGTGCCAAGCACCTCT acacaatatTTGTGCC	(SEQ ID NO:347)
TRAV30.L1.HP	GTTCTCCTTTacactctttccctacacgacgctct tccgatctTGAAGCACCCGTCTTCCTGATGATAcc taaaGGAGAAC	(SEQ ID NO:348)
TRAV34.L1.HP	TGGGCTGTTGacactctttccctacacgacgctct tccgatctAGAAAAAGCAGCAAAGTTCCTGCATA catacaaCAGCCCA	(SEQ ID NO:349)
TRAV35.L1.HP	CTTTCTGGAAacactctttccctacacgacgctct tccgatctACCTCAAATGGAAGACTGACTGCTCAT caccttCCAGAAAG	(SEQ ID NO:350)
TRAV36.L1.HP	GAGTCTCGTTacactctttccctacacgacgctct	(SEQ ID NO:351)

	tccgatctAGCATCCTGAACATCACAGCCACaatt tataacGAGACTC	
TRAV38.L1.HP	CGATTCTCGTAacactctttccctacacgacgctc ttccgatctCGTTATTCGCCAAGAAGCTTATAAGC AACActtattacGAGAATCG	(SEQ ID NO:352)
TRAV39.L1.HP	GGTATCAAGATGacactctttccctacacgacgct cttccgatctGCAGTGAAGCAGGAGGGACGATTAA actaaaacatCTTGATACC	(SEQ ID NO:353)
TRAV40.L1.HP	GAGTTTTTGTcactctttccctacacgacgctc ttccgatctAGCAAAAACCTTCGGAGGCGGAAtctc acctttGACAAAACCTC	(SEQ ID NO:354)
TRAV41.L1.HP	TCTGGGAATTacactctttccctacacgacgctct tccgatctCACAGCTCCCTGCACATCACaaca aatTCCCAGA	(SEQ ID NO:355)
TRBV02.L1.HP	GCTGAGTAAAGacactctttccctacacgacgctc ttccgatctTCACTCTGAAGATCCGGTCCACAAtc aatctttACTCAGC	(SEQ ID NO:356)
TRBV03.L1.HP	GATGTGAAGTactctttccctacacgacgctct tccgatctCCAAATCGMTTCTCACCTAAATCTCCA GACAAtcttaCTTCACATC	(SEQ ID NO:357)
TRBV04.L1.HP	GAGAGCATAAacactctttccctacacgacgctct tccgatctGCCAAGTCGTTCTCACCTGAAacaat tatGCTCTC	(SEQ ID NO:358)
TRBV05.L11.HP	CCTGAGATAGTTacactctttccctacacgacgct cttccgatctTCAGTGAGACACAGAGAAACAAAGG AAACTcaactaTCTCAGG	(SEQ ID NO:359)
TRBV05.L12.HP	CGAGTCTAGAAAGGacactctttccctacacgacg ctcttccgatctCTCTGAGCTGAATGTGAACGCCT TcctttctaGACTCG	(SEQ ID NO:360)
TRBV06.L11.HP	GGAGACAAATacactctttccctacacgacgctct tccgatctGTACCACTGRCAAAGGAGAAGTCCCat tattTGTCTCC	(SEQ ID NO:361)
TRBV06.L12.HP	TCAGCCcactctttccctacacgacgctcttcc gatctACATGTMCTGGTATCGACAAGACCCAtcat actcatacGGGCTGA	(SEQ ID NO:362)
TRBV07.L11.HP	TGTCGGTTAGacactctttccctacacgacgctct tccgatctTGATCCAATTTAGGTCATACTGCCCT catacacctaACCGACA	(SEQ ID NO:363)
TRBV07.L12.HP	CATGACCATGTGacactctttccctacacgacgct cttccgatctGACRGGATGTAGCTCTCAGGTGTGA	(SEQ ID NO:364)

	TaattcacatGGTCATG	
TRBV07.L13.HP	GATCCCTAGTTAGacactctttccctacacgacgc tcttccgatctGCYCAGTGATCGGTTCTCTGCAct ctaactAGGGATC	(SEQ ID NO:365)
TRBV07.L14.HP	GATCCCTAGTTAGacactctttccctacacgacgc tcttccgatctTGCTCAGTGATCGGATCTCTGCAC tctaactAGGGATC	(SEQ ID NO:366)
TRBV09.L1.HP	GCAAGTCGacactctttccctacacgacgctcttc cgatctACATTCTTGAACGATTCTCCGCACAAatc acaaacGACTTGC	(SEQ ID NO:367)
TRBV10.L11.HP	AACACCATGAacactctttccctacacgacgctct tccgatctCATGGGCTGAGGCTGATCCAcatacac atcATGGTGTT	(SEQ ID NO:368)
TRBV10.L12.HP	AGCAGCTTAcactctttccctacacgacgctcttc cgatctGGACATGGGCTGAGGCTGATCTAactaca tcaAGCTGCT	(SEQ ID NO:369)
TRBV10.L13.HP	AACACCATGAacactctttccctacacgacgctct tccgatctGCATGGGCTGAGGCTAATCCAcataca catcATGGTGTT	(SEQ ID NO:370)
TRBV11.L1.HP	CTACTCCAAAacactctttccctacacgacgctct tccgatctCAGTTGCCTAAGGATCGATTTTCTGCA tcttttGGAGTAG	(SEQ ID NO:371)
TRBV12.L11.HP	GAATCGAAGGAGTAcactctttccctacacgacgc tcttccgatctACAACGTTCCGATAGATGATTCAG GGATcaaactcctTCGATTC	(SEQ ID NO:372)
TRBV12.L12.HP	AGTGGCTTGacactctttccctacacgacgctctt ccgatctTCGATTCTCAGCAGAGATGCCTGATcat tataacaAGCCACT	(SEQ ID NO:373)
TRBV13.L1.HP	GAGCTGATTGacactctttccctacacgacgctct tccgatctGCAGAGCGATAAAGGAAGCATCCCTtt cactcaaTCAGCTC	(SEQ ID NO:374)
TRBV14.L1.HP	CTCCAGTAGAacactctttccctacacgacgctct tccgatctTCCGGTATGCCCAACAATCGATTCTTA caacttttctACTGGAG	(SEQ ID NO:375)
TRBV15.L1.HP	GTGTTCGTGacactctttccctacacgacgctctt ccgatctAGCAGACACCCCTGATAACTTCCAAaaa ttccaCGAACAC	(SEQ ID NO:376)
TRBV16.L1.HP	CACTTAGCATAcactctttccctacacgacgctct tccgatctCTTTGATGAAACAGGTATGCCCAAGGA AtctacacatGCTAAGTG	(SEQ ID NO:377)

TRBV18.L1.HP	GGGAAATTCTTGacactctttccctacacgacgct cttccgatctGAGTCAGGAATGCCAAAGGAACGAT caaacaacaaGAATTTCCC	(SEQ ID NO:378)
TRBV19.L1.HP	GATTCCTTCAAGacactctttccctacacgacgct cttccgatctGAGATATAGCTGAAGGGTACAGCGT CTacactcttGAAGGAATC	(SEQ ID NO:379)
TRBV20.L1.HP	CATGGTTGGAacactctttccctacacgacgctct tccgatctGCAAGGCGTCGAGAAGGACAAatacaac atcCAACCATG	(SEQ ID NO:380)
TRBV24.L1.HP	GAGAATTTAGacactctttccctacacgacgctct tccgatctTCTCTGATGGATACAGTGTCTCTCGAC ActatatctaCTAAATTCTC	(SEQ ID NO:381)
TRBV25.L1.HP	GAAAATGCAAacactctttccctacacgacgctct tccgatctCTTTCCTCTGAGTCAACAGTCTCCAGA ActtcacttGCATTTTC	(SEQ ID NO:382)
TRBV27.L1.HP	GGAAATTCAAGacactctttccctacacgacgctc ttccgatctTTCCTGAAGGGTACAAAGTCTCTCGA AttcttcttGAATTTCC	(SEQ ID NO:383)
TRBV28.L1.HP	TCCTTTTTCAacactctttccctacacgacgctct tccgatctGGGCTACGGCTGATCTATTTCTCATAT GATtcatttGAAAAAGGA	(SEQ ID NO:384)
TRBV29.L1.HP	TGATGGGTTGacactctttccctacacgacgctct tccgatctAGGCCACATATGAGAGTGGATTTGTCA TcttatcaaCCCATCA	(SEQ ID NO:385)
TRBV30.L1.HP	GTCCTGGTTAacactctttccctacacgacgctct tccgatctTGAGGTGCCCCAGAATCTCTCAtaact aaCCAGGAC	(SEQ ID NO:386)
ACTB.L1.HP	TTGGGAGTTTGacactctttccctacacgacgct cttccgatctCCCCACTTCTCTCTAAGGAGAATGG aatccaaaCTCCCAA	(SEQ ID NO:387)
BCL6.L1.HP	TTCCGTGTGTacactctttccctacacgacgctct tccgatctCGTACAACGTGTCCTCACGGTtaacca acaCACGGAA	(SEQ ID NO:388)
BTLA.L1.HP	TTTGAGAGTGacactctttccctacacgacgctct tccgatctCAGATGAACACACGAAATTGAACAGTT CCacatcttcaCTCTCAAA	(SEQ ID NO:389)
CBLB.L1.HP	TTCCCAGTGGacactctttccctacacgacgctct tccgatctTCAGGTACTACCTGTTGACCTGGAtaa ccaCTGGGAA	(SEQ ID NO:390)
CCL22.L1.HP	TTCTCACTTATacactctttccctacacgacgctc	(SEQ ID NO:391)

	ttccgatctACTCCCTGAACCCAGCCTGtatttca taAGTGAGAA	
CCL3.L1.HP	TTAAGAAGATAAGAGacactctttccctacacgac gctcttccgatctGGTTGTTGCCAAACAGCCACAa acctcttaTCTTCTTAA	(SEQ ID NO:392)
CCL4.L1.HP	TGCGGAGacactctttccctacacgacgctcttcc gatctCCATGAGACACATCTCCTCCATACTCtctt acaaaCTCCGCA	(SEQ ID NO:393)
CCL5.L1.HP	TTGACAAAGTGacactctttccctacacgacgctc ttccgatctCCCAACTAAAGCCTAGAAGAGCTTCT ttctacacaCTTTGTCAA	(SEQ ID NO:394)
CCR1.L1.HP	TTTGGGTATAacactctttccctacacgacgctct tccgatctAGAAAAACCCCAAACACTGACATTACC TAataccaataTACCCAAA	(SEQ ID NO:395)
CCR10.L1.HP	TTGTCCCTTTacactctttccctacacgacgctct tccgatctCTCCCACGGAGACCCACAtcaaacaaa GGGACAA	(SEQ ID NO:396)
CCR2.L1.HP	TTGGGAGAGAacactctttccctacacgacgctct tccgatctGGCTTCTAGAACCAGGCAACTTGtctt actctCTCCCAA	(SEQ ID NO:397)
CCR3.L1.HP	GAAGCAAATGacactctttccctacacgacgctct tccgatctGCAGCGTACTCATCATCAACCCTtttt caTTTGCTTC	(SEQ ID NO:398)
CCR4.L1.HP	TTGCCAGTacactctttccctacacgacgctcttc cgatctGGCAGCTTTTTCTCTCCCACTAGACTtcc aatcaCTGGCAA	(SEQ ID NO:399)
CCR5.L1.HP	TTGCTTCCATacactctttccctacacgacgctct tccgatctCTGAATATGAACGGTGAGCATTTGTGGa accatcatGGAAGCAA	(SEQ ID NO:400)
CCR7.L1.HP	TTTCCCTTTAacactctttccctacacgacgctct tccgatctCTCTTGCTCCACTGGGATGGttcttt aAAGGGAAA	(SEQ ID NO:401)
CCR8.L1.HP	TTTACACAGTGacactctttccctacacgacgct cttccgatctGGCATGCTAGTAGCAGTGAGCAttc tccactGTGTGAAA	(SEQ ID NO:402)
CCR9.L1.HP	TAGAGTAAGTTGGacactctttccctacacgacgc tcttccgatctGCTGGAAGGCTATTTACTTCCATG CTaacaaccaaCTTACTCTA	(SEQ ID NO:403)
CD160.L1.HP	TTGTAGTCGTGTTacactctttccctacacgacgc tcttccgatctGGAAGAGGAAGATTTGTGCAGACC	(SEQ ID NO:404)

	AAGtcataaacacGACTACAA	
CD244.L1.HP	TGGCCTGTGTacactctttccctacacgacgctct tccgatctTGGAATCTAGACACTATGCTGGGTTC aacttaacaCAGGCCA	(SEQ ID NO:405)
CD27.L1.HP	TGACAAGATTacactctttccctacacgacgctct tccgatctCTGGCAGCCACAACCTGCAtcaaataca TCTTGTCa	(SEQ ID NO:406)
CD274.L1.HP	TTACAAATCAactctttccctacacgacgctcttc cgatctAGTCTATTCCCTAAGTCCTAACTCCTCCTT GatcacacaatGATTTGTAA	(SEQ ID NO:407)
CD276.L1.HP	TTCCTGGAGACTacactctttccctacacgacgct cttccgatctGCAGAAAAGGCAGAGCCTGGtagtc tCCAGGAA	(SEQ ID NO:408)
CD28.L1.HP	TTGAGCAATTacactctttccctacacgacgctct tccgatctTGGCTTGCCTCGTCACCCacattccaa TTGCTCAA	(SEQ ID NO:409)
CD3E.L1.HP	TTACCCAGAAacactctttccctacacgacgctct tccgatctCCCCTTTTGCAGCCCTCTCTtccttac ttCTGGGTAA	(SEQ ID NO:410)
CD4.L1.HP	TTGTAACCAAacactctttccctacacgacgctct tccgatctCCTCCAGACCATTCAGGACACAGtctt tcattGGTTACAA	(SEQ ID NO:411)
CD40LG.L1.HP	TAGGGACATTacactctttccctacacgacgctct tccgatctAGGCCGTTGCTAGTCAGTTCTCaagaa aTGTCCCTA	(SEQ ID NO:412)
CD44.L1.HP	TACACAAAGactctttccctacacgacgctcttcc gatctAGAATAAGAATAACATGGTCCATTACCTT TATGTaccctaCTTTGTGTA	(SEQ ID NO:413)
CD5.L1.HP	TTCCCAGATAacactctttccctacacgacgctct tccgatctAGCCCAGGTCACAGATCTTCCaagatt atCTGGGAA	(SEQ ID NO:414)
CD69.L1.HP	TATTTCTAGATacactctttccctacacgacgct cttccgatctGGAAAATGTGCAATATGTGATGTGG CAttcacaaatcTAGGAAATA	(SEQ ID NO:415)
CD80.L1.HP	TTACACAGTGacactctttccctacacgacgctct tccgatctGCTACCTCACTATGCTGCTTCACAAta acacacaCTGTGTAA	(SEQ ID NO:416)
CD8A.L1.HP	TAAGCAAGTacactctttccctacacgacgctctt ccgatctGCCCAAACCTGCTGTCCCAAACtccatac aaCTTGCTTA	(SEQ ID NO:417)

