CELLECTA

USER MANUAL

DriverMap™ AIR RNA TCR-BCR Spike-in Controls (Human)

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Cellecta, Inc.

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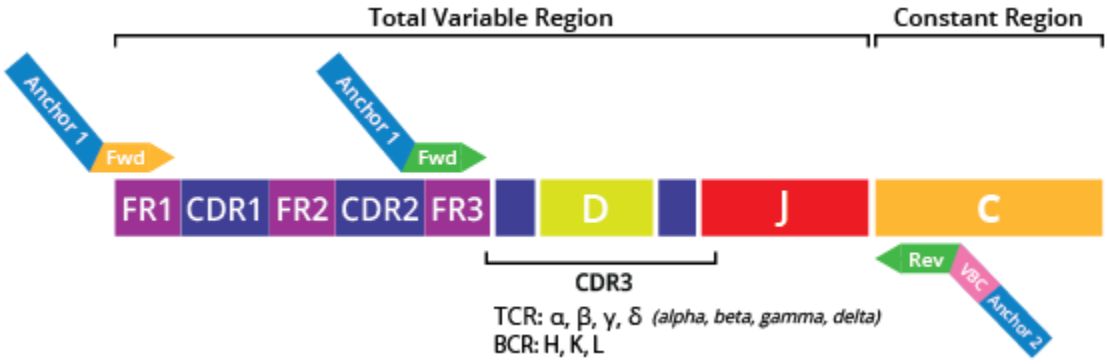
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1. DriverMap™ AIR Spike-in Controls

The DriverMap Adaptive Immune Receptor (AIR) Spike-in controls are a set of synthetic T-Cell Receptor (TCR) and B-Cell Receptor (BCR) constructs with unique CDR3 sequences in RNA format. These controls contain nearly full-length V(D)JC structures (Fig. 1), which represent all seven TCR (TRB, TRA, TRG, TRD) and BCR (IGH, IGK, and IGL) chains. They are designed as universal RNA positive controls for validating, calibrating, and standardizing commercial and home-brewed AIR sequencing technologies based on multiplex RT-PCR or 5'-RACE (SMART) PCR assays. Please refer to Appendix (A and B) for the sequences of all immune receptor constructs, the concentration of template molecules, and the ratio between different constructs provided in the AIR Control Mix and Isoform Pools.



As reviewed by the AIRR consortium Truck et al. (doi: 10.7554/eLife.66274), there is a general need for controls to standardize Adaptive Immune Receptor Repertoire Profiling assays. The DriverMap AIR Synthetic Spike-in controls help address this need by providing synthetic TCR and BCR isoforms that can be added to any PCR-based AIR assay to measure the following:

- Errors in TCR or BCR sequences, introduced at reverse transcription, amplification, or NGS steps.
- Sensitivity, linearity, and specificity in detecting AIR clonotypes affected by enzymatic inefficacy, off-target activity, low sequencing depth, and non-sufficient or excessive template sample size.
- Cross-contamination between samples due to sample mixing, PCR contaminations, or index hopping/jumping in the NGS step.
- Recombination between clonotypes with similar structures, generated at amplification or NGS steps.
- Data processing errors are introduced during filtering, error correction, and calculation of consensus sequences.
- Sample quality control due to potential degradation (e.g., FFPE), loss or unbalanced composition of RNA templates, or presence of inhibiting impurities.
- Batch effects and reproducibility in overall data quality generated by differences introduced at multiple workflow stages.

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. Design of AIR Spike-in Controls

DriverMap AIR Spike-in controls are synthetic constructs for TCR or BCR in RNA format. They are designed with several unique combinations of V(D)JC genes (isoforms), each construct having a unique CDR3 sequence. Each isoform is represented by three different constructs/variants (a, b, and c) with the same V(D)JC structure but different CDR3 sequences.

AIR Spike-in Controls are available in two formats:

1. **Premixed Control Mixes:** Premade mixes of 48 BCR and 39 TCR synthetic RNA constructs that contain all 16 BCR V(D)JC Isoform with 3 CDR3 variants for each isoform (i.e., 48 synthetic BCR constructs) or all 13 TCR V(D)JC isoforms with 3 CDR3 variants for each isoform (i.e., 39 TCR constructs), respectively. In the Premixed Controls, the three CD3 variants from each V(D)JC Isoform Pool are mixed in a 16:4:1 ratio as described in Appendix A, so the three CD3 variants of each isoform are present in three discrete concentrations 4000, 1000 and 250 molecules per microliter.

2. **Triplex Isoform Pools:** Set of the 16 BCR and 13 TCR pools have three synthetic RNA constructs with the same V(D)JC isoform receptor structure, but each construct has a different unique CD3 sequence. The sequences of the three CD3 variant constructs within each Pool differ from each other by 3 unique single-nucleotide mutations, and the three variants are mixed at a 1:1:1 ratio with 2,500 molecules/µl each. Please refer to the Appendix section for specific TCR and BCR construct sequences, concentrations, and ratios for Triplex Isoform Pools and AIR Control Mix.

DriverMap AIR Spike-in Control Mix Kit contains TCR/BCR isoform pools or mixes at different concentrations calculated based on the A260 OD measurement of individual RNAs. The “theoretical” concentration is the number of molecules per microliter. However, based on our experimental validation results using digital-droplet PCR (ddPCR), this theoretical concentration is approximately 2x higher than the concentration measured by ddPCR. We observed that the number of construct-specific molecules detected in the AIR-RNA profiling experiment by data alignment software such as MiXCR (MiLabs Inc.) is approximately 20-60% of the “theoretical” number of molecules spiked in the RNA sample.

For example, if you spike in 5,000 “theoretical” control RNA molecules in an experimental RNA sample and run the DriverMap AIR-RNA assay, it is expected to reveal approximately 1,000-3,000 molecules at the NGS-data analysis step. The number of detected molecules for each V(D)JC-specific isoform control construct can be different (usually +/- 2-fold), which reflects differences in the performance of PCR primers used in AIR assay. The difference between theoretical and measured concentration can be attributed to losing control molecules at the library prep stage, such as purification with SPRIselect magnetic beads, pipetting, and enzymatic steps, and contamination of short RNA products in the final control RNAs.

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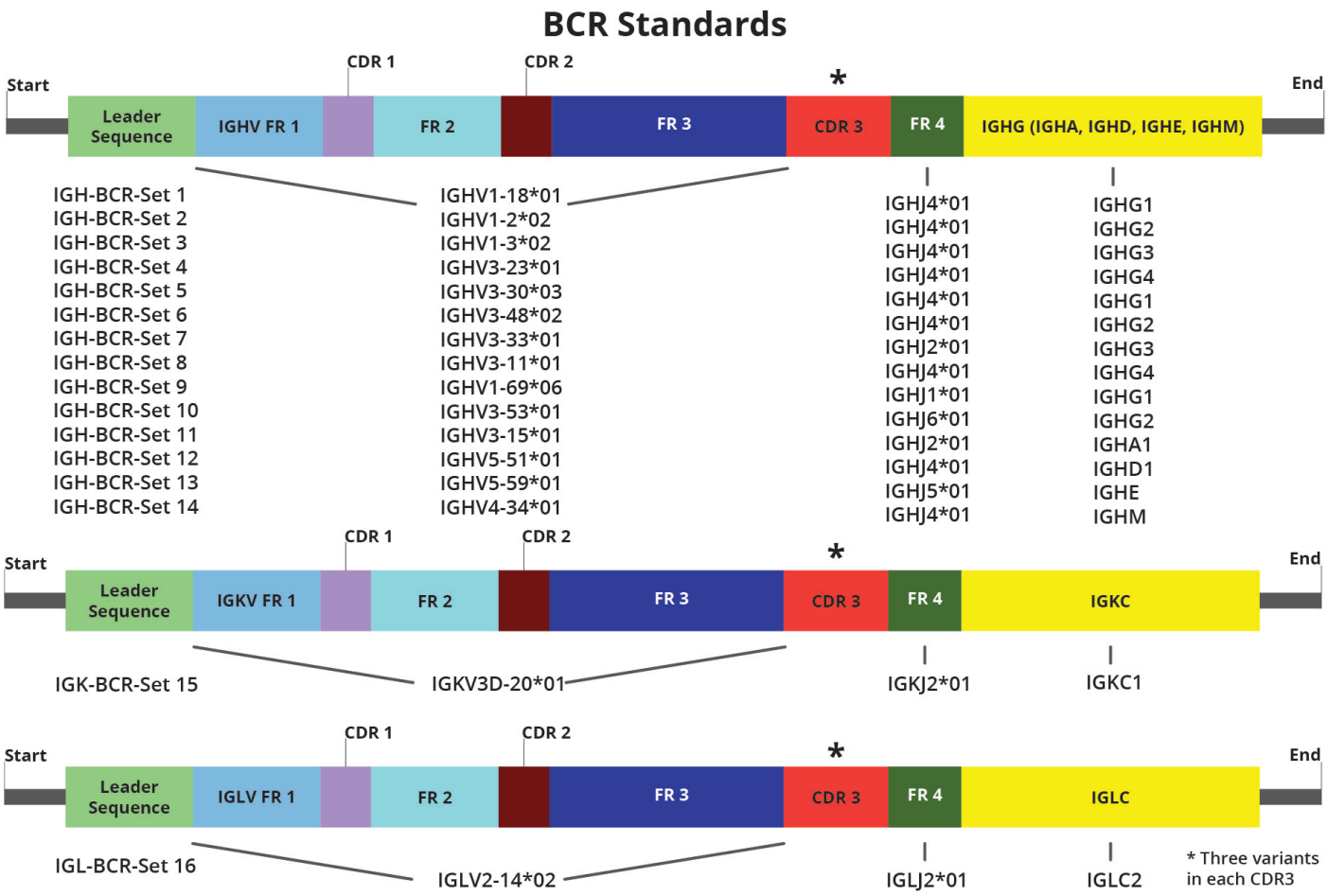


