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Universal synthetic TCR/BCR spike-in controls to evaluate immune receptor profiling assay and next-generation sequencing performance

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Abstract

Adaptive immune receptor (AIR) repertoire diversity assays are susceptible to biases arising from variations in conditions in the RT-PCR and next-generation sequencing (NGS) steps. We designed synthetic TCR and BCR spike-in controls to mitigate these biases and to serve as universal standards for any PCR-based immune receptor profiling assay. We synthesized 48 BCR constructs representing different IGH, IGK, and IGL genes and 39 TCR constructs for TRB, TRA, TRG, and TRD genes. The spike-in controls were tested as (16x3) BCR constructs and (13x3) TCR Triplex isoform pools added to multiple samples in the same batch to detect cross-contamination across samples. We successfully discriminated between controls and background sequences by combining a unique molecular identifier (UMI)-based correction strategy with spike-in controls at the data analysis step. We also tested 48 BCR and 39 TCR Premixed Controls by spiking into peripheral blood mononuclear cells (PBMC) RNA samples before reverse transcription using Cellecta's DriverMap™ Adaptive Immune Receptor Profiling Assay that uses a multiplex RT-PCR approach with gene-specific primers and UMIs. We successfully used it to evaluate assay performance by adding premixed controls at different concentrations. Results showed a linear trend as the number of spike-in molecules increased. Our analysis revealed an average sequencing error rate of 0.4%-0.8% per base, aligning with the reported error rate range of Illumina sequencing. This suggests the reliability of our spike-in controls, which can be used to rectify biases in the AIR protocol and accurately estimate error and mutation rates for the DriverMap™ AIR assay or any other sequencing-based immune receptor profiling assay. This innovative approach enhances the robustness of immune receptor profiling technology, facilitating more accurate assessments of repertoire diversity.

Introduction

- Synthetic TCR/BCR controls work as a calibration standard to measure and compensate for biases caused by the amplification and sequencing of immune receptors.
- Cellecta developed BCR and TCR controls with unique CDR3 regions that mimic the 29 most abundant immune receptor RNA isoforms.
- TCR/BCR RNA calibration standards spiked at different concentrations to experimental RNA samples allowed us to measure sensitivity and linearity in the quantitation of target immune receptor mRNAs.
- Synthetic TCR/BCR control RNAs with nearly full-length V(D)JC structures are designed to be compatible with any RT-PCR-based receptor profiling assays.

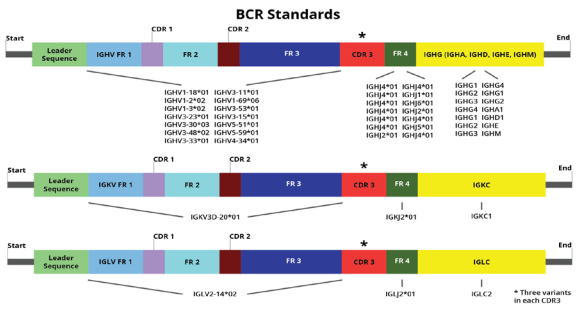


Fig 1: 48 BCR mRNA spike-in constructs represent 10 different IGHs; 1 for each IGHA, IGHD, IGHE, IGHM, IGK and IGL genes.

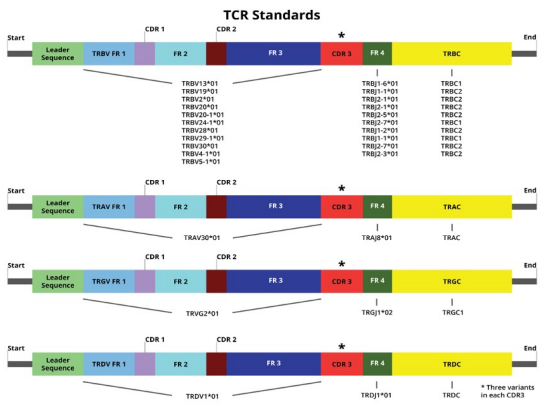
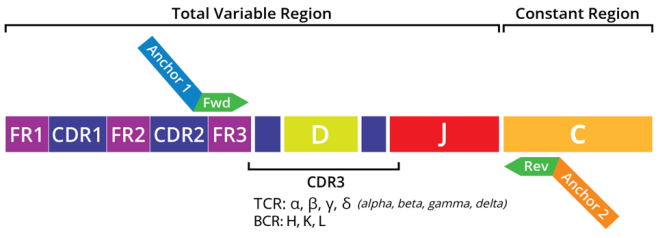


Fig 2: 39 TCR spike-in constructs represent 10 different TRBs, 1 for each of TRA, TRG and TRD genes.