CSF2.L1.HP	TTGGTCCAAAGGacactctttccctacacgacgct cttccgatctACAAGAGCTAGAAACTCAGGATGGT CtcaacctttGGACCAA	(SEQ ID NO:418)
CTLA4.L1.HP	TTCCTAGAGTTAGacactctttccctacacgacgc tcttccgatctGCTTACTCCAGGAGACCCACAGtc tactaacTCTAGGAA	(SEQ ID NO:419)
CXCL8.L1.HP	TAGTTCTATGAGTacactctttccctacacgacgc tcttccgatctTTGGTAGTGCTGTGTTGAATTACG GAtcttactcaTAGAACTA	(SEQ ID NO:420)
CXCR3.L1.HP	TTGGCAGacactctttccctacacgacgctcttcc gatctTCTGAGGACTGCACCATTGCTtacaacatt CTGCCAA	(SEQ ID NO:421)
CXCR4.L1.HP	TTCTAGCTAAGacactctttccctacacgacgctc ttccgatctCTGAACATTCCAGAGCGTGTAGTGAt cataccttAGCTAGAA	(SEQ ID NO:422)
CXCR5.L1.HP	TTCCTCGAATacactctttccctacacgacgctct tccgatctGCTCACGTGAGAGTGTCTTCACGtatt ctattCGAGGAA	(SEQ ID NO:423)
CXCR6.L1.HP	TTGCACATAacactctttccctacacgacgctctt ccgatctGCTGATAATTCCAGTGGTCCATGGAtca tcctATGTGCAA	(SEQ ID NO:424)
EBI3.L1.HP	TTCTCAGCacactctttccctacacgacgctcttc cgatctCCGACTTTTCCCTTTGAGCCTCtcaacaa tGCTGAGAA	(SEQ ID NO:425)
EGR2.L1.HP	TAGGCAGTGacactctttccctacacgacgctctt ccgatctATCACTCCCTGAGTTTAGTATGGCTGat ctccatccaCTGCCTA	(SEQ ID NO:426)
EGR3.L1.HP	TTGGCATTGacactctttccctacacgacgctctt ccgatctTCACCAACATTTTCATTTGCTCCTTTGTC tctacceaatcAATGCCAA	(SEQ ID NO:427)
EOMES.L1.HP	TAAGAAGTATTGacactctttccctacacgacgct cttccgatctGGAGAGTTTCATCATCCCCATGATA TTTGGctccaaTACTTCTTA	(SEQ ID NO:428)
FAS.L1.HP	TGCTTTCATAacactctttccctacacgacgctct tccgatctAGGCTATTTGCAGAAGGAGCTCAatct cataTGAAAGCA	(SEQ ID NO:429)
FOXP3.L1.HP	TGTGCGTTacactctttccctacacgacgctcttc cgatctCCTGTTAGAATTCACCTGTGTATCTCACG atctccataACGCACA	(SEQ ID NO:430)
GATA3.L1.HP	TACCCTCTTacactctttccctacacgacgctctt	(SEQ ID NO:431)

	ccgatctCATCTGTCTTGTCCCTATTCCTGCAtaa ctacaaGAGGGTA	
GGT1.L1.HP	TTGCACCTGTacactctttccctacacgacgctct tccgatctCCAGATCGCGTCCACCTTctcacacaa caGGTGCAA	(SEQ ID NO:432)
GZMA.L1.HP	TTCCAGAAAGacactctttccctacacgacgctct tccgatctCCGAGGTGGAAGAGACTCGTGattctt tctTTCTGGAA	(SEQ ID NO:433)
HAVCR2.L1.HP	TTCTGTGATGGacactctttccctacacgacgctc ttccgatctACTTCTGTATTCTGTGGACCAAACCTGA AGactataaccaTCACAGAA	(SEQ ID NO:434)
HLA-DRA.L1.HP	TTAGAGTACTTGTacactctttccctacacgacgc tcttccgatctCCCCAAGTGTGGATATGCCTCTTC ttcatacaaGTACTCTAA	(SEQ ID NO:435)
ICOS.L1.HP	TGCAAACATGacactctttccctacacgacgctct tccgatctGACTGGCTTTGCACAGGTGTcaaactt caTGTTTGCA	(SEQ ID NO:436)
IFNG.L1.HP	TAAACACACacactctttccctacacgacgctctt ccgatctGCAGCCAACCTAAGCAAGATCCatctct ccaaGTGTGTTTA	(SEQ ID NO:437)
IL10.L1.HP	TTCCATCTGAATAAGacactctttccctacacgac gctcttccgatctAGGCTGAGGCAAGAGAATTGCT ccttattcAGATGGAA	(SEQ ID NO:438)
IL12A.L1.HP	TTAAAGTGAAactctttccctacacgacgctcttc cgatctGCTAAGAAGGGAAAATATCCATCCTGAAG GatcaaccatTTCACTTTAA	(SEQ ID NO:439)
IL12B.L1.HP	TTCTCACATTacactctttccctacacgacgctct tccgatctTCAGAATGGCAGGTGGCTTCTcttatc aaaTGTGAGAA	(SEQ ID NO:440)
IL13.L1.HP	TTCTGCCAGAAcactctttccctacacgacgctct tccgatctCCTCATCCGAGGCAGGGTatcttctct GGCAGAA	(SEQ ID NO:441)
IL15.L1.HP	TTAGTTTTCTGacactctttccctacacgacgctc ttccgatctCCAGGGAAATCAAAAGATTGGATGCC aactcatcaGAAAACCTAA	(SEQ ID NO:442)
IL16.L1.HP	TTAGGGATTGTAAGacactctttccctacacgacg ctcttccgatctGCGTGCTCAGTTCAGAATCACTT CactcttacaATCCCTAA	(SEQ ID NO:443)
IL17A.L1.HP	TTAATCTCCGacactctttccctacacgacgctct tccgatctGTCCAGTTTCTCCCCTAGACTCAGtac	(SEQ ID NO:444)

	caacacGGAGATTAA	
IL2.L1.HP	TAGAAGGCGacactctttccctacacgacgctctt ccgatctACTGACTTGATAATTAAGTGCTTCCCAC TTctcgtcGCCTTCTA	(SEQ ID NO:445)
IL2RA.L1.HP	TGAGATTCGAacactctttccctacacgacgctct tccgatctCTCCCCCTTCAGGTATATGTTTTCTGA GcttttctGAATCTCA	(SEQ ID NO:446)
IL2RB.L1.HP	TTCCATTTGTTTactctttccctacacgacgct cttccgatctCCTCTCTGCAAGTCGGTCTCCactc aaaaaCAAATGGAA	(SEQ ID NO:447)
IL4.L1.HP	TTCAGGAAAGTTactctttccctacacgacgct cttccgatctCAGTTCCACAGGCACAAGCAGaact caactTTCCTGAA	(SEQ ID NO:448)
IL5.L1.HP	TCTGGCATGAacactctttccctacacgacgctct tccgatctACTTCAGAGGGAAAGTAAATATTTTCAG GCActactcaTGCCAGA	(SEQ ID NO:449)
IL6.L1.HP	TGGCTCTGTactctttccctacacgacgctct tccgatctTGGAAGTGGCTATGCAGTTTGAATAT CCcaaccacacaAGAGCCA	(SEQ ID NO:450)
IL7.L1.HP	TGACAATGGTTcactctttccctacacgacgctct tccgatctTCACCTAGTCTAAGGATGCTAAACCTT AGTtaccttaacCATTGTCA	(SEQ ID NO:451)
IL7R.L1.HP	TGCATCATAAAGacactctttccctacacgacgct cttccgatctGCCAGGAAGAATATGTGGCAGAGCa aatacctttATGATGCA	(SEQ ID NO:452)
IL9.L1.HP	TTGCCTGacactctttccctacacgacgctcttcc gatctCCTGTGAACAGCCATGCAACCTttaatacc CAGGCAA	(SEQ ID NO:453)
ITCH.L1.HP	TTTGGTCTTTTGTactctttccctacacgacgc tcttccgatctACAGCCCATCTGGATTGTGGTtac cacaaaAGACCAA	(SEQ ID NO:454)
ITGA2.L1.HP	TACAAACATTactctttccctacacgacgctct tccgatctTGTTTTGCCTCTTTCCACAAAACTGT ttcctaATGTTTGTA	(SEQ ID NO:455)
ITGAE.L1.HP	TGATAGCCATTAGacactctttccctacacgacgc tcttccgatctCGAAGAAGAGAATTAGGACCTGCT ATCCtcactaatGGCTATCA	(SEQ ID NO:456)
ITGAL.L1.HP	TTCTTGATGacactctttccctacacgacgctct tccgatctTGGGCAACACAGCGAGACaaaccacaa tcaTCAAGGAA	(SEQ ID NO:457)

ITGAX.L1.HP	TAGAGGGATGacactctttccctacacgacgctct tccgatctGGCAACATTGCTGGCTGGAAGtcttct catCCCTCTA	(SEQ ID NO:458)
KLRG1.L1.HP	TTGAGGCGacactctttccctacacgacgctcttc cgatctTGTGGTTGGATCACCAAATTATCTTAGGT tacttctacttcGCCTCAA	(SEQ ID NO:459)
LCK.L1.HP	TTGTACAGATAGacactctttccctacacgacgct cttccgatctTGTACATGTGTAGCCTGTGCATGTA TGaacatctatCTGTACAA	(SEQ ID NO:460)
NEDD4.L1.HP	TGTCCTTGGAacactctttccctacacgacgctct tccgatctGCGGGACAGTCAGTAGGATCAacactt cCAAGGACA	(SEQ ID NO:461)
PECAM1.L1.HP	TGACCTGAacactctttccctacacgacgctcttc cgatctGTGCATGCGACCCCTTCCTtcacaactCA GGTCA	(SEQ ID NO:462)
PPIA.L1.HP	TTGATTGCAacactctttccctacacgacgctctt ccgatctCCCACCTTAACAGACCTCTAGGGTTacc ttaaatGCAATCAA	(SEQ ID NO:463)
PRDM1.L1.HP	TTCACATGATacactctttccctacacgacgctct tccgatctTCACCATCCATCCTTCTCTTTTCTGCa caactacacatCATGTGAA	(SEQ ID NO:464)
PRF1.L1.HP	TTGGCCTAacactctttccctacacgacgctcttc cgatctAGATCACAGCTTCAGCCAGGAGacttcat ctAGGCCAA	(SEQ ID NO:465)
PTPRC.L1.HP	TAATGGTTGTacactctttccctacacgacgctct tccgatctACCAGGAATGGATGTCGCTAATCATtt cttttcaCAACCATTA	(SEQ ID NO:466)
RNF128.L1.HP	TAGCTCTAGacactctttccctacacgacgctctt ccgatctGGTGCCTTAATATAAAGTTTGAAGCTTC ATCCAaaccttcTAGAGCTA	(SEQ ID NO:467)
RORC.L1.HP	TGTCCTCAGAACacactctttccctacacgacgctct tccgatctGTCATTGGGCACCGCTGACcaatcatc tGAGGACA	(SEQ ID NO:468)
RPS3.L1.HP	TTGACCCTacactctttccctacacgacgctcttc cgatctCCAAGGGATTTGCATCTGTGGATTCAtaa ttaccaGGGTCAA	(SEQ ID NO:469)
RUNX1.L1.HP	TATCTAGAATGGacactctttccctacacgacgct cttccgatctTGAGAGAATATCCCAGAACCCTCTC TaattctccaTTCTAGATA	(SEQ ID NO:470)
RUNX3.L1.HP	TATGTCACTGGacactctttccctacacgacgctc	(SEQ ID NO:471)

	ttccgatctTGAGCCTGTCAGTAGTGGGTACattc aaccaGTGACATA	
SELL.L1.HP	TTCATTGTactctttccctacacgacgctcttccg atctTGGAAAGAGTTAAAACAGGTGGAGAAATTCCa cctcaaactcaCACAAATGAA	(SEQ ID NO:472)
SNCA.L1.HP	TTGGGAGGTATGacactctttccctacacgacgct cttccgatctGGATCAATCCAGTCCTAGGTTTATT TTGCAttacatacCTCCCAA	(SEQ ID NO:473)
TBX21.L1.HP	TTTGAGCATTTTTGacactctttccctacacgacg ctcttccgatctGACTTTGGACAGCTGGCCTGtca caaaaaTGCTCAAA	(SEQ ID NO:474)
TGFB1.L1.HP	TTCATTAATGacactctttccctacacgacgctct tccgatctGAGCACCTTGGGCACTGTTGttcaaaa tCATTAATGAA	(SEQ ID NO:475)
TIGIT.L1.HP	TGGTGGAAGTactctttccctacacgacgctct tccgatctGACCATTTCATAGTTGGATTCCTGGAG AatctacactTCCACCA	(SEQ ID NO:476)
TNF.L1.HP	TTACGGGTGTactctttccctacacgacgctct tccgatctGGAGCTGCCTTGGCTCAGAataccaca caCCGTGAA	(SEQ ID NO:477)
TNFRSF18.L1.HP	TGGTCACATATGTactctttccctacacgacgc tcttccgatctAGCAGAAAGTGGGTGCAGGAAtcat cacatatGTGACCA	(SEQ ID NO:478)
TNFRSF9.L1.HP	TAGTCATCATGacactctttccctacacgacgctc ttccgatctGGAAGATATCACTCTGACGGAAAGTT TTGActatcatGATGACTA	(SEQ ID NO:479)
UBB.L1.HP	TTCCAGCATGacactctttccctacacgacgctct tccgatctCCAGCAGAGGCTCATCTTTGctccatt catGCTGGAA	(SEQ ID NO:480)
VEGFA.L1.HP	TGTATATTGTGacactctttccctacacgacgctc ttccgatctTCCCCAGCACACATTCCTTTGAttct tccaCAATATACA	(SEQ ID NO:481)
VTCN1.L1.HP	TTCAAAGTGacactctttccctacacgacgctct tccgatctGGCAACAAACATATACCTTCCATGAAG CtatatatatcACTTTTGAA	(SEQ ID NO:482)
P5.rcRBC001.Rd1x. HP	tcgtgtaggAATGATACGGCGACCACCGAGATCTA CACgcaacgtaagACACTCTTTCCCTACACGA	(SEQ ID NO:483)
P5.rcRBC002.Rd1x. HP	tcgtgtaggAATGATACGGCGACCACCGAGATCTA CACgctgctcgaaACACTCTTTCCCTACACGA	(SEQ ID NO:484)
P5.rcRBC003.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:485)

