Technology Guide

DriverMap™ AIR Technology Guide

v1 — Last update: 26 January 2024

Cellecta, Inc.

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1. DriverMap™ AIR Profiling Assay

Immune Receptor Profiling is a powerful tool for characterizing adaptive immune responses to cancer, autoimmune and infectious diseases, allergies, vaccinations,and therapeutic treatments. The unique sequences of the T-cell and B-cell receptors (TCRs and BCRs), and antibody variable regions (CDR3) that recognize foreign antigens define the individual differences in adaptive immune responses. Profiling the TCR and BCR variable regions using RT-PCR and NGS provides critical data for the discovery of novel, disease-associated immunity biomarkers.

Cellecta offers two types of DriverMap™ Adaptive Immune Receptor (AIR) repertoire profiling assays: AIR RNA and AIR DNA. Both assays are based on targeted multiplex PCR amplification and next-generation sequencing (NGS) analysis of variable T-cell receptor (TCR) and B-cell receptor (BCR) sequences using RNA and/or DNA purified from biological samples as the starting material. AIR RNA is our front-line recommended assay which provides the highest performance in immune receptor profiling. The AIR DNA assay can be used separately or in combination with AIR RNA, as discussed below.

Main Advantages of the DriverMap AIR Assays:

- An easy-to-use protocol that allows comprehensive, unbiased immune repertoire profiling from small samples (e.g., sorted cells, FFPE, biopsy or blood microsamples) or larger bulk samples (e.g., whole blood or tissue biopsies).
- A novel, multiplex RT-PCR protocol that provides the highest sensitivity in the detection of functional or productive CDR3 clonotypes and also eliminates non-specific, off-target, and primer-dimer amplification products.
- Built-in Unique Molecular Identifiers (UMIs) to identify and correct amplification biases and sequencing errors for both the AIR RNA and AIR DNA assays. The UMIs accurately quantitate copy number of cDNA / DNA molecules used in amplification steps and detect low-abundance clonotypes above the background level.
- A dual-index amplicon labeling strategy that minimizes the potential NGS shortcoming of index-hopping when using the Illumina Paired-End kit for CDR3 sequencing.
- Open-source software such as MiXCR (preferred choice), IMGT/HighV-QUEST, IgBLAST, IMSEQ, LymAnalyzer software can be used for AIR assay NGS data analysis. Cellecta also provides custom data analysis services with MiXCR software. We can generate interactive reports that include comprehensive somatic mutation profiling across the antigen-recognition CDR3 region, accurate clonotype quantitation, CDR3 length distribution, V(D)J segment usage, and isotype composition for BCRs.

Last modified: 8 March 2023

1.1. DriverMap™ AIR RNA Profiling

The DriverMap™ AIR RNA profiling assay is a next-generation targeted, multiplex RT-PCR technology designed for bulk expression profiling of all CDR3 TCR (TRA, TRB, TRD, TRG) and/or BCR (IGH, IGK, and IGL) clonotypes in a single reaction using RNA as the starting material. The assay is based on 315 forward primers designed for the FR3 variable region and 19 reverse primers designed for the conservative C-region of TCR/BCR mRNAs. These primers have been experimentally validated and optimized for the best performance. An additional set of primers is designed for the FR1 region, which allows (in combination with C region primers) the user to amplify full-length CDR1-CDR2-CDR3 V regions of all seven immune receptor genes (Fig. 1).

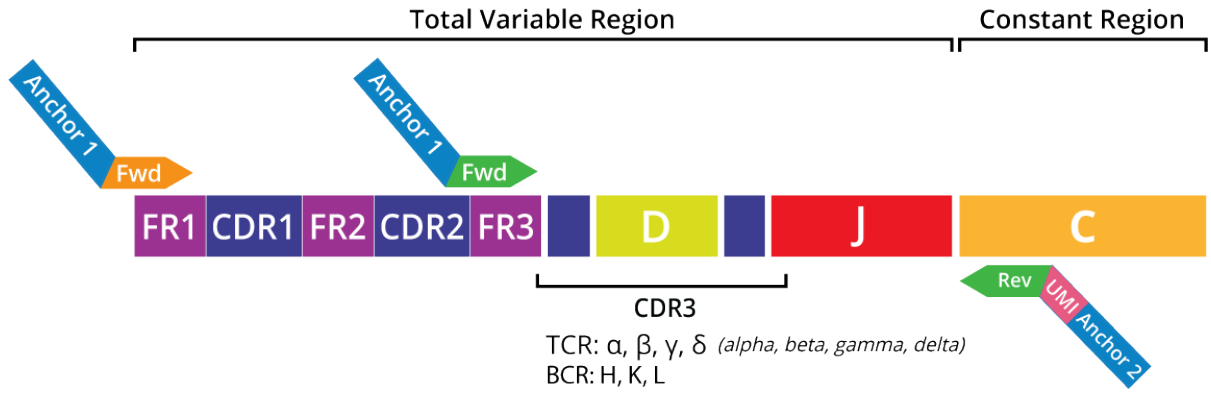

Fig 1. mRNA structure for TCR (alpha, beta, gamma, delta) and BCR (heavy, kappa, lambda) chains, and positions of forward and reverse PCR primers to amplify the CDR3 regions or CDR1-CDR2-CDR3 regions in AIR RNA assay.

The AIR RNA assay profiles all functional or productive isoforms and excludes non-functional pseudogenes and ORFs (as defined by the IMGT database https://www.imgt.org/IMGTrepertoire/) (Fig. 2). The AIR RNA assay is designed to profile the TCR and BCR repertoire together (preferred strategy) or separately (if necessary).

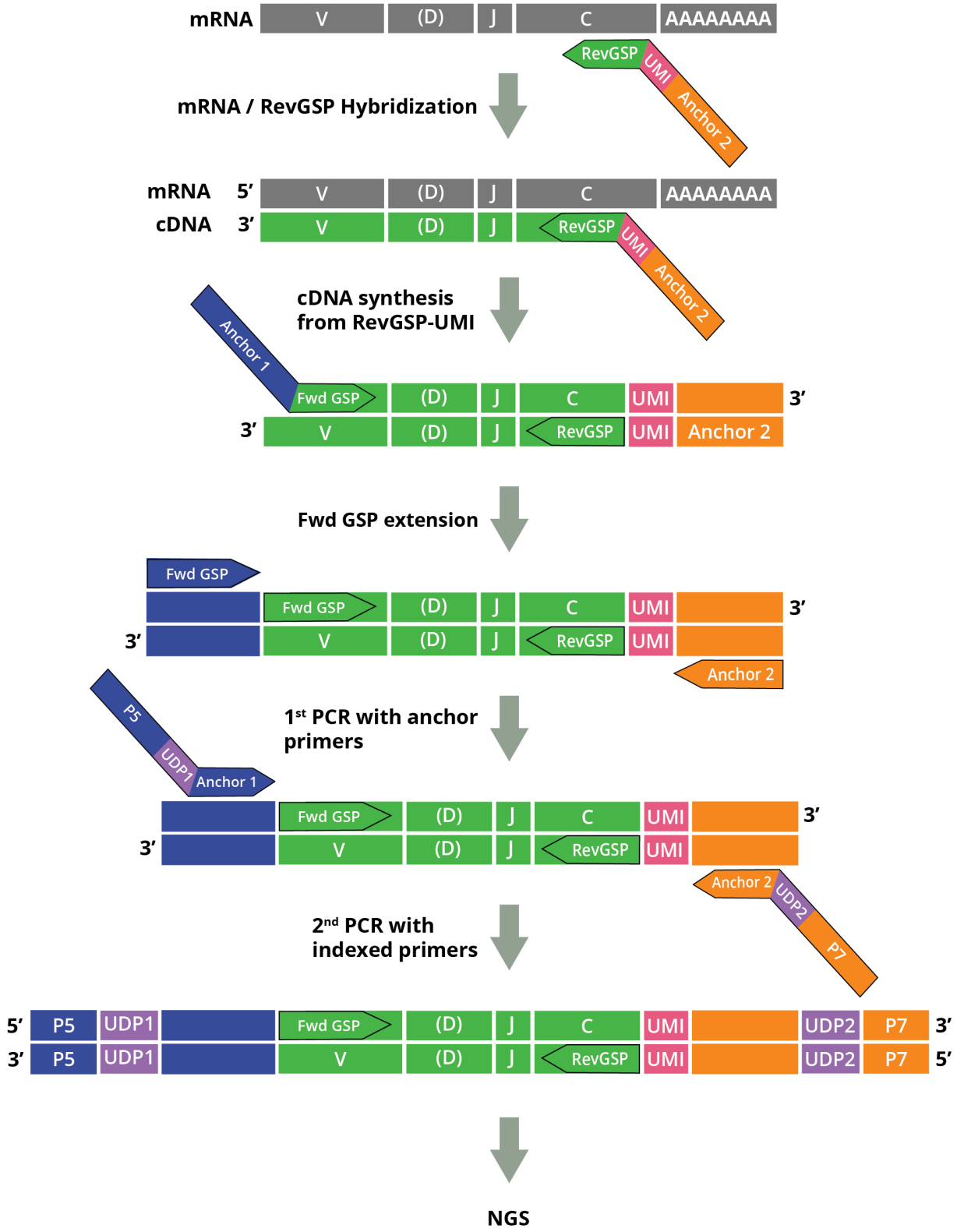

Fig. 2. Outline of DriverMap Multiplex RT-PCR Technology. Step 1: Reverse gene-specific primers (GSP) with UMI targeting all TCR and BCR C-region isoforms, are hybridized with mRNA and extended with reverse transcriptase. Step 2: Forward GSPs are annealed with cDNA template and extended by DNA polymerase. Step 3: In the first PCR step, anchored universal primers (AP1 and AP2) are amplified CDR3 (or CDR1-CDR2-CDR3) cDNA fragments. Step 4: Second PCR amplifies CDR3 fragments using indexed primers. The indexed amplified products are then analyzed by NGS.

Last modified: 28 February 2023

1.2. DriverMap™ AIR DNA Profiling

The DriverMap™ AIR DNA profiling assay is a multiplex PCR assay that uses the same set of 315 forward primers designed for the FR3 region and 100 reverse primers designed for the J region of seven immune receptor genes (Fig. 3).

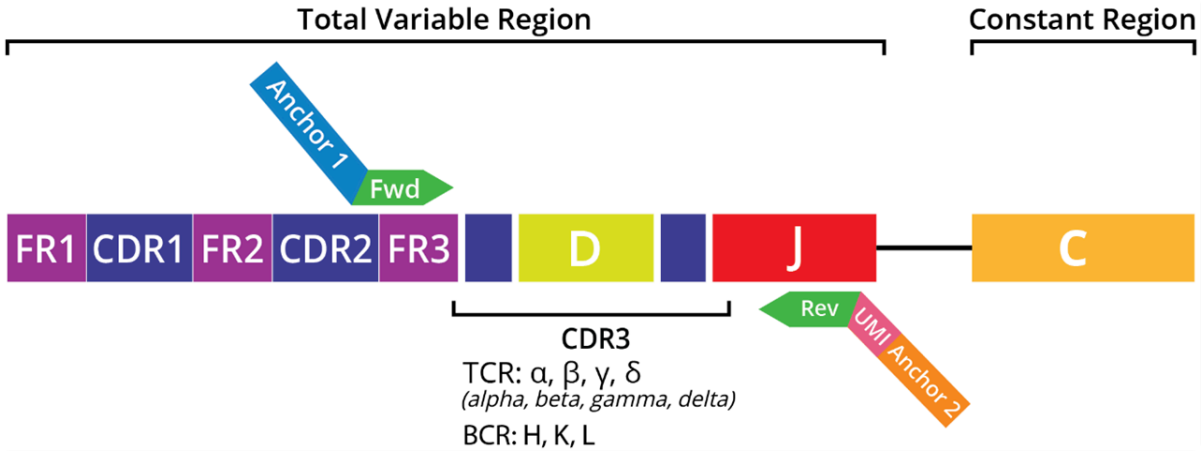

Fig 3. gDNA structure and position of PCR primers to amplify CDR3 regions from gDNA template in the AIR DNA assay.

