CELLECTA

USER MANUAL

DriverMap™ AIR TCR-BCR Profiling Kit (RNA)

v1 — Last update: 26 January 2024

Cellecta, Inc.

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1. DriverMap™ AIR RNA Profiling Assay

The DriverMap™ AIR TCR-BCR Profiling Kit combines multiplexed RT-PCR amplification with the depth and precision of Next-Generation Sequencing (NGS) to quantitatively measure gene expression and analyze RNA sequences of all functional immune receptor mRNA isoforms for TCR chains (TRA, TRB, TRD, TRG) and BCR chains (IGH, IGK, and IGL) with the exclusion of non-functional pseudogenes and ORFs (as defined by IMGT database http://www.imgt.org/IMGTrepertoire/).

The AIR assay allows profiling of the repertoire of antigen-binding CDR3 regions using a set of experimentally validated forward primers (designed for variable FR3 region), and reverse primers (designed for the conserved C region) of TCR and BCR mRNAs. Two sets of AIR primers specific for TCR (TRA, TRB, TRD, and TRG) and BCR (IGH, IGL, and IGK) chains are provided separately in AIR reagent kits to allow profiling AIR repertoires individually for TCRs or BCRs or together in a single reaction.

Main advantages of DriverMap™ AIR Assay:

- An easy-to-run, one-tube, single-day assay allows profiling of target transcripts from total RNA isolated from whole blood, PBMC, tissue, or directly from a small number (e.g., 100-50,000) of purified immune cells without prior RNA isolation.
- The assay provides robust, quantitative, and reproducible measurements of the copy number of clonotype mRNAs over as many as 4-orders of magnitude differences in expression level.
- To assay provides increased sensitivity from a small amount of RNA (1-50 ng), the DriverMap protocol employs several mechanisms to minimize amplification of off-target and primer dimer background products.
- No mRNA enrichment, rRNA, mitochondrial, beta-globin depletion, or other processing required.
- The primers are designed using Illumina's DNA/RNA UD Indexes labeling strategy to minimize NGS index-swapping background issues and amplified products are compatible with 300-n paired-end NGS sequencing using Illumina's NextSeq/NovaSeq instruments.
- The reverse gene-specific primers are designed with Unique Molecular Identifiers (UMIs), which facilitates the accurate quantification of the copy number of cDNA molecules used in amplification steps and detection of low abundance clonotypes above background level.
- NGS data can be analyzed with the MiXCR software package available online. Cellecta also provides custom data analysis services on request.

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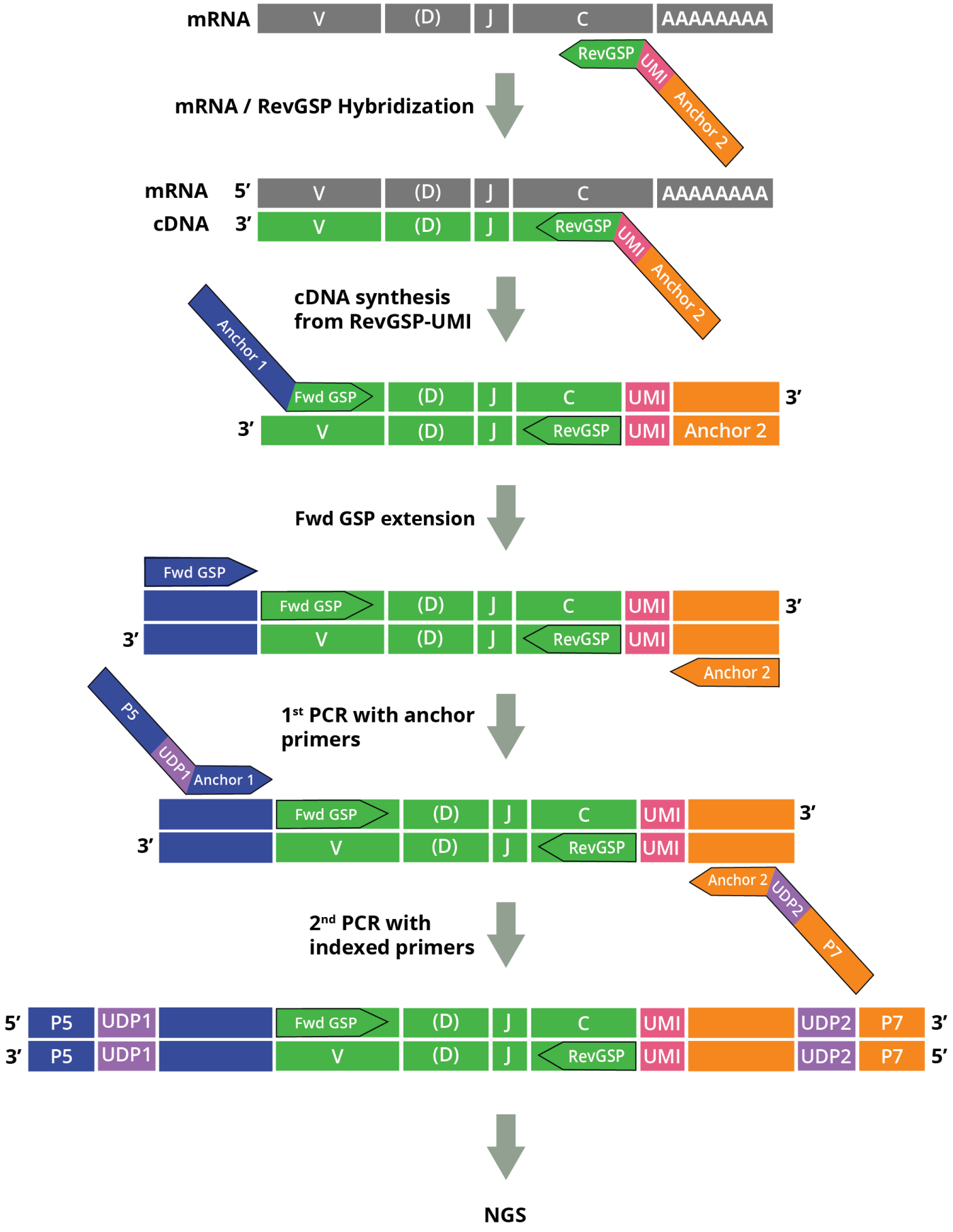

Fig. 1. Outline of DriverMap AIR RT-PCR-NGS assay for expression and sequence analysis of immune receptor mRNA regions

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Additional Profiling Assays Compatible with the AIR RNA Assay:

- **DriverMap™ AIR TCR/BCR Profiling Kit (human DNA)** allows comprehensive CDR3 repertoire analysis from gDNA for all four TCR chains (TRA, TRB, TRD, and TRG) and/or three BCR chains (IGH, IGK, and IGL) using primers designed for FR3 and J regions. Although AIR RNA provides higher sensitivity than AIR DNA assay, both assays could be effectively used together for the identification of antigen-induced clonotypes. For more details, please refer to AIR Technical Guide.
- **DriverMap™ EXP T/B500 Immune Marker Profiling Kit** is designed for deep characterization of T and B immune cell fractions (e.g., naïve, effector, memory, exhausted, etc.) using a set of primers designed for 500 highly informative T-cell and B-cell subtyping and activation marker genes. Combined profiling of AIR repertoire and T/B marker genes in isolated (e.g., by FACS or magnetic beads) immune cell fractions provide a comprehensive phenotypic characterization of T/B cell subtypes associated with different TCR/BCR clonotypes.
- DriverMap™ EXP Genome-Wide Profiling Kit is a convenient single-tube RT-PCR-NGS protocol that robustly measures the expression level of all 19,000 human protein-coding genes starting from a small amount of total RNA (down to single-cell level). Combined EXP and AIR profiling can be used for comprehensive phenotypic characterization of RNA samples and unbiased discovery of novel biomarkers associated with specific CDR3 clonotypes.

Related Products and Services:

- AIR profiling assay in whole blood microsamples. AIR repertoire analysis could be performed without RNA purification directly in 30 µl of whole blood collected, stabilized, and dried using microsampling technology tips from Neoteryx.

For more detailed recommendations related to the design of your experiment, please refer to the AIR Guide brochure. For more information on Cellecta's DriverMap kits and services, please visit the DriverMap Adaptive Immune Receptor (AIR) Profiling Service webpage.

Please read the entire user manual before proceeding with your experiment.

Click the ? Download as PDF link located at the bottom of the left menu to download the PDF version of this user manual.

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2. DriverMap™ AIR TCR-BCR Profiling Kit Components

DriverMap™ AIR TCR-BCR RNA Profiling Kit contains enough reagents to prepare NGS samples starting from total RNA from blood, tissue, cells, or other biological samples, including Positive Control RNA. AIR TCR and BCR kits have the same reagents but different TCR or BCR-specific primer sets.