Fig 2. 48 BCR mRNA spike-in constructs representing ten different IGHG isoforms; 1 for each of IGHA, IGHD, IGHE, IGHM, and 1 for each of IGK and IGL genes.

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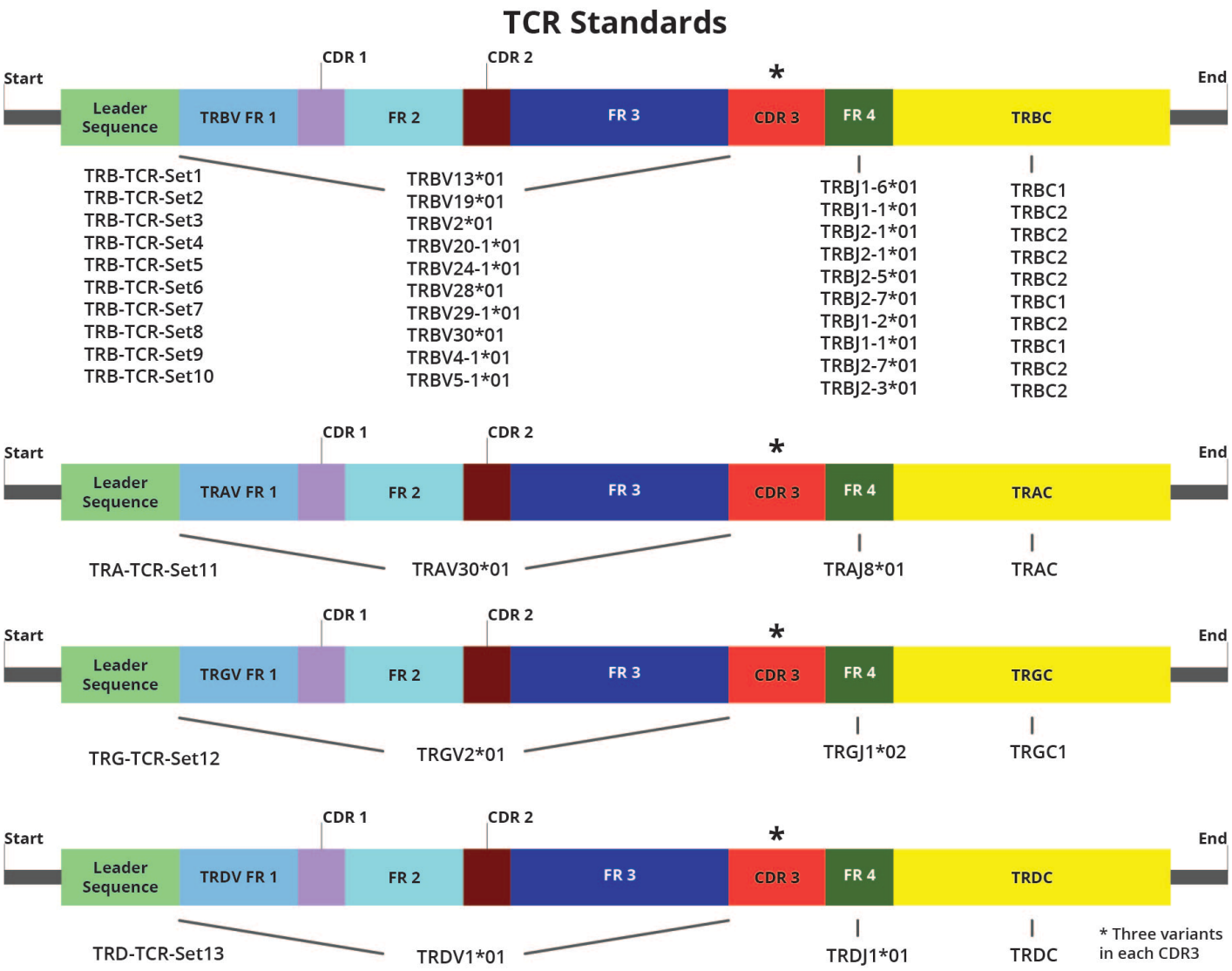


Fig 3. 39 TCR spike-in constructs represent ten different TRB isoforms, 1 for each of TRA, TRG, and TRG genes.

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3. DriverMap AIR Control Products

Product Configuration:

Separate DriverMap AIR Spike-In Control Mixes Kits and Triplex Isoform Pools Kits are offered for the TCR and BCR Controls, as described in Table 1. Each TCR/BCR Control Isoform Pool or AIR Control Mix contains enough reagents for ~50 RNA experimental samples when using the DriverMap™ AIR TCR-BCR Profiling Kits.

This product is offered in four kit configurations:

Catalog No.	Name	Description
DMAIR-HBR-SC-P	DriverMap AIR BCR Spike-in Triplex Isoform Pools (Human RNA)	Set of 16 BCR isoforms, each BCR isoform is a pool of three constructs (a+b+c) mixed at 1:1:1 molar ratio.
DMAIR-HTR-SC-P	DriverMap AIR TCR Spike-in Triplex Isoform Pools (Human RNA)	Set of 13 TCR isoforms, each isoform is a pool of three constructs (a+b+c) mixed at 1:1:1 molar ratio.
DMAIR-HBR-SC-M	DriverMap AIR BCR Spike-in Premixed Controls (Human RNA)	Premade mix of 16 unique triplex isoform sub-pools. Mixed at 16:4:1 molar ratio.
DMAIR-HTR-SC-M	DriverMap AIR TCR Spike-in Premixed Controls (Human RNA)	Premade mix of 13 unique triplex isoform sub-pools. Mixed at 16:4:1 molar ratio.

Individual Triplex Isoform Pools can also be purchased separately.

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3.1. Premixed Controls Components

There are two DriverMap AIR Spike-in Controls Kits—one for the TCR Controls, and one for the BCR controls, as described below:

DriverMap AIR BCR Spike-in Premixed Controls (Human RNA)

Label Name	Volume
BCR48-RNA Premix	100 µl
Dilution Buffer	500 µl
PBMC Control RNA	5 µl

DriverMap AIR TCR Spike-in Premixed Controls (Human RNA)

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Label Name	Volume
TCR39-RNA Premix	100 µl
Dilution Buffer	500 µl
PBMC Control RNA	5 µl

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3.2. Triplex Isoform Pools Components

The DriverMap AIR BCR Spike-in Triplex Isoform Pool Kit has 16 BCR Isoform Pools:

Label Name	Description	Concentration	Volume
IGH-RNA-BS01	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS02	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS03	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS04	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS05	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS06	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS07	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS08	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS09	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS10	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS11	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS12	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS13	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGH-RNA-BS14	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGK-RNA-BS15	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
IGL-RNA-BS16	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
Dilution Buffer	18S rRNA carrier in distilled water	2 ng/µl	500 µl

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PBMC Control RNA	Normal reference PBMC control total RNA	50 ng/µl	5 µl

The DriverMap AIR TCR Spike-in Triplex Isoform Pools has 13 TCR Isoform Pools:

Label Name	Description	Concentration (AD260 OD)	Volume
TRB-RNA-TS01	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRB-RNA-TS02	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRB-RNA-TS03	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRB-RNA-TS04	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRB-RNA-TS05	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRB-RNA-TS06	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRB-RNA-TS07	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRB-RNA-TS08	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRB-RNA-TS09	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRB-RNA-TS10	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRA-RNA-TS11	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRG-RNA-TS12	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
TRD-RNA-TS13	Pool of three constructs mixed at 1:1:1 molar ratio.	2.5K molecules/µl of each construct	50 µl
Dilution Buffer	18S rRNA carrier in distilled water	2 ng/µl	500 µl
PBMC Control RNA	Normal Reference PBMC control total RNA	50 ng/µl	5 µl

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4. Additional Materials Required

No additional reagents or equipment are required to run DriverMap AIR Spike-in controls with your experiment. When using DriverMap™ AIR TCR-BCR Profiling Kit or other TCR-BCR profiling assay, please follow the protocol for the respective assay.