Similar to the AIR-RNA assay, it allows you to amplify all these seven chains encoded by the TCR and BCR genes. As there is a significant difference in the number of T and B cells present in biological samples, the preferred strategy for balanced immune receptor profiling from DNA is to amplify the TCR and BCR chains separately (Fig 4).

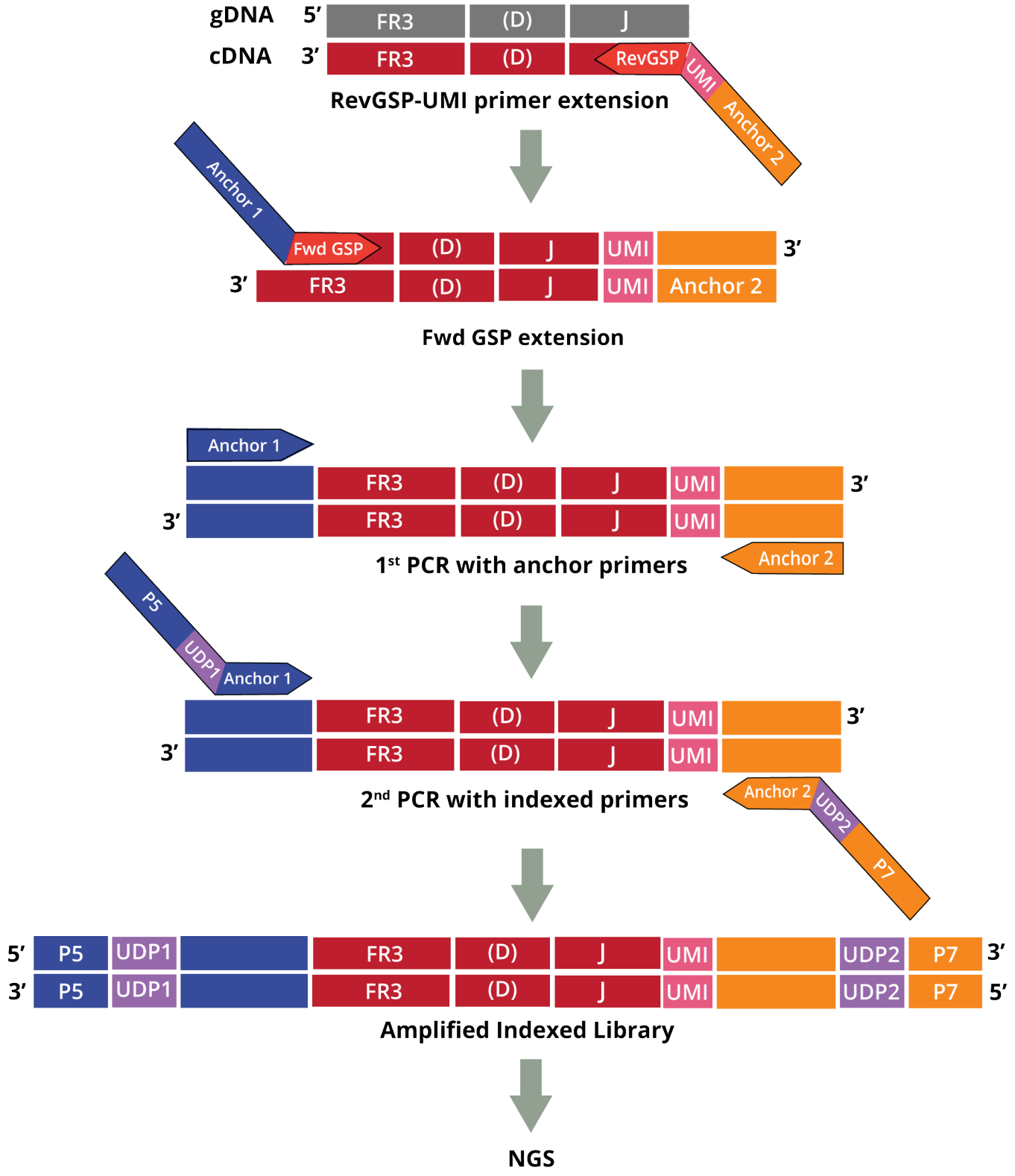

Fig.4. Outline of AIR DNA multiplex-PCR technology. Step1: Reverse gene-specific primers (GSP) with UMI targeting TCR or BCR J-region isoforms (in separate reactions) are annealed to single, complementary DNA strand (top strand) and extended with DNA polymerase. Step 2: Forward GSPs are annealed to extended DNA template and after extension by DNA polymerase generate CDR3 regions flanking from both sides by universal anchor sequences (AP1 and AP2). Step 3: In the first PCR step, anchored universal primers (AP1 and AP2) amplify CDR3 DNA fragments. Step 4: The second PCR step amplifies CDR3 fragments using indexed primers. The indexed amplified products (separately for TCR and BCR) are combined in equal amounts and analyzed by NGS.

Last modified: 23 January 2024

1.3. Comparing Immune Profiling Technologies

For reproducible and comprehensive immune profiling analysis, it is essential to use a technology that provides the highest efficiency for amplification of both high and low-abundant clonotypes and can accomplish this with a small amount of input template (e.g., 10-100 ng for RNA or 0.5-5 ug for DNA). Currently, multiplex PCR and 5'-RACE (SMART) are two commonly used targeted CDR3 amplification technologies for immune receptor repertoire analysis [1-5].

Recent benchmark studies of commercially available immune receptor profiling assays show that 5' RACE (SMART) for RNA and multiplex PCR for gDNA have the best sensitivity for comprehensive repertoire analysis [8,9]. However, Cellecta has developed a novel, ultra-sensitive, multiplex RT-PCR DriverMap AIR assay that detects three-fold more clonotypes than the conventional SMART-based assay with the same input amount (50 ng of PBMC RNA), as shown in Fig 5. Both technologies identify a similar set of the top abundant TCR clonotypes with less reproducible detection of medium to low-abundant clonotypes. Importantly, sensitivity and reproducibility are significantly higher with the DriverMap AIR RNA multiplex technology when compared to SMART technology (Fig 6).

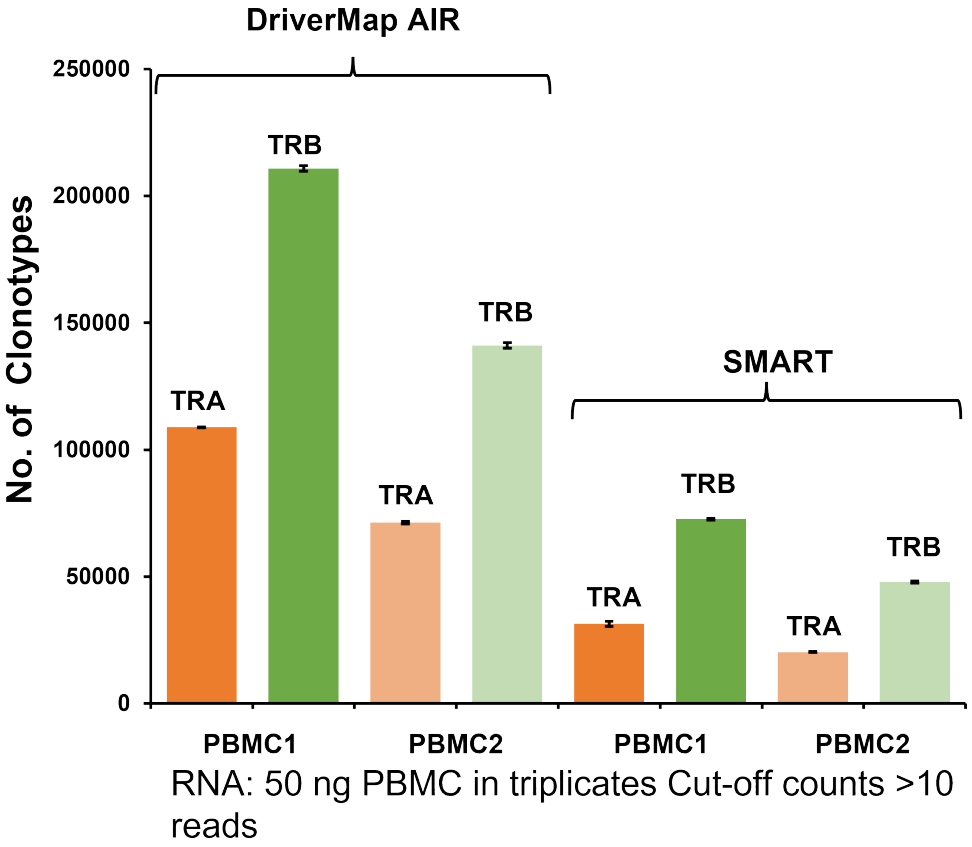

Fig 5. The DriverMap AIR RNA assay detects 3-fold more clonotypes than the conventional SMART-based immune profiling assay, and demonstrates better reproducibility in detecting abundant clonotypes in duplicate samples.

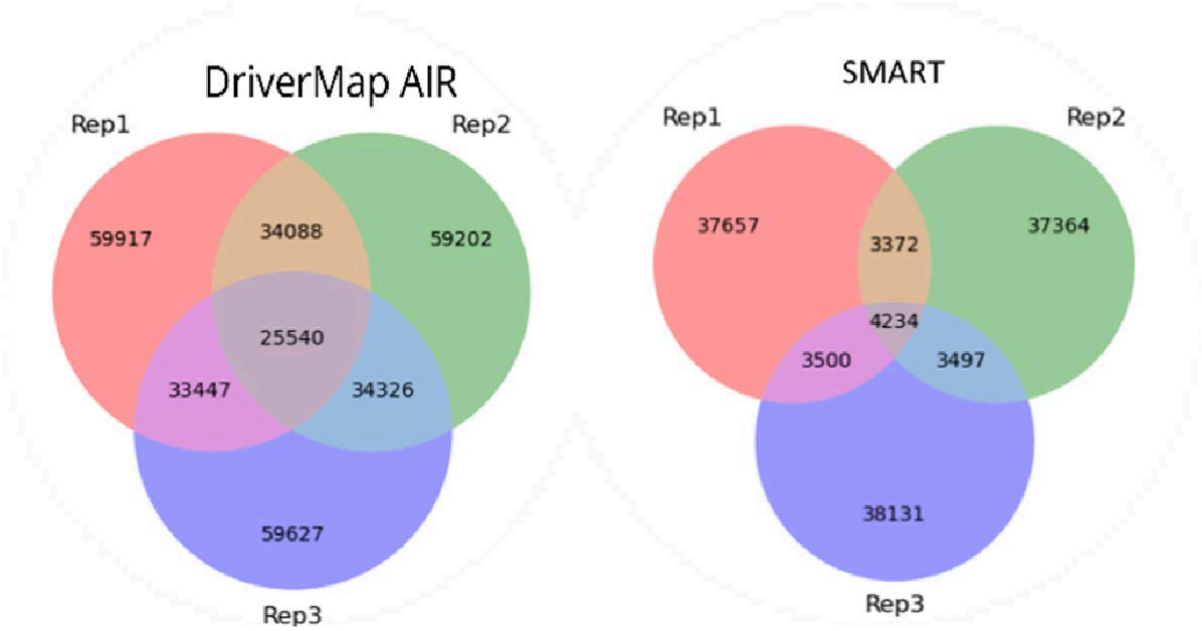

Fig 6. DriverMap AIR RNA reproducibly profiles more overlapping clonotypes in triplicate than the SMART-based assay.

