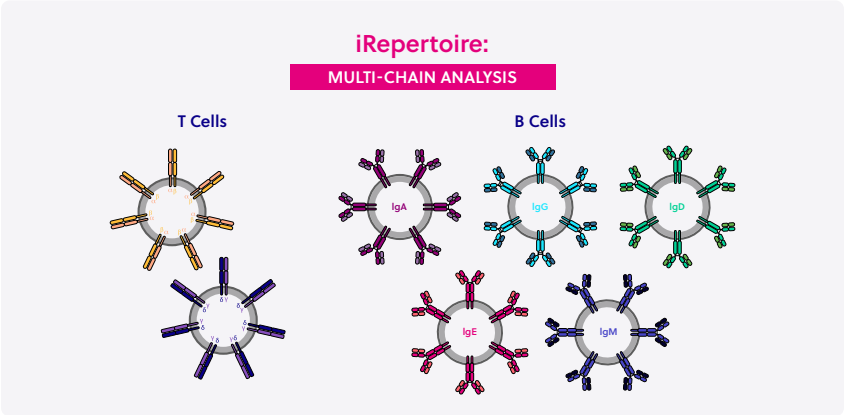
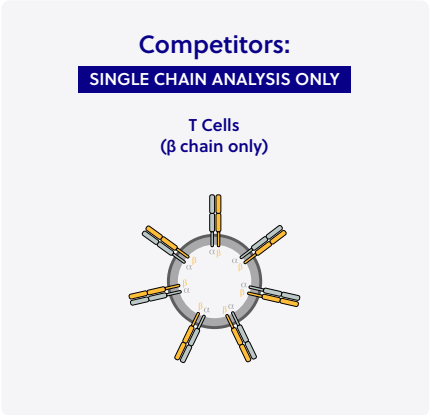
Achieving Quantitative and Inclusive Immune Repertoire Analysis

Capturing the **full clonal diversity of the immune repertoire** can reveal important information about the pathology and prognosis of disease. Compared to normal individuals, the immune repertoire of patients with different diseases may be quantitatively or qualitatively different from healthy controls in terms of composition and diversity.

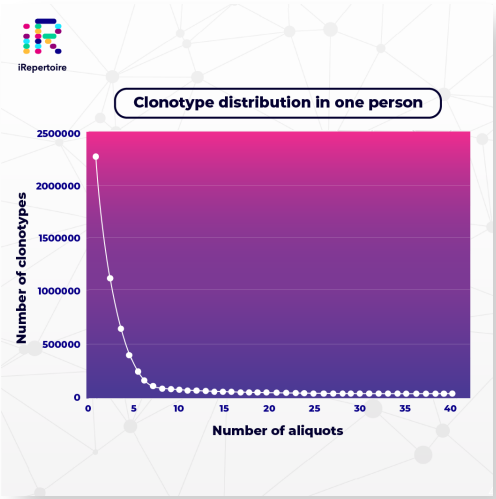
Qualitative changes may present as increased sharing of disease-specific CDR3s in T or B cells. Quantitative changes may manifest as increases and decreases in repertoire diversity. Capturing both qualitative and quantitative changes requires a **repertoire sequencing approach** that is both sensitive (so as to include all CDR3s) and unbiased (to allow for relative quantitation of all CDR3s).





CHALLENGES OF IMMUNE REPERTOIRE SEQUENCING

While there are hundreds of thousands of different clonotypes in one person's immune system, only a small number of clonotypes make up the bulk of the immune repertoire. Sampling the CDR3 clonotypes in a single person would thus produce a distribution like the one shown in the graph below.

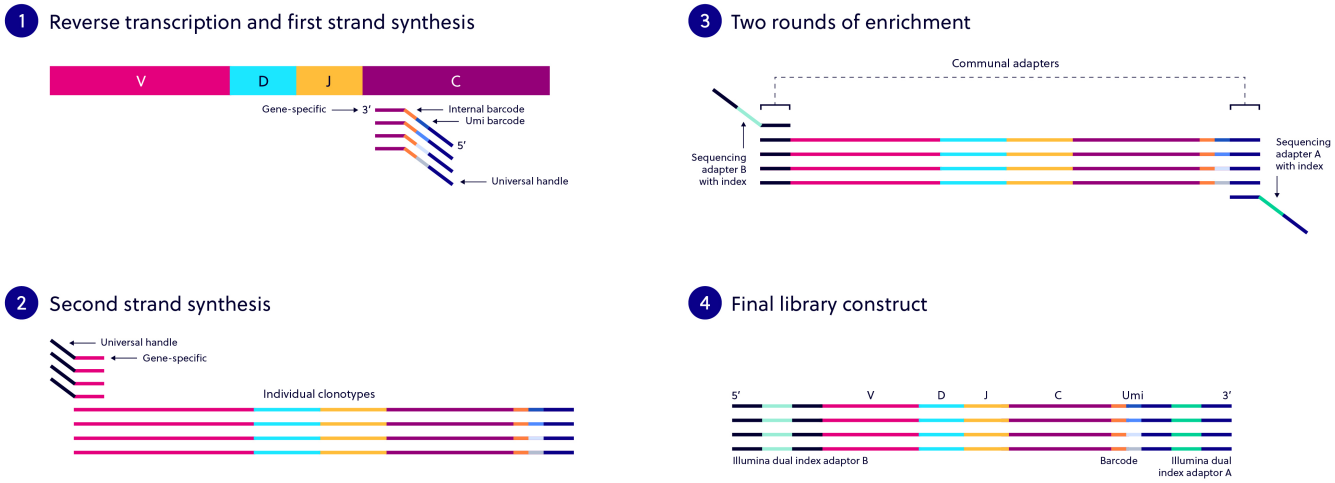


This long-tailed distribution results in a low signal-to-noise ratio, as the more frequent CDR3s will be overrepresented in a sequencing library. To effectively capture the full breadth and depth of the immune repertoire, the sequencing methods must, therefore, be inclusive and quantitative.

