Targeted T-cell receptor beta immune repertoire sequencing in several FFPE tissue types – applications in profiling the tumor microenvironment.

G. Lowman[1], T. Looney[2], E. Linch[1], L. Miller[1], D. Topacio-Hall[1], J. Zheng[3], F. Hyland[3], M. Andersen[1]

[1]Thermo Fisher Scientific, Life Sciences Solutions, 5781 Van Allen Way, Carlsbad, California, U.S.A. 92008
[2]Thermo Fisher Scientific, Life Sciences Solutions, 2130-2150 Woodward St, Austin, TX U.S.A. 78701
[3]Thermo Fisher Scientific, Life Sciences Solutions, 180 Oyster Point Blvd, South San Francisco, California, U.S.A. 94080

ABSTRACT

T-cell receptor beta (TCRβ) immune repertoire analysis by next-generation sequencing is a valuable tool for studies of the tumor microenvironment and potential immune responses to cancer immunotherapy. Here we describe a TCRβ sequencing assay that leverages the low sample input requirements of AmpliSeq library preparation technology to extend the capability of targeted immune repertoire sequencing to include FFPE samples which can often be degraded and in short supply.

This assay targets the highly diverse CDR3 region which allows for T-cell clone identification and frequency measurement which, when combined, can provide a broad view of the immune landscape within archived tissue samples.

To evaluate assay accuracy, we sequenced libraries including known amounts of 29 well-studied T-cell lymphoma rearrangements, as well as samples comprised of sorted T cells. T-cell repertoires were successfully evaluated from as low as 5 ng to as large as 1µg of input from samples of varying T-cell repertoire diversity, such as sorted T cells, peripheral blood leukocytes, fresh-frozen tissue, and FFPE tissue from a variety of normal and cancerous tissues including lung, colon, brain, spleen, lymph node, and thymus. In addition, we demonstrate use of a qPCR assay for quantification of sample T cell content to guide sample input for TCRB immune repertoire sequence experiments.

These data represent a T-cell immune repertoire sequencing solution for application in a wide range of sample types, in particular, challenging FFPE preserved samples. We find that the assay is capable of profiling repertoire metrics from FFPE samples over a large range of input amounts from several normal and tumor tissue types.

INTRODUCTION

The Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific, Catalog No. Q32852) is used to quantify and evaluate RNA integrity. Due to FFPE quality and variation in T-cell content in different tissue types, standardized inputs lead to inconsistent assay performance. For RNA samples with biologically variable or low T-cell content, or for samples that may be degraded, we developed a functional CD3 RNA qualification assay to determine the minimum acceptable input amount. For DNA samples, we use the TaqMan® RNase P assay to check for sample degradation.

Here we present the Oncomine™ TCR-Beta-SR assay. A high-throughput next-generation sequencing (NGS) assay that interrogates the complementarity determining region 3 (CDR3) of the gene that codes for the T-cell receptor beta chain, and is optimized for convenient but difficult to sequence formalin-fixed paraffin-embedded (FFPE) tissue samples. These assays identify unique T-cell clones through interrogation of the diverse complementarity-determining region 3 (CDR3) of the T-cell receptor (TCR) gene locus in genomic DNA or RNA. The nucleotide sequence of the CDR3 region is unique to each T-cell clone and codes for the part of the TCR beta chain that is involved in antigen recognition.

MATERIALS AND METHODS

The **Ion Oncomine™ TCR Beta-SR Assay** leverages Ion AmpliSeq™ technology to profile the TCR repertoire through the enrichment of the highly diverse CDR3 of the TCR beta gene. By utilizing multiplex primers to target the framework 3 (FR3) region and the joining (J) region that flank the CDR3 this method produces an 80bp amplicon thus enabling the use of both genomic DNA and RNA templates and high-throughput sequencing on Ion 530™, 540™ and 550™ chips. The Oncomine™ TCR Beta-SR Assay is compatible with the new Ion Torrent™ Dual Barcode Kit 1-96 which significantly increases the assay specificity to enable deep TCR sequencing with multiplexed samples.

Figure 1. Assay Design

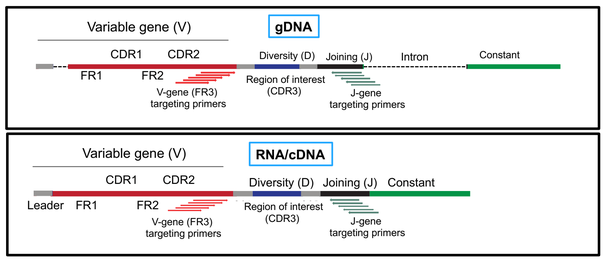


Figure 1. Oncomine™ TCR Beta-SR Assay consists of Multiplex AmpliSeq primers that target the framework region 3 (FR3) and joining (J) regions of the TCRβ locus producing a ~80bp amplicon which covers the CDR3 region. The assay utilizes both RNA and genomic DNA input from blood, tissue (fresh frozen or FFPE), or sorted T cells and has a flexible input range between 10ng – 1µg.