We also compared the performance of the AIR RNA assay with a leading commercial gDNA-based multiplex immune receptor profiling assay. DNA and RNA were extracted from sorted T and B cells from metastatic tumor samples. Profiling studies of both immune receptors were then conducted in parallel [20]. Fig. 7 shows that the AIR RNA assay identifies 1.5 to 2-fold more clonotypes than the leading gDNA-based multiplex PCR assay.

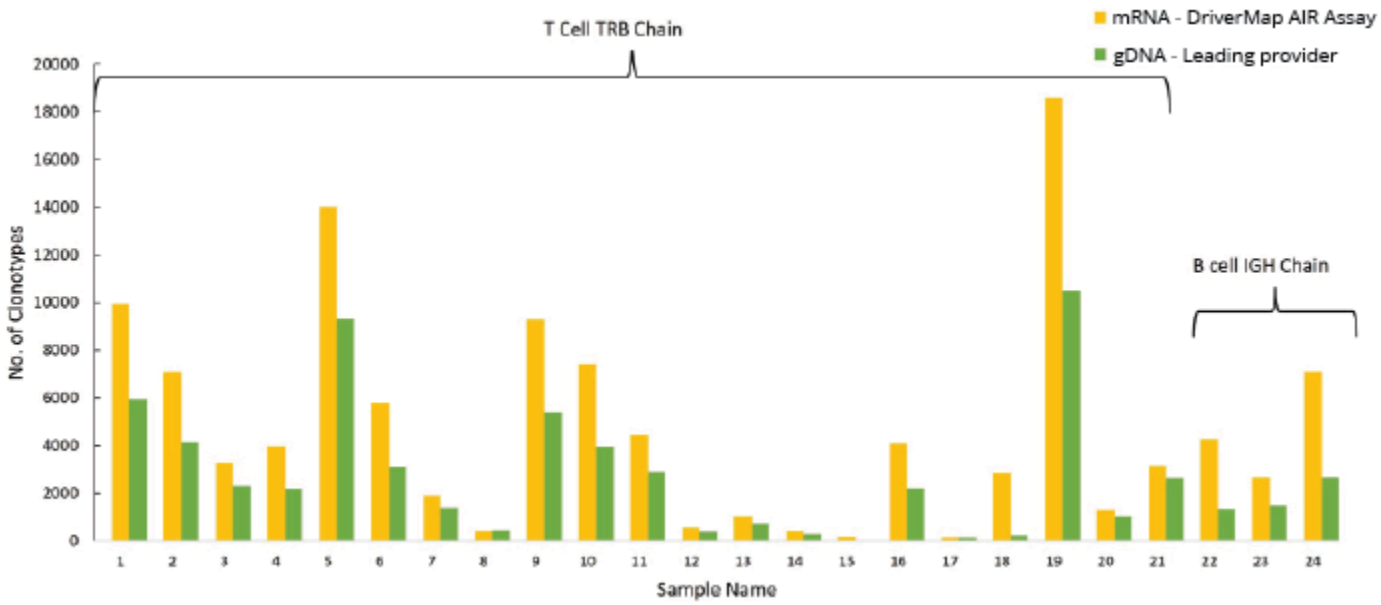

Fig 7. The AIR RNA assay identifies 1.5 to 2-fold more TRB and IGH clonotypes than a gDNA-based multiplex PCR immune receptor profiling assay in both T and B cells (1,000-100,000 cells per sample) sorted from several metastatic tumor samples.

Last modified: 22 February 2023

2. Experimental Design Strategies

The main goal of the DriverMap AIR assays is to:

- Characterize the global AIR repertoire in normal and pathological conditions
- Identify activated subsets of TCR and BCR clonotypes in the adaptive immune response
- Identify disease- or pathogen-specific TCR and BCR clonotypes (both CDR3 (or CDR1-CDR2-CDR3) sequences and chain-pairing)
- Characterize the phenotype of antigen-specific, activated T and B cells

Currently, most available AIR technologies are based on the amplification of TCR and BCR sequences followed by NGS but provide only partial immune receptor repertoire information [1-7,18] and are characterized by significant systematic biases [8, 14]. The following sections will provide practical guidelines for selecting the best design strategy for AIR experiments.

Last modified: 22 February 2023

2.1. Combined TCR and BCR Analysis

In most clinical responses, T and B cells work synergistically in the adaptive immune response [13,18]. Therefore, for comprehensive analysis of the adaptive immune response and unbiased discovery of antigen-induced immunity receptors, we recommend profiling the repertoire of both TCR (TRA, TRB, TRD, TRG) and BCR (IGH, IGK, and IGL) genes simultaneously.

Hence, the AIR RNA assay is designed to profile all TCR and BCR isoforms in a single reaction without compromising sensitivity for robust repertoire detection. It is important to note that TRA and TRB clonotypes have similar complexity and representation at the RNA level in blood samples, which allows them to be profiled together, as illustrated in Fig.8. TRD and TRG clonotypes have significantly lower complexity but can easily be detected together with other TCR and BCR chains. In studies designed for immune receptor repertoire analysis of only T or B cells or in the samples with significant (e.g., 5-10-fold) differences in T/B content, Cellecta also offers primer sets to amplify only the TCR or BCR chains in separate reactions.

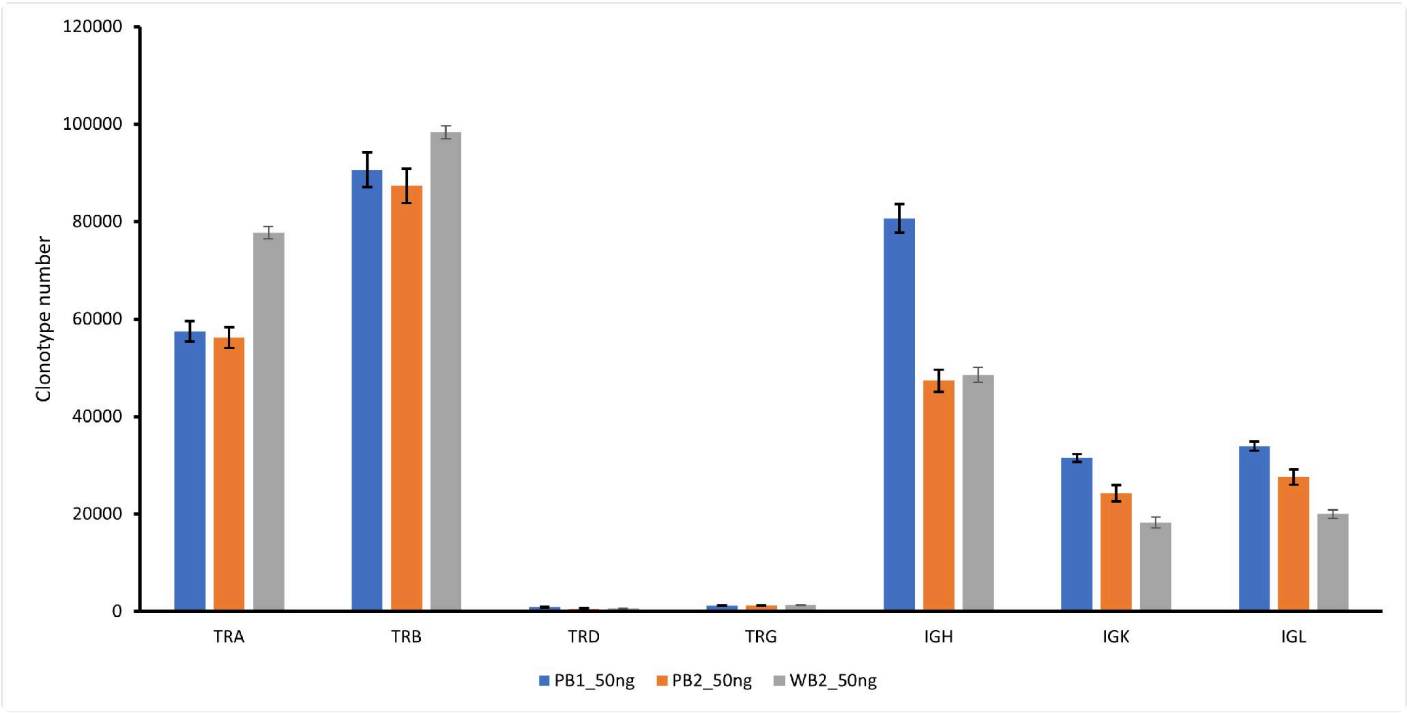

Fig 8. Number of clonotypes for 7 TCR/BCR chains identified in 50 ng of normal PBMC or whole blood RNA using the AIR RNA assay. 10x10^6 reads per sample, each sample run in triplicates.

Similarly, in the AIR DNA assay, we recommend amplifying the CDR3 regions separately for TCR and BCR, considering the significant differences (e.g., 4-5-fold in the blood) in the abundance of T and B cells in many biological samples. Furthermore, the number of clonotypes identified for TRA, IGK, and TRG are usually higher than for other matched chains, as illustrated in Fig.9. One of the reasons for these differences is that a significant fraction of lymphocytes have two rearranged TRA, IGK, and TRG genes wherein one copy is non-productive and silenced or expressed at a very low level [20-22]. An extreme case is seen with TRG rearranged genes which are present not only in gamma-delta T cells but also present but silenced in alpha-beta T cells [22].