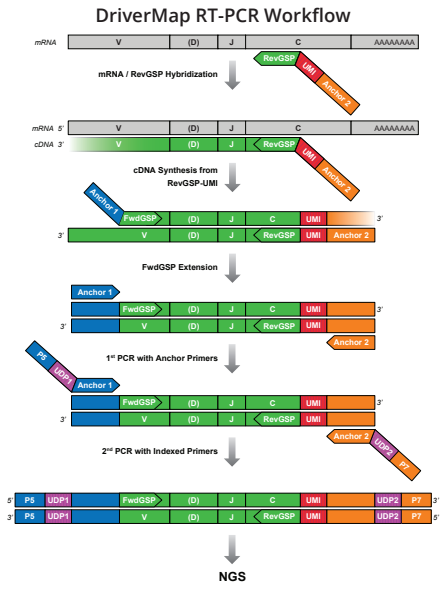
*Each construct has 3 variations in the CDR3 region that differ by 3 nucleotides in a fixed position

DriverMap™ Adaptive Immune Receptor (AIR) Profiling Assays

AIR-RNA Assay Design



DriverMap RT-PCR Workflow

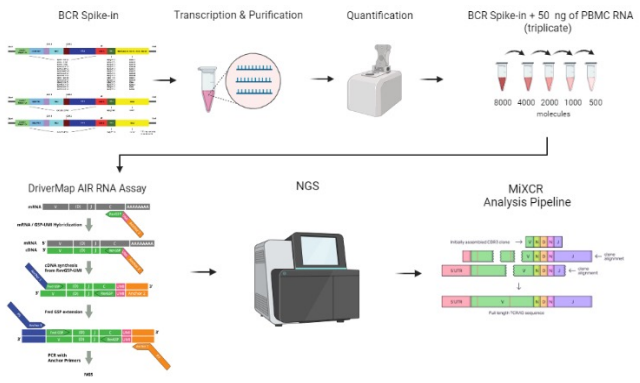


Method

Synthetic TCR/BCR mRNA Control Constructs

- Linear TCR and BCR plasmids were transcribed to make total RNA for each construct.
- A mixture of 48 BCR constructs and 39 TCR constructs was spiked at different concentrations to 50 ng PBMC RNA at a 1:1 ratio.
- DriverMap™ AIR-RNA TCR/BCR assay was run for immune receptor profiling of all TCR (TRA, TRB, TRD, and TRG) and BCR (IGH, IGK, and IGL) chains.
- Pooled library was sequenced on Illumina NextSeq 500, 300-n paired-end kit for 10M reads per sample.
- Data was analyzed using the MiXCR 3.0 software.

Workflow for RNA Spike-in



Data Analysis Pipeline

Demultiplex samples
bcl2fastq v2.20.0.422 is used for demultiplexing

NGS Data QC
FastQC v0.11.9 is used for Data QC

Clonotype extraction
MiXCR 3.0 and later is used for UMI extraction, indels, mismatches correction, alignment, clonotypes assembly with additional PCR and sequencing errors correction, final clonotypes extraction.

TCR/BCR spike-in Calibration correlation analysis
Customized Python scripts and R scripts were used to analyze TCR/BCR spike in the Calibration dataset.