Box 1: Kit Components

Component	Description	Cap Color	Conc.	Volume (24 rxn)*	Volume (96 rxn)
Reverse TCR-C primer mix (human/mouse)	Mix of reverse gene-specific primers for C-region of TRA, TRB, TRG and TRD	n/a	20X	30 µl	120 µl
Reverse BCR- C primer mix (human/mouse)	Mix of reverse gene-specific primers for C-region of IGH, IGL and IGK	n/a	20X	30 µl	120 µl
Forward TCR-FR3 primer mix (human/mouse)	Mix of forward gene-specific primers for FR3-region of TRA, TRB, TRG and TRD	n/a	20X	60 µl	240 µl
Forward BCR-FR3 primer mix (human/mouse)	Mix of forward gene-specific primers for FR3-region of IGH, IGL and IGK	n/a	20X	60 µl	240 µl
Positive Control RNA (human/mouse)	Total PBMC RNA	white	50 ng/µl	5 µl	5 µl
Hybridization Buffer	RNA-RevGSP Hybridization Buffer, 4X	yellow	4X	1 ml	1 ml
Water	Milli-Q purified, water for reaction mixes	white	NA	3 ml	12 ml
dNTP Mix	Mix of dATP, dGTP, dCTP and dTTP each	white	10 mM	125 µl	600 µl
RT-EXT Buffer	cDNA synthesis and forward gene-specific primer extension reaction buffer, 5X	green	5X	250 µl	1 ml
Reverse Transcriptase	Multiplex-optimized M-MLV Reverse Transcriptase (RT)	green	20X	30 µl	120 µl
Hot-start RT Aptamer	Aptamer which inactivates RT at room temperature but activates RT at 50°C	green	20X	30 µl	120 µl
PCR Buffer	Reaction Buffer for 1st and 2nd PCR steps, 5X	red	5X	600 µl	2×1.2 ml
DNA Polymerase	Multiplex-optimized thermostable hot-start DNA polymerase	red	100X	50 µl	200 µl
Anchor Primer Mix	Forward/Reverse Anchor-specific Primers for 1st PCR, 10X	red	10X	300 µl	1.25 ml
Primer Removal Enzyme	Enzyme Mix for primer removal	orange	20X – 50X	90 µl	360 µl
Forward SeqDNA NGS Primer	Forward NGS Primer for cDNA sequencing	blue	100 µM	30 µl	90 µl
Reverse SeqDNA NGS Primer	Reverse NGS Primer for cDNA sequencing	blue	100 µM	30 µl	90 µl
Forward SeqIND NGS Primer	Forward NGS Primer for Index sequencing	blue	100 µM	30 µl	90 µl

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Reverse SeqIND NGS Primer	Reverse NGS Primer for Index sequencing	blue	100 µM	30 µl	90 µl

Box 2: Kit Components

Component	Description	Conc	Volume (24 reactions) *	Volume (96 reactions)
Dual Index Primer Set Plate	96 Dual DNA/RNA UD Indexes primer pairs, pre-aliquoted and dried in a 96-well plate	N/A	Dried One 96 well plate	Dried Two 96 well plates

Kit contents should be stored at -20°C. Shelf life is 1 year.

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3. Additional Materials Required

Depending on the type of samples and procedural options followed in the protocol, several of the following items will be required to run the assay.

For General RNA Preparation

Name	Recommended Manufacturer	Catalog #	Purpose
RNAeasy Plus Micro/Mini Kit	QIAGEN	74034/74134	RNA isolation from PBMC, sorted cells or tissues
Tempus Blood RNA tubes/Spin RNA isolation kit	Thermo Fisher	4342792/ 4380204	RNA isolation from whole blood
RNA 6000 Pico Kit for 2100 Bioanalyzer	Agilent	5067-1511	RNA quality control calculation
HS RNA Analysis Kit for Fragment Analyzer	Agilent	DNF-472_0502	RNA quality control calculation

Additional Reagents for Hybridization, cDNA Synthesis, Amplification, and Amplified Libraries QC

Name	Recommended Manufacturer	Catalog #	Purpose
N-Lauroyl Sarcosine, sodium salt, 20%	Sigma	L7414	Detergents for hybridization step in DirectCell protocol
Agencourt® AMPure® XP (Magnetic Beads)	Beckman-Coulter	A63881	RNA-RevGSP hybrid and PCR product purification
Dynabeads® MPC®-S (Magnetic Particle Concentrator) for 1.5 ml Test Tubes Magnetic Stand-96 for 96-well PCR plates	Thermo Fisher	A13346/ AM10027	RNA-RevGSP hybrid and PCR product purification
96-well PCR plates	Thermo-Fisher	A43673	RT-PCR reactions
Clear Adhesive Film for 96-well plates	Bio-Rad	MSB1001	PCR plate seal
High Sensitivity DNA Kit for Bioanalyzer	Agilent	5067-4626	PCR product QC/ quantification
High Sensitivity NGS Fragment Analysis Kit (1 bp – 6000 bp) for Fragment Analyzer	Agilent	DNF-474-1000	PCR product QC/ quantification
Qubit® dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher	Q32851	Quantify Indexed Libraries for NGS
NextSeq® 500/550 High Output Kit v2.5 (300 cycles) /NovaSeq	Illumina	20024908 (NextSeq)	Next-Gen Sequencing

Recommended Instrumentation

This protocol is optimized for the instruments specified below. Some modifications and optimization to the protocol may be necessary if using different models or instruments from other manufacturers.

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Description	Model	Manufacturer
Spectrophotometer (NanoDrop or equivalent)	NanoDrop® ND-2000	Thermo Fisher
Thermal Cycler (Veriti 96-Well or equivalent)	Veriti® 96-Well	Thermo Fisher
Capillary Instrument or Slab Gel Electrophoresis System to analyze amplified DNA products	2100 Bioanalyzer®	Agilent
	Fragment Analyzer™	Agilent
	Agarose gel electrophoresis/imaging system	many suppliers
DNA Quantification	Qubit 3.0 Fluorometer	Thermo Fisher
Next-Generation Sequencer	NextSeq, NovaSeq	Illumina
Thermomixer R (for microsampling AIR profiling)	Thermomixer R with heat block for 1.5-ml test tubes	Eppendorf/Fisher Scientific

Other than the specific reagents and instruments listed above, the protocol assumes the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (tabletop centrifuges, pipettes), and common reagents (e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

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4. General Procedural Recommendations

- Keep enzyme components on ice during use. All other components, including primer pools, may be placed at room temperature. Thaw components, gently vortex, and spin down before use. Please be sure to dissolve any precipitate if visible.

- Use Good Laboratory Practices to minimize cross-contamination of products. If possible, perform the first part of the procedure (from cDNA synthesis through first PCR setup) in a location set aside for RNA work, and use a set of equipment, pipettes, test tubes, and other consumables dedicated for work involving RNA. Then run the PCR amplification reactions in a separate lab or area designated for PCR. Always change pipette tips for adding components to new samples.

- For a small number of samples (1-12) we recommend using 0.2-ml, 0.5-ml test tubes or tube strips to run the assay. To minimize the hands-on time and mistakes in liquid deposition for large numbers of samples (e.g., 24-96), we recommend running the assay in a 96-well plate using a 8-channel (or 12-channel) pipet. After adding all the necessary reagents, seal the plate well with Clear Adhesive Film and use a Compression Pad to minimize evaporation from experimental samples. Only use each Clear Adhesive Film once. Do not reuse them.

- Do not reuse clear adhesive films to seal 96-well plates.

- Pipet viscous enzyme solutions slowly. After adding them to the reaction mix, ensure complete mixing by vortexing or pipetting up and down several times.

- For several steps in the procedure, we recommend making a Master Mix by combining adequate amounts of the key reagents into a single tube, and then aliquoting the appropriate portion from this combined mix into each sample. This approach helps ensure a consistent amount of each reagent is added to all samples. For these Master Mix reactions, first calculate the volume of each component you need for all the reactions you are running. Then, add 5% to each volume to cover pipetting variance, and pool the appropriate volumes of reagents together into a single tube or well. Mix the pooled reagents, then pipette the required portion into each sample. If you are using a multichannel pipette, aliquot the Master Mix in a tube strip first, then pipette from this strip into each sample.

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5. Sample Preparation

Depending on the sample amount, either total RNA or isolated immune cells (i.e., DirectCell Protocol) can be used as input for the DriverMap AIR Assay. No preliminary poly(A) selection or ribosomal/mitochondrial/globin RNA depletion is required:

- For bulk tissue, blood, or cell samples with more than ~50,000 cells, follow the RNA Preparation Protocol.
- For biological samples with a small number of cells—less than ~50,000 cells (e.g., sorted immune cells), or blood and tissue microsamples—we recommend using the immune cells directly as input. Follow the DirectCell Protocol.

Last modified: 21 June 2023

5.1. General Guidelines

AIR Repertoire Profiling:

- AIR TCR-BCR profiling Kit provides reagents for unbiased, comprehensive profiling of all seven immune receptor chains in one reaction and could be used for a wide range of biological samples containing both T and B cells. AIR TCR-BCR kit is a good starting assay for samples with unknown lymphocyte compositions. For samples that contain only T or B cells (e.g., purified immune cell fractions), we recommend using individual TCR or BCR profiling kits.

Starting Amount of RNA:

The table below provides a general guideline for starting amount of RNA for AIR TCR/BCR profiling assay:

Sample Type	Optimal	Range (total RNA)
Whole blood	100 ng	5-200 ng
PBMC	50 ng	2-100 ng
T/B Cell fractions	25 ng	1-100 ng
Lymphoid Tissue (fresh/frozen)	100 ng	5-100 ng
Cancer biopsies	500 ng	20-1,000 ng
Non-lymphoid tissues	1,000 ng	50-2,000 ng
FFPE tissues	1,000 ng	50-2,000 ng

- The optimal range for AIR assay is 25-100 ng of total RNA from a large number of immune cells e.g., PBMC, buffy coat, whole blood, purified immune cell fractions (more than 50,000), or lymphoid tissues with a high percentage of immune cells. For PBMC RNA we recommend using 50 ng, and for whole blood RNA, we recommend 100 ng.

