CASE STUDY

iRepertoire,Inc.

Mitigating AIRRseq Challenges in FFPE Tissue Samples with the RepSeq+ Immune Repertoire Intelligence Platform

KEY TAKEAWAYS:

- FFPE RNA samples offer unique challenges—nucleic acid fragmentation, cross-linking, and induced artifacts—that can introduce errors into PCR and sequencing data.
- Specialized FFPE sample preparation, multiplex amplification, and sequencing protocols are required to generate reliable and reproducible TCRseq and BCRseq data.
- iRepertoire's optimized FFPE RNA extraction methods and proprietary multiplex, seven-chain RepSeq+ library preparation mitigate the challenges of working with FFPE to produce highly reproducible and reliable AIRRseq data from the tumor microenvironment.

INTRODUCTION

Formalin-fixation paraffin-embedding (FFPE) is the gold standard for tissue sample preservation, enabling long-term storage at room temperature while maintaining tissue morphology and compatibility with analytical techniques. This makes FFPE samples valuable for adaptive immune receptor repertoire sequencing (AIRRseq) applications, including characterizing tumor-infiltrating lymphocytes, monitoring immune evasion mechanisms, discovering prognostic and predictive immunotherapy biomarkers, and conducting retrospective studies using biobank specimens.

However, FFPE processing presents technical challenges for AIRRseq due to cross-linking and chemical modifications that compromise nucleic acid integrity, combined with progressive DNA and RNA degradation during storage. Despite these limitations, recent methodological advances have enabled increasingly reliable TCR and BCR sequencing from archived clinical specimens, expanding the utility of FFPE samples for immune repertoire analysis.

CHALLENGES IN WORKING WITH FFPE SAMPLES FOR AIRRseq

Challenge 1: FFPE Processing Degrades RNA and Introduces Chemical Alterations

Formalin fixation causes extensive cross-linking[1] and fragmentation of nucleic acid molecules, with these effects being particularly pronounced when acidic, non-buffered formalin is used.[2] The cross-linking phenomenon creates significant difficulties during nucleic acid extraction and subsequent amplification steps, as the covalent bonds formed between nucleic acids and proteins must be reversed to obtain amplifiable templates.[2]

This issue proves especially problematic for AIRRseq analysis, where successful TCR and BCR sequencing depends on capturing relatively intact V(D)J sequences to accurately assess clonotype diversity and immune repertoire composition. The degradation limits PCR amplification efficiency and reduces read lengths, ultimately producing incomplete or biased repertoire profiles that may not reflect true immune sequence diversity.

FFPE processing also induces chemical alterations including cytosine deamination events that introduce sequencing artifacts and compromise sequence fidelity.[3] These chemical modifications can complicate downstream analyses such as allele calling and complementarity-determining region 3 (CDR3) sequence analysis, both of which are fundamental requirements for accurate clonotype identification and immune repertoire characterization.

Challenge 2: Low Nucleic Acid Yields from FFPE Extraction

The extraction process from FFPE specimens often yields low amounts of usable nucleic acids.[4] This limitation poses particular challenges for TCRseq and BCRseq applications, where immune receptor transcripts represent low-abundance targets within the complex background of total RNA from tissue. The reduced input material availability can lead to underrepresentation of T and B cell clones within the final sequencing library, with low-frequency clonotypes at risk of being missed entirely.

Challenge 3: Detection Bias Toward Highly Abundant Clones

The combination of RNA degradation and low extraction yields creates a detection bias that favors only the most highly abundant clones, fundamentally skewing both clonality and diversity analyses.[3] Short and damaged RNA templates are more prone to biased amplification during PCR[3], with certain V or J gene segments being preferentially amplified over others based on their relative stability or primer binding efficiency rather than their true biological abundance.