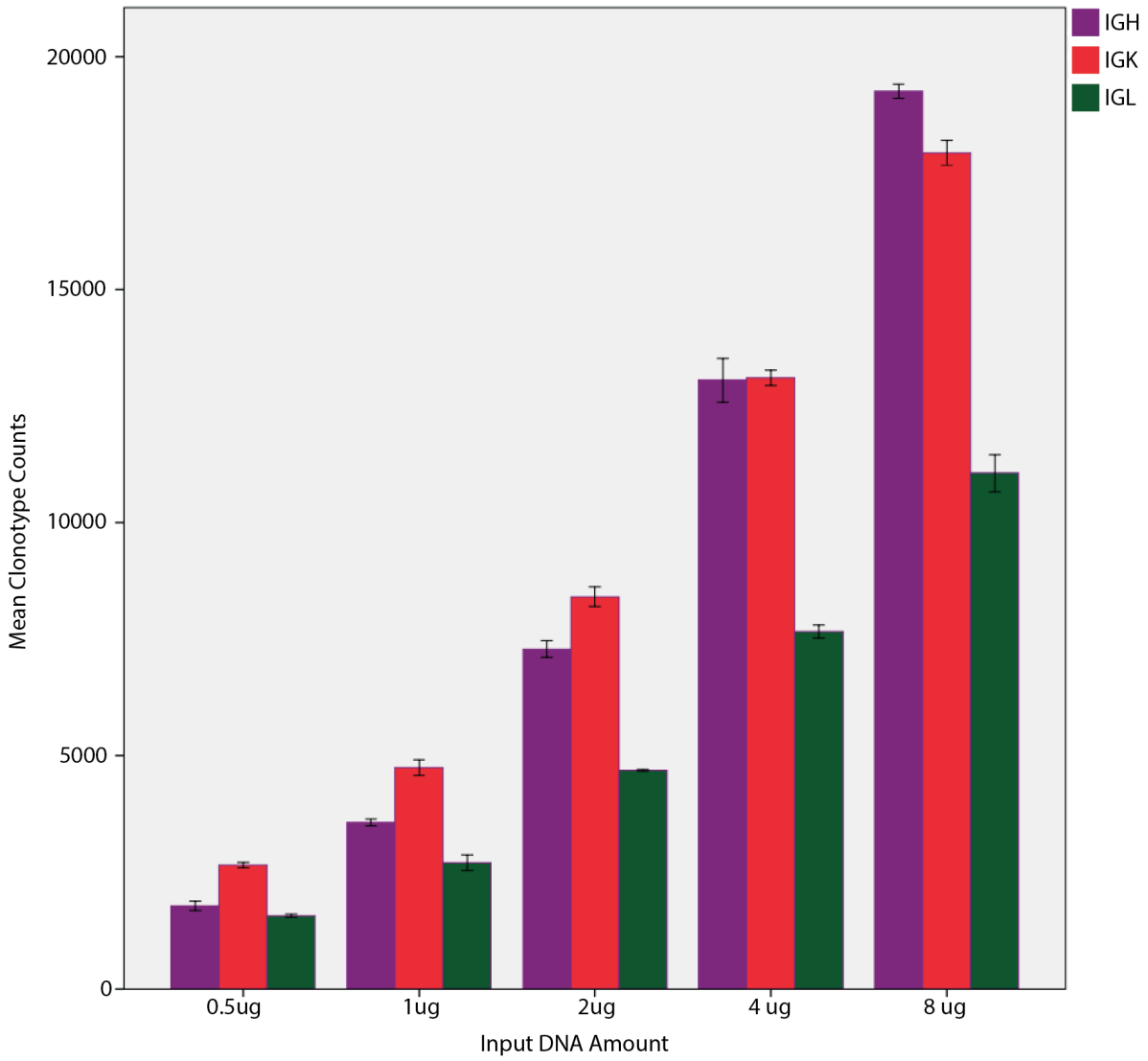

Fig 9A. AIR DNA repertoire analysis in all three BCR chains (IGH, IGK and IGL). PBMC gDNA in triplicates, 5x10^6 reads per sample.

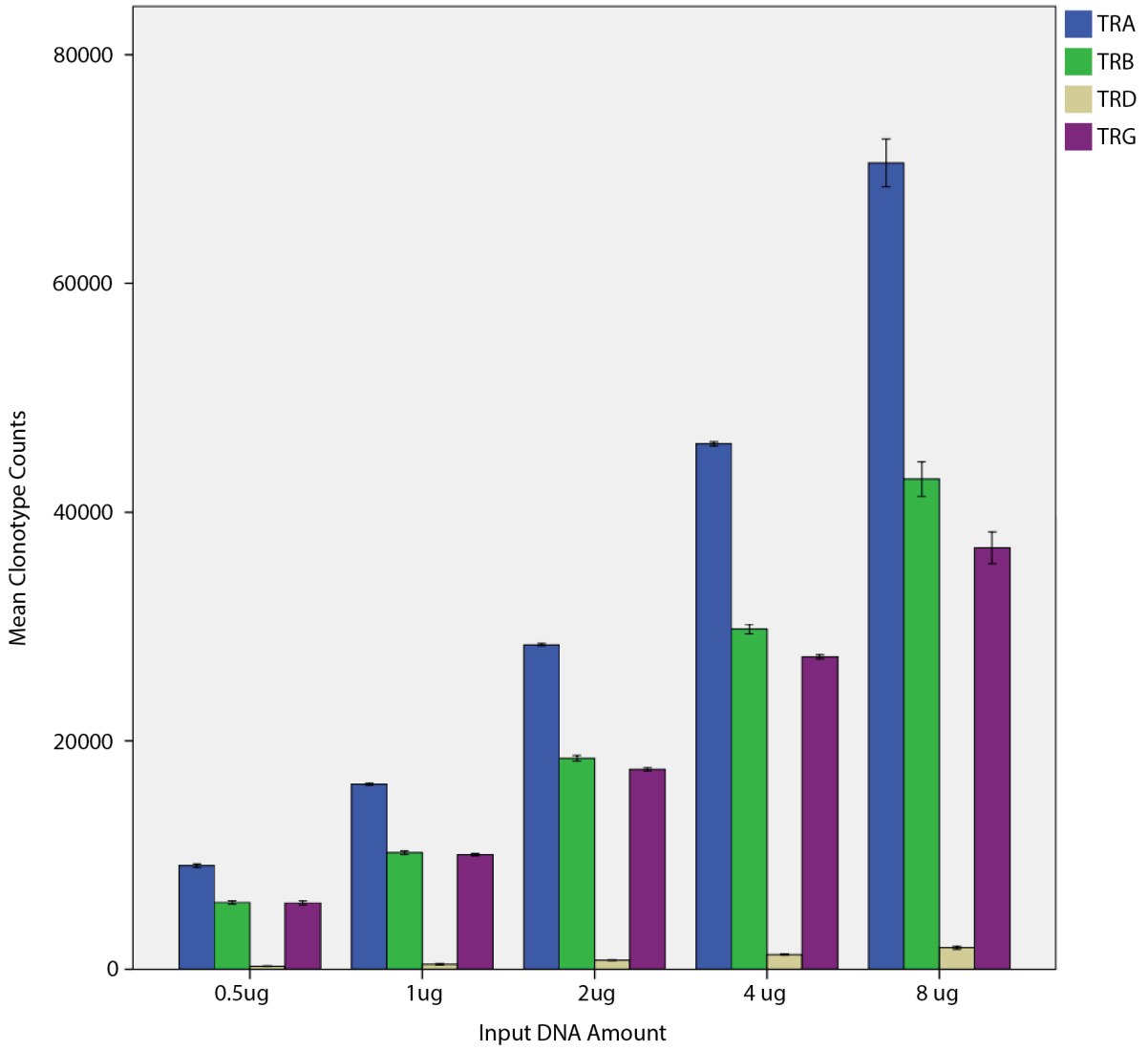

Fig 9B. . AIR DNA repertoire analysis of four TCR chains (TRB, TRA, TRD and TRG) .PBMC gDNA in triplicates, 5x10^6 reads per sample.

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2.2. RNA vs. DNA Template

Both genomic DNA (gDNA) and mRNA are commonly used as starting material for immune receptor profiling, and each has advantages and disadvantages. Cellecta offers both an RNA-based and a DNA-based AIR assay. Based on our experience, the AIR RNA assay has a significant advantage over the AIR DNA assay for the following reasons:

- **Sensitivity:** The copy number of mRNA per cell is at least 10 to 100-fold greater than gDNA, significantly increasing sensitivity due to the higher number of starting RNA template copies [4]. The increased AIR sensitivity from using RNA templates is an essential advantage for reliable profiling of TCR and BCR clonotypes in small biological samples (e.g., sorted cells, tumors biopsy, FFPE, etc.) (Fig. 10).

- **Functionality:** Using RNA as a template limits amplification to only the receptor chains which are functional and productively expressed. AIR profiling from gDNA amplifies many additional sequences, including non-rearranged and rearranged but non-functional (usually expressed at low level) receptor genes, which increases undesirable background in the NGS data, especially in tissue samples with low immune cell content.

- **Coverage:** BCR repertoire analysis using gDNA as a template does not allow identification of the Ig isotype, as V(D)J and C regions are separated by an intron and cannot be effectively amplified by a multiplex-PCR reaction.

The drawbacks of using RNA include requiring reasonable RNA template integrity (RIN > 5). Furthermore, if your research needs to accurately quantify the T and B cell number for each clonotype, a gDNA-based assay based on UMI (Cellecta AIR DNA assay) or in combination with calibration standards is a better choice. Please note that precise quantitation of cell number for each CDR3 clonotype (based on receptor gene copy number) is not critical if the goal is the detection of “up-regulated” disease-associated, activated clonotypes (similar to differential gene-expression analysis), e.g., in serial / time course studies in blood samples.

Identification of disease-activated CDR3 clonotypes

The most common strategy for discovering antigen-induced clonotypes is AIR repertoire profiling in multiple samples collected in serial/time course studies of infection, immunization, immune therapy treatment, etc. [1-5]. Antigen-activated clonotypes are detected by an increase in the copy number of RNA or DNA CDR3-specific sequences compared to control non-activated samples.
In another application, the identification of overlapping, transcriptionally activated CDR3 clonotypes in the tumor, blood, or tumor-draining lymph nodes can be used for identifying tumor-reactive T cells [24]. Unfortunately, for many human diseases, only a single sample (e.g., a heterogeneous tumor biopsy) is available for AIR profiling.

Studies demonstrate that adaptive immunity activation induces significant up-regulation of both BCR and TCR transcription in antigen-specific clonotypes (e.g., up to 1,000-fold for plasma B cells [13]) as illustrated in Fig.10. Furthermore, the transcriptional up-regulation of TCR genes in large CD8+ effector memory clonotypes in peripheral blood samples was associated with durable responses to immune checkpoint blockade in patients with metastatic melanoma [23].

As a result of transcriptional activation, an RNA-based immune receptor repertoire is usually dominated by a low number of abundant clonotypes, some of which are antigen-induced or disease-specific. Up-regulation of TCR and BCR receptor gene expression in activated effector/memory T and B cells can identify and differentiate antigen-induced CDR3 sequences from non-activated, bystander CDR3 sequences commonly present in tumor samples [20].