- Although the standard AIR protocol works reliably in the 1 ng-1,000 ng range, for deep, quantitative AIR repertoire profiling, we recommend using 25-100 ng of total RNA (from whole blood, PBMC samples, or isolated immune cells). Using larger amounts of total RNA can improve the detection of rare clonotypes, but it requires increasing the sequencing depth (and NGS cost) which can affect expression levels of high-medium abundant clonotypes. Using smaller amounts of total RNA will reduce the number of clonotypes that can be reliably profiled. For example, running 5-10 ng of total whole blood/PBMC RNA instead of 50 ng will profile ~200-500 highly abundant clonotypes (i.e., transcripts with >10 target CDR mRNA molecules) as opposed to ~1,000 clonotypes.

- For RNA isolated from tissue samples with a low percentage of immune cells (e.g., cancer biopsies, non-lymphoid tissues), the amount should be increased to use 200-1,000 ng of total RNA. Due to the high specificity of the AIR

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assay, the total RNA can be used directly without purification of immune cell fraction.

Replicates:

- For samples with enough total RNA, we recommend running replicates or increasing the amount of total RNA at the hybridization step (e.g., 3-fold) and splitting the samples after the Forward Primer extension step, and running the first and second amplification steps in triplicate reactions for each sample. This allows estimation of variability and expands confident quantitation from ~500 clonotypes if a single replicate is used to ~1,000-3,000 clonotypes (in PBMC) based on the statistical power gained with triplicate analyses (see also the Guide to AIR profiling for more details).

- For purified immune cell samples, we do not recommend splitting the samples into triplicates since the number of cells is usually limited. We recommend collecting multiple samples for each cell fraction so the biological replicates can be run in parallel.

PBMC RNA is supplied with the kit as a positive control. We recommend running at least one positive control sample with a similar concentration to your experimental samples. This will help with troubleshooting, data analysis, and normalization across different sample runs.

When running DriverMap AIR and T/B500 Immune Marker assays on the same immune cell sample (e.g. in sorted T/B cell fractions), we suggest running them separately by splitting the sample by approximately 5:1 ratio as this will ensure the highest sensitivity for each assay. At NGS step, amplified AIR and T/B marker fractions could be mixed (at different ratios, e.g., 1:1 molar ratio) to provide balanced, high sensitivity detection of both AIR CDR and T/B marker gene expression levels.

Last modified: 20 July 2023

5.2. RNA Preparation Protocol

For larger bulk tissue, blood, or cell samples as shown in the Table above, follow the guidelines below to isolate, quantify, and QC total RNA. Generally, deep, quantitative AIR profiling of the most abundant clonotypes (with more than 3 mRNA/UMI copies of CDR-specific mRNA per sample) requires using 50-100 ng of total RNA isolated from whole blood (preferred choice) or PBMC samples.

1. Use the appropriate method to isolate total RNA. We recommend the following RNA isolation kits.

    - Whole blood: we recommend using the Tempus (Thermo-Fisher) or PAXgene (QIAGEN) for total RNA isolation. Based on our experience, the collection of 3 ml of whole blood in Tempus RNA Blood Tubes followed by RNA isolation using Tempus Spin RNA Isolation kit provides the most simple and reliable protocol for purification of RNA from whole blood.
    - PBMC, buffy coat, or fresh/frozen tissue: for more than 50,000 cells we recommend using the QIAGEN RNAeasy Micro/Mini Plus Kit for total RNA isolation.
    - FFPE tissue: we recommend using the Thermo Fisher's RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Cat.# AM1975). With FFPE, the yield and quality of isolated RNA can vary significantly, depending on the age and method used to fix the sample. It is critical to assess the quality of the RNA before proceeding with the DriverMap protocol.

2. Ensure the extracted RNA is free of almost all DNA.

    - Small amounts of DNA (i.e, <5% genomic DNA contamination) do not interfere with the assay. With the Tempus and RNeasy Plus RNA Kits, a separate DNase treatment is not necessary. When using other RNA

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isolation protocols, a separate DNase treatment of RNA samples is recommended before starting the procedure. Follow the DNase treatment instructions in the manufacturer's RNA isolation kit manual.

3. Quantify total RNA with the Thermo-Fisher NanoDrop (or equivalent), and confirm the integrity of RNA in each sample prior to starting the assay by one of the following methods:

    - Determine the RIN number using the Agilent Bioanalyzer and Agilent RNA 6000 Pico Kit (Cat.# 5067-1511)
    - Determine the RIN number using the Agilent Fragment Analyzer and High Sensitivity RNA Analysis Kit (Cat.# DNF-472-1000)
    - Using a gel imager, calculate the 28S:18S rRNA ratio after running the RNA samples on an agarose gel.

The RIN number for your RNA samples should be no less than 5. If you are using FFPE RNA samples, you should additionally check the samples to ensure that a significant level (at least 50%) of RNA fragments is larger than 300 nt.

Last modified: 20 July 2023

5.3. DirectCell™ Protocol

Sample Type	Optimal	Range (total RNA)
Cell fractions	N/A	500 – 50,000 cells
Tissue (fresh/frozen)	N/A	up to 1 mg
Blood Microsamples	N/A	30 ul

As shown in the Table above, for a small number of cells, such as immune cell fractions isolated by FACS or magnetic beads (e.g. 5,000-50,000 cells), and tissue samples (up to ~1 mg), we recommend using cells directly without RNA purification step. Using sorted cells/tissues increases the sensitivity of CDR clonotype detection as compared to RNA-isolated samples. Refer to the guidelines in this section for the preparation of these samples.

To use small samples directly in the assay, follow the procedure below:

1. Purify immune cells or prepare dissociated tissue samples (e.g., by collagenase treatment). Adjust the volume of each sample to a total of 14 ul. Depending on your cell collection method, this may require spinning down the cells and removing excess supernatant over 14 ul.

2. When you are ready to start the DriverMap AIR Assay, prepare the Hybridization Buffer Master Mix as described in Step 1 of the Hybridization Procedure, add N-Lauroyl Sarcosine to 0.3% (0.7 µl of 20% N-Lauroyl Sarcosine), and then set up the Hybridization reaction with your sample as described.

3. Follow the standard protocol for hybridization, AMP purification, cDNA synthesis, and amplification steps.

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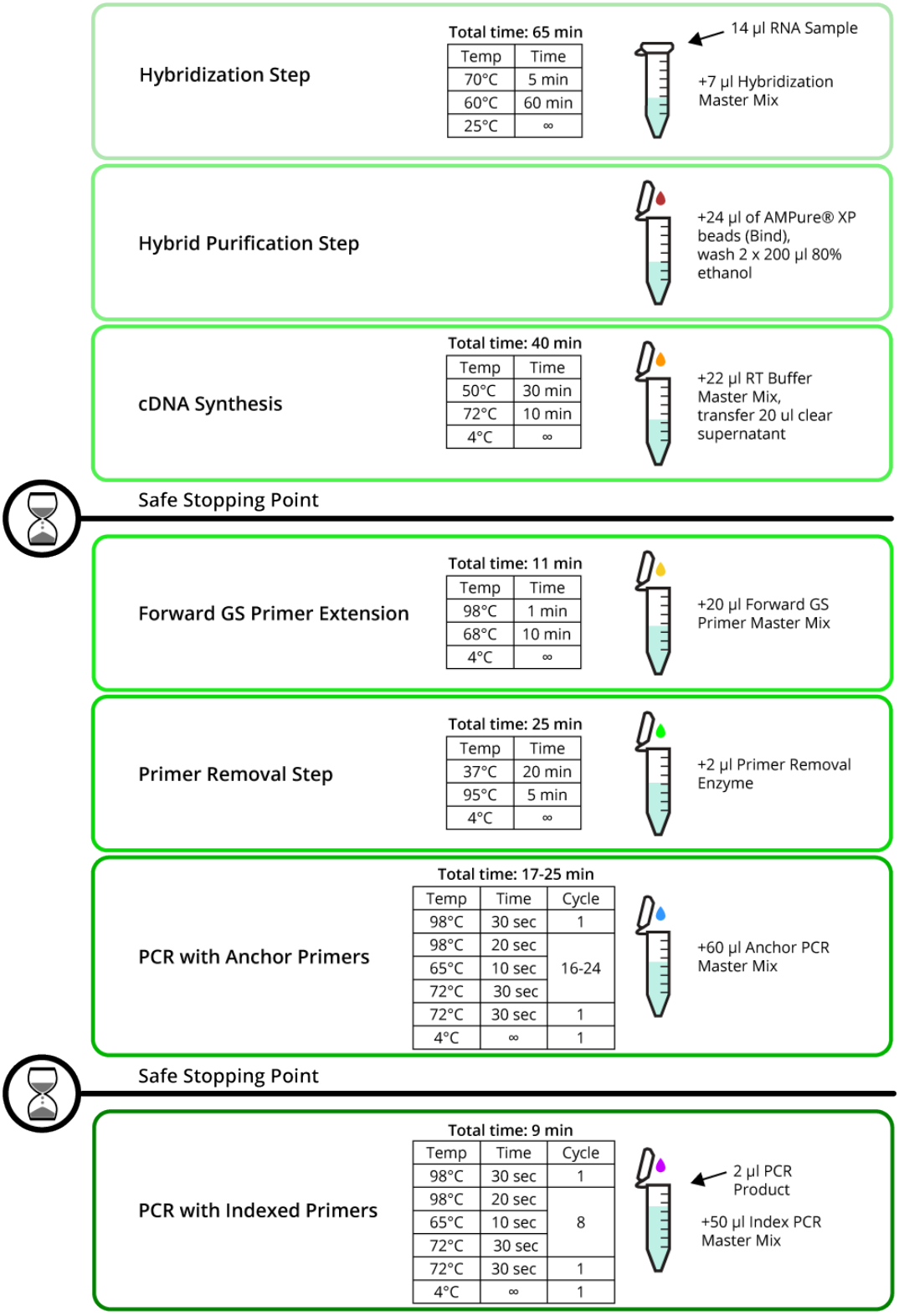
6. Outline of DriverMap™ AIR RNA Protocol