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HP	CACtagaacaacgACACTCTTTCCCTACACGA	
P5.rcRBC004.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:486)
HP	CACacgtcggataACACTCTTTCCCTACACGA	
P5.rcRBC005.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:487)
HP	CACtcagagaatgACACTCTTTCCCTACACGA	
P5.rcRBC006.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:488)
HP	CACatagctcgaaACACTCTTTCCCTACACGA	
P5.rcRBC007.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:489)
HP	CACgacacggataACACTCTTTCCCTACACGA	
P5.rcRBC008.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:490)
HP	CACattgttgcaACACTCTTTCCCTACACGA	
P5.rcRBC009.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:491)
HP	CACcgatagaagaACACTCTTTCCCTACACGA	
P5.rcRBC010.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:492)
HP	CACtaccgtatgaACACTCTTTCCCTACACGA	
P5.rcRBC011.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:493)
HP	CACatggagtctaACACTCTTTCCCTACACGA	
P5.rcRBC012.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:494)
HP	CACagctccgtagACACTCTTTCCCTACACGA	
P5.rcRBC013.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:495)
HP	CACgaatctacagACACTCTTTCCCTACACGA	
P5.rcRBC014.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:496)
HP	CACcaatacgctaACACTCTTTCCCTACACGA	
P5.rcRBC015.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:497)
HP	CACcaacatgagaACACTCTTTCCCTACACGA	
P5.rcRBC016.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:498)
HP	CACggcaacagaaACACTCTTTCCCTACACGA	
P5.rcRBC017.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:499)
HP	CACagagtgcataACACTCTTTCCCTACACGA	
P5.rcRBC018.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:500)
HP	CACcactgtcgggaACACTCTTTCCCTACACGA	
P5.rcRBC019.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:501)
HP	CACgatcgtaacaACACTCTTTCCCTACACGA	
P5.rcRBC020.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:502)
HP	CACacatgctccaACACTCTTTCCCTACACGA	
P5.rcRBC021.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:503)
HP	CACgcacattcgaACACTCTTTCCCTACACGA	
P5.rcRBC022.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:504)
HP	CACggcgatactcACACTCTTTCCCTACACGA	
P5.rcRBC023.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:505)

HP	CACacgctacatcACACTCTTTCCCTACACGA	
P5.rcRBC024.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:506)
HP	CACacaatgctcgACACTCTTTCCCTACACGA	
P5.rcRBC025.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:507)
HP	CACtacacgacgcACACTCTTTCCCTACACGA	
P5.rcRBC026.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:508)
HP	CACagatgcactcACACTCTTTCCCTACACGA	
P5.rcRBC027.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:509)
HP	CACtcatctaggaACACTCTTTCCCTACACGA	
P5.rcRBC028.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:510)
HP	CACtggtatacagACACTCTTTCCCTACACGA	
P5.rcRBC029.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:511)
HP	CACgcactcaacgACACTCTTTCCCTACACGA	
P5.rcRBC030.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:512)
HP	CACactgtatgtgACACTCTTTCCCTACACGA	
P5.rcRBC031.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:513)
HP	CACattcgtgcacACACTCTTTCCCTACACGA	
P5.rcRBC032.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:514)
HP	CACcataatgcgACACTCTTTCCCTACACGA	
P5.rcRBC033.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:515)
HP	CACtagattgtcgACACTCTTTCCCTACACGA	
P5.rcRBC034.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:516)
HP	CACtaagaagacgACACTCTTTCCCTACACGA	
P5.rcRBC035.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:517)
HP	CACacattgagacACACTCTTTCCCTACACGA	
P5.rcRBC036.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:518)
HP	CACagtccgactcACACTCTTTCCCTACACGA	
P5.rcRBC037.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:519)
HP	CACgcaatcaatcACACTCTTTCCCTACACGA	
P5.rcRBC038.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:520)
HP	CACtacgattgagACACTCTTTCCCTACACGA	
P5.rcRBC039.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:521)
HP	CACatatcgctgaACACTCTTTCCCTACACGA	
P5.rcRBC040.Rd1x.	tcgtgtaggAATGATACGGCGACCACCGAGATCTA	(SEQ ID NO:522)
HP	CACaccacgtgacACACTCTTTCCCTACACGA	
P7.rcCBC001.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATcg	(SEQ ID NO:523)
HP	cgactgaaGTGACTGGAGTTCAGACGT	
P7.rcCBC002.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATag	(SEQ ID NO:524)
HP	catcgataGTGACTGGAGTTCAGACGT	
P7.rcCBC003.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATcg	(SEQ ID NO:525)

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HP	acacatggGTGACTGGAGTTCAGACGT	
P7.rcCBC004.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATcg	(SEQ ID NO:526)
HP	actacgcaGTGACTGGAGTTCAGACGT	
P7.rcCBC005.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATca	(SEQ ID NO:527)
HP	ctgctgagGTGACTGGAGTTCAGACGT	
P7.rcCBC006.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATtc	(SEQ ID NO:528)
HP	gctgtacaGTGACTGGAGTTCAGACGT	
P7.rcCBC007.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATcg	(SEQ ID NO:529)
HP	ctgcagtaGTGACTGGAGTTCAGACGT	
P7.rcCBC008.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATag	(SEQ ID NO:530)
HP	acttgcagGTGACTGGAGTTCAGACGT	
P7.rcCBC009.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATgc	(SEQ ID NO:531)
HP	gaactaagGTGACTGGAGTTCAGACGT	
P7.rcCBC010.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATgc	(SEQ ID NO:532)
HP	tagaacagGTGACTGGAGTTCAGACGT	
P7.rcCBC011.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATta	(SEQ ID NO:533)
HP	cgctaacgGTGACTGGAGTTCAGACGT	
P7.rcCBC012.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATca	(SEQ ID NO:534)
HP	gctatccgGTGACTGGAGTTCAGACGT	
P7.rcCBC013.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATgt	(SEQ ID NO:535)
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P7.rcCBC014.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATat	(SEQ ID NO:536)
HP	gatgcatgGTGACTGGAGTTCAGACGT	
P7.rcCBC015.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATga	(SEQ ID NO:537)
HP	atgcatcaGTGACTGGAGTTCAGACGT	
P7.rcCBC016.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATgt	(SEQ ID NO:538)
HP	ctcgacaaGTGACTGGAGTTCAGACGT	
P7.rcCBC017.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATag	(SEQ ID NO:539)
HP	cacgctcaGTGACTGGAGTTCAGACGT	
P7.rcCBC018.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATct	(SEQ ID NO:540)
HP	atcgaacaGTGACTGGAGTTCAGACGT	
P7.rcCBC019.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATaa	(SEQ ID NO:541)
HP	gtcatcagGTGACTGGAGTTCAGACGT	
P7.rcCBC020.Rd2x.	acgtctgaaCAAGCAGAAGACGGCATAACGAGATtt	(SEQ ID NO:542)
HP	cgtcgtgaGTGACTGGAGTTCAGACGT	
P5	AATGATACGGCGACCACCGAGATCTACAC	(SEQ ID NO:543)
P7	CAAGCAGAAGACGGCATAACGAGAT	(SEQ ID NO:544)

CLAIMS

What is claimed is:

1. A method for preparing DNA templates for single-cell transcript sequencing of RNA from a population of cells, the method comprising:
 - 5 distributing cells from the population into separate reaction volumes so that a plurality of separate reaction volumes each comprise a single cell, wherein the cells have been treated with a fixative prior to distribution, wherein the fixative comprises a cell-permeant fixative that enables the production of DNA templates from transcripts derived from single cells with greater efficiency than in the absence of said cell-permeant
 - 10 fixative;
permeabilizing or disrupting each cell in each separate reaction volume;
reverse transcribing cDNA from RNA in each separate reaction volume; and
 - 15 amplifying the cDNA to produce DNA templates, wherein the amplification incorporates one or more nucleotide sequences that facilitate DNA sequencing of the DNA templates.
2. The method of claim 1, wherein the fixative stabilizes the cell nucleus and/or stabilizes RNA.
- 20 3. The method of claim 1, wherein the method comprises the prior treatment of the cells with the fixative.
4. The method of claims 1 or 3, wherein the fixative comprises biomarker and histology preservative (BHP).
5. The method of claims 1 or 3, wherein the fixative comprises
- 25 dithiobis(succinimyl propionate) (DSP).
6. The method of any of claims 1-5, wherein the DNA templates are recovered from the separate reaction volumes in one or more pools of DNA templates.

7. The method of any of claims 1-6, wherein the DNA templates are further amplified after recovery.

8. The method of any of claims 1-7, wherein the method additionally comprises subjecting the DNA templates to DNA sequencing.

5 9. The method of any of claims 1-8, wherein the method comprises preparing DNA templates for single-cell transcript sequencing of T-cell receptor or immunoglobulin RNA from the population, wherein:

the cells comprise T cells or B cells; and

the DNA templates are generated from T-cell receptor or

10 immunoglobulin RNA, respectively.

10. The method of claim 9, wherein the cells are T cells.

11. The method of claim 9, wherein the cells are B cells.

12. The method of claims 10 or 11, wherein the cells are activated.

13. A primer combination for producing DNA templates from RNA
15 encoding T-cell receptor or immunoglobulin chains, the primer combination comprising:

a first reverse transcription (RT) primer having a portion specific for the Constant segment (CS) of an RNA encoding a first chain of a T-cell receptor or immunoglobulin, the T-cell receptor or immunoglobulin also comprising a second chain;

a second reverse transcription (RT) primer having a portion specific
20 for the Constant segment (CS) of an RNA encoding the second chain of the T-cell receptor or immunoglobulin, respectively;

wherein the first and second RT primers each additionally
comprise a first nucleotide tag comprising a first primer binding site for a first
DNA sequencing primer, the first nucleotide tag being 5' of the CS-specific
25 portion; and

a first barcode primer that comprises, from 3' to 5', a portion specific for the first primer binding site, a first barcode nucleotide sequence, and a first sequencing adaptor;

a plurality of first amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the first chain of the T-cell receptor or immunoglobulin;

5 a plurality of second amplification primers, each having a portion specific for a different Variable segment (VS) of the RNA encoding the second chain of the T-cell receptor or immunoglobulin, respectively;

wherein the first and second amplification primers each additionally comprise a second nucleotide tag comprising a second primer binding site for a second DNA sequencing primer, the second nucleotide tag being 5' of the
10 VS-specific portion; and

a second barcode primer that comprises, from 3' to 5', a portion specific for the second primer binding site, a second barcode nucleotide sequence, and a second sequencing adaptor.

14. A primer combination for producing DNA templates from RNA
15 encoding T-cell receptor or immunoglobulin chains, the primer combination comprising:

a first reverse transcription (RT) primer having a portion specific for the Constant segment (CS) of an RNA encoding a first chain of a T-cell receptor or immunoglobulin, the T-cell receptor or immunoglobulin also comprising a second chain;

a second reverse transcription (RT) primer having a portion specific
20 for the Constant segment (CS) of an RNA encoding the second chain of the T-cell receptor or immunoglobulin, respectively;

wherein the first and second RT primers each additionally comprise a first nucleotide tag comprising a first primer binding site for a first DNA sequencing primer, the first nucleotide tag being 5' of the CS-specific
25 portion; and

a first barcode primer that comprises, from 3' to 5', a portion specific for the first primer binding site, a first barcode nucleotide sequence, and a first sequencing adaptor;

a 5' oligonucleotide comprising, from 5' to 3', the second primer
30 binding site for the second DNA sequencing primer, a unique molecular identifier (UMI), and an oligo-riboG sequence; and

a second barcode primer that comprises, from 3' to 5', a portion specific for the second primer binding site, a second barcode nucleotide sequence, and a second sequencing adaptor.

15. A kit comprising the primer combination of claim 13 or claim 14
5 and a matrix-type microfluidic device comprising:
- capture sites arranged in a matrix of R rows and C columns, wherein R and C are integers greater than 1, and wherein the capture sites can be fluidically isolated from one another after distribution of cells to the capture sites;
 - 10 a set of R first input lines configured to deliver the first reagent(s) to capture sites in a particular row;
 - a set of C second input lines configured to deliver second reagent(s) to capture sites in a particular column, wherein said delivery is separate from the delivery first reagent(s),
 - wherein, after a reaction, reaction products can be recovered from
15 the microfluidic device in pools of reaction products from individual rows or columns.