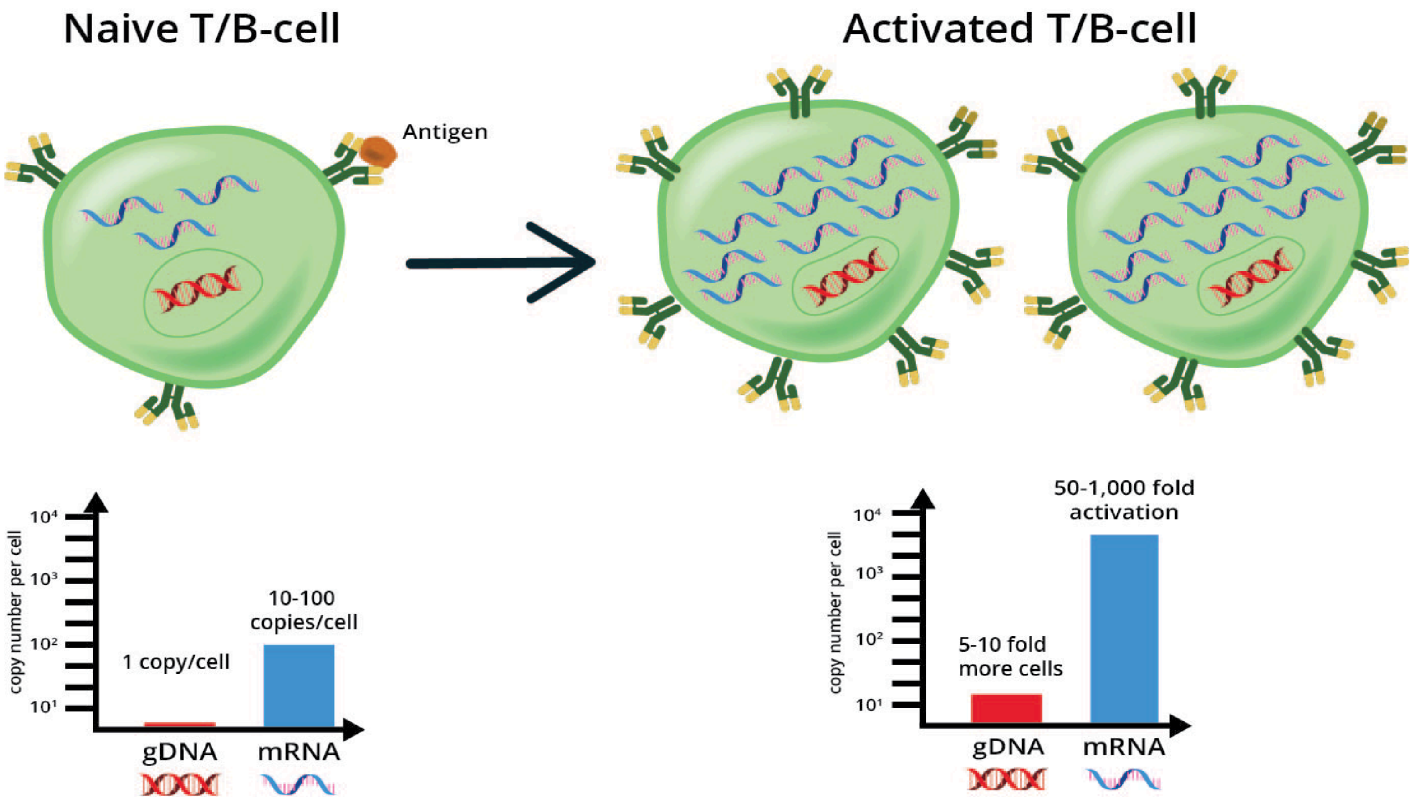

Fig 10. Expression of TCR and BCR genes in naive and activated T and B cells. Normalization of RNA to DNA copy number allows effective discrimination of antigen-activated vs naive cells.

Combining mRNA and gDNA-based assay technology

A simple and efficient strategy to differentiate antigen-induced clonotypes from abundant naive or bystander clonotypes commonly present in clinical samples is to normalize RNA-based CDR3 profiling to similar data from a DNA-based assay. To facilitate this approach, we developed UMI-based AIR RNA and AIR DNA assays, which can be combined to identify antigen-activated clonotypes.

As a practical guideline, we suggest that AIR profiling be performed using both RNA and DNA isolated from the same biological sample (e.g., PBMC with high content of immune cells). In another approach, RNA-based and DNA-based AIR profiling could be performed directly in T and/or B cells sorted from tissue or blood samples (see below). Furthermore, to reveal disease-associated, activated CDR3 clonotypes, RNA-based data can be normalized to DNA-based AIR profiling data for overlapping clonotypes, as illustrated in Fig.11.

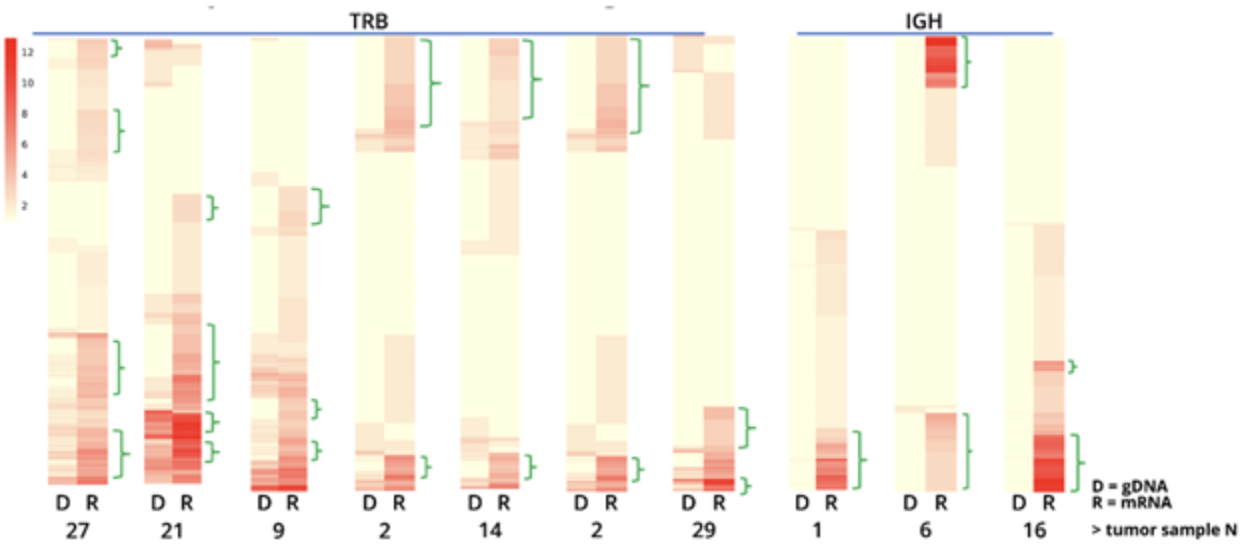

Fig 11. Comparison of RNA-based to DNA-based immune receptor repertoire profiling data from RNA and DNA purified from the T and B cells sorted from metastatic brain tumor samples [20]. Results show the presence of 5-10% tumor-activated immune receptor clones (marked by brackets) that can be activated by up to 1000-fold (B cells) and to 50-fold (T cells) by transcriptional activation of immune receptor genes.

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2.3. Bulk vs. Single-cell Analysis

Bulk Analysis

Bulk analysis is the current front-line, high-throughput experimental strategy for comprehensive and sensitive disease or treatment-associated clonotypes detection. It can be performed using RNA isolated from either small or large samples of frozen or lysed blood, cell fractions, or tissues. The main advantage of bulk analysis is a simple protocol that can be conducted with standard lab equipment and scaled for hundreds of samples in 96-well plates. The cost of sequencing is reasonable, and multiple open-source software tools are available for downstream data analysis. The drawback of bulk analysis is the inability to provide information about the pairing of receptor chains and integration of CDR3 sequences with the phenotype of T and B cells for each clonotype. Although cell-type deconvolution algorithms applied to RNA-seq data can potentially link cell subtypes with the most abundant clonotypes, in practice, the application of this strategy is currently limited [10].

Single-cell Analysis

Single-cell immune receptor profiling is a revolutionary approach that allows investigators to combine clonotype repertoire with paired-chain information and the phenotype of cells (e.g., cell subtype). Single-cell immune receptor profiling assays can be performed at low throughput (96-384 cells) using sorted cells in 96-well plates (e.g., AIR RNA assay) or at medium throughput (1,000-5,000 cells) using microwell arrays (BD Rhapsody) or droplet microfluidics (10x Genomics) technologies [1-5, 9]. However, single-cell immune receptor profiling assays are more complicated and expensive (for both reagents and sequencing) than bulk assays. They typically require live cells, which is not practical for many applications. Furthermore, single-cell immune receptor repertoire analysis can only profile (in a non-quantitative fashion) the most abundant clonotypes due to the low number of cells analyzed in the assay.

Immune Cell Fractions

To address the limitations of single-cell analysis, Cellecta offers a promising strategy to profile immune cell fractions (e.g., T/

B naive, memory, effector, exhausted cells) isolated by antibody-loaded magnetic beads or FACS sorting from blood or tissue samples using conventional cell typing-specific antibodies. FACS-purified immune cell fractions (e.g., 5K-50K cells) can be used directly without purification for DriverMap AIR RNA and a DriverMap T/B immune marker assay developed for expression profiling the 500 top T and B subtyping and activation marker genes (see section 4 below). By combining AIR profiling with expression profiling of key immunity genes in the same sorted cells, you can link the clonotype information with the cellular phenotype of cells (Fig. 12). [13,18]

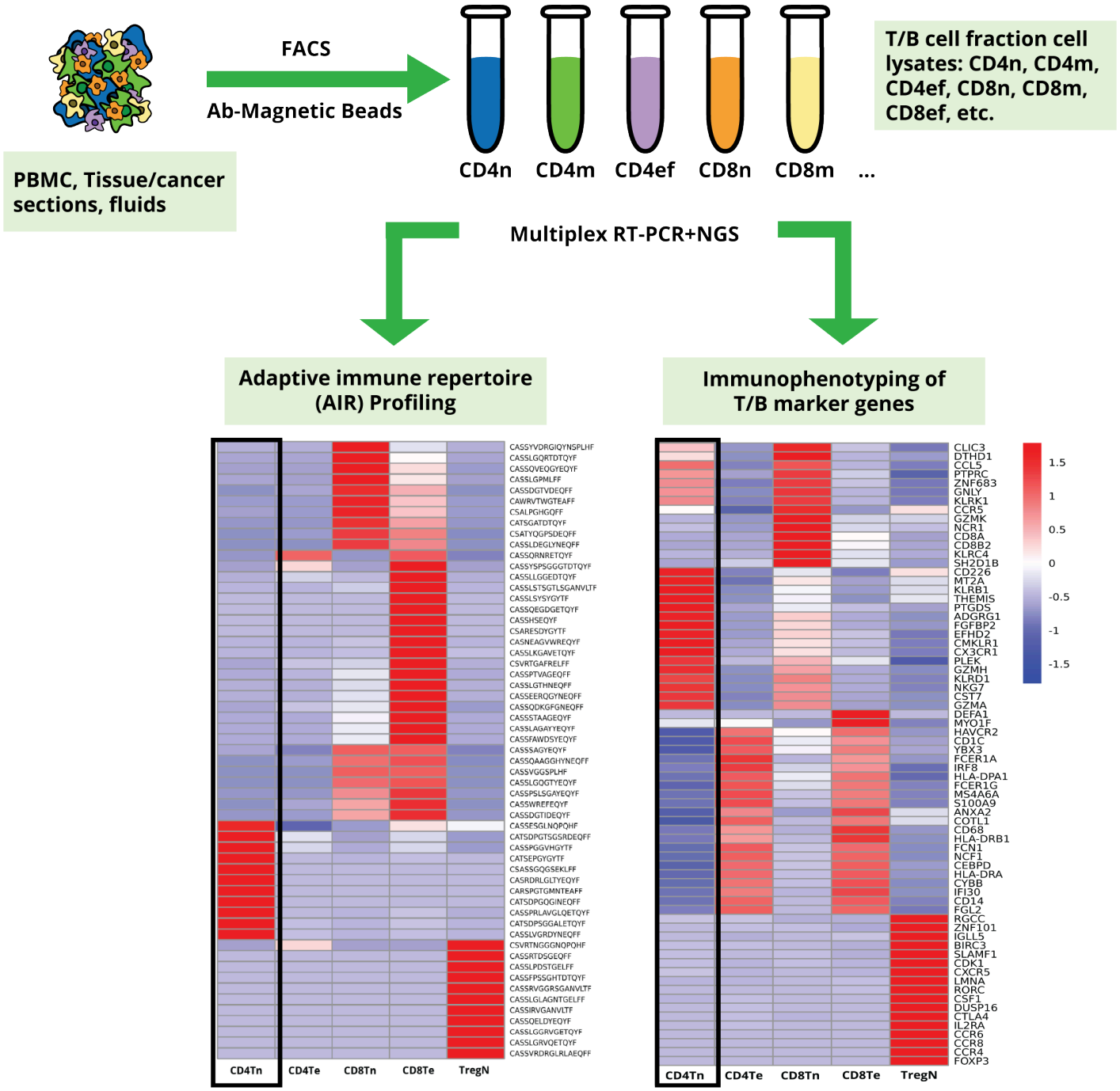

Fig 12. In FACS-sorted PBMC fractions of CD4 naive, CD4 effector, CD8 naive, CD8 effector, and Treg cells (25,000 cells each), parallel profiling of TRB clonotypes and phenotypic characterization using the DriverMap T/B Immune Marker Panel reveals links between specific CDR3 clonotypes and comprehensive immunophenotypes of sorted cell fractions based on expression of key immune cell markers.