Last modified: 8 March 2023

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6.1. Hybridization of mRNA with Reverse C-region Gene-Specific Primers

In this step, mRNA-Rev C GSP hybrids are generated and then purified from non-hybridized primers by nuclease treatment and AMPure magnetic beads (Outline). The protocol is written assuming the reactions will be set off in a 96-well plate. For small numbers of samples, reactions can be done in tubes or strips also. Note: Hybridization, cDNA synthesis, and Forward Primer Extension steps should be set up in a PCR-free room.

1. Prepare a Hybridization Master Mix as described below for all samples and controls (make 5% extra to account for pipetting error) and aliquot 7 µl in the wells of a 96-well plate:

Hybridization Master Mix Component	Volume per sample, µl
Hybridization Buffer, 4X	5
Reverse TCR-C primer mix, 20X*	1
Reverse BCR-C primer mix, 20X*	1
Total	7

Note: If you need to run only TCR or BCR repertoire analysis, use only TCR-C or BCR-C primer mix and adjust the total volume to 7 µl by water. If you decide to run biological or amplification triplicates, increase the number of tubes, volume, and amount of RNA allocated for each sample respectively. For the DirectCell protocol, add N-Lauroyl Sarcosine to 0.3% (0.7 µl of 20% N-Lauroyl Sarcosine). We recommend running positive control RNA (50 ng), and negative control (water) for troubleshooting and comparing batch effects in different experiments.

2. Adjust the volume of each RNA Sample (e.g., 100 ng of whole blood or 50 ng of PBMC RNA) to 14 µl with water as shown in the table.

Component	Volume per sample, µl
Total RNA (~50-100 ng) of immune cell-rich sample	1 – 14*
Water, to 14 µl final volume	0 – 13

Note: For tissue samples with a low content of immune cells, the recommended amount is 200-1,000 ng of total RNA). For AIR profiling directly in immune cell fractions, use 5,000-50,000 cells in 14 µl of 1xPBS buffer.

3. Add 14 µl of RNA Sample to each well with pre-aliquoted 7 µl of Hybridization Master Mix and mix contents by pipetting 3 times. Seal the plate with adhesive film, and spin down to collect droplets. Load the plate in the thermal cycler, and run the following program to hybridize mRNAs with Reverse TCR/BCR-C primers:

Temperature	Time
70°C	5 min
60°C	60 min
25°C	∞

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4. Add 24 µl (1.2x volume) of Agencourt AMPure® XP Reagent (adjusted to room temperature) to each reaction well, and pipet up and down 5 times to thoroughly mix the bead suspension with the hybridization reaction mix. Check that the whole volume in each well has a uniform brown color.

5. Incubate the mixture for 5 minutes at room temperature. While waiting, prepare the Reverse Transcriptase Buffer Master Mix based on protocol Step 6.2.1. Store on ice before use.

6. Place the plate in the Magnetic Stand for 96-well plates for 1-2 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.

7. Add 200 µl of freshly prepared 80% ethanol in each reaction well without removal of plate from Magnetic Stand, wait for 2 minutes, carefully remove, and discard the supernatant without disturbing the bead pellets.

8. Repeat 80% ethanol washing in step 9.

9. Briefly centrifuge the plate at low speed and place the plate in the Magnetic Stand. Use a 20-µl pipette to remove the residual ethanol droplets from reaction wells and air-dry the beads at room temperature for approximately 5 minutes.

! Immediately proceed to the next step (cDNA Synthesis)

Last modified: 8 September 2023

6.2. cDNA Synthesis

In this step, the purified mRNA-Reverse GS primer hybrids eluted from AMPure beads are extended by Reverse Transcriptase to generate cDNA (antisense strand of mRNA).

1. Prepare the Reverse Transcriptase Buffer Master Mix as follows for each sample plus 5% extra volume of all components:

RT Buffer Master Mix Component	Volume per sample, µl
RT-EXT Buffer, 5x	4.4
dNTP Mix	1.1
Water	14.3
Hot-start RT Aptamer	1.1
Reverse Transcriptase	1.1
Total	22

2. Gently vortex Master Mix and spin down briefly to collect droplets. Add 22 µl of the RT Buffer Master Mix in each reaction well and resuspend AMPure beads attached to the well surface by pipetting or in the plate using an Eppendorf shaker. Briefly centrifuge the plate at low speed and place the plate in Magnetic Stand for 1 minute. Transfer 20 µl of clear supernatant without beads from each reaction well to the new plate and seal the plate.

3. Load the plate in the thermal cycler and start running the following program:

Temperature	Time

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50°C	30 min
72°C	10 min
4°C	∞

Stopping Point in procedure: cDNA samples may be stored at 4°C overnight or at –20°C for longer periods.

Last modified: 3 October 2022

6.3. Forward Gene-Specific Primer Extension

In this step, the pool of Forward Gene-Specific Primers with adjoining Anchor 1 sequences (Fig. 1) generates sense strands of the target amplicons flanked from both sides by Anchor 1 and Anchor 2 sequences using the cDNA generated in the previous step as a template and purified from non-extended primers by nuclease treatment.

1. Prepare the Forward GS Primer Extension Master Mix as follows for all samples plus 5% extra volume of all components:

Forward GS Primer Extension Master Mix Component	Volume per sample, µl
RT-EXT Buffer, 5X	4
Water	11.6
Forward TCR-FR3 Primer Mix, 20X*	2
Forward BCR-FR3 Primer Mix, 20X*	2
DNA Polymerase	0.4
Total	20

Note: If you decide to run only TCR or BCR repertoire profiling, use only a single corresponding forward TCR or BCR FR3 primer mix and adjust by water total volume to 20 µl.

2. Gently vortex Master Mix and spin down briefly to collect droplets. Spin down the cDNA plate, remove the seal, then add 20 µl of the Forward GS Primer Extension Master Mix to each reaction well of the plate:

Component	Volume, µl
cDNA (step above)	20
Forward GS Primer Extension Master Mix	20
Total	40

3. Mix contents by pipetting three times, seal the plate with a new adhesive film, and spin down to collect droplets.

Note: A new adhesive film is required to avoid cross-contamination.

4. Load the plate in the thermal cycler, and run the following program:

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Temperature	Time
98°C	1 min
68°C	10 min
4°C	∞

5. Spin down the plate, remove the seal from the plate, then add 2 µl of the Primer Removal Enzyme to each reaction well of the plate. Mix contents by pipetting 3 times, seal the plate, and spin down to collect droplets

6. Load the plate in the thermal cycler, and run the following program:

Temperature	Time
37°C	20 min
95°C	5 min
4°C	∞

! Immediately proceed to the next step (PCR with Anchor Primers)

Last modified: 8 March 2023

6.4. First PCR with Anchor Primers

This step utilizes universal Anchor PCR primers to amplify the target cDNA fragments flanked with the Anchor 1 and Anchor 2 sequences generated during the previous **Forward Gene-Specific Primer Extension** step (Outline).

1. Prepare the Anchor PCR Master Mix as shown below for all samples and controls plus 5% extra volume of all components:

Anchor PCR Master Mix Component	Volume per sample, µl
PCR Buffer, 5X	12
dNTP Mix	1.2
Anchor Primer Mix, 10X	10
Water	36.2
DNA Polymerase	0.6
Total	60

2. Gently vortex Master Mix and spin down briefly to collect droplets. Spin down the Forward GS Primer Extension plate, remove the seal, then add 60 µl of Anchor PCR Master Mix to each reaction well:

Component	Volume per sample, µl
Forward GS Primer Extension DNA (after primer removal step)	42
Anchor PCR Master Mix (prepared above)	60
Total	102

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3. Mix content by pipetting 3 times. Seal the plate with new adhesive film and spin down to collect droplets.

✻ Note: Take the plate from the PCR-free room and perform all follow-up Amplification, Purification, and NGS steps in the location dedicated to PCR work. Never bring amplified library products back to the PCR-free room.