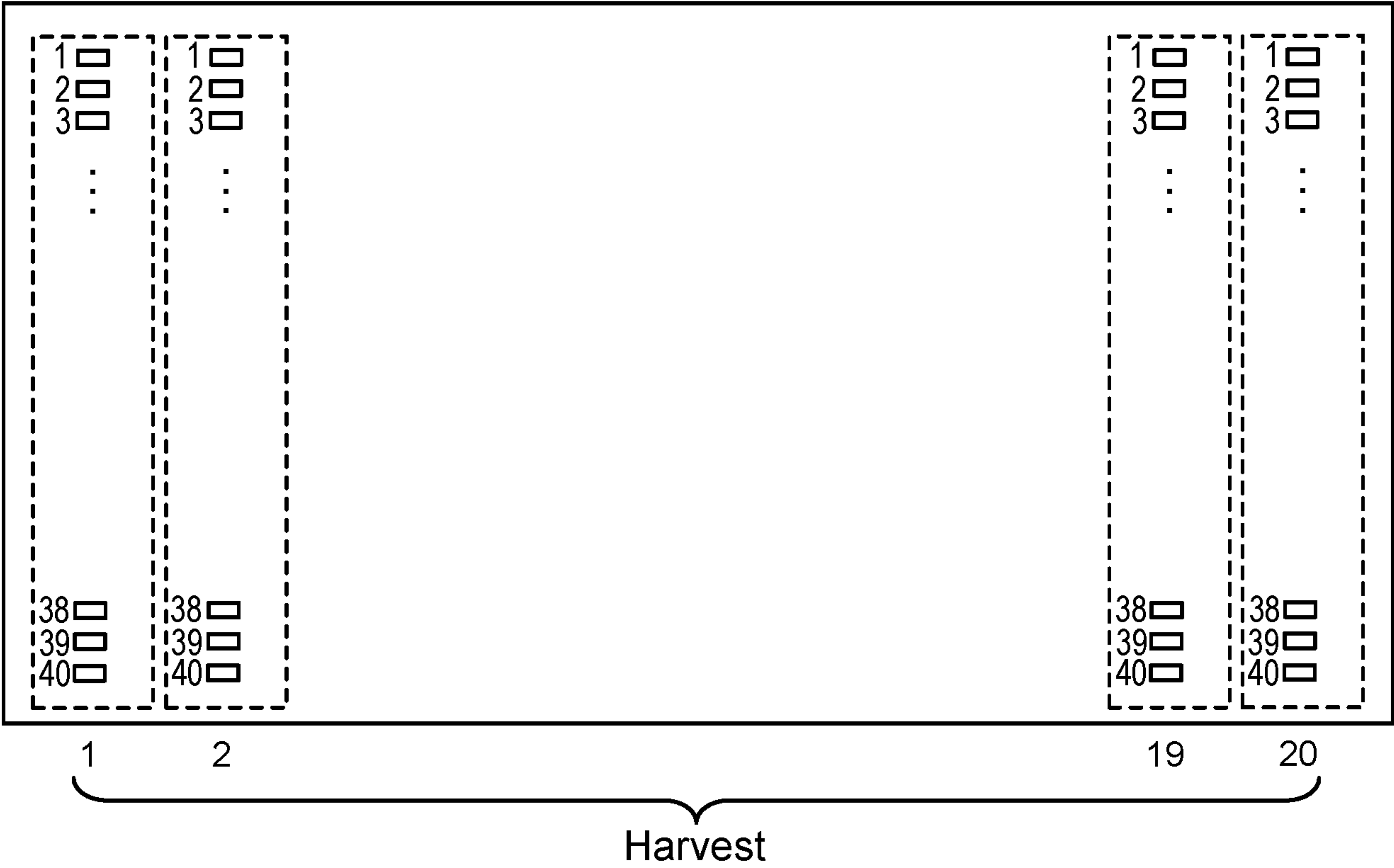


Figure 1A

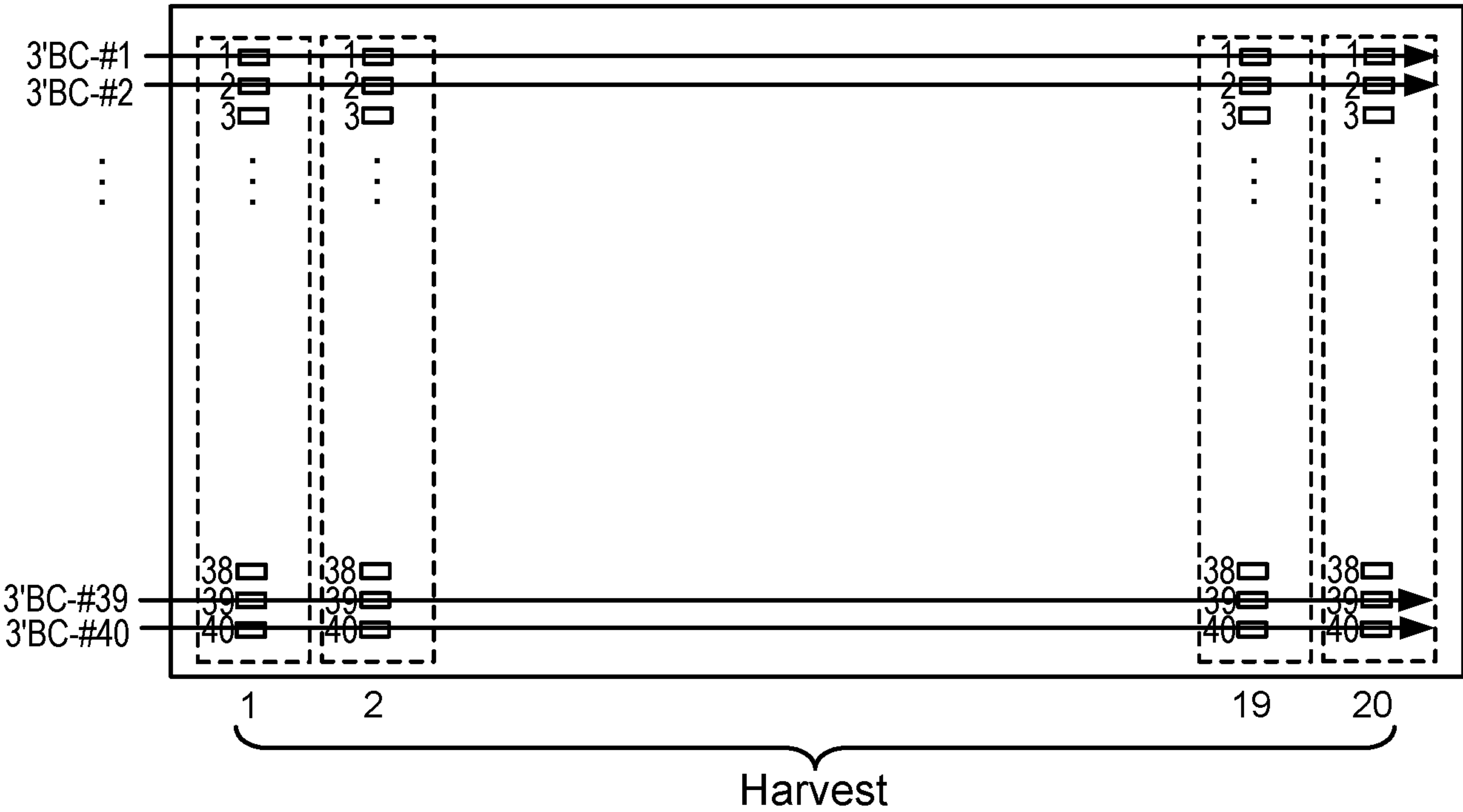


Figure 1B

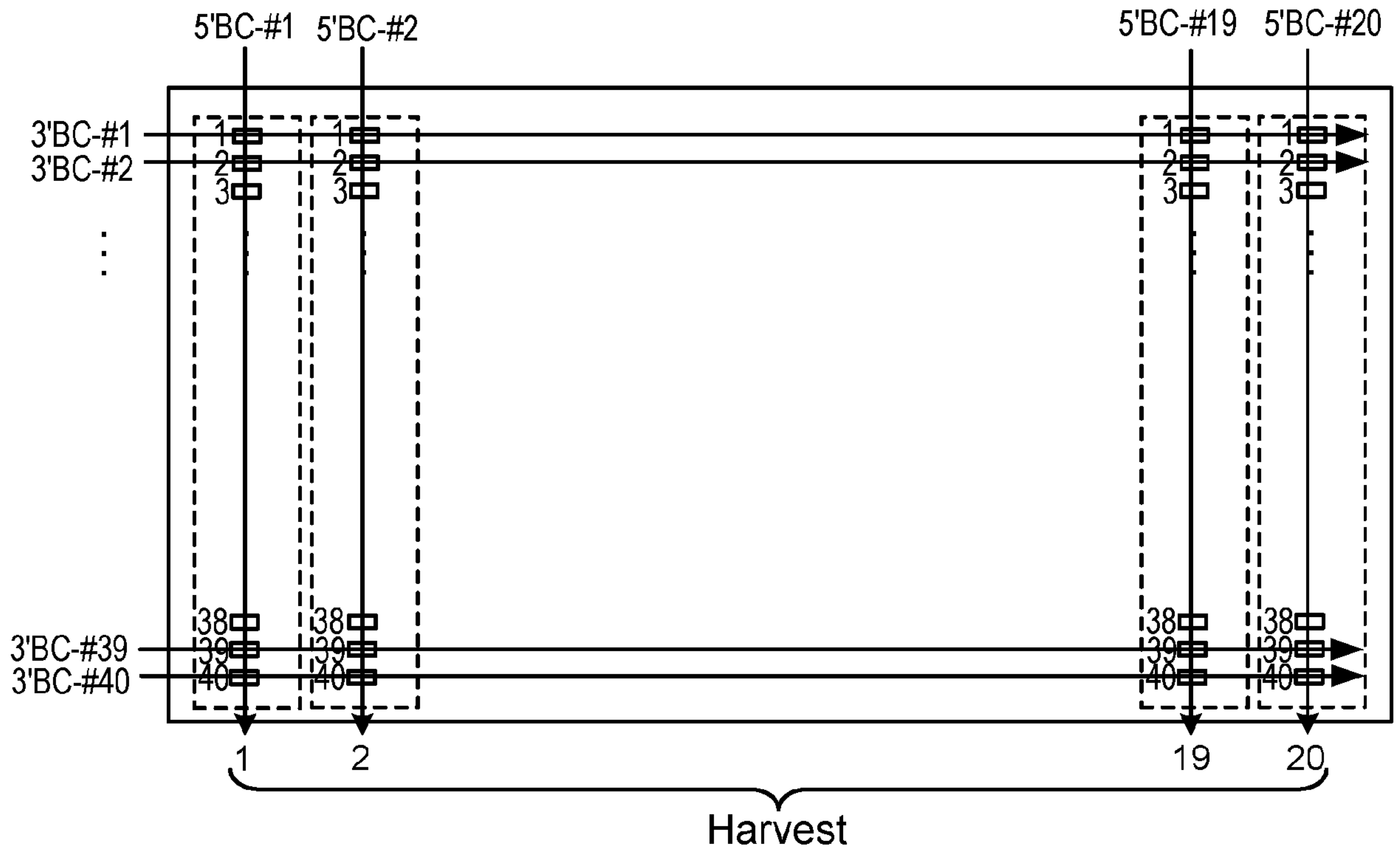


Figure 1C

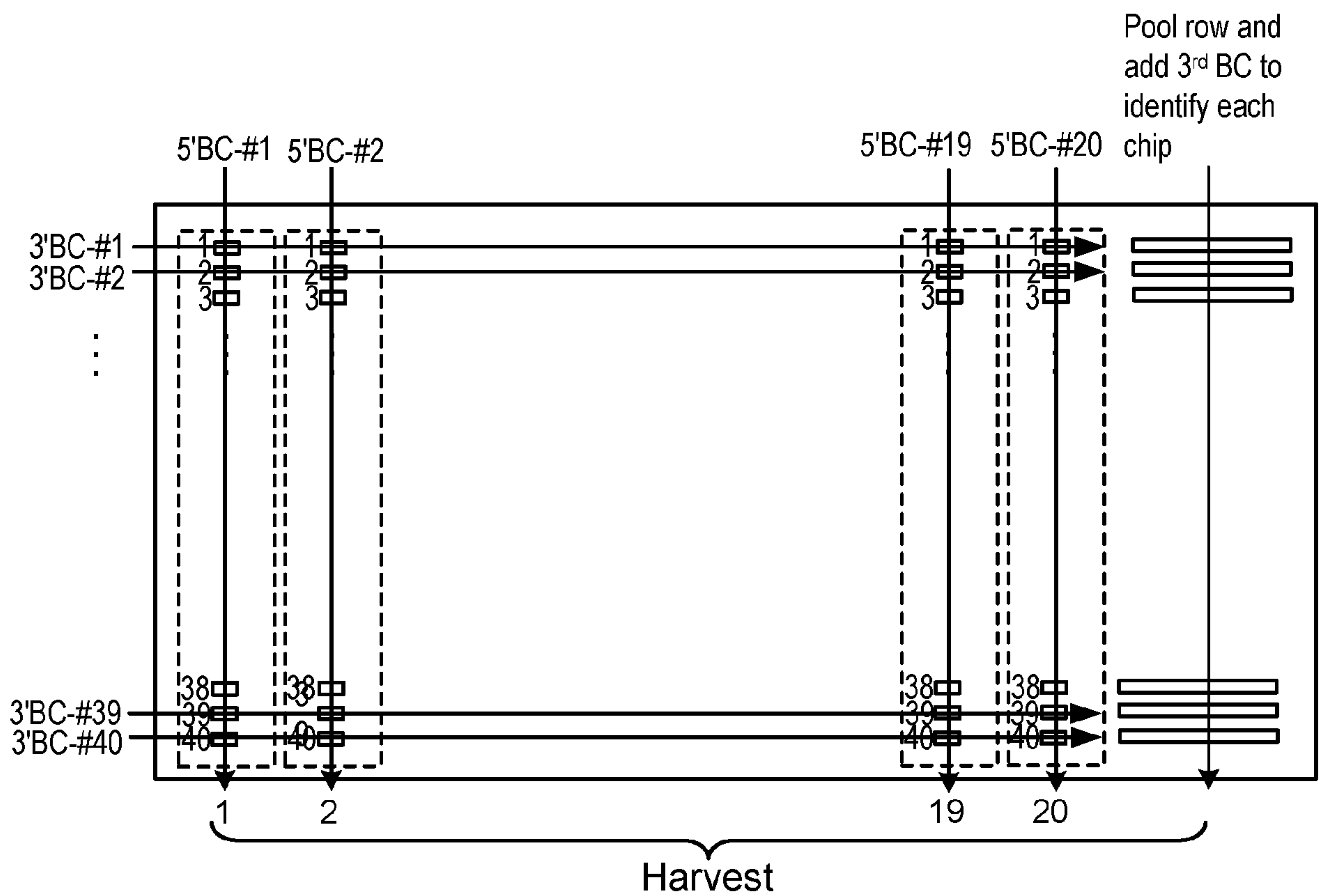


Figure 1D

40 ROWS, each cell gets a unique barcode x 20 units (columns) get