Last modified: 3 April 2023

2.4. Technical Reproducibility

For reliable detection of clonotypes activated during infection, pathological processes, or treatment conditions, it is critical to measure the accuracy of AIR repertoire profiling. To achieve this goal, we recommend running each sample (or at least a few samples) if possible, in technical replicates to estimate reproducibility in the detection of high- and medium-abundant clonotypes. For samples with a limited amount of starting RNA, you can split and amplify in replicates the same cDNA sample after the initial forward primer extension step [18].

In addition to technical or amplification replicates, it is desirable to repeat AIR RNA profiling from at least two to three RNA samples independently purified from the same biological sample (e.g., different tissue sections of tumor samples) whenever possible. AIR profiling without replicates may only identify high-abundant clonotypes with a significant loss(false-negative) of medium-abundant clonotypes.
To provide a more quantitative measurement of clonotype abundance level and compensate for variability in the amplification steps (PCR duplicates), both AIR RNA-based and AIR DNA-based assays incorporate UMIs in reverse gene-specific primers. Using UMIs allows one to calibrate and normalize the AIR profiling data (read number of each clonotype) to the copy number of CDR3-specific target mRNA or DNA molecules in the biological sample. Furthermore, to use UMIs for reliable detection and quantitation of high- to medium-abundant clonotypes in the AIR RNA assay, it is important to achieve at least 20-40 reads per UMI, which corresponds to approximately 5-10 million reads/ sample.

In a typical AIR profiling experiment using PBMC or whole blood RNA samples, as illustrated in Fig.13, you should expect to detect the following clonotype classes:

- About 100-500 highly abundant clonotypes (approximately 20% of reads) are amplified from at least 10 mRNA molecules (>10 UMI copies). They can be reproducibly profiled (Fig. 13) from even a single RNA sample without biological triplicates.

- About 3,000-5,000 medium-abundant clonotypes are amplified from at least three mRNA molecules. They could have some significant variation (including false negatives) in measured abundance level due to the low number of CDR3-specific mRNA molecules used in the assay. To reliably measure medium-abundant clonotypes, technical replicates are necessary.

- Approximately 50,000-500,000 clonotypes are present in low abundance, randomly amplified from a large pool of low abundant (1-2 copies) mRNA TCR/BCR molecules (in PBMC or blood samples), and cannot be reproducibly profiled. Comparison of technical AIR repertoire replicates for low abundant clonotypes usually reveals thousands of clonotypes with a minimum overlap in triplicate samples.

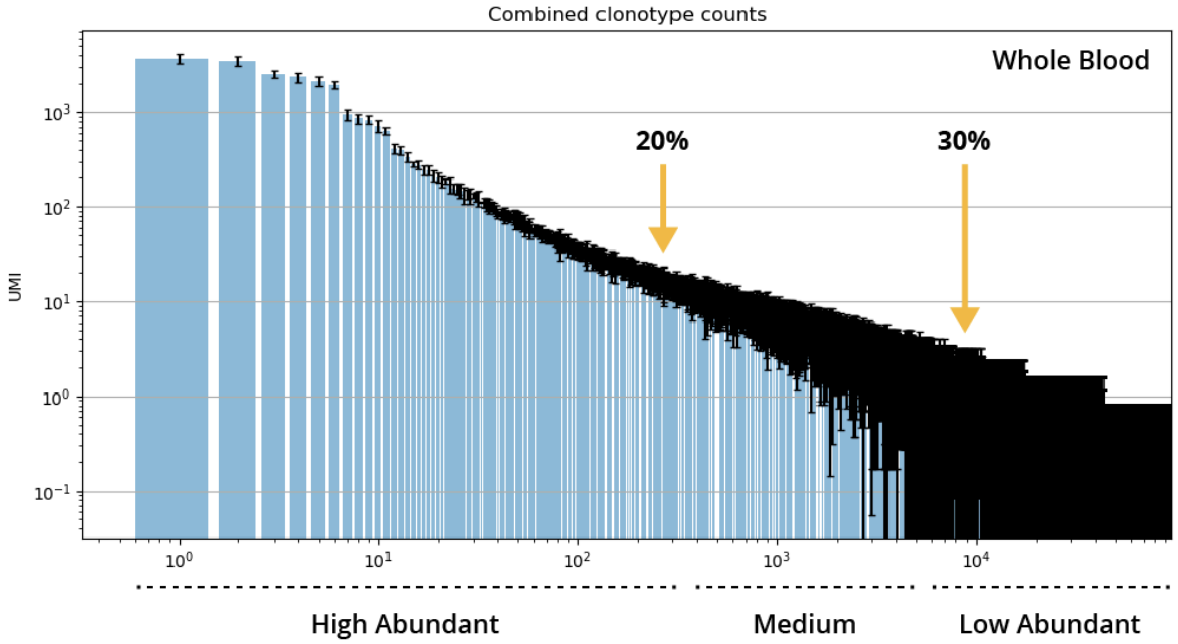

Fig 13. Analysis of reproducibility of high-, medium-, and low-abundant clonotypes in whole blood samples measured in triplicates by AIR RNA-based assay in 50 ng of whole blood RNA (10x10^6 reads per sample). Y axis shows the number of CDR3-specific mRNA molecules (based on UMI measurement) amplified for each clonotype with number of unique clonotypes in each class shown on the X axis.

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2.5. Challenges of AIR Profiling

Studies show that healthy individuals have at least 100-200 million different TCR and BCR clonotypes circulating in peripheral blood, and about 1-10% of these clonotypes are shared between individuals [15-17]. As a result, comprehensive characterization of the complete immune repertoire would require at least 10 billion lymphocyte cells (e.g., from leukapheresis blood samples) which is not practical for most applications. Therefore, comprehensive AIR profiling is more challenging than conventional RNA-seq due to the enormous complexity of different clonotype sequences (100-200 million) compared to the number of protein-coding genes (around 20,000).

AIR profiling data shows a long-tailed clonotype distribution, with most reads corresponding to low-abundant clonotypes (present at single-cell levels), resulting in reduced efficiency of detecting medium-abundant clonotypes only a few molecules may represent in the RNA sample. Therefore, with a limited amount of total RNA, we can reliably detect only the most abundant (high- to medium-abundant) clonotypes while losing a significant fraction of low-abundant clonotypes, which are present at a single mRNA molecule level (Fig. 13). For biological samples with a very small number of lymphocytes (e.g., specific tumor samples, some immune cell fractions, FFPE, etc.) or single-cell AIR assays, comprehensive quantitation and detection of both high and medium-abundant clonotypes represent a severe technical challenge and in most cases is not feasible.

Additionally, the cost of NGS sequencing can be a challenge. When working with a large number of samples, if the goal of the experiment is to profile the most frequent clonotypes, you can run more samples in the flow cell with lower reads per sample, as shown in the table below:

Instrument	Reads per flow cell	Number of samples for multiplexing per flow cell	Reads per AIR sample
NextSeq 500/	120 million (medium	12-20	6-10M reads/sample

550	throughput)		
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

For detailed instructions for sequencing, please refer to the Product User Manual

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3. Applications of AIR Profiling

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

- For the AIR RNA assay, the table below describes the optimal input amount and range of total RNA purified from different biological samples. As illustrated in Fig. 14, using less than the optimal amount of RNA could reduce reproducibility in detecting medium-abundant clonotypes.

- For bulk tissue, blood, or cell samples with more than ~50,000 cells, purify RNA and DNA using conventional protocols (e.g., as recommended in the AIR manual). 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 as described in the DirectCell Protocol.

- For the AIR DNA assay, the general rule for quantitating high- and medium-abundant clonotypes is to use at least 5-10 µg of DNA corresponding to approximately 1 million cells. Using a smaller amount of DNA (0.5-2 µg) would reduce the detection of medium-abundant clonotypes, and even lower amounts (0.1-0.5 µg) would also reduce the sensitivity of high-abundant clonotypes (Fig. 14). Considering the reduced sensitivity of the AIR DNA assay due to the low copy number of starting DNA molecules, technical replicates are highly recommended.

RNA Prep Protocol > 50,000 cells		
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

DirectCell AIR RNA Protocol < 50,000 cells		
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

Last modified: 8 March 2023

3.1. Profiling Sample with High Amount of Lymphocytes

When working with a sample containing abundant lymphocyte cells (e.g., at least 1-2 million) such as whole blood, buffy coats, PBMC, lymphoid tissues, immune cell fractions, and bone marrow aspirates, we recommend purifying both total RNA and DNA as recommended in the AIR manual. Using RNA as the starting material for AIR RNA profiling increases reproducibility in detecting medium-abundant clonotypes.

The optimal input amount for the DriverMap AIR RNA assay is 50-100 ng of total PBMC RNA, with which you can get quantitative profiling of about 1,000 high-abundant TCR and BCR clonotypes (Fig. 14). Increasing the input amount to 200 ng increases the number of rare clonotypes detected but it does not improve the detection of medium- to high-abundant clonotypes due to restrictions in sequencing depth (cost of sequencing) and a lower consensus in CDR3 sequence alignment efficiency. However, lower levels of PBMC RNA (e.g., 10 ng) reduce the accuracy of profiling medium-abundant clonotypes. We recommend using the DirectCell Protocol for direct AIR profiling from a small number of immune cells, as described in Section 3.2

If using the AIR DNA profiling assay, the DNA used in the assay corresponds to the number of cells (6.6 ug is 1 million cells). As a result, to achieve high detection sensitivity of high- to medium-abundant clonotypes, it is necessary to run replicates and use at least 2-5 ug of DNA per reaction.