This amplification bias greatly impairs the ability to accurately assess the full immune repertoire and detect clinically relevant subclonal expansions that are particularly important in disease contexts such as cancer immunotherapy response or autoimmune disorders. Furthermore, the high PCR cycle numbers required to successfully amplify degraded templates introduce errors that can be misinterpreted as true BCR/TCR sequence diversity, leading to overestimation of repertoire complexity and potential misidentification of clonotypes.

Despite these technical challenges, pre-analytical and analytical solutions have been developed to enable more reliable AIRRseq results from archived clinical samples.

AN AIRRseq WORKFLOW DESIGNED TO ADDRESS THE CHALLENGES TIED TO FFPE SAMPLES

Successful immune sequencing from FFPE samples requires a comprehensive approach that addresses challenges at multiple stages of the analytical workflow—from optimized sample collection and preservation to specialized extraction methods and refined analytical approaches—that maximize the quality and reliability of TCRseq and BCRseq data from archived specimens.

Optimized Sample Collection and FFPE Preservation Procedures

Sample quality can directly impact sequencing results. By implementing FFPE tissue preparation best practices, nucleic acid integrity is better protected, enabling greater recovery from the sample and improving the accuracy of PCR amplification and TCRseq and BCRseq analysis. Recommended best practices[5] include:

- Fixing tissues within one hour of surgical recovery
- Limiting fixation time to 12 to 24 hours, depending on tissue type and thickness
- Using a neutral buffered formalin
- Completely drying tissues before embedding with paraffin[6]
- Avoid oxidized tissue by excluding external pieces (standard practice is to discard first slice) and sectioning tissues just before purification
- Storing FFPE samples at 4°C to significantly slow degradation rates; -20°C or -80°C is preferred
- Sectioning tissues to a thickness of 10 to 20 µm, with a surface area of 50 to 300 mm^2 to provide adequate input material for downstream processing
- Removing excess paraffin prior to nucleic acid purification

Specialized Extraction Methods to Mitigate Processing Effects

Extraction methods can either compound or mitigate damage introduced during biospecimen collection, preservation, and storage. At iRepertoire, nucleic acid extraction from FFPE specimens is performed using Covaris acoustic sonication technology, which helps increase the yield of intact RNA by efficiently disrupting

cross-links and paraffin matrices while minimizing additional mechanical shearing.

This approach enables extraction of longer RNA templates, which proves particularly beneficial for immune sequencing applications requiring intact V(D)J sequences for accurate clonotype identification. The semi-automated, high-throughput nature of the Covaris system also enhances reproducibility and reduces operator-dependent variability across sample batches.

RepSeq+: A Comprehensive, Quantitative Seven-Chain Multiplex Assay

RepSeq+ is a sensitive, quantitative, high-throughput assay that combines multiplex PCR amplification with next-generation sequencing to simultaneously analyze any combination of all seven chains of the adaptive immune system (TCRα, TCRβ, TCRγ, TCRδ, BCR-IgH, BCR-IgK, BCR-IgL) within a single reaction.

This integrated approach maximizes the utility of limited FFPE-derived nucleic acid input while providing comprehensive immune repertoire characterization.

Dam-PCR Technology

The core innovation underlying RepSeq+ performance with FFPE samples is iRepertoire's proprietary dimer avoided multiplex PCR (dam-PCR) technology **(Figure 1)**. The dam-PCR method enables simultaneous amplification of any combination of TCR and BCR chains through a controlled series of single-cycle binding and extension steps with stringent cleanup protocols that eliminate primer-dimer formation. The process involves sequential addition of 3′ primers, followed by binding and extension, complete removal of unincorporated primers, then introduction of 5′ primers for a second cycle before final multi-cycle exponential amplification using universal primers. This controlled amplification strategy proves particularly beneficial for FFPE samples by minimizing non-specific amplification products that compete with legitimate immune receptor targets.