4. Load the plate in the thermal cycler in a location dedicated to PCR work. Run the following program using the recommended number* of PCR cycles:

Temperature	Time	Cycles
98°C	30 sec	1
98°C	20 sec	16-24* Note below
65°C	10 sec	
72°C	30 sec	
72°C	30 sec	1
4°C	∞	1

✻ Note: To avoid bias in gene expression levels by over-cycling samples, we recommend starting with 18 PCR cycles for samples that contained 25 ng or more RNA (e.g., 50 ng of PBMC or 100 ng of whole blood or lymphocyte-rich samples) or at least 25,000 immune cells. For samples with less RNA or cells (or low lymphocyte RNA content), you may have to add extra cycles, but in general, we recommend not exceeding 20 cycles. For very small RNA samples (1-5 ng) or experiments with less than ~1000 immune cells, 22-26 cycles may be required. The recommended number of cycles needs to be optimized and adjusted based on specific cell types, sample types, RNA quality, and the level of TCR/BCR mRNAs, etc. In order to optimize cycle number we recommend analyzing 5 ul of amplified products in a 3% agarose gel or fragment analyzer. For optimal cycle number, you will detect weak cDNA amplification products in the range of 300-500 bp. If you can't see any products, add 3 more cycles and analyze the yield of PCR products again.

! Stopping Point in procedure: PCR products may be stored at 4°C overnight.

Last modified: 8 September 2023

6.5. Second PCR with Indexed Primers

This step adds a dual unique DNA/RNA UDP index combination to each Anchored PCR Product generated in the previous **PCR with Anchor Primers** step as well as universal flanking P5 and P7 sequences needed for cluster formation on the Illumina NGS flow cell (Outline).

The Index PCR Plate in the kit contains a unique combination of Forward and Reverse DNA/RNA UDP index primers in each well. The primers have been dried onto the bottom of each well and will be dissolved when the PCR reaction mix with the sample is added. One well should be used for each sample (triplicate samples are different samples) being sequenced. See **Appendix D. List of Dual DNA/RNA UDP Indexes** for the sequences of each Forward and Reverse Index combination.

For the 24 samples, use any two 2 lines (e.g., A1-B12) or 3 columns (e.g., 1A-3H) of well for the samples. If you are

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processing less than 24 samples, you can use scissors to cut the desired number of wells from the index plate (e.g., cut 1-2 columns for 8 samples), then store the rest of the plate for later use.

NOTE: If you run samples on a cut-out portion of the plate, be sure to reference the well location relative to the whole plate when entering the sample names at each plate index.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

Arrangement of the index pairs for 96 samples

1. Prepare enough of the Index PCR Master Mix, following the formulation below for all samples and controls plus 5% extra volume of all components:

Index PCR Master Mix Component	Volume per sample, µl
PCR Buffer, 5X	10
dNTP Mix	1
Water	38.5
DNA Polymerase	0.5
Total	50

Note: If you are using a multichannel pipette, aliquot the master mix in a tube strip to minimize pipetting steps.

2. Gently vortex the Index PCR Master Mix, and spin down briefly to collect droplets. Remove the plate seal from the Index PCR Plate. Set up the Index Primer PCR Reactions as follows:

3. Aliquot 50 µl of the Index PCR Master Mix into appropriate wells of the 96-well Index PCR Plate (or cut-off portion of the plate) provided in the kit. To avoid index- to-index contamination, add Index PCR Master Mix using a new tip for each well.

4. Spin down, then remove the seal from the Anchor Primer PCR plate (plate from **PCR with Anchor Primers** step). Transfer 2 µl of Anchored PCR products to each of the Index Primer PCR reactions on the Index PCR Plate. To avoid mistakes, ensure that samples in the Anchor Primer PCR plate are arranged in the same format as the Index PCR Primer pair mixes in the Index PCR Plate (e.g. Sample 1A is aliquoted to well 1A). We recommend that you record the Sample name and well number (e.g., Sample 1 in well 1A) for all the samples, including the positive control. This will help minimize mistakes in the NGS deconvolution step.

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Component	Volume per sample, µl
Anchored PCR Product (step above)	2
Index PCR Master Mix (above)	50
Total	52

5. Seal the plate with new adhesive film and spin down to collect droplets.

6. Load the plate in the thermal cycler, and run the following program:

Temperature	Time	Cycles
98°C	30 sec	1
98°C	20 sec	8
65°C	10 sec	
72°C	30 sec	
72°C	30 sec	1
4°C	∞	1

! Stopping Point in procedure: PCR products may be stored at 4°C overnight.

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7. NGS Prep and Sequencing

The product from the **PCR with Indexed Primers** step contains both P5 and P7 sequences and dual DNA/RNA UDP Indexes for Next-Gen Sequencing (NGS) on Illumina instruments. The protocol in this section provides the instructions to normalize the amount of each Amplified Indexed Library to obtain similar reads for each sample, to combine and clean up samples before loading onto the instrument, and guidelines for sequencing and data analysis.

Last modified: 23 June 2023

7.1. QC, Quantify and Combine Samples for NGS

In this step, the yield of products from the Index Primer PCR Reaction—the Amplified Indexed Libraries of transcripts from each sample—are analyzed (adjusted if necessary), measured, and then pooled in equimolar amounts for sequencing.

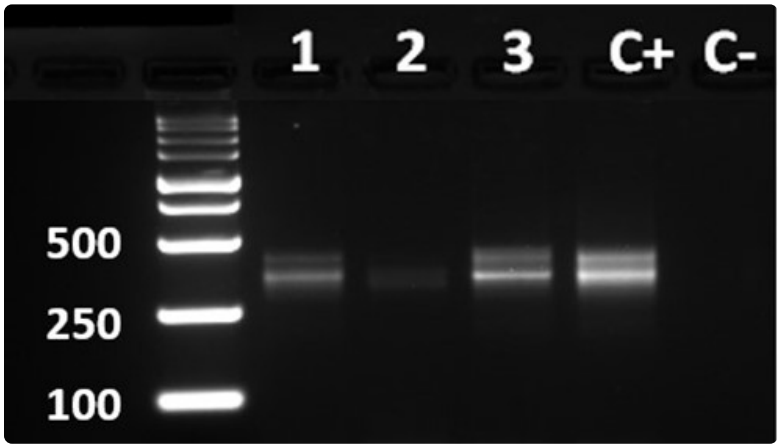
QC and Quantify Amplified Indexed Libraries

1. Analyze the Amplified Indexed Libraries using one of the following methods:

- Standard Method: Separate 5 µl of Amplified Indexed Libraries on a 3% agarose-TAE gel and analyze the size distribution of NGS probes by UV transilluminator. To minimize the sample number, you could run only one sample from each triplicate set. See below for the expected results of amplified libraries generated from good-quality whole blood RNA samples. For AIR TCR-BCR assay, the smear with several bright bands should be in the 220-420 bp range.

- Alternative Method: Analyze 1 µl of each of the Amplified Indexed Libraries on either an Agilent Bioanalyzer with the Agilent High Sensitivity DNA Kit (Cat.# 5067-4626) or Fragment Analyzer using the High Sensitivity NGS Analysis Kit (Cat.# DNF-473-1000) using the manufacturer's protocol.

✻ Note: In some cases, excess PCR primers compromise resolution and complicate data analysis with the BioAnalyzer or Fragment Analyzer systems. For such cases, we recommend running a conventional gel to look at the samples.



Gel electrophoresis of Amplified Indexed Libraries. Gel image of amplicons after Index Primer PCR Reaction. Lanes 1-3: Libraries prepared from total whole blood RNA of different samples; Lane 4: Positive control (C+) PBMC RNA; Lane 5: Negative Control (C-) water

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2. Analyze yields of the Amplified Indexed Libraries. The yield should be roughly the same for all experimental samples within +/- 2-3-fold levels and similar to the Positive Control RNA sample. Negative control sample should not generate any significant yield of amplified products. If some samples show a significantly lower yield of amplification products, it could indicate differences in the amount, quality of RNA, or content of TCR/BCR mRNAs used in AIR assay. For the experimental RNA samples with a significantly lower yield of PCR product (e.g., >5-10-fold) than other samples or positive control RNA, you can re-run the lower yield samples in the thermal cycler for 2-5 additional cycles (see Note below). After cycling, quantify the products again relative to the Positive Control RNA. **Note:** Do not include the Positive Control RNA sample in additional cycles. Remove the positive control sample from the plate and keep it as a reference to assess the number of your PCR samples.

> Note: We recommend adjusting the cycle number for each sample separately. Adjustments for small differences in the yield between samples will be made when the samples are combined for the sequencing step following this quantification procedure.

Remove Excess PCR Primers and Combine Samples

1. Remove excess primers from the completed PCR reactions by adding 1 µl of Primer Removal Enzyme to each of the Amplified Indexed Libraries and the Negative Control sample, then incubate at 37°C for 30 minutes.

2. To ensure accurate quantification for sequencing, you should repeat the quantification procedure of the Amplified Indexed Libraries and the Positive Control RNA. The preferred method is to analyze 2 µl of each of the Amplified Indexed Libraries using either an Agilent Bioanalyzer with the Agilent High Sensitivity DNA Kit (Cat.# 5067-4626) or Fragment Analyzer using the High Sensitivity NGS Analysis Kit (Cat.# DNF-473-1000) using the manufacturer's protocol. Quantifying the PCR products after removing PCR primers is more accurate than quantifying before the primer removal clean-up step.