To evaluate the linearity of the Oncomine™ TCR Beta-SR Assay, we sequenced control samples with known TCRβ rearrangement sequences[1] spiked into peripheral blood leukocyte cDNA.

Figure 2. Assay Linearity

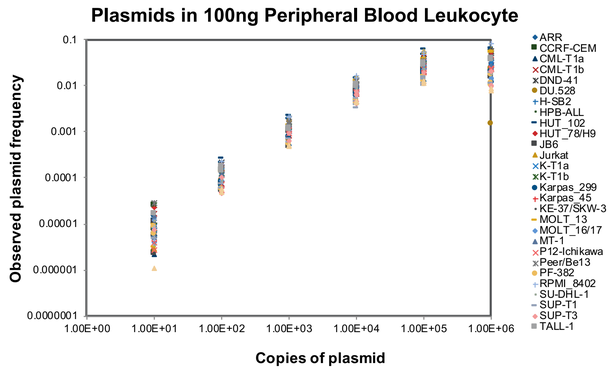


Figure 2. Linearity was established using reference rearrangements. Libraries were prepared using a pool of 29 unique plasmids, cloned with known T-cell lymphoma rearrangement sequences[1], spiked into a background of 100ng peripheral blood leukocyte cDNA at known inputs (10 to 1,000,000 copies per reference plasmid). We observed strong linearity across 6 orders of magnitude and were able to detect the plasmid sequences when spiked-in at a level as low as 10 copies of each plasmid.

RESULTS

While a valuable source for retrospective studies of archival tissues, the modifications that occur during the fixation-process of formalin-fixed-paraffin-embedded (FFPE) tissues pose challenges for next-generation sequencing applications. NGS based TCRβ profiling in FFPE tissue has the additional difficulty of the biological variability of T cell recruitment and tissue infiltration. RNA quality and relative T cell content in a tissue sample significantly affects assay input requirements. To address this need, we developed a qPCR assay that guides the template input for the Oncomine TCR Beta-SR Assay by taking into account sample quality in the context of T cell content.

Figure 3. Development of a functional qPCR assay to guide sample input

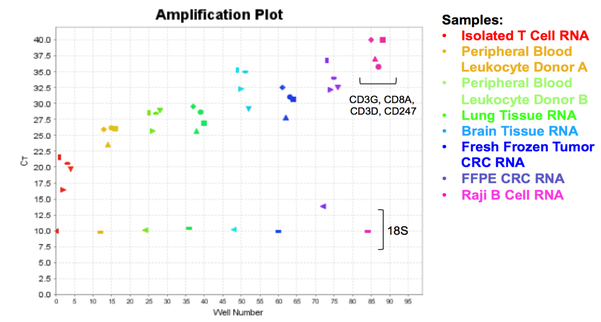


Figure 3. CD247 TaqMan® Gene Expression Assay measures RNA quality as it relates to the relative T cell content in a particular sample. The CD247 TaqMan® probe targets the CD247 gene which expresses the T-cell receptor T3 zeta chain of the T-cell receptor-CD3 complex.

CD247 TaqMan® Gene Expression Assay

(A)

Required materials for cDNA qualification assay		
Standard	T Cell Leukemia (Jurkat) Total RNA	Cat. No. AM7858
Probe	TaqMan™ Gene Expression Assay, CD247 (20X, Hs00167901_m1)	Cat. No. 4331182
Master Mix	TaqMan™ Fast Advanced Master Mix	Cat. No. 4444556

Oncomine TCR Beta-SR (RNA) Library Yields before and after cDNA input adjustment

(B)

Following standard workflow, without input adjustment:

	Sample	Input (ng)	Library Yield (pM)
1	Isolated T-Cell	25	16,590
2	PBL RNA B710019	25	863
3	PBL RNA B707173	25	355
4	Normal Tissue (Lung) RNA	25	147
5	Normal Tissue (Brain) RNA	25	63
6	Fresh Frozen CRC RNA	25	63

Following standard workflow, after input adjustment:

	Sample	Quantity Mean	Input (ng)	Library Yield (pM)
1	Jurkat Control RNA	1	5	300
2	CAR-T 11 Pre-Expansion	21.44	0.23	204
3	PBL RNA B710019	0.8667	5.77	209
4	PBL RNA B707173	0.1332	37.5	296
5	Normal Tissue (Lung) RNA	0.07061	70.8	209
6	Normal Tissue (Brain) RNA	0.04227	118.3	211
7	Fresh Frozen CRC RNA	0.005625	888.9	269