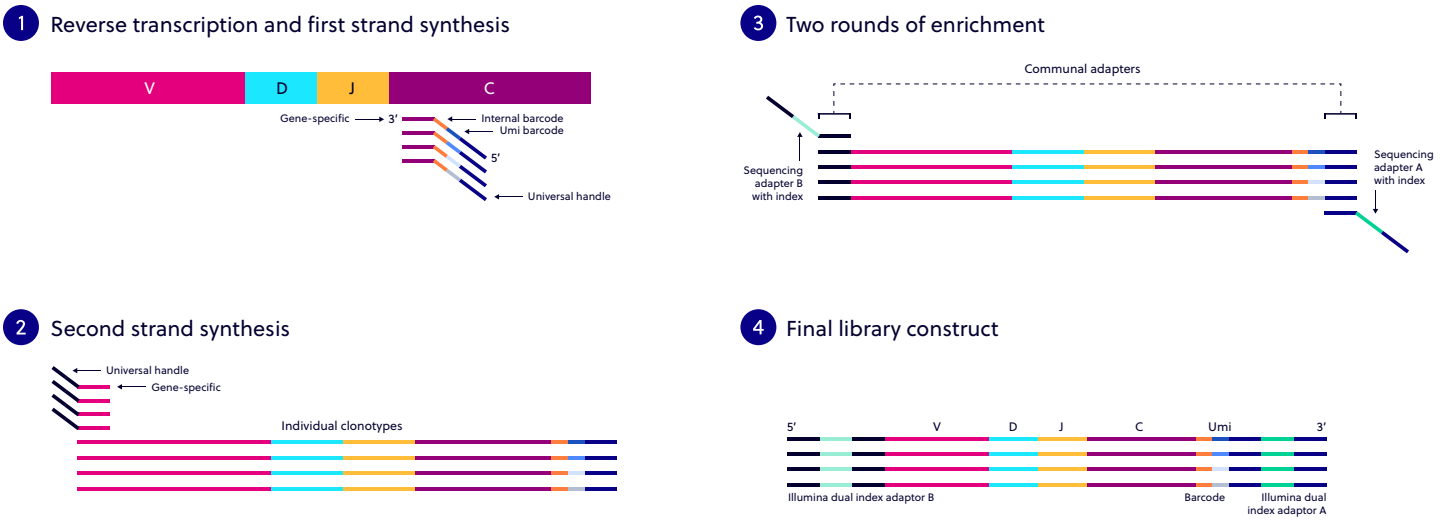


Figure 1. An illustration of iRepertoire's proprietary dam-PCR technology.

Adaptive Primer Strategy for Degraded Samples

RepSeq+ incorporates a flexible primer strategy specifically designed to accommodate varying degrees of RNA degradation in FFPE specimens. The primary approach utilizes short read primers optimized for samples with RNA Integrity Number (RIN) values between 3 and 5, which represents the quality range commonly observed in FFPE materials. The short read primer strategy employs targeted amplification of immunologically informative short regions, particularly focusing on CDR3 sequences that contain the most critical information for clonotype identification. This approach maximizes sequencing success even when longer RNA templates are unavailable due to degradation.

For FFPE samples with superior preservation (RIN >5), long read primers can capture more extensive sequence information, while samples with severe degradation (RIN <3) are typically considered too compromised for reliable analysis.

Specialized Bioinformatics Tools for Degraded Inputs

iRepertoire utilizes bioinformatics tools specifically tailored for processing low-quality, degraded inputs characteristic of FFPE specimens. These computational approaches account for increased error rates, shortened read lengths, and potential sequence artifacts associated with chemically modified and fragmented RNA templates. The bioinformatics pipeline applies enhanced quality filtering algorithms to distinguish genuine immune receptor sequences from degradation-induced artifacts, while specialized error correction algorithms improve the accuracy of sequencing data.

Advanced analytical tools generate data that allows us to not only map FFPE clones but also track the clonal frequencies over treatment. This comprehensive analytical solution enables reliable immune repertoire sequencing from FFPE specimens.