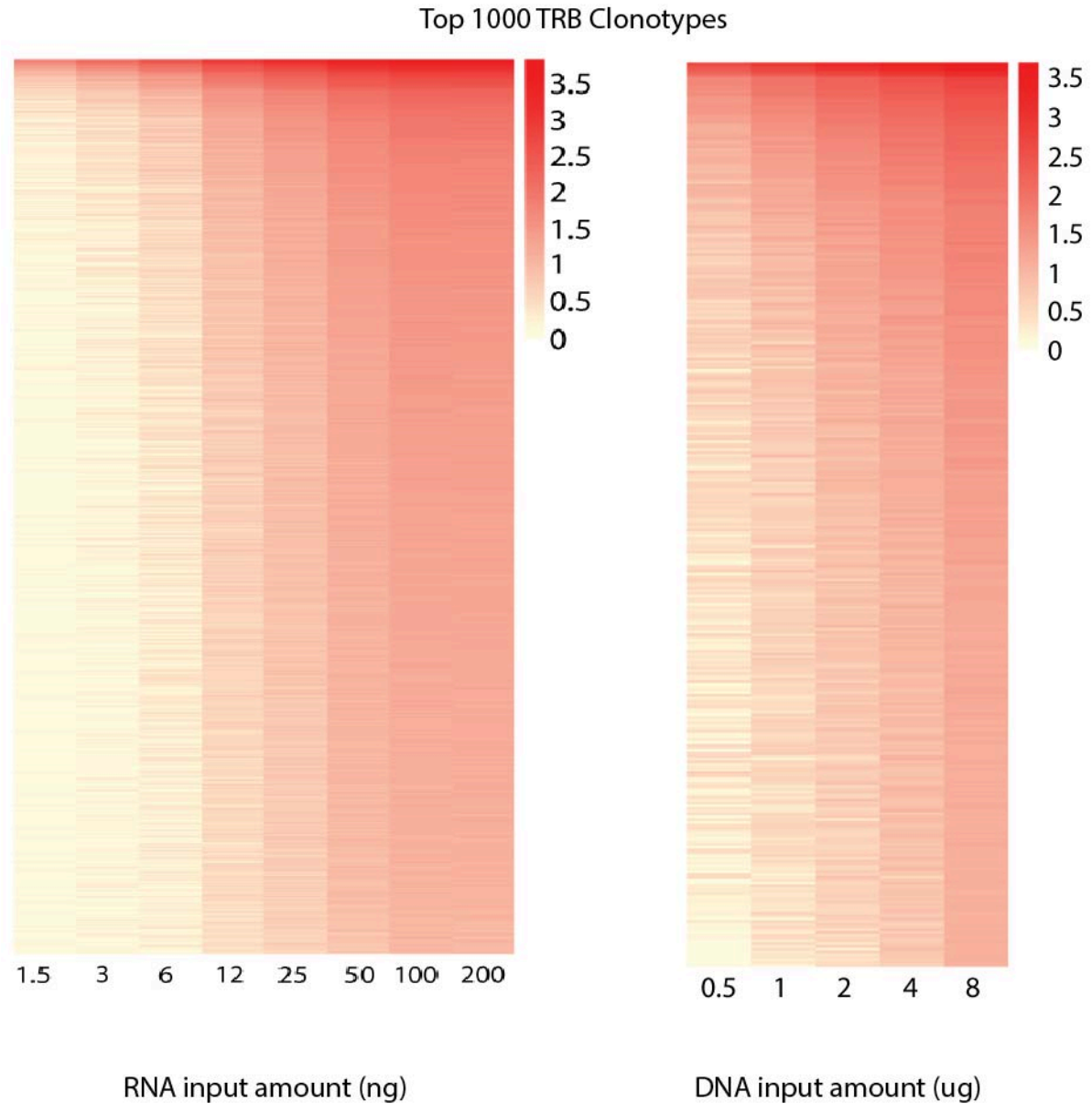

Fig 14. Heatmap of Reproducibility of AIR RNA and AIR DNA assays for top 1,000 medium-high abundance CDR3 clonotypes for TRB gene using different starting amounts of RNA or DNA. Both AIR RNA and AIR DNA assays were performed for all 7 chains in triplicates, 5x10^6 reads per sample. Median UMI value was calculated for each clonotype (horizontal lines), sorted by abundance, and shown in heatmap with color corresponding log10 UMI (TRB template) copy number used in the assay.

Last modified: 3 April 2023

3.2. Profiling Samples with Low Amount of Lymphocyte

When working with a small number of lymphocyte cells, such as cancer biopsy, non-lymphoid tissue, biological fluid, tissue or blood microsamples and FFPE samples, we recommend purifying RNA from at least 1 mg of the tissue sample. For bulk tissue with a small number of lymphocytes (e.g., 100-10,000), we recommend using as much RNA as possible (up to 1,000-2,000 ng of total RNA) to capture most of the receptor diversity (most abundant clonotypes) (Fig.12). Using a lower input amount will reduce the detection efficiency of medium-abundant clonotypes. For the AIR DNA assay, lymphocyte DNA will be diluted by DNA from other cell types, and as a result, the detection sensitivity of TCR and BCR clonotypes will be significantly compromised. If possible, please consider purifying the lymphocyte cell fraction prior to DNA isolation to increase the sensitivity of the AIR DNA assay.

If the size of the tissue sample is less than 1 mg (e.g., micro-dissected tumor samples or FFPE slide) or using immune fractions purified by FACS or antibody-magnetic beads from samples with lower lymphocyte counts (e.g., tissue, blood, fluid, or tumor cells), we recommend using the DirectCell™ Protocol to directly profile immune receptors from cell lysate without purifying RNA or DNA. Based on our experience, direct profiling of isolated immune cells increases the detection sensitivity of the AIR assay by 5- to 10-fold, which can be explained by avoiding the loss of RNA/DNA during purification steps. Due to the lower complexity of TCR/BCR clonotypes in these samples, we recommend using approximately 2 million reads per sample (or at least 20 reads per UMI).

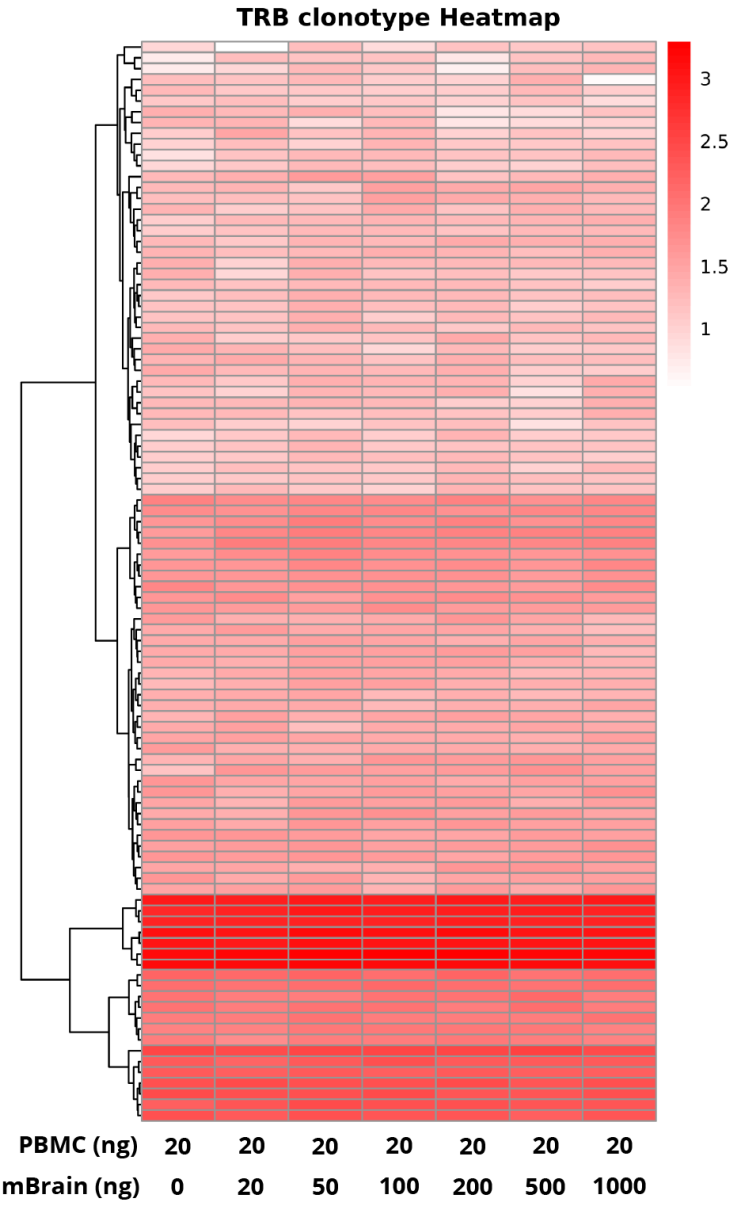

Fig. 15.Reproducibility of AIR RNA assay in profiling top 100 TRB clonotypes in 20 ng of PBMC RNA mixed with up to 50-fold excess total RNA from mouse brain.

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3.3. Profiling Whole Blood Samples

We have demonstrated that AIR RNA profiling can be done directly from whole blood samples (after purification of RNA from Tempus or PAXgene test tubes) without isolating PBMC. A comparative analysis conducted with the same amount of whole blood and PBMC samples shows that both assays detect a comparable number and similar repertoire of high- to medium-abundant clonotypes.

Based on these results, we recommend using whole blood (stabilized in Tempus) instead of PBMC for deep AIR profiling. This reduces the variation in batch effects caused by the preparation, storage, and transportation of PBMC samples and simplifies the blood collection process, especially when samples need to be collected at multiple time points [12].

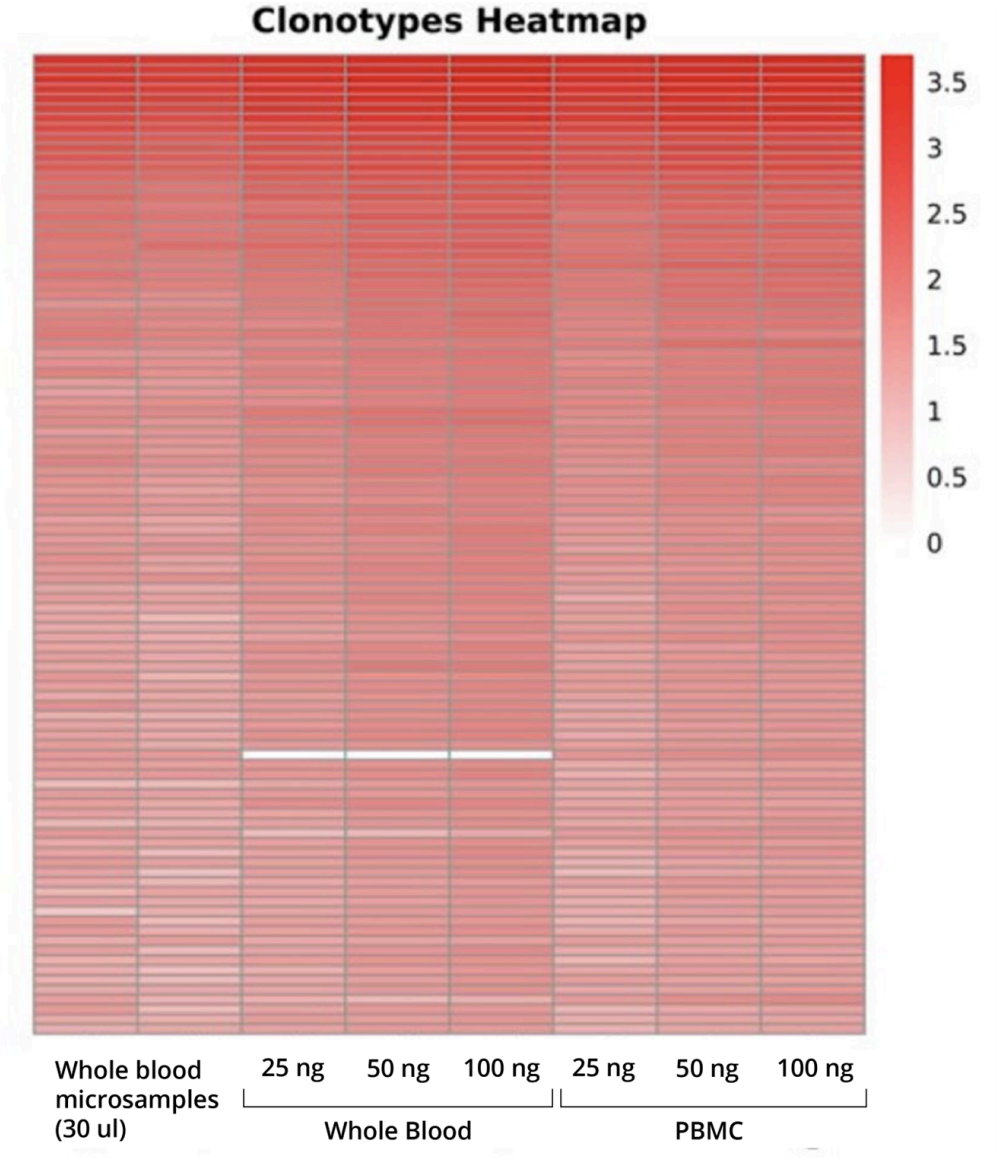

Fig. 16 Similar TRB V gene usage for whole blood microsamples (30ul dried blood), whole blood, and PBMC samples (range of 25ng -100ng of total RNA). Median log10 UMI counts of 3 replicates.