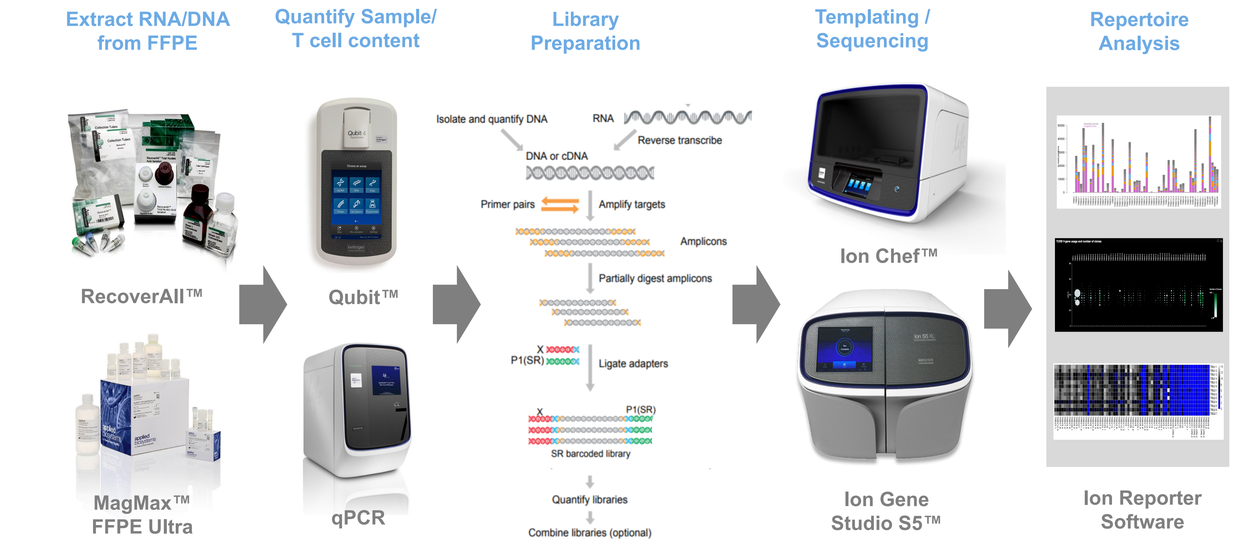
5ng Jurkat typically yields ~300pM library

If desired yield is ~200-300 pM, then recommended input = **5ng**/Quantity Mean

PCR cycle number can be scaled up or down by 3 cycles with every increase or decrease in input amount by a factor of 10

25ng required at 20 cycles
2.5ng required at 23 cycles

Figure 4. (A) Components used in CD247 TaqMan® Gene Expression Assay. (B) When 25ng input is used with samples of varied T-cell content, library yield ranged from 60pM to 16nM, making it difficult to assess assay performance. After calculating recommended input using the Quantity Mean and Jurkat correction factor, the samples with varied T-cell content were prepared with different input amounts to obtain similar library yields.



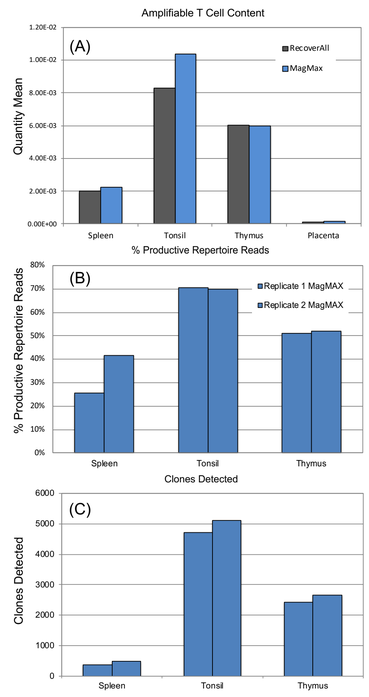


Figure 5. Total RNA was extracted from various FFPE tissue samples with high (tonsil), medium (thymus), and low (spleen and placenta) levels of T cell content. Two purification methods were compared in this experiment: RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE and MagMax™ FFPE DNA/RNA Ultra Kit. (A) Extraction kits were compared by measuring amplifiable T cell content using CD247 TaqMan® gene expression Assay. We observe similar RNA yields [not shown] and amplifiable T cell content between extraction kits. (B-C) Productive read percentage and clone richness are highly correlated in replicate experiments using FFPE RNA from spleen, tonsil, and thymus tissue. The productive read percentage and number of clones detected also follow the same trend predicted from the CD247 TaqMan® gene expression assay.