QUANTITATIVE IMMUNE REPERTOIRE PROFILING FROM FFPE

Case Study 1: Reproducible Seven-Chain AIRRseq Analysis in Lung Cancer

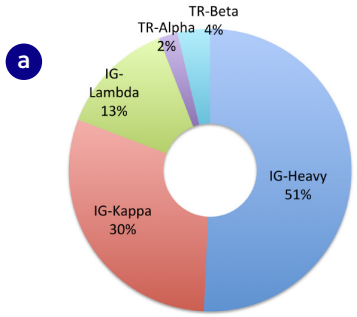
Validation of the RepSeq+ workflow to yield highly reproducible, quantitative seven-chain AIRRseq data from FFPE lung cancer RNA samples was successfully conducted using a specimen obtained from a lung cancer patient that was responding to anti-PD1 therapy.[7]

Data demonstrated high reproducibility of data obtained from the seven-chain multiplex amplification of the immune repertoire of the FFPE-derived RNA sample **(Figure 2)**.

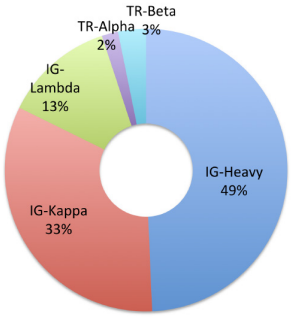
Case Study 2: Predicting and Evaluating Treatment Response in Renal Cancer Using AIRRseq Data

Research was conducted to profile the adaptive immune repertoire of renal cancer patients undergoing hydroxychloroquine and IL-2 treatment.[8] FFPE RNA samples were processed using the RepSeq+ workflow as described above.

Data showed that both TCRα and TCRβ diversity prior to treatment along with the expression ratio between B cells and T cells are good predictors of treatment efficacy, suggesting that the evaluation of the multi-chain immune repertoire composition can be valuable for predicting treatment response and evaluating treatment protocols **(Figure 3)**.



