ion torrent

Oncomine™ Human Immune Repertoire

USER GUIDE

For use in hematology research

using:
Oncomine™ BCR Pan-Clonality Assay
Oncomine™ IGH FR3(d)-J Assay
Oncomine™ IGH FR2-J Assay
Oncomine™ IGH FR1-J Assay
Oncomine™ IGHV Leader-J Assay
Oncomine™ TCR Pan-Clonality Assay

Catalog Numbers A51547, A51559, A51560, A51561, A51562, A51563, A51564

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

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0019231

Revision	Date	Description
B.0	24 September 2021	• Added section for recommended materials for quantification of clone cell numbers (page 20). • Added information for the Ion Torrent™ Dual Barcode Kit 1–96 (page 26). • Updated the Torrent Suite™ Software version and run plan template names (page 85). • Added a new troubleshooting topic (page 134). • Updated multiplexing level for Ion 530™ Chip and moved SMH analysis information to a separate section (page 146). • Created separate section for SMH analysis and updated multiplexing level for Ion 530™ Chip (page 147). • Added *Oncomine™ Human Immune Repertoire User Guide*, MAN0017438 (page 153).
A.0	15 July 2021	New user guide for Oncomine™ Human Immune Repertoire assays. • Oncomine™ BCR Pan-Clonality Assay • Oncomine™ IGH FR3(d)-J Assay • Oncomine™ IGH FR2-J Assay • Oncomine™ IGH FR1-J Assay • Oncomine™ IGHV Leader-J Assay • Oncomine™ TCR Pan-Clonality Assay

Contents

1 Product information

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

Product description

The Oncomine™ portfolio of immune repertoire assays for hematology-oncology research represent highly sensitive, next-generation sequencing (NGS) assays that accurately measure B- and T cell repertoire diversity and clonality in a variety of sample types. All Oncomine™ Immune Repertoire Assays use Ion AmpliSeq™ library construction technology.

The Oncomine™ BCR Pan-Clonality Assay and the Oncomine™ TCR Pan-Clonality Assay each represent a step forward in repertoire assessment by NGS, by combining multiple B- and T cell receptor targets in a single library construction reaction. Multiple receptor assays allow for simpler determination of clonality from DNA samples using fewer secondary tests and conserving sample material.

The Oncomine™ BCR Pan-Clonality Assay targets the B cell heavy chain (IGH) and light chains – kappa and lambda (IgK/IgL). The Oncomine™ TCR Pan-Clonality Assay covers both the TCR beta and gamma chains. Both assays use primers targeting the framework 3 (FR3) and joining (J) regions of the specified receptor, producing an amplicon between 60–120 bp in length. This enables measurement from DNA extracted from sample types including whole blood, peripheral blood leukocytes (PBL), peripheral blood momonuclear cells (PBMC), bone marrow aspirates (BMA) and bone marrow mononuclear cells (BMMC), as well as formalin-fixed paraffin-embedded (FFPE) preserved tissue.

DNA samples which include B cells containing a high rate of somatic hypermutation (SHM) may employ the Oncomine™ IGH FR3(d)-J Assay and Oncomine™ IGH FR2-J Assay. The Oncomine™ IGH FR3(d)-J Assay targets the distal portion of the framework 3 (FR3(d)) and J regions of the rearranged heavy chain resulting in amplicons 150–200 bp in length. The Oncomine™ IGH FR2-J Assay produces longer (200–250 bp) amplicons by targeting the framework 2 (FR2) and J regions of the IGH chain.

Accuracy in determination of somatic hypermutation rates depend on sufficient coverage of the IGH variable gene (IGHV). The Oncomine™ IGH FR1-J Assay and the Oncomine™ IGHV Leader-J Assay were each designed to accurately assess the extent of SHM in the IGHV gene region using DNA input from blood or bone marrow derived samples. This pair of assays use primers targeting either the framework 1 (FR1) or leader regions and J gene of the IGH chain, producing ~300–350 bp and ~480–500 bp reads, respectively.

This guide covers the following products:

- Oncomine™ BCR Pan-Clonality Assay
- Oncomine™ IGH FR3(d)-J Assay
- Oncomine™ IGH FR2-J Assay
- Oncomine™ IGH FR1-J Assay
- Oncomine™ IGHV Leader-J Assay
- Oncomine™ TCR Pan-Clonality Assay

Each of these assay kits include components of the Ion AmpliSeq™ Library Kit Plus. The Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802, purchased separately) is required for library quantification.

These kits are designed for use with the Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360, purchased separately) so that multiple libraries, using separate barcodes, can be combined and loaded onto a single Ion 530™, Ion 540™, or Ion 550™ Chip to reduce the per-sample sequencing cost.

Ion GeneStudio™ S5 Series instrument reference

In this document, Ion GeneStudio™ S5 Series Sequencer or Ion GeneStudio™ S5 Series System refers generically to the 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)

Oncomine™ BCR Pan-Clonality Assay

The Oncomine™ BCR Pan-Clonality Assay is a highly sensitive, single reaction, DNA-based NGS assay that enables the characterization of B cell heavy chain (IGH) and light chain (IgK and IgL) receptor sequences, including each complementarity-determining region 3 (CDR3). The assay accurately measures B cell repertoire metrics such as repertoire diversity and clonality in multiple receptors in a single reaction.

Table 1 Oncomine™ BCR Pan-Clonality Assay sample types

Sample types		Assay Input	Supported Chips
Peripheral blood	Whole blood	gDNA	Ion 520™ Chip[1] Ion 530™ Chip Ion 540™ Chip Ion 550™ Chip[2]
	Periperal Blood Leukocytes (PBL)	gDNA	
	Periperal Blood Mononuclear Cells (PBMC)	gDNA	
Tissue	Fresh frozen	gDNA	
	Formalin-Fixed Paraffin-Embedded (FFPE)	gDNA	
Isolated cells	Sorted B cells	gDNA	

Table 1 Oncomine BCR Pan-Clonality Assay sample types *(continued)*

Sample types		Assay Input	Supported Chips
Bone marrow	Bone marrow aspirate (BMA)	gDNA	Ion 520™ Chip[1] Ion 530™ Chip Ion 540™ Chip Ion 550™ Chip[2]
	Bone marrow mononuclear cells (BMMC)	gDNA	

[1] if using an Ion 520™ Chip contact support for assistance.

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

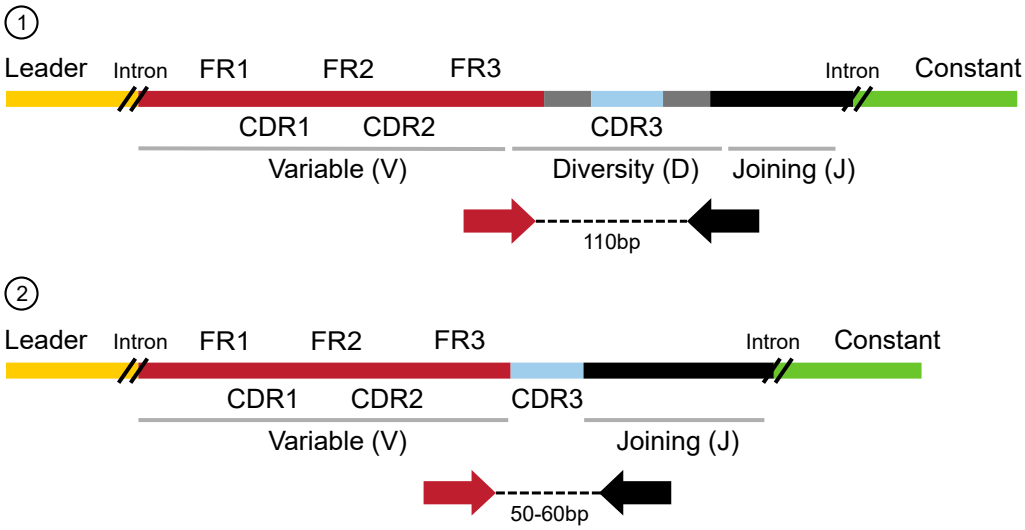


Figure 1 Oncomine™ BCR Pan-Clonality Assay

The Oncomine™ BCR Pan-Clonality Assay uses multiplex Ion AmpliSeq™ primers to target the FR3 region of the variable gene and the joining gene segments of IGH, IGK, and IGL rearrangements including rearrangements containing the kappa deletion element (KDE) in genomic DNA producing 50–110 bp amplicons.

① IGH rearrangement

② IGK/IGL rearrangement

Oncomine™ IGH FR3(d)-J Assay and Oncomine™ IGH FR2-J Assay

The Oncomine™ IGH FR3(d)-J Assay and Oncomine™ IGH FR2-J Assay are highly sensitive, DNA-based NGS assays that enable the characterization of B cell heavy chain (IGH) sequences, including complementarity-determining region 3 (CDR3). The assays accurately measure B cell repertoire metrics such as repertoire diversity and clonality.

Table 2 Oncomine™ IGH FR3(d)-J Assay and Oncomine™ IGH FR2-J Assay sample types

Sample types		Assay Input	Supported Chips
Peripheral blood	Whole blood	gDNA	Ion 520™ Chip[1] Ion 530™ Chip Ion 540™ Chip Ion 550™ Chip[2]
	Peripheral Blood Leukocytes (PBL)	gDNA	
	Peripheral Blood Mononuclear Cells (PBMC)	gDNA	
Tissue	Fresh frozen	gDNA	
	Formalin-Fixed Paraffin-Embedded (FFPE)	gDNA	
Isolated cells	Sorted B cells	gDNA	
Bone marrow	Bone marrow aspirate (BMA)	gDNA	
	Bone marrow mononuclear cells (BMMC)	gDNA	

[1] if using an Ion 520™ Chip contact support for assistance.

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

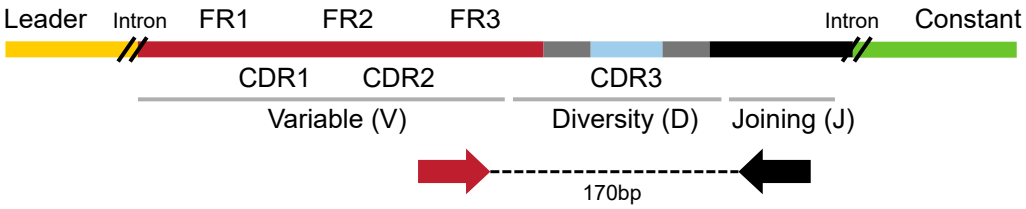


Figure 2 Oncomine™ IGH FR3(d)-J Assay

The Oncomine™ IGH FR3(d)-J Assay uses multiplex Ion AmpliSeq™ primers to target the distal section of the FR3 region of the variable gene and the joining gene segment of IGH rearrangements in genomic DNA producing 150–200 bp amplicons.