This protocol assumes the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (tabletop centrifuges, pipettes), and common reagents (e.g., Milli-Q water, TE buffer, ethanol) and buffers used in a typical life science laboratory.

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5. General Recommendations

The DriverMap AIR Controls are designed for use with PCR-based assays. For accurate results, it is essential to use Good Laboratory Practices to minimize cross-contamination of products. If possible, perform the first part of the procedure (from cDNA synthesis through the first PCR setup) in a location set aside for RNA/DNA purification work and use a set of equipment, pipettes, test tubes, and other consumables dedicated to work involving RNA/DNA. 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 small numbers of samples (1-12), we recommend using 0.2-ml or 0.5-ml test tubes. Test tubes allow for minimal levels of cross-contamination between different samples. To minimize the hands-on time and mistakes in a liquid deposition for large numbers of samples (e.g., 24-96), we recommend running the assay in tube strips or a 96-well plate using an 8-channel (or 12-channel) pipette. 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.

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5.1. Procedural Guidelines

Guidelines for Use of the Spike-In Control Mixes

The DriverMap TCR/TCR RNA Spike-in controls have been validated using Cellecta's DriverMap™ AIR TCR-BCR Profiling Kit. The AIR assay is a multiplex PCR-based NGS assay that quantitatively profiles all functional immune receptor isoforms, i.e., TCR chains (TRA, TRB, TRD, TRG) and BCR chains (IGH, IGK, and IGL). With the AIR assay, you can profile the CDR3 region or full-length receptor regions (see Fig. 2). For more information on the DriverMap AIR Assay, please visit Cellecta's website. When using AIR-seq assays different than the DriverMap AIR assay, the efficiency of Spike-in controls detection may vary so some adjustments to the protocol may be necessary.

The AIR TCR and BCR Spike-In Control Mixes contain 3 different construct variants of each of the 13 TCR isoforms or 16 BCR isoforms, respectively. The 3 variants of each isoform are present at 250, 1000, and 4000 molecules per microliter. As a result, 2 µl of the mix contains 500, 2000, and 8000 theoretical molecules of each isoform variant. These 16-fold differences in concentrations enable a quantitative estimation in a 16-fold dynamic range of the absolute number of molecules of each TCR or BCR isoform in the profiled sample.

It is also important to include in each experiment the PBMC RNA reference standard provided in the kit. Cellecta's PBMC control RNA sample acts as a universal control (with Spike-in Control mix) to measure and sometimes compensate for batch effects in experiments. Using the same reference universal positive control RNA sample in different experiments is the best strategy to evaluate and potentially compensate for batch effects due to differences in assay conditions.

To accurately measure the number of control constructs in the DriverMap AIR assay, it is recommended to sequence at a higher depth (appr. 5-10M reads per sample) to achieve an average of at least 10-20 per cDNA molecule. Sequencing at a lower depth could reduce control constructs' measured copy number.

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5.2. Instructions for Spike-in Controls

- Before using AIR Spike-In Controls Mixes or Isoform Pools, heat the whole test tube(s) containing controls at 70°C for 3 minutes, cool down to room temperature, and pipet the whole content of the test tube(s) by pipet several (5-10) times. The heating/mixing step is important to achieve uniformity in the concentration of Spike-In Controls aliquots.

- Directly add an aliquot of Spike-In Control Mix to the RNA sample before performing any processing step using AIR-RNA or similar AIR-seq assays. We recommend using a positive control RNA sample in every experiment to help troubleshoot, analyze data, and normalize across different sample runs. To achieve high reproducibility, avoid adding volumes of less than 1 µl. For RNA samples with low-content immune cells, it may be necessary to dilute Spike-In Controls with the dilution buffer provided in the kit.

✻ Heating and pipetting are recommended after each freezing cycle to generate homogeneous RNA control concentration.

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5.3. Spike-in Control Workflow Summary

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Outline for Spike-in Protocol

Heat Spike-in Control Mix or Isoform Pool Tube at 70C for 3 min. Cool down at room temp.

Pipette up and down (x5) to mix the content of the tube uniformly

Aliquot 2 ul Spike-In Control Mix or Pool (refer to Table 1) to the RNA sample before performing any processing step

Run PCR based immune receptor profiling assay (such as DriverMap AIR assay) following the manufactures protocol

Quantify & Combine Samples for NGS

Analyze results with data alignment software (such as MiXCR) to quantify clonotypes and spike-in control sequences

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6. Premixed Controls

Determine the amount of Spike-In Mix to add.

For typical AIR-RNA assay protocols using 25-100 ng of total RNA for optimal clonotype detection of lymphocyte-rich samples, like whole blood, PBMC, lymphoid tissues, or isolated immune cells, we recommend spiking in 2 µl of TCR/BCR control mix (or control from TCR/BCR set) in a 20 µl AIR-RNA hybridization reaction mix (step 1, AIR protocol). Under these conditions, you could expect that the AIR-RNA profiling assay will detect approximately 100 to 1,600 spike-in cDNA molecules for different control constructs present in the AIR Spike-In Control mix.

If you use less than 25-100 ng of RNA in your AIR assay, reduce the spike-in control constructs to 0.2-0.5 µl. To avoid adding small volumes, dilute the Spike-In Controls in the provided dilution buffer. Some assays, such as those of lymphocyte-deficient samples (e.g., non-lymphoid tissues or cancer samples), often require more than 100 ng of total RNA. The optimal amount of TCR/BCR control of spiking into RNA samples with a low number of lymphocyte cells needs to be adjusted in proportion based on the approximate content of lymphocytes as compared to more typical samples, such as PBMC samples.

In general, the maximum number of spike-in control molecules should be significantly less than the number of TCR/BCR molecules in the sample (e.g., below 20%), and the number of molecules of the most abundant clonotypes (e.g., below 10,000 mRNA molecules). The minimum number of spike-in molecules for any construct should be enough to be measured in the AIR assay, e.g., more than 10-20 spike-in mRNA molecules. The complexity and diversity of TCR/BCR molecules significantly depend on the starting RNA amount used in the assay and the immune cell composition of experimental samples. To predict the appropriate Spike-in volume for your experimental samples, we recommend running an immune receptor profiling assay for a few pilot samples to measure the immune receptor template complexity and diversity for your experimental samples.

Table 1 below provides guidelines for the starting amount of RNA for AIR TCR-BCR profiling assay, expected complexity (similar to the number of TCR/BCR molecules), and recommended amount of spike-in controls:

Sample Type	Optimal Input Amount	Measured Complexity of TCR/BCR molecules	Recommended Spike-in Mix Volume
Whole blood	100 ng	50-200K	2 µl
PBMC	50 ng	50-200K	2 µl
T/B Cell fractions	10-25 ng	10-20K	2 µl
Lymphoid Tissue (fresh/ frozen)	100 ng	50-200K	2 µl
Cancer biopsies	500 ng	1-20K	0.5 µl*
Non-lymphoid tissues	1000 ng	1-50K	0.5 µl*
FFPE Tissue	1000 ng	0-5K	0.5 µl*