Correlation of Clones from 25ng of FFPE-Preserved Tonsil RNA

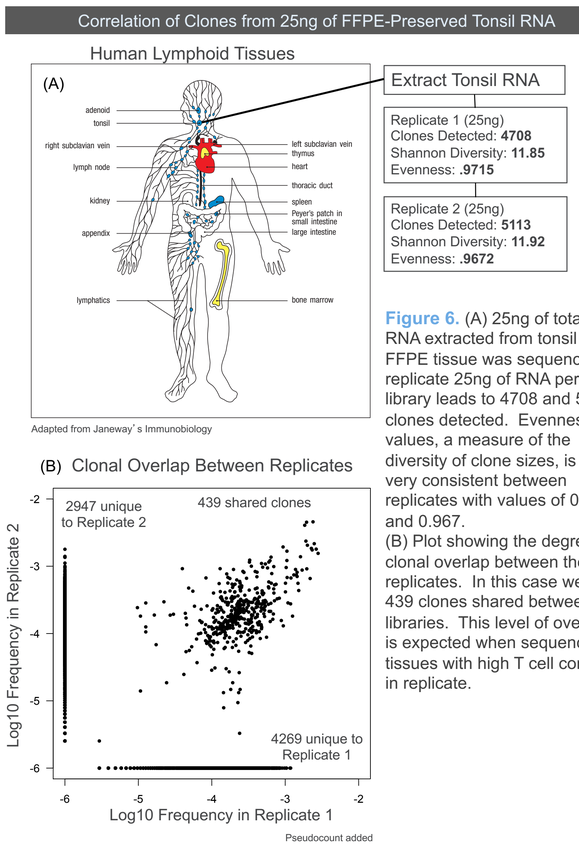
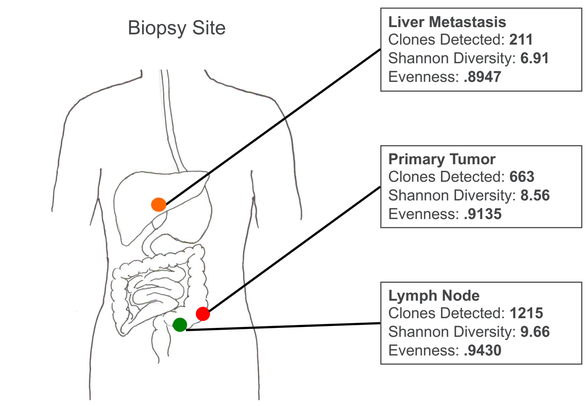


Figure 6. (A) 25ng of total RNA extracted from tonsil FFPE tissue was sequenced in replicate 25ng of RNA per library leads to 4708 and 5513 clones detected. Evenness values, a measure of the diversity of clone sizes, is also very consistent between replicates with values of 0.972 and 0.967.
(B) Plot showing the degree of clonal overlap between the replicates. In this case we see 439 clones shared between libraries. This level of overlap is expected when sequencing tissues with high T cell content in replicate.

Clonal Overlap in an Individual with Colorectal Cancer (FFPE-DNA Input)
Data Courtesy Josè Luis Costa, IPATIMUP, Porto, Portugal



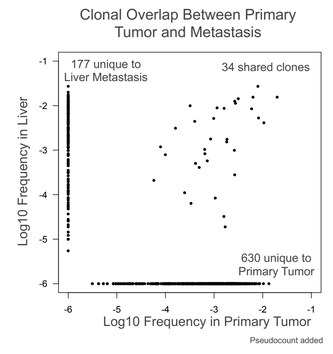


Figure 7. (A) 100ng of genomic DNA extracted from primary CRC FFPE tissue, liver metastasis FFPE tissue, and lymph node adjacent to the primary tumor was sequenced. We observed 1215 clones in the lymph node tissue, 663 clones in the primary tumor, and 211 clones in the liver metastasis tissue. We observe greater evenness in the lymph node tissue than either the primary tumor or the metastasis. (B) Plot showing the degree of clonal overlap between the replicates. We observe 34 clones that are detected in both the primary tumor and liver metastasis (often at elevated frequency). These shared clones may be at an increased probability to have arisen from a tumor neoantigen, and further study, given their detection within both primary and met tissue.

CONCLUSIONS

To summarize, the Oncomine Immune Repertoire-SR Assay profiles the T cell repertoire by enriching the CDR3 region of the TCR beta gene in both RNA and gDNA templates. An assay optimized for difficult FFPE tissue samples of variable T cell content, the Oncomine TCR Beta-SR Assay is an ideal choice for applications that profile the tumor microenvironment. We have also developed the TaqMan® CD247 qPCR assay to help guide assay input for particular sample types and research objectives.

REFERENCES

1. Y. Sandberg et al. Leukemia 21, 230-237 (2007)

ACKNOWLEDGEMENTS

The authors would like to acknowledge the work of all who participated in this program, particularly: Lifeng Lin, Grace Lui, Gauri Ganpule, Lakshmi Sampath, Sonny Sovan, Tyler Stine, Lawreen Asuncion, Rob Bennett, and Jim Godsey.

For Research Use Only. Not for use in diagnostic procedures.