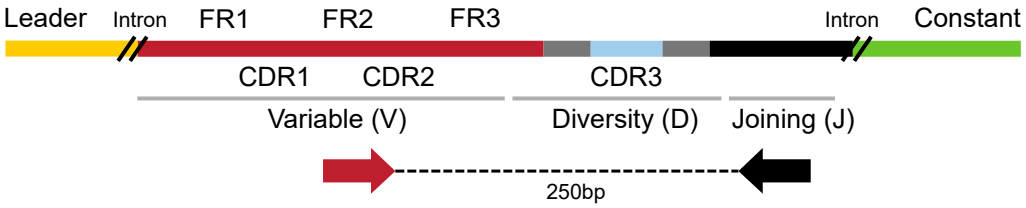


Figure 3 Oncomine™ IGH FR2-J Assay

The Oncomine™ IGH FR2-J Assay uses multiplex Ion AmpliSeq™ primers to target the FR2 region of the variable gene and the joining gene segment of IGH rearrangements in genomic DNA producing 200–250 bp amplicons.

Oncomine™ IGH FR1-J Assay and Oncomine™ IGHV Leader-J Assay

The Oncomine™ IGH FR1-J Assay and Oncomine™ IGHV Leader-J Assay are highly sensitive, DNA-based NGS assays that enable the characterization of B cell heavy chain (IGH) sequences, including complementarity-determining region 3 (CDR3). These assays accurately measure B cell clonality and somatic hypermutation rate.

Table 3 Oncomine™ IGH FR1-J Assay and Oncomine™ IGHV Leader-J Assay sample types

Sample types		Assay Input	Supported Chips
Peripheral blood	Whole blood	gDNA	Ion 520™ Chip[1] Ion 530™ Chip
	Peripheral Blood Leukocytes (PBL)	gDNA	
	Peripheral Blood Mononuclear Cells (PBMC)	gDNA	
Isolated cells	Sorted B-cells	gDNA	
Bone marrow	Bone marrow aspirate (BMA)	gDNA	
	Bone marrow mononuclear cells (BMMC)	gDNA	

[1] if using an Ion 520™ Chip contact support for assistance.

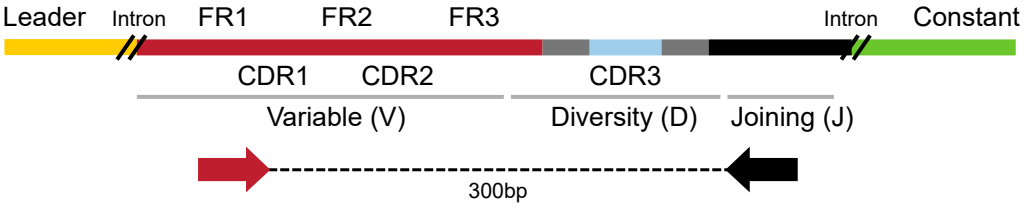


Figure 4 Oncomine™ IGH FR1-J Assay

The Oncomine™ IGH FR1-J Assay uses multiplex Ion AmpliSeq™ primers to target the FR1 region of the variable gene and the joining gene segment of IGH rearrangements in genomic DNA producing 300–350 bp amplicons.

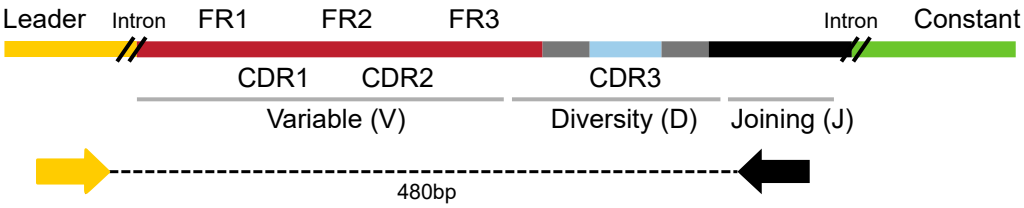


Figure 5 Oncomine™ IGHV Leader-J Assay

The Oncomine™ IGHV Leader-J Assay uses multiplex Ion AmpliSeq™ primers to target the Leader region and the joining gene segment of IGH rearrangements in genomic DNA producing 480–500 bp amplicons.

Oncomine™ TCR Pan-Clonality Assay

The Oncomine™ TCR Pan-Clonality Assay is a highly sensitive, DNA-based NGS assay that enables the characterization of T cell receptor beta and gamma chain sequences including complementarity-determining region 3 (CDR3).

Table 4 Oncomine™ TCR Pan-Clonality Assay sample types

Sample types		Assay Input	Supported Chips
Peripheral blood	Whole blood	gDNA	Ion 520™ Chip[1] Ion 530™ Chip
	Periperal Blood Leukocytes (PBL)	gDNA	
	Periperal Blood Mononuclear Cells (PBMC)	gDNA	
Isolated cells	Sorted T-cells	gDNA	
Bone marrow	Bone marrow aspirate (BMA)	gDNA	
	Bone marrow mononuclear cells (BMMC)	gDNA	

[1] if using an Ion 520™ Chip contact support for assistance.

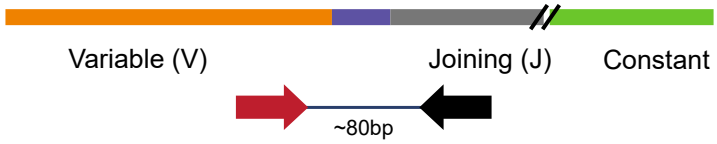


Figure 6 Oncomine™ TCR Pan-Clonality Assay

The Oncomine™ TCR Pan-Clonality Assay uses multiplex Ion AmpliSeq™ primers to target the FR3 region of the variable gene and the joining gene segment of TCR beta and TCR gamma rearrangemnets in genomic DNA producing ~75–100 bp amplicons.

Contents and storage

Oncomine™ BCR Pan-Clonality Assay Kit

The Oncomine™ BCR Pan-Clonality Assay (Cat. Nos. A51559 & A51547) consists of the Oncomine™ BCR Pan-Clonality Assay panel (Part No. A51040), the Ion AmpliSeq™ Library Kit Plus (Cat. Nos. 4488990 & A35907), and premixed dNTPs (Part No. R1121). Sufficient reagents are provided for the rapid preparation of up to 24 or 96 sample libraries.

In addition, the Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360) is required for the rapid preparation of barcoded sample libraries from DNA that can be combined and sequenced simultaneously. The Ion Torrent™ Dual Barcode Kit 1–96 is included with the Oncomine™ BCR Pan-Clonality Assay (Cat. No. A51547) but must be ordered separately with (Cat. No. A51559).

Contents	Amount		Storage
	Cat. No. A51559 (24 reactions)	Cat. No. A51547 (96 reactions)	
Oncomine™ BCR Pan-Clonality Assay			
5X Oncomine™ BCR Pan-Clonality Assay Panel	96 µL	384 µL	–25°C to –15°C
dNTP Mix (25 mM each)	1.0 mL	1.0 mL	
Ion AmpliSeq™ Library Kit Plus			
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	480 µL	–30°C to –10°C
FuPa Reagent (brown cap)	48 µL	192 µL	
Switch Solution (yellow cap)	96 µL	384 µL	
DNA Ligase (blue cap)	48 µL	192 µL	
25X Library Amp Primers (pink cap)	48 µL	192 µL	
1X Library Amp Mix (black cap)	1.2 mL	4 x 1.2 mL	
Low TE (clear cap)	6 mL	2 x 6 mL	15°C to 30°C[1]

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

Oncomine™ IGH FR3(d)-J Assay Kit

The Oncomine™ IGH FR3(d)-J Assay (Cat. No. A51560) consists of the Oncomine™ IGH FR3(d)-J Assay panel (Part No. A51041), the Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990), and premixed dNTPs (Part No. R1121). Sufficient reagents are provided for the rapid preparation of up to 24 sample libraries.

In addition, the Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360) is required for the rapid preparation of barcoded sample libraries from DNA that can be combined and sequenced simultaneously, and must be ordered separately.

Contents	Amount	Storage
Oncomine™ IGH FR3(d)-J Assay (Cat. No. A51560)		
5X Oncomine™ IGH FR3(d)-J Assay Panel	96 µL	–25°C to –15°C
dNTP Mix (25 mM each)	1.0 mL	
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)	6 mL	15°C to 30°C[1]

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

Oncomine™ IGH FR2-J Assay Kit

The Oncomine™ IGH FR2-J Assay (Cat. No. A51561) consists of the Oncomine™ IGH FR2-J Assay panel (Part No. A51042), the Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990), and premixed dNTPs (Part No. R1121). Sufficient reagents are provided for the rapid preparation of up to 24 sample libraries.

In addition, the Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360) is required for the rapid preparation of barcoded sample libraries from DNA that can be combined and sequenced simultaneously, and must be ordered separately.

Contents	Amount	Storage
Oncomine™ IGH FR2-J Assay (Cat. No. A51561)		
5X Oncomine™ IGH FR2-J Assay Panel	96 µL	–25°C to –15°C
dNTP Mix (25 mM each)	1.0 mL	

Contents	Amount	Storage
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)	6 mL	15°C to 30°C[1]

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

Oncomine™ IGH FR1-J Assay Kit

The Oncomine™ IGH FR1-J Assay (Cat. No. A51564) consists of the Oncomine™ IGH FR1-J Assay panel (Part No. A51043), the Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990), and premixed dNTPs (Part No. R1121). Sufficient reagents are provided for the rapid preparation of up to 24 sample libraries.

In addition, the Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360) is required for the rapid preparation of barcoded sample libraries from DNA that can be combined and sequenced simultaneously, and must be ordered separately.

Contents	Amount	Storage
Oncomine™ IGH FR1-J Assay (Cat. No. A51564)		
5X Oncomine™ IGH FR1-J Assay Panel	96 µL	–25°C to –15°C
dNTP Mix (25 mM each)	1.0 mL	
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)	6 mL	15°C to 30°C[1]

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

Oncomine™ IGHV Leader-J Assay Kit

The Oncomine™ IGHV Leader-J Assay (Cat. No. A51563) consists of the Oncomine™ IGHV Leader-J Assay panel (Part No. A51045), the Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990), and premixed dNTPs (Part No. R1121). Sufficient reagents are provided for the rapid preparation of up to 24 sample libraries.