Results: TCR spike-in controls

Evaluate Assay Performance

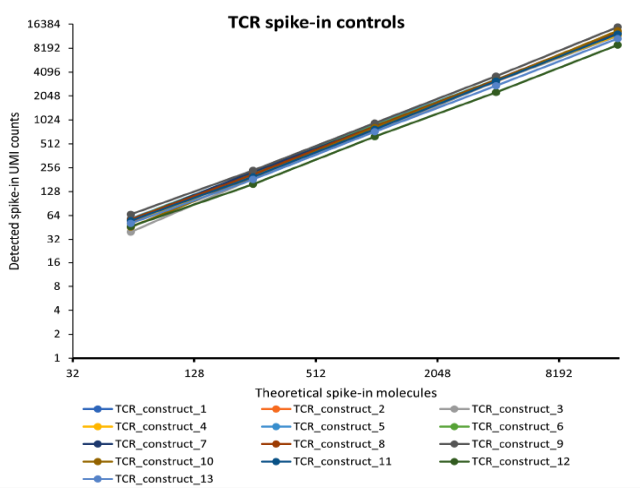


Fig 3: 39 TCR spike-in control constructs detection at different number of molecules. Figure illustrates the results of linearity, and dynamic range of AIR-RNA assay in PBMC control RNA sample spiked in with Control Mix at different input amounts.

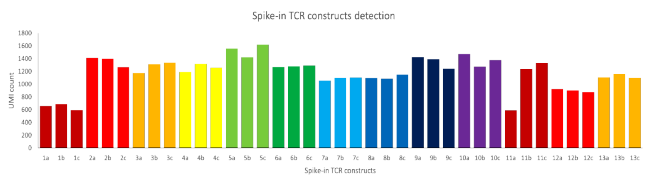


Fig 4: UMI count of 39 TCR control constructs spike-in at 8000 mole-cules in 50 ng of PBMC RNA. Efficiency of detection of control molecules is in 30-80% range which aligns with the efficiency of multiplex RT-PCR technology.

Measure Cross-Contamination

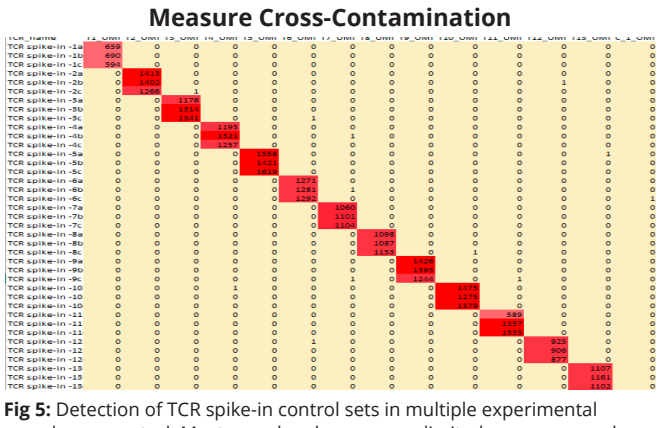


Fig 5: Detection of TCR spike-in control sets in multiple experimental samples vs. control. Most samples show zero or limited cross-over and only detect the respective spiked-in control set.

Results: BCR spike-in controls

Evaluate Batch Effects and Reproducibility

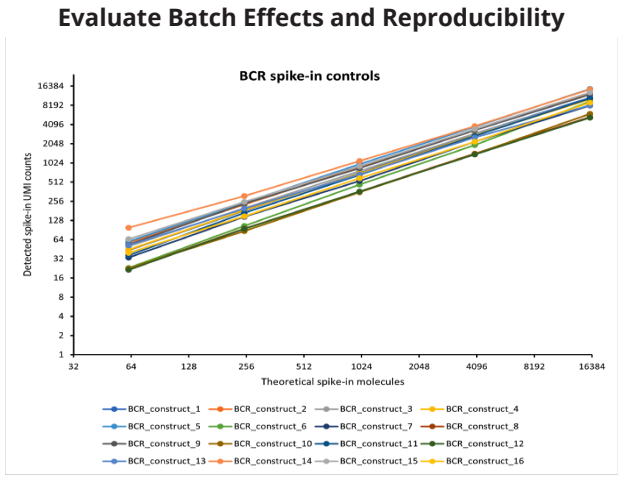


Fig 6: 48 BCR spike-in control constructs at different number of molecules. Results show a linear trend as the number of spike-in molecules increase.