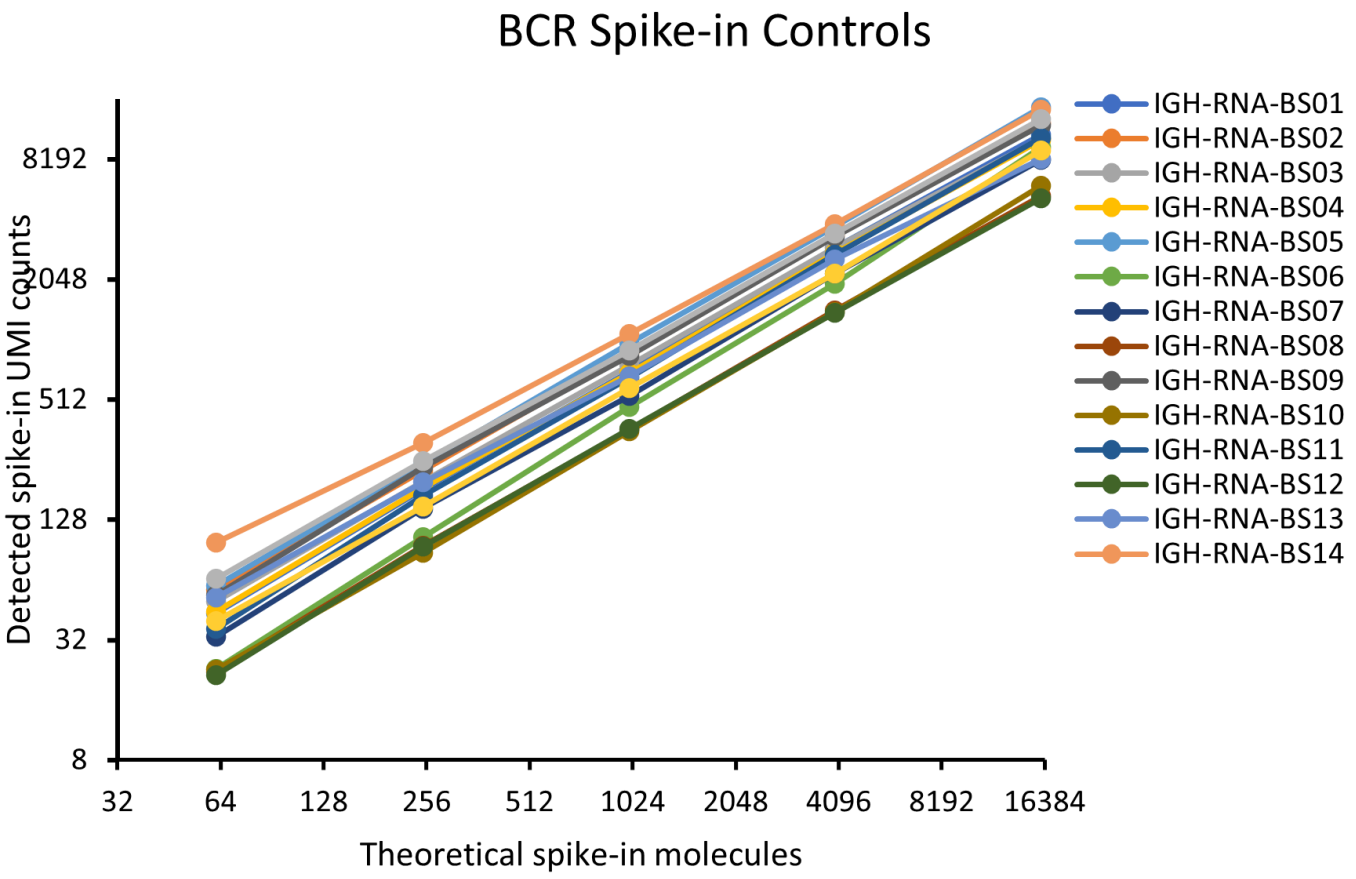
Note: (*) To accurately add small volumes, we recommend diluting Spike-In Control with the dilution buffer provided in the kit.

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Use cases for AIR Control Mixes:

1. Assessing Assay Performance: To assess the linearity, dynamic range, and sensitivity (Fig 4) of the DriverMap AIR Assay or similar AIR-seq assays, we recommend adding 2 µl of either the AIR TCR or BCR Spike-In Control Mix. Depending on the specifics of your assay, you can add more or less amount of Control Mix to additional samples to extend the assay's linear range, as shown in the table above. The quality of AIR profiling data could be different for experiments performed in different batches. This can be investigated by high-complexity standard reference control samples (e.g., PBMC or spleen) spiked with synthetic AIR control mixes.

Spike-in of the control to just one experimental sample and positive control RNA sample may be sufficient for quantification of immune samples with similar lymphocyte content, such as RNA from PBMC or whole blood. We recommend including spike-in control in multiple samples for samples with different lymphocyte content (e.g., PBMC and tumor samples) or potentially different template quality (e.g., frozen and FFPE samples) to evaluate assay performance. Please refer to Table 1 (above) for detailed input volumes. If you aim to evaluate reproducibility, measurements should be done in replicates.



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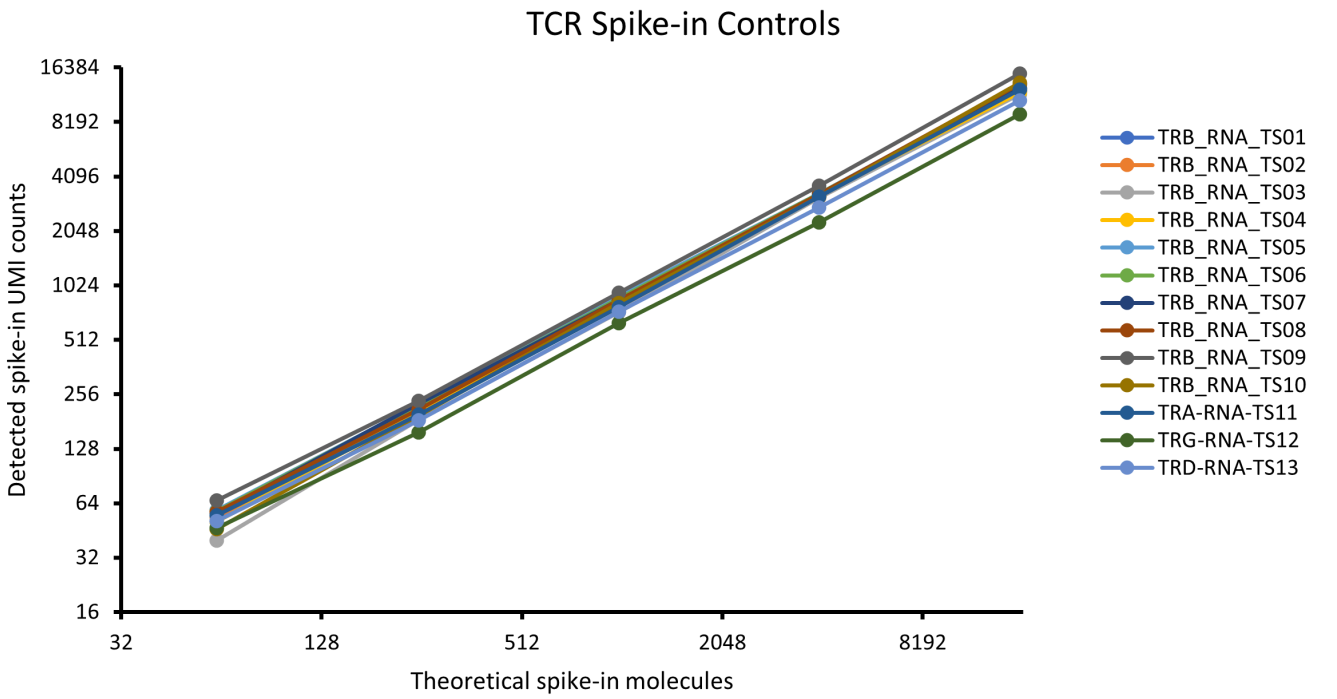


Fig 4. illustrates the results of linearity and dynamic range of AIR-RNA assay in PBMC control RNA sample spiked in with Spike-in Control Mix at different input amounts. AIR-RNA CDR3 assay was performed in triplicates using 50 ng of positive control PBMC RNA spiked with 0.5, 2 or 8 µl of control RNA mixes. Amplified TCR/BCR CDR3 library was sequenced at depth of 20x10^6 reads per sample and analyzed by MiXCR software to calculate the number of detected molecules or each RNA in comparison with number of theoretical RNA molecules spiked in PBMC RNA.