a unit BC

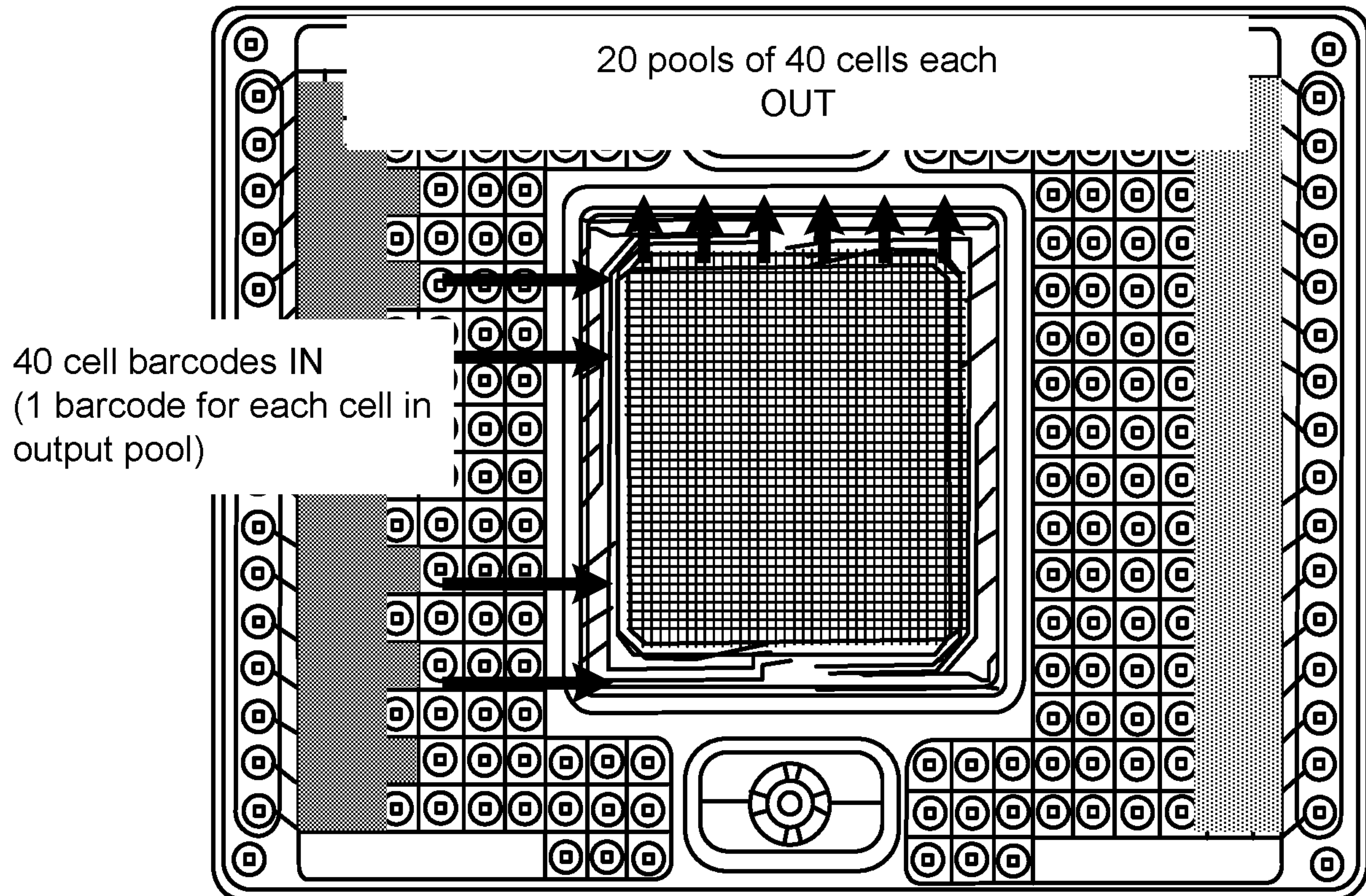
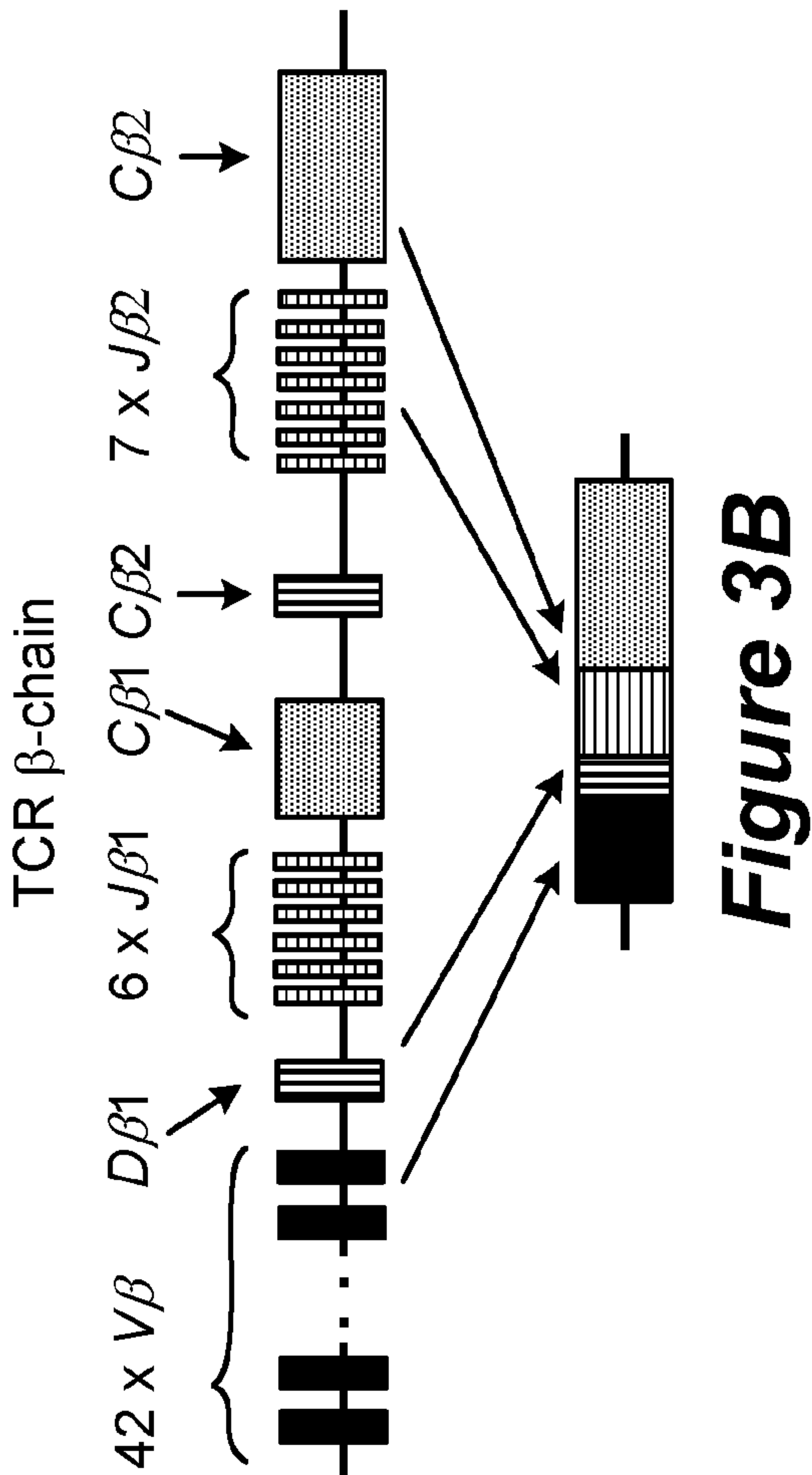
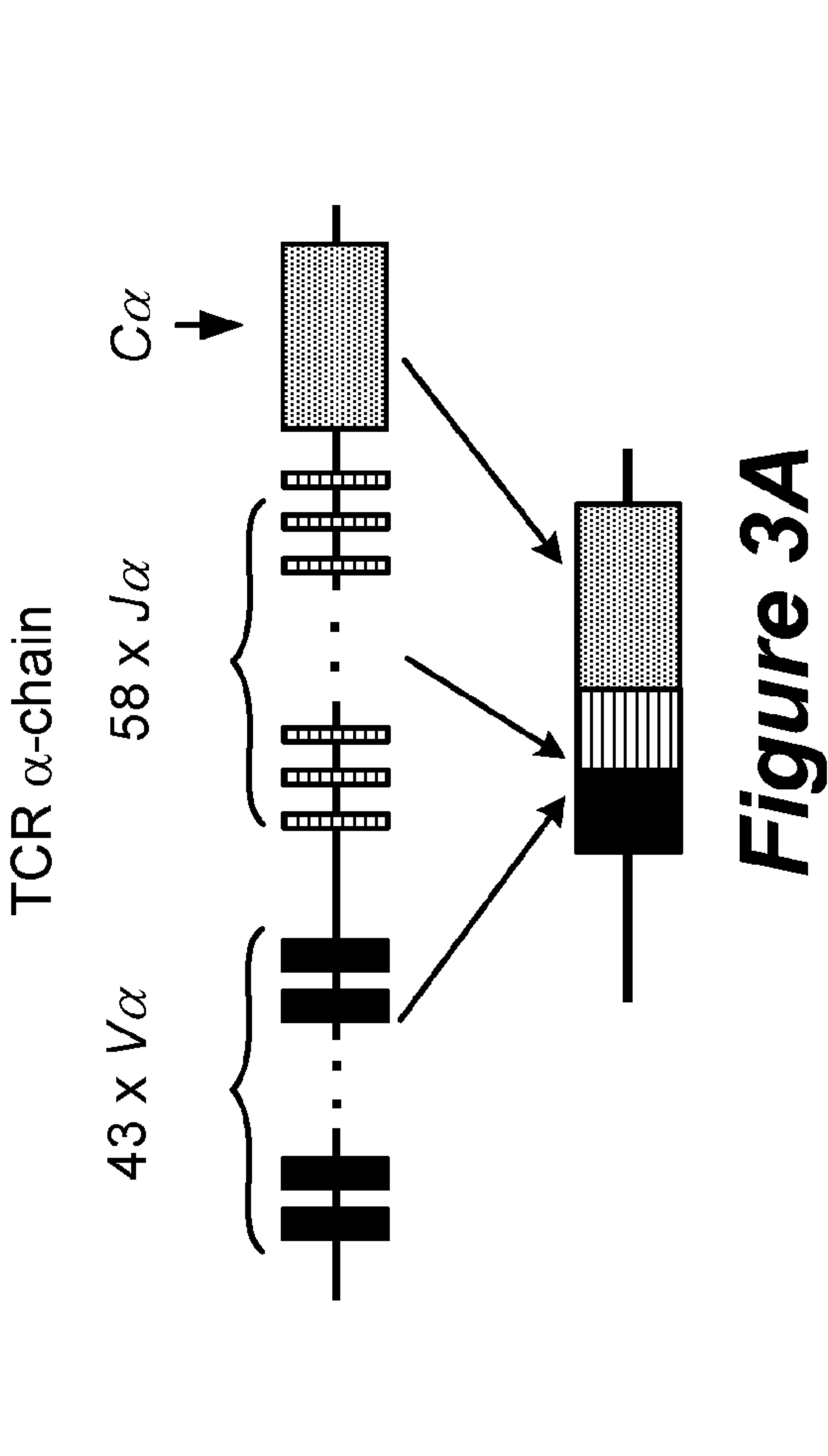
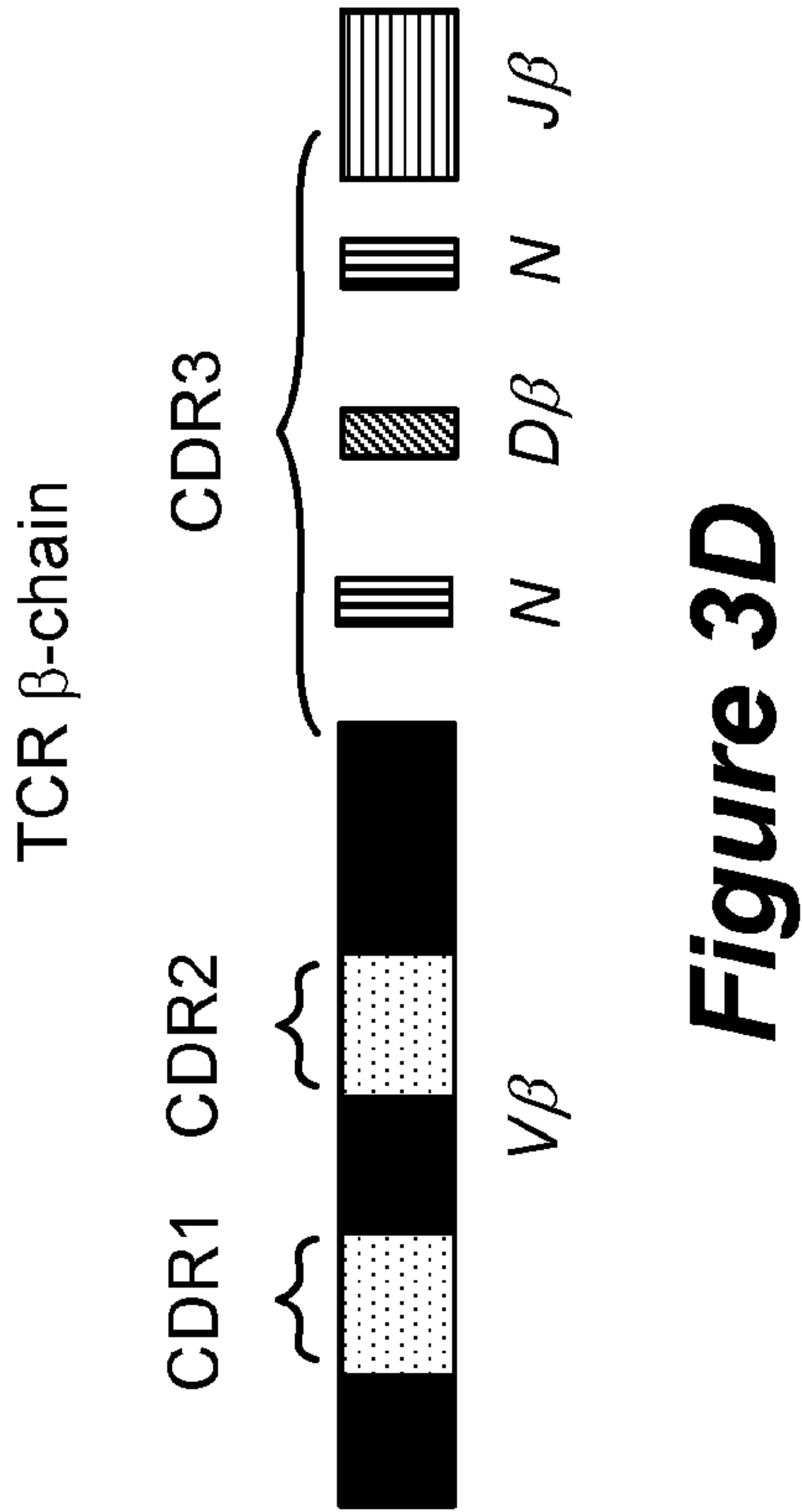
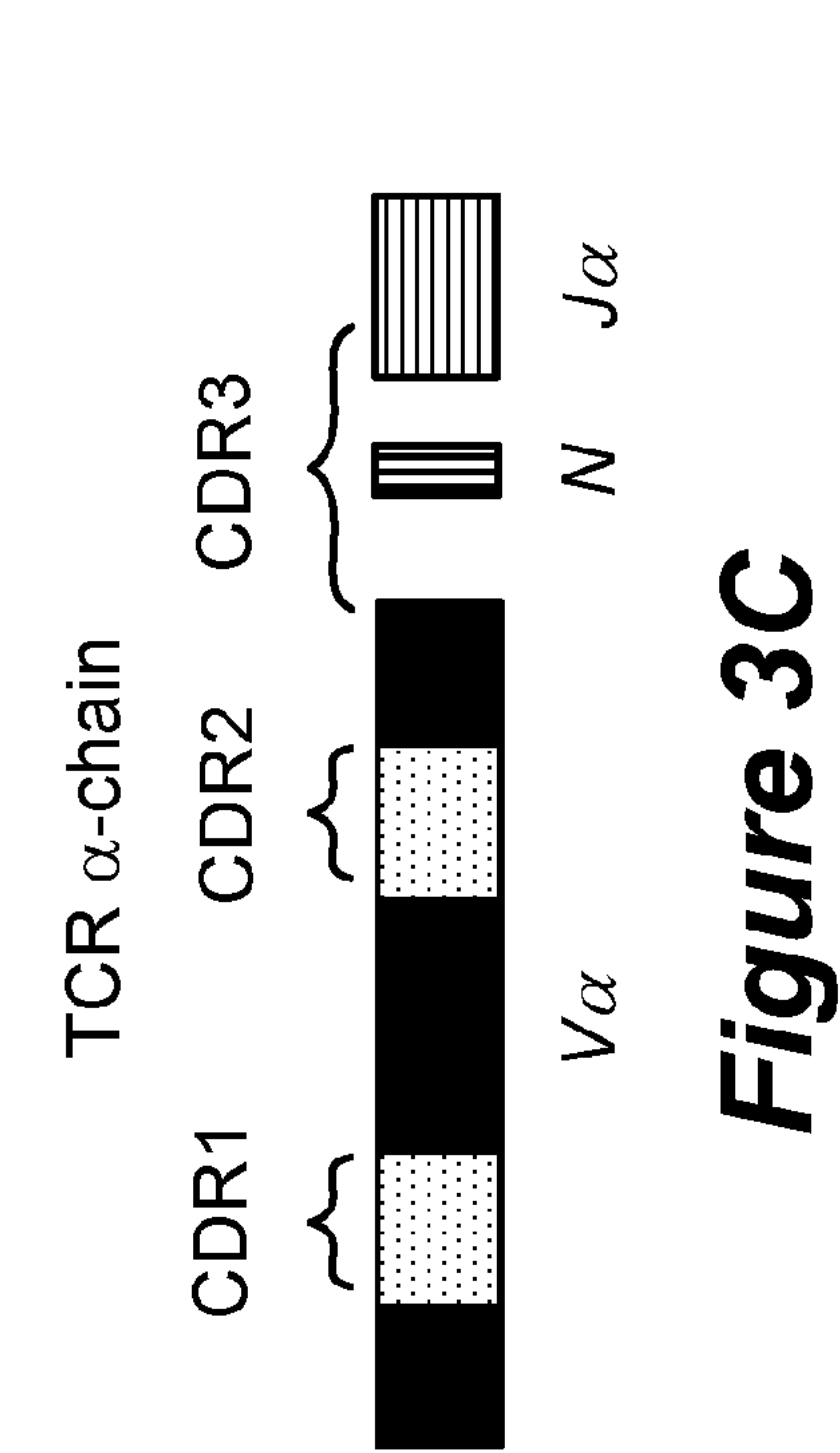