iRepertoire's multiplex PCR amplification technologies were specially designed to amplify the B cell receptor (BCR) and T cell receptor (TCR) chains of the immune system with both inclusivity and quantitation in mind.

iRepertoire offers two different multiplex PCR approaches, both of which amplify all of the V(D)Js in human or mouse samples, including the highly variable CDR3 region. With dam-PCR (dimer avoided multiplex PCR, patent pending), you can amplify any or all of the BCR and TCR chains in a much more quantitative manner than competing approaches. With arm-PCR (amplicon rescued multiplex PCR, patent 7,999,092) you can amplify the chain of your choosing with superior sensitivity so as to include all the diversity present in a sample, even for extremely rare clonotypes.

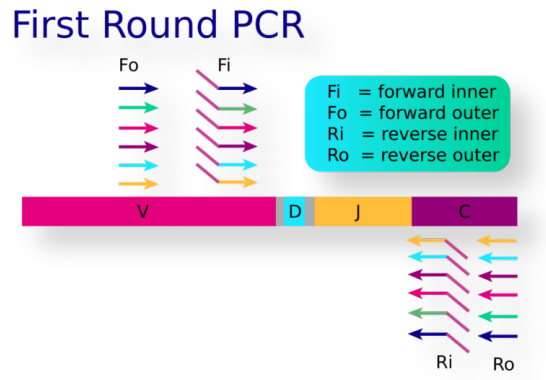
DIMER AVOIDED MULTIPLEX PCR (DAM-PCR)



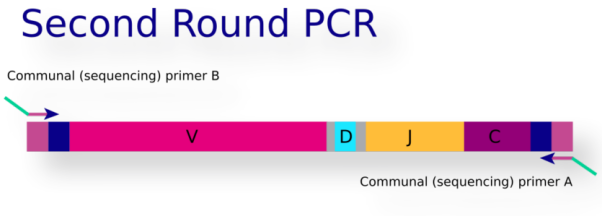
iRepertoire's dam-PCR technology lets you select any combination of TCR and BCR chains for simultaneous amplification in one reaction. This is made possible by the unique single-cycle binding and extension steps and stringent clean up steps in-between, which omit harmful dimer formation. First, only the 3' primer is added and one binding and extension step is performed. The unincorporated 3' primers are washed away, and then the 5' primers are added. After another single cycle binding and extension protocol, the 5' primers are washed away. Primers that bind to the communal primer sites introduced in the first two steps are added for multi-cycle, exponential amplification.

Dam-PCR also allows for the inclusion of unique molecular indices (UMI) so that each strand of RNA can be tagged for direct quantification and both PCR and/or sequencing error removal. This increases confidence in the sequenced targets, and the quantification allows for investigation of interchain ratios in the immune adaptome. Thus, while arm-PCR provides an inclusive, semi-quantitative overview of the immune repertoire with respect to particular chains, dam-PCR provides a more quantitative look at the frequency of particular clonotypes of interest for any or all BCR and TCR chains.

AMPLICON RESCUED MULTIPLEX PCR (ARM-PCR)



In the first round of arm-PCR, inside and outside TCR/BCR-specific primers amplify the targets of interest, increasing sensitivity and appending communal primer binding sites.



In the second round of arm-PCR, communal primers exponentially amplify the target amplicons generated in the first round

Arm-PCR uses a high concentration of **hundreds of nested inside and outside gene-specific primers** in the initial combined reverse transcription (RT) plus PCR round one. Both the reverse outside and inside primers can contribute to first strand synthesis during RT, which is especially important if there are any secondary RNA structures that make the inner primer binding site inaccessible during RT.

The outside primers help to improve the sensitivity of the reaction by increasing target template abundance for the inside primer to bind. Because the nested primer mix goes through many binding and extension cycles, **arm-PCR is a great technological solution for rare clonotype discovery**. When RT-PCR1 is complete, target amplicons are rescued, and PCR round two is performed using fresh enzymes. For PCR2, communal primers that recognize the shared tag sequence introduced during the first round of amplification are used for further amplification.

DECIDING WHAT TO SEQUENCE

Depending on the goals of the study, an experimenter wishing to sequence an immune repertoire will need to determine what region(s) of the T cell and/or B cell receptor (TCR and BCR) genes to target, whether to use DNA or RNA as starting material, and whether to perform bulk repertoire analysis or single-cell V(D)J pairing and immunophenotyping.

The regions of TCRs and BCRs that play the most important role in antigen recognition are also the most variable. These variable regions are constructed from a random shuffling of gene segments, and, in the case of BCRs, somatic mutations. The most variable portion of the variable region is the CDR3 region. It is, therefore, critically important to target the CDR3 region in immune repertoire sequencing. All of **iRepertoire's primer systems** cover the highly variable CDR3 region. Sequencing the full variable region can provide more information, but that extra information comes at the cost of increased sequencing reads or decreased sequencing coverage. While both RNA and DNA sequencing can provide a picture of the immune repertoire, only RNA sequencing will reflect the library of TCR and BCR sequences that are actually expressed.

Finally, bulk sequencing is required to analyze immune repertoire diversity, but information about the cognate pairing of BCR/TCR chains within a given B or T cell is lost in bulk sequencing. Researchers that wish to examine the relationships between paired chains, in particular clonotypes, should perform single cell sequencing. iRepertoire's single cell sequencing service iPair uses arm-PCR for high-resolution immune repertoire insights at the single cell level.

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iRepertoire, Inc.
www.irepertoire.com
(256) 327-0948
Info@irepertoire.com