2. Measuring sequencing errors: Due to the enormous complexity and variability in TCR and BCR repertoire sequences, any biases generated in enzymatic, NGS, or data processing steps could significantly affect AIR profiling results. Using Spike-In Control Mixes with defined reference sequences is the best strategy to accurately measure sequence errors and evaluate mechanisms responsible for generating these errors. Data Analysis Section 8.4 describes the alignment of raw sequencing data to reference control sequences to accurately quantify error rate measurement. Importantly, Spike-In Control Mixes only allows measuring but can't correct these sequencing errors. Cellecta's DriverMap AIR-RNA assay incorporates eight Validator Barcodes (VBCs) in reverse primers at the reverse transcription or primer extension steps. VBCs similar to UMIs are designed to identify and correct amplification biases and sequencing errors for AIR RNA assay. The MiXCR software incorporates an efficient algorithm for VBC-based error correction by analyzing the distribution of reads per cDNA molecule ratio. However, errors introduced at reverse transcription or in the first PCR cycle cannot be corrected by VBC-based analysis, especially for rare clonotypes. However, these errors can be measured by spike-in control constructs, and this information can be used to validate the analysis of the detected actual complexity of immune receptor RNA molecules.

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7. Triplex Isoform Pools

Guidelines for using AIR Control Isoform Pools

For the Spike-In Control Isoform Pools, typically, 2 µl of each isoform-specific control should be added to each experimental sample. This recommended volume will generate approximately 1,000 detected spike-in cDNA molecules for each of the 3 spike-in constructs, providing a high sensitivity level for detecting potential cross-contamination levels between different samples.

Directly spike in an aliquot of Spike-In Control Isoform Pools to the RNA sample before performing any processing step using AIR-RNA or similar AIR-seq assays. We recommend using a positive control RNA sample in every experiment to help troubleshoot, analyze data, and normalize across different sample runs. To achieve high reproducibility, avoid adding volumes of less than 1 µl and dilute Spike-In Controls with the dilution buffer provided in the kit.

Use cases for AIR Control Isoform Pools

1. Assess cross-contamination: Cross-contamination between different samples is a serious problem for any PCR-based technology. Bench-level cross-contamination due to sample mixing, contamination by PCR products from previous experiments, or barcode jumping during sequencing are the most common reasons that could significantly affect AIR experimental results. Cellecta's Spike-In Control Isoform Pools provide the best strategy for measuring cross-contamination levels between different samples. There are 13 different TCR isoforms or 16 different BCR isoforms with three CDR3-specific constructs for each isoform available to spike-in different experimental samples. The level of cross-contamination could be easily measured by comparing control-specific spike-in cDNA molecules for different samples. You can measure the contamination by any two PCR products generated by previous experiments by detecting common/shared clonotypes in all experimental samples, as illustrated in Figure 5.

BCR_name	B1_1_UMI	B2_1_UMI	B3_1_UMI	B4_1_UMI	B5_1_UMI	B6_1_UMI	B7_1_UMI	B8_1_UMI	B9_1_UMI	B10_1_UMI	B11_1_UMI	B12_1_UMI	B13_1_UMI	B14_1_UMI	B15_1_UMI	B16_1_UMI	C_2_UMI
BCR Spike-in-1a	1183	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
BCR Spike-in-1b	1170	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BCR Spike-in-1c	978	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
BCR Spike-in-2a	0	1497	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BCR Spike-in-2b	0	1274	0	1	0	1	0	0	0	0	0	2	0	0	0	0	0
BCR Spike-in-2c	0	1529	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
BCR Spike-in-3a	0	0	739	0	0	0	0	0	0	0	0	1	0	0	0	0	0
BCR Spike-in-3b	0	0	717	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BCR Spike-in-3c	0	0	815	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BCR Spike-in-4a	0	0	0	1304	0	0	0	0	0	0	0	0	0	0	0	0	0
BCR Spike-in-4b	1	0	0	1260	0	0	0	1	0	0	0	0	0	0	0	0	0
BCR Spike-in-4c	0	1	0	1171	0	1	0	0	0	0	0	0	0	0	0	0	0
BCR Spike-in-5a	0	1	0	0	1411	0	0	0	0	0	0	0	0	0	0	0	0
BCR Spike-in-5b	0	0	0	0	1538	0	0	0	0	0	1	1	13	0	0	0	0
BCR Spike-in-5c	0	0	0	0	1439	0	0	0	0	0	0	0	0	0	0	0	0
BCR Spike-in 6a	0	0	0	0	0	2107	0	0	0	0	0	0	1	0	0	0	2
BCR Spike-in 6b	0	0	0	0	0	1409	0	0	0	0	1	1	0	0	0	2	0
BCR Spike-in 6c	1	0	0	0	0	1395	0	0	1	0	0	0	0	0	0	0	0
BCR Spike-in 7a	0	0	0	0	0	0	1386	0	0	0	0	0	0	0	0	0	0
BCR Spike-in 7b	0	0	0	0	0	0	1532	0	0	0	0	0	0	0	0	0	0
BCR Spike-in 7c	1	0	0	0	0	0	1476	0	0	0	0	0	0	0	0	0	0
BCR Spike-in 8a	0	0	0	0	0	0	0	832	0	0	0	0	0	0	0	0	0
BCR Spike-in 8b	0	0	0	0	0	0	0	912	0	0	0	0	0	0	0	0	0
BCR Spike-in 8c	0	0	0	0	0	0	0	936	0	0	1	0	0	0	0	0	0
BCR Spike-in 9a	0	0	0	0	0	0	0	0	1521	2	0	0	0	0	0	0	0
BCR Spike-in 9b	0	0	0	0	0	0	0	0	1709	0	0	0	0	1	0	0	0
BCR Spike-in 9c	0	0	0	0	0	0	0	0	1602	0	0	0	0	0	0	0	0
BCR Spike-in 10a	0	0	0	0	0	0	0	0	0	863	0	0	0	0	0	0	0
BCR Spike-in 10b	0	0	0	0	0	0	0	0	0	691	0	0	0	0	0	0	0
BCR Spike-in 10c	0	0	0	0	0	0	0	0	0	809	0	0	0	0	0	0	0
BCR Spike-in 11a	0	0	0	0	0	0	1	0	1	0	1270	0	0	0	0	0	0
BCR Spike-in 11b	0	0	0	0	0	0	0	0	0	0	1245	0	0	0	0	0	0
BCR Spike-in 11c	0	0	0	0	0	0	0	0	0	0	1230	0	0	0	0	0	0
BCR Spike-in 12a	0	0	0	0	0	0	0	0	0	0	0	656	0	0	0	0	0
BCR Spike-in 12b	0	0	0	0	0	0	0	0	0	0	0	689	0	0	1	0	0
BCR Spike-in 12c	0	0	0	0	0	0	0	0	0	0	0	634	0	0	0	0	0
BCR Spike-in 13a	0	0	0	0	0	0	0	0	0	0	0	0	947	1	1	0	0
BCR Spike-in 13b	0	0	0	0	0	0	0	0	0	0	0	0	1314	0	1	0	0
BCR Spike-in 13c	0	0	0	0	0	0	0	0	0	0	0	0	1200	0	1	0	0
BCR Spike-in 14a	0	0	0	0	0	0	0	0	0	0	0	0	0	1472	0	0	0
BCR Spike-in 14b	1	0	1	3	0	3	0	3	1	1	0	1	0	1478	1	0	0
BCR Spike-in 14c	0	0	0	0	0	0	0	0	0	0	1	0	0	2055	0	0	0
BCR Spike-in 15a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1418	0	0
BCR Spike-in 15b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1357	0	0
BCR Spike-in 15c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1426	0	0
BCR Spike-in 16a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2137	0
BCR Spike-in 16b	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1683	0
BCR Spike-in 16c	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1	1710	0