Figure 2



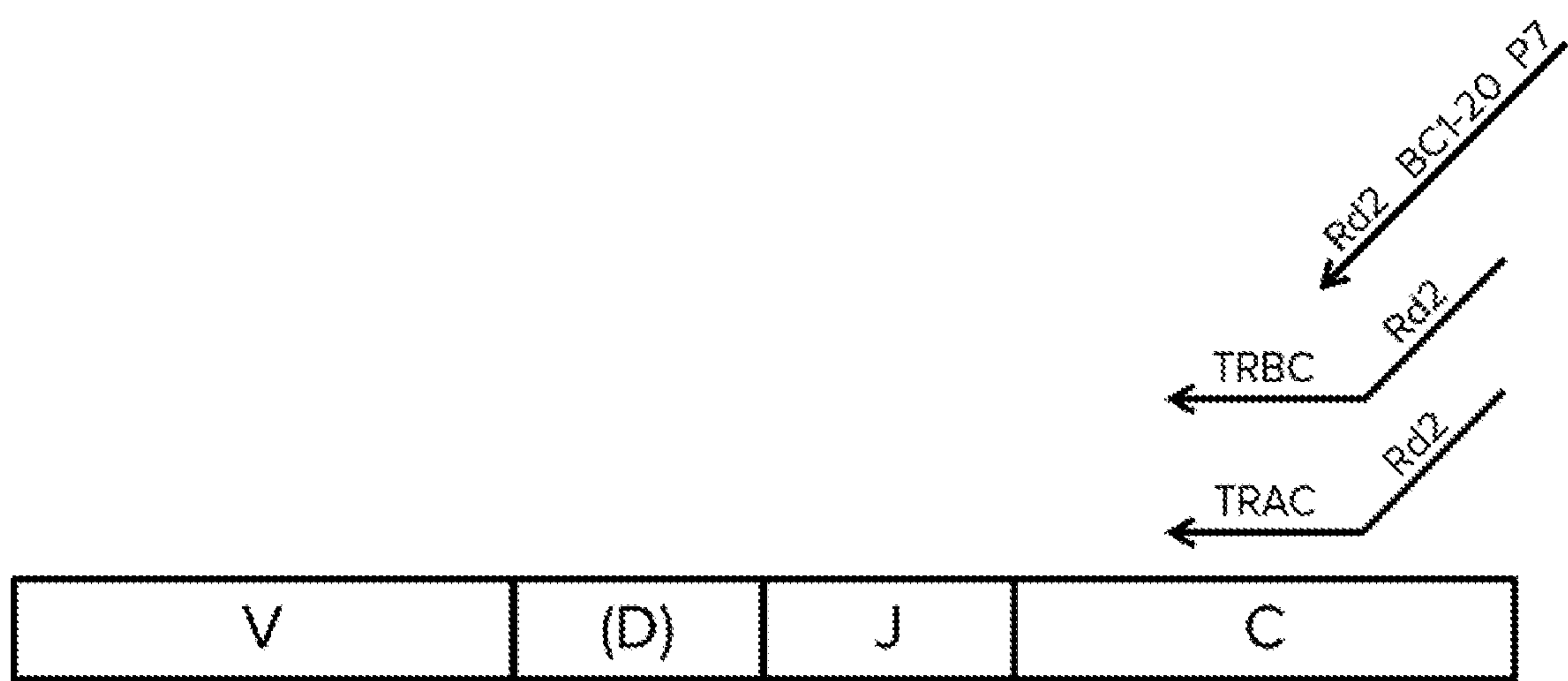


Figure 4A

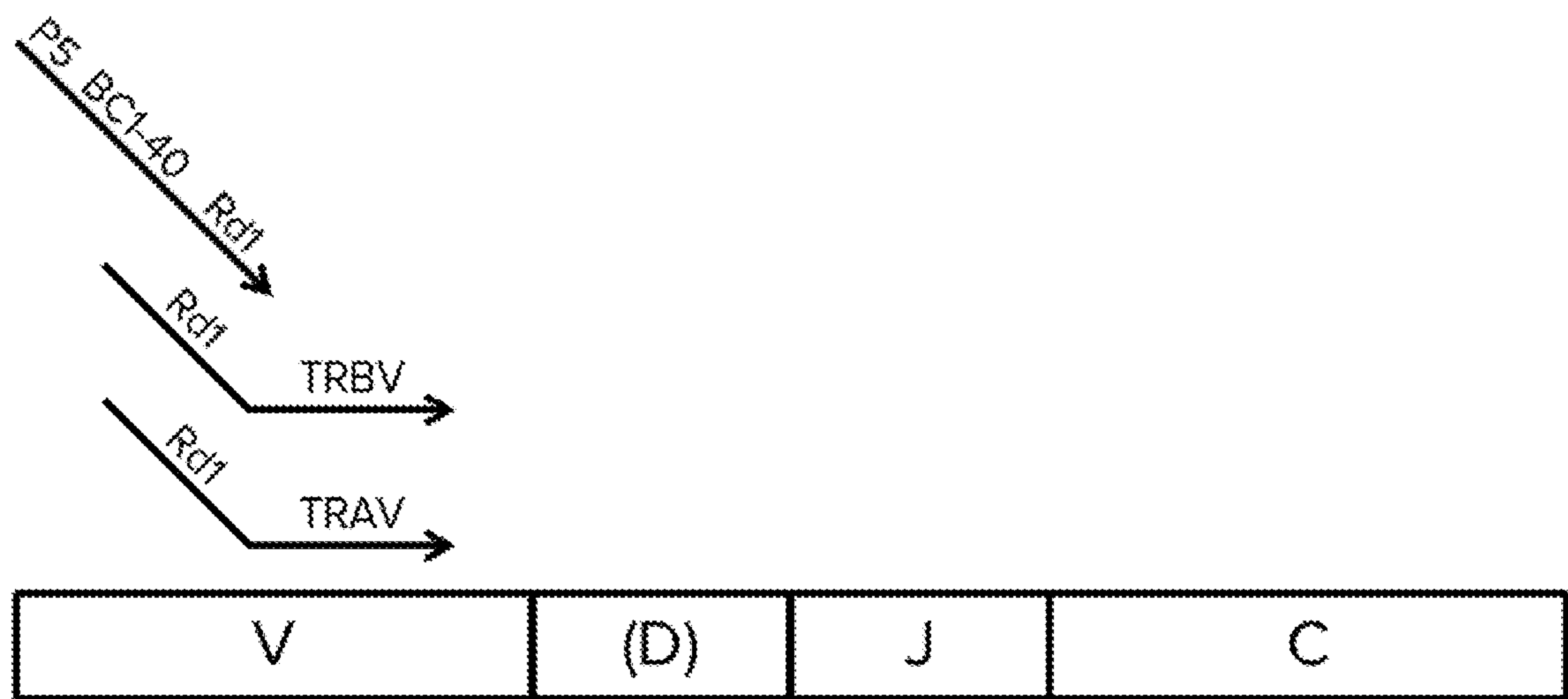


Figure 4B

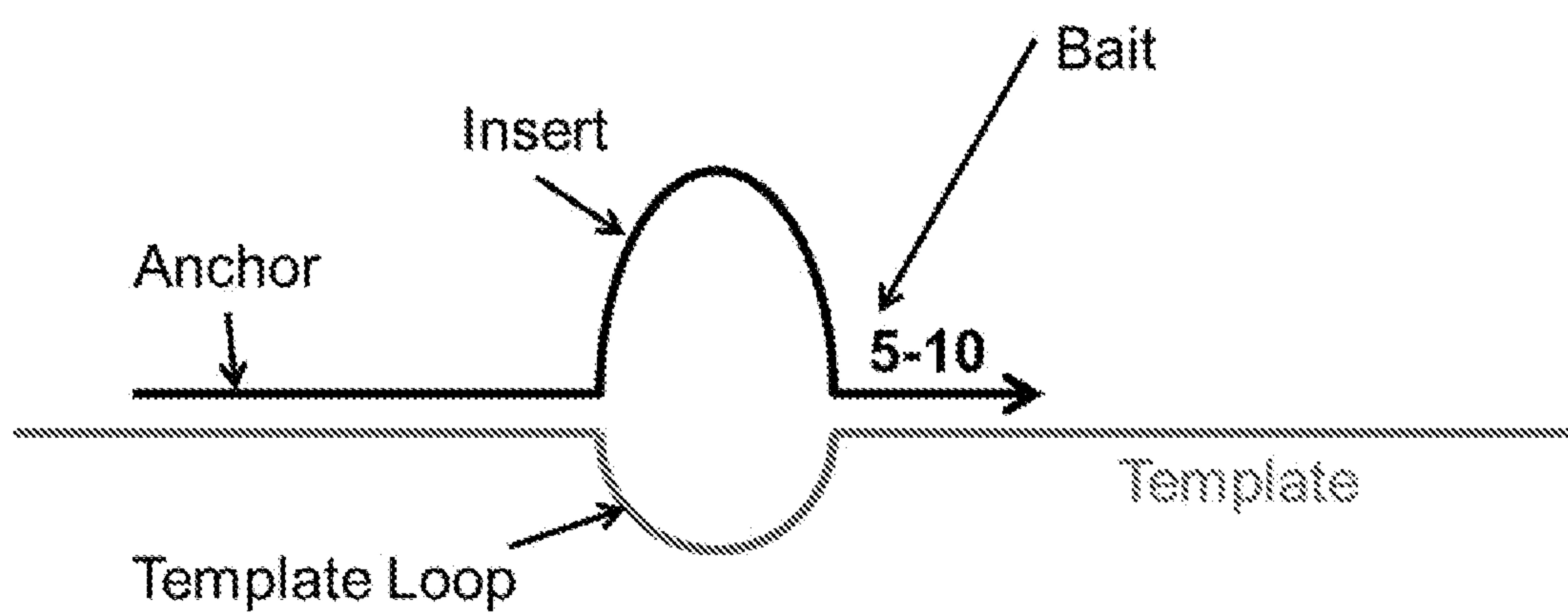


Figure 5

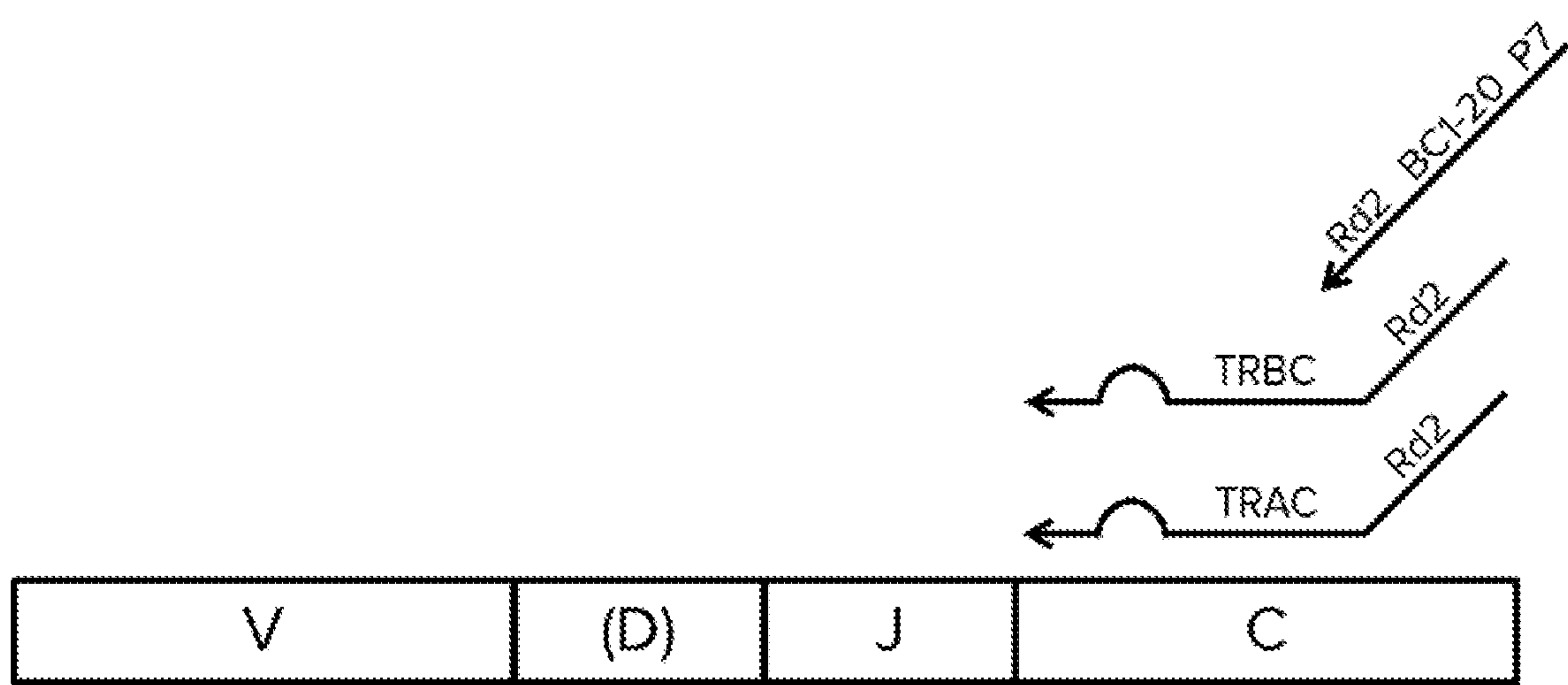


Figure 6A