In addition, the Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360) is required for the rapid preparation of barcoded sample libraries from DNA that can be combined and sequenced simultaneously, and must be ordered separately.

Contents	Amount	Storage
Oncomine™ IGHV Leader-J Assay (Cat. No. A51563)		
5X Oncomine™ IGHV Leader-J Assay Panel	96 µL	–25°C to –15°C
dNTP Mix (25 mM each)	1.0 mL	
Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990)		
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	–30ºC to –10ºC
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)	6 mL	15°C to 30°C[1]

[1] Can be stored at –30ºC to –10ºC for convenience.

Oncomine™ TCR Pan-Clonality Assay Kit

The Oncomine™ TCR Pan-Clonality Assay (Cat. No. A51562) consists of the Oncomine™ TCR Pan-Clonality Assay panel (Part No. A51044), the Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990), and premixed dNTPs (Part No. R1121). Sufficient reagents are provided for the rapid preparation of up to 24 or 96 sample libraries.

In addition, the Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360) is required for the rapid preparation of barcoded sample libraries from DNA that can be combined and sequenced simultaneously, and must be ordered separately.

Contents	Amount	Storage
Oncomine™ TCR Pan-Clonality Assay (Cat. No. A51562)		
5X Oncomine™ TCR Pan-Clonality Assay Panel	96 µL	–25°C to –15°C
dNTP Mix (25 mM each)	1.0 mL	
Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990)		
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	–30ºC to –10ºC
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)	6 mL	15°C to 30°C[1]

[1] Can be stored at –30ºC to –10ºC for convenience.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another 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: • 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™ Clear Adhesive Film	4306311
MicroAmp™ Optical Film 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" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item		Source
Recommended additional equipment		
One of the following Applied Biosystems™ real-time PCR instruments: • 7500 Real-Time PCR System • 7900HT Fast Real-Time PCR System[1] • StepOne™ Real-Time PCR System • StepOnePlus™ Real-Time PCR System • ViiA™ 7 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	See web product pages
Qubit™ 4 Fluorometer[2]		Q33238
Fisher Scientific™ Mini Plate Spinner Centrifuge, or equivalent 96-well plate centrifuge		MLS
BD Vacutainer® Plastic Blood Collection Tubes with K_2EDTA: 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
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
Recommended for quantification		
Qubit™ dsDNA HS Assay Kit		Q32851
Recommended for functional quantification of DNA samples		
TaqMan™ RNase P Detection Reagents Kit		4316831
TaqMan™ Fast Advanced Master Mix		4444556
TaqMan™ Control Genomic DNA		4312660
GUSB TaqMan™ Gene Expression Assay		Hs04944335_cn

(continued)

Item	Source
Recommended for quantification of clone cell numbers	
Jurkat, Clone E6-1	ATCC® TIB-152 **www.atcc.org**
TALL-104	ATCC® CRL-11386 **www.atcc.org**
HuT 78	ATCC® TIB-161 **www.atcc.org**
CA46	ATCC® CRL-1648 **www.atcc.org**
Toledo	ATCC® CRL-2631 **www.atcc.org**
BDCM	ATCC® CRL-2740 **www.atcc.org**
TMM	DSMZ ACC-95 **dsmz.de**

[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.

2 Before you begin

Immune repertoire sequencing overview

The immune repertoire refers to the collection of B and T-cell receptors present in an individual. Human T-cell receptors (TCR) are heterodimers that fall into two classes: TCR-αβ, and TCR-γδ. TCR-αβ T-cells constitute most of the T-cell receptor repertoire (typically >90% of T-cells) and are the primary contributors to cellular adaptive immunity. The human 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 (IgK and 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 B and T-cell receptors, the CDR3 region of the TCRβ and IGH chain contains the greatest sequence diversity. This sequence diversity may be used to measure immune repertoire diversity, and clonality.

Procedural guidelines

- Minimize freeze-thaw cycles of Oncomine™ 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.
- Bring the Agencourt™ AMPure™ XP Reagent to room temperature.

IMPORTANT! Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

GUSB qPCR DNA sample quantification

GUSB is a useful gene for quantifying amplifiable DNA in low-quality or FFPE DNA samples when used in conjunction with a standard curve generated from a genomic DNA standard of known concentration.

Note: Alternatively, quantify amplifiable DNA with the TaqMan™ RNase P Detection Reagents Kit. For more information, see “Related documentation” on page 153.

1. Place a new 96-well MicroAmp™ Optical Reaction Plate in a pre-chilled 4°C cold block or on ice.
2. Prepare a 2-fold dilution of each sample DNA. Pipet 5 µL of each sample DNA into an individual well in the 96-well plate. To each well add 5 µL nuclease-free water, then pipet up and down 5X to mix.
3. Prepare four 5-fold serial dilutions of TaqMan™ Control Genomic DNA (10 ng/µL) at 2.0 ng/µL, 0.4 ng/µL, 0.08 ng/µL and 0.016 ng/µL.
4. Calculate, then prepare sufficient master mix for all samples.

Component	Volume/Sample
Nuclease-free water	2.5 µL
2X TaqMan™ Fast Advanced Master Mix	5 µL
Primer/Probe Mix (GUSB)	0.5 µL

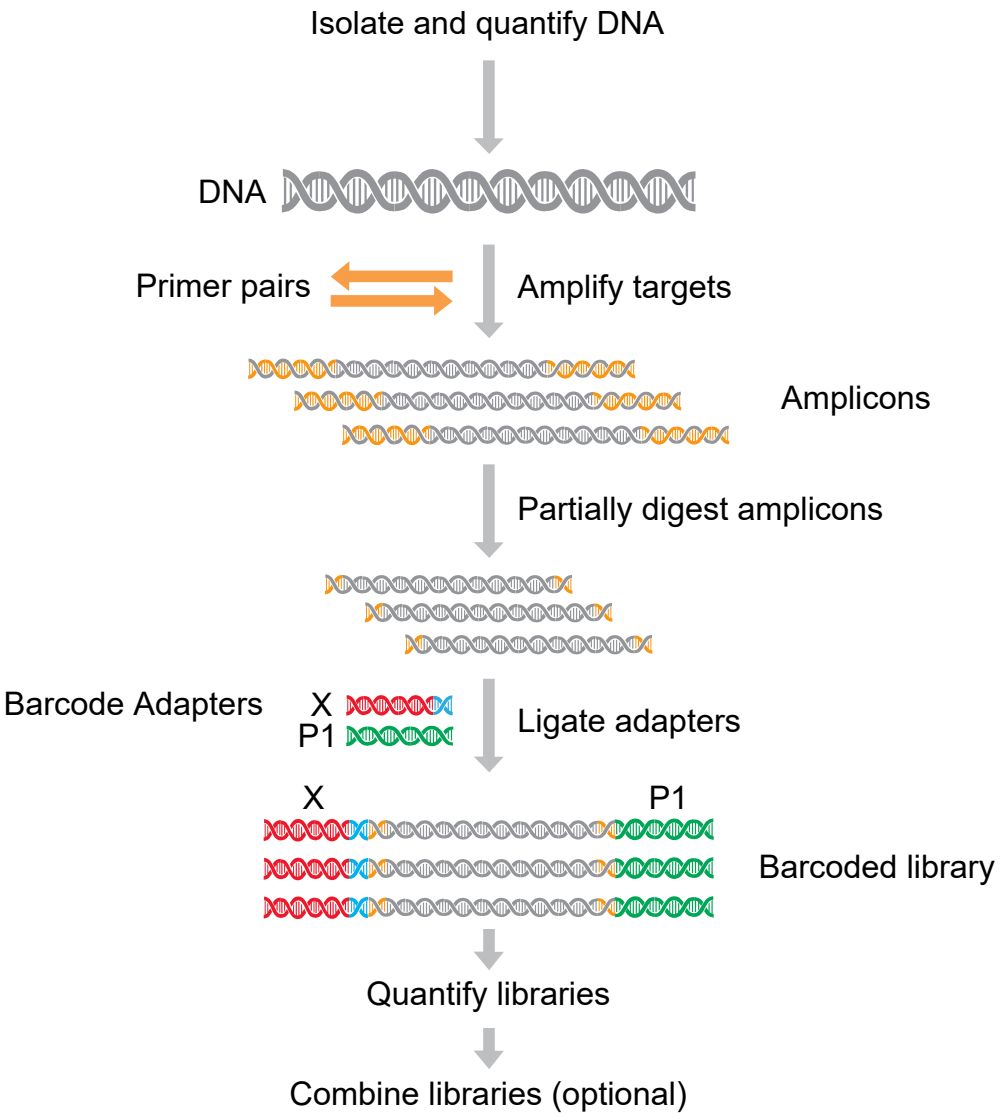
5. Pipet 8 µL of master mix into each reaction well.
6. To each reaction well add 2 µL 2-fold diluted sample DNA or 2 µL standard.
7. Seal plate with a MicroAmp™ Optical Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. .

8. Program your Real-Time PCR instrument.

Stage	Temperature	Time
Hold (UDG incubation)	50°C	2 minutes
Hold (polymerase activation)	95°C	20 seconds
40 cycles	95°C	3 seconds
	60°C	60 seconds

9. Following qPCR, calculate the average concentration of the sample DNA by multiplying the determined concentration × 2.

Workflow


Isolate and quantify DNA
DNA
Primer pairs
Amplify targets
Amplicons
Partially digest amplicons
Barcode Adapters
X
P1
Ligate adapters
X
P1
Barcoded library
Quantify libraries
Combine libraries (optional)

3 Oncomine™ BCR Pan-Clonality Assay library preparation

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.

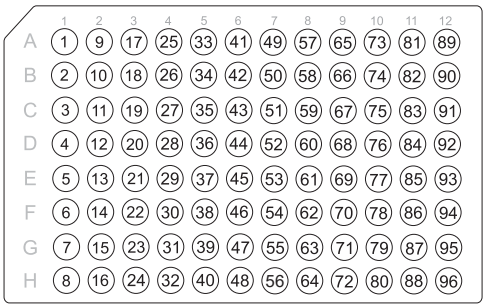
Proceed to the target amplification procedure specific to the assay and sample type you are performing.

Guidelines for DNA isolation, quantification, and input

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

- Use kits described in "Required materials not supplied" for isolating high quality gDNA from research samples for use in library preparation.
- We recommend the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) for quantifying human genomic DNA.
- Quantification methods such as densitometry (for example, using a NanoDrop™ spectrophotometer) are not recommended, because they are not specific for DNA. Use of these methods can lead to gross overestimation of the sample DNA concentration, under-seeding of the target amplification reaction, and low library yields.
- 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 plastic BD Vacutainer® blood collection tube with K_2EDTA: BD Hemogard™ Closure. See "Recommended materials" on page 20.
- Sample input amount and the target amplification cycling program vary with sample type. In general, DNA from sorted cell samples includes a greater fraction of relevant template molecules. The fraction of relevant molecules is higher when using PBMC or PBL compared to whole blood.
- Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown.
- In general, library yield from high-quality DNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.

