

Ion AmpliSeq™ Mouse Immune Repertoire

USER GUIDE

for use with:

Ion AmpliSeq™ Mouse TCR Beta-SR (RNA)

Ion AmpliSeq™ Mouse TCR Beta-SR (DNA)

Ion AmpliSeq™ Mouse BCR IGH-SR (RNA)

Ion AmpliSeq™ Mouse BCR IGH-SR (DNA)

Catalog Numbers A45489, A45488, A45487, A45486

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Revision	Date	Description
B.0	19 November 2019	<ul style="list-style-type: none">Added instructions and guidelines for the following assays:<ul style="list-style-type: none">– Ion AmpliSeq™ Mouse TCR Beta-SR (RNA)– Ion AmpliSeq™ Mouse BCR IGH-SR (RNA)Added the following saturation analysis sections:<ul style="list-style-type: none">– “Saturation analysis of mouse gDNA” on page 107– “Saturation analysis of Mouse spleen FFPE tissue” on page 108– “Saturation analysis of Mouse thymus FFPE tissue” on page 111
A.0	08 October 2019	New user guide describing library preparation, templating, sequencing, and results analysis for the Ion AmpliSeq™ Mouse Immune Repertoire Panels.

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IMPORTANT! Before using this product, read and understand the information in the “Safety” appendix in this document.

Product description

The Ion AmpliSeq™ Mouse Immune Repertoire Assays are highly sensitive, next-generation sequencing (NGS) assays that accurately measure T- and B-cell repertoire diversity and clonal expansion in a wide variety of mouse sample types.

This guide covers the following products:

- Ion AmpliSeq™ Mouse TCR Beta-SR (RNA) (Cat. No. A45489)
- Ion AmpliSeq™ Mouse TCR Beta-SR (DNA) (Cat. No. A45268)
- Ion AmpliSeq™ Mouse BCR IGH-SR (RNA) (Cat. No. A45487)
- Ion AmpliSeq™ Mouse BCR IGH-SR (DNA) (Cat. No. A45486)

Each of assay kit includes the Ion AmpliSeq™ Library Kit Plus, and either the Ion Torrent™ NGS Reverse Transcription Kit (RNA assays only), or 25 mM dNTP mix (DNA assays only). The Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802, purchased separately) is required for library quantification.

Each of assay kit includes the Ion AmpliSeq™ Library Kit Plus, and the Ion Torrent™ NGS Reverse Transcription Kit. The Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802, purchased separately) is required for library quantification.

These kits are designed for use with Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360, purchased separately) adapters so that multiple libraries can be combined and loaded onto a single Ion Chip to minimize the per-sample sequencing cost.

Sequencer compatibility

Ion GeneStudio™ S5 Series Sequencer or Ion GeneStudio™ S5 Series System refers generically to following systems, unless otherwise specified.

- Ion GeneStudio™ S5 System (Cat. No. A38194)
- Ion GeneStudio™ S5 Plus System (Cat. No. A38195)
- Ion GeneStudio™ S5 Prime System (Cat. No. A38196)

Ion AmpliSeq™ Mouse TCR Beta-SR Assay

The Ion AmpliSeq™ Mouse TCR Beta-SR Assay is a highly sensitive RNA or DNA-based NGS assay that enables the characterization of the T-cell receptor β (TCR β) complementarity-determining region 3 (CDR3) sequences of the rearranged receptor. The assay accurately measures T-cell repertoire diversity and clonal expansion in a wide array of sample types.

Table 1 Ion AmpliSeq™ Mouse TCR Beta-SR Assay sample types

Sample types		Assay Input	Supported Chips
Peripheral blood	Whole blood	gDNA, cDNA	Ion 530™ Chip Ion 540™ Chip Ion 550™ Chip ^[1]
	Peripheral Blood Leukocytes (PBL)	gDNA, cDNA	
	Peripheral Blood Mononuclear Cells (PBMC)	gDNA, cDNA	
Tissue	Fresh frozen	gDNA, cDNA	
	FFPE	gDNA, cDNA	
Isolated cells	Sorted T-cells	gDNA, cDNA	

^[1] The Ion 550™ Chip is only compatible with the Ion GeneStudio™ S5 Plus and Ion GeneStudio™ S5 Prime model sequencers.

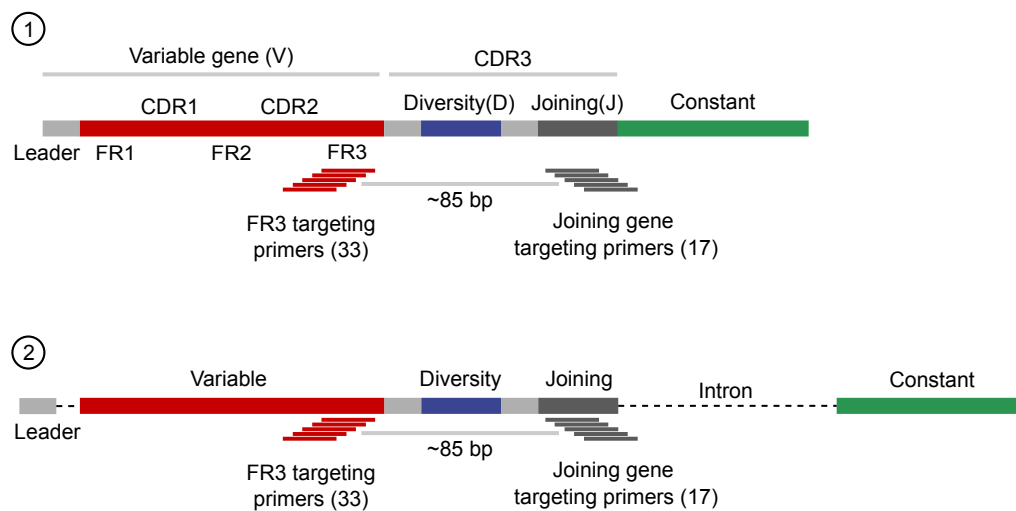


Figure 1 Ion AmpliSeq™ Mouse TCR Beta-SR Assay

- ① RNA/cDNA input
- ② Genomic DNA input

The Ion AmpliSeq™ Mouse TCR Beta-SR Assay uses multiplex Ion AmpliSeq™ primers to target the FR3 region of the variable gene and the joining gene segments of the TCR β rearrangements in genomic DNA or cDNA, producing an ~85-bp amplicon.

Ion AmpliSeq™ Mouse BCR IGH-SR Assay

The Ion AmpliSeq™ Mouse BCR IGH-SR Assay is a highly sensitive RNA or DNA-based NGS assay that enables the characterization of the immunoglobulin heavy chain complementarity-determining region 3 (CDR3) sequences. The assay accurately measures B cell repertoire diversity and clonal expansion in a wide array of sample types.

Table 2 Ion AmpliSeq™ Mouse BCR IGH-SR Assay sample types

Sample types		Assay Input	Supported Chips
Peripheral blood	Whole blood	gDNA, cDNA	Ion 530™ Chip Ion 540™ Chip Ion 550™ Chip ^[1]
	Peripheral Blood Leukocytes (PBL)	gDNA, cDNA	
	Peripheral Blood Mononuclear Cells (PBMC)	gDNA, cDNA	
Tissue	Fresh frozen	gDNA, cDNA	
	FFPE	gDNA, cDNA	
	Bone Marrow	gDNA, cDNA	
Isolated cells	Sorted B cells	gDNA, cDNA	

^[1] The Ion 550™ Chip is only compatible with the Ion GeneStudio™ S5 Plus and Ion GeneStudio™ S5 Prime model sequencers.

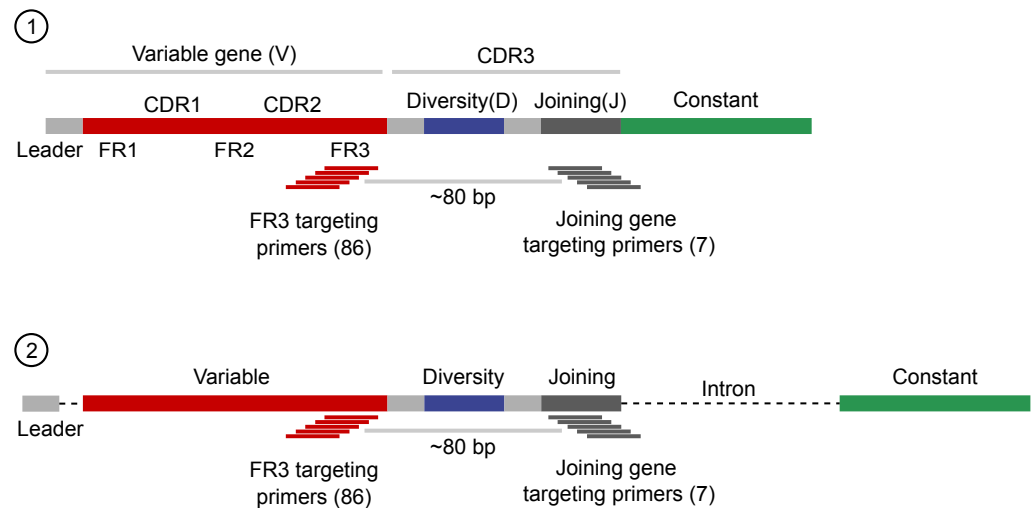


Figure 2 Ion AmpliSeq™ Mouse BCR IGH-SR Assay

- ① RNA/cDNA input
- ② Genomic DNA input

The Ion AmpliSeq™ Mouse BCR IGH-SR Assay uses multiplex Ion AmpliSeq™ primers to target the FR3 region of the variable gene and the joining gene segments of IGH-VDJ rearrangements in genomic DNA or cDNA, producing an ~80-bp amplicon.

Contents and storage

Ion AmpliSeq™ Mouse TCR Beta-SR (RNA) Assay Kit

The Ion AmpliSeq™ Mouse TCR Beta-SR (RNA) Assay kit (Cat. No. A45489) consists of the Ion AmpliSeq™ Mouse TCR Beta-SR Panel, the Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990), and the Ion Torrent™ NGS Reverse Transcription Kit (Cat. No. A45003). Sufficient reagents are provided for the rapid preparation of 24 sample libraries.

Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360, purchased separately) adapters are required for the rapid preparation of barcoded sample libraries, which you can then combine and sequence simultaneously.

Contents	Amount	Storage
Ion AmpliSeq™ Mouse TCR Beta-SR (RNA) (Cat. No. A45489)		
5X Ion AmpliSeq™ Mouse TCR Beta-SR Panel	96 µL	-25°C to -15°C
Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990)		
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	-30°C to -10°C
FuPa Reagent (brown cap)	48 µL	
Switch Solution (yellow cap)	96 µL	
DNA Ligase (blue cap)	48 µL	
25X Library Amp Primers (pink cap)	48 µL	
1X Library Amp Mix (black cap)	1.2 mL	
Low TE (clear cap)	1 each	15°C to 30°C ^[1]
Ion Torrent™ NGS Reverse Transcription Kit (Cat. No. A45003)		
10X Ion Torrent™ NGS 10X RT Enzyme Mix	32 µL	-30°C to -10°C
5X Ion Torrent™ NGS 5X Reaction Buffer	64 µL	

^[1] Can be stored at -30°C to -10°C for convenience.

**Ion AmpliSeq™
Mouse TCR
Beta-SR (DNA)
Assay Kit**

The Ion AmpliSeq™ Mouse TCR Beta-SR (DNA) Assay kit (Cat. No. A45488) consists of the Ion AmpliSeq™ Mouse TCR Beta-SR Panel, the Ion AmpliSeq™ Library Kit Plus (Part No. 4488990), and premixed dNTPs. Sufficient reagents are provided for the rapid preparation of 24 sample libraries.

Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360, ordered separately) adapters are required for the rapid preparation of barcoded sample libraries, which you can then combine and sequence simultaneously.

Contents	Amount	Storage
Ion AmpliSeq™ Mouse TCR Beta-SR (DNA)		
5X Ion AmpliSeq™ Mouse TCR Beta-SR Panel	96 µL	-25°C to -15°C
dNTP Mix (25 mM each) ^[1]	1.0 mL	
Ion AmpliSeq™ Library Kit Plus		
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	-30°C to -10°C
FuPa Reagent (brown cap)	48 µL	
Switch Solution (yellow cap)	96 µL	
DNA Ligase (blue cap)	48 µL	
25X Library Amp Primers (pink cap)	48 µL	
1X Library Amp Mix (black cap)	1.2 mL	
Low TE (clear cap)	1 each	15°C to 30°C ^[2]

^[1] Provided with the Ion AmpliSeq™ Mouse TCR Beta-SR (DNA) (Cat. No. A45488) only.

^[2] Can be stored at -30°C to -10°C for convenience.

Ion AmpliSeq™ Mouse BCR IGH-SR (RNA) Assay Kit

The Ion AmpliSeq™ Mouse BCR IGH-SR (RNA) Assay kit (Cat. No. A45487) consists of the Ion AmpliSeq™ Mouse BCR IGH-SR Panel, the Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990), and the Ion Torrent™ NGS Reverse Transcription Kit (Cat. No. A45003). Sufficient reagents are provided for the rapid preparation of 24 sample libraries.

Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360, purchased separately) adapters are required for the rapid preparation of barcoded sample libraries, which you can then combine and sequence simultaneously.

Contents	Amount	Storage
Ion AmpliSeq™ Mouse BCR IGH-SR (RNA) (Cat. No. A45487)		
5X Ion AmpliSeq™ Mouse BCR IGH-SR Panel	96 µL	-25°C to -15°C
Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990)		
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	-30°C to -10°C
FuPa Reagent (brown cap)	48 µL	
Switch Solution (yellow cap)	96 µL	
DNA Ligase (blue cap)	48 µL	
25X Library Amp Primers (pink cap)	48 µL	
1X Library Amp Mix (black cap)	1.2 mL	
Low TE (clear cap)	1 each	15°C to 30°C ^[1]
Ion Torrent™ NGS Reverse Transcription Kit (Cat. No. A45003)		
10X Ion Torrent™ NGS 10X RT Enzyme Mix	32 µL	-30°C to -10°C
5X Ion Torrent™ NGS 5X Reaction Buffer	64 µL	

^[1] Can be stored at -30°C to -10°C for convenience.

Ion AmpliSeq™ Mouse BCR IGH-SR (DNA) Assay Kit

The Ion AmpliSeq™ Mouse BCR IGH-SR (DNA) Assay kit (Cat. No. A45486) consist of the Ion AmpliSeq™ Mouse BCR IGH-SR Panel, the Ion AmpliSeq™ Library Kit Plus (Part No. 4488990), and premixed dNTPs. Sufficient reagents are provided for the rapid preparation of 24 sample libraries.

Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360, ordered separately) adapters are required for the rapid preparation of barcoded sample libraries, which you can then combine and sequence simultaneously.

Contents	Amount	Storage
Ion AmpliSeq™ Mouse BCR IGH-SR (DNA)		
5X Ion AmpliSeq™ Mouse BCR IGH-SR Panel	96 µL	-25°C to -15°C
dNTP Mix (25 mM each) ^[1]	1.0 mL	
Ion AmpliSeq™ Library Kit Plus		
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	-30°C to -10°C
FuPa Reagent (brown cap)	48 µL	
Switch Solution (yellow cap)	96 µL	
DNA Ligase (blue cap)	48 µL	
25X Library Amp Primers (pink cap)	48 µL	
1X Library Amp Mix (black cap)	1.2 mL	
Low TE (clear cap)	1 each	15°C to 30°C ^[2]

^[1] Provided with the Ion AmpliSeq™ Mouse BCR IGH-SR (DNA) (Cat. No. A45486) only.

^[2] Can be stored at -30°C to -10°C for convenience.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**.
MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Required for library preparation	
Agencourt™ AMPure™ XP Kit	A63880 or A63881, Beckman Coulter
Ion Library TaqMan™ Quantitation Kit	4468802
Ion Torrent™ Dual Barcode Kit 1–96	A39360
One of the following: <ul style="list-style-type: none"> • Veriti™ 96-Well Thermal Cycler • ProFlex™ 96-well PCR System • SimpliAmp™ Thermal Cycler • GeneAmp™ PCR System 9700 	See web product pages
MicroAmp™ Optical 96-well Reaction Plate	N8010560 4306737 (with barcode)
MicroAmp™ Fast Optical 96-Well Reaction Plate	4346907
MicroAmp™ Optical Adhesive Film	4311971
MicroAmp™ Adhesive Film	4306311
MicroAmp™ Compression Pad	4312639
RNase-Free Microfuge Tubes (1.5 mL)	AM12400
DynaMag™ –96 Side Magnet, or other plate magnet	12331D
Low TE buffer	12090015
Nuclease-free Water	AM9932
Absolute ethanol	MLS
Pipettors, 2–200 µL, and low-retention filtered pipette tips	MLS

Recommended materials

Unless otherwise indicated, all materials are available through **thermofisher.com**.
MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Recommended additional equipment	
One of the following Applied Biosystems™ real-time PCR instruments: <ul style="list-style-type: none"> • 7500 Real-Time PCR System • 7900HT Fast Real-Time PCR System^[1] • StepOne™ Real-Time PCR System • StepOnePlus™ Real-Time PCR System • QuantStudio™ 3 Real-Time PCR System • QuantStudio™ 5 Real-Time PCR System • QuantStudio™ 7 Flex Real-Time PCR System • QuantStudio™ 12K Flex Real-Time PCR System • ViiA™ 7 Real-Time PCR System 	See web product pages
Qubit™ 4 Fluorometer ^[2]	Q33226
Fisher Scientific™ Mini Plate Spinner Centrifuge, or equivalent 96-well plate centrifuge	MLS
BD Vacutainer® Plastic Blood Collection Tubes with K ₂ EDTA: Hemogard™ Closure	02-683-99A fisherscientific.com
Recommended for nucleic acid isolation from fresh frozen tissue samples	
RecoverAll™ Multi-Sample RNA/DNA Workflow	A26069
MagMAX™ DNA Multi-Sample Ultra 2.0 Kit (DNA only)	A36570
MagMAX™ -96 Total RNA Isolation Kit (RNA only)	AM1830
Recommended for nucleic acid isolation from FFPE tissue samples	
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
Ion AmpliSeq™ Direct FFPE DNA Kit (DNA only)	A31133
MagMAX™ FFPE DNA/RNA Ultra Kit (DNA & RNA)	A31881
Recommended for nucleic acid isolation from whole blood, cells (buffy coat, PBMCs, Isolated T-cells)	
MagMAX™ DNA Multi-Sample Ultra 2.0 Kit (DNA only)	A36570
Mouse RiboPure™ -Blood RNA Purification Kit	AM1951
Recommended for quantification	
Qubit™ RNA HS Assay Kit	Q32852
Qubit™ dsDNA HS Assay Kit	Q32851
Recommended positive control	
Universal Mouse Reference RNA	QS0640

^[1] Supported but no longer available for purchase.

^[2] The Qubit™ 2.0 Fluorometer & Qubit™ 3.0 Fluorometer are supported but no longer available for purchase.



Before you begin

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Immune repertoire sequencing overview

The immune repertoire refers to the collection of B- and T-cell receptors present in an individual. Murine T-cell receptors (TCR) are heterodimers that fall into two classes: TCR- $\alpha\beta$, and TCR- $\gamma\delta$. TCR- $\alpha\beta$ T-cells constitute most the T-cell receptor repertoire (typically >90% of T-cells) and are the primary contributors to cellular adaptive immunity. The murine B cell receptor (BCR) complex consists of an antigen-binding subunit that is known as the membrane immunoglobulin. The membrane immunoglobulin is composed of two immunoglobulin light chains (IGL) and two immunoglobulin heavy chains (IGH) as well as two heterodimer subunits of Ig- α and Ig- β . The TCR β -chains and BCR IGHs are made up of variable (V), diversity (D), joining (J), and constant regions. Recombination of these regions, with additional nucleotide additions and/or deletions, yields a high diversity of rearranged receptors to enable the recognition of millions of antigens. The complementarity-determining region 3 (CDR3) straddles the V(D)J junction and is the primary site of antigen contact. Among the components of the T and B cell receptors, the CDR3 region of the TCR β and IGH chain contains the greatest sequence diversity. This sequence diversity may be used to track T or B cells across longitudinal samples, measure immune repertoire diversity and clonal expansion, and identify amino acid motifs linked to antigen exposure. The Ion AmpliSeq™ Mouse TCR Beta-SR Assay measures the murine T-cell receptor (TCR) immune repertoire by interrogating the TCR β -chain locus. The Ion AmpliSeq™ Mouse BCR IGH-SR Assay measures the murine B-cell receptor (BCR) immune repertoire by interrogating the B cell heavy chain (IGH) locus.

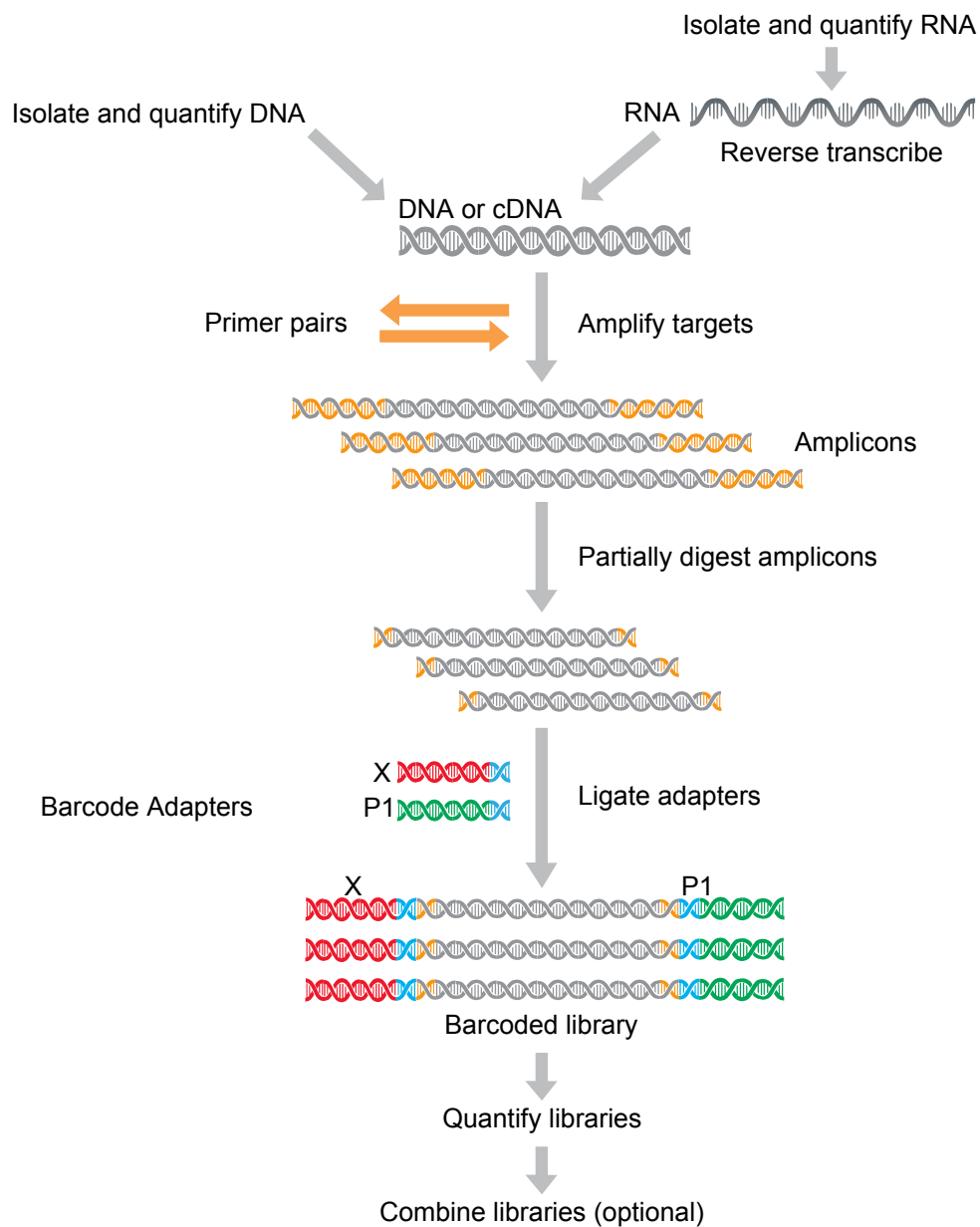
Procedural guidelines

- Minimize freeze-thaw cycles of Ion AmpliSeq™ Mouse Immune Repertoire Panels by aliquoting as needed for your experiments. Panels can be stored at 4°C for one year.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform PCR setup in an area or room that is free of amplicon contamination. Always change pipette tips between samples.
- Use a calibrated thermal cycler that is specified in “Required materials not supplied”.
- Pipet viscous solutions, such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, Switch Solution, DNA Ligase, and panels, slowly and ensure complete mixing by vortexing or pipetting up and down several times.
- Arrange samples in alternating columns on the plate for easier pipetting with multichannel pipettes during purification with the DynaMag™ Side Magnet.

Before each use of the kit

- Thaw components that contain enzymes—such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, DNA Ligase, and 1X Library Amp Mix—on ice, and keep on ice during procedure. All other components, including primer pools, can be thawed at room temperature. Gently vortex and centrifuge before use.
- If there is visible precipitate in the Switch Solution after thawing, vortex or pipet up and down at room temperature to resuspend.

Workflow



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Guidelines for nucleic acid isolation and quantification

IMPORTANT! Sample input amount and the target amplification cycling program vary with sample type.

- Use kits in “Recommended materials” on page 15 for isolating total RNA.
- We recommend the Qubit™ RNA HS Assay Kit (Cat. No. Q32852) for quantifying RNA.
- We recommend the Agilent™ 2100 Bioanalyzer™ and Agilent™ RNA 6000 Nano Kit to quantify and evaluate RNA integrity. We recommend RIN values derived from the Agilent™ 2100 Bioanalyzer™ software be ≥ 4 , for more information see “Quality assessment of heat degraded RNA samples” on page 115.
- For best results, use blood that is collected in EDTA tubes that have been centrifuged to separate the plasma from cellular blood components as soon as possible after blood collection. We recommend the BD Vacutainer® Plastic Blood Collection Tubes with K₂EDTA: Hemogard™ Closure used during verification testing. See “Recommended materials” on page 15.
- Each reverse transcription reaction requires at least 25 ng of DNase-treated total RNA. For RNA samples <6.25-ng/ μ L concentration, we recommend using the DNAclean™ Purification Kit (Cat. No. AM1756) to concentrate the sample after performing reverse transcription. We recommend using ≥ 25 -ng DNase-treated total RNA depending on sample type, ranging from 25–2,500 ng.
- If the amount of RNA sample is not limiting, we recommend performing reverse transcription with 2 μ g RNA, use the resultant cDNA for multiple library preparations.
- Sample input amount and the target amplification cycling program vary with sample type. In general, DNA and RNA from sorted cell samples includes a greater fraction of relevant template molecules. The fraction of relevant molecules is higher when using mRNA instead of total RNA, and PBMC or PBL compared to whole blood.
- In general, library yield from high-quality DNA or RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.

Prepare Ion AmpliSeq™ Mouse TCR Beta-SR (RNA) libraries

Reverse-transcribe RNA

IMPORTANT! For RNA samples <6.25 ng/μL, the desired target amplification input amount can be achieved by scaling up the reverse transcription reaction. After reverse transcription, use the DNAClear™ Purification Kit (Cat. No. AM1756) to purify and concentrate the cDNA.

1. If the RNA was prepared from FFPE tissue and not previously heat-treated, heat at 80°C for 10 minutes, then cool to room temperature.
2. For each sample, add the following components into a single well of a 96-well PCR plate on ice or in a pre-chilled 4°C cold block. Prepare a master mix without sample RNA for multiple reactions.

Note: We recommend that you reverse-transcribe a sample of control total RNA (Universal Mouse Reference RNA, sold separately) in parallel with your sample RNA. Parallel reverse transcription of the control RNA sample can help answer questions concerning overall reverse transcription performance, PCR inhibition due to RT carryover, PCR inhibitors present in the sample, contamination, and so forth.

Component	Volume
Ion Torrent™ NGS 5X Reaction Buffer	2 μL
Ion Torrent™ NGS 10X RT Enzyme Mix	1 μL
Total RNA (25 ng to 1 μg) ^[1]	≤7 μL
Nuclease-free Water	to 10 μL
Total volume per well	10 μL

^[1] Substitute an equal volume of nuclease-free water or Low TE to prepare a no-template control (NTC).

3. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.
4. Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program to synthesize cDNA.

Temperature	Time
25°C	10 minutes
50°C	10 minutes
85°C	5 minutes
10°C	Hold

Note: Samples can be stored at 10°C for up to 16 hours in the thermal cycler.

5. Briefly centrifuge the plate to collect any droplets at the bottom of the wells.

STOPPING POINT For long-term storage, transfer the cDNA to an RNase-free microcentrifuge tube and store at -20°C.

Prepare target amplification reactions - TCR Beta - SR - RNA

IMPORTANT!

- The target amplification cycling program and sample input vary with the sample type and assay. Performing the wrong procedure can result in suboptimal sequencing read depth and decreased number of clones detected.
- Primer pools and HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place the 96-well reverse transcription plate with 10-µL cDNA in a pre-chilled cold block or on ice.
2. Place the 5X Ion AmpliSeq™ HiFi Mix on ice, gently vortex to mix, then centrifuge briefly to collect.
3. If needed, remove the seal from the plate, then add the following components to each 10-µL cDNA synthesis reaction. Prepare a master mix for multiple reactions.

Component	Volume
cDNA (from reverse transcription)	10 µL
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Ion AmpliSeq™ Mouse TCR Beta-SR Panel	4 µL
Nuclease-Free Water	2 µL
Total volume per well (includes 10 µL from cDNA synthesis)	~20 µL

4. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
5. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.
6. Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	2 minutes
23 cycles ^[1]	Denature	95°C	30 seconds
	Anneal	60°C	45 seconds
	Extend	72°C	45 seconds
Hold	Final extension	72°C	10 minutes
Hold	—	10°C	Hold

^[1] Adjust cycle number according to the following table to accommodate variable sample input amounts.

Amount of cDNA starting material	Recommended adjustment to cycle number
2.5 ng	+3
25 ng to 2 µg	0

Note:

- Cycle number can be adjusted when input material quality or quantity is questionable. Increase number by +3 when starting from FFPE samples.
- Libraries determined to be <25 pM by qPCR quantification can be rescued with library amplification. For more information, see “Rescue amplification and library purification” on page 99.

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that the input across all samples is equivalent so that the selected cycle number is optimal for all the samples in the run.

7. Remove the plate from the thermal cycler, then briefly centrifuge the plate to collect the contents.

STOPPING POINT Target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer periods, store at -20°C.

Partially digest amplicons

IMPORTANT! Keep the plate on ice or in a pre-chilled 4°C cold block while preparing the reactions.

1. Keep the FuPa Reagent (brown cap) on ice, gently vortex to mix, then briefly centrifuge to collect.
 2. Carefully remove the adhesive film from the plate.
-
- IMPORTANT!** Be careful when removing the film to minimize contamination.
-
3. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.
 4. Seal the plate with a new clear adhesive film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
 5. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

Temperature	Time
50°C	10 min
55°C	10 min
60°C	20 min
10°C	Hold (for up to 1 hour)

6. Briefly centrifuge the plate to collect the contents to the bottom of the wells.

IMPORTANT! Proceed immediately to “Perform the ligation reaction” on page 23. Do not store the partially digested amplicons over night.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single chip, you *must* ligate a different barcode adapter to each library.

IMPORTANT! When handling barcoded adapters, be careful to avoid cross contamination by changing gloves frequently and opening one tube at a time.

Perform the ligation reaction

IMPORTANT! When using Ion Torrent™ Dual Barcode Kit 1–96 adapters, we recommend that you remove the foil seal from only the specific wells being used to prevent potential cross-contamination. After adding dual barcode adapters to your reaction, cut a portion of a MicroAmp™ Clear Adhesive Film to size, then reseal the used wells. Each well of Ion Torrent™ Dual Barcode adapter is single use only.

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
2. Briefly centrifuge the plate to collect droplets, then carefully remove the plate seal.
Note: Centrifuge the plate of dual barcode adapters at the same time, to collect the contents.
3. Add the following components in the order listed to each well containing digested amplicons.

IMPORTANT! Add the DNA Ligase last. Do not combine DNA Ligase and barcode adapters before adding to the digested amplicons.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Ion Torrent™ Dual Barcode Kit 1–96 adapters	2 µL
3	DNA Ligase (blue cap)	2 µL
—	Total volume (including ~22 µL of digested amplicon)	~30 µL

4. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
5. Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 1 hour)

STOPPING POINT Samples can be stored for longer periods at –20°C.

Purify the library

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
 - Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.
-

1. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
 2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
- Note:** Visually inspect each well to ensure that the mixture is homogeneous.
3. Incubate the mixture for 5 minutes at room temperature.
 4. Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
 5. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.

Note: If your magnet does not have two positions for shifting the beads. Remove the plate from the magnet, gently pipet up and down 5 times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

6. Repeat step 5 for a second wash.
 7. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. Do not overdry.
-

IMPORTANT! Residual ethanol inhibits library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads. Under conditions of low relative humidity, the beads air-dry rapidly. Do not overdry.

Library amplification (Optional)

When starting material quality or quantity is questionable amplification of the purified library can be performed immediately following purification before elution from the Agencourt™ AMPure™ XP beads. In this procedure you elute the library directly into amplification mix, then perform 5 cycles of amplification. If not performing library amplification, proceed directly to “Elute the library” on page 27.

1. To each well containing purified library and air-dried Agencourt™ AMPure™ XP beads add the following components.

Note: If processing multiple samples, prepare a reaction master mix (+ 5–10% overage), then add 52 µL to each well.

Component	Volume
1X Library Amp Mix (black cap)	50 µL
25X Library Amp Primers (pink cap)	2 µL
Total Volume	52 µL

2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Place the plate back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant from each well to a new well or a new plate without disturbing the pellet.
4. Seal the plate with MicroAmp™ Adhesive Film, place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 minutes
5 cycles ^[1]	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

^[1] 5 cycles should be sufficient, but you may perform up to 9 cycles library amplification.

STOPPING POINT Samples can be stored at –20°C.

Purify the amplified library

Perform a two-round purification process with the Agencourt™ AMPure™ XP Reagent:

- **First round at 0.5X bead-to-sample-volume ratio:** High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant.**
- **Second round at 1.2X bead-to-original-sample-volume ratio:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
 - Use freshly prepared 70% ethanol for the next steps. Combine 230 µL of ethanol with 100 µL of Nuclease-free Water per sample.
 - Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.
-

First-round purification

1. Tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, then remove the plate seal.
2. **Add 25 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent** to each plate well containing ~50 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnet such as the DynaMag™-96 Side Magnet for at least 5 minutes, or until the solution is clear.
5. Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

IMPORTANT! The **supernatant** contains the desired amplicons. Do not discard!

Second-round purification

1. To the supernatant from step 5, **add 60 µL (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent**. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

IMPORTANT! The amplicons are bound to the beads. **Save the bead pellet.**

4. Add 150 μL of freshly prepared 70% ethanol to each well, then move the plate side to side in the magnet to wash the beads. Remove, then discard the supernatant without disturbing the pellet.

Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down five times (with the pipettor set at 100 μL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

5. Repeat step 4 for a second wash.
6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. **Do not overdry.**

Elute the library

1. Remove the plate with purified libraries from the plate magnet, then add 50 μL of Low TE to the pellet to disperse the beads.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Incubate at room temperature for at least 2 minutes.
4. Place the plate on the magnet for at least 2 minutes.
5. Prepare a 100-fold dilution for quantification. Remove 2 μL of supernatant, containing the library, then combine with 198 μL of Nuclease-free Water.
6. If you performed library amplification. Prepare a further 100-fold dilution of the 100X dilution for quantification. Remove 2 μL of 100X dilution, then combine with 198 μL of Nuclease-free Water.
Use this 10,000-fold dilution for quantification of amplified libraries.

Proceed to “Quantify the library by qPCR and calculate the dilution factor” on page 50.

Prepare Ion AmpliSeq™ Mouse TCR Beta-SR (DNA) libraries

Prepare target amplification reactions - TCR Beta - SR - DNA

IMPORTANT!

- The target amplification cycling program and sample input vary with the sample type and assay. Performing the wrong procedure can result in suboptimal sequencing read depth and decreased number of clones detected.
- Primer pools and HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place a new 96-well plate in a pre-chilled cold block or on ice.
2. Dilute the dNTP Mix (25 mM each) to 6 mM each.
 - a. Add the following components to a nuclease-free tube on ice.

Component	Volume
Nuclease-free Water	19 µL
dNTP Mix (25 mM each)	6 µL
Total volume	25 µL

- b. Close the tube, then vortex to mix.
3. Place the 5X Ion AmpliSeq™ HiFi Mix on ice, gently vortex to mix, then centrifuge briefly to collect.
 4. Add the following components to each sample well. Prepare a master mix for multiple reactions.

Component	Volume
gDNA (200 ng)	X µL
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Ion AmpliSeq™ Mouse TCR Beta-SR Panel	4 µL
dNTP Mix (6 mM each dNTP, prepared in step 2)	2 µL
Nuclease-Free Water	10-X µL
Total volume per well	~20 µL

5. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
6. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.

7. Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	2 minutes
25 cycles ^[1]	Denature	95°C	30 seconds
	Anneal	60°C	45 seconds
	Extend	72°C	45 seconds
Hold	Final extension	72°C	10 minutes
Hold	—	10°C	Hold

^[1] Adjust cycle number according to the following table to accommodate variable sample input amounts.

Amount of DNA starting material	Recommended adjustment to cycle number
≤100 ng	+3
200 ng to 2 µg	0

Note:

- Cycle number can be adjusted when input material quality or quantity is questionable. Increase number by +3 when starting from FFPE samples.
- Libraries determined to be <25 pM by qPCR quantification can be rescued with library amplification. For more information, see “Rescue amplification and library purification” on page 99.

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that the input across all samples is equivalent so that the selected cycle number is optimal for all the samples in the run.

8. Remove the plate from the thermal cycler, then briefly centrifuge the plate to collect the contents.

STOPPING POINT Target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer periods, store at -20°C.

Partially digest amplicons

IMPORTANT! Keep the plate on ice or in a pre-chilled 4°C cold block while preparing the reactions.

1. Keep the FuPa Reagent (brown cap) on ice, gently vortex to mix, then briefly centrifuge to collect.
2. Carefully remove the adhesive film from the plate.

IMPORTANT! Be careful when removing the film to minimize contamination.

3. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.

4. Seal the plate with a new clear adhesive film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
5. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

Temperature	Time
50°C	10 min
55°C	10 min
60°C	20 min
10°C	Hold (for up to 1 hour)

6. Briefly centrifuge the plate to collect the contents to the bottom of the wells.

IMPORTANT! Proceed immediately to “Perform the ligation reaction” on page 30. Do not store the partially digested amplicons over night.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single chip, you *must* ligate a different barcode adapter to each library.

IMPORTANT! When handling barcoded adapters, be careful to avoid cross contamination by changing gloves frequently and opening one tube at a time.

Perform the ligation reaction

IMPORTANT! When using Ion Torrent™ Dual Barcode Kit 1–96 adapters, we recommend that you remove the foil seal from only the specific wells being used to prevent potential cross-contamination. After adding dual barcode adapters to your reaction, cut a portion of a MicroAmp™ Clear Adhesive Film to size, then reseal the used wells. Each well of Ion Torrent™ Dual Barcode adapter is single use only.

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
2. Briefly centrifuge the plate to collect droplets, then carefully remove the plate seal.

Note: Centrifuge the plate of dual barcode adapters at the same time, to collect the contents.

- Add the following components in the order listed to each well containing digested amplicons.

IMPORTANT! Add the DNA Ligase last. Do not combine DNA Ligase and barcode adapters before adding to the digested amplicons.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Ion Torrent™ Dual Barcode Kit 1–96 adapters	2 µL
3	DNA Ligase (blue cap)	2 µL
—	Total volume (including ~22 µL of digested amplicon)	~30 µL

- Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
- Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 1 hour)

STOPPING POINT Samples can be stored for longer periods at –20°C.

Purify the library

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
- Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

- Briefly centrifuge the plate to collect the contents in the bottom of the wells.
- Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
Note: Visually inspect each well to ensure that the mixture is homogeneous.
- Incubate the mixture for 5 minutes at room temperature.
- Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.

5. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.

Note: If your magnet does not have two positions for shifting the beads. Remove the plate from the magnet, gently pipet up and down 5 times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

6. Repeat step 5 for a second wash.
7. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. Do not overdry.

IMPORTANT! Residual ethanol inhibits library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads. Under conditions of low relative humidity, the beads air-dry rapidly. Do not overdry.

Library amplification (Optional)

When starting material quality or quantity is questionable amplification of the purified library can be performed immediately following purification before elution from the Agencourt™ AMPure™ XP beads. In this procedure you elute the library directly into amplification mix, then perform 5 cycles of amplification. If not performing library amplification, proceed directly to “Elute the library” on page 34.

1. To each well containing purified library and air-dried Agencourt™ AMPure™ XP beads add the following components.

Note: If processing multiple samples, prepare a reaction master mix (+ 5–10% overage), then add 52 µL to each well.

Component	Volume
1X Library Amp Mix (black cap)	50 µL
25X Library Amp Primers (pink cap)	2 µL
Total Volume	52 µL

2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Place the plate back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant from each well to a new well or a new plate without disturbing the pellet.

- Seal the plate with MicroAmp™ Adhesive Film, place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 minutes
5 cycles ^[1]	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

^[1] 5 cycles should be sufficient, but you may perform up to 9 cycles library amplification.

STOPPING POINT Samples can be stored at –20°C.

Purify the amplified library

Perform a two-round purification process with the Agencourt™ AMPure™ XP Reagent:

- First round at 0.5X bead-to-sample-volume ratio:** High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant.**
- Second round at 1.2X bead-to-original-sample-volume ratio:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
 - Use freshly prepared 70% ethanol for the next steps. Combine 230 µL of ethanol with 100 µL of Nuclease-free Water per sample.
 - Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.
-

First-round purification

- Tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, then remove the plate seal.
- Add 25 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent** to each plate well containing ~50 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
- Incubate the mixture for 5 minutes at room temperature.
- Place the plate in a magnet such as the DynaMag™-96 Side Magnet for at least 5 minutes, or until the solution is clear.
- Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

IMPORTANT! The **supernatant** contains the desired amplicons. Do not discard!

Second-round purification

1. To the supernatant from step 5, **add 60 µL (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent**. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

IMPORTANT! The amplicons are bound to the beads. **Save the bead pellet.**

4. **Add 150 µL of freshly prepared 70% ethanol** to each well, then move the plate side to side in the magnet to wash the beads. Remove, then discard the supernatant without disturbing the pellet.

Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down five times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

5. Repeat step 4 for a second wash.
6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. **Do not overdry.**

Elute the library

1. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Incubate at room temperature for at least 2 minutes.
4. Place the plate on the magnet for at least 2 minutes.
5. Prepare a 100-fold dilution for quantification. Remove 2 µL of supernatant, containing the library, then combine with 198 µL of Nuclease-free Water.
6. If you performed library amplification. Prepare a further 100-fold dilution of the 100X dilution for quantification. Remove 2 µL of 100X dilution, then combine with 198 µL of Nuclease-free Water.
 Use this 10,000-fold dilution for quantification of amplified libraries.

Proceed to “Quantify the library by qPCR and calculate the dilution factor” on page 50.

Prepare Ion AmpliSeq™ Mouse BCR IGH-SR (RNA) libraries

Reverse-transcribe RNA

IMPORTANT! For RNA samples <6.25 ng/μL, the desired target amplification input amount can be achieved by scaling up the reverse transcription reaction. After reverse transcription, use the DNAClear™ Purification Kit (Cat. No. AM1756) to purify and concentrate the cDNA.

1. If the RNA was prepared from FFPE tissue and not previously heat-treated, heat at 80°C for 10 minutes, then cool to room temperature.
2. For each sample, add the following components into a single well of a 96-well PCR plate on ice or in a pre-chilled 4°C cold block. Prepare a master mix without sample RNA for multiple reactions.

Note: We recommend that you reverse-transcribe a sample of control total RNA (Universal Mouse Reference RNA, sold separately) in parallel with your sample RNA. Parallel reverse transcription of the control RNA sample can help answer questions concerning overall reverse transcription performance, PCR inhibition due to RT carryover, PCR inhibitors present in the sample, contamination, and so forth.

Component	Volume
Ion Torrent™ NGS 5X Reaction Buffer	2 μL
Ion Torrent™ NGS 10X RT Enzyme Mix	1 μL
Total RNA (25 ng to 1 μg) ^[1]	≤7 μL
Nuclease-free Water	to 10 μL
Total volume per well	10 μL

^[1] Substitute an equal volume of nuclease-free water or Low TE to prepare a no-template control (NTC).

3. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.
4. Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program to synthesize cDNA.

Temperature	Time
25°C	10 minutes
50°C	10 minutes
85°C	5 minutes
10°C	Hold

Note: Samples can be stored at 10°C for up to 16 hours in the thermal cycler.

5. Briefly centrifuge the plate to collect any droplets at the bottom of the wells.

STOPPING POINT For long-term storage, transfer the cDNA to an RNase-free microcentrifuge tube and store at -20°C.

Prepare target amplification reactions - BCR IGH - SR - RNA

IMPORTANT!

- The target amplification cycling program and sample input vary with the sample type and assay. Performing the wrong procedure can result in suboptimal sequencing read depth and decreased number of clones detected.
- Primer pools and HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place the 96-well reverse transcription plate with 10- μ L cDNA in a pre-chilled cold block or on ice.
2. Place the 5X Ion AmpliSeq™ HiFi Mix on ice, gently vortex to mix, then centrifuge briefly to collect.
3. If needed, remove the seal from the plate, then add the following components to each 10- μ L cDNA synthesis reaction. Prepare a master mix for multiple reactions.

Component	Volume
cDNA (from reverse transcription)	10 μ L
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 μ L
5X Ion AmpliSeq™ Mouse BCR IGH-SR Panel	4 μ L
Nuclease-Free Water	2 μ L
Total volume per well (includes 10 μ L from cDNA synthesis)	~20 μL

4. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
5. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.
6. Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	2 minutes
23 cycles ^[1]	Denature	95°C	30 seconds
	Anneal	60°C	45 seconds
	Extend	72°C	45 seconds
Hold	Final extension	72°C	10 minutes
Hold	—	10°C	Hold

^[1] Adjust cycle number according to the following table to accommodate variable sample input amounts.

Amount of cDNA starting material	Recommended adjustment to cycle number
2.5 ng	+3
25 ng to 2 μ g	0

Note:

- Cycle number can be adjusted when input material quality or quantity is questionable. Increase number by +3 when starting from FFPE samples.
- Libraries determined to be <25 pM by qPCR quantification can be rescued with library amplification. For more information, see “Rescue amplification and library purification” on page 99.

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that the input across all samples is equivalent so that the selected cycle number is optimal for all the samples in the run.

7. Remove the plate from the thermal cycler, then briefly centrifuge the plate to collect the contents.

STOPPING POINT Target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer periods, store at -20°C.

Partially digest amplicons

IMPORTANT! Keep the plate on ice or in a pre-chilled 4°C cold block while preparing the reactions.

1. Keep the FuPa Reagent (brown cap) on ice, gently vortex to mix, then briefly centrifuge to collect.
2. Carefully remove the adhesive film from the plate.

IMPORTANT! Be careful when removing the film to minimize contamination.

3. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.
4. Seal the plate with a new clear adhesive film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
5. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

Temperature	Time
50°C	10 min
55°C	10 min
60°C	20 min
10°C	Hold (for up to 1 hour)

6. Briefly centrifuge the plate to collect the contents to the bottom of the wells.

IMPORTANT! Proceed immediately to “Perform the ligation reaction” on page 38. Do not store the partially digested amplicons over night.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single chip, you *must* ligate a different barcode adapter to each library.

IMPORTANT! When handling barcoded adapters, be careful to avoid cross contamination by changing gloves frequently and opening one tube at a time.

Perform the ligation reaction

IMPORTANT! When using Ion Torrent™ Dual Barcode Kit 1–96 adapters, we recommend that you remove the foil seal from only the specific wells being used to prevent potential cross-contamination. After adding dual barcode adapters to your reaction, cut a portion of a MicroAmp™ Clear Adhesive Film to size, then reseal the used wells. Each well of Ion Torrent™ Dual Barcode adapter is single use only.

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
2. Briefly centrifuge the plate to collect droplets, then carefully remove the plate seal.
Note: Centrifuge the plate of dual barcode adapters at the same time, to collect the contents.
3. Add the following components in the order listed to each well containing digested amplicons.

IMPORTANT! Add the DNA Ligase last. Do not combine DNA Ligase and barcode adapters before adding to the digested amplicons.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Ion Torrent™ Dual Barcode Kit 1–96 adapters	2 µL
3	DNA Ligase (blue cap)	2 µL
—	Total volume (including ~22 µL of digested amplicon)	~30 µL

4. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
5. Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 1 hour)

STOPPING POINT Samples can be stored for longer periods at –20°C.

Purify the library

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
 - Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.
-

1. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
Note: Visually inspect each well to ensure that the mixture is homogeneous.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.
Note: If your magnet does not have two positions for shifting the beads. Remove the plate from the magnet, gently pipet up and down 5 times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.
6. Repeat step 5 for a second wash.
7. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. Do not overdry.

IMPORTANT! Residual ethanol inhibits library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads. Under conditions of low relative humidity, the beads air-dry rapidly. Do not overdry.

**Library
amplification
(Optional)**

When starting material quality or quantity is questionable amplification of the purified library can be performed immediately following purification before elution from the Agencourt™ AMPure™ XP beads. In this procedure you elute the library directly into amplification mix, then perform 5 cycles of amplification. If not performing library amplification, proceed directly to “Elute the library” on page 42.

1. To each well containing purified library and air-dried Agencourt™ AMPure™ XP beads add the following components.

Note: If processing multiple samples, prepare a reaction master mix (+ 5–10% overage), then add 52 µL to each well.

Component	Volume
1X Library Amp Mix (black cap)	50 µL
25X Library Amp Primers (pink cap)	2 µL
Total Volume	52 µL

2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Place the plate back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant from each well to a new well or a new plate without disturbing the pellet.
4. Seal the plate with MicroAmp™ Adhesive Film, place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 minutes
5 cycles ^[1]	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

^[1] 5 cycles should be sufficient, but you may perform up to 9 cycles library amplification.

STOPPING POINT Samples can be stored at –20°C.

Purify the amplified library

Perform a two-round purification process with the Agencourt™ AMPure™ XP Reagent:

- **First round at 0.5X bead-to-sample-volume ratio:** High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant.**
- **Second round at 1.2X bead-to-original-sample-volume ratio:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
 - Use freshly prepared 70% ethanol for the next steps. Combine 230 µL of ethanol with 100 µL of Nuclease-free Water per sample.
 - Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.
-

First-round purification

1. Tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, then remove the plate seal.
2. **Add 25 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent** to each plate well containing ~50 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnet such as the DynaMag™-96 Side Magnet for at least 5 minutes, or until the solution is clear.
5. Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

IMPORTANT! The **supernatant** contains the desired amplicons. Do not discard!

Second-round purification

1. To the supernatant from step 5, **add 60 µL (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent.** Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

IMPORTANT! The amplicons are bound to the beads. **Save the bead pellet.**

4. **Add 150 µL of freshly prepared 70% ethanol** to each well, then move the plate side to side in the magnet to wash the beads. Remove, then discard the supernatant without disturbing the pellet.

Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down five times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

5. Repeat step 4 for a second wash.
6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. **Do not overdry.**

Elute the library

1. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Incubate at room temperature for at least 2 minutes.
4. Place the plate on the magnet for at least 2 minutes.
5. Prepare a 100-fold dilution for quantification. Remove 2 µL of supernatant, containing the library, then combine with 198 µL of Nuclease-free Water.
6. If you performed library amplification. Prepare a further 100-fold dilution of the 100X dilution for quantification. Remove 2 µL of 100X dilution, then combine with 198 µL of Nuclease-free Water.
Use this 10,000-fold dilution for quantification of amplified libraries.

Proceed to “Quantify the library by qPCR and calculate the dilution factor” on page 50.

Prepare Ion AmpliSeq™ Mouse BCR IGH-SR (DNA) libraries

Prepare target amplification reactions - BCR IGH - SR - DNA

IMPORTANT!

- The target amplification cycling program and sample input vary with the sample type and assay. Performing the wrong procedure can result in suboptimal sequencing read depth and decreased number of clones detected.
- Primer pools and HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place a new 96-well plate in a pre-chilled cold block or on ice.
2. Dilute the dNTP Mix (25 mM each) to 6 mM each.
 - a. Add the following components to a nuclease-free tube on ice.

Component	Volume
Nuclease-free Water	19 µL
dNTP Mix (25 mM each)	6 µL
Total volume	25 µL

- b. Close the tube, then vortex to mix.
3. Place the 5X Ion AmpliSeq™ HiFi Mix on ice, gently vortex to mix, then centrifuge briefly to collect.
 4. Add the following components to each sample well. Prepare a master mix for multiple reactions.

Component	Volume
gDNA (200 ng) ^[1, 2]	X µL
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Ion AmpliSeq™ Mouse BCR IGH-SR Panel	4 µL
dNTP Mix (6 mM each dNTP, prepared in step 2)	2 µL
Nuclease-Free Water	10-X µL
Total volume per well	~20 µL

^[1] Sample input can vary up to 2 µg dependent upon sample type.

^[2] If sample input is ≤100 ng we recommend performing "Library amplification (Optional)" on page 47.

5. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
6. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.

7. Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	2 minutes
28 cycles ^[1]	Denature	95°C	30 seconds
	Anneal	60°C	45 seconds
	Extend	72°C	45 seconds
Hold	Final extension	72°C	10 minutes
Hold	—	10°C	Hold

^[1] Cycle number can be adjusted to accommodate variable sample input amounts.

Note:

- Cycle number can be adjusted when input material quality or quantity is questionable. Increase number by +3 when starting from FFPE samples.
- Libraries determined to be <25 pM by qPCR quantification can be rescued with library amplification. For more information, see “Rescue amplification and library purification” on page 99.

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that the input across all samples is equivalent so that the selected cycle number is optimal for all the samples in the run.

8. Remove the plate from the thermal cycler, then briefly centrifuge the plate to collect the contents.

STOPPING POINT Target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer periods, store at -20°C.

Partially digest amplicons

IMPORTANT! Keep the plate on ice or in a pre-chilled 4°C cold block while preparing the reactions.

1. Keep the FuPa Reagent (brown cap) on ice, gently vortex to mix, then briefly centrifuge to collect.
2. Carefully remove the adhesive film from the plate.
IMPORTANT! Be careful when removing the film to minimize contamination.
3. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.
4. Seal the plate with a new clear adhesive film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

- Place a compression pad on the plate, load in the thermal cycler, then run the following program:

Temperature	Time
50°C	10 min
55°C	10 min
60°C	20 min
10°C	Hold (for up to 1 hour)

- Briefly centrifuge the plate to collect the contents to the bottom of the wells.

IMPORTANT! Proceed immediately to “Perform the ligation reaction” on page 45. Do not store the partially digested amplicons over night.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single chip, you *must* ligate a different barcode adapter to each library.

IMPORTANT! When handling barcoded adapters, be careful to avoid cross contamination by changing gloves frequently and opening one tube at a time.

Perform the ligation reaction

IMPORTANT! When using Ion Torrent™ Dual Barcode Kit 1–96 adapters, we recommend that you remove the foil seal from only the specific wells being used to prevent potential cross-contamination. After adding dual barcode adapters to your reaction, cut a portion of a MicroAmp™ Clear Adhesive Film to size, then reseal the used wells. Each well of Ion Torrent™ Dual Barcode adapter is single use only.

- If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
- Briefly centrifuge the plate to collect droplets, then carefully remove the plate seal.

Note: Centrifuge the plate of dual barcode adapters at the same time, to collect the contents.

- Add the following components in the order listed to each well containing digested amplicons.

IMPORTANT! Add the DNA Ligase last. Do not combine DNA Ligase and barcode adapters before adding to the digested amplicons.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Ion Torrent™ Dual Barcode Kit 1–96 adapters	2 µL
3	DNA Ligase (blue cap)	2 µL
—	Total volume (including ~22 µL of digested amplicon)	~30 µL

4. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
5. Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 1 hour)

STOPPING POINT Samples can be stored for longer periods at –20°C.

Purify the library

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
 - Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.
-

1. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

Note: Visually inspect each well to ensure that the mixture is homogeneous.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.

Note: If your magnet does not have two positions for shifting the beads. Remove the plate from the magnet, gently pipet up and down 5 times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.
6. Repeat step 5 for a second wash.

- Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. Do not overdry.

IMPORTANT! Residual ethanol inhibits library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads. Under conditions of low relative humidity, the beads air-dry rapidly. Do not overdry.

Library amplification (Optional)

When starting material quality or quantity is questionable amplification of the purified library can be performed immediately following purification before elution from the Agencourt™ AMPure™ XP beads. In this procedure you elute the library directly into amplification mix, then perform 5 cycles of amplification. If not performing library amplification, proceed directly to “Elute the library” on page 49.

- To each well containing purified library and air-dried Agencourt™ AMPure™ XP beads add the following components.

Note: If processing multiple samples, prepare a reaction master mix (+ 5–10% overage), then add 52 µL to each well.

Component	Volume
1X Library Amp Mix (black cap)	50 µL
25X Library Amp Primers (pink cap)	2 µL
Total Volume	52 µL

- Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
- Place the plate back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant from each well to a new well or a new plate without disturbing the pellet.
- Seal the plate with MicroAmp™ Adhesive Film, place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 minutes
5 cycles ^[1]	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

^[1] 5 cycles should be sufficient, but you may perform up to 9 cycles library amplification.

STOPPING POINT Samples can be stored at –20°C.

Purify the amplified library

Perform a two-round purification process with the Agencourt™ AMPure™ XP Reagent:

- **First round at 0.5X bead-to-sample-volume ratio:** High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant.**
- **Second round at 1.2X bead-to-original-sample-volume ratio:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
 - Use freshly prepared 70% ethanol for the next steps. Combine 230 µL of ethanol with 100 µL of Nuclease-free Water per sample.
 - Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.
-

First-round purification

1. Tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, then remove the plate seal.
2. **Add 25 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent** to each plate well containing ~50 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnet such as the DynaMag™-96 Side Magnet for at least 5 minutes, or until the solution is clear.
5. Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

IMPORTANT! The **supernatant** contains the desired amplicons. Do not discard!

Second-round purification

1. To the supernatant from step 5, **add 60 µL (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent.** Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

IMPORTANT! The amplicons are bound to the beads. **Save the bead pellet.**

4. Add 150 μL of freshly prepared 70% ethanol to each well, then move the plate side to side in the magnet to wash the beads. Remove, then discard the supernatant without disturbing the pellet.

Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down five times (with the pipettor set at 100 μL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

5. Repeat step 4 for a second wash.
6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. **Do not overdry.**

Elute the library

1. Remove the plate with purified libraries from the plate magnet, then add 50 μL of Low TE to the pellet to disperse the beads.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Incubate at room temperature for at least 2 minutes.
4. Place the plate on the magnet for at least 2 minutes.
5. Prepare a 100-fold dilution for quantification. Remove 2 μL of supernatant, containing the library, then combine with 198 μL of Nuclease-free Water.
6. If you performed library amplification. Prepare a further 100-fold dilution of the 100X dilution for quantification. Remove 2 μL of 100X dilution, then combine with 198 μL of Nuclease-free Water.
Use this 10,000-fold dilution for quantification of amplified libraries.

Proceed to “Quantify the library by qPCR and calculate the dilution factor” on page 50.



Quantify, combine, and sequence the libraries

- Quantify the library by qPCR and calculate the dilution factor 50
- Combine libraries 52
- Guidelines for templating and sequencing 53

Quantify the library by qPCR and calculate the dilution factor

Determine the concentration of each library by qPCR with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802). Libraries that have not undergone a second round of amplification typically have yields of 50–500 pM. However, yield is not indicative of library quality. After quantification, determine the dilution factor that results in a concentration of ~25 pM. Analyze each sample, standard, and negative control in duplicate reactions.

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Ion Control Library (~68 pM, from the Ion Library TaqMan™ Quantitation Kit) at 6.8 pM, 0.68 pM, and 0.068 pM. Mark these tubes as standards, then use these concentrations in the qPCR instrument software.
2. Calculate, then prepare the required volume of PCR reaction mix for duplicate reactions of each library sample, standard, and NTC using the following table. Include a 5–10% overage to accommodate pipetting errors.

Component	Volume per reaction	
	96-well plate	384-well plate
Ion Library qPCR Master Mix, 2X	10 µL	5 µL
Ion Library TaqMan™ Quantitation Assay, 20X	1 µL	0.5 µL
Total	11 µL	5.5 µL

3. In a MicroAmp™ Optical Reaction Plate, set up duplicate PCR reactions for each sample, standard, and NTC. Add the following components to each well.

Component	Volume per reaction	
	96-well plate	384-well plate
PCR Reaction Mix	11 µL	5.5 µL
1:100 dilution of the sample ^[1]	9 µL	4.5 µL

^[1] Substitute E. coli DH10B standards prepared in step 1 for standards. Substitute nuclease-free water for NTC.

4. Seal the plate with a MicroAmp™ Optical Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
5. Program your real-time instrument.

Note: The fast cycling program was developed using the StepOnePlus™ Real-Time PCR System in **Fast** mode.

- Enter the concentrations of the control library standards.
- Select ROX™ Reference Dye as the passive reference dye.
- Select a reaction volume of 20 µL.
- Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

IMPORTANT! We recommend using standard cycling conditions for the Ion AmpliSeq™ Mouse Immune Repertoire Panels. Fast cycling can result in inaccurate quantification.

Reaction plate format	Run mode	Stage	Temperature	Time
96-well Standard OR 384-well Standard	Standard	Hold (UDG incubation)	50°C	2 min
		Hold (polymerase activation)	95°C	2 min
		Cycle (40 cycles)	95°C	15 sec
			60°C	1 min
48- / 96-well Fast OR 384-well Standard	Fast	Hold (UDG incubation)	50°C	2 min
		Hold (polymerase activation)	95°C	20 sec
		Cycle (40 cycles)	95°C	1 sec
			60°C	20 sec

6. Following qPCR, calculate the average concentration of the undiluted library by multiplying the determined concentration × 100.

7. Based on the calculated library concentration, determine the dilution that results in a concentration of ~25 pM for template preparation on the Ion Chef™ System.

Note:

- Good results have been observed with libraries ≤ 25 pM. Proceed to “Combine libraries” on page 52, then perform template preparation, without further dilution.
- Libraries that yield significantly < 25 pM as determined by qPCR can be rescued with library amplification. For more information, see “Rescue amplification and library purification” on page 99.
- Dilute libraries to 50 pM if sequencing on an Ion 550™ Chip.

For example:

- The undiluted library concentration is 300 pM.
 - The dilution factor is $300 \text{ pM} / 25 \text{ pM} = 12$.
 - Therefore, 10 μL of library that is mixed with 110 μL of Low TE (1:12 dilution) yields approximately 25 pM.
8. Proceed to “Combine libraries” on page 52, then template preparation, or store libraries as described below.

STOPPING POINT Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C. We recommend transferring the supernatant to an RNase-free microcentrifuge tube for long-term storage. Alternatively, seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

Combine libraries

Multiple barcoded libraries can be sequenced on a single chip by combining equal volumes of each library before template preparation.

IMPORTANT! Do NOT combine libraries that are prepared with different barcode adapter sets (for example, do not mix Ion Torrent™ Dual Barcode Kit 1–96 adapter libraries with Ion Select barcode adapter libraries).

We recommend combining up to a maximum of 48 libraries for the following Ion sequencing chips.

Ion sequencing chip	Suggested number of libraries (X)
Ion 530™ Chip	8
Ion 540™ Chip	8–32 ^[1]
Ion 550™ Chip	12–48

^[1] Libraries prepared from less input material are often smaller and do not require as high sequencing depth.

Prepare a combined library as follows.

1. Dilute all individual barcoded libraries to 25 pM concentration (50 pM if using an Ion 550™ Chip).
2. Combine 10 µL of each library in a single 1.5-mL Eppendorf LoBind™ tube.
3. After adding the last library, pipet up and down 5 times to mix, then centrifuge briefly to collect in the bottom of the tube.

STOPPING POINT Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C.

Proceed to templating and sequencing, see “Guidelines for templating and sequencing” for more information.

Guidelines for templating and sequencing

Proceed to template preparation and sequencing using the following kits.

Chip	Template System	Sequencing System	Kit	User Guide
Ion 530™ Chip	Ion Chef™ System	Ion GeneStudio™ S5 Series	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Cat. No. A34461)	<i>Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide</i> (Pub. No. MAN0016854)
Ion 540™ Chip			Ion 540™ Kit – Chef (Cat. No. A27759)	<i>Ion 540™ Kit – Chef User Guide</i> (Pub. No. MAN0010851)
Ion 550™ Chip		Ion GeneStudio™ S5 Plus System or Ion GeneStudio™ S5 Prime System	Ion 550™ Kit – Chef (Cat. No. A34541)	<i>Ion 550™ Kit – Chef User Guide</i> (Pub. No. MAN0017275)

To create a specific Run Plan for use in templating and sequencing see “Create a Run Plan” on page 57. Refer to the appropriate user guide listed in the table for more information.



Create an assay-specific Planned Run

- About Run Plans 55
- Create a custom Run Plan template 55
- Create a Run Plan 57

IMPORTANT! These kits are compatible with Torrent Suite™ Software 5.12 or later and Ion Reporter™ Software 5.12 or later. Before proceeding, check for updates to the Torrent Suite™, Ion Reporter™, and Ion Chef™ System software, and install the updates if available. For more information, see “Enable off-cycle product updates” on page 98.

About Run Plans

Run Plans contain all the settings that are used in a sequencing run, including number of flows, kit types, barcodes, sample information, and reference files (if any). Run Plans are used to track samples, chips, and reagents throughout the sequencing workflow, from template preparation through sequencing and subsequent data analysis.

The Torrent Suite™ Software 5.12 contains 2 pre-installed Run Plan templates for use with the Ion AmpliSeq™ Mouse Immune Repertoire Panels.

Application	Torrent Suite™ Software 5.12 templates ^[1]	Description
Immune Repertoire	Ion AmpliSeq™ Mouse BCR IGH-SR for S5	RNA (cDNA) only input Run Plan template for template preparation, and sequencing of the Ion AmpliSeq™ Mouse BCR IGH-SR Panel using the Ion Chef™ and Ion GeneStudio™ S5 Series Systems. RNA (cDNA) or DNA only input Run Plan template for template preparation, and sequencing of the Ion AmpliSeq™ Mouse BCR IGH-SR Panel using the Ion Chef™ and Ion GeneStudio™ S5 Series Systems.
	Ion AmpliSeq™ Mouse TCRB-SR for S5	RNA (cDNA) only input Run Plan template for template preparation, and sequencing of the Ion AmpliSeq™ Mouse TCR Beta-SR Panel using the Ion Chef™ and Ion GeneStudio™ S5 Series Systems. RNA (cDNA) or DNA only input Run Plan template for template preparation, and sequencing of the Ion AmpliSeq™ Mouse TCR Beta-SR Panel using the Ion Chef™ and Ion GeneStudio™ S5 Series Systems.

^[1] Users running earlier versions of Torrent Suite™ Software must create a custom Run Plan template, see page 55 for more information.

Create a custom Run Plan template

We recommend setting up a customized Run Plan template for reuse when the same conditions will be used for multiple runs. For more information about creating Run Plans manually or from the generic application template, see the *Torrent Suite™ Software Help* available through the **Help ▶ Software Help** menu in the Torrent Suite™ Software.

1. Sign in to the Torrent Suite™ Software for the Ion Torrent™ Server connected to your Ion Chef™ System.
2. In the **Plan** tab, in the **Templates** screen, click **Immune Repertoire** in the research application list.

3. In the **Immune Repertoire** list, find the **Template Name** that corresponds to your assay, then click **⚙️ (Actions) ▶ Copy**.
The copy template workflow bar opens to the **Save** step.
4. Enter a name for the Run Plan template.
Note: The **Reference Library** and **Target Regions** fields are not required for the Ion AmpliSeq™ Mouse Immune Repertoire Panels.
5. In the **Ion Reporter** step, set up the transfer of the completed run results to your Ion Reporter™ Server.
 - a. Select your Ion Reporter™ account.
 - b. In the **Existing Workflow** dropdown list, select the appropriate Ion Reporter™ workflow for your Planned Run.
 - c. Click **Next**.**Note:** If the Ion Reporter™ Software is not configured, configure it through Ion Reporter Configure settings (see page 97).
6. In the **Research Application** step, ensure that **Immune Repertoire** is selected under **Research Application** and that **Mixed Samples (DNA/RNA)** is selected under **Target Technique**, then click **Next**.
7. In the **Kits** step, select the **Ion Chef Template Kit** radio button, then complete the following fields.

Field	Assay
Instrument	Ion GeneStudio™ S5 System
Library Kit Type	Ion AmpliSeq™ Library Kit Plus
Template Kit	See the following table.
Sequencing Kit	Ion S5™ Sequencing Kit
Base Calibration Mode	Default Calibration
Chip Type	Ion 520™ Chip <i>or</i> Ion 530™ Chip <i>or</i> Ion 540™ Chip <i>or</i> Ion 550™ Chip
Barcode Set	Ion Torrent™ Dual Barcode Kit 1–96
Flows	500

Chip size used	Templating kit used
Ion 520™ Chip	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef
Ion 530™ Chip	
Ion 540™ Chip	Ion 540™ Kit – Chef
Ion 550™ Chip	Ion 550™ Kit – Chef

8. Select or edit the optional information fields appropriately for your run, then click **Next**.
9. Review the **Projects** step and make selections appropriate to your run, then click **Next**.
10. In the **Save** tab, click **Copy Template** to save the new Run Plan template.

The customized template is now available in the **Immune Repertoire** list of templates.

Create a Run Plan

1. Sign in to the Torrent Suite™ Software for the Ion Torrent™ Server connected to your Ion Chef™ System.
2. In the **Plan** tab, in the **Templates** screen, click **Immune Repertoire** in the research application list.
3. In the **Immune Repertoire** list, click on your customized Run Plan template name. Alternatively click **⚙ (Actions) ▶ Plan Run**.
The **Create Plan** workflow bar opens to the **Plan** step.
4. Enter a **Run Plan Name**.
5. In the **Number of barcodes** field, enter the number of barcodes that will be used in this run, then click the check mark button to the right of this field.
6. In the **Sample Tube Label** field(s), enter or scan the barcode of the Ion Chef™ Library Sample Tube that will be used in the run.
7. For each sample select the **Barcode** that will identify it, then enter or select from the available dropdown list the appropriate information for each field (all fields are required except **Sample Description** and **Sample ID**).

IMPORTANT! **Sample Names** should be unique to each sample. The Ion Reporter™ Software will group all BAM files with the same sample name for aggregate analysis and be analyzed together. This feature can be used to increase sequencing read depth by combining results if desired. Sample BAM files may also be designated in the Ion Reporter™ Software for aggregate analysis at a later time.

8. Click **Plan Run**.

The run is listed in the **Planned Runs** screen under the name that you specified and is automatically used by the Ion Chef™ System when the associated Ion Chef™ Library Sample Tube is loaded.

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Analysis workflows in Ion Reporter™ Software

If the appropriate Ion Reporter™ Software workflow was selected in your Planned Run in the Torrent Suite™ Software, automated analysis was already performed and you can view the analysis results in the Ion Reporter™ Software. For instructions on manually launching an analysis, see “Manually launch an analysis in Ion Reporter™ Software” on page 64.

Note: Microsoft™ Excel™, or other spreadsheet tool, is required for viewing VCF, CSV, and TSV files.

The Ion Reporter™ Software 5.12 contains the following workflows for use with the Ion AmpliSeq™ Mouse Immune Repertoire Assays:

Analysis Workflow	Description
For use with Ion GeneStudio™ S5 Series Systems	
Ion AmpliSeq Mouse TCRB-SR - w1.2 - DNA - Single Sample	Analyze mouse T-cell receptor beta chains amplified from gDNA or cfDNA using Framework 3 and Joining Gene targeting primers. The short amplicon (80 bp average length) provides coverage of the CDR3 region to enable clone tracking and measurements of T-cell diversity from degraded material including FFPE-treated gDNA and cfDNA. Reports the frequency and sequence features of clonotypes and performs secondary analyses of repertoire features. Uses the IMGT® database of variable, diversity, and joining genes as a reference. Released with: Ion Reporter™ Software 5.12. Workflow Version: 1.2

Analysis Workflow	Description
Ion AmpliSeq Mouse TCRB-SR - w1.2 - RNA - Single Sample	Analyze mouse T-cell receptor beta chains amplified from fresh or FFPE-treated RNA/cDNA using Framework 3 and Joining Gene targeting primers. The short amplicon (80 bp average length) provides coverage of the CDR3 region to enable clone tracking and measurements of T-cell diversity from degraded material including FFPE-treated RNA. Reports the frequency and sequence features of clonotypes and performs secondary analyses of repertoire features. Uses the IMGT® database of variable, diversity, and joining genes as a reference. Released with: Ion Reporter™ Software 5.12. Workflow Version: 1.2
Ion AmpliSeq Mouse BCR IGH-SR - w1.2 - DNA - Single Sample	Analyze mouse B cell receptor IGH chains amplified from gDNA or cfDNA using Framework 3 and Joining Gene targeting primers. The short amplicon (80 bp average length) provides coverage of the CDR3 region to enable clone tracking and measurements of B-cell diversity from degraded material including FFPE-treated gDNA and cfDNA. Reports the frequency and sequence features of clonotypes, identifies clonal lineages, and performs secondary analyses of repertoire features. Uses the IMGT® database of variable, diversity, and joining genes as a reference. Released with: Ion Reporter™ Software 5.12. Workflow Version: 1.2
Ion AmpliSeq Mouse BCR IGH-SR - w1.2 - RNA - Single Sample	Analyze mouse B cell receptor IGH chains amplified from non-FFPE RNA/cDNA using Framework 3 and Joining Gene targeting primers. The short amplicon (80 bp average length) provides coverage of the CDR3 region to enable clone tracking and measurements of B-cell diversity from degraded material including FFPE-treated RNA. Reports the frequency and sequence features of clonotypes, identifies clonal lineages, and performs secondary analyses of repertoire features. Uses the IMGT® database of variable, diversity, and joining genes as a reference. Released with: Ion Reporter™ Software 5.12. Workflow Version: 1.2

Clonotype identification

Immune repertoire analysis is performed in the Ion Reporter™ Software by selecting an immune repertoire assay workflow that reports the frequency and sequence features of clonotypes, and performs secondary analyses of repertoire features.

If, when setting up your Planned Run, you selected and configured your Ion Reporter™ account and the proper immune repertoire assay workflow, and selected the option to **Automatically upload to Ion Reporter™ after run completion**, your assay results will be available to view when you sign in to the Ion Reporter™ Software.

Alternatively, you can use the IonReporterUploader plugin to manually upload your sequencing results to Ion Reporter™ (see page 62 for more information) following completion of a sequencing run. When the data transfer is complete, manually launch the workflow analysis (see “Manually launch an analysis in Ion Reporter™ Software” on page 64 for more information).

The Immune Repertoire Results Report includes sample and quality control results for each sample that is included in the analysis.

Note: Assay samples in the Ion Reporter™ Software are listed as being of "Fusions" sample type instead of "RNA" sample type.

Data analysis workflow

Low quality and off-target reads are removed from the analysis



Error containing reads are corrected or removed from the analysis



VDJ rearrangements are reported



Secondary analysis of repertoire features

Review sequencing run results

After your sequencing run completes, review the results and quality metrics in the **Run Summary** page.

1. Under the **Data** tab, in the **Completed Runs & Reports** screen, use the search, filter, and sort functions to find your run.
2. Click the **Report Name** link to open the **Run Summary** page for your run.
3. View the Read length histogram.

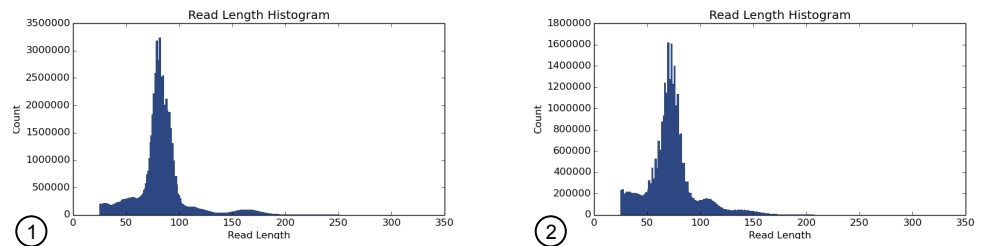


Figure 3 Read length histograms

- ① Ion AmpliSeq™ Mouse TCR Beta-SR Assay. Confirm that the peak is centered at approximately 85 nt.
 - ② Ion AmpliSeq™ Mouse BCR IGH-SR Assay. Confirm that the peak is centered at approximately 75 nt.
4. (Optional) If you haven't set your data to automatically upload to your Ion Reporter™ server. Click **Upload to IR**, select your Ion Reporter™ server, then click **Upload just BAM**.

IMPORTANT! Before uploading your data to your Ion Reporter™ server, we recommend that you update the IonReporterUploader plugin in your Torrent Suite™ Software to the latest version. See “Configure the IonReporterUploader 5.12 plugin in Torrent Suite™ Software” on page 97 for more information.

Upload sequence files with IonReporterUploader

Ion Reporter™ Software uses the Torrent Suite™ Software output BAM file for analysis. You can configure the IonReporterUploader plugin to run as part of a Planned Run or Planned Run template. You can also manually run the IonReporterUploader plugin from a completed Run Summary to transfer the BAM output files to Ion Reporter™ Software.

1. In the **Data** tab, click **Completed Runs & Reports**, then click the **Report Name** link for your completed sequencing run.
2. From the **Run Summary** page, you can upload the entire sequencing run or select results for individual barcodes to be uploaded.

To	Steps
Upload the entire sequencing run	<ol style="list-style-type: none"> 1. Click the Upload to IR button in the run report header. 2. Select your Ion Reporter™ Server from the dropdown list. 3. Click the button to Upload just BAM files, the upload begins automatically.
Select individual barcodes for upload	<ol style="list-style-type: none"> 1. Click the Plugins link, or scroll down to the Plugins section. 2. Click Plugins ▶ Select Plugins to Run, then select IonReporterUploader from the list. 3. Click to expand the list of barcodes under Barcode Sample Settings, then select the checkbox for each barcoded sample to upload. By default, all samples are selected for uploading. 4. Select the type of output file (BAM) to transfer from the Upload Options list. 5. Click Launch IRU in the row next to the Ion Reporter™ Server account that you want to upload the files to. 6. Click Yes to confirm that you want to upload the data.

When the upload completes, **Sign in** to your Ion Reporter™ Software, then launch an analysis on the new datasets.

Define multiple BAM files as a sample

Sequence results from multiple libraries prepared from the same sample or multiple sequencing results from the same library can be combined for increased analytical power. To define the sample:

1. Sign in to the Ion Reporter™ Software.
2. In the **Samples** tab, in the **Overview** screen, click **Define Samples**, then select **Manual** from the dropdown list.
3. (Optional) Click **⊕ Upload BAM**, click **Select File**, then navigate to and select the desired BAM file. Click **Open**.
4. Select one or more BAM files from the list of available files, then click **Add to Sample**.
5. Enter a **Sample Name**, then click **Add to Sample List**.
6. (Optional) To define additional samples, repeat step 3 to step 5.
7. Click **Next**.
8. Click **Add Attribute**, then select the **Percent Cellularity** attribute from the dropdown list. Select additional attributes as desired.
Note: The **Percent Cellularity** attribute is required. Other attributes are optional.
9. Enter or select attribute values in the respective **Attribute** fields, then click **Next**.
10. Ensure the sample information is correct, then click **Next**.


The new sample is added to the **Samples** list.

Manually launch an analysis in Ion Reporter™ Software

1. Sign in to the Ion Reporter™ software.
2. Launch the analysis.

From the...	Directions
Home tab	<ol style="list-style-type: none"> 1. In the Dashboard screen, click Launch analysis. 2. In the Launch Analysis screen, select Immune Repertoire from the Research Application dropdown list. 3. Click Next.
Analyses tab	<ol style="list-style-type: none"> 1. Click Launch Analysis, then select Manual from the dropdown list. 2. In the Launch Analysis screen, select Immune Repertoire from the Research Application dropdown list. 3. Click in the appropriate row, <ul style="list-style-type: none"> • Ion AmpliSeq Mouse TCRB-SR - w1.2 - DNA - Single Sample • Ion AmpliSeq Mouse TCRB-SR - w1.2 - RNA - Single Sample • Ion AmpliSeq Mouse BCR IGH-SR - w1.2 - DNA - Single Sample • Ion AmpliSeq Mouse BCR IGH-SR - w1.2 - RNA - Single Sample then click Next. <p>Note: Workflows designated w1.0 may be available if upgrading from Ion Reporter™ Software 5.10.</p>
Workflows tab	<ol style="list-style-type: none"> 1. Select Immune Repertoire from the Research Application dropdown list. 2. Click in the appropriate row, <ul style="list-style-type: none"> • Ion AmpliSeq Mouse TCRB-SR - w1.2 - DNA - Single Sample • Ion AmpliSeq Mouse TCRB-SR - w1.2 - RNA - Single Sample • Ion AmpliSeq Mouse BCR IGH-SR - w1.2 - DNA - Single Sample • Ion AmpliSeq Mouse BCR IGH-SR - w1.2 - RNA - Single Sample then select Launch Analysis from the Actions dropdown list in the Details pane. <p>Note: Workflows designated w1.0 may be available if upgrading from Ion Reporter™ Software 5.10.</p>

3. Search by any unique identifier you used to label your samples during setup, select one or more samples to include in the analysis, then click **Next**.

4. (Optional) Select plugins to run with your analysis.
 - a. Click  (**Actions**) in the row of the selected plugin.
 - b. Configure the plugin parameters as necessary, click **Submit**, then click **Close**.
5. Click **Next**.
6. Enter an **Analysis Name** and **Description (Optional)** , then click **Launch Analysis**.

The Ion Reporter™ Software performs the analysis and the results will appear on the **Analyses** page.

Multi-sample analysis

The Ion Reporter™ Software can perform multi-sample (or cross-sample) analyses to compare the immune repertoire between samples. To perform a multi-sample analysis:

1. Under the **Analyses** tab, in the **Overview** screen, select the appropriate workflows from the Workflow filter.
2. Click the adjacent checkboxes to select 2 or more results files from the available list of analyses, then click **Visualize**.
3. Select the **Join Type** from the dropdown list. Selections provide increasing stringency from top to bottom of the list.
 - CDR3NT
 - Variable+CDR3NT
 - Variable+CDR3NT+Variable Gene SHM positions
4. Select 2 or more samples for multi-sample analysis, then click **Compare Samples**.
5. Click **Comparison Table** or **Comparison Plots** to view results of the comparison.

Result output	Description
Comparison Table	Multi-sample analysis results are summarized with each row representing an individual clone in rank order from the most frequently occurring to least frequent. For more information, see "Clone summary table" on page 77. You can also download the multi-sample clone summary table or the multi-sample metrics file (see "Download the metrics file" on page 79).
Comparison Plots	
Correlation Scatterplot	Scatterplot indicating the frequency of clones across two samples. For more information see "Comparison plots" on page 78.
Jaccard Similarity Index	The Jaccard similarity index is determined for each pairwise comparison and displayed in heatmap form. For more information see "Comparison plots" on page 78.



Results – Ion AmpliSeq™ Mouse TCR Beta-SR Assay

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View the Ion Reporter™ analysis results

If you selected an Ion Reporter™ workflow when setting up your Planned Run in Torrent Suite™ Software, automated analysis has already been performed and you can view the analysis results in the Ion Reporter™ Software. For instructions on manually launching an analysis, see “Manually launch an analysis in Ion Reporter™ Software” on page 64.

Note: Assay analyses in the Ion Reporter™ Software are searchable by Analysis name, Sample name, and Project, but not by barcode.

1. Sign in to the Ion Reporter™ Software.
2. Click the **Analyses** tab.
The **Analyses** tab opens to the **Overview** screen that displays the **Analyses** table.
3. In the **Workflow** dropdown list above the **Analyses** table, search for "TCRB" to limit the table to display Immune Repertoire analyses results.
You can further refine the list of analyses by using filters, or clicking column headings to sort the list.
4. Select a row of the analysis of interest to view the analysis details in the **Details** pane.
5. Click **Visualize**. Alternatively, in the **Analyses** table, click the link in the **Analysis** column to open the **Immune Repertoire Results** summary screen.
Note: See “Multi-sample analysis” on page 65 to perform a multi-sample analysis.
6. In the **Immune Repertoire Results** summary screen, click the link in the **Sample** column to open the **Immune Repertoire Results** summary for that sample.
7. Click either the **Sample Results** or **Sample QC** tab, then select the graphical representation of the data from the **Views** dropdown list.

Sample QC tab

The Ion AmpliSeq™ Mouse TCR Beta-SR Assay results are represented graphically on the **Sample QC** tab.

Select the QC metric to view from the **Views** list. The QC metrics are explained below.

Read classification

After the first stage of data processing, raw sequencing reads are classified and proportionally represented in a stacked barplot. Actual read counts for each classification are listed below the figure in the results report.

Read classification	Description	Example
Off-target/low-quality (dark gray)	Reads that are of low quality or represent the product of an off-target amplification.	
Unproductive (gray)	Reads that have uncorrectable sequencing or PCR errors that lead the rearrangement to have out-of-frame variable and joining genes or a premature stop codon.	
Rescued productive (light blue)	Reads that have an in-frame variable and joining gene, and no stop codons after INDEL error correction.	
Productive (blue)	Reads that have an in-frame variable and joining gene, and no stop codons.	

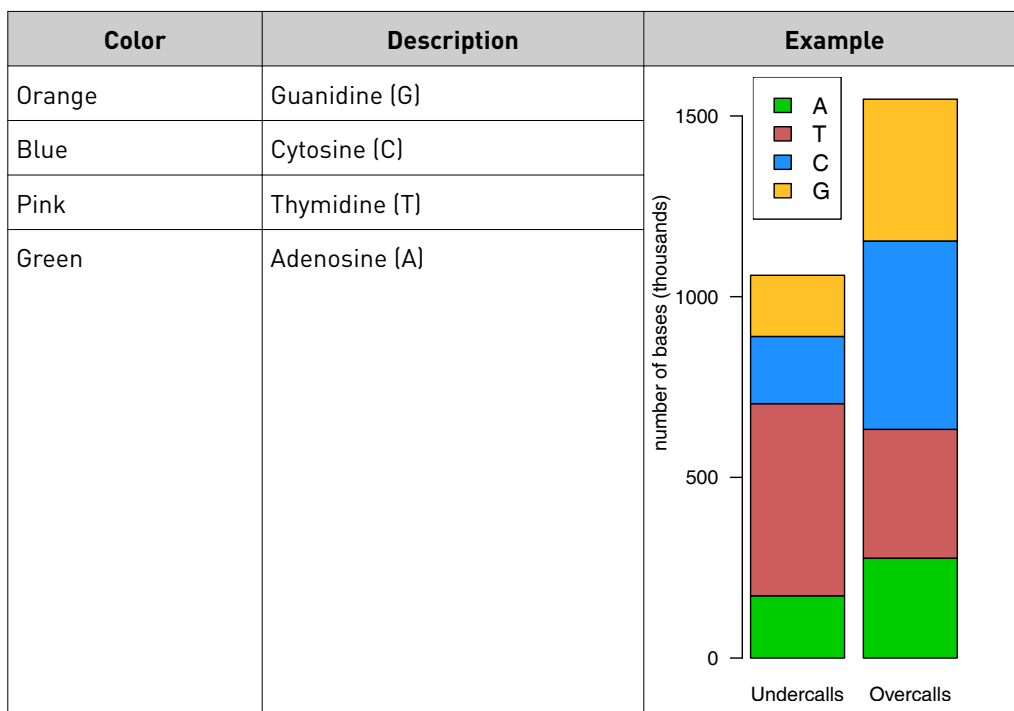
Proportion of full length, quality trimmed and reads lacking P1-key, by read classification

This stacked barplot indicates the frequency of quality trimming for reads classified as productive, rescued productive, unproductive, and off-target/low-quality. Full-length reads categorized as low quality/off-target likely represent off-target amplifications.



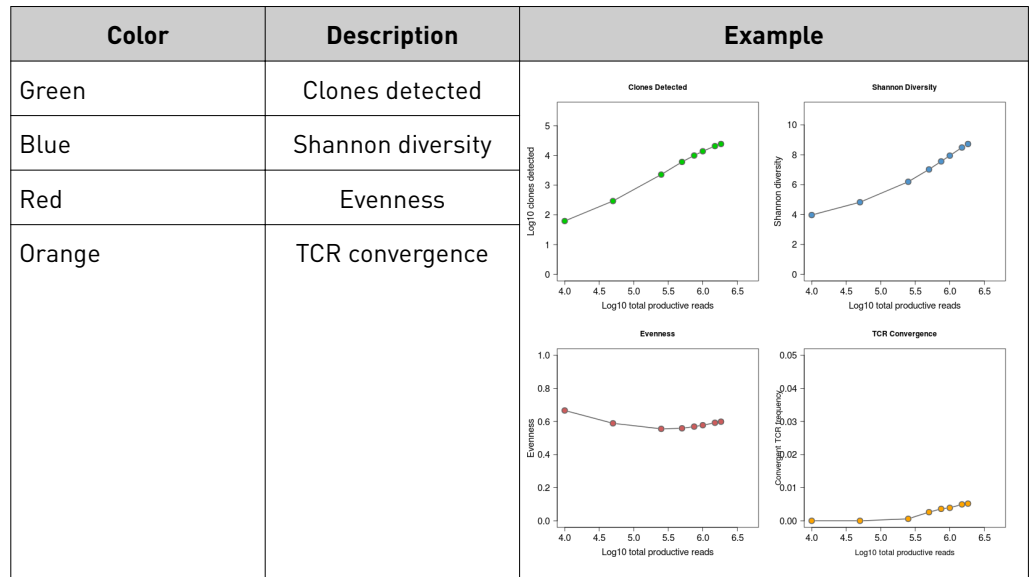
Base composition of overcalled and undercalled homopolymers

This stacked barplot indicates the nucleotide composition of overcalled bases (base insertion sequencing errors) and undercalled bases (base deletion sequencing errors). Highly-skewed nucleotide composition may indicate lower quality sequencing or low library diversity.



Downsampling analysis

Downsampling is achieved by repeating clone identification and measurement of repertoire features using 10 K, 50 K, 250 K, 500 K, 750 K, 1 M, 1.5 M, 2 M, and 5 M randomly selected productive and rescued productive reads, contingent on sequencing depth. The graphs show the effect of sequencing depth on select repertoire features: number of clones detected, clone evenness, Shannon diversity, and the convergent TCR frequency. Values for these repertoire metrics that are displayed in this plot are provided in the metrics file. Clone summary and lineage summary files that are derived from downsampled data are provided in the 'downsampling' subdirectory of the zipped results download file. If insufficient reads are available for a particular downsampling depth, the corresponding fields are assigned a 'NA' value in the metrics file.



QC metrics

The QC metrics include the read classification counts and strand QC metrics.

Category	Description
Read classification	
Total productive reads	Productive + rescued productive reads.
Productive reads	Reads having an in-frame variable and joining gene, and no stop codons.
Rescued productive reads	Reads having an in-frame variable and joining gene, and no stop codons after INDEL error correction.
Unproductive reads	Reads that have uncorrectable sequencing or PCR errors that lead the rearrangement to have out-of-frame variable and joining genes or a premature stop codon.
Off-target/ low-quality reads	Reads which are of low quality or represent the product of an off-target amplification.

Category	Description
Strand QC metrics	
Plus strand (v-side) read counts	Number of sequence read counts from the plus (+) strand.
Minus strand (c-side) read counts	Number of sequence read counts from the minus (-) strand.
Plus strand CDR3 avg PHRED	Average PHRED score for plus (+) strand reads.
Minus strand CDR3 avg PHRED	Average PHRED score for minus (-) strand reads.



Sample Results tab

The assay results are represented graphically on the **Sample Results** tab.

Select the desired results output to view from the **Views** list.

Spectratyping plots

The immune repertoire within a sample is represented in each spectratyping plot by the range of CDR3 lengths and their pattern of distribution. Reads for identified clones are arranged along the X-axis according to the variable gene identity and the Y-axis according to the CDR3 nucleotide length. The variable gene order reflects the gene position within the TCR β locus. Dots are separated vertically along the Y-axis by 3 nucleotides (one codon), the higher up the Y-axis the longer the CDR3 region. Circle size indicates the frequency of a particular variable gene-CDR3 nucleotide length combination within the dataset. Circle color represents a fourth metric specific to each graph (for example, Shannon Diversity, evenness, clone frequency). Key repertoire metrics are displayed along the lower margin of the plot.

In the Ion Reporter™ Software, the spectratyping plots are interactive, allowing you to adjust the data and access clone details. Drag the ends of the horizontal bar below the X-axis to limit the region (v-genes) to view in the plot. Drag the ends of the color range up or down to limit the clones that are viewed. Hover your cursor over any dot to view the details of an individual clone. Click  to restore the default plot view, click  to download a static image of the plot (if you have adjusted the plot view, the adjusted plot is downloaded).

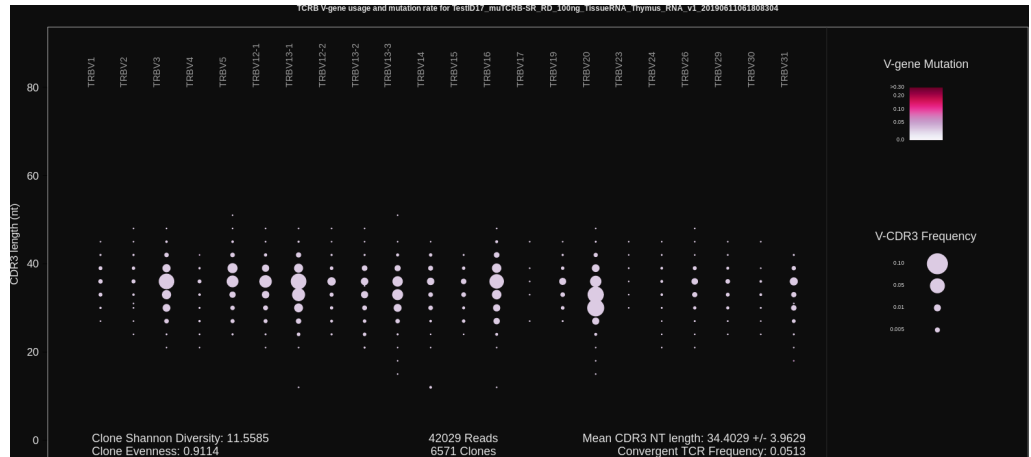


Figure 4 TCRB V-gene usage and mutation rate

Spectratyping plot highlighting frequency of mutated bases over the variable gene of identified clones. Circle color indicates the average frequency of mutated bases for clones having a particular variable gene-CDR3 nucleotide length combination. The two large circles in the TRBV3-1 column indicate the presence of expanded clones having that particular variable gene. The light color of the circles indicates that the variable gene sequences of the sample closely match reference variable gene sequences in the IMGT database. Systematic differences for reference may indicate the presence of polymorphism within the variable gene that is not captured by the IMGT database.

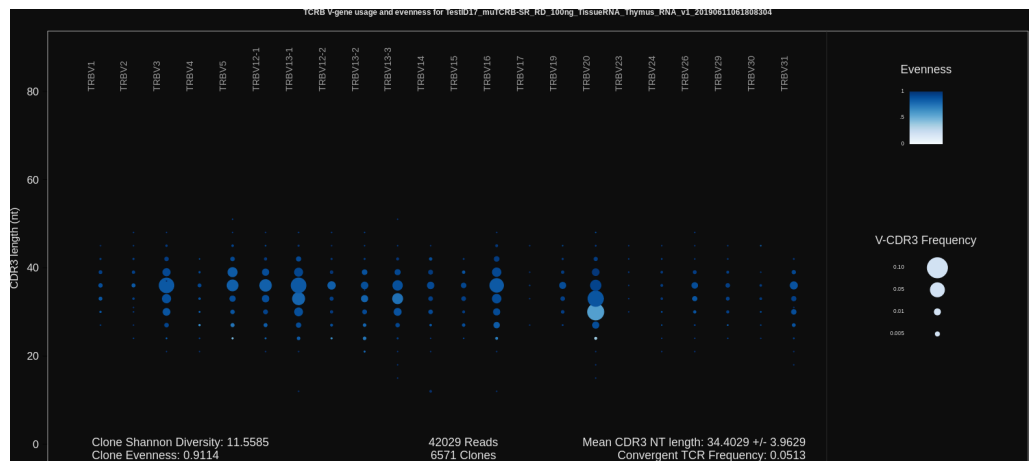


Figure 5 TCRB V-gene usage and evenness

Spectratyping plot highlighting evenness of identified clone sizes (Normalized Shannon Entropy). Circle color indicates the evenness of clone sizes for clones from 0 to 1, with 1 indicating most even clone sizes. In this representation, portions of the repertoire containing highly expanded clones appear white.

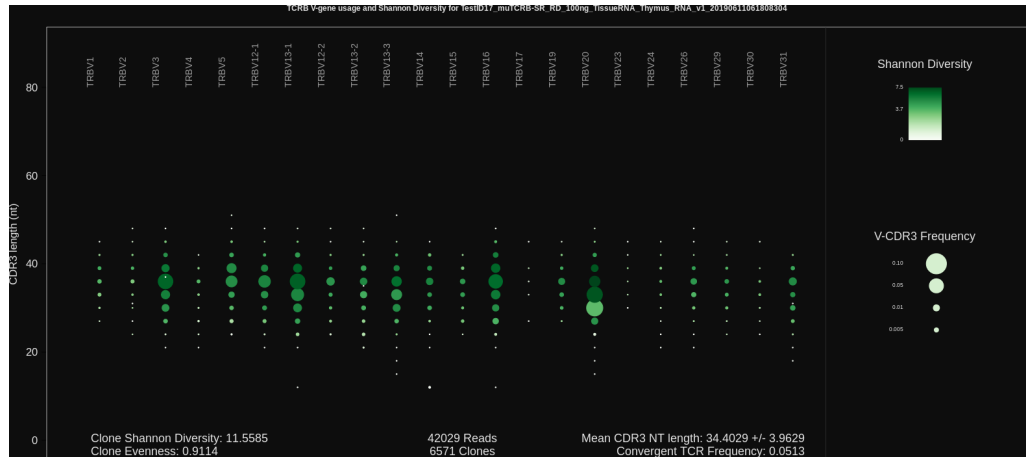


Figure 6 TCRB V-gene usage and Shannon Diversity

Spectratyping plot highlighting Shannon Diversity (entropy) of identified clones. Circle color indicates the Shannon Diversity of clones having a particular variable gene-CDR3 nucleotide length combination. Portions of the repertoire containing highly expanded clones typically have a corresponding low Shannon Diversity value.

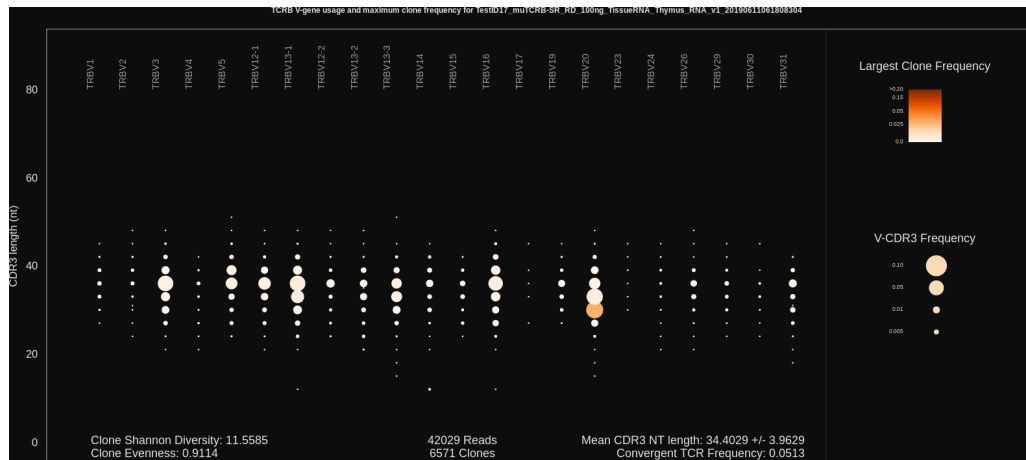


Figure 7 TCRB V-gene usage and largest clone frequency

Spectratyping plot highlighting the frequency of the largest clone for each variable gene-CDR3 nucleotide length combination. Circle color indicates the frequency of the largest clone having a particular variable gene-CDR3 nucleotide length combination. Dark color indicates the presence of expanded clones. These expanded clones may indicate a response to antigen.

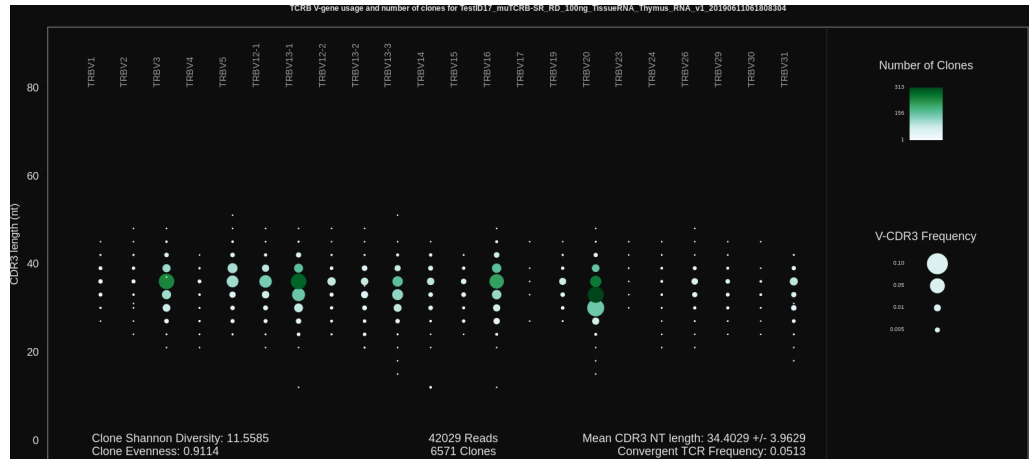


Figure 8 TCRB V-gene usage and number of clones

Spectratyping plot highlighting the number of clones that are identified for each variable gene-CDR3 nucleotide length combination. Circle color indicates the number of clones having a particular variable gene-CDR3 nucleotide length combination. Specific variable genes may more frequently participate in VDJ recombination, leading to an enrichment in distinct clones for those variable genes.

V-gene usage

The Ion AmpliSeq™ Mouse TCR Beta-SR Assay produces a graphical representation of the frequency of Variable genes and alleles in a sample. Select **V gene usage** from the **Views** list. The stacked barplots indicate the representation of Variable genes among identified clones. Ordering of Variable genes reflects position within the TCRβ locus.

Note: Variable gene and allele identification is most accurate when using long-amplicon sequencing covering all three CDR domains.

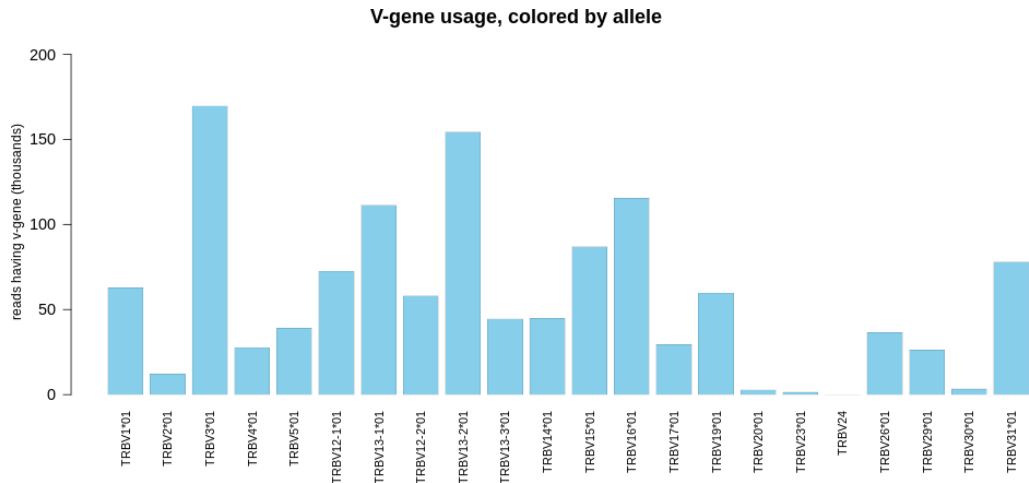


Figure 9 V-gene usage highlighting alleles

Color segments within each bar indicate the frequency of particular Variable gene alleles, arranged by frequency from rarest (top) to most common (bottom).

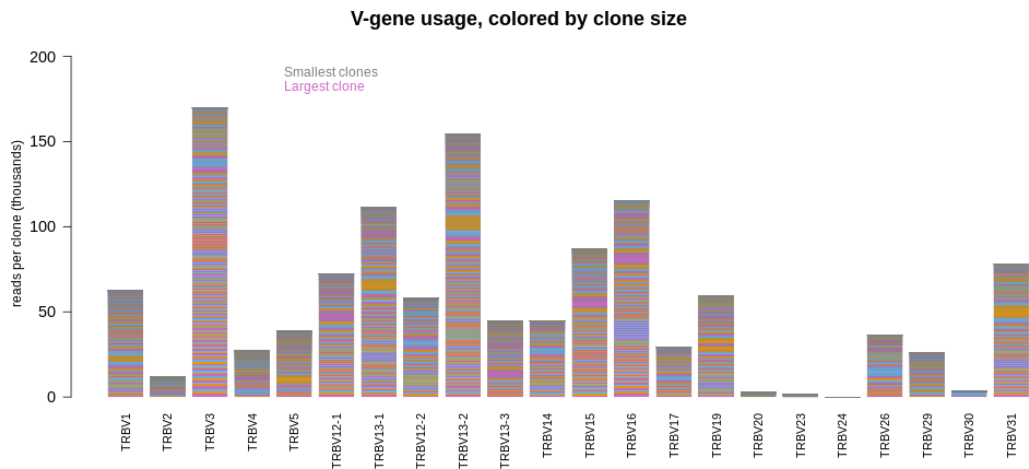


Figure 10 V-gene usage

VJ-gene usage heatmap

The Ion AmpliSeq™ Mouse TCR Beta-SR Assay produces a graphical representation of the frequency of each Variable gene - Joining gene combination for identified clones. Select **VJ-gene usage heatmap** from the **Views** dropdown list to see heatmaps that represent the results.

Note: Variable gene and allele identification is most accurate when using long-amplicon sequencing covering all three CDR domains.

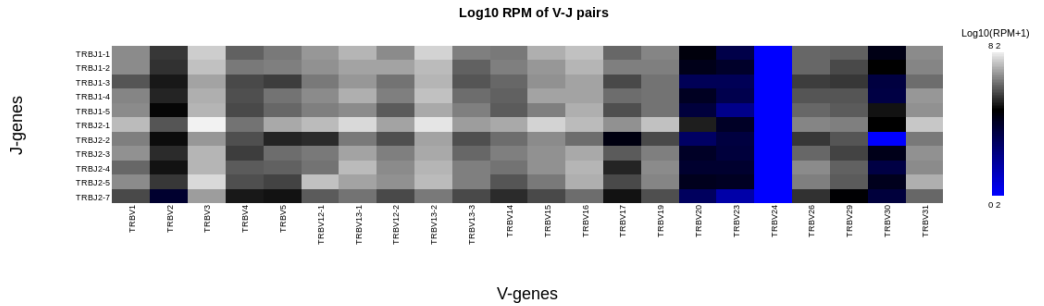


Figure 11 VJ-gene usage heatmap

Heatmap illustrating the frequency of particular Variable gene-Joining gene combinations for identified clones. Frequencies are log transformed with pseudocount added. Over-represented Variable-Joining gene combinations (white) may indicate presence of a highly expanded clone. Ordering of variable genes reflects position within the TCRB locus.

Clones detected per variable gene

The Ion AmpliSeq™ Mouse TCR Beta-SR Assay produces a graphical representation of the Variable genes usage among detected clones. Select **Clones detected per variable gene** from the **Views** list.

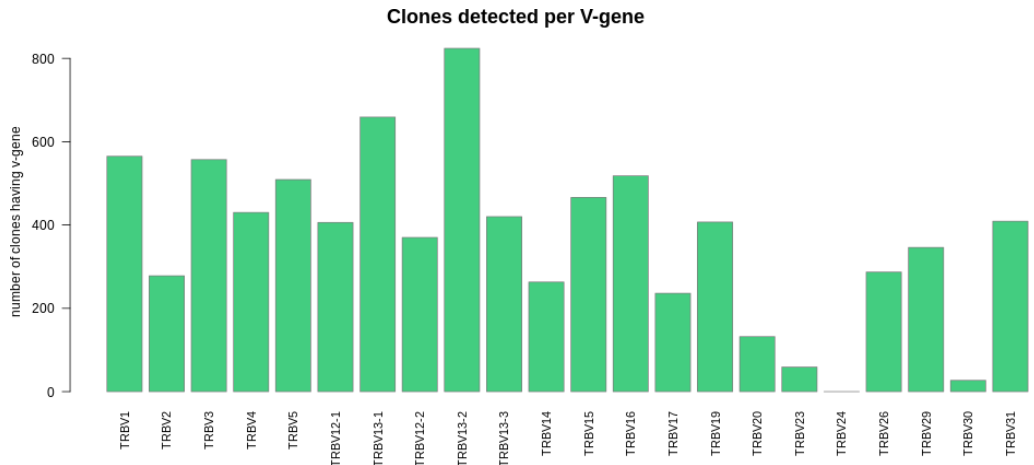


Figure 12 Clones detected per V-gene

The barplot indicates the number of identified clones having a particular Variable gene. Ordering of Variable genes reflects position within the TCRB locus.

CDR3 histogram

The Ion AmpliSeq™ Mouse TCR Beta-SR Assay produces a graphical representation of the CDR3 lengths of clones that are detected in a sample. Select **CDR3 histogram** from the **Views** list.

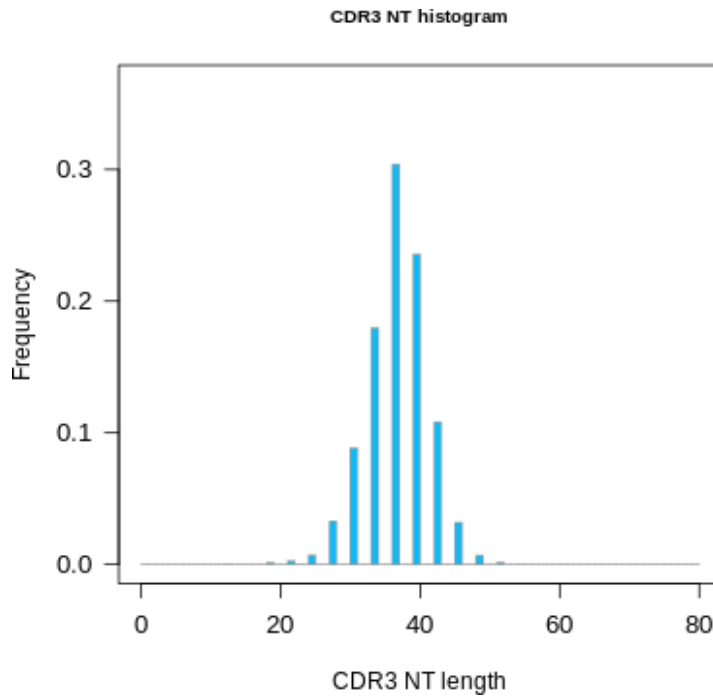


Figure 13 Relative frequency vs CDR3 nucleotide length of identified clones

The histogram indicates the distribution of CDR3 lengths for clones identified in the sample.

Clone summary table

The **Clone Summary** table lists the identified clones in rank order from the most frequently occurring to least frequent. Each row represents an individual clone. For each clone the identified **Variable** and **Joining** region are listed as well as the amino acid (**CDR3 AA**) and nucleotide (**CDR3 NT**) sequences of the CDR3 region.

The screenshot shows the 'Immune Repertoire Results' page with a navigation bar (Home, Samples, Analyses, Workflows, Admin) and a sub-navigation bar (Overview, Launch, My Variants). The main content area includes a search bar, a 'Download Clone Summary' button, and a table of clone data. The table has columns for Variable, Joining, CDR3 AA, CDR3 NT, Count, Frequency, and Rank. The first five rows of the table are as follows:

Variable	Joining	CDR3 AA	CDR3 NT	Count	Frequency	Rank
TRBV3-1	TRBJ2-3	ASSQDGGQNTDTQY	GCCAGCAGCCAAGATGGGGGACAGAACACAGATACGCAGTAT	93237	0.1032433	1
TRBV3-1	TRBJ2-1	ASSQQLGEQF	GCCAGCAGCCAACAATTAGGTGAGCAGTTC	53472	0.0592107	2
TRBV7-4	TRBJ2-5	SDPSRETQY	AGTGACCCCGCAGCAGAGACCCAGTAC	29161	0.0322906	3
TRBV10-1	TRBJ1-5	ASSRDGKGSNQPQH	GCCAGCAGCAGAGATGGAAAGGGCAGCAATCAGCCCCAGCAT	24237	0.0268382	4
TRBV11-1	TRBJ2-3	ASSLGTDTQY	GCCAGCAGCCTTGGGACAGATACGCAGTAT	20009	0.0221564	5

Below the table is a pagination control showing '1 - 5 of 35833 items' and a '5 items per page' dropdown.

Click a column heading to sort the table. Frequency = # of reads for the identified clone (**Count**) / total reported reads (sum of **Count** column).

For multi-sample analyses the **Clone Summary** table lists the frequency of each clone identified in any of the samples—sorted in descending order based on the frequency of the clones in the leftmost column—for each sample in the analysis. While you can compare across repertoire workflows, in some instances individual results may include multiple variable gene assignments.

The screenshot shows the 'Immune Repertoire Results' page with a search bar and a 'Download' button. The table displays clone data across multiple samples. The first two rows of the table are as follows:

Variable	Joining	CDR3 AA	CDR3 NT	B212050_RNA_v1_c825_201...	B707172_RNA_v1_c753_201...
TRBV5-6	TRBJ2-2	ASSLGSSELF	GCCAGCAGCTTAGGTTCCGGGGAGCTGTTT	0.0874930	0.0000125
TRBV3-1	TRBJ1-3	ASSQKWTGNTY	GCCAGCAGCCAAGTGGCAGACCCGAAACACCATATAT	0.0183406	0

Annotations 1 and 2 are placed below the table, pointing to the 'B212050_RNA_v1_c825_201...' and 'B707172_RNA_v1_c753_201...' columns respectively.

① Sample 1

② Sample 2

The following information is provided in the **Clone Summary** table.

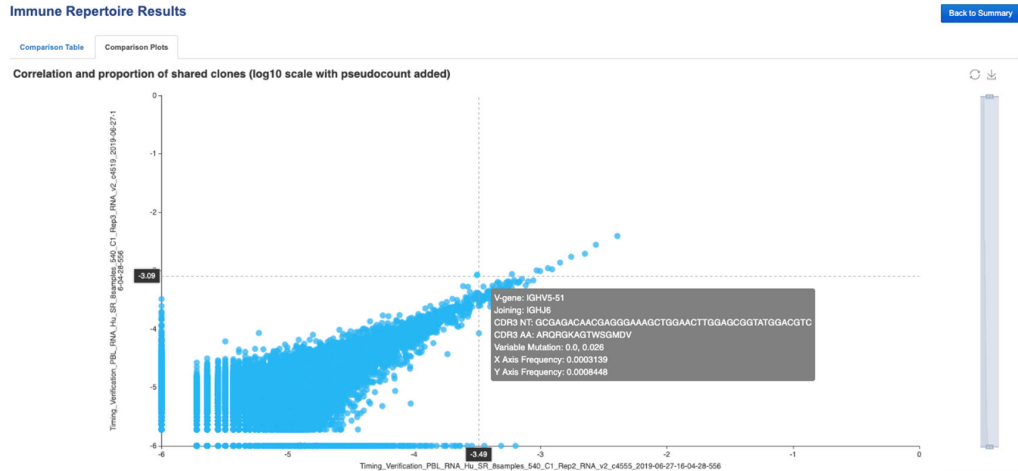
Column name	Description
Variable	The best matching IMGT variable gene of the rearrangement.
Joining	The best matching IMGT joining gene of the rearrangement.
CDR3 AA	The CDR3 amino acid sequence of the rearrangement, denoted using the IMGT definition of the CDR3 region.
CDR3 NT	The CDR3 nucleotide sequence of the rearrangement, denoted using the IMGT definition of the CDR3 region.
Count	The total number of reads mapping to the rearrangement after quality filtering.
Frequency	The frequency of the rearrangement as a proportion of total reads passing quality filtering.
Rank	The frequency rank of the rearrangement.

Note: Additional details are available by downloading the **Clone Summary** table.

Comparison plots

Correlation scatterplot

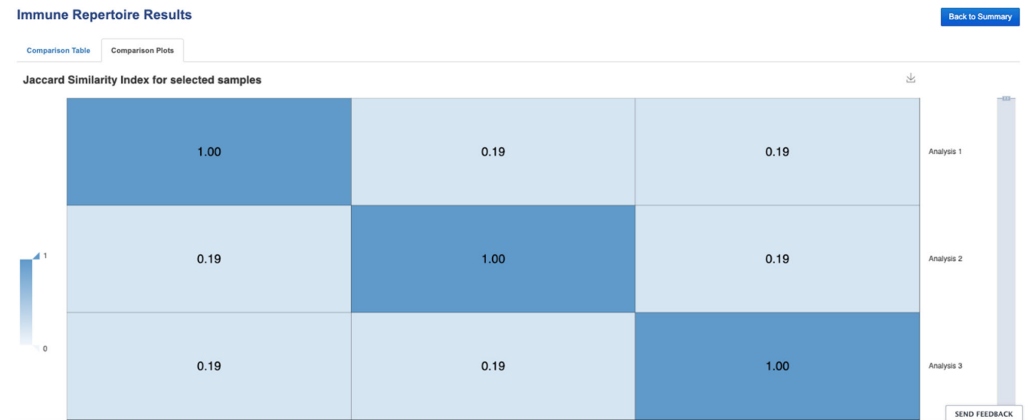
The results of comparing of 2 samples include a scatterplot indicating the frequency of clones across both samples.



Frequency values are log10 transformed with a pseudocount frequency of 1E-6 added to each value. Hovering over a point will reveal the CDR3NT and AA sequence of a clone, the Variable mutation, and the frequency in either sample.

Jaccard similarity index

The results of comparing of 3 or more samples include a Jaccard similarity index plot.



The Jaccard similarity index is determined for each pairwise comparison and displayed in heatmap form. The Jaccard similarity index ranges from 0 to 1 and is calculated as the total number of shared clones divided by the total number of unique clones across two samples.

Download the metrics file

1. In the **Immune Repertoire Results** screen, **Sample Results** tab, select **Clone Summary** from the **Views** dropdown list.
2. Click **Download Clone Summary**.
The clone summary CSV file downloads automatically.
3. Open the clone summary CSV file to view the additional information that is included in the spreadsheet.

See the **Glossary** page 123 for column header descriptions.

Note: The Convergent TCR Frequency metric is new to Ion Reporter™ Software 5.10 or later. TCR convergence refers to a "focusing" of the T cell repertoire that can occur following chronic antigen stimulation. Convergent TCRs are identical in amino acid space but different in nucleotide space (Variable gene + CDR3AA shared with at least one other clone in a sample). The convergent TCR frequency is calculated as the aggregate frequency of clones that are identified as convergent. See also ([bioRxiv doi: 665612](https://doi.org/10.1101/066561)) for a discussion of the potential use of TCR convergence as a predictive biomarker for response to immunotherapy for cancer.



Results – Ion AmpliSeq™ Mouse BCR IGH-SR Assay

- View the Ion Reporter™ analysis results 80
- Sample QC tab 81
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- Clonal lineage identification 90

View the Ion Reporter™ analysis results

If you selected an Ion Reporter™ workflow when setting up your Run Plan in Torrent Suite™ Software, automated analysis has already been performed and you can view the analysis results in the Ion Reporter™ Software. For instructions on manually launching an analysis, see “Manually launch an analysis in Ion Reporter™ Software” on page 64. To view the results:

Note: Assay analyses in the Ion Reporter™ Software are searchable by Analysis name, Sample name, and Project, but not by barcode.

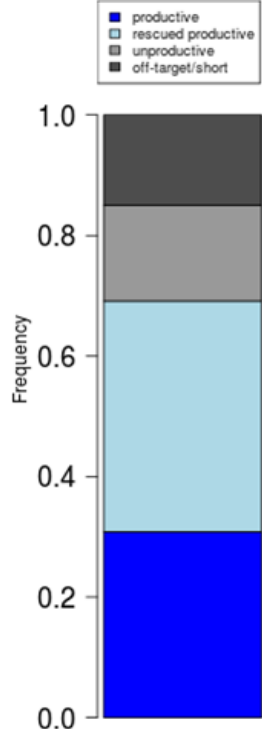
1. Sign in to the Ion Reporter™ Software.
2. Click the **Analyses** tab.
The **Analyses** tab opens to the **Overview** screen that displays the **Analyses** table.
3. In the **Workflow** dropdown list above the **Analyses** table, search for "BCR-IGH" to limit the results displayed.
You can further refine the list of analyses by using filters, or clicking column headers to sort the list.
4. Select a row of the analysis of interest to view the analysis details in the **Details** pane.
5. Click **Visualize**. Alternatively, in the **Analyses** table, click the link in the **Analysis** column to open the **Immune Repertoire Results** summary screen.
Note: See “Multi-sample analysis” on page 65 to perform a multi-sample analysis.
6. In the **Immune Repertoire Results** summary screen, click the link in the **Sample** column to open the **Immune Repertoire Results** summary for that sample.
7. Click either the **Sample Results** or **Sample QC** tab, then select the graphical representation of the data from the **Views** dropdown list.

Sample QC tab

The Ion AmpliSeq™ Mouse BCR IGH-SR Assay results are represented graphically. Select the QC metric to view from the **Views** list.

Read classification

After the first stage of data processing, raw sequencing reads are classified and proportionally represented in a stacked barplot. Actual read counts for each classification are listed below the figure in the results report.

Read classification	Description	Example
Off-target/low-quality (dark gray)	Reads that are of low quality or represent the product of an off-target amplification.	
Unproductive (gray)	Reads that have uncorrectable sequencing or PCR errors that lead the rearrangement to have out-of-frame variable and joining genes or a premature stop codon.	
Rescued productive (light blue)	Reads that have an in-frame variable and joining gene, and no stop codons after INDEL error correction.	
Productive (blue)	Reads that have an in-frame variable and joining gene, and no stop codons.	

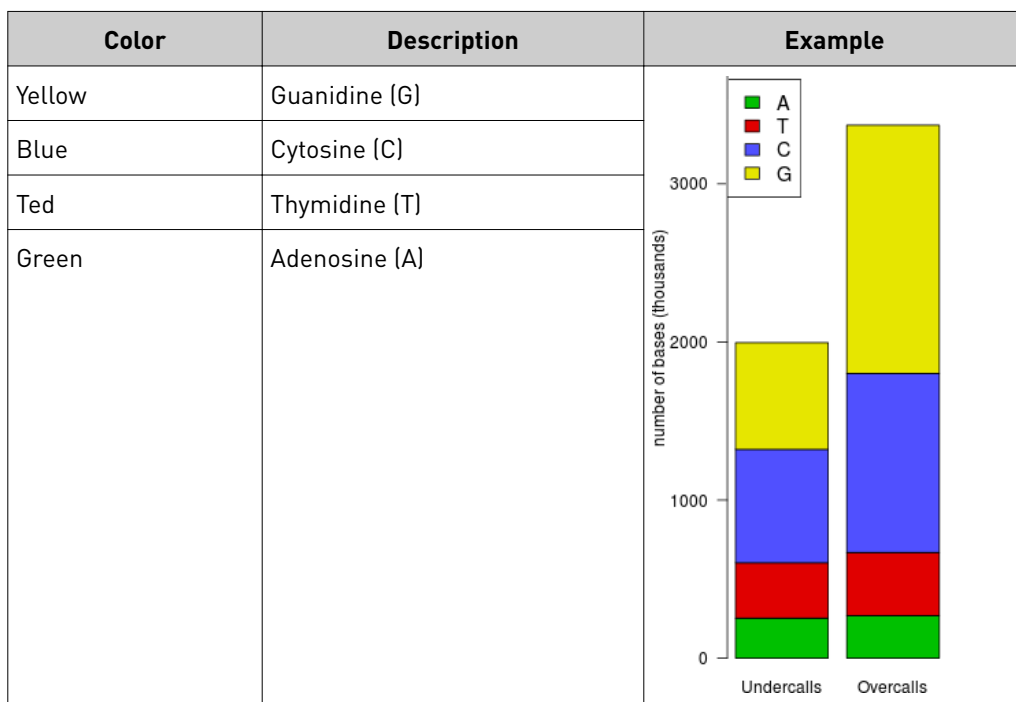
Proportion of full length, quality trimmed and reads lacking P1-key, by read classification

Stacked barplot indicating the frequency of quality trimming for reads classified as productive, rescued productive, unproductive, and off-target/low-quality. Full length reads categorized as low quality/off-target likely represent off-target amplifications.



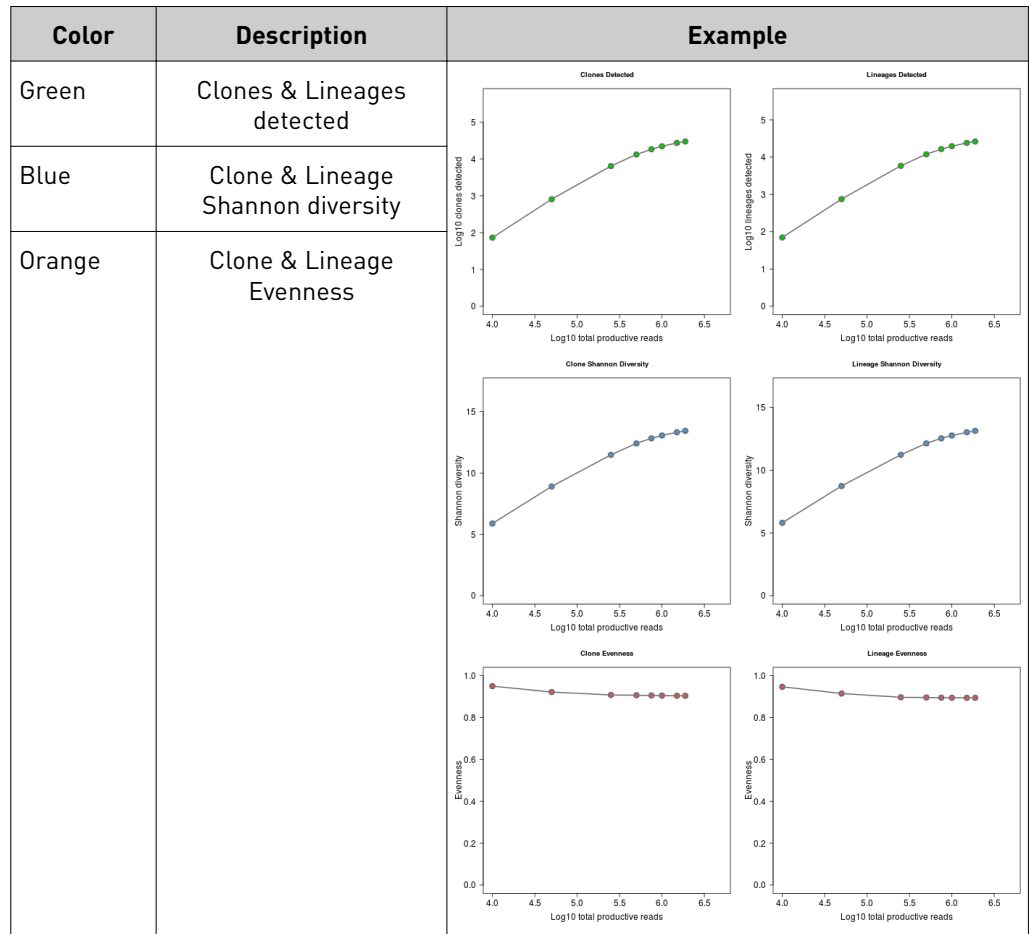
Base composition of overcalled and undercalled homopolymers

Stacked barplot indicating the nucleotide composition of overcalled bases (base insertion sequencing errors) and undercalled bases (base deletion sequencing errors). Highly skewed nucleotide composition may indicate lower quality sequencing or low library diversity.



Downsampling analysis

Downsampling is achieved by repeating clone identification and measurement of repertoire features using 10 K, 50 K, 250 K, 500 K, 750 K, 1 M, 1.5 M, 2 M, and 5 M randomly selected productive and rescued productive reads, contingent on sequencing depth. The graphs show the effect of sequencing depth on select repertoire features: number of clones detected, lineages detected, clone & lineage evenness, and the clone & lineage Shannon diversity. Values for these repertoire metrics that are displayed in this plot are provided in the metrics file. Clone summary and lineage summary files that are derived from downsampled data are provided in the 'downsampling' subdirectory of the zipped results download file. If insufficient reads are available for a particular downsampling depth the corresponding fields are assigned a 'NA' value in the metrics file.



QC metrics

The QC metrics include the read classification counts and strand QC metrics.

Category	Description
Read classification	
Total productive reads	Productive + rescued productive reads.
Productive reads	Reads having an in-frame variable and joining gene, and no stop codons.
Rescued productive reads	Reads having an in-frame variable and joining gene, and no stop codons after INDEL error correction.
Unproductive reads	Reads that have uncorrectable sequencing or PCR errors that lead the rearrangement to have out-of-frame variable and joining genes or a premature stop codon.
Off-target/ low-quality	Reads which are of low quality or represent the product of an off-target amplification.
Strand QC metrics	
Plus strand (v-side) read counts	Number of sequence read counts from the plus (+) strand.
Minus strand (c-side) read counts	Number of sequence read counts from the minus (-) strand.
Plus strand CDR3 avg PHRED	Average PHRED score for plus (+) strand reads.
Minus strand CDR3 avg PHRED	Average PHRED score for minus (-) strand reads.



Sample Results tab

The assay results are represented graphically on the **Sample Results** tab.

Select the desired results output to view from the **Views** list.

Spectratyping plots

The immune repertoire in a sample is represented in each spectratyping plot by the range of CDR3 lengths and their pattern of distribution. Reads for identified clones are arranged along the X-axis according to the variable gene identity and the Y-axis according to the CDR3 nucleotide length. The variable gene order reflects the gene position within the IGH locus. Dots are separated vertically along the Y-axis by 3 nucleotides (one codon), the higher up the Y-axis the longer the CDR3 region. Circle size indicates the frequency of a particular variable gene-CDR3 nucleotide length combination in the dataset. Circle color represents a fourth metric specific to each graph (for example, Shannon Diversity, evenness, clone frequency). Key repertoire metrics are displayed along the lower margin of the plot.

In the Ion Reporter™ Software the spectratyping plots are interactive, allowing you to adjust the data and access clone details. Drag the ends of the horizontal bar below the X-axis to limit the region (v-genes) to view in the plot. Drag the ends of the color range up or down to limit the clones that are viewed. Hover your cursor over any dot to view the details of an individual clone. Click  to restore the default plot view, click  to download a static image of the plot (if you have adjusted the plot view, the adjusted plot is downloaded).

Note: Sequencing of a B cell line such as Ramos will reveal a single dominating clonal lineage, indicated by a single spot on the spectratyping plot.

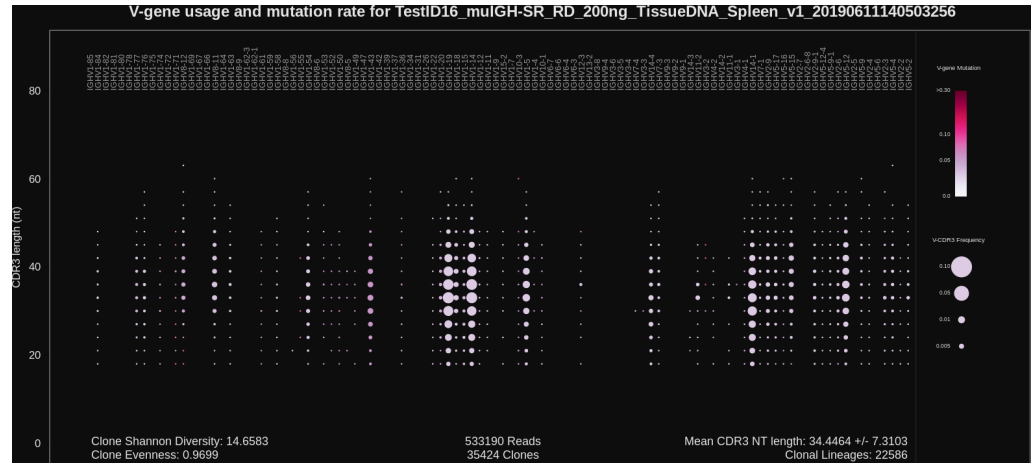


Figure 14 IGH V-gene usage and mutation rate

Spectratyping plot highlighting frequency of mutated bases over the variable gene of identified clones. Circle color indicates the average frequency of mutated bases for clones having a particular variable gene-CDR3 nucleotide length combination. B cells that have undergone isotype switching tend to have a higher frequency of somatic hypermutation than B cells expressing IgM or IgD isotypes, which tend to represent naive B cells. Systematic differences with respect to reference may indicate the presence of polymorphism within the variable gene that is not captured by the IMGT database.

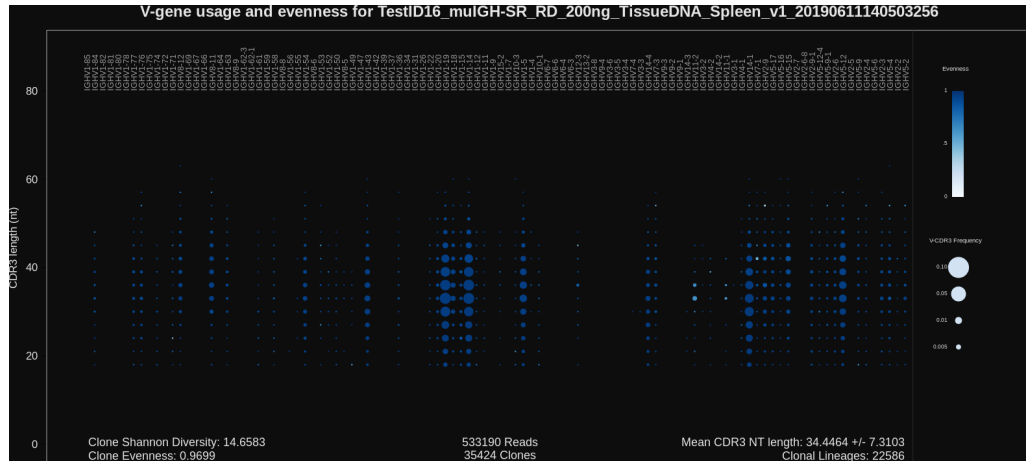


Figure 15 IGH V-gene usage and evenness

Spectratyping plot highlighting evenness of identified clone sizes (Normalized Shannon Entropy). Circle color indicates the evenness of clone sizes for clones having a particular variable gene-CDR3 nucleotide length combination. Values range from 0 to 1, with 1 indicating most even clone sizes. In this representation, portions of the repertoire containing highly expanded clones appear white.

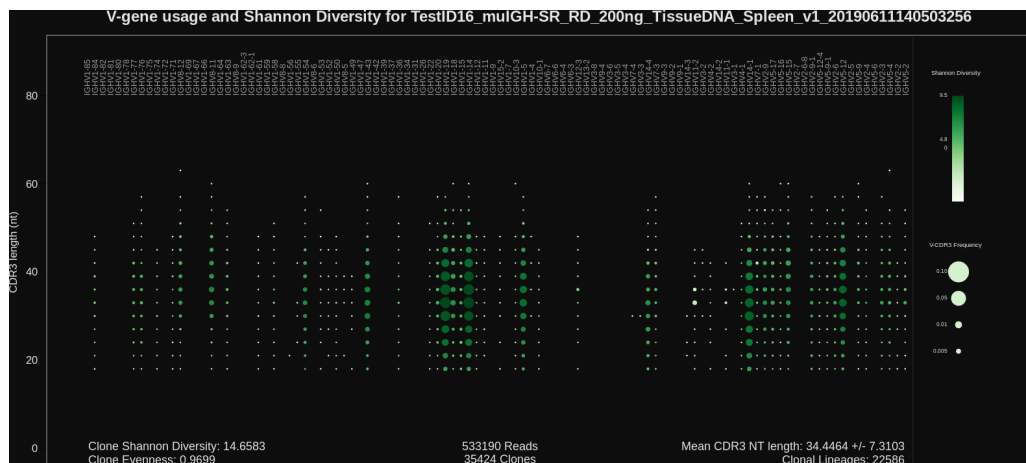


Figure 16 IGH V-gene usage and Shannon Diversity

Spectratyping plot highlighting Shannon Diversity (entropy) of identified clones. Circle color indicates the Shannon Diversity of clones having a particular variable gene-CDR3 nucleotide length combination. Portions of the repertoire containing highly expanded clones typically have a corresponding low Shannon Diversity value.

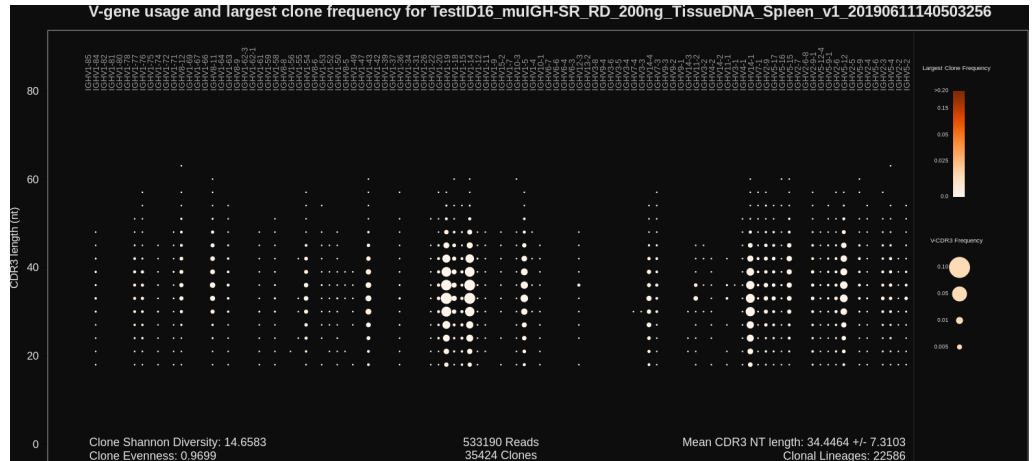


Figure 17 IGH V-gene usage and largest clone frequency

Spectratyping plot highlighting the frequency of the largest clone for each variable gene-CDR3 nucleotide length combination. Circle color indicates the frequency of the largest clone having a particular variable gene-CDR3 nucleotide length combination. Dark color indicates the presence of expanded clones. This is a polyclonal repertoire that lacks highly expanded clones.



Figure 18 IGH V-gene usage and number of clones

Spectratyping plot highlighting the number of clones that are identified for each variable gene-CDR3 nucleotide length combination. Circle color indicates the number of clones having a particular variable gene-CDR3 nucleotide length combination. Specific variable genes may more frequently participate in VDJ recombination, leading to an enrichment in distinct clones for those variable genes.

V-gene usage

The Ion AmpliSeq™ Mouse BCR IGH-SR Assay produces a graphical representation of the frequency of Variable genes and alleles in a sample. Select **V-gene usage** from the **Views** dropdown list. The stacked barplots indicate the representation of variable genes among identified clones. Ordering of variable genes reflects position in the IGH locus.

Note: Variable gene and allele identification is most accurate when using long-amplicon sequencing covering all three CDR domains.

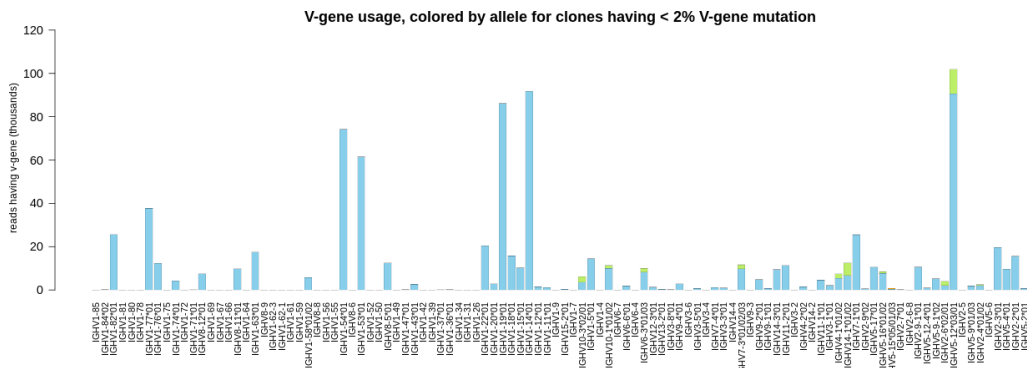


Figure 19 V-gene usage highlighting alleles

Color segments within each bar indicate the frequency of particular variable gene alleles, arranged by frequency from rarest (top) to most common (bottom), for clones having <2% variable gene mutation. This cutoff is used to avoid noise in allele identification caused by somatic hypermutation.

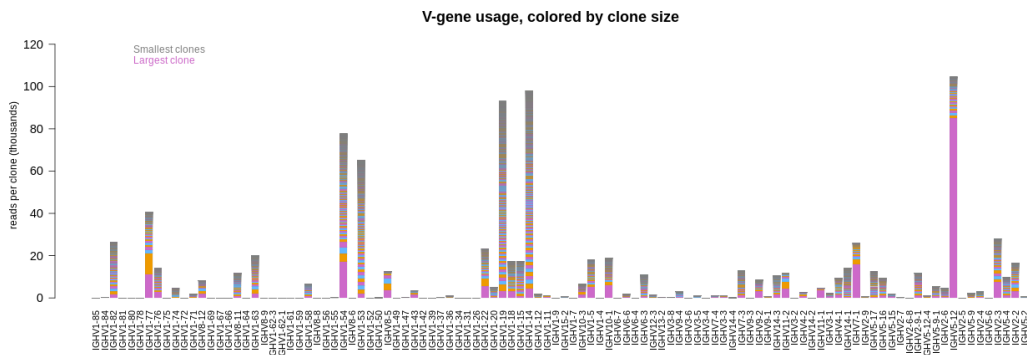


Figure 20 V-gene usage highlighting clone sizes

Color segments in each bar indicate the contribution of individual clones, arranged from smallest clones (top) to largest clones (bottom).

VJ-gene usage heatmap

The Ion AmpliSeq™ Mouse BCR IGH-SR Assay produces a graphical representation of the frequency of each Variable gene - Joining gene combination for identified clones. Select **VJ-gene usage heatmap** from the **Views** dropdown list to see heatmaps that represent the results.

Note: Variable gene and allele identification is most accurate when using long-amplicon sequencing covering all three CDR domains.

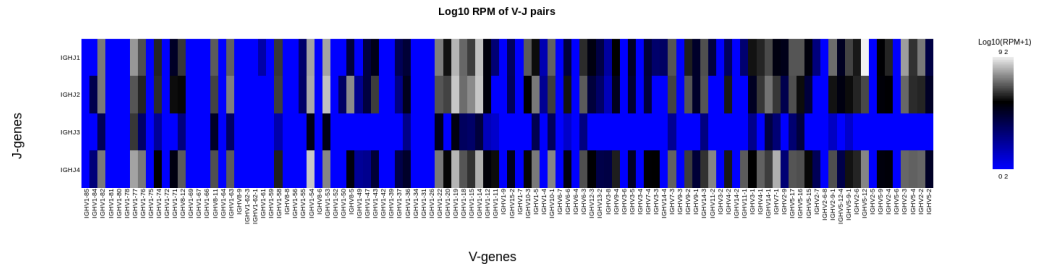
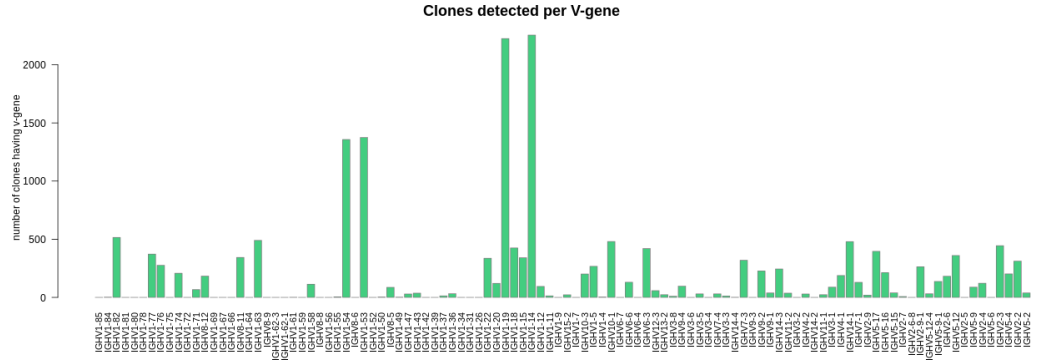


Figure 21 VJ-gene usage heatmap

Heatmap illustrating the frequency of particular Variable gene-Joining gene combinations for identified clones. Frequencies are log transformed with pseudocount added. Over-represented Variable-Joining gene combinations (white) may indicate presence of a highly expanded clone. Ordering of variable genes reflects position within the IGH locus.

Clones detected per variable gene

The Ion AmpliSeq™ Mouse BCR IGH-SR Assay produces a graphical representation of the Variable genes usage among detected clones. Select **Clones detected per variable gene** from the **Views** dropdown list.



The barplot indicates the number of identified clones having a particular variable gene. Ordering of variable genes reflects position within the IGH locus.

CDR3 histogram

The Ion AmpliSeq™ Mouse BCR IGH-SR Assay produces a graphical representation of the CDR3 lengths of clones that are detected in a sample. Select **CDR3 histogram** from the **Views** dropdown list.

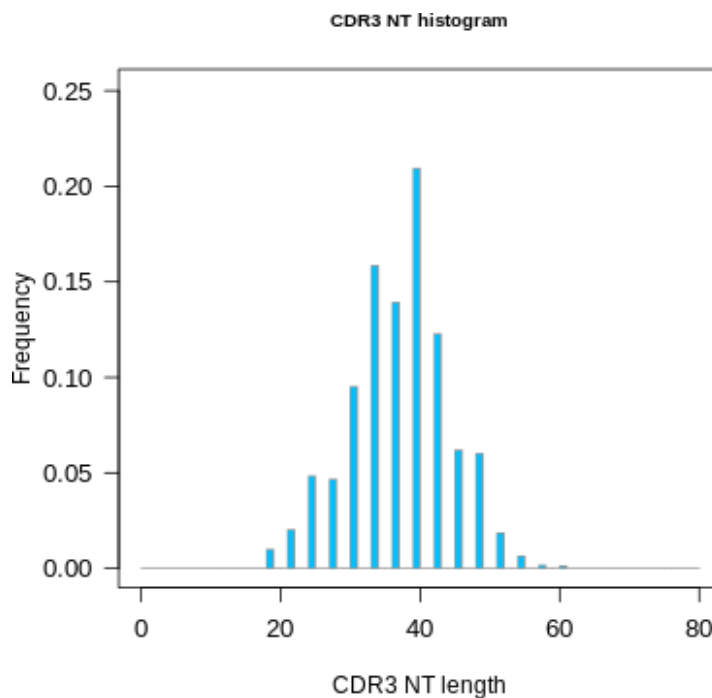


Figure 22 Relative frequency (Y-axis) of identified clones with a given CDR3 nucleotide length (X-axis)

The histogram indicates the distribution of CDR3 lengths for clones identified in the sample.

Clonal lineage identification

A B cell clonal lineage represents a set of B cells that are related by descent, arising from the same VDJ rearrangement event. B cells in a clonal lineage may differ at the sequence level owing to somatic hypermutation or isotype switching. Members of a clonal lineage are more likely to have a shared antigen specificity than members of different clonal lineages. Analysis of patterns of somatic hypermutation within clonal lineages may be used to infer patterns of isotype switching and identify IGH residues important for antigen recognition. For these reasons, the clonal lineage is a fundamental unit of B cell repertoire analysis. Ion Reporter™ Software automatically groups B cell clones into clonal lineages such that lineage members:

- Have the same variable and joining genes, excluding allele information.
- Have CDR3 regions of the same nucleotide sequence length.
- Have ≥85% CDR3 region sequence homology with one another.

Each clone is assigned a Lineage ID. The ordering of the Lineage ID reflects the abundance ranking of the clonal lineage, which is calculated by determining the sum of the frequencies of all members of the clonal lineage. Lineage 1 corresponds to the most abundant lineage, followed by Lineage 2, until the least abundant lineage is reached.

Clone summary table

The Lineage ID is displayed in the leftmost column of the clone summary and lineage summary views.

The **Clone Summary** table lists the identified clones in rank order from the most frequently occurring to least frequent. Each row represents an individual clone. For each clone, the identified **Variable** and **Joining** region are listed as well as the amino acid (**CDR3 AA**) and nucleotide (**CDR3 NT**) sequences of the CDR3 region. The variable gene mutation, and the clonal lineage assignment (Lineage ID) are also listed.

Lineage ID	Variable	Joining	CDR3 AA	CDR3 NT	Variable Mutation	Count	Frequency	Rank	Isotype
1	IGHV4-39	IGHJ3	ARQVSGYDPS...	GCGAGACAG...		0.1	6528	0.0069297	1 IGHA2
2	IGHV1-3	IGHJ5	ARDLFAAVGH...	GCGAGAGATC...		0.069	4004	0.0042504	2 IGHA1
3	IGHV3-48	IGHJ4	ARDYVTGALD	GCGAGAGATT...		0.066	3051	0.0032387	3 IGHA2
6	IGHV4-31	IGHJ4	AKTSNPNNHV...	GCGAAAACCT...		0.243	2117	0.0022473	4 IGHA1
5	IGHV2-5	IGHJ5	VHRPPVYSFW...	GTTACACAGAC...		0.11	2038	0.0021634	5 IGHA1

Click the column header cells to sort the table. Frequency = # of reads for the identified clone (**Count**) / total reported reads (sum of **Count** column).

For multi-sample analyses the **Clone Summary** table lists the frequency of each clone that is identified in any of the samples. The table is sorted in descending order based on the frequency of the clones in the leftmost column for each sample in the analysis.

Variable	Joining	CDR3 AA	CDR3 NT	B212060_RNA_v1_c826_201...	B707172_RNA_v1_c753_201...
TRBV5-6	TRBJ2-2	ASSLGSSELF	GCCAGCAGCTTAGGTTCCGGGGAGCTGTTT	0.0674630	0.0000125
TRBV3-1	TRBJ1-3	ASSRWGTGNTY	GCCAGCAGCCAAAGTGGCAGACCAGAACACCATATAT	0.0163406	0

① Sample 1

② Sample 2

Table 3 Clone summary information fields

Column header	Description
Lineage ID	Lineage ID represents the rank order of the clonal lineage abundance. Calculated as the sum of the frequencies of all members of the clonal lineage. Lineage 1 corresponds to the most abundant lineage, followed by Lineage 2, until the least abundant lineage is reached.
Variable	The best matching IMGT variable gene of the rearrangement.
Joining	The best matching IMGT joining gene of the rearrangement.
CDR3 AA	The CDR3 amino acid sequence of the rearrangement, denoted using the IMGT definition of the CDR3 region.

Coloumn header	Description
CDR3 NT	The CDR3 nucleotide sequence of the rearrangement, denoted using the IMGT definition of the CDR3 region.
Variable Mutation	The fraction of bases within the variable gene that differ from the best-matching IMGT variable gene. In B cells, such mismatching bases are largely derived from somatic hypermutation.
Count	The total number of reads mapping to the rearrangement after quality filtering.
Frequency	The frequency of the rearrangement as a proportion of total reads passing quality filtering.
Rank	The frequency rank of the rearrangement.

Note: Additional details are available by downloading the **Clone Summary** table.

Lineage summary table

The **Lineage Summary** table lists the identified clones in rank order from the most frequently occurring to least frequent. Each row represents an individual clone with its clonal lineage assignment (Lineage ID). Key features that are identified for each clone are the **Variable** region, **Top CDR3 AA**, **Lineage Frequency**, minimum and maximum v-gene somatic hypermutation (SHM) level, as well as the number of clones.

Sample Results
Sample QC

Views: Lineage Summary
Download Lineage Summary

Go

Lineage Summary Table

Summary metrics for clonal lineages detected in a sample. Clonal lineages represent groups of B cell clones that are believed to be related by descent (shared VDJ rearrangement) but have distinct VDJ sequences owing to somatic hypermutation and isotype switching. Clonal lineages are identified as sets of IGH rearrangements having the same variable gene identity, the same CDR3 length, and CDR3 nucleotide similarity greater than the user-defined threshold (default: minimum 85% homology). The lineage ID column is also presented in the clone summary file. [Learn more...](#)

Lineage ID	Variable	Top CDR3AA	Lineage Frequency	Number of Clones	Isotypes	Minimum V-gene SHM	Maximum V-gene SHM	Minimum Clone Frequency	Maximum Clone Frequency
1	IGHV4-39	ARQVSGYDPSGSA...	0.0069297	1	IGHA2	0.1	0.1	0.0069297	0.0069297
2	IGHV1-3	ARDLFAAVGHGFDP	0.0051813	4	IGHA1;IGHG2;IGHM	0.069	0.082	0.0000212	0.0042504
3	IGHV3-48	ARDYVTGALD	0.0050030	10	IGHA1;IGHA2;IGHG2	0.041	0.091	0.0000096	0.0032387
4	IGHV3-23	AKRGLLQCTGAN...	0.0032780	8	IGHA1;IGHA2	0.087	0.145	0.0000127	0.0015297
5	IGHV2-5	VHRPPVYSFWSTA...	0.0029012	4	IGHA1	0.04	0.11	0.0000085	0.0021634

1 2 3 4 5 6 7 8 9 10 ...

5 items per page

1 - 5 of 26235 items

Click the column header cells to sort the table.

Table 4 Lineage summary information fields

Column header	Description
Lineage ID	Lineage ID represents the rank order of the clonal lineage abundance. Calculated as the sum of the frequencies of all members of the clonal lineage. Lineage 1 corresponds to the most abundant lineage, followed by Lineage 2, until the least abundant lineage is reached.
Variable	The best matching IMGT variable gene of the rearrangement.
Top CDR3 AA	CDR3 amino acid sequence of the most abundant clone within the clonal lineage.

Column header	Description
Lineage Frequency	Aggregate frequency of clonal lineage members. Lineage IDs are assigned in ascending order based on the lineage frequency such that Lineage ID 1 corresponds to the most abundant clonal lineage.
Number of Clones	Number of clones within the clonal lineage.
Minimum V-gene SHM	The minimum variable gene mutation value for members of the clonal lineage.
Maximum V-gene SHM	The maximum variable gene mutation value for members of the clonal lineage.
Minimum Clone Frequency	The frequency of the least abundant clone within the clonal lineage.
Maximum Clone Frequency	The frequency of the most abundant clone within the clonal lineage.

Note: Additional details are available by downloading the **Clone Summary** table.

Download a metrics file

- To download the **Clone Summary** metrics file.
 - a. In the **Immune Repertoire Results** screen, **Sample Results** tab, select **Clone Summary** from the **Views** dropdown list.
 - b. Click **Download Clone Summary**.
The clone summary CSV file downloads automatically.
 - c. Open the `.clone_summary` CSV file to view the additional information that is included in the spreadsheet.
- To download the **Lineage Summary** metrics file.
 - a. In the **Immune Repertoire Results** screen, **Sample Results** tab, select **Lineage Summary** from the **Views** dropdown list.
 - b. Click **Download Lineage Summary**.
The lineage summary CSV file downloads automatically.
 - c. Open the `.lineage_summary` CSV file to view the additional information that is included in the spreadsheet.

See the **Glossary** on page 123 for column header descriptions.



Troubleshooting

Library yield and quantification

Observation	Possible cause	Recommended action
Library concentration is low (Library concentration is NOT indicative of quality.)	Sample DNA or RNA was mis-quantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit; quantify RNA with Qubit™ Fluorometer.
	Residual ethanol in sample DNA or RNA inhibited target amplification.	Incubate uncapped tube in hood for 1 hour.
		Speed-vac tube at room temperature for 5 minutes.
	RNA concentration is low.	<ol style="list-style-type: none"> 1. Dry RNA completely in a centrifugal vacuum concentrator at low or medium heat (≤ 40 °C) for 10–20 minutes. 2. Resuspend in an appropriate volume of Nuclease-free Water, then proceed with library preparation.
	Reverse transcription of RNA sample yields cDNA that is difficult to amplify.	After reverse transcription of the RNA, perform a sample rescue purification on your cDNA (see page 103 for more information).
	Residual ethanol from AMPure™ purification inhibited library amplification.	Carefully remove all drops of ethanol before library amplification, then centrifuge plate, if necessary.
	Sample DNA or RNA quality was low.	Add more DNA/RNA or increase target amplification cycles.
		Perform additional library amplification immediately following post-ligation purification.
	PCR, digestion, or ligation was inefficient.	Ensure proper dispensing and mixing of viscous components at each step.
	Library was discarded during two-round bead purification of the amplified library.	Be sure to save the supernatant during first-round purification, and save the bead pellet during the second round.
	AMPure™ XP Beads were over-dried.	Do not dry the AMPure™ XP Beads more than 5 minutes.
	AMPure™ XP Beads inhibited library amplification.	Transfer library off of beads prior to amplification.
qPCR cycling time is too short.	Use standard qPCR cycling for library designs >175 bp instead of Fast cycling.	
FFPE RNA was not heat treated before reverse transcription.	Heat FFPE RNA at 80°C for 10 minutes, then cool to room temperature before reverse transcribing.	



Observation	Possible cause	Recommended action
Library concentration is too high	Sample DNA or RNA was mis-quantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit; quantify RNA with a Qubit™ Fluorometer.
	More than 100 ng of sample DNA/RNA was used.	Add less DNA/RNA, or decrease target amplification cycles.

Other

Observation	Possible cause	Recommended action
The number of on-target reads is lower than expected	Unknown.	Increase the number of target amplification cycles by two, or increase the anneal/extend temperature of the target amplification reaction from 60°C to 62°C or 64°C for the first two cycles of the target amplification reaction.
		Lower the DNA input.
Barcode representation is uneven	Library quantification was inaccurate.	Use the Ion Library TaqMan™ Quantitation Kit for the most specific and accurate library quantification.
	Library combination was inaccurate.	Dilute libraries to 100 pM, then combine equal volumes.
Percentage of polyclonal ISPs is high (>40%)	Library input was too high.	Decrease amount of library added to the template preparation reaction by 50%.
	Library was mis-quantified.	Ensure that library was quantified accurately.
	Other.	Check the appropriate template preparation user guide for more information.
Low quality ISPs are present at high percentage (>15%)	Library input was too low.	Double the volume of library used in template preparation.
		Use a fresh dilution of library prepared in a low-bind tube.
	Other.	Check the appropriate template preparation user guide for more information.



Supplemental information

- Install the IonReporterUploader plugin in Ion Reporter™ Software 96
- Configure the IonReporterUploader 5.12 plugin in Torrent Suite™ Software . . 97
- Enable off-cycle product updates 98
- Install off-cycle bundles without Internet access 98
- Rescue amplification and library purification 99
- Library clean up (off-target products) 102
- Sample rescue purification and cDNA amplification 103

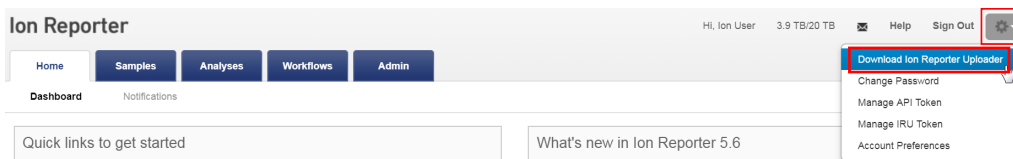
Install the IonReporterUploader plugin in Ion Reporter™ Software

The IonReporterUploader 5.12 plugin is automatically installed in Ion Reporter™ Software when you update to a new software release.

The latest IonReporterUploader plugin software is available for download from within the Ion Reporter™ Software. If your Ion Reporter™ Server is not connected to the internet download a ZIP or DEB file of the IonReporterUploader 5.12 plugin from <http://iru.ionreporter.thermofisher.com/>. To reinstall or update IonReporterUploader 5.12 plugin in Torrent Suite™ Software 5.2 or later.

Note: An administrative **ionadmin** account is not required for this procedure.

1. Sign in to Ion Reporter™ Software, then click **⚙ (Settings) ▶ Download Ion Reporter Uploader**.



2. Click the Torrent Suite Plugin filename **IonReporterUploader.zip** to download the file to your local machine.
3. Sign in to Torrent Suite™ Software, then click **⚙ (Settings) ▶ Plugins**.

4. Click **Install or Upgrade Plugin**.



5. In the **Install or Upgrade Plugin** dialog, click **Select File**, then browse to the file location. Select the downloaded **IonReporterUploader.zip** file, click **Open**, then click **Upload and Install**.

Configure the IonReporterUploader 5.12 plugin in Torrent Suite™ Software

1. Sign in to the Torrent Suite™ Software.
2. Click **⚙ (Settings) ▶ Ion Reporter Configure**.
3. In the **Ion Reporter Uploader Account Configuration** screen, click **+ Add Account ▶ Ion Reporter**.
4. In the **Add Ion Reporter account** screen, enter the following information into the fields:

Field	Directions
Server Type	Select: ^[1]
Display Name	Enter a meaningful name of your choice. This name is used in the run plan template wizard and is seen by other Torrent Suite™ Software users. Use only alphanumeric characters, spaces, and underscores.
Server	Enter: ^[1]
Port	Enter: 443
Username	Enter your Ion Reporter™ Software username (your email address)
Password	Enter your Ion Reporter™ Software password

^[1] Ask your Ion Reporter™ Server administrator for these values.

5. The "Default Account" is the account that is configured by default in run templates and run plans. If this account is the main account to be used for file transfers, enable the **Default Account** checkbox.


Note: You can always change this selection in the Planned Run template workflow bar and in the Upload to IR quick link.

6. Click **Get Versions**, select **Ion Reporter 5.12 or later**, then click **✓Add**.

Enable off-cycle product updates


An administrator can add new kits, chips, templates, plugins and Ion Chef™ scripts that are released outside of the regular software release cycle.

When you learn of a new product that you would like to use, check to see if a software update is available.

1. Click  **(Settings)** ▶ **Updates**.
2. Scroll down to the **Update Products** section at the bottom of the screen.
3. Select the desired new product and click **Update**.
Your installed version of Torrent Suite™ Software is updated to include the new products that you selected.

Install off-cycle bundles without Internet access

If the site Ion Torrent™ Server is not connected to the Internet, an administrator can manually install updates.

1. Request a USB device that contains the updates from your local Field Service Engineer or Technical Support.
2. Insert the USB device into your Ion Torrent™ Server.
3. In Torrent Suite™ Software, click  **(Settings)** ▶ **Updates**.
4. On the **Updates** screen, under **Manual Upload**, click **Click to Upload and Install**.
5. Follow the prompts to upload the compressed folder.

Rescue amplification and library purification

If you have a low-yield library, you can perform rescue amplification to improve the library yield. After rescue amplification, purify the amplified library using the two-round purification process.

IMPORTANT! Rescue amplification uses reagents provided in the assay kit. Sufficient reagents are provided in the kit to prepare 24 libraries. Performing rescue amplifications can reduce the number of sample libraries you are able to prepare with the kit.

Amplify the library

Yields increase with extra library amplification cycles, however percent productive reads, clone Shannon diversity, and normalized evenness does not change significantly. We recommend that you keep the number of library amplification cycles to a minimum.

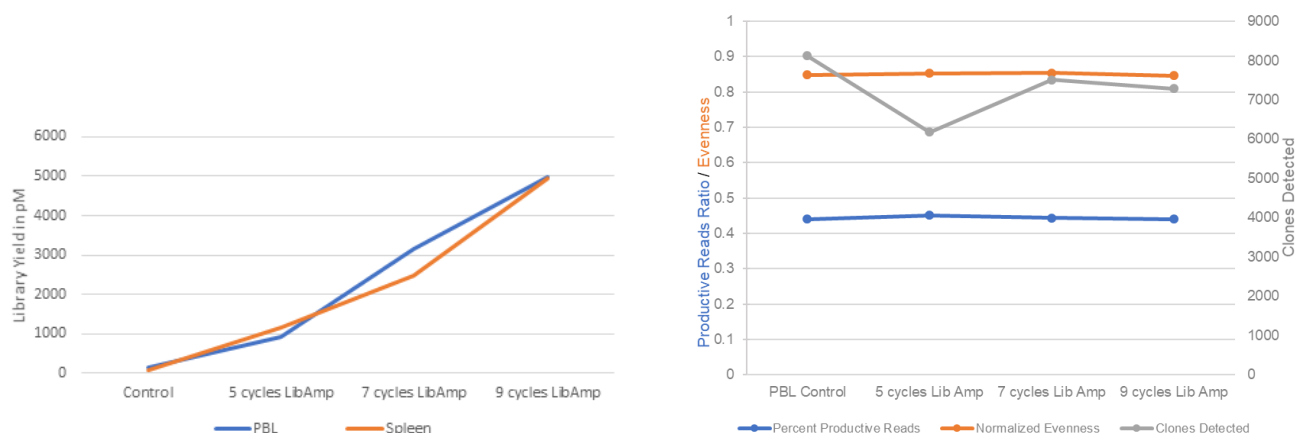


Figure 23 Effect of library amplification on yield

The example presented in these graphs was generated using human samples. It is representative of what can be expected with murine samples.

1. Place a 96-well plate on ice or in a pre-chilled 4°C cold block.
2. Prepare separate rescue amplification reactions for each library. Add the following components to individual wells of the 96-well PCR plate.

Note: If processing multiple samples, prepare a reaction master mix (+ 5–10% overage) without template for each purified library.

Component	Volume
Purified library	25 µL
25X Library Amp Primers (pink cap)	1.6 µL
5X Ion AmpliSeq™ HiFi Mix (red cap)	8 µL
Nuclease-free Water	5.4 µL
Total Volume	40 µL

- Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
- Place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 minutes
5 cycles ^[1]	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

^[1] 5 cycles should be sufficient, but you may perform up to 9 cycles library amplification.

STOPPING POINT Samples can be stored at -20°C.

Purify the amplified library

Perform a two-round purification process with the Agencourt™ AMPure™ XP Reagent:

- First round at 0.5X bead-to-sample-volume ratio:** High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant.**
- Second round at 1.2X bead-to-original-sample-volume ratio:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
 - Use freshly prepared 70% ethanol for the next steps. Combine 230 µL of ethanol with 100 µL of Nuclease-free Water per sample.
 - Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.
-

First-round purification

- Tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, then remove the plate seal.
- Add 20 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent** to each plate well containing ~40 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
- Incubate the mixture for 5 minutes at room temperature.
- Place the plate in a magnet such as the DynaMag™ -96 Side Magnet for at least 5 minutes, or until the solution is clear.
- Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

IMPORTANT! The **supernatant** contains the desired amplicons. Do not discard!

Second-round purification

1. To the supernatant from step 4 above, **add 48 μ L (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent**. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

IMPORTANT! The amplicons are bound to the beads. **Save the bead pellet.**

4. **Add 150 μ L of freshly prepared 70% ethanol** to each well, then move the plate side to side in the magnet to wash the beads. Remove, then discard the supernatant without disturbing the pellet.

Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down five times (with the pipettor set at 100 μ L), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

5. Repeat step 4 for a second wash.
6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. **Do not overdry.**
7. Remove the plate with purified libraries from the plate magnet, then **add 50 μ L of Low TE** to the pellet to disperse the beads.
8. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
9. Incubate at room temperature for at least 2 minutes.
10. Place the plate on the magnet for at least 2 minutes.

IMPORTANT! The supernatant contains the desired amplicons. Do not discard!

STOPPING POINT Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C. We recommend transferring the supernatant to a 1.5-mL Eppendorf LoBind™ tube for long-term storage.

Proceed to Chapter 4, “Quantify, combine, and sequence the libraries”. For quantification, prepare a 10,000-fold dilution of each purified library by performing serial dilutions. Combine 2 μ L of supernatant with 198 μ L Nuclease-free Water to prepare a 100-fold dilution. Then, combine 2 μ L of 100-fold dilution with 198 μ L Nuclease-free Water to prepare a 10,000-fold dilution of each purified library.

Library clean up (off-target products)

Perform library clean up for libraries that contain a large amount of off-target products.

IMPORTANT! Incubate the AMPure™ XP reagent at room temperature for at least 30 minutes, then vortex thoroughly to disperse the beads before use. Pipet the solution slowly. We recommend using low-retention pipette tips.

1. Use a 100- μ L pipette to measure the reaction volume in each well, then add nuclease-free water to bring the volume in each well to 30 μ L.
2. To 30 μ L purified library, add 30 μ L (1X sample volume) of Agencourt™ AMPure™ XP Reagent.
3. Vortex thoroughly, centrifuge briefly to collect droplets, then incubate at room temperature for 5 minutes.

IMPORTANT! Thorough mixing of beads with samples is very important. After vortexing, check that the contents of each well is homogeneous in color.

4. Place in a magnetic rack for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Add 150 μ L of freshly prepared 70% ethanol, then pipet up and down 5X to mix. Place in a magnetic rack for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
6. Repeat step 5 for a second wash.
7. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. Do not overdry.
8. Add 30 μ L Low TE buffer to the pellet, then pipet up and down 5 times to mix.
9. Incubate at room temperature for at least 2 minutes, then place the plate on the magnet for at least 2 minutes or until the solution clears.
10. Carefully transfer the supernatant to a new 1.5-mL Eppendorf LoBind™ tube.

Proceed to Chapter 4, “Quantify, combine, and sequence the libraries”. For quantification, prepare a 100-fold dilution of each library by combining 2 μ L of supernatant with 198 μ L Nuclease-free Water.

Sample rescue purification and cDNA amplification

For difficult-to-amplify RNA samples or for samples with low starting concentrations, it can be beneficial to perform an Agencourt™ AMPure™ XP cleanup step on your cDNA, following the reverse transcription reaction. Perform the following procedure after RNA reverse transcription and in place of cDNA target amplification.

Note:

- If your RNA sample has a low starting concentration, see the *SuperScript™ IV Reverse Transcriptase User Guide* (Pub. No. MAN0013443) to increase your RT reaction volume, and thus the volume of template RNA allowed.
- To recover maximum yield, cDNA dilution is not necessary or advisable before rescue purification.

IMPORTANT! Incubate the AMPure™ XP reagent at room temperature for at least 30 minutes, then vortex thoroughly to disperse the beads before use. Pipet the solution slowly. We recommend using low-retention pipette tips.

1. Following reverse transcription, briefly centrifuge the plate to collect the contents, then carefully remove the plate seal.
2. Use a 100- μ L pipette to measure the reaction volume in each well, then add nuclease-free water to bring the volume in each well to 30 μ L.
3. Add 30 μ L (1.0 \times sample volume) of Agencourt™ AMPure™ XP Reagent to each sample, seal the plate with a new MicroAmp™ Clear Adhesive Film.
4. Vortex thoroughly, then incubate at room temperature for 5 minutes.

IMPORTANT! Thorough mixing of beads with samples is very important. After vortexing, check that the contents of each well is homogeneous in color.

5. Centrifuge briefly (1 minute at 300 \times g) to collect the contents, place the plate on the splash-free 96-well base, then carefully remove the seal.

Note: Do not centrifuge the plate too fast with beads in the wells, as this may cause damage to the beads.

6. Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
7. Add 150 μ L of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.

Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down 5 times (with the pipettor set at 100 μ L), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

8. Repeat step 7 for a second wash.

9. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. Do not overdry.

IMPORTANT! Residual ethanol inhibits library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads. Under conditions of low relative humidity, the beads air-dry rapidly. Do not overdry.

10. Remove the plate with purified cDNA from the plate magnet, then add 14 μL of nuclease-free water to the pellet to disperse the beads.
11. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
12. Incubate at room temperature for at least 2 minutes, then place the plate on the magnet for at least 2 minutes.
13. Transfer 12 μL of the purified cDNA to a well in a new plate for cDNA target amplification.

IMPORTANT! PCR amplification can be inhibited by small amounts of carryover beads. Remove any beads before proceeding to cDNA target amplification. If you see beads in the pipette tip containing supernatant, slowly pipet the supernatant and beads back into their respective well on the side of the well adjacent to the magnet so that the beads pass over the magnet. Then repeat step 12, repelleting the beads for an additional 1 minute.

14. Add the following components to each cDNA synthesis reaction. Prepare a master mix without sample RNA for multiple reactions.

Component	Volume
Purified cDNA (from step 13)	12 μL
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 μL
5X Ion AmpliSeq™ Mouse TCR Beta-SR Panel	4 μL
Total volume	20 μL

15. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

16. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then run the following program to synthesize cDNA.

Stage	Temperature	Time
Hold	99°C	2 minutes
23 cycles	97°C	15 seconds
	60°C	4 minutes
Hold	10°C	Hold

STOPPING POINT cDNA amplification reactions may be stored at 10°C overnight on the thermal cycler. For longer periods, store at -20°C.

17. Remove the plate from the thermal cycler, then briefly centrifuge the plate to collect the contents.

Proceed to "Partially digest amplicons" to prepare libraries.



General sample input and sequencing depth guidelines

- RNA samples <6.25 ng/μL 106
- Guidelines for using sorted T-cells 106
- Saturation analysis of mouse gDNA 107
- Saturation analysis of Mouse spleen FFPE tissue 108
- Saturation analysis of Mouse thymus FFPE tissue 111

RNA samples <6.25 ng/μL

For low concentration RNA samples the desired input amount can be achieved by scaling up the reverse transcription reaction. After reverse transcription, you can use the DNAClear™ Purification Kit (Cat. No. AM1756) to purify and concentrate the cDNA following the reverse transcription reaction.

Immediately following the reverse transcription reaction, use the DNAClear™ Purification Kit to purify up to 100 μL of cDNA, eluting in a volume as low as 10 μL for use in library preparation.

Note: You can assume 100% reverse transcription reaction efficiency.

Guidelines for using sorted T-cells

Sample input amount and the target amplification cycling program vary with sample type. In general RNA from sorted T-cell samples will include a greater fraction of relevant template molecules. The fraction of relevant molecules is even higher when using mRNA instead of total RNA. If starting with RNA isolated from sorted T-cells, we recommend performing reverse transcription with 2 ng mRNA.



Saturation analysis of mouse gDNA

The Ion AmpliSeq™ Mouse TCR Beta-SR Assay libraries and the Ion AmpliSeq™ Mouse BCR IGH-SR Assay libraries were each prepared from 200 ng of mouse gDNA. The libraries were sequenced to obtain up to 10 M raw reads per sample. Using downsampling, we evaluated the effect of sequencing depth on clone and lineage detection and obtained the following results and conclusions.

- Sequencing coverage of the library greatly influences the number of clones that are reported.
- It is better to sequence a small library to low depth than a large library to low depth.

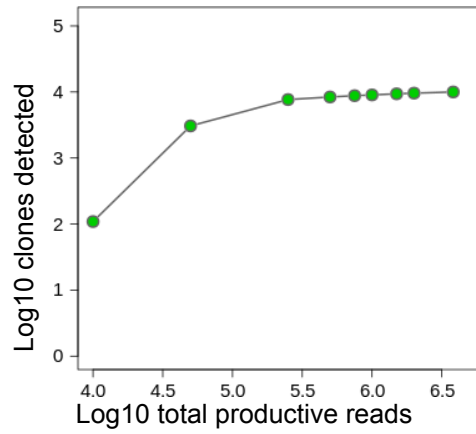


Figure 24 Clones detected from 200 ng of mouse gDNA as a function of read depth – Ion AmpliSeq™ Mouse TCR Beta-SR Assay

Table 5 Clones detected from 200 ng of mouse gDNA as a function of read depth – Ion AmpliSeq™ Mouse TCR Beta-SR Assay

Downsampled read count	Clones detected
10 K	109
50 K	3062
250 K	7677
500 K	8396
750 K	8788
1 M	9031
1.5 M	9383
2 M	9601

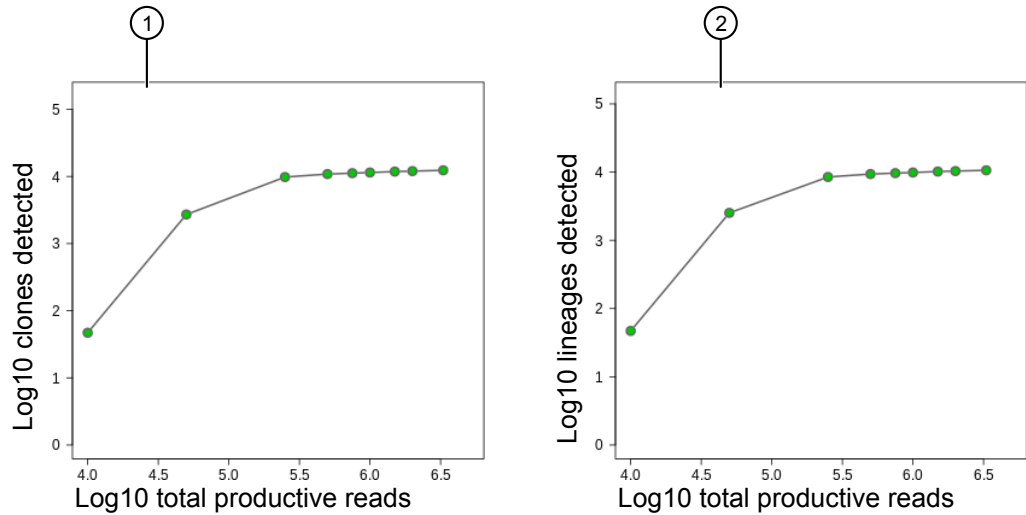


Figure 25 Clones and clonal lineages detected from 200 ng of mouse gDNA as a function of read depth – Ion AmpliSeq™ Mouse BCR IGH-SR Assay

① Clones detected

② Lineages detected

Table 6 Clones and clonal lineages detected from 200 ng of mouse gDNA as a function of read depth – Ion AmpliSeq™ Mouse BCR IGH-SR Assay

Downsampled read count	Clones detected	Lineages detected
10 K	47	47
50 K	2712	2525
250 K	9789	8475
500 K	10855	9363
750 K	11199	9655
1 M	11442	9859
1.5 M	11823	10188
2 M	12010	10343

Saturation analysis of Mouse spleen FFPE tissue

The Ion AmpliSeq™ Mouse BCR IGH-SR Assay libraries were each prepared from 200 ng of gDNA and 50 ng of RNA extracted from FFPE-preserved mouse spleen tissue. The libraries were sequenced to obtain up to 10 M raw reads per sample. Using downsampling, we evaluated the effect of sequencing depth on clone and lineage detection and obtained the following results and conclusions.

- Sequencing coverage of the library greatly influences the number of clones that are reported.
- It is better to sequence a small library to low depth than a large library to low depth.

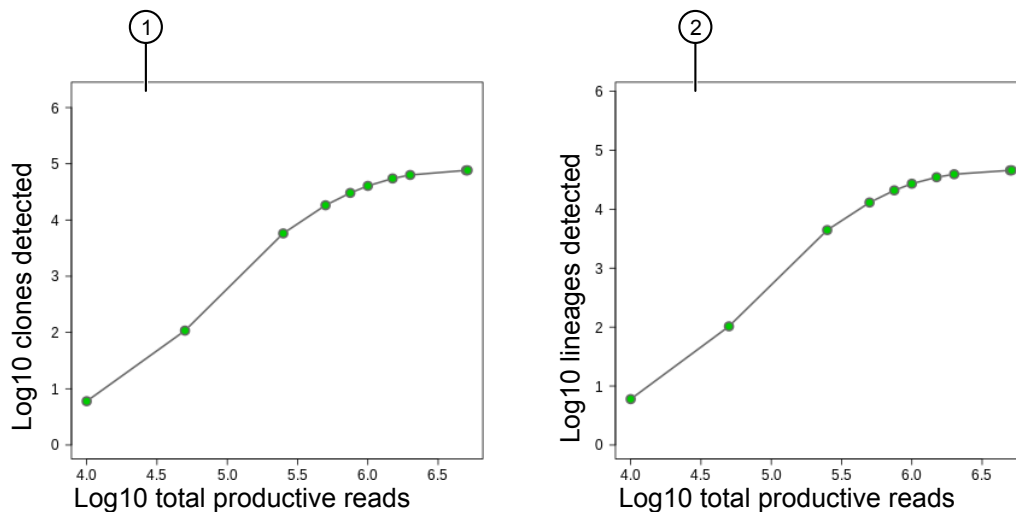


Figure 26 Clones and clonal lineages detected from 200 ng of gDNA extracted from FFPE-preserved mouse spleen tissue as a function of read depth – Ion AmpliSeq™ Mouse BCR IGH-SR Assay

① Clones detected

② Lineages detected

Table 7 Clones and clonal lineages detected from 200 ng of gDNA extracted from FFPE-preserved mouse spleen tissue as a function of read depth – Ion AmpliSeq™ Mouse BCR IGH-SR Assay

Downsampled read count	Clones detected	Lineages detected
10 K	6	6
50 K	108	103
250 K	5776	4426
500 K	18305	12991
750 K	30322	20873
1 M	40452	27080
1.5 M	54779	34856
2 M	63213	39214
5 M	76418	45665

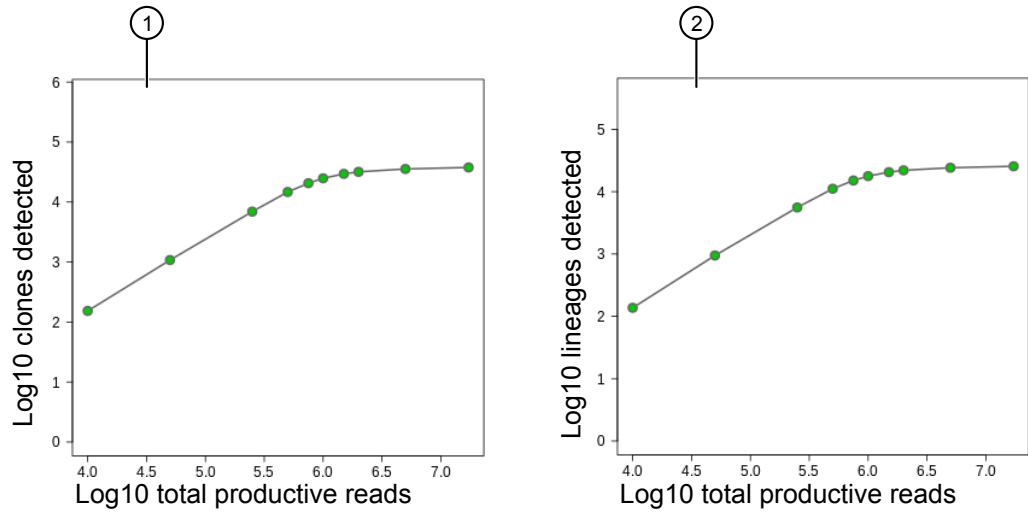


Figure 27 Clones and clonal lineages detected from 50 ng of RNA extracted from FFPE-preserved mouse spleen tissue as a function of read depth – Ion AmpliSeq™ Mouse BCR IGH-SR Assay

① Clones detected

② Lineages detected

Table 8 Clones and clonal lineages detected from 50 ng of RNA extracted from FFPE-preserved mouse spleen tissue as a function of read depth – Ion AmpliSeq™ Mouse BCR IGH-SR Assay

Downsampled read count	Clones detected	Lineages detected
10 K	144	125
50 K	1094	949
250 K	6841	5521
500 K	14687	11045
750 K	21188	15393
1 M	25637	18186
1.5 M	31098	21499
2 M	33761	23111
5 M	38021	25601



Saturation analysis of Mouse thymus FFPE tissue

The Ion AmpliSeq™ Mouse TCR Beta-SR Assay libraries were each prepared from 200 ng of gDNA and 50 ng of RNA extracted from FFPE-preserved mouse thymus tissue. The libraries were sequenced to obtain up to 10 M raw reads per sample. Using downsampling, we evaluated the effect of sequencing depth on clone detection and obtained the following results and conclusions.

- Sequencing coverage of the library greatly influences the number of clones that are reported.
- It is better to sequence a small library to low depth than a large library to low depth.

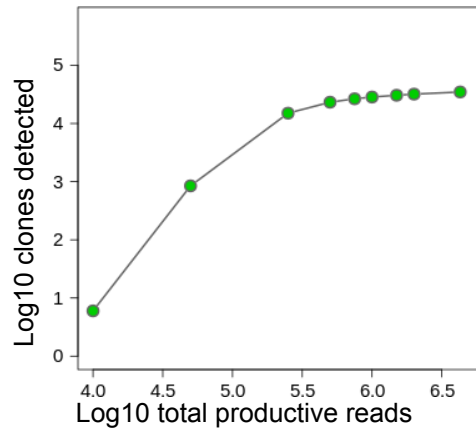


Figure 28 Clones detected from 200 ng of gDNA extracted from FFPE-preserved mouse thymus tissue as a function of read depth – Ion AmpliSeq™ Mouse TCR Beta-SR Assay

Table 9 Clones detected from 200 ng of gDNA extracted from FFPE-preserved mouse thymus tissue as a function of read depth – Ion AmpliSeq™ Mouse TCR Beta-SR Assay

Downsampled read count	Clones detected
10 K	6
50 K	846
250 K	15022
500 K	23221
750 K	26566
1 M	28463
1.5 M	30542
2 M	31957

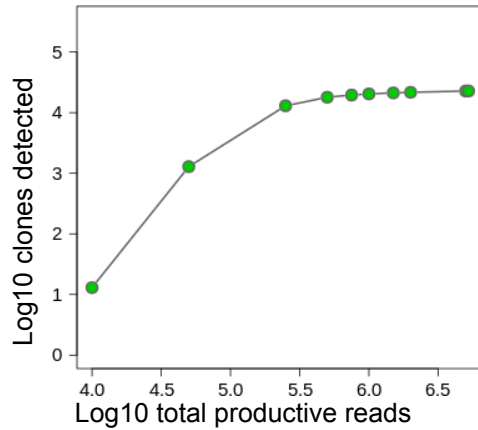


Figure 29 Clones detected from 50 ng of RNA extracted from FFPE-preserved mouse thymus tissue as a function of read depth – Ion AmpliSeq™ Mouse TCR Beta-SR Assay

Table 10 Clones and clonal lineages detected from 50 ng of RNA extracted from FFPE-preserved mouse spleen tissue as a function of read depth – Ion AmpliSeq™ Mouse BCR IGH-SR Assay

Downsampled read count	Clones detected
10 K	13
50 K	1287
250 K	12971
500 K	17958
750 K	19499
1 M	20371
1.5 M	21189
2 M	21650
5 M	22723



Effect of RNA quality on assay output

- Amplify TBP cDNA for use as a positive control 113
- Quality assessment of heat degraded RNA samples 115

To assess the effect of RNA quality on assay results we systematically degraded PBL RNA (BioChain) prior to library preparation. RNA quality was analyzed using an Agilent™ 2100 Bioanalyzer™ and independently assessed by semi-quantitative PCR of the TBP housekeeping gene (see page 113 for more information). Libraries were then sequenced to 5 M raw reads per sample and analyzed as described and evaluated for the effect of sequencing read depth on clone detection, Shannon Diversity, and Evenness.

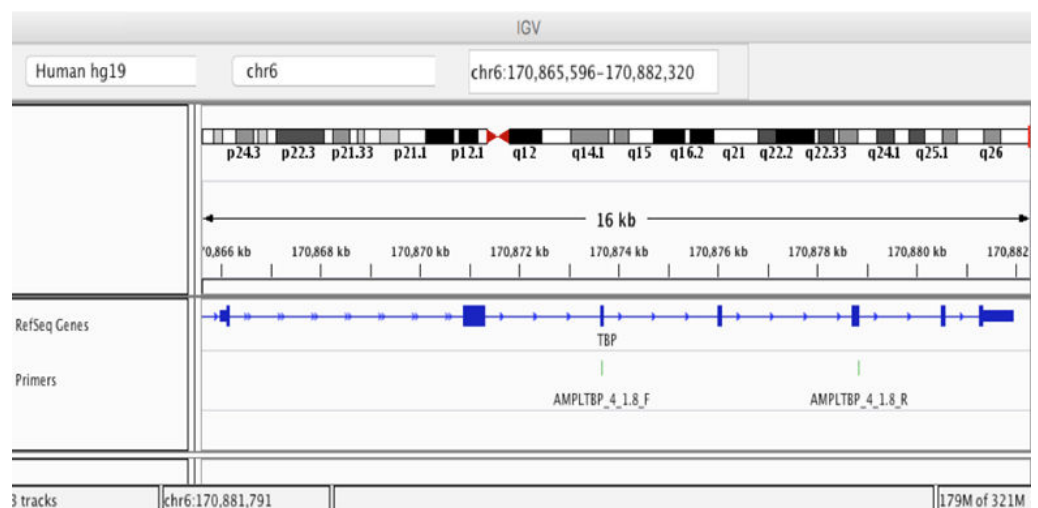
Amplify TBP cDNA for use as a positive control

Required primers (purchased separately):

TBP_300bp_Fwd: 5'-GTAAACT TGACCTAAAG ACCATTGCAC T-3'

TBP_300bp_Rev: 5'-TGAGCAC AAGGCCTTCT AACC-3'

Note: Primers can be ordered at 25-nM scale with standard desalting, then diluted to 5- μ M working stock.



Visualization of primers used for amplification of the TBP housekeeping gene from cDNA using the Integrative Genomics Viewer. The ~300bp amplicon spans two introns.

1. Thaw the 5X Ion AmpliSeq™ HiFi Mix on ice, gently vortex to mix, then centrifuge briefly to collect.
2. Place a new 96-well plate in a pre-chilled cold block or on ice.
3. Add the following components to each well in the order listed. Prepare a master mix for multiple reactions.

Component	Volume
cDNA (10 ng) ^[1]	X µL
5X Ion AmpliSeq™ HiFi Mix (red cap)	2 µL
Primer mix (2.5 µM each Fwd & Rev)	2 µL
Nuclease-Free Water	6-X µL
Total volume per well	10 µL

^[1] Quantify the cDNA as described in Appendix B, "Supplemental information", then dilute to 5–10 ng/µL.

4. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
5. Place a MicroAmp™ Compression Pad on the plate, then load the plate into a Veriti™ 96-Well Thermal Cycler.
6. Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	2 minutes
30 cycles	Denature	95°C	10 seconds
	Anneal and extend	60°C	30 seconds
Hold	—	10°C	Hold

7. Remove the plate from the thermal cycler, then briefly centrifuge the plate to collect the contents.

STOPPING POINT Target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer periods, store at -20°C.

8. Visualize the amplification product on a 2% agarose gel.

TBP housekeeping gene amplification products

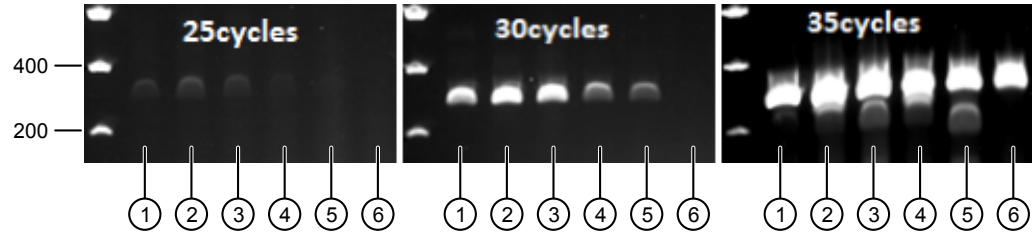


Figure 30 2% E-gels of TBP PCR products

Analysis of 300-bp amplification products of the TBP housekeeping gene on 2% E-gel using the designed primer pair. Results for the indicated number of PCR cycles are displayed. We recommend using 30 PCR cycles to evaluate RNA quality; good quality RNA will yield visible bands on the gel. Input RNA corresponds to heat-degraded RNA Figure 31 with the following RIN values.

- | | |
|-------|-------|
| ① 8.3 | ④ 2.2 |
| ② 5.7 | ⑤ 2.1 |
| ③ 4.2 | ⑥ 2.1 |

Quality assessment of heat degraded RNA samples

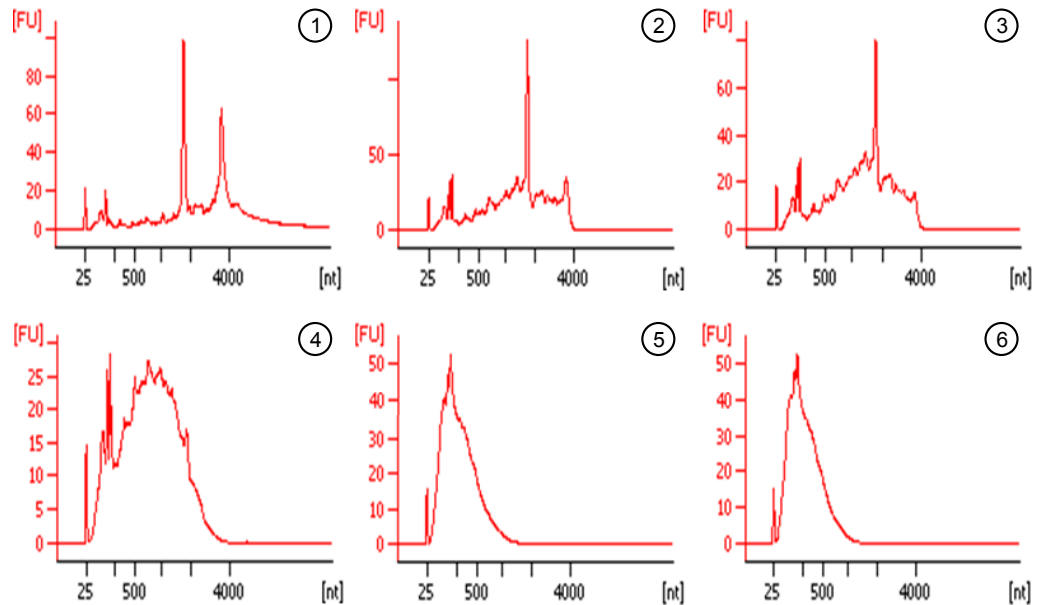


Figure 31 Agilent™ 2100 Bioanalyzer™ analysis of heat-degraded RNA samples.

Total RNA from PBL was systematically degraded by 90°C heat-treatment, then analyzed via Agilent™ 2100 Bioanalyzer™ using 20 µL of each sample at 50 ng/µL. Temperature and time for heat treatment are indicated. RIN values are derived from the Agilent™ 2100 Bioanalyzer™ software.

- | | |
|---------------------------------|----------------------------------|
| ① No heat treatment, RIN = 8.3 | ④ 30 minutes at 90°C, RIN = 2.2 |
| ② 5 minutes at 90°C, RIN = 5.7 | ⑤ 90 minutes at 95°C, RIN = 2.1 |
| ③ 10 minutes at 90°C, RIN = 4.2 | ⑥ 100 minutes at 95°C, RIN = 2.1 |

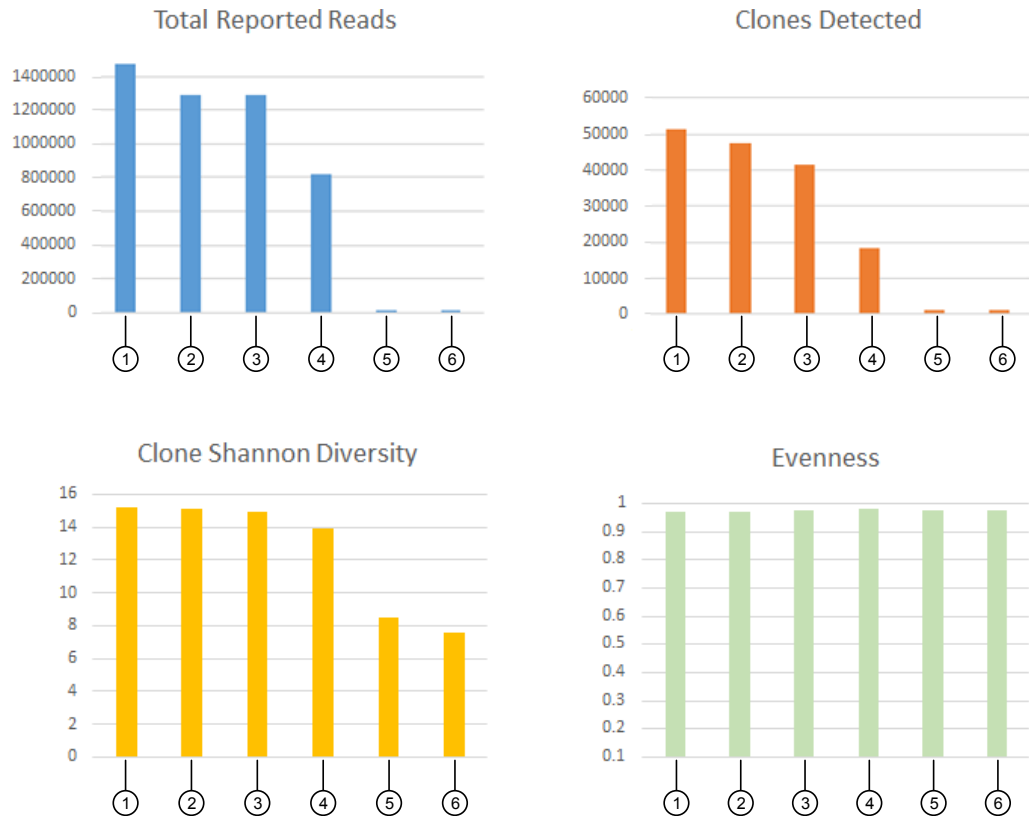


Figure 32 Effect of RNA degradation on Immune Repertoire metrics

The Total Reported Reads, number of Clones Detected, Shannon Diversity values, and Evenness for libraries derived from heat-treated RNA are represented. RNA quality affects the number of identified clones and thus diversity estimates. By contrast, measurements of clone size Evenness are more robust to differences in RNA quality.

- | | |
|---------------------------------|----------------------------------|
| ① No heat treatment, RIN = 8.3 | ④ 30 minutes at 90°C, RIN = 2.2 |
| ② 5 minutes at 90°C, RIN = 5.7 | ⑤ 90 minutes at 95°C, RIN = 2.1 |
| ③ 10 minutes at 90°C, RIN = 4.2 | ⑥ 100 minutes at 95°C, RIN = 2.1 |

Table 11 Clones detected from PBL as a function of RNA quality and read depth

- The number of clones detected correlates with RNA quality.
- RIN number may not accurately reflect the RNA fragment length.
- Sequencing of libraries derived from very low quality RNA (RIN <2.2) yielded few reads.

Note: The number of T-cells detected in tumor biopsy material may vary greatly depending on the tumor type and tumor micro-environment.

Heat treatment	RIN value	Number of raw reads							
		10 K	50 K	100 K	250 K	500 K	1 M	2 M	5 M
—	8.3	0	45	184	1280	6071	20736	40499	55854
5 minutes @ 90°C	5.7	0	52	194	1389	7000	22306	40431	53596
10 minutes @ 90°C	4.2	0	63	257	2161	9355	24620	38601	46560
30 minutes @ 90°C	2.2	3	73	403	3334	9415	16073	18939	20306
90 minutes @ 95°C	2.1	154	385	419	N/A	N/A	N/A	N/A	N/A
100 minutes @ 95°C	2.1	20	90	183	N/A	N/A	N/A	N/A	N/A

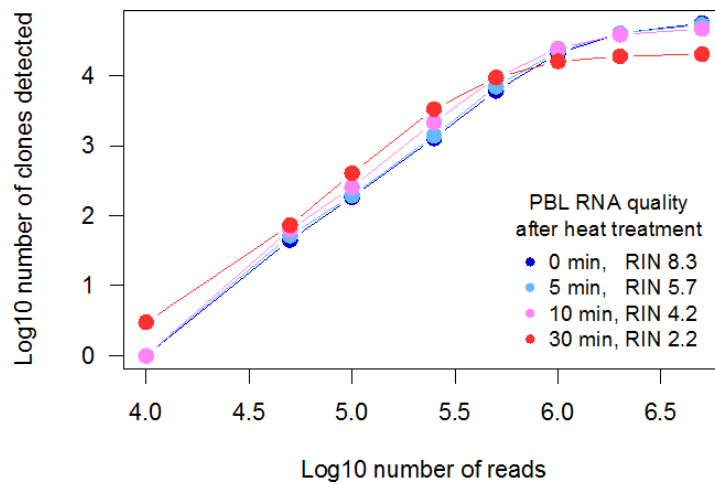


Figure 33 Clones detected from 25 ng PBL RNA as a function of RNA quality (RIN value)



Safety



WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
 - Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the “Documentation and Support” section in this document.
-

Chemical safety



WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.



WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.



WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety



WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.



WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
<https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf>
 - World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf
-

Documentation and Support

Related documentation

Document	Pub. No.
<i>Ion AmpliSeq™ Immune Repertoire Assay Plus, TCRB User Guide</i>	MAN0016953
<i>Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide</i>	MAN0013432
<i>Ion AmpliSeq™ Library Kit Plus User Guide</i>	MAN0017003
<i>Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide</i>	MAN0016854
<i>Ion 540™ Kit – Chef User Guide</i>	MAN0010851
<i>Ion 550™ Kit – Chef User Guide</i>	MAN0017275
<i>Ion Library TaqMan™ Quantitation Kit User Guide</i>	MAN0015802
<i>Demonstrated Protocol: Sample Quantification for Ion AmpliSeq™ Library Preparation Using the TaqMan™ RNase P Detection Reagents Kit</i>	MAN0007732

Note: For additional documentation, see “Customer and technical support” on page 122.

Customer and technical support

Visit thermofisher.com/support for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
 - Product FAQs
 - Software, patches, and updates
 - Training for many applications and instruments
- Order and web support
- Product documentation
 - User guides, manuals, and protocols
 - Certificates of Analysis
 - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at www.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.

Glossary

CDR1 AA	Column indicating the nucleotide and amino acid sequence for each framework and CDR region of the rearrangement.
CDR1 NT	Column indicating the nucleotide and amino acid sequence for each framework and CDR region of the rearrangement.
CDR2 AA	Column indicating the nucleotide and amino acid sequence for each framework and CDR region of the rearrangement.
CDR3 length stdev	Standard deviation of nucleotides in CDR3 region of identified clones.
Clone count	Number of clones found in the sample. Also known as clone richness.
Clone gini index	Measurement of the evenness of clone sizes. Calculated as:

$$\frac{A}{A + B}$$

Where, for a set of clone frequencies, A represents the difference between the total area under the line of equality and the area under the Lorenz Curve, and B represents the area under the Lorenz Curve. Ranges from 0 to 1. Higher value indicates dissimilar clone sizes while lower value indicates more similar clone sizes.

Clone Shannon diversity	Measurement of the clonal diversity of a repertoire. Also known as Shannon Entropy. Calculated as:
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$$-\sum_{i=1}^R p_i \log_2(p_i)$$

Where p_i indicates the frequency of the i^{th} clone and R indicates the total number of clones.

Samples having many clones of similar frequencies will have a high Shannon Diversity

convergent_BCR_frequency	Aggregate frequency of clones that have the same variable gene and the same CDR3 AA sequence, but derive from two or more clonal lineages.
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convergent_BCR_frequency_all_CDRs	Aggregate frequency of clones that have the same variable gene and the same CDR1, 2 and 3 AA sequences, but derive from two or more clonal lineages.
Convergent TCR Frequency <i>(new in Ion Reporter™ Software 5.10)</i>	Frequency of clonotypes that are identical in amino acid space but different in nucleotide space. Clonotypes are deemed to be identical in amino acid space if they have a shared variable gene (excluding allele information) and identical CDR3 amino acid sequence. Convergent TCRs can preferentially arise owing to T-cell responses to chronic antigen stimulation instead of acute but transient antigen stimulation typical of infectious disease. For this reason, the frequency of convergent TCRs may have utility as an indicator of T-cell responses to tumor antigen or auto-antigen associated with chronic autoimmune disease.
Downsampling statistics	<p>The following repertoire features are reported following downsampling of data to 10 K, 50 K, 250 K, 500 K, 750 K, 1 M, 1.5 M, 2 M, and 5 M productive plus rescued productive reads:</p> <ul style="list-style-type: none"> <li style="display: inline-block; width: 45%;">• Number of clones <li style="display: inline-block; width: 45%;">• Lineage Shannon Diversity <li style="display: inline-block; width: 45%;">• Number of lineages <li style="display: inline-block; width: 45%;">• Convergent BCR Frequency <li style="display: inline-block; width: 45%;">• Clone evenness <li style="display: inline-block; width: 45%;">• Convergent BCR Frequency- all CDRs <li style="display: inline-block; width: 45%;">• Lineage evenness <li style="display: inline-block; width: 45%;">• Clone Shannon Diversity <p>In cases where insufficient reads are available for a particular downsampling depth the corresponding fields will assume an 'NA' value.</p>
Evenness	<p>Also known as the normalized Shannon Diversity. Measurement of the similarity of clone sizes. Calculated as:</p> $\frac{-\sum_{i=1}^R p_i \log_2(p_i)}{\log_2(R)}$ <p>Where p_i indicates the frequency of the i^{th} clone and R indicates the total number of clones. Evenness values range from 0 to 1. Samples where all clones are of equal frequency have an Evenness of 1, while samples having clones of unequal sizes have Evenness <1.</p>
FR1 AA	Column indicating the nucleotide and amino acid sequence for each framework and CDR region of the rearrangement.
FR1 NT	Column indicating the nucleotide and amino acid sequence for each framework and CDR region of the rearrangement.
FR2 AA	Column indicating the nucleotide and amino acid sequence for each framework and CDR region of the rearrangement.
FR2 NT	Column indicating the nucleotide and amino acid sequence for each framework and CDR region of the rearrangement.

FR3 AA	Column indicating the nucleotide and amino acid sequence for each framework and CDR region of the rearrangement.
FR3 NT	Column indicating the nucleotide and amino acid sequence for each framework and CDR region of the rearrangement.
lineage_evenness	Normalized Shannon entropy, calculated using the set of lineage frequencies.
lineage_gini_index	The Gini index, calculated using the set of lineage frequencies.
Max CDR3 length	Maximum number of nucleotides in CDR3 region of identified clones.
Max read length	Maximum length of productive or rescued productive reads identified after initial processing of rearrangements.
Mean CDR3 length	Average number of nucleotides in CDR3 region of identified clones.
Mean read length	Average length of productive or rescued productive reads identified after initial processing of rearrangements.
Median CDR3 length	Median number of nucleotides in CDR3 region of identified clones.
Median read length	Median length of productive or rescued productive reads identified after initial processing of rearrangements.
Min CDR3 length	Minimum number of nucleotides in CDR3 region of identified clones.
Min read length	Minimum length of productive or rescued productive reads identified after initial processing of rearrangements.
Minus strand counts	Number of reads mapping to the negative strand of the VDJ rearrangement (proceeding from C to FR1 region) after initial processing of rearrangements.
Minus strand CDR3 phred avg	Average PHRED score over the CDR3 region of reads mapping to the negative strand of the VDJ rearrangement.
Minus strand CDR3 phred stdev	Standard deviation of PHRED scores for reads mapping to the negative strand of the VDJ rearrangement.
Mode CDR3 length	Mode number of nucleotides in CDR3 region of identified clones.
Mode read length	Mode length of productive or rescued productive reads identified after initial processing of rearrangements.
Off target reads	Reads which are of low quality or represent the product of an off-target amplification.
Overcalled A bases	Number of A nucleotide insertion errors in on-target reads.

Overcalled C bases	Number of C nucleotide insertion errors in on-target reads.
Overcalled G bases	Number of G nucleotide insertion errors in on-target reads.
Overcalled T bases	Number of T nucleotide insertion errors in on-target reads.
Plus strand counts	Number of reads mapping to the positive strand of the VDJ rearrangement (proceeding from FR1 to C region) after initial processing of rearrangements.
Plus strand CDR3 phred avg	Average PHRED score over the CDR3 region of reads mapping to the positive strand of the VDJ rearrangement.
Plus strand CDR3 phred stdev	Standard deviation of PHRED scores for reads mapping to the positive strand of the VDJ rearrangement.
Productive reads	Reads having in-frame variable and joining genes and no stop codons.
Proportion germline reads	Proportion of productive or rescued productive reads have <1% base differences over the variable gene with respect to reference.
Proportion perfect reads	Proportion of productive or rescued productive reads having no mismatches to reference over the variable gene with respect to reference.
Proportion reads having mismatches	Proportion of productive or rescued productive reads having mismatches to reference over the variable gene with respect to reference.
Read length stdev	Standard deviation of lengths of productive or rescued productive reads identified after initial processing of rearrangements.
Repertoire metrics file	The Ion Reporter™ Software Analysis produces a file containing a comma-separated list of secondary repertoire metrics (metrics.csv) including indicators of TCR richness, diversity, evenness, variable gene representation, and various quality-control metrics. The metrics file is included in the zip folder containing the output from a single sample analysis, and can also be downloaded via the "download aggregate metrics" selection appearing following use of the "compare samples" function. A full description of the metrics found in this file is included in the glossary at the end of the user guide.
Reported read count	Number of reads reported after indel correction, quality filtering, and elimination of reads representing clones with insufficient coverage.
Rescued productive reads	Reads having in-frame variable and joining genes and no stop codons after indel error correction.
Undercalled A bases	Number of A nucleotide deletion errors in on-target reads.

Undercalled C bases	Number of C nucleotide deletion errors in on-target reads.
Undercalled G bases	Number of G nucleotide deletion errors in on-target reads.
Undercalled T bases	Number of T nucleotide deletion errors in on-target reads.
Unproductive reads	Reads having uncorrectable sequencing errors or PCR errors which lead the rearrangement to have out-of-frame variable and joining genes or a premature stop codon.
Variable Gene SHM Locations	Semicolon delimited list of differences in the variable gene sequence with respect to the best matching IMGT [®] reference. These differences are presumed to arise from somatic hypermutation, though mismatching positions found in a plurality of clones from different clonal lineages could indicate the presence of an unannotated (non-IMGT) variable gene allele. The notation is provided as the number of bases from the start of the CDR3 sequence (0-based numbering) along with the identity of the base at the mismatched position. For example, '7A' indicates the presence of an A mismatch with respect to the reference at base 7 from the start of the CDR3. The variable gene SHM locations can be independently derived by pairwise comparison of the sequences in the 'Sequence' and 'Reference Sequence' columns of the clone summary file.
Vgene_evenness	Also known as variable gene normalized Shannon Diversity. Measurement of the similarity of variable gene frequencies in a repertoire. Ranges from 0 to 1. Samples having variable genes represented at equal frequencies will have a variable gene Evenness value of 1, while samples having variable genes of unequal frequencies have Evenness <1.
Vgene gini index	Measurement of the evenness of variable gene frequencies. Ranges from 0 to 1. Higher value indicates dissimilar clone sizes while lower value indicates more similar clone sizes.
vgene shannon diversity	Variable gene Shannon Diversity. Measurement of the diversity of variable genes present in a repertoire. Calculated using the frequency of each variable gene within the repertoire. Samples having variable genes represented at similar frequencies will have a high variable gene Shannon Diversity.