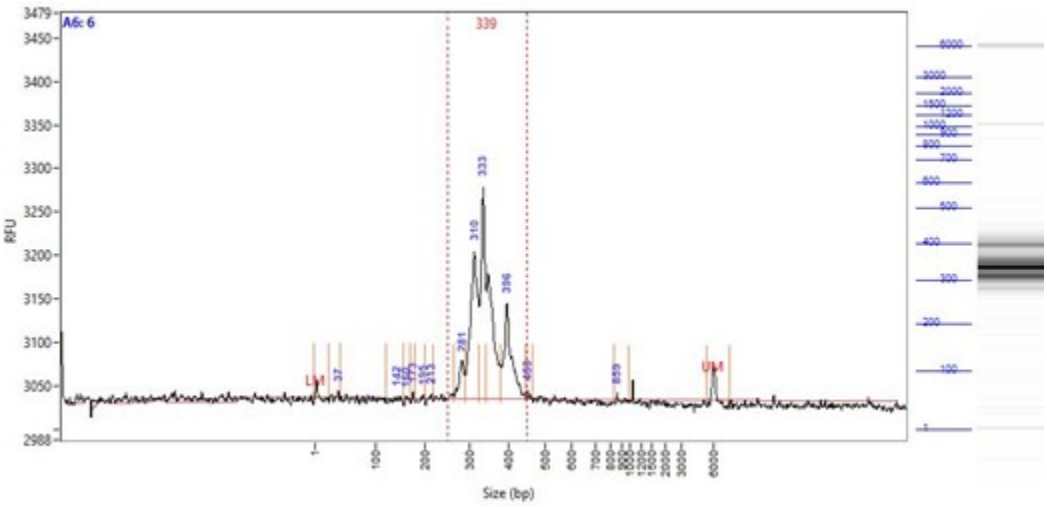

Fig. 4. Size distribution of Amplified Indexed Library after Primer Removal step analyzed by Fragment Analyzer.

3. After primer removal and quantification, use the yield assessment of the Amplified Indexed Libraries as a basis to combine equimolar amounts of each of the Amplified Index Libraries into a single pool for NGS. For example, if the yield of Library 1 is twice that of Library 2, then mix 5 µl of Library 1 with 10 µl of Library 2. To minimize sample-to-sample sequencing variations, combine and load all experimental samples onto one flow cell.

For the DriverMap AIR Assay, which generates a complex pool of amplified TCR/BCR CDR regions (100K-200K from 50 ng of total whole blood, PBMC RNA), refer to the table below for guidelines on how many samples may be combined for different

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instruments and read depths. Generally, you should aim for 5-10 million reads per AIR sample (starting from 50 ng of PBMC RNA) or at least 20 reads per UMI. For Illumina instruments, AIR profiling assays require 300-n paired-end reagent kits.

Instrument	Reads per flow cell	Number of samples for multiplexing per flow cell	Reads per AIR sample
NextSeq 500/ 550	120 million (medium throughput)	12-20	6-10M reads/sample
NextSeq 500/ 550	400 million (high throughput)	40-72	6-10M reads/sample
NextSeq 2000	1200 million (P3 flow cell)	96	10M reads/sample
NovaSeq Series	6000 million (S2)	500	10M reads/sample

! Stopping Point in procedure: PCR products may be stored at 4°C overnight or at -20°C for a few days.

Last modified: 20 July 2023

7.2. Purification of Amplified Indexed Library

The purpose of this step is to remove any residual primers and reagents from the pooled Amplified Indexed Libraries so that the preparations are ready for NGS.

1. Add 1.5x volume of Agencourt® AMPure® XP Reagent (at room temperature) to pooled Amplified Indexed Libraries, mix in an Eppendorf tube, and pipet up and down 5 times to thoroughly mix the bead suspension with the pooled Amplified Indexed Libraries.

2. Incubate the mixture for 5 minutes at room temperature.

3. Place the tube in the Magnetic Stand for 1 minute or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.

4. Add 500 µl of freshly prepared 80% ethanol to the tube.

5. Place the tube in the Magnetic Stand for 2 minute or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.

6. Repeat steps 4 and 5 for a second wash.

7. Briefly centrifuge tubes at low speed and place the tubes in the Magnetic Stand. Use a 200-µl pipette to remove the residual ethanol droplets from the tube and air-dry the beads at room temperature for 2 minutes.

8. Add 45 µl of TE buffer to the pellet to disperse the beads and let stand for 1 minute.

9. Place the tube on the Magnetic Stand for 1 minute. Transfer 40 µl of the supernatant to a new Eppendorf tube.

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! Stopping Point in procedure: PCR products may be stored at 4°C overnight or at -20°C for a few days.

Last modified: 26 September 2023

7.3. Next Generation Sequencing

The Amplified AIR NGS Index Libraries made from each total RNA sample should be run on an Illumina sequencer following the manufacturer's instructions. Generally, we recommend setting up the sequencing reactions to generate 5-10 million reads per sample (read depth per sample). This depth works out to produce 20-50 reads, on average, per UMI.

✻ Note: The procedure below is based on our experience with the NextSeq 500/550 using the 300-cycle NextSeq 500/550 High Output Kit v2.5 (Illumina Cat.# 20024908). For other Illumina instruments, please contact Illumina Tech Support Team for optimal NGS instructions.

The multiplexing level may be modified to meet your experimental needs. For example, multiplex fewer samples together to generate more sequencing reads per sample (i.e., more depth) for increased and more sensitive detection of clonotypes present across a broader dynamic range of abundance/expression levels, or, if you are mostly interested only in highly abundant/expressed clonotypes, you can sequence more samples together which will be less expensive.

Follow the standard Illumina procedures for Cluster Generation, starting with 10 nM of the purified PCR sample. We recommend adding 15% of PhiX to the library.

1. Add 6 µl of each of the custom sequencing primers into the appropriate wells of the Illumina reagent cartridge, as follows:

    - Forward SeqDNA NGS Primer (Read 1 Sequencing Primer) into well #20
    - Reverse SeqDNA NGS Primer (Read 2 Sequencing Primer) into well #21
    - Forward SeqIND NGS Primer (Index 1 Sequencing Primer) into well #22
    - Reverse SeqIND NGS Primer (Index 2 Sequencing Primer) into well #22

✻ Note: We do not recommend adding primers to the "Custom" wells of the reagent cartridge. We add our in-house custom sequencing primers into the Illumina premixed primer wells to take advantage of the PhiX internal control. Addition of our in-house sequencing primers does not affect the performance of the flow cell.

2. Perform the NGS run using 300-nt paired-end reads on the NextSeq. Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit; refer to the manufacturer's instructions for this step. The optimal seeding concentration for cluster amplification of indexed libraries is approximately 1.8 pM for NextSeq500/550. Use the following program for the sequence run:

Program	Custom Primer	Cycles
Read 1:	Forward SeqDNA	148
Index 1:	Forward SeqIND	10
Index 2:	Reverse SeqIND	10

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Read 2:	Reverse SeqDNA	148

Note: The program has a total of 316 cycles. There are enough reagents in the 300-cycle kit (Illumina Cat.# 20024908) to run at least 316 cycles.

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8. Data Processing and Analysis

The protocols in this section describe how to extract T- and B- Cell receptor repertoire from NGS data generated from the Cellecta DriverMap AIR kit. MiXCR is used to analyze NGS data, extract clonotype and obtain vector plots and tabular results.

Even though MiXCR is widely used for immune repertoires data analysis. Other bioinformatics tools/frameworks are also developed to analyze immune repertoires.

Tools	Published year	Aligner	Website
IMGT/HighV-QUEST	2012	GPA (NW)	https://www.imgt.org/IMGTindex/IMGTHighV-QUEST.php
IgBLAST	2013	BLAST	https://www.ncbi.nlm.nih.gov/igblast/
IMSEQ	2015	SCF matching	https://www.imtools.org/
LymAnalyzer	2015	Fast-tag	https://sourceforge.net/projects/lymanalyzer/

MiXCR is free for academic and non-profit use (see License). The commercial license could be requested at https://licensing.milaboratories.com or by email to licensing@milaboratories.com or https://milaboratories.com/contacts. Please refer to Appendix C for installing instructions. Before downloading or accessing the software, please carefully read the License Agreement available at https://github.com/milaboratory/mixcr/blob/develop/LICENSE.

Mac OS and Linux OS are recommended for data processing and analysis.

Cellecta does not own any of these software packages and is not responsible for maintaining them. In case you don't have the license, Cellecta also provides custom data analysis services using the MiXCR software package. Please email tech@cellecta.com for more information.

Last modified: 12 January 2024

8.1. Extract T- and B- Cell receptor repertoire workflow

The following bioinformatics workflow is recommended for DriverMap AIR assay:

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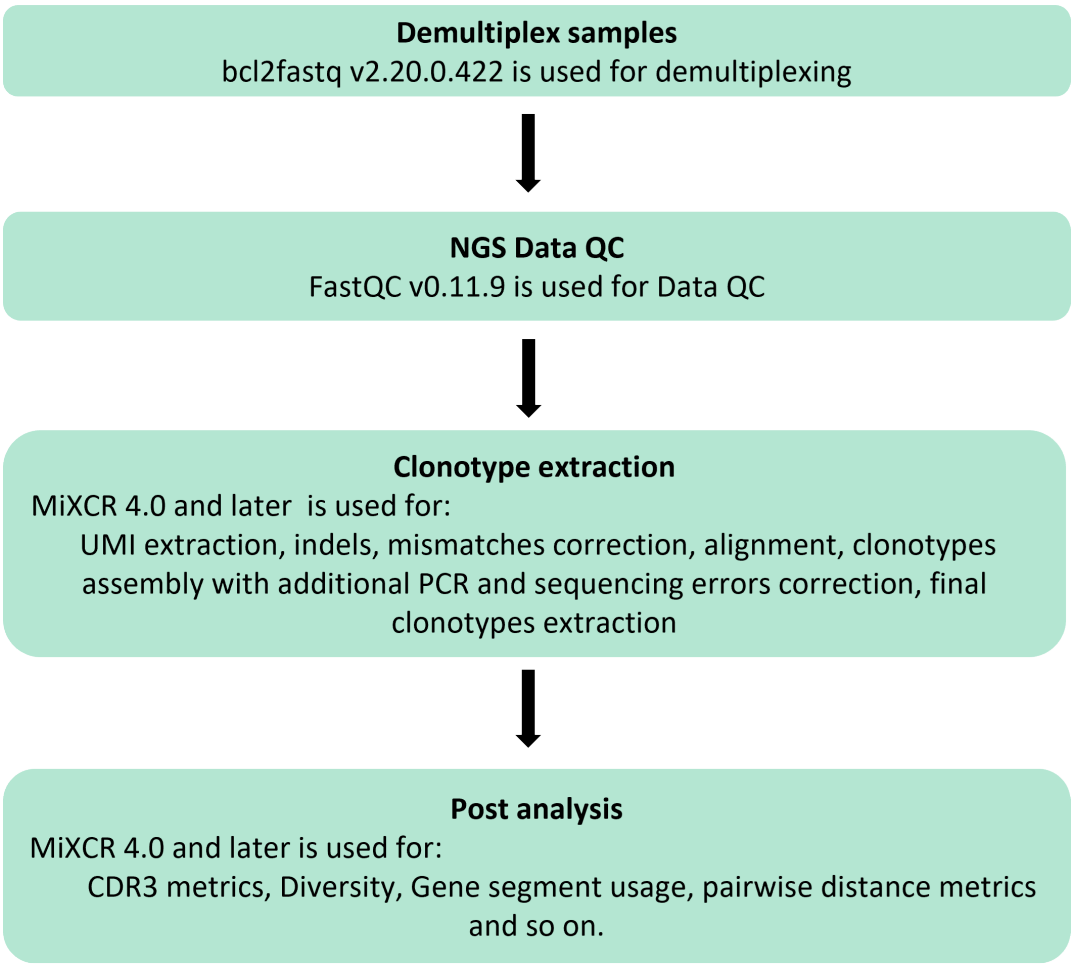

Last modified: 30 September 2022

8.2. Convert Sequencing Data to FASTQ Format

bcl2fastq2 converter is used to demultiplex sequencing data and convert base call (BCL) files to FASTQ files. The user manual can be downloaded from the following link:

https://support.illumina.com/content/dam/illumina-support/documents/documentation/software_documentation/bcl2fastq/bcl2fastq2-v2-20-software-guide-15051736-03.pdf

Refer to Appendix D or download Example sample sheet *(Right-click link and select Save Link As… to save .txt file)*

This step can be overlooked if demultiplexed fastq files were generated from NGS sequencing core.

Last modified: 8 March 2023

8.3. NGS Data QC

FastQC provides a modular set of analyses that you can use to give a quick impression of whether your data has any problems of which you should be aware before doing any further analysis. The user manual can be found at the following link:

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https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Last modified: 30 September 2022

8.4. Alignment and clonotypes assembly using MiXCR

Clonotype extraction can be performed using MiXCR. The pipeline includes alignment of raw sequencing reads using **align**, error correction and UMI grouping using **correctAndSortTags**, assembly of aligned sequences into clonotypes using assemble, and exporting the resulting clonotypes into tab-delimited file using **exportClones**.

The pattern used to detect UMI is:

```
^(R1:*)\^(UMI:N{18})(R2:*)
```

You can use the following presets for the MiXCR analysis:

For the DriverMap AIR RNA Kit:
cellecta-human-rna-xcr-umi-drivermap-air

For the DriverMap AIR DNA Kit:
cellecta-human-dna-xcr-umi-drivermap-air

For the DriverMap AIR RNA Full-length Kit:
cellecta-human-rna-xcr-full-length-umi-drivermap-air

For the DriverMap AIR Mouse Kit:
cellecta-mouse-rna-xcr-umi-drivermap-air

More details can be found on the MiXCR official website: Cellecta AIR Assay Guide.

Last modified: 24 January 2024

8.5. Advance Statistical Analysis

The downstream analysis also can be performed by MiXCR. MiXCR V4.0 and later provide new features to export results in tabular format and vector plots with various statistical comparisons. More details can also be found on MiXCR's official website (https://docs.milaboratories.com/).

Last modified: 8 March 2023

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9. Appendices

Last modified: 30 September 2022

9.1. Appendix A. Tips and Troubleshooting

Observation	Possible Cause	Recommended Action
Yield of PCR products for DNA samples is low, but is good for Positive Control DNA sample — or — Low yield of PCR products for both experimental and Positive Control DNA samples	Error in quantitation of input DNA, or DNA is degraded	Re-analyze the amount and quality of input DNA using Agilent Bioanalyzer, Agilent Fragment Analyzer, or Thermo Fisher NanoDrop.
	DNA input is too low	Add more DNA, or increase target amplification cycles (Refer to **PCR with Anchor Primers** for cycle numbers recommended based on input amounts).
	RT step is not optimal	Please check for procedural mistakes. Make master mixes where possible. For individual reaction setups, make sure that the correct volume of RT-EXT Buffer and RT enzyme is added into each reaction.
	PCR cycling conditions are not optimal	Increase PCR amplification cycles. Ensure proper dispensing and mixing of viscous components at each step.

Observation	Possible Cause	Recommended Action
Low Cluster Density.	Inefficient purification from low molecular weight DNA products or low yield of PCR products	Re-purify PCR products using different technology (e.g. QIAGEN QIAquick PCR Purification Kit), and/or increase the concentration of pooled PCR products in the cluster generation step.

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Observation	Possible Cause	Recommended Action
Yields of PCR products are significantly higher than Positive Control	Incorrect quantitation of input DNA amount	Re-analyze the amount and quality of input DNA using Agilent Bioanalyzer, Agilent Fragment Analyzer, or Thermo Fisher NanoDrop.
	DNA input is too high	Add less DNA or decrease number of PCR cycles in the **PCR with Indexed Primers** step.
Low fluorescence readings on Illumina sequencer — or — Lower than expected number of on-target reads — or — High mutation rate in the Index reads	PCR primers from amplified indexed library quantification step (**Quantify and Combine Samples for NGS**) were not removed	Check that you added Primer Removal Master Mix in the **Quantify and Combine Samples for NGS** step. If it had been added, use a new vial of Primer Removal Reagent and repeat.
Lower than expected number of on-target reads	DNA input too low or PCR cycle number too low	Add more DNA or increase number of PCR cycles in target amplification step (**PCR with Anchor Primers**).
	DNA is degraded	Use highest quality DNA possible. For degraded DNA, increase the number of PCR cycles.
Uneven representation of Indexed Libraries	Inaccurate Indexed Library quantification	Check that you correctly calculate the molar concentration of each indexed library sample.
	Inaccurate Indexed Library mixing	Re-quantify the indexed library samples and mix them in equimolar amounts.
Inconsistent library yields from replicate DNA samples	Sample evaporation in thermal cycler	Seal 96-well plates well with an Adhesive Film Applicator and use a Compression Pad. Fill empty wells with water to minimize evaporation from experimental samples.

Last modified: 14 February 2023

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9.2. Appendix B. Structure of Amplified Indexed Library

Last modified: 30 September 2022

9.3. Appendix C. Obtaining and Installing Software for Data Analysis

MiXCR Installation on different operation systems (More details can be obtained from the following link: https://mixcr.readthedocs.io/en/master/install.html).

MiXCR installation on Mac OS / Linux using Homebrew

Homebrew is a simple package manager developed for Mac OS and also ported to Linux. To install MiXCR using Homebrew just type the following commands:

```
$ brew tap milaboratory/all
$ brew install mixcr
```

MiXCR Installation on Mac OS X / Linux / FreeBSD from zip distribution

- Check that you have Java 1.8+ installed on your system by typing in the command line:
  `$ java -version`
- Download latest binary distributaion of MiXCR from the release page on GitHub
- Unzip the archive
- Add extracted folder of MiXCR distribution to your PATH variable or add a symbolic link for MiXCR script to your bin/ folder (e.g. ~/bin/ in Ubuntu and many other popular Linux distributions)

MiXCR Installation on Windows

Currently, there is no execution script or installer for Windows. Still, MiXCR can easily be used by direct execution from the jar file.

- Check that you have Java 1.8+ installed on your system by typing in the command line:
  `$ java -version`
- Download latest binary distribution of MiXCR from the release page on GitHub
- Unzip the archive

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- Use mixcr.jar from the archive in the following way:
  ```
  > java -Xmx4g -Xms3g -jar path_to_mixcr\jar\mixcr.jar ...
  ```

Bcl2fastq2 and FastQC Installation

Details on how to download, install, and execute bcl2fastq2 and FastQC can be obtained from the links below:
Download and install bcl2fastq2: https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software/downloads.html
Download FastQC: https://www.bioinformatics.babraham.ac.uk/projects/download.html#fastqc
Install FastQC: https://raw.githubusercontent.com/s-andrews/FastQC/master/INSTALL.txt

> Note: FastQC can also be installed and run as a “click-and-go” Desktop application on your Windows/Linux FastQC v0.11.9 (Win/Linux zip file) or MacOS personal computer FastQC v0.11.9 (Mac DMG image).

Last modified: 3 October 2022

9.4. Appendix D. List of Dual DNA/RNA UDP Indexes

Example Sample Sheet *(Right-click link and select Save Link As… to save .txt file)*

Sample_ID	Sample_Name	I7_Index_ID	index	I5_Index_ID	index2
A1	1	UDP0001	GAACTGAGCG	UDP0001	CGCTCCACGA
A2	2	UDP0002	AGGTCAGATA	UDP0002	TATCTTGTAG
A3	3	UDP0003	CGTCTCATAT	UDP0003	AGCTACTATA
A4	4	UDP0004	ATTCCATAAG	UDP0004	CCACCAGGCA
A5	5	UDP0005	GACGAGATTA	UDP0005	AGGATAATGT
A6	6	UDP0006	AACATCGCGC	UDP0006	ACAAGTGGAC
A7	7	UDP0007	CTAGTGCTCT	UDP0007	TACTGTTCCA
A8	8	UDP0008	GATCAAGGCA	UDP0008	ATTAACAAGG
A9	9	UDP0009	GACTGAGTAG	UDP0009	CACTATCAAC
A10	10	UDP0010	AGTCAGACGA	UDP0010	TGTCGCTGGT
A11	11	UDP0011	CCGTATGTTC	UDP0011	ACAGTGTATG
A12	12	UDP0012	GAGTCATAGG	UDP0012	AGCGCCACAC
B1	13	UDP0013	CTTGCCATTA	UDP0013	CCTTCGTGAT
B2	14	UDP0014	GAAGCGGCAC	UDP0014	AGTAGAGCCG
B3	15	UDP0015	TCCATTGCCG	UDP0015	TCGTGCATTC
B4	16	UDP0016	CGGTTACGGC	UDP0016	CTATAGTCTT
B5	17	UDP0017	GAGAATGGTT	UDP0017	TTGCTGCCGA
B6	18	UDP0018	AGAGGCAACC	UDP0018	CCATCATTAG
B7	19	UDP0019	CCATCATTAG	UDP0019	AGAGGCAACC
B8	20	UDP0020	GATAGGCCGA	UDP0020	GCCATGTGCG
B9	21	UDP0021	ATGGTTGACT	UDP0021	AGGACAGGCC

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B10	22	UDP0022	TATTGCGCTC	UDP0022	CCTAACACAG
B11	23	UDP0023	ACGCCTTGTT	UDP0023	ACGTTCCTTA
B12	24	UDP0024	TTCTACATAC	UDP0024	TTACAGTTAG
C1	25	UDP0025	AACCATAGAA	UDP0025	CCATCTCGCC
C2	26	UDP0026	GGTTGCGAGG	UDP0026	TTGCTCTATT
C3	27	UDP0027	TAAGCATCCA	UDP0027	AATGGATTGA
C4	28	UDP0028	ACCACGACAT	UDP0028	CCGCATACGA
C5	29	UDP0029	GCCGCACTCT	UDP0029	CGAGGTCGGA
C6	30	UDP0030	CCACCAGGCA	UDP0030	ATTCCATAAG
C7	31	UDP0031	GTGACACGCA	UDP0031	GTCCGTAAGC
C8	32	UDP0032	ACAGTGTATG	UDP0032	CCGTATGTTC
C9	33	UDP0033	TGATTATACG	UDP0033	TGTAATCGAC
C10	34	UDP0034	CAGCCGCGTA	UDP0034	CACGGCTAGT
C11	35	UDP0035	GGTAACTCGC	UDP0035	TCACCAACTT
C12	36	UDP0036	ACCGGCCGTA	UDP0036	AATATTGCCA
D1	37	UDP0037	TGTAATCGAC	UDP0037	CGCGGTGATC
D2	38	UDP0038	GTGCAGACAG	UDP0038	ACGGATGGTA
D3	39	UDP0039	CAATCGGCTG	UDP0039	TTCCTACAGC
D4	40	UDP0040	TATGTAGTCA	UDP0040	CATTAGTGCG
D5	41	UDP0041	ACTCGGCAAT	UDP0041	TTCAGTTGTC
D6	42	UDP0042	GTCTAATGGC	UDP0042	CCTGACCACT
D7	43	UDP0043	CCATCTCGCC	UDP0043	AACCATAGAA
D8	44	UDP0044	CTGCGAGCCA	UDP0044	TGGCCGGATT
D9	45	UDP0045	CGTTATTCTA	UDP0045	AACCTTATGG
D10	46	UDP0046	AGATCCATTA	UDP0046	TGGTAGAGAT
D11	47	UDP0047	GTCCTGGATA	UDP0047	TTCGCCACCG
D12	48	UDP0048	CAGTGGCACT	UDP0048	CCTATTGTTA
E1	49	UDP0049	AGTGTTGCAC	UDP0049	CGTGTACCAG
E2	50	UDP0050	GACACCATGT	UDP0050	TACACGTTGA
E3	51	UDP0051	CCTGTCTGTC	UDP0051	TCACAACAGT
E4	52	UDP0052	TGATGTAAGA	UDP0052	AAGGACGCAC
E5	53	UDP0053	GGAATTGTAA	UDP0053	AGGATGTGCT
E6	54	UDP0054	GCATAAGCTT	UDP0054	TGCGACGGAA
E7	55	UDP0055	CTGAGGAATA	UDP0055	AGTGGTTAAG
E8	56	UDP0056	AACGCACGAG	UDP0056	TATCCGAGGC
E9	57	UDP0057	TCTATCCTAA	UDP0057	CCAGTCGACG
E10	58	UDP0058	CTCGCTTCGG	UDP0058	TTGACTAGTA
E11	59	UDP0059	CTGTTGGTCC	UDP0059	AACGGTCTAT
E12	60	UDP0060	TTACCTGGAA	UDP0060	CTGGAACTGT
F1	61	UDP0061	TGGCTAATCA	UDP0061	CTACATGCCT

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F2	62	UDP0062	AACACTGTTA	UDP0062	TGAGACTTGC
F3	63	UDP0063	ATTGCGCGGT	UDP0063	GCGGAGCCAA
F4	64	UDP0064	TGGCGCGAAC	UDP0064	AGTATCAGTT
F5	65	UDP0065	TAATGTGTCT	UDP0065	TATGCCTTAC
F6	66	UDP0066	ATACCAACGC	UDP0066	CGCAGCAATT
F7	67	UDP0067	AGGATGTGCT	UDP0067	GGAATTGTAA
F8	68	UDP0068	CACGGAACAA	UDP0068	GTGCTAGGTT
F9	69	UDP0069	TGGAGTACTT	UDP0069	TCCACACAGA
F10	70	UDP0070	GTATTGACGT	UDP0070	TTGGAATTCC
F11	71	UDP0071	CTTGTACACC	UDP0071	AAGCGCGCTT
F12	72	UDP0072	ACACAGGTGG	UDP0072	ACAACGCTCA
G1	73	UDP0073	CCTGCGGAAC	UDP0073	AGCCTATGAT
G2	74	UDP0074	TTCATAAGGT	UDP0074	CCTTCTAACA
G3	75	UDP0075	CTCTGCAGCG	UDP0075	TACATCCATC
G4	76	UDP0076	CTGACTCTAC	UDP0076	TGACGGCCGT
G5	77	UDP0077	TCTGGTATCC	UDP0077	GTAAGCAACG
G6	78	UDP0078	CATTAGTGCG	UDP0078	TATGTAGTCA
G7	79	UDP0079	ACGGTCAGGA	UDP0079	AACGAGGCCG
G8	80	UDP0080	GGCAAGCCAG	UDP0080	CGGATGCTTG
G9	81	UDP0081	TGTCGCTGGT	UDP0081	AGTCAGACGA
G10	82	UDP0082	ACCGTTACAA	UDP0082	TCGCTATGAG
G11	83	UDP0083	TATGCCTTAC	UDP0083	TAATGTGTCT
G12	84	UDP0084	ACAAGTGGAC	UDP0084	AACATCGCGC
H1	85	UDP0085	TGGTACCTAA	UDP0085	AGTACTCATG
H2	86	UDP0086	TTGGAATTCC	UDP0086	GTATTGACGT
H3	87	UDP0087	CCTCTACATG	UDP0087	AGGAGGTATC
H4	88	UDP0088	GGAGCGTGTA	UDP0088	ACTTACGGAT
H5	89	UDP0089	GTCCGTAAGC	UDP0089	AAGATACACG
H6	90	UDP0090	ACTTCAAGCG	UDP0090	TTCATGGTTC
H7	91	UDP0091	TCAGAAGGCG	UDP0091	TATGATGGCC
H8	92	UDP0092	GCGTTGGTAT	UDP0092	GGAAGTATGT
H9	93	UDP0093	ACATATCCAG	UDP0093	ATTGCACATA
H10	94	UDP0094	TCATAGATTG	UDP0094	CACCTTAATC
H11	95	UDP0095	GTATTCCACC	UDP0095	TTGTCTACAT
H12	96	UDP0096	CCTCCGTCCA	UDP0096	CACCGATGTG

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