- The Ion Torrent™ Dual Barcode Kit 1–96 is arrayed by column with barcode 1 in position A1, barcode 8 in position H1, and barcode 96 in position H12.



Set up DNA target amplification reactions

Prepare DNA target amplification reactions

IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place a new 96-well plate in a pre-chilled 4°C cold block or on ice.
2. Dilute the dNTP mix (25 mM each) to 6.0 mM each.

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

3. Gently vortex the 5X Ion AmpliSeq™ HiFi Mix, centrifuge briefly to collect, then keep on ice.
4. For each sample, add the following components to each sample well. Prepare a master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.0 µL
5X Oncomine™ BCR Pan-Clonality Assay panel	4.0 µL
dNTP Mix (6 mM each dNTP, prepared in step 2)	2.0 µL
gDNA (200 ng–2.0 µg)[1]	≤10 µL
Nuclease-free Water	to 20 µL

[1] Substitute an equal volume of nuclease-free water or low TE to prepare a No-Template Control (NTC).

Note: Avoid using columns on the periphery of the plate.

5. Seal the plate with a 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.

Amplify the targets

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that equivalent amounts of sample is used in each well so that the selected cycle number is optimal for all the samples in the run.

1. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.
2. 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] Increase number by +3 when starting from FFPE samples. Cycle number can be increased when input material quality or quantity is questionable.

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

Partially digest the amplicons

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

1. Thaw the FuPa Reagent (brown cap) on ice, gently vortex to mix, then centrifuge briefly to collect.
2. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.

   IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly.
3. Seal the plate with a 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.
4. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

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

Proceed immediately to “Perform the ligation reaction“.

IMPORTANT! Do not store the partially digested amplicons overnight.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single run, you *must* ligate a different barcode 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 sample 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 24 hours)

STOPPING POINT Samples can be stored for up to 24 hours at 10°C on the thermal cycler. For longer periods, store 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.
- Agencourt™ AMPure™ XP Reagent is viscous, pipet the solution slowly.
- Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

1. Prepare 70% ethanol (350 µL × # of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. 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.

4. Incubate the mixture for 5 minutes at room temperature.
5. 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.
6. 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.

7. Repeat step 6 for a second wash.

8. 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.

   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.

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.

Proceed to "Quantify the library by qPCR and calculate the dilution factor" on page 31.

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 PCR instrument.

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

   a. Enter the concentrations of the control library standards.

   b. Select ROX™ Reference Dye as the passive reference dye.

   c. Select a reaction volume of 20 µL.

   d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

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 83, 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 140.
- 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 pM/25 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 83, 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, transfer the supernatant to a new well and seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

4 Oncomine™ IGH FR3(d)-J Assay library preparation

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.

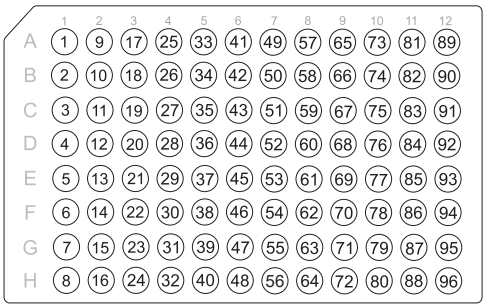
Proceed to the target amplification procedure specific to the assay and sample type you are performing.

Guidelines for DNA isolation, quantification, and input

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

- Use kits described in "Required materials not supplied" for isolating high quality gDNA from research samples for use in library preparation.
- We recommend the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) for quantifying human genomic DNA.
- Quantification methods such as densitometry (for example, using a NanoDrop™ spectrophotometer) are not recommended, because they are not specific for DNA. Use of these methods can lead to gross overestimation of the sample DNA concentration, under-seeding of the target amplification reaction, and low library yields.
- 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 plastic BD Vacutainer® blood collection tube with K_2EDTA: BD Hemogard™ Closure. See "Recommended materials" on page 20.
- Sample input amount and the target amplification cycling program vary with sample type. In general, DNA from sorted cell samples includes a greater fraction of relevant template molecules. The fraction of relevant molecules is higher when using PBMC or PBL compared to whole blood.
- Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown.
- In general, library yield from high-quality DNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.

- The Ion Torrent™ Dual Barcode Kit 1–96 is arrayed by column with barcode 1 in position A1, barcode 8 in position H1, and barcode 96 in position H12.



Set up DNA target amplification reactions

Prepare DNA target amplification reactions

IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place a new 96-well plate in a pre-chilled 4°C cold block or on ice.
2. Dilute the dNTP mix (25 mM each) to 6.0 mM each.

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

3. Gently vortex the 5X Ion AmpliSeq™ HiFi Mix, centrifuge briefly to collect, then keep on ice.
4. For each sample, add the following components to each sample well. Prepare a master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.0 µL
5X Oncomine™ IGH FR3(d)-J Assay panel	4.0 µL
dNTP Mix (6 mM each dNTP, prepared in step 2)	2.0 µL
gDNA (200 ng–2.0 µg)[1]	≤10 µL
Nuclease-free Water	to 20 µL

[1] Substitute an equal volume of nuclease-free water or low TE to prepare a No-Template Control (NTC).

Note: Avoid using columns on the periphery of the plate.

5. Seal the plate with a 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.

Amplify the targets

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that equivalent amounts of sample is used in each well so that the selected cycle number is optimal for all the samples in the run.

1. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.
2. 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	45 seconds
	Anneal	60°C	45 seconds
	Extend	72°C	90 seconds
Hold	Final extension	72°C	10 minutes
Hold	—	10°C	Hold

[1] Increase number by +3 when starting from FFPE samples. Cycle number can be increased when input material quality or quantity is questionable.

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

Partially digest the amplicons

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

1. Thaw the FuPa Reagent (brown cap) on ice, gently vortex to mix, then centrifuge briefly to collect.
2. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.

   IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly.
3. Seal the plate with a 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.
4. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

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

Proceed immediately to “Perform the ligation reaction“.

IMPORTANT! Do not store the partially digested amplicons overnight.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single run, you *must* ligate a different barcode 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 sample 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 24 hours)

STOPPING POINT Samples can be stored for up to 24 hours at 10°C on the thermal cycler. For longer periods, store 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.
- Agencourt™ AMPure™ XP Reagent is viscous, pipet the solution slowly.
- Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

1. Prepare 70% ethanol (350 µL × # of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. 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.

4. Incubate the mixture for 5 minutes at room temperature.
5. 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.
6. 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.

7. Repeat step 6 for a second wash.

8. 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.

   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.

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.

Proceed to "Quantify the library by qPCR and calculate the dilution factor" on page 39.

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 PCR instrument.

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

   a. Enter the concentrations of the control library standards.

   b. Select ROX™ Reference Dye as the passive reference dye.

   c. Select a reaction volume of 20 µL.

   d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

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 83, 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 140.
- 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 pM/25 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 83, 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, transfer the supernatant to a new well and seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

5 Oncomine™ IGH FR2-J Assay library preparation

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.

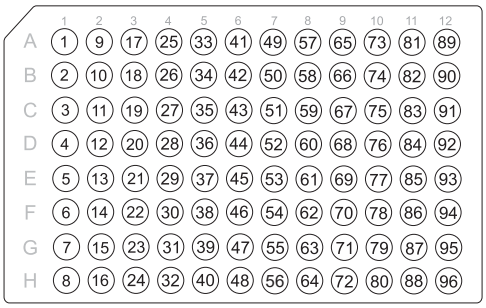
Proceed to the target amplification procedure specific to the assay and sample type you are performing.

Guidelines for DNA isolation, quantification, and input

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

- Use kits described in "Required materials not supplied" for isolating high quality gDNA from research samples for use in library preparation.
- We recommend the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) for quantifying human genomic DNA.
- Quantification methods such as densitometry (for example, using a NanoDrop™ spectrophotometer) are not recommended, because they are not specific for DNA. Use of these methods can lead to gross overestimation of the sample DNA concentration, under-seeding of the target amplification reaction, and low library yields.
- 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 plastic BD Vacutainer® blood collection tube with K_2EDTA: BD Hemogard™ Closure. See "Recommended materials" on page 20.
- Sample input amount and the target amplification cycling program vary with sample type. In general, DNA from sorted cell samples includes a greater fraction of relevant template molecules. The fraction of relevant molecules is higher when using PBMC or PBL compared to whole blood.
- Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown.
- In general, library yield from high-quality DNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.

- The Ion Torrent™ Dual Barcode Kit 1–96 is arrayed by column with barcode 1 in position A1, barcode 8 in position H1, and barcode 96 in position H12.



Set up DNA target amplification reactions

Prepare DNA target amplification reactions

IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place a new 96-well plate in a pre-chilled 4°C cold block or on ice.
2. Dilute the dNTP mix (25 mM each) to 7.0 mM each.

Component	Volume
Nuclease-free Water	18 µL
dNTP Mix (25 mM each)	7 µL
Total volume	25 µL

3. Gently vortex the 5X Ion AmpliSeq™ HiFi Mix, centrifuge briefly to collect, then keep on ice.
4. For each sample, add the following components to each sample well. Prepare a target amplification master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.0 µL
5X Oncomine™ IGH FR2-J Assay panel	4.0 µL
dNTP Mix (7 mM each dNTP, prepared in step 2)	2.0 µL
gDNA (200 ng–2.0 µg)[1]	≤10 µL
Nuclease-free Water	to 20 µL

[1] Substitute an equal volume of nuclease-free water or low TE to prepare a No-Template Control (NTC).

Note: Avoid using columns on the periphery of the plate.

5. Seal the plate with a 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.

Amplify the targets

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that equivalent amounts of sample is used in each well so that the selected cycle number is optimal for all the samples in the run.

1. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.
2. Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	2 minutes
32 Cycles[1]	Denature	95°C	45 seconds
	Anneal	62°C	45 seconds
	Extend	72°C	3 minutes 15 seconds
Hold	Final extension	72°C	10 minutes
Hold	—	10°C	Hold

[1] Increase number by +3 when starting from FFPE samples. Cycle number can be increased when input material quality or quantity is questionable.

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

Partially digest the amplicons

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

1. Thaw the FuPa Reagent (brown cap) on ice, gently vortex to mix, then centrifuge briefly to collect.
2. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.

   IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly.
3. Seal the plate with a 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.
4. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

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

Proceed immediately to “Perform the ligation reaction“.

IMPORTANT! Do not store the partially digested amplicons overnight.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single run, you *must* ligate a different barcode 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 sample 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 24 hours)

STOPPING POINT Samples can be stored for up to 24 hours at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the library

IMPORTANT!

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

1. Prepare 70% ethanol (350 µL × # of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. Carefully remove the plate seal, then add 30 µL (1X 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.
4. Incubate the mixture for 5 minutes at room temperature.
5. 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.
6. 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.
7. Repeat step 6 for a second wash.

8. 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.

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.
- For FFPE and low quality samples proceed immediately to "Additional amplification and library purification (Optional)" on page 49.

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.

Proceed to "Quantify the library by qPCR and calculate the dilution factor" on page 47.

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 made from high quality (non-FFPE) samples that have not undergone a second round of amplification typically have yields of 100–1,000 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.

Note: If quantifying libraries prepared using the optional library amplification prepare a 10,000-fold dilution for quantification.

4. Seal the plate with a MicroAmp™ Optical Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.

5. Program your Real-Time PCR instrument.

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

   a. Enter the concentrations of the control library standards.

   b. Select ROX™ Reference Dye as the passive reference dye.

   c. Select a reaction volume of 20 µL.

   d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

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 83, 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 140.
- If short off-target, possible primer-dimer or adapter-dimer products are observed in the histogram data, perform rescue amplification (see "Rescue amplification and library purification" on page 140) and re-purify the library with Agencourt™ AMPure™ XP Reagent twice (1.0X sample volume) and elute in a final volume of 30–50 µL.
- 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 pM/25 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 83, 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, transfer the supernatant to a new well and seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

Additional amplification and library purification *(Optional)*

With FFPE samples, or if you have a low-yield library, we recommend that you perform additional target amplification to improve the library yield. After amplification, purify the amplified library using the two-round purification process.

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

Amplify FFPE (or low quality) libraries

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.

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

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 a MicroAmp™ Optical Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.

3. Prepare separate 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 library amplification 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

4. 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.

5. 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 the plate seal, then transfer 50 µL supernatant to a new well (or new plate) without disturbing the pellet.

6. Seal the plate with a new MicroAmp™ Optical Adhesive Film, then briefly centrifuge to collect droplets.

7. 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
7 cycles[1]	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

[1] 7 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 twice

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use.
- Agencourt™ AMPure™ XP Reagent is viscous, pipet the solution slowly.
- Use freshly prepared 70% ethanol for the next steps. Combine 490 µL of ethanol with 210 µL of Nuclease-free Water per sample.
- Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

Perform two-rounds of library purification with the Agencourt™ AMPure™ XP Reagent.

1. Prepare 70% ethanol (700 µL × # of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. Carefully remove the plate seal, then add 40 µL (1X 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.

4. Incubate the mixture for 5 minutes at room temperature.
5. 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.

6. 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.

7. Repeat step 6 for a second wash.
8. 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.
9. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.
10. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
11. Incubate at room temperature for at least 2 minutes, then place the plate on the magnet for at least 2 minutes.
12. Carefully remove the plate seal, then transfer 50 µL of supernatant containing the library to a clean well or new plate.
13. Add 50 µL (1X 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.
14. Repeat step 4 through step 8 to complete the second round of purification.

Elute the amplified 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 10,000-fold dilution for quantification.
   a. Remove 2 µL of supernatant, containing the library, then combine with 198 µL of Nuclease-free Water.
   b. Prepare a further 1:100 serial dilution of the first dilution to achieve a 10,000-fold dilution.

Proceed to “Quantify the amplified library by qPCR and calculate the dilution factor” on page 53.

Quantify the amplified 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). Depending on the initial FFPE sample quality, the ampllified libraries 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
1:10,000 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 PCR instrument.

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

   a. Enter the concentrations of the control library standards.

   b. Select ROX™ Reference Dye as the passive reference dye.

   c. Select a reaction volume of 20 µL.

d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

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 × the dilution factor.

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 83, then perform template preparation, without further dilution.
- 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 pM/25 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 83, 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, transfer the supernatant to a new well and seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

6 Oncomine™ IGH FR1-J Assay library preparation

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.

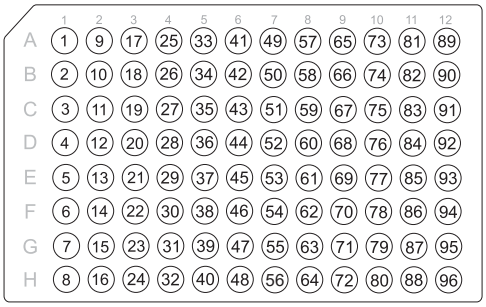
Proceed to the target amplification procedure specific to the assay and sample type you are performing.

Guidelines for DNA isolation, quantification, and input

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

- Use kits described in "Required materials not supplied" for isolating high quality gDNA from research samples for use in library preparation.
- We recommend the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) for quantifying human genomic DNA.
- Quantification methods such as densitometry (for example, using a NanoDrop™ spectrophotometer) are not recommended, because they are not specific for DNA. Use of these methods can lead to gross overestimation of the sample DNA concentration, under-seeding of the target amplification reaction, and low library yields.
- 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 plastic BD Vacutainer® blood collection tube with K_2EDTA: BD Hemogard™ Closure. See "Recommended materials" on page 20.
- Sample input amount and the target amplification cycling program vary with sample type. In general, DNA from sorted cell samples includes a greater fraction of relevant template molecules. The fraction of relevant molecules is higher when using PBMC or PBL compared to whole blood.
- Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown.
- In general, library yield from high-quality DNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.

- The Ion Torrent™ Dual Barcode Kit 1–96 is arrayed by column with barcode 1 in position A1, barcode 8 in position H1, and barcode 96 in position H12.



Set up DNA target amplification reactions

Prepare DNA target amplification reactions

IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place a new 96-well plate in a pre-chilled 4°C cold block or on ice.
2. Dilute the dNTP mix (25 mM each) to 6.7 mM each.

Component	Volume
Nuclease-free Water	18.3 µL
dNTP Mix (25 mM each)	6.7 µL
Total volume	25 µL

3. Gently vortex the 5X Ion AmpliSeq™ HiFi Mix, centrifuge briefly to collect, then keep on ice.
4. For each sample, add the following components to each sample well. Prepare a target amplification master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.0 µL
5X Oncomine™ IGH FR1-J Assay panel	4.0 µL
dNTP Mix (6.7 mM each dNTP, prepared in step 2)	2.0 µL
gDNA (200 ng–2.0 µg)[1]	≤10 µL
Nuclease-free Water	to 20 µL

[1] Substitute an equal volume of nuclease-free water or low TE to prepare a No-Template Control (NTC).

Note: Avoid using columns on the periphery of the plate.

5. Seal the plate with a 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.

Amplify the targets

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that equivalent amounts of sample is used in each well so that the selected cycle number is optimal for all the samples in the run.

1. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.
2. Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	2 minutes
32 Cycles[1]	Denature	95°C	45 seconds
	Anneal	62°C	45 seconds
	Extend	72°C	3 minutes 15 seconds
Hold	Final extension	72°C	10 minutes
Hold	—	10°C	Hold

[1] Increase number by +3 when starting from FFPE samples. Cycle number can be increased when input material quality or quantity is questionable.

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

Partially digest the amplicons

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

1. Thaw the FuPa Reagent (brown cap) on ice, gently vortex to mix, then centrifuge briefly to collect.
2. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.

   IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly.
3. Seal the plate with a 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.
4. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

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

Proceed immediately to "Perform the ligation reaction".

IMPORTANT! Do not store the partially digested amplicons overnight.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single run, you *must* ligate a different barcode 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 sample 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 24 hours)

STOPPING POINT Samples can be stored for up to 24 hours at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the library

IMPORTANT!

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

1. Prepare 70% ethanol (350 µL × # of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. Carefully remove the plate seal, then add 30 µL (1X 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.

4. Incubate the mixture for 5 minutes at room temperature.
5. 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.
6. 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.

7. Repeat step 6 for a second wash.

8. 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.

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.
- For FFPE and low quality samples proceed immediately to "Additional amplification and library purification (Optional)" on page 49.

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.

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

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 in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove the plate seal, then transfer 50 µL supernatant to a new well (or new plate) without disturbing the pellet.

4. Seal the plate with a new MicroAmp™ Optical Adhesive Film, then briefly centrifuge to collect droplets.

5. 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
7 cycles[1]	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

[1] 7 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 twice

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use.
- Agencourt™ AMPure™ XP Reagent is viscous, pipet the solution slowly.
- Use freshly prepared 70% ethanol for the next steps. Combine 490 µL of ethanol with 210 µL of Nuclease-free Water per sample.
- Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

Perform two-rounds of library purification with the Agencourt™ AMPure™ XP Reagent.

1. Prepare 70% ethanol (700 µL × # of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. Carefully remove the plate seal, then add 50 µL (1X 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.

4. Incubate the mixture for 5 minutes at room temperature.
5. 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.
6. 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.

7. Repeat step 6 for a second wash.
8. 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.
9. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.
10. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
11. Incubate at room temperature for at least 2 minutes, then place the plate on the magnet for at least 2 minutes.

12. Carefully remove the plate seal, then transfer 50 µL of supernatant containing the library to a clean well or new plate.

13. Repeat step 3 through step 8 to complete the second round of purification.

Elute the amplified 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 10,000-fold dilution for quantification.
   a. Remove 2 µL of supernatant, containing the library, then combine with 198 µL of Nuclease-free Water.

   b. Prepare a further 1:100 serial dilution of the first dilution to achieve a 10,000-fold dilution.

Proceed to “Quantify the amplified library by qPCR and calculate the dilution factor” on page 62.

Quantify the amplified 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 undergone amplification typically have yields of 2,000–20,000 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
1:10,000 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 PCR instrument.

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

   a. Enter the concentrations of the control library standards.

   b. Select ROX™ Reference Dye as the passive reference dye.

   c. Select a reaction volume of 20 µL.

   d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

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 × the dilution factor.

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 83, then perform template preparation, without further dilution.
- 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 pM/25 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 83, 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, transfer the supernatant to a new well and seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

7 Oncomine™ IGHV Leader-J Assay library preparation

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.