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3.4. Profiling Whole Blood Microsamples

We have developed a protocol that can reliably profile 30 µl of dried whole blood (approximately 50,000 – 100,000 lymphocyte cells) collected by micro-sampling technologies such as Neoteryx tubes (Fig. 17). The micro-sampling technology allows home-based sampling of precise volumes (30 µl) from fingertip blood. The dried blood sample is then mailed to labs at room temperature, where the AIR assay can be used for TCR-BCR analysis. This technique is extensively used in clinical research to monitor metabolites, drugs, and other analytes. It can be conveniently used for AIR RNA repertoire analysis across multiple time points, such as monitoring response to drug treatment in patients with cancer with a simple home-based test [19].

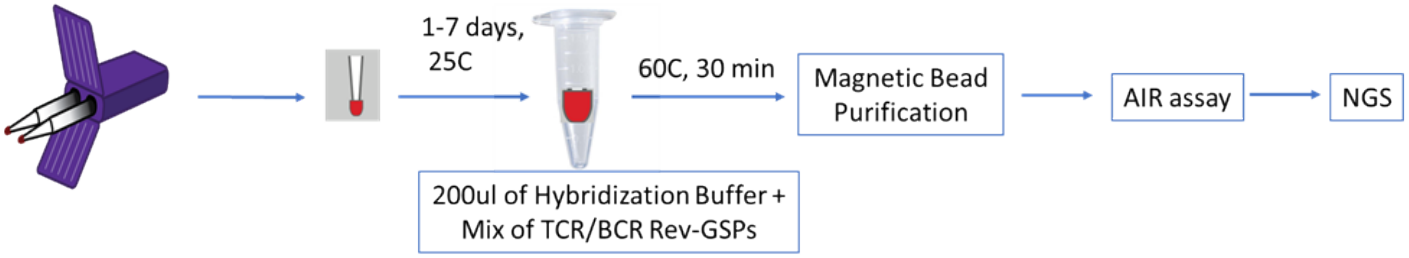

Fig. 17. Processing outline of dried blood samples using Neoterix Microsampling technology.

The AIR RNA Microsample workflow can reliably profile up to 200 of the most abundant clonotypes (with at least 20 UMI counts) and some medium-abundant clonotypes (e.g., 500 with UMI 10 or more). To generate reproducible results, we recommend running the blood microsamples in triplicate (Fig. 16).

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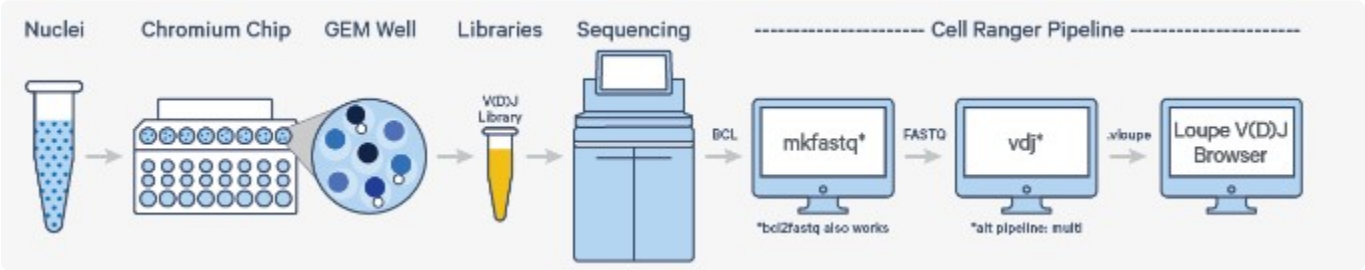
4. Single-Cell Immune Profiling Service

In addition to bulk AIR profiling (kit and services), Cellecta offers single-cell T and B immune profiling custom service using the 10x Genomics Chromium V(D)J assay. The Chromium Single Cell 5' Library Construction Kit and V(D)J Enrichment Kit offer comprehensive, scalable solutions for measuring gene expression and immune repertoire information from the same cell. This protocol can generate an enriched T-cell library, an enriched B-cell library, and a 5' Gene Expression library from amplified cDNA from the same cells.

This service can profile full-length (5' UTR to constant region), paired T-cell receptor (TCR), or B-cell immunoglobulin (Ig) transcripts from 100-10,000 individual cells per sample. A pool of ~750,000 barcodes is sampled separately to index each cell's transcriptome. It does so by partitioning thousands of cells into nanoliter-scale Gel Beads-in-emulsion (GEMs), where all generated cDNAs share a common 10x Barcode. Libraries are generated and sequenced, and 10x Barcodes associate individual reads back to the individual partitions. Detailed information about experimental design, sample preparation, and downstream data analysis may be found at: www.10xgenomics.com/support/single-cell-immune-profiling.

Single-cell V(D)J immune profiling and data analysis services are available for cryopreserved (in DMSO) lymphocyte cell samples (e.g., PBMC or sorted immune cells). For single-cell AIR experiments, it is desirable to use samples in which lymphocytes are enriched by centrifugation (PBMC), magnetic beads, or FACS sorting. It is recommended to use low-pressure sorting (if possible, or a nozzle > 100 um). It is also highly recommended for all immune cell samples undergo viability testing using live-dead staining to confirm that at least 70% of cells are alive. Upon receiving the cells, they will be processed using the Chromium controller, and 5'-RNA expression profiling libraries will be constructed using the RNA V(D)J protocol. The samples will be sequenced and analyzed using Cell Ranger V(D)J and Loupe V(D)J Browser tools.



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5. T/B Immune Marker Panel

Immunophenotyping of CDR3 clonotypes with T/B Immune Marker Assay

In addition to AIR RNA profiling, sorted immune cell subfractions can be phenotypically characterized by an additional targeted multiplex RT-PCR DriverMap T/B Immune Marker assay. To develop the T/B Immune Marker assay, we combined a list of more than 3,000 T and B-specific cell subtyping and activation markers from more than 100 different public databases, commercial assays, and scientific publications. We also designed several sets of redundant primers and validated the performance of these primers against a panel of 40 different immune cell types and activated PBMC and whole blood samples. As a result of these validation studies, we arrived at approximately 500 highly expressed T/B subtyping and activation marker genes that confirm the quality of cell sorting and could phenotypically characterize different immune cell subtypes.

In addition to this, we also offer the DriverMap EXP assay, a genome-wide expression profiling assay to measure the expression levels of 19,000 human protein-coding genes. If the research goal is the unbiased discovery of novel immunity biomarkers, we recommend using a genome-wide expression profiling panel.

Combining AIR profiling with the T/B Immune Marker assay in sorted immune cells will link specific CDR3 sequences with the comprehensive phenotype of different immune subfractions (e.g., CD4. CD8, naïve, memory, cytotoxic, effector, etc.) as illustrated in Fig. 18. When performing a combined AIR assay and T/B Immune Marker assay, we recommend splitting each sorted cell fraction in a 5:1 ratio (e.g., 25,000 and 5,000 cells) and mixing amplified samples at a 1:1 ratio at the NGS step to account for the differences in the complexity of CDR3 sequences and genes in the T/B Immune Marker Panel.

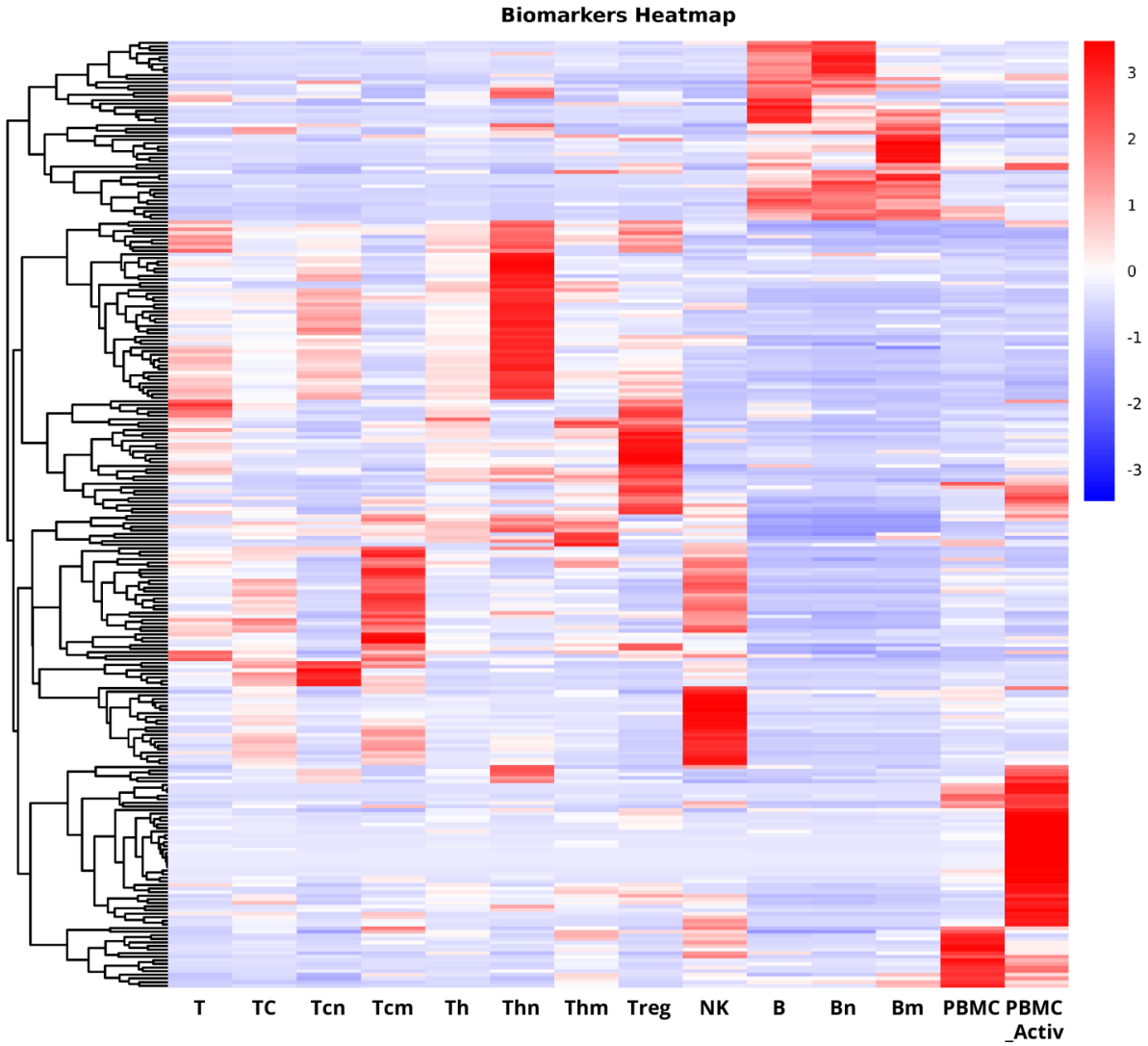

Fig. 18. Characterization of different T and B cell types (T, CD8, CD8 naive, CD8 memory, CD4, CD4 naive, CD4 memory, Treg, NK, B, B naive, B memory) with DriverMap T/B Immune Marker Panel

Last modified: 22 February 2023

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