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TCR_name	T1_UMI	T2_UMI	T3_UMI	T4_UMI	T5_UMI	T6_UMI	T7_UMI	T8_UMI	T9_UMI	T10_UMI	T11_UMI	T12_UMI	T13_UMI	C_1_UMI
TCR spike-in -1a	659	0	0	0	0	0	0	0	0	0	0	0	0	0
TCR spike-in -1b	690	0	0	0	0	0	0	0	0	0	0	0	0	0
TCR spike-in -1c	594	0	0	0	0	0	0	0	0	0	0	0	0	0
TCR spike-in -2a	0	1413	0	0	0	0	0	0	0	0	0	0	0	0
TCR spike-in -2b	0	1402	0	0	0	0	0	0	0	0	0	1	0	0
TCR spike-in -2c	0	1266	1	0	0	0	0	0	0	0	0	0	0	0
TCR spike-in -3a	0	0	1176	0	0	0	0	0	0	0	0	0	0	0
TCR spike-in -3b	0	0	1314	0	0	0	0	0	0	0	0	0	0	0
TCR spike-in -3c	0	0	1341	0	0	1	0	0	0	0	0	0	0	0
TCR spike-in -4a	0	0	0	1193	0	0	0	0	0	0	0	0	0	0
TCR spike-in -4b	0	0	0	1321	0	0	1	0	0	0	0	0	0	0
TCR spike-in -4c	0	0	0	1257	0	0	0	0	0	0	0	0	0	0
TCR spike-in -5a	0	0	0	0	1558	0	0	0	0	0	0	0	1	0
TCR spike-in -5b	0	0	0	0	1421	0	0	0	0	0	0	0	0	0
TCR spike-in -5c	0	0	0	0	1619	0	0	0	0	0	0	0	0	0
TCR spike-in -6a	0	0	0	0	0	1271	0	0	0	0	0	0	0	0
TCR spike-in -6b	0	0	0	0	0	1281	1	0	0	0	0	0	0	0
TCR spike-in -6c	0	0	0	0	0	1292	0	0	0	0	0	0	0	1
TCR spike-in -7a	0	0	0	0	0	0	1060	0	0	0	0	0	0	0
TCR spike-in -7b	0	0	0	0	0	0	1101	0	0	0	0	0	0	0
TCR spike-in -7c	0	0	0	0	0	0	1104	0	0	0	0	0	0	0
TCR spike-in -8a	0	0	0	0	0	0	0	1098	0	0	0	0	0	0
TCR spike-in -8b	0	0	0	0	0	0	0	1087	0	0	0	0	0	0
TCR spike-in -8c	0	0	0	0	0	0	0	1153	0	1	0	0	0	0
TCR spike-in -9a	0	0	0	0	0	0	0	0	1426	0	0	0	0	0
TCR spike-in -9b	0	0	0	0	0	0	0	0	1393	0	0	0	0	0
TCR spike-in -9c	0	0	0	0	0	0	1	0	1244	0	1	0	0	0
TCR spike-in -10	0	0	0	1	0	0	0	0	0	1475	0	0	0	0
TCR spike-in -10	0	0	0	0	0	0	0	0	0	1275	0	0	0	0
TCR spike-in -10	0	0	0	0	0	0	0	0	0	1379	0	0	0	0
TCR spike-in -11	0	0	0	0	0	0	0	0	0	0	589	0	0	0
TCR spike-in -11	0	0	0	0	0	0	0	0	0	0	1237	0	0	0
TCR spike-in -11	0	0	0	0	0	0	0	0	0	0	1335	0	0	0
TCR spike-in -12	0	0	0	0	0	1	0	0	0	0	0	925	0	0
TCR spike-in -12	0	0	0	0	0	0	0	0	0	0	0	906	0	0
TCR spike-in -12	0	0	0	0	0	0	0	0	0	0	0	877	0	0
TCR spike-in -13	0	0	0	0	0	0	0	0	0	0	0	0	1107	0
TCR spike-in -13	0	0	0	0	0	0	0	0	0	0	0	0	1161	0
TCR spike-in -13	0	0	0	0	0	0	0	0	0	0	0	0	1102	0

Fig 5: Detection of cross-contamination level between different experimental samples in AIR-RNA assay. Individual TCR/BCR triplex isoform control pools (2 µl each) of T1-T13 and B1-B16 were spiked in each of different whole blood RNA samples (50 ng each), and control PBMC RNA (C). AIR-RNA assay for each experimental RNA sample was performed in separate test tubes and spike-in control cDNA molecule numbers for each spike-in control construct were calculated by MiXCR. Most samples show zero or limited crossover and only detect the respective spiked-in control set allowing us to measure cross contamination or crossover between multiple samples.

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8. Spike-in Correlation Analysis

Customized Python scripts and R scripts were used to analyze the TCR/BCR spike-in dataset; you can access the code by clicking the link below:

- Python/R Code
- Demo dataset
- Fastq files are deposited in NCBI BioProject ID PRJNA975292
- To generate the table for the clonotype sequence and the spike-in sequences, the Excel Vlookup function can be used to create an output as shown in Appendix B.

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9. Data Processing and Analysis

The protocol in this section describes the procedure to extract T- and B- Cell receptor repertoire from NGS data generated from the Cellecta DriverMap AIR profiling kit using MiXCR or Platforma computational tool.

MiXCR is the leading software used for immune repertoire data analysis. It is free for academic and non-profit use (see License). For commercial use, a license can be requested at MiLaboratories-Licensing Information or by email to licensing@milaboratories.com. Please refer to Appendix C for installing instructions.

There are also other open-source bioinformatic tools/frameworks used to analyze immune repertoires. Please refer to these papers for a comparison of these computational tools:

- Rigorous benchmarking of T-cell receptor repertoire profiling methods for cancer RNA sequencing
- Benchmarking immunoinformatic tools for the analysis of antibody repertoire sequences

Tools	Published year	Aligner	Website
TRUST4	2008	IMGT	TRUST4 Github
IMGT/HighV-QUEST	2012	GPA (NW)	IMGT
IgBLAST	2013	BLAST	NCBI
IMSEQ	2015	SCF matching	IMTools
LymAnalyzer	2015	Fast-tag	LymAnalyzer

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

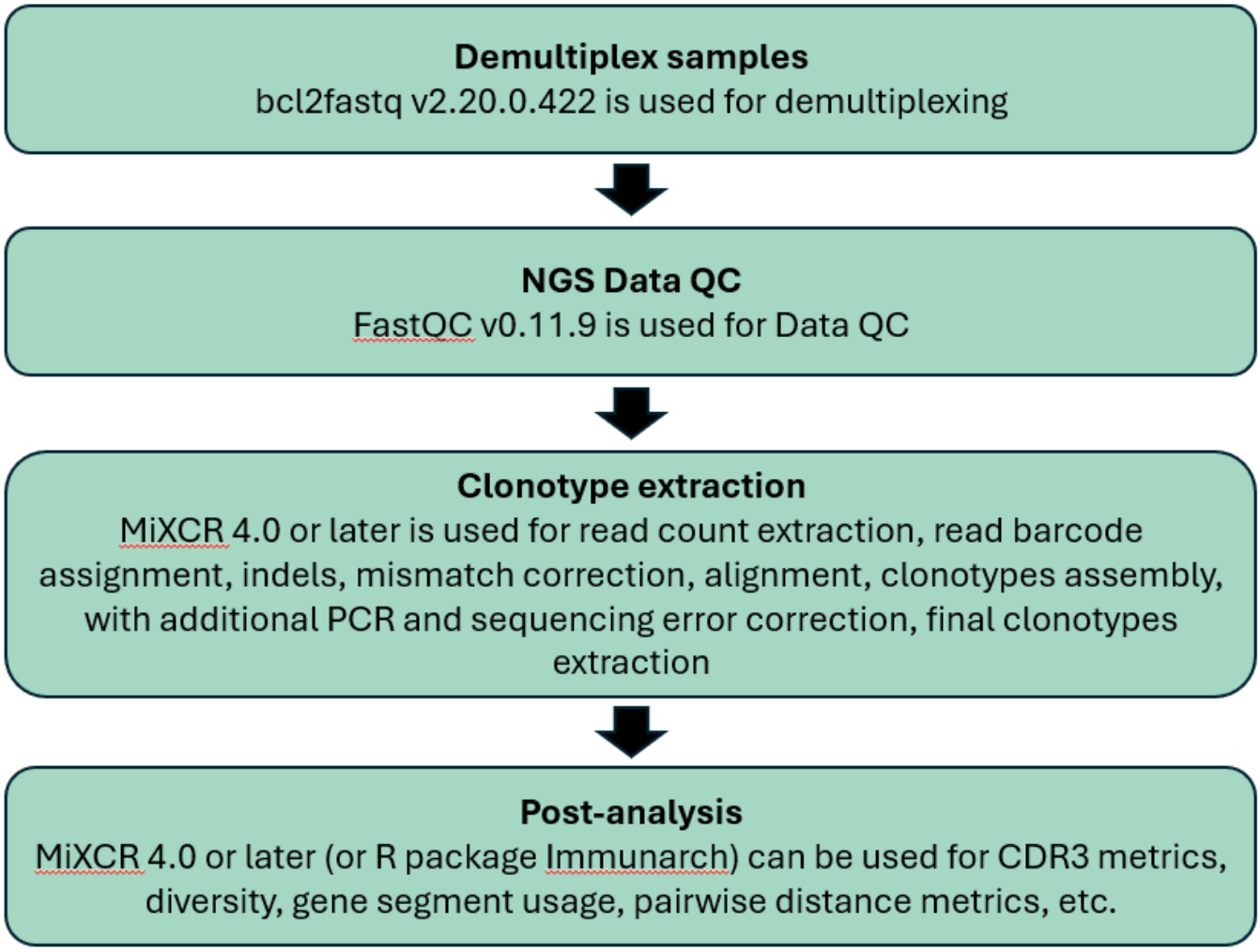
Cellecta does not own any of these software packages and is not responsible for maintaining them. In case you don't have the license, Cellecta also provides custom data analysis services using the MiXCR software package. Please email tech@cellecta.com for more information.