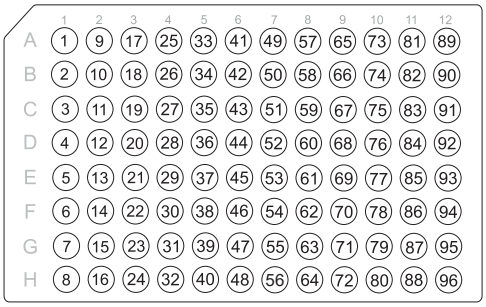
Proceed to the target amplification procedure specific to the assay and sample type you are performing.

Guidelines for DNA isolation, quantification, and input

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

- Use kits described in "Required materials not supplied" for isolating high quality gDNA from research samples for use in library preparation.
- We recommend the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) for quantifying human genomic DNA.
- Quantification methods such as densitometry (for example, using a NanoDrop™ spectrophotometer) are not recommended, because they are not specific for DNA. Use of these methods can lead to gross overestimation of the sample DNA concentration, under-seeding of the target amplification reaction, and low library yields.
- 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 plastic BD Vacutainer® blood collection tube with K_2EDTA: BD Hemogard™ Closure. See "Recommended materials" on page 20.
- Sample input amount and the target amplification cycling program vary with sample type. In general, DNA from sorted cell samples includes a greater fraction of relevant template molecules. The fraction of relevant molecules is higher when using PBMC or PBL compared to whole blood.
- Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown.
- In general, library yield from high-quality DNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.

- The Ion Torrent™ Dual Barcode Kit 1–96 is arrayed by column with barcode 1 in position A1, barcode 8 in position H1, and barcode 96 in position H12.



Set up DNA target amplification reactions

Prepare DNA target amplification reactions

IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place a new 96-well plate in a pre-chilled 4°C cold block or on ice.
2. Dilute the dNTP mix (25 mM each) to 6.5 mM each.

Component	Volume
Nuclease-free Water	18.5 µL
dNTP Mix (25 mM each)	6.5 µL
Total volume	25 µL

3. Gently vortex the 5X Ion AmpliSeq™ HiFi Mix, centrifuge briefly to collect, then keep on ice.
4. For each sample, add the following components to each sample well. Prepare a target amplification master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.0 µL
5X Oncomine™ IGHV Leader-J Assay panel	4.0 µL
dNTP Mix (6.5 mM each dNTP, prepared in step 2)	2.0 µL
gDNA (200 ng–2.0 µg)[1]	≤10 µL
Nuclease-free Water	to 20 µL

[1] Substitute an equal volume of nuclease-free water or low TE to prepare a No-Template Control (NTC).

Note: Avoid using columns on the periphery of the plate.

5. Seal the plate with a 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.

Amplify the targets

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that equivalent amounts of sample is used in each well so that the selected cycle number is optimal for all the samples in the run.

1. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.
2. Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	2 minutes
32 Cycles[1]	Denature	95°C	45 seconds
	Anneal	62°C	45 seconds
	Extend	72°C	3 minutes 15 seconds
Hold	Final extension	72°C	10 minutes
Hold	—	10°C	Hold

[1] Increase number by +3 when starting from FFPE samples. Cycle number can be increased when input material quality or quantity is questionable.

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

Partially digest the amplicons

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

1. Thaw the FuPa Reagent (brown cap) on ice, gently vortex to mix, then centrifuge briefly to collect.
2. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.

   IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly.
3. Seal the plate with a 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.
4. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

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

Proceed immediately to “Perform the ligation reaction“.

IMPORTANT! Do not store the partially digested amplicons overnight.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single run, you *must* ligate a different barcode 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 sample 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 24 hours)

STOPPING POINT Samples can be stored for up to 24 hours at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the library

IMPORTANT!

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

1. Prepare 70% ethanol (350 µL × # of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. Carefully remove the plate seal, then add 24 µL (0.8X 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.

4. Incubate the mixture for 5 minutes at room temperature.
5. 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.
6. 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.

7. Repeat step 6 for a second wash.

8. 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.

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.

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.

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

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 in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove the plate seal, then transfer 50 µL supernatant to a new well (or new plate) without disturbing the pellet.

4. Seal the plate with a new MicroAmp™ Optical Adhesive Film, then briefly centrifuge to collect droplets.

5. 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
7 cycles[1]	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

[1] 7 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 twice

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use.
- Agencourt™ AMPure™ XP Reagent is viscous, pipet the solution slowly.
- Use freshly prepared 70% ethanol for the next steps. Combine 490 µL of ethanol with 210 µL of Nuclease-free Water per sample.
- Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

Perform two-rounds of library purification with the Agencourt™ AMPure™ XP Reagent.

1. Prepare 70% ethanol (700 µL × # of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. Carefully remove the plate seal, then add 30 µL (0.6X 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.

4. Incubate the mixture for 5 minutes at room temperature.
5. 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.
6. 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.

7. Repeat step 6 for a second wash.
8. 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.
9. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.
10. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
11. Incubate at room temperature for at least 2 minutes, then place the plate on the magnet for at least 2 minutes.

12. Carefully remove the plate seal, then transfer 50 µL of supernatant containing the library to a clean well or new plate.

13. Repeat step 3 through step 8 for a second round of purification.

Elute the amplified 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 1,000-fold dilution for quantification.
   a. Remove 2 µL of supernatant, containing the library, then combine with 18 µL of Nuclease-free Water.

   b. Prepare a further 1:100 serial dilution of the first dilution to achieve a 1,000-fold dilution.

Proceed to “Quantify the amplified library by qPCR and calculate the dilution factor” on page 72.

Quantify the amplified 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 undergone amplification typically have yields of 500–5,000 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
1:1,000 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 PCR instrument.

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

   a. Enter the concentrations of the control library standards.

   b. Select ROX™ Reference Dye as the passive reference dye.

   c. Select a reaction volume of 20 µL.

   d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

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 × the dilution factor.

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 83, then perform template preparation, without further dilution.
- 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 pM/25 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 83, 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, transfer the supernatant to a new well and seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

8 Oncomine™ TCR Pan-Clonality Assay library preparation

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.

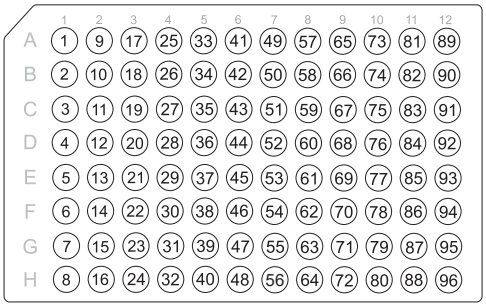
Proceed to the target amplification procedure specific to the assay and sample type you are performing.

Guidelines for DNA isolation, quantification, and input

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

- Use kits described in "Required materials not supplied" for isolating high quality gDNA from research samples for use in library preparation.
- We recommend the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) for quantifying human genomic DNA.
- Quantification methods such as densitometry (for example, using a NanoDrop™ spectrophotometer) are not recommended, because they are not specific for DNA. Use of these methods can lead to gross overestimation of the sample DNA concentration, under-seeding of the target amplification reaction, and low library yields.
- 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 plastic BD Vacutainer® blood collection tube with K_2EDTA: BD Hemogard™ Closure. See "Recommended materials" on page 20.
- Sample input amount and the target amplification cycling program vary with sample type. In general, DNA from sorted cell samples includes a greater fraction of relevant template molecules. The fraction of relevant molecules is higher when using PBMC or PBL compared to whole blood.
- Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown.
- In general, library yield from high-quality DNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.

- The Ion Torrent™ Dual Barcode Kit 1–96 is arrayed by column with barcode 1 in position A1, barcode 8 in position H1, and barcode 96 in position H12.



Set up DNA target amplification reactions

Prepare DNA target amplification reactions

IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place a new 96-well plate in a pre-chilled 4°C cold block or on ice.
2. Dilute the dNTP mix (25 mM each) to 7.5 mM each.

Component	Volume
Nuclease-free Water	14 µL
dNTP Mix (25 mM each)	6 µL
Total volume	20 µL

3. Gently vortex the 5X Ion AmpliSeq™ HiFi Mix, centrifuge briefly to collect, then keep on ice.
4. For each sample, add the following components to each sample well. Prepare a target amplification master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.0 µL
5X Oncomine™ TCR Pan-Clonality Assay panel	4.0 µL
dNTP Mix (7.5 mM each dNTP, prepared in step 2)	2.0 µL
gDNA (200 ng–2.0 µg)[1]	≤10 µL
Nuclease-free Water	to 20 µL

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

Note: Avoid using columns on the periphery of the plate.

5. Seal the plate with a 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.

Amplify the targets

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that equivalent amounts of sample is used in each well so that the selected cycle number is optimal for all the samples in the run.

1. Place a MicroAmp™ Compression Pad on the plate, then load the plate into the thermal cycler.
2. 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] Increase number by +3 when starting from FFPE samples. Cycle number can be increased when input material quality or quantity is questionable.

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

Partially digest the amplicons

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

1. Thaw the FuPa Reagent (brown cap) on ice, gently vortex to mix, then centrifuge briefly to collect.
2. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.

   IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly.
3. Seal the plate with a 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.
4. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

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

Proceed immediately to "Perform the ligation reaction".

IMPORTANT! Do not store the partially digested amplicons overnight.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single run, you *must* ligate a different barcode 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 sample 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 24 hours)

STOPPING POINT Samples can be stored for up to 24 hours at 10°C on the thermal cycler. For longer periods, store 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.
- Agencourt™ AMPure™ XP Reagent is viscous, pipet the solution slowly.
- Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

1. Prepare 70% ethanol (350 µL × # of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. 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.

4. Incubate the mixture for 5 minutes at room temperature.
5. 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.
6. 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.

7. Repeat step 6 for a second wash.

8. 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.

   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.

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.

Proceed to "Quantify the library by qPCR and calculate the dilution factor" on page 80.

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 PCR instrument.

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

   a. Enter the concentrations of the control library standards.

   b. Select ROX™ Reference Dye as the passive reference dye.

   c. Select a reaction volume of 20 µL.

   d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

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 83, 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 140.
- If short off-target, possible primer-dimer or adapter-dimer products are observed in the histogram data, perform rescue amplification (see "Rescue amplification and library purification" on page 140) and re-purify the library with Agencourt™ AMPure™ XP Reagent twice (1.0X sample volume) and elute in a final volume of 30–50 µL.
- 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 pM/25 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 83, 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, transfer the supernatant to a new well and seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

9 Combine and sequence the libraries

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 24 libraries for the following Ion sequencing chips, see "Guidelines for clonality testing" and "Guidelines for SHM analysis" on page 147 for more information.