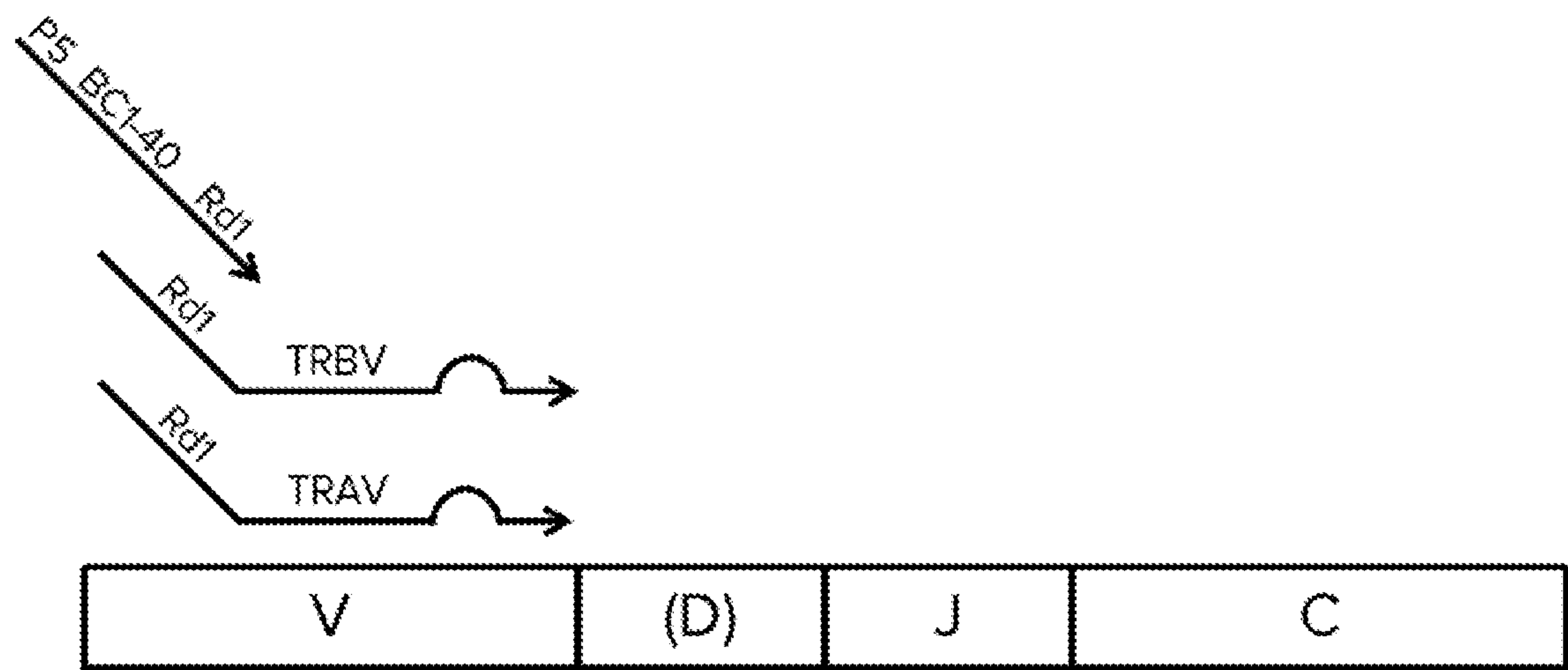


Figure 6B

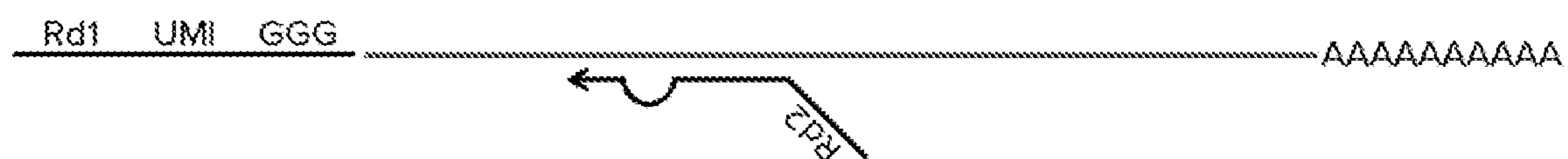


Figure 7

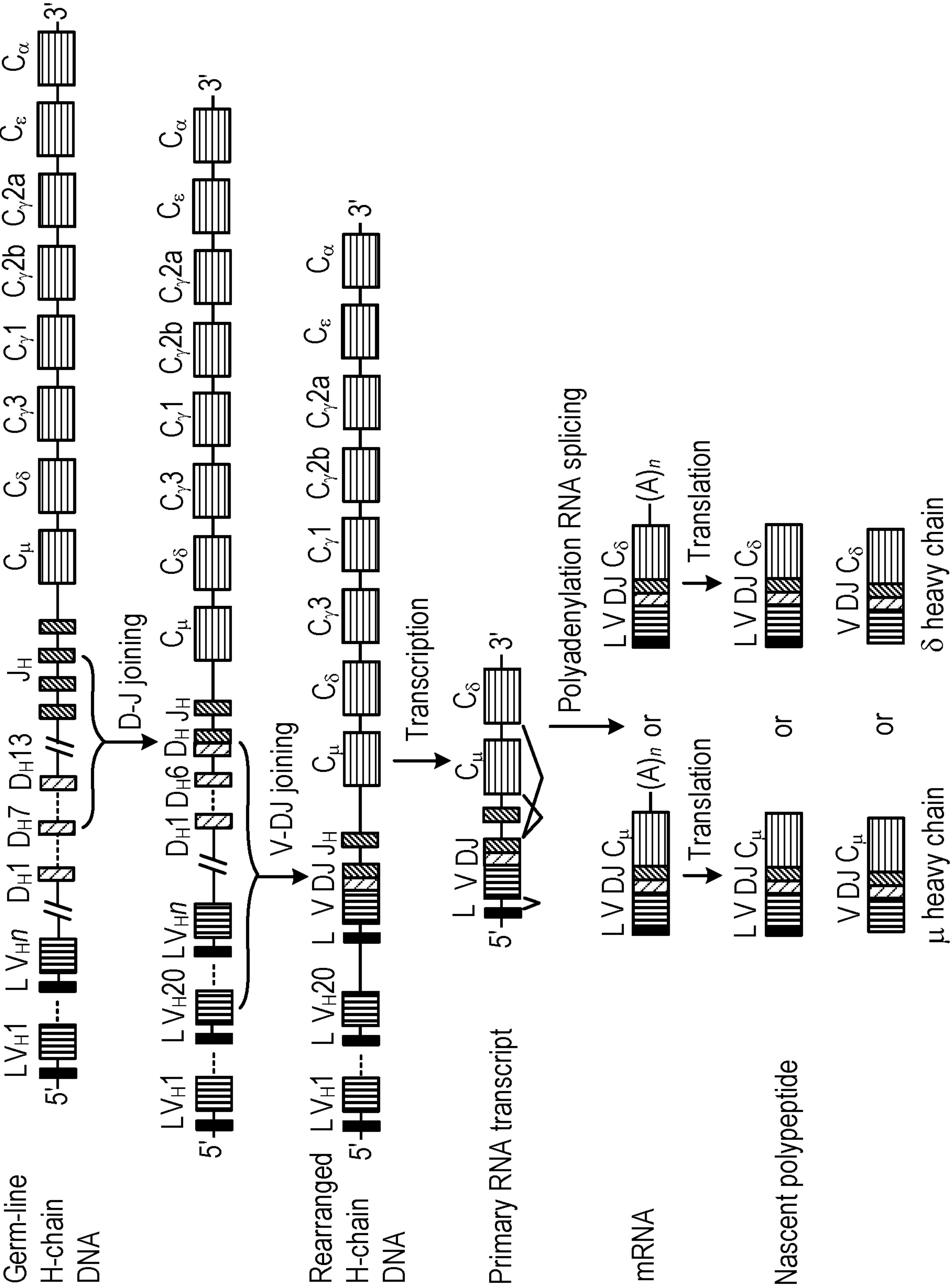


Figure 8A

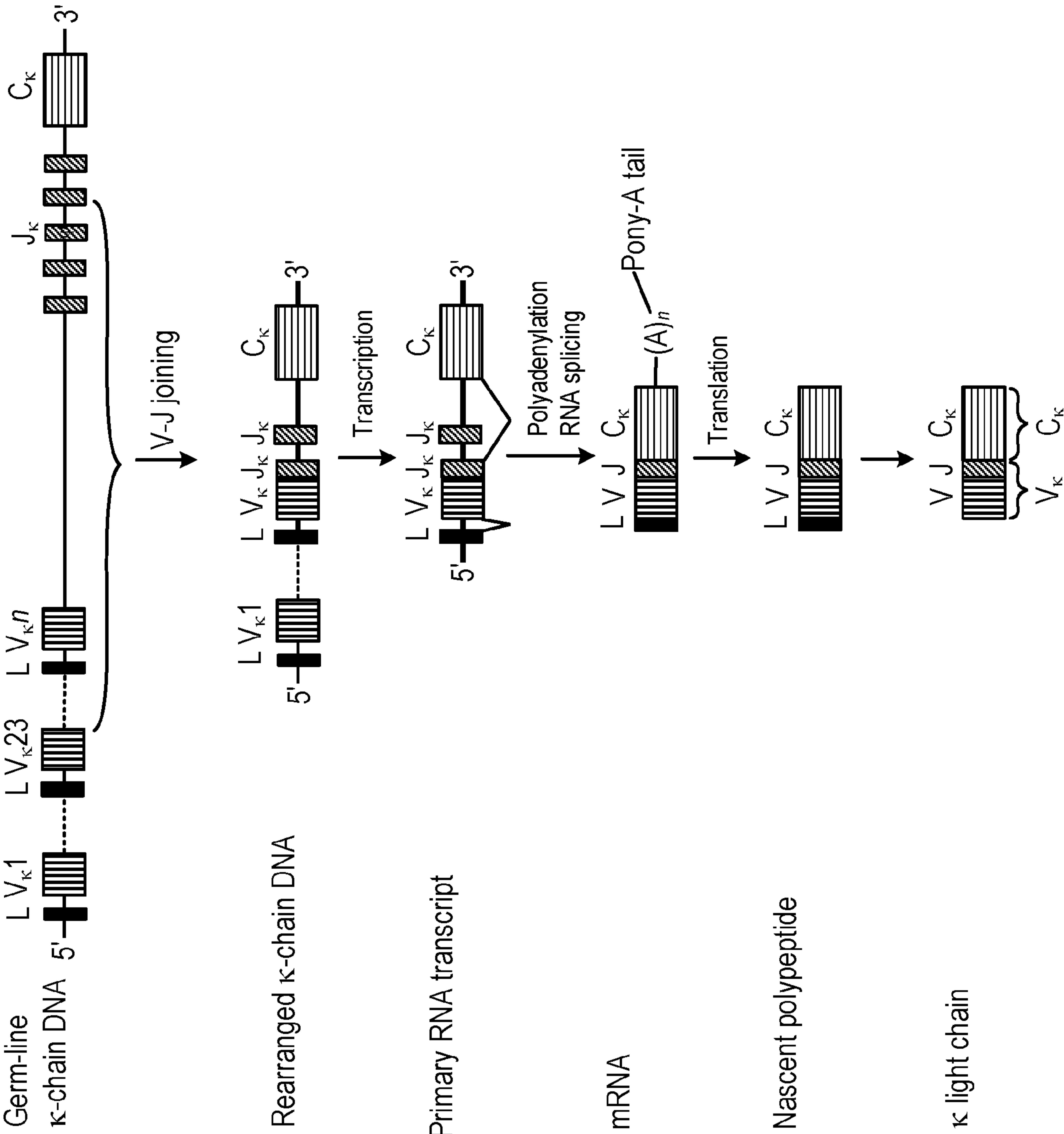
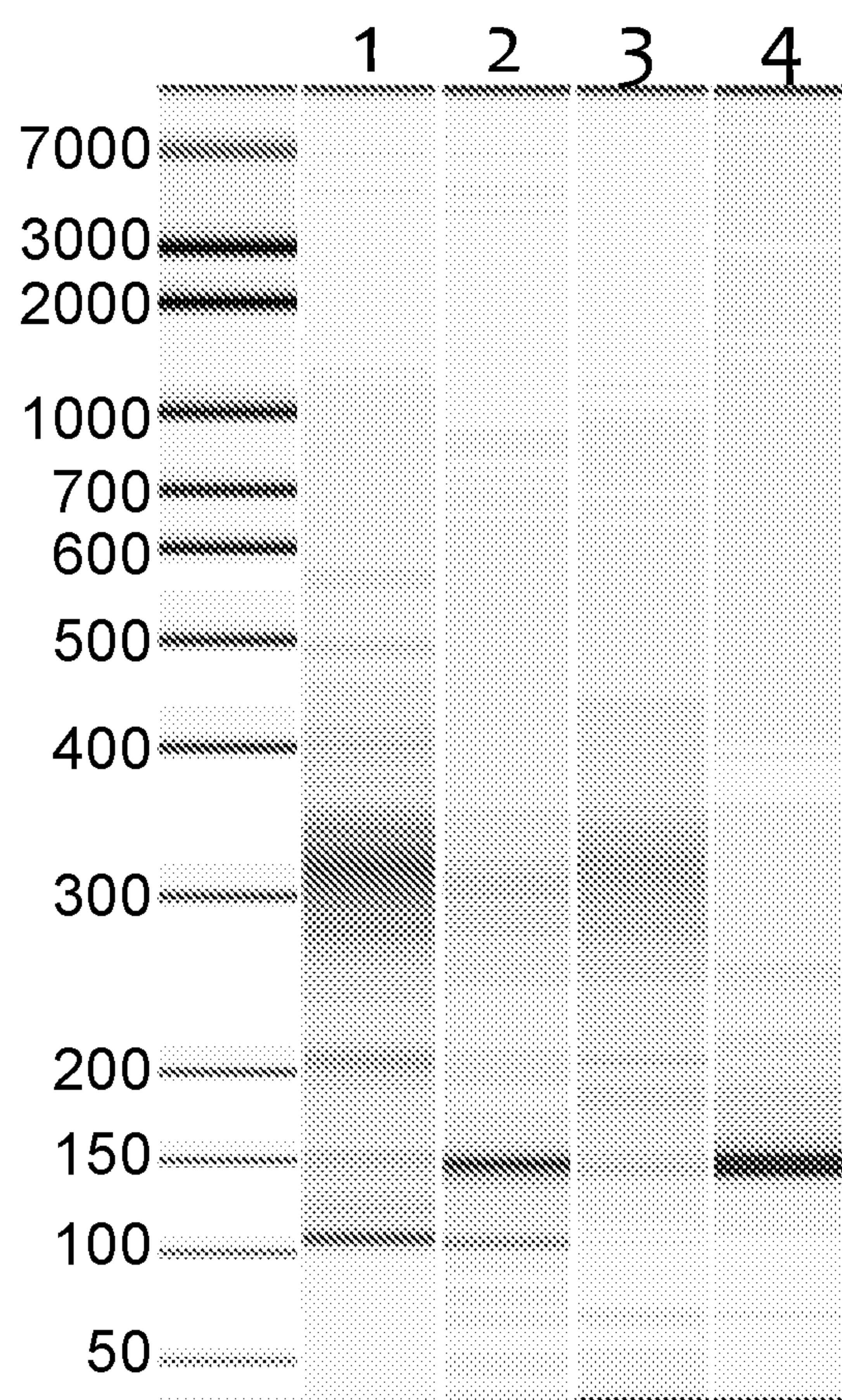