Last modified: 15 May 2025

9.1. Bioinformatic Workflow

The following bioinformatics workflow is recommended for DriverMap AIR assay:

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9.2. 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:Illumina Documentation

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.

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9.3. 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

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link:FastQC Guide

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9.4. MiXCR Alignment and Clonotypes Assembly

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

```
mixcr analyze local:cellecta-new-bulk-kit \
--species $SPECIES \
--assemble-clonotypes-by $ASSEMBLY_TYPE \
$SPLIT_CLONES_CONFIG \
--$MOLECULE \
--tag-pattern $TAG_PATTERN \
$LEFT_BOUNDARY \
--floating-right-alignment-boundary $BOUNDARY \
--export-productive-clones-only \
$READ_1 \
$READ_2 \
$OUTPUT_NAME
```

where:

```
$SPECIES = hsa (human) or mmu (mouse)
$ASSEMBLY_TYPE = CDR3 (CDR3 amplicon) or {CDR1Begin:FR4End} (Full length amplicon)
$SPLIT_CLONES_CONFIG = --split-clones-by C (if working with B cells to distinguish isotypes)
or otherwise skip this option
$MOLECULE = rna (for RNA) or dna (for DNA)
$LEFT_BOUNDARY = --rigid-left-alignment-boundary (for RNA) or --floating-left-alignment-bound
ary (for DNA)
$BOUNDARY = J (for DNA) or C (for RNA)
$READ_1, $READ_2 = paired end read file names (e.g. sample_a_R1.fastq.gz, sample_a_R2.fastq.g
z)
$OUTPUT_NAME = name of output files (e.g. sample_a)
```

The $TAG_PATTERN is always:

```
'^(R1:*)\^(MIVBC:GCATCAN)(R2:*)|^(MIVBC:AGTCGTN)(R2:*)|^(MIVBC:TCGCATCN)(R2:*)|^(MIVBC:AGCGTA
GN)(R2:*)|^(MIVBC:TACGACTN)(R2:*)|^(MIVBC:CTGATGAN)(R2:*)|^(MIVBC:CATAGCATN)(R2:*)|^(MIVBC:GT
AGGCTAN)(R2:*)'
```

is specified by the 8 validator barcodes.

The preset can be accessed by saving the file cellecta-new-bulk-kit.yaml in the directory of the MiXCR script that can be obtained here.

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

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9.5. 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/).

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10. Appendices

Appendix A. Tips and Troubleshooting Experiment
Appendix B. Download AIR Spike-in Control and Mix Sequences
Appendix C. Obtaining and Installing Software for Data Analysis
Appendix D. List of Dual DNA/RNA UDP Indexes for DriverMap AIR Profiling Kit

Last modified: 24 January 2024

10.1. Appendix A.Tips and Troubleshooting

Premixed Controls

Observation	Possible Cause	Recommended Action
Detected cDNA molecule numbers for Spike-in controls are significantly higher (e.g.,>5x) than cDNA molecule numbers for most abundant RNA/DNA clonotypes.	Low content or high diversity of immune receptor RNA/DNA genes in the sample, RNA/DNA is degraded or has a wrong concentration.	Reduce the amount of Spike-in control mix added to experimental RNA/DNA samples. Measure RNA/DNA concentration, re-purify, replace or use more amount of RNA/DNA samples.
Abundant Spike-in control cDNA molecule numbers are less than 100, low yield of amplified products for both experimental and positive control samples.	Possible manual error in the protocol.	Add extra cycles in 2nd PCR step, and repeat the AIR protocol using positive control RNA/DNA.
Abundant Spike-in control cDNA molecule numbers are less than 100 for experimental samples but higher (e.g., 500-1,000) for positive control RNA/DNA samples.	Presence of inhibitors in RNA samples	Add extra cycles in 2nd PCR for experimental samples, use alternative source, or re-purify RNA/DNA samples
Lower than expected 16:4:1 ratio in cDNA molecules for different Spike-in controls.	PCR overcycling, background in measurement of AIR control constructs, and not enough NGS reads.	Reduce PCR cycle number (in 1st PCR), use control RNA without Spike-in controls to measure Spike-in control background level, replace AIR reagents, use PCR-free area/conditions to re-run AIR assay, use separate test tubes rather than plate to minimize cross-contamination level, run NGS with more reads per sample, optimize parameters for reduction background level based on ration reads per cDNA molecule.
Lower reproducibility	Not equal concentration of	Heat Spike-in controls at 70°C for 2 min and pipet the whole

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in cDNA molecule number between different samples or in triplicates.	Spike-in controls, Spike-In RNA controls are not fully dissolved (RNA molecules could “stick” to each other), not equal primer extension/ amplification conditions for different samples.	volume several (e.g., 10) times before use, optimize liquid handling/PCR conditions to have an equal yield of PCR products in triplicate samples and use master mixes for common reagents.

Triplex Isoform Pools

Observation	Possible Cause	Recommended Action
Low level of cross-contamination between different experimental samples	<0.1% or less cross-contamination	Based on our experience, a level of cross-contamination less than or equal to 0.1% is commonly detected due to the high sensitivity of AIR assay
High level of cross-contamination between different experimental samples	>1% cross-contamination	Mistakes in AIR assay conditions or sample handling. Use PCR-free area/conditions to re-run AIR assay, use separate test tubes rather than plate to minimize cross-contamination level, replace AIR reagents, and use control RNA without added Spike-in control as a “negative” control to monitor the level of sample cross-contamination.

Last modified: 24 February 2025

10.2. Appendix B. Downloading AIR Spike-in Sequences

Download the Excel sheet for sequence information:

Control Mix:

- BCR Premixed Controls
- TCR Premixed Controls

Triplex Isoform Pools

- BCR Triplex Isoform Pools
- TCR Triplex Isoform Pools

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10.3. 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.com/mixcr/getting-started/installation/”).

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
- 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).

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11. Frequently Asked Questions

Please refer to the AIR Technology Guide for recommendations for experimental design, sample-input amounts, and assay design.

Assay:

Q1: What is included in the AIR profiling kit?
A1: The kit includes all the necessary reagents for reverse transcription, amplification, and primers for next-generation sequencing.

Q2: What is the difference between TCR/BCR CDR3 RNA kits for human and mouse?
A2: The TCR/BCR gene-specific primers for the human and mouse kits are different but all other reagents and protocols are the same.

Q3: Does the AIR profiling assay have Unique Molecular Identifiers (UMIs)?
A3: Cellecta's DriverMap AIR RNA and AIR DNA profiling assays do not have UMIs. The AIR RNA and AIR DNA profiling assays incorporate a redundant set of Validator Barcodes (VBCs) which enable easy identification of and correction for amplification and sequencing biases and error. The VBCs also provide a reference for normalization and copy number quantitation of amplified RNA or DNA molecules to facilitate detection of low-abundant clonotypes.

Q4: What is the difference between AIR RNA CDR3 and Full-length Receptor Kit?
A4: The CDR3 kit amplifies approximately 250-300 bp of the CDR3 region, whereas the full-length kit amplifies the full variable region ~600 bp (CDR1-CDR2-CDR3). For more information on sequencing instructions and applications for the two kits, please visit the AIR-RNA webpage to view the table Comparison of CDR3 vs Full-Length Variable Region Repertoire Profiling.

Q5: What sample types are compatible with the assay?
A5: Our assay is compatible with various sample types, including RNA or/and DNA samples isolated from whole blood, PBMC, immune cell fractions, tissue/cancer biopsies, or FFPE tissue samples. For more information on sample types and preparation guidelines, please refer to the Technology Guide: Applications of AIR profiling.

Q6: What is the recommended protocol for profiling a small number of cells, such as sorted cells or fresh frozen tissue?
A6: For a small number of cells (e.g., 5,000-50,000 cells), e.g., isolated by FACS, tissue or blood microsamples, we recommend using the Cellecta DirectCell™ Protocol which profiles directly from the cell lysate without the need to extract total RNA or DNA from the sample.