Ion sequencing chip	Suggested number of libraries (X)					
	Oncomine™ BCR Pan-Clonality Assay	Oncomine™ TCR Pan-Clonality Assay	Oncomine™ IGH FR3(d)-J Assay	Oncomine™ IGH FR2-J Assay	Oncomine™ IGH FR1-J Assay	Oncomine™ IGHV Leader-J Assay
Ion 530™ Chip	4	4	4	8	4–8	4–8
Ion 540™ Chip	8–16	8–16	8–16	8–16	Not recommended	Not recommended
Ion 550™ Chip	12–24	12–24	12–24	Not recommended	Not recommended	Not recommended

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" on page 84 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 System	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Cat. No. A34461)	*Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0016854)
Ion 540™ Chip			Ion 540™ Kit – Chef (Cat. No. A30011)	*Ion 540™ Kit – Chef User Guide* (Pub. No. MAN0010851)
Ion 550™ Chip		Ion GeneStudio™ S5 Plus System or Ion GeneStudio™ S5 Prime System	Ion 550™ Kit – Chef (Cat. No. A34541)	*Ion 550™ Kit – Chef User Guide* (Pub. No. MAN0017275)

To create a specific Run Plan for use in templating and sequencing, see Chapter 10, "Create an assay-specific Planned Run". Refer to the appropriate user guide listed in the table for more information.

10 Create an assay-specific Planned Run

IMPORTANT! These kits are compatible with Torrent Suite™ Software 5.16 or later and Ion Reporter™ Software 5.18 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 139.

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 5.18 contains 6 pre-installed Run Plan templates for use with the Oncomine™ Human Immune Repertoire assays.

Application	Run Plan Templates	Description
Immune Repertoire	Oncomine™ BCR Pan-Clonality Assay	DNA only input Run Plan templates for template preparation, and sequencing using the Ion Chef™ and Ion GeneStudio™ S5 Series Systems.
	Oncomine™ IGH FR3(d)-J Assay	
	Oncomine™ IGH FR2-J Assay	
	Oncomine™ IGH FR1-J Assay	
	Oncomine™ IGHV Leader-J Assay	
	Oncomine™ TCR Pan-Clonality Assay	

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 any of the Oncomine™ immune repertoire assays"
5. In the Copy Template workflow bar, click **Research Application**, verify that the **Research Application** is set to Immune Repertoire and that the **Target Technique** is set to Mixed Samples (DNA/RNA). Click **Next**.

6. In the **Kits** step, select the **Ion Chef Template Kit** radio button, then complete the following fields.

Field	Assay		
	Oncomine™ BCR Pan-Clonality Assay Oncomine™ IGH FR3(d)-J Assay Oncomine™ TCR Pan-Clonality Assay	**Oncomine™ IGH FR2-J Assay**	**Oncomine™ IGH FR1-J Assay or Oncomine™ IGHV Leader-J Assay**
Instrument	Ion GeneStudio™ S5 System		
Library Kit Type	Ion AmpliSeq™ Library Kit Plus		
Template Kit	See the following table		Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef
Sequencing Kit	Ion S5™ Sequencing Kit		
Base Calibration Mode	Default Calibration		
Chip Type	• Ion 530™ Chip or • Ion 540™ Chip or • Ion 550™ Chip	• Ion 530™ Chip or • Ion 540™ Chip or	• Ion 530™ Chip
Barcode Set	Ion Dual Barcode Kit 1–96		
Flows	550	550	1100

Chip size used	Templating kit used	
	Oncomine™ BCR Pan-Clonality Assay Oncomine™ IGH FR3(d)-J Assay Oncomine™ TCR Pan-Clonality Assay	**Oncomine™ IGH FR2-J Assay**
Ion 530™ Chip	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef
Ion 540™ Chip	Ion 540™ Kit – Chef	Ion 540™ Kit – Chef
Ion 550™ Chip	Ion 550™ Kit – Chef	—

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

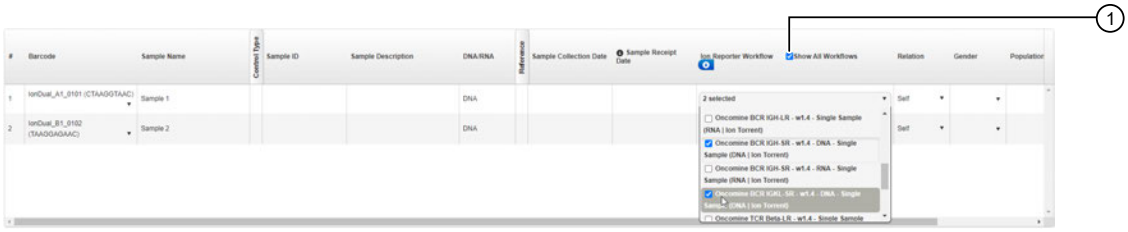
The customized template is now available in the **Immune Repertoire** list of templates.

Create a Run Plan

Both the Oncomine™ BCR Pan-Clonality Assay and Oncomine™ TCR Pan-Clonality Assay profile multiple receptor chains and require multiple Ion Reporter™ Softwareanalysis workflows for complete analysis. Torrent Suite™ Software version 5.16 or later includes functionality to automatically launch multiple Ion Reporter™ Softwareanalysis workflows from within the provided run plan template for each assay.

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. In the **Samples Table**, select the **Barcode** that will identify each sample, set the nucleic acid type to **DNA**, 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. For each sample, in the **Ion Reporter Workflow** column, select each analysis workflow that is to be automatically launched upon upload.
   a. Select the **Show All Workflows** checkbox.



① Show All Workflows enabled

b. Click in the cell, then select from the dropdown list of available options.

Assay	Analysis workflow
Oncomine™ BCR Pan-Clonality Assay	Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample Oncomine BCR IGKL-SR - w1.4 - DNA - Single Sample
Oncomine™ TCR Pan-Clonality Assay	Oncomine TCR Beta-SR - w1.4 - DNA - Single Sample Oncomine TCR Gamma-SR - w1.4 - DNA - Single Sample
Oncomine™ IGH FR3(d)-J Assay	Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample
Oncomine™ IGH FR2-J Assay	Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample
Oncomine™ IGH FR1-J Assay	Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample
Oncomine™ IGHV Leader-J Assay	Oncomine BCR IGH Leader-J - w1.4 - DNA - Single Sample

9. 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.

11 Data analysis

Analysis workflows in Ion Reporter™ Software

If the appropriate Ion Reporter™ Software analysis 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 98. To create an analysis workflow with non-default paramters, see "Modify analysis workflow parameters" on page 96.

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

The following Ion Reporter™ Software 5.16 workflows are available for analysis of Immune Repertoire assay results.

Analysis Workflow	Description
For use with Ion GeneStudio™ S5 Series Systems	
Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample	Analyze human B cell receptor IGH chains amplified from gDNA using Framework 3 (FR3), Framework 2 (FR2), or Framework 1 (FR1), and Joining Gene targeting primers. The amplicon provides coverage of the CDR3 region to enable clone identification and measurements of B cell diversity from a variety of sample types. Reports the frequency and sequence features of clonotypes, identifies clonal lineages, somatic hypermutation rate, 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 5.16. Workflow Version: 1.4

(continued)

Analysis Workflow	Description
Oncomine BCR IGKL-SR - w1.4 - DNA - Single Sample	Analyze human B cell receptor IgK and IgL chains, and KDE-containing rearrangements amplified from gDNA using Framework 3 and Joining Gene targeting primers. The short amplicon provides coverage of the CDR3 region to enable clone identification and measurements of B cell diversity from a variety of sample types. 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 5.16. Workflow Version: 1.4
Oncomine BCR IGH Leader-J - w1.4 - DNA - Single Sample	Analyze human B cell receptor IGH chains amplified from gDNA using Leader and Joining Gene targeting primers. The amplicon provides coverage of the CDR3 region to enable clone identification and measurements of B cell diversity from a variety of sample types. Reports the frequency and sequence features of clonotypes, identifies clonal lineages, somatic hypermutation rate, 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 5.16. Workflow Version: 1.4
Oncomine TCR Beta-SR - w1.4 - DNA - Single Sample	Analyze human T-cell receptor beta chains amplified from gDNA using Framework 3 (FR3) and Joining Gene targeting primers. The short amplicon provides coverage of the CDR3 region to enable clone identification 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 5.16. Workflow Version: 1.4
Oncomine TCR Gamma-SR - w1.4 - DNA - Single Sample	Analyze human T-cell receptor gamma chains amplified from gDNA using Framework 3 (FR3) and Joining Gene targeting primers. The short amplicon provides coverage of the CDR3 region to enable clone identification 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 5.16. Workflow Version: 1.4

Assay	Analysis workflow[1]
Oncomine™ BCR Pan-Clonality Assay	Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample Oncomine BCR IGKL-SR - w1.4 - DNA - Single Sample
Oncomine™ TCR Pan-Clonality Assay	Oncomine TCR Beta-SR - w1.4 - DNA - Single Sample Oncomine TCR Gamma-SR - w1.4 - DNA - Single Sample
Oncomine™ IGH FR3(d)-J Assay	Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample
Oncomine™ IGH FR2-J Assay	Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample
Oncomine™ IGH FR1-J Assay	Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample
Oncomine™ IGHV Leader-J Assay	Oncomine BCR IGH Leader-J - w1.4 - DNA - Single Sample

[1] For assays which require multiple Ion Reporter™ Software analysis workflows see "Create a Run Plan" on page 88 for instructions on launching multiple workflows from a single run plan.

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 95 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 98 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.