7 Chain Reads Frequency Pie Chart



7 Chain Reads Frequency Pie Chart on repeat amplification

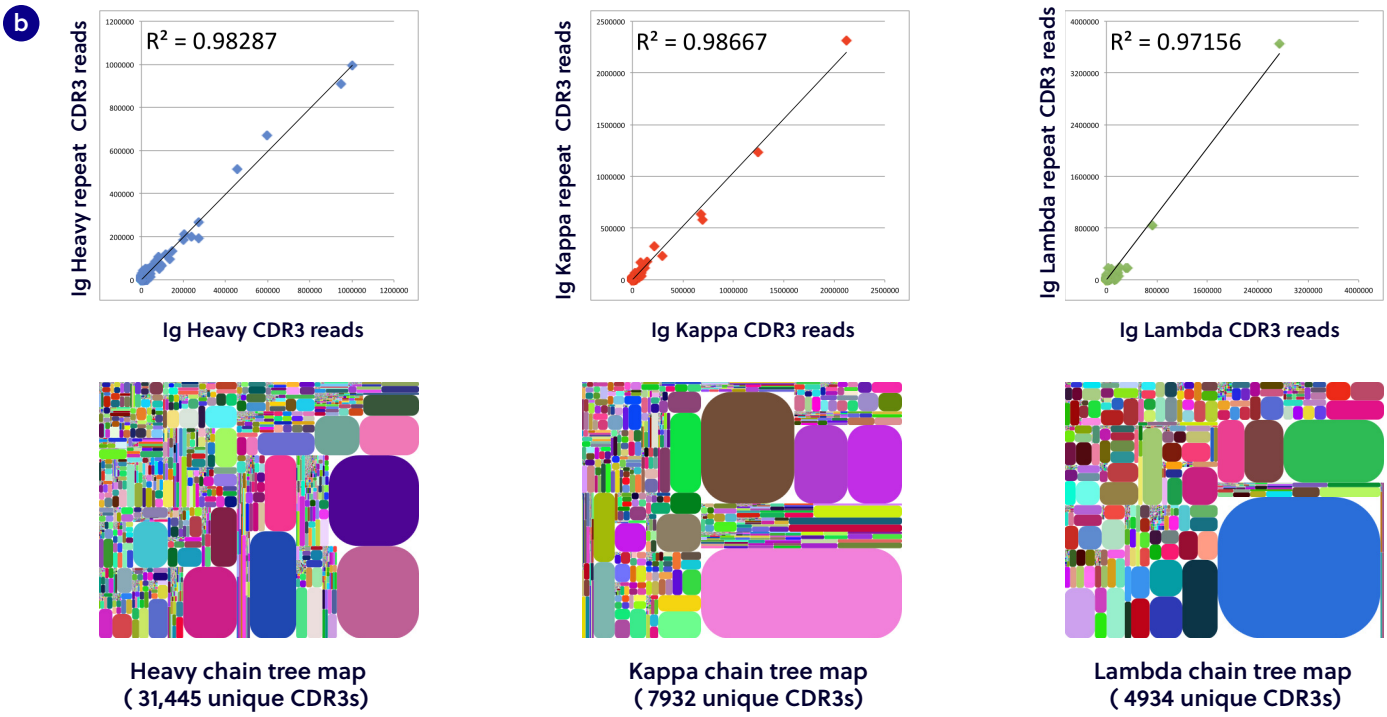


Figure 2. **(a) Reproducible seven-chain repertoire data from a lung cancer FFPE sample is demonstrated across technical replicates. (b) B cell CDR3 profile repeatability for IgH, IgK, and IgL between samples is shown as scatter plots with correlations between 0.97-0.98. Tree maps shown for each BCR chain demonstrate the high diversity of CDR3 regions detected from RNA isolated from FFPE using the RepSeq+ workflow.[7]**

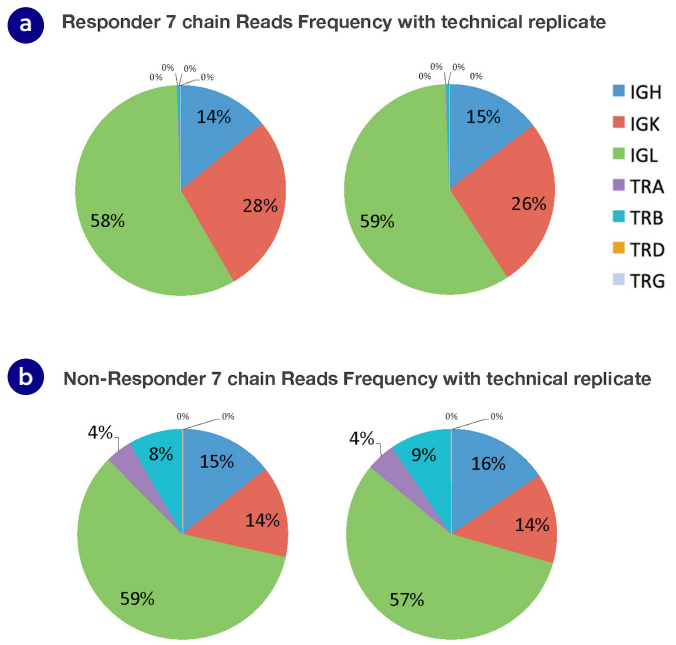


Figure 3. FFPE samples of two renal cancer patients undergoing treatment were profiled. The ratio of the 7 chains for the responding patient (a) and non-responding patient (b) are shown.[8]

CONCLUSION

RepSeq+ enables cost-effective, all-inclusive, and quantitative immune profiling analysis of immune repertoires from challenging FFPE samples, unlocking opportunities to extract repertoire data from preserved biospecimens, even when RNA quality is not optimal. These insights can help advance our understanding of the host immune response to disease, predict treatment response, or drive the development of more effective therapeutics.

Learn more about our RepSeq+ services for FFPE samples or contact us to discuss your project.

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