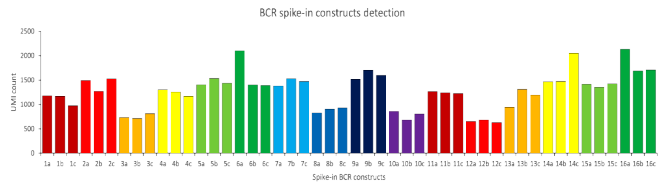


Fig 7: UMI count of 48 BCR control constructs spike-in at 8000 molecules in 50 ng of PBMC RNA. Efficiency of detection of control molecules is in 30-80% range which aligns with the efficiency of multiplex RT-PCR technology.

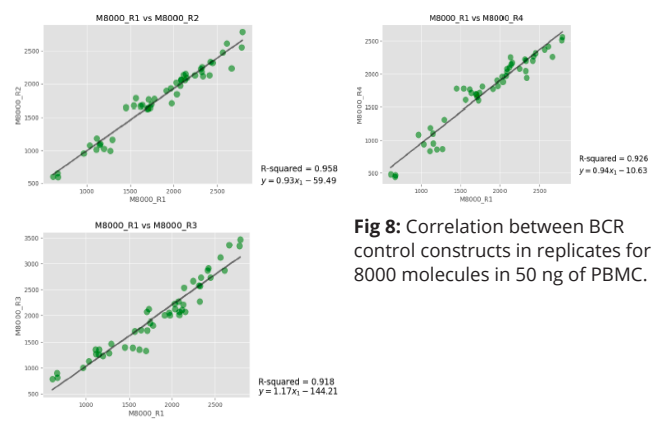


Fig 8: Correlation between BCR control constructs in replicates for 8000 molecules in 50 ng of PBMC.

Measure Cross-Contamination

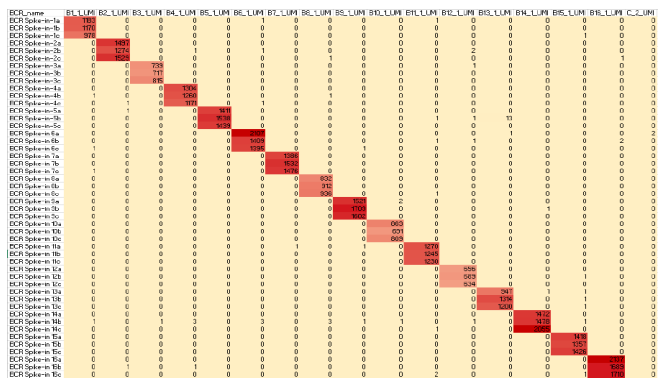


Fig 9: Detection of BCR spike-in control sets in multiple experimental samples vs. control. Most samples show zero or limited cross-over and only detect the respective spiked-in control set.

Discussion

- Cellecta developed a panel of synthetic, near full-length TCR/BCR RNA spike-in control constructs for universal application in immune receptor profiling assays.
- TCR/BCR spike-in can be used for measuring the sensitivity and accuracy (by mutation rate analysis) of multiplex RT-PCR AIR-RNA profiling data using UMI-powered MiXCR analysis in universal PCR-based immune receptor profiling assay.
- Spike-in controls can be used to detect contamination between different samples.
- Spike-in controls provides a basis to measure primer performance in PCR-based AIR assays.
- The design of the control constructs enables them to be used with any standard PCR-based immune receptor profiling assay.

Kit Format

AIR Spike-in controls are available in two formats:

1) Control sets: Set of 16 BCR or 13 TCR individual V(D)JC-specific isoforms provided in separate test tubes, where each isoform has a unique combination of V(D)JC genes, and each isoform is represented by three different CDR3 constructs different from each other by three mutations in CDR3 region, mixed at 1:1:1 ratio.

2) Control mix: Mix of 48 constructs for 16 different BCR isoforms or 39 TCR constructs for 13 TCR isoforms. Each isoform is represented by three constructs with different CDR3 sequences and is mixed at 16:4:1 ratio.

Download Demo Results

Access on GitHub

Access on NCBI: BioProject ID PRJNA975292