Figure 8B

***Figure 9***

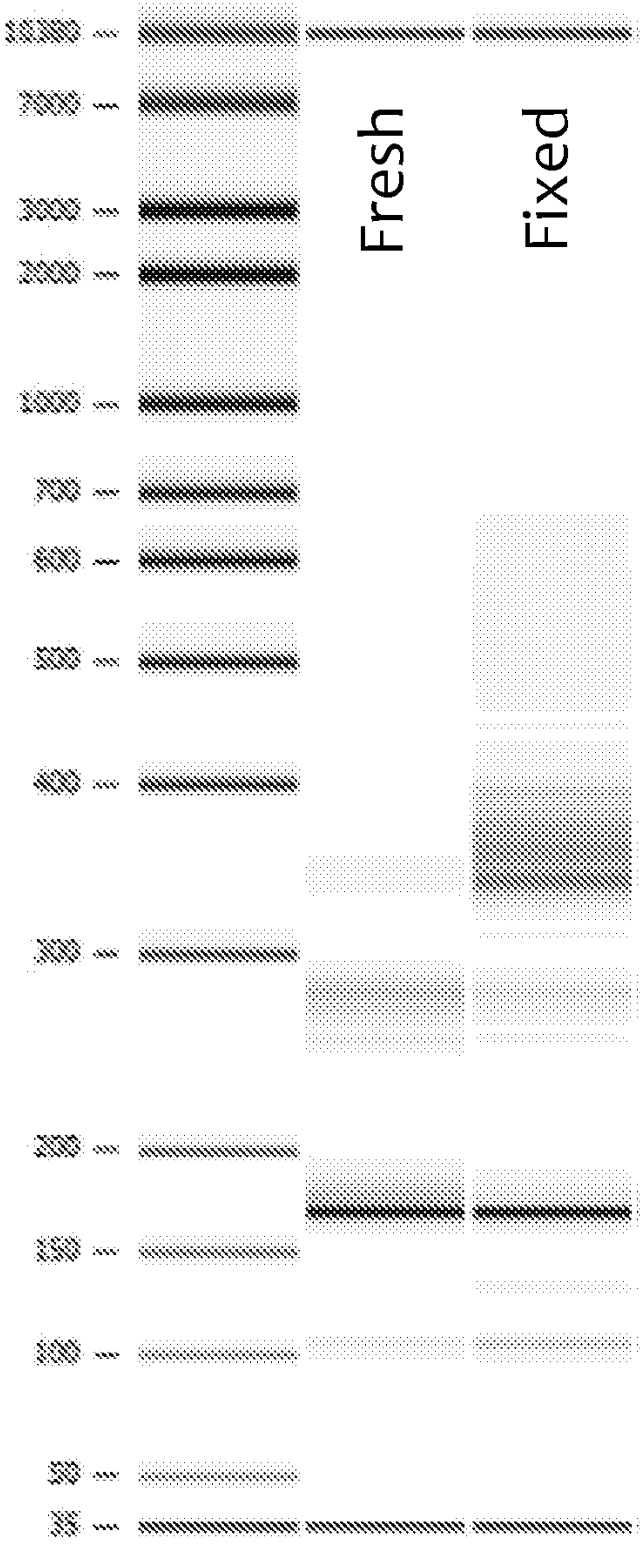


Figure 10

Figure 11A

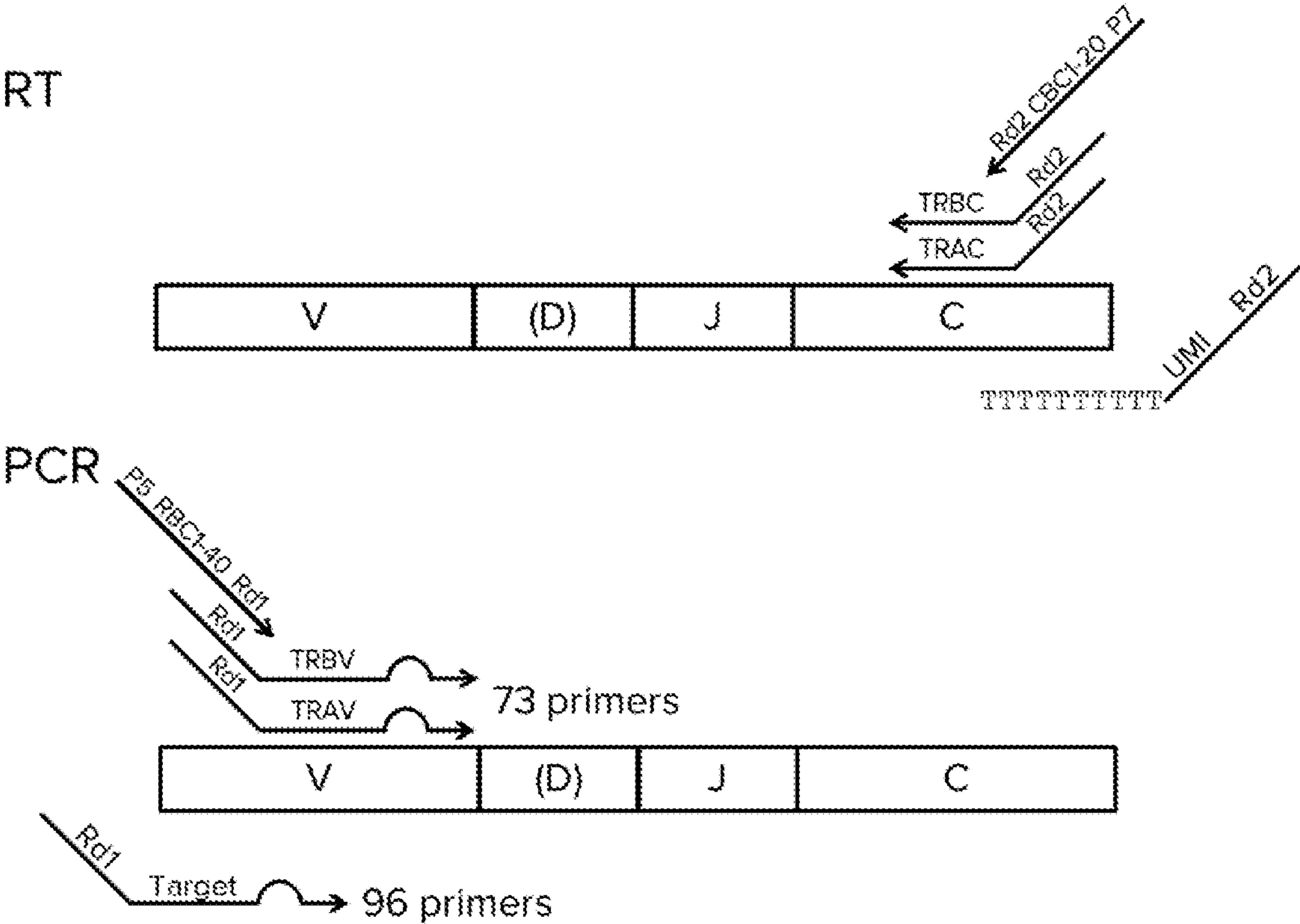
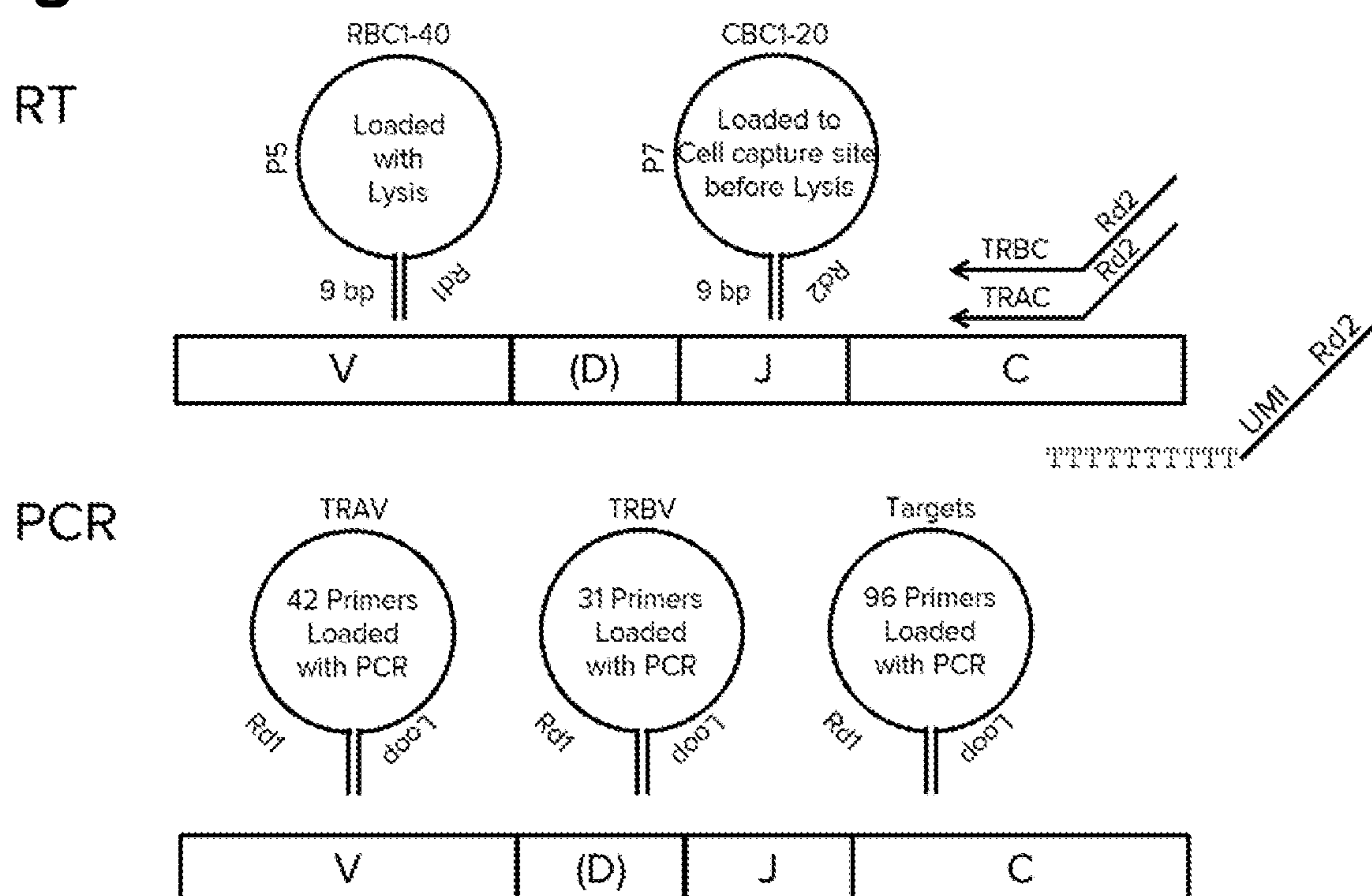


Figure 11B

Figure 12A

After Harvest, Pool All Columns & Perform P5/P7 PCR

Figure 12B

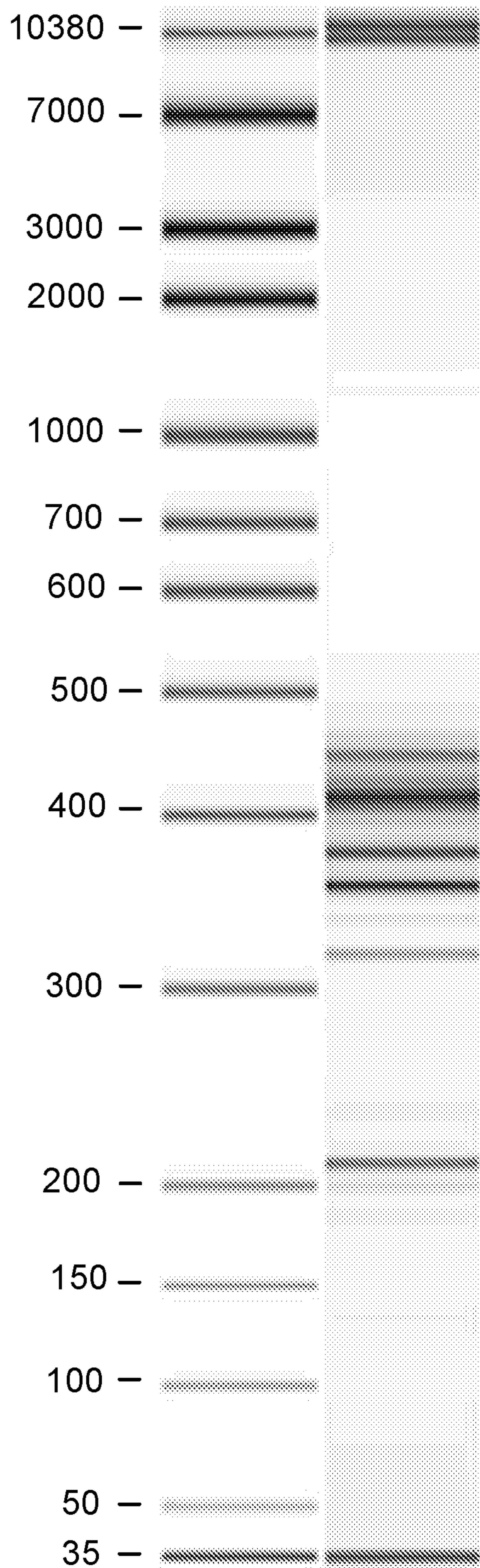


Figure 13

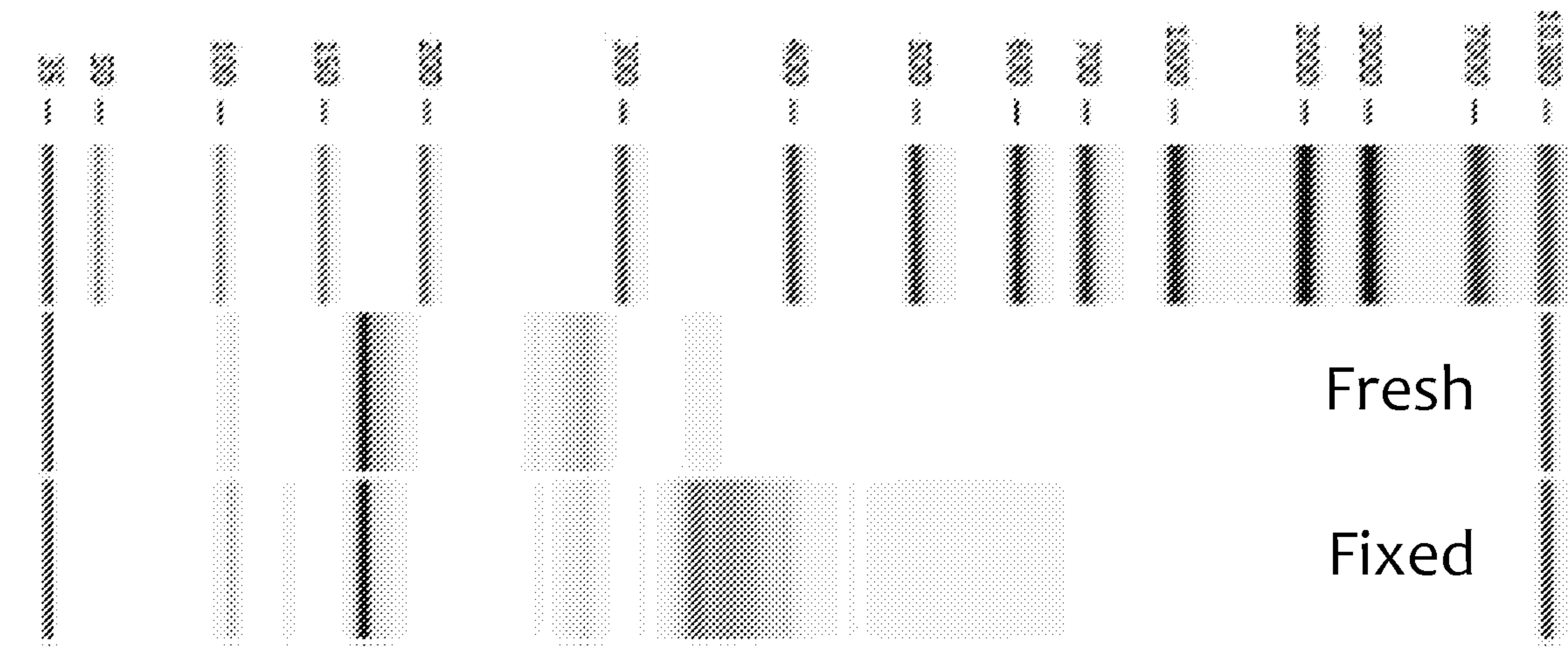


Figure 10