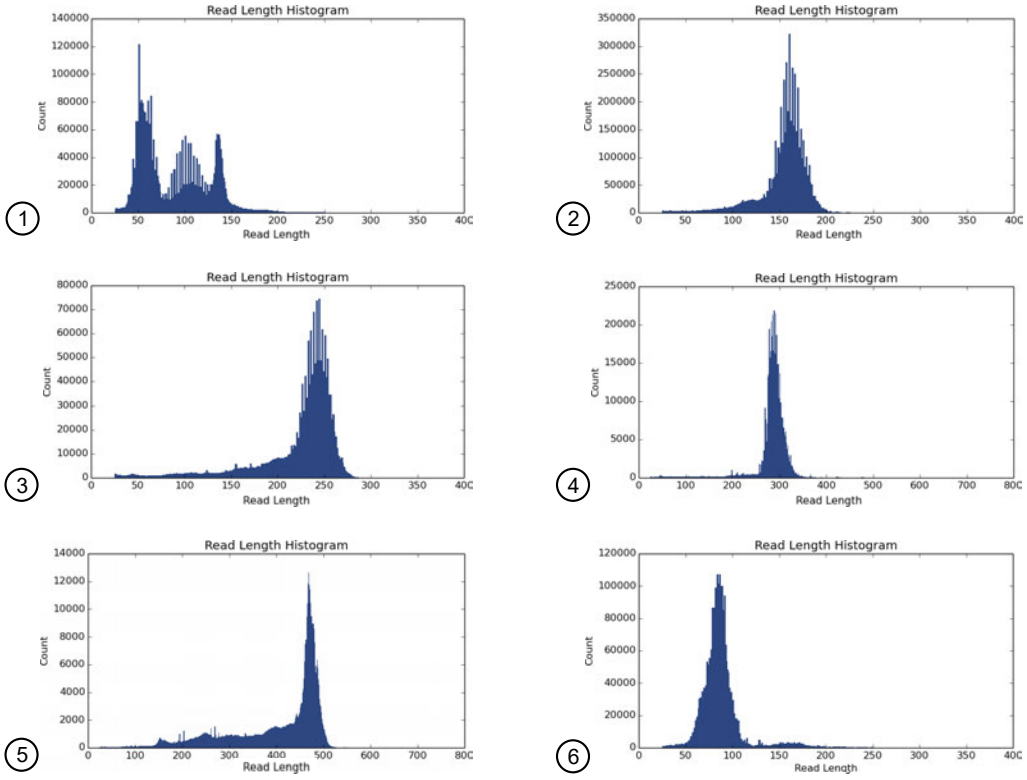


Figure 7 Read length histograms—PBL samples

① Oncomine™ BCR Pan-Clonality Assay. Confirm that the peaks are centered at approximately 60 nt, 110 nt, and a possible third peak at 135 nt.

② Oncomine™ IGH FR3(d)-J Assay. Confirm that the peak is centered at approximately 160 nt.

③ Oncomine™ IGH FR2-J Assay. Confirm that the peak is centered at approximately 240 nt.

④ Oncomine™ IGH FR1-J Assay. Confirm that the peak is centered at approximately 285 nt.

⑤ Oncomine™ IGHV Leader-J Assay . Confirm that the peak is centered at approximately 465 nt.

⑥ Oncomine™ TCR Pan-Clonality Assay. Confirm that the peak is centered at approximately 80 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 plugin in Torrent Suite™ Software” on page 138 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	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	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.

Modify analysis workflow parameters

IMPORTANT! Consult your local field support representative before copy editing an analysis workflow.

The Ion Reporter™ Software contains a collection of analysis workflows with pre-configured parameter settings that are recommended for most situations. After consultation with your local field support representative you can modify the default parameter settings and create copies of workflows with these modifications. For example, turn bidirectional reads support off and include clones on either strand.

To create a copy of a default workflow with edited parameters:

1. Sign in to the Ion Reporter™ Software.
2. Under the **Workflows** tab, in the **Overview** screen, select the appropriate workflow from the Workflow filter.
   The analysis workflow **Details** pane is populated to the right of the workflow list.
3. In the **Details** pane, click **(Actions)** ▸ **Copy**.
   In the **Create** screen, the **Edit** workflow opens to the **Research Application** step.
4. Click **Next** twice to advance to the **Parameters** step.
   For more information on each parameter, see the *Ion Reporter™ Software 5.16 User Guide* (Pub. No. MAN0019148).
5. To turn off bidirectional read support, under **Bidirectional support required**, select **False**. Click **Next**.

6. In the **Confirm** step, enter a **Workflow Name** and **Description** *(optional)*.

   Note: We recommend that the copied workflow have a unique **Workflow Name** , different from the original analysis workflow.

7. Click **Save Workflow** to confirm and save the changes.

The new modified workflow can now be found in the **Workflows** list of the **Overview** screen.

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	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	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, • Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample • Oncomine BCR IGKL-SR - w1.4 - DNA - Single Sample • Oncomine BCR IGH Leader-J - w1.4 - DNA - Single Sample • Oncomine TCR Beta-SR - w1.4 - DNA - Single Sample • Oncomine TCR Gamma-SR - w1.4 - DNA - Single Sample then click **Next**. **Note:** Workflows designated w1.0 may be available if upgrading from Ion Reporter™ Software 5.10.
Workflows tab	1. Select **Immune Repertoire** from the **Research Application** dropdown list. 2. Click in the appropriate row, • Oncomine BCR IGH-SR - w1.4 - DNA - Single Sample • Oncomine BCR IGKL-SR - w1.4 - DNA - Single Sample • Oncomine BCR IGH Leader-J - w1.4 - DNA - Single Sample • Oncomine TCR Beta-SR - w1.4 - DNA - Single Sample • Oncomine TCR Gamma-SR - w1.4 - DNA - Single Sample then select **Launch Analysis** from the **Actions** dropdown list in the **Details** pane. **Note:** Workflows designated w1.0 may be available if upgrading from Ion Reporter™ Software 5.10.

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 (IGH SR/Leader-J only)

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 113. You can also download the multi-sample clone summary table or the multi-sample metrics file (see “Download the metrics file” on page 115).
Comparison Plots	
Correlation Scatterplot	Scatterplot indicating the frequency of clones across two samples. For more information see “Comparison plots” on page 114.
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 114.

12 Results – Oncomine™ TCR Beta Assay

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 98.

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

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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 "*TCR beta*" or "*TCR gamma*" 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 99 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.

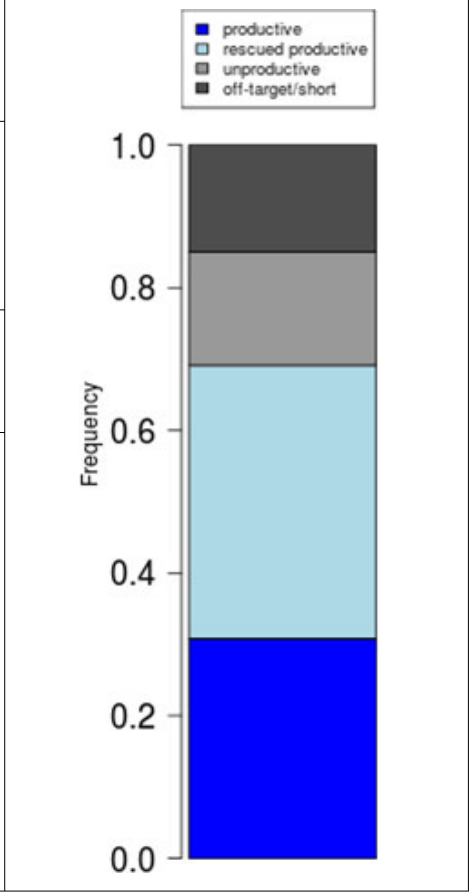
Sample QC tab

The Oncomine™ TCR Pan-Clonality Assay (TCR beta and TCR gamma) 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.

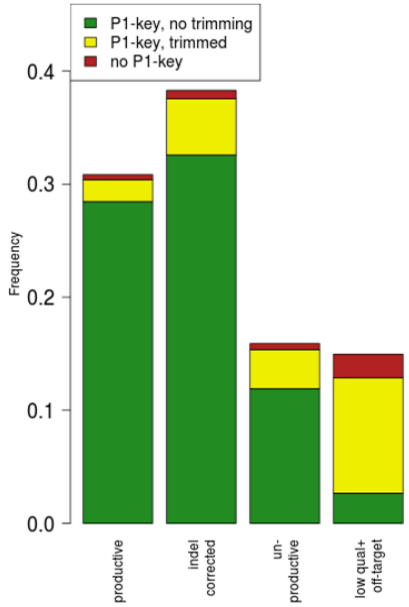
Read classification

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 an out-of-frame variable or joining gene or premature stop codon arising naturally (in gDNA samples) or from uncorrectable sequencing or PCR errors.	
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.	

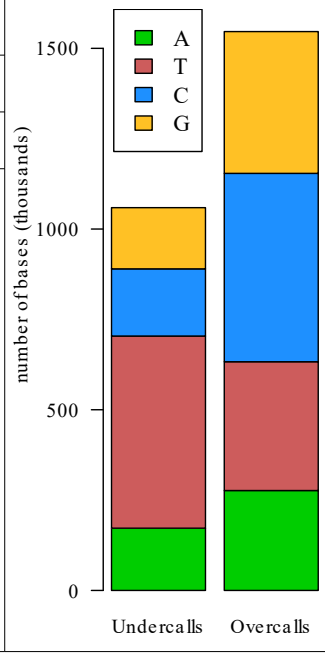
Proportion of full length, quality trimmed and reads lacking P1-key, by read classification

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.

Color	Description	Example
Red	Quality trimmed reads lacking an identifiable P1-key.	
Yellow	Quality trimmed reads having an identified P1-key.	
Green	Full length reads having an identified P1-key, and no quality trimming.	

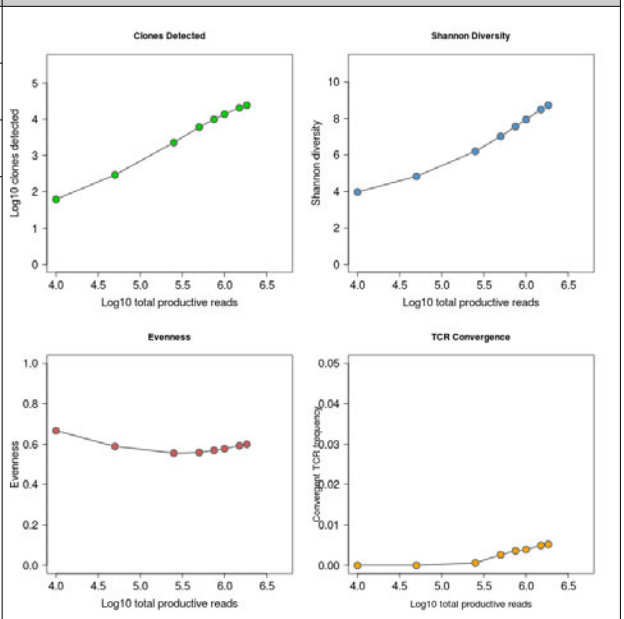
Base composition of overcalled and undercalled homopolymers

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.

Color	Description	Example
Orange	Guanidine (G)	
Blue	Cytosine (C)	
Pink	Thymidine (T)	
Green	Adenosine (A)	

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.

Color	Description	Example
Green	Clones detected	
Blue	Shannon diversity	
Red	Evenness	
Orange	TCR convergence	

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 an out-of-frame variable or joining gene or premature stop codon arising naturally (in gDNA samples) or from uncorrectable sequencing or PCR errors.
Off-target/ low-quality reads	Reads which are of low quality