Q7: What is the optimal input amount for DNA as a starting material?
A7: To determine the optimal input amount for your samples, we recommend testing amplification efficiency using different starting amounts of DNA, e.g. 0.5 µg, 1 µg, 2 µg, 5 µg. Some samples such as FFPE blocks, or tumor biopsies contain impurities that inhibit the activity of DNA polymerase, so a maximum of 2 µg is recommended.

If you have enough input amount, we recommend running samples in triplicates, e.g. using 2×3=6 µg of DNA for each sample to get better quantitative data, as shown in Technology Guide Section 2.1, Fig. 9, which lists the number of clonotypes you can obtain from different amounts of input DNA material.

Q8: What is the better choice of starting material for FFPE samples, DNA or RNA?
A8: In our experience, DNA, due to its stability, generally provides more robust data with FFPE samples. However, RNA can lead to a more sensitive profiling of clonotypes provided that the RNA is not significantly degraded. For FFPE AIR-RNA

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profiling, we recommend using RNA with a RIN Score > 5, or RNA with at least 20% of fragments above 300-n. For best results, we recommend purifying both RNA and DNA from FFPE samples and running both AIR-RNA and AIR-DNA assays.

Q9: Can less than 500 ng of DNA be used for the AIR DNA Profiling Kit?
A9: As shown in Technology Guide Section 2.1, Fig. 9, profiling with 500 ng or lower is possible, but the number of detected clonotypes will be reduced. Furthermore, only top abundant clonotypes (with clonotype-specific cells numbering more than 10) could be quantitated.

Sequencing:

Q10: What is the recommended number of reads for the AIR protocol?
A10: For sequencing, we recommend around 5-10 million reads per sample, depending on the number of samples in one flow cell and the required sensitivity. More information about sequencing can be found in the User Manuals' NGS Prep and Sequencing Section.

Q11: How much PhiX is needed for the sequencing run?
A11: We recommend adding 15% of PhiX to the library (anywhere in the range 10-20% is acceptable).

Q12: Can AIR RNA and AIR DNA samples be sequenced together in the same lane?
A12: Yes, AIR-RNA and AIR-DNA samples can be combined for NGS, but due to differences in amplicon sizes (approximately 350 bp for RNA vs. approximately 250 bp for DNA), we suggest mixing AIR-RNA with AIR-DNA at a 1.5:1 ng ratio (e.g., 150 ng of AIR-RNA with 100 ng of AIR-DNA). Be sure to use the different indexing primers for RNA and DNA samples. NGS sequencing primers are the same for both AIR-RNA and AIR-DNA.

Q13: Which instruments can be used for CDR3 or full-length receptor sequencing?
A13: For most applications, we recommend using the Illumina NextSeq 2000 instrument with SBS-Leap sequencing chemistry kits due to low NGS cost and wide range of flow cells (between 100M to 1.8B reads for both 300-cycle and 600-cycle Paired-End reads). The NextSeq 500/550 could still could be used for CDR3 profiling, but the cost of sequencing is higher than when using the NextSeq 2000. The MiSeq instrument allows sequencing for the 600 cycles (full-length receptor sequence) but its scale is limited to 5-10 samples. Our kits use the reverse complement workflow, so for MiSeq, you would need to use the protocol with one index primer and the V2/V3 Kits. To optimize your sequencing protocol, please contact techsupport@illumina.com.

Q14: Can you sequence DriverMap AIR and DriverMap Human Genome-Wide Targeted RNA-Seq Expression (EXP) Profiling Kits in the same flow cell?
A14: Yes, the custom sequencing primers (Reverse and Forward SeqDNA and SeqIND primers) are the same in the DriverMap AIR and DriverMap Human Genome Wide Expression Profiling Kits. Please note that AIR and Expression profiling samples need to be amplified with different indexing primers to be sequenced in the same flow cell.

Q15: Can the libraries be sequenced on the MiSeq? Do you have any recommendations?
A15: Yes, the AIR library can be sequenced on the MiSeq instrument. Our kits use the reverse complement workflow, so only the V2/V3 kits are compatible with the MiSeq and you would need to use the protocol with one index primer. Attached is the Illumina Guide for Indexed Sequencing Overview. To optimize your sequencing protocol please contact techsupport@illumina.com.

Q16: Is it recommended to do sequencing on the NovaSeq 6000 or NovaSeq X Platform?
A16: As NGS with NovaSeq instruments provides a minimum of 8B reads per flow cell, NovaSeq instruments could be used for NGS analysis of AIR samples that are done on a large scale (i.e.> 500 samples).

Q17: Can sequencing be performed on NovaSeq 6000?

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A17: Yes, it is possible to use two custom indexing primers with the NovaSeq 6000 Reagent Kit v1.5, as the NovaSeq 6000 machine works with a mixture of Ind1 and Ind2 sequencing primers from one well. The recommended approach is to spike Cellecta primers in the wells with Illumina primers, as described in the Spiking custom primers into the Illumina sequencing primers | Illumina Knowledge. If you prefer not to spike in the primers, you can use the custom primers protocol described in the NovaSeq Series Custom Primers Guide (illumina.com).

We have a customer that used the SP flow cell and loaded 2nM with the protocol with 148-10-10 -148 cycles as follows:

Sequencing setup - NovaSeq 6000					
Kit version	**Primer name**	**Cartridge positition**	**Total volume (mL)**	**Custom primer final conc. (uM)**	**Volume Primer (uL)**
S1 and S2 100/200/300 cycles	Read 1 (Seq DNA1)	24	4	0.3	12
S1 and S2 100/200/300 cycles	Read 2 (Seq DNA2)	13	2	0.3	6
S1 and S2 100/200/300 cycles	Index 1 (Seq Ind Fw)	23	5	0.3	15
S1 and S2 100/200/300 cycles	Index 2 (Seq Ind Rv)	23	5	0.3	15
Cellecta primers (uM):	**100**				

Please note that Cellecta has not internally validated this protocol, so to optimize your sequencing protocol please contact techsupport@illumina.com.

Q18: What are the recommended concentration and sequencing primer setup for NovaSeq 6000 kits?
A18: For NovaSeq 6000, we generally recommend starting at 100 pM (same as PhiX) if the library type loading concentration is unknown, then optimizing in 50-100 pM increments based on results. Here is Illumina resource for determining optimal loading based on PF and occupancy. To optimize your sequencing protocol please contact techsupport@illumina.com.

Q19: Can sequencing be performed on NovaSeq X?
A19: Yes, our kits use Dual DNA/RNA UDP Indexes and follow the reverse-complement workflow. For loading on the NovaSeq X, follow Illumina's protocol for NovaSeq X Custom Primers Protocol. To optimize your sequencing protocol please contact techsupport@illumina.com.

Data Analysis:

Q20: How can I analyze the data for TCR and BCR analysis?
A20: We recommend using the MiXCR software tool (MiLaboratories) for alignment and AIR repertoire data analysis. Here are the options for our customers:
- **Academic customers:** Academics may use the free, user-intuitive MiXCR software available for download on Platforma.bio.
- **Industry customers:** MiLaboratories offers a free trial period to test our kits on Platforma.bio or MiXCR command line version, starting with a 1-month free trial onwards. MiXCR packages include special presets for AIR kit analysis, the complete workflow can be set up with the name of the AIR Kit eg: MiXCR Guides Cellecta Kit
- **Cellecta bioinformatics services:** Additionally, Cellecta offers bioinformatics services, including running the MiXCR pipeline and providing output reports. Custom analysis can also be performed upon request. Here is an example report: 2024-02-13 Final Report. Request a quote or more information at quotes@cellecta.com.

Other bioinformatics tools/frameworks are also available to analyze immune repertoires as follows:

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/

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Q21: How do I obtain a MiXCR license?
A21: For an academic license go to https://licensing.milaboratories.com/, for a business license email licensing@milaboratories.com and provide a Cellecta kit reference.

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12. Technical Support

Email Addresses

Technical Support: tech@cellecta.com
General information: info@cellecta.com

Phone Numbers

Phone: +1 650 938-3910
Toll-free (USA): (877) 938-3910

For the latest technical news and updates, visit Cellecta's blog at: https://cellecta.com/blogs/news

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13. Contact Us

Email Addresses

General information: info@cellecta.com
Sales: sales@cellecta.com
Orders: orders@cellecta.com
Technical Support: tech@cellecta.com

Mailing Address

Cellecta, Inc.
320 Logue Ave.
Mountain View, CA 94043
USA

Phone Numbers

Phone: +1 650 938-3910
Toll-free (USA): (877) 938-3910
Fax: +1 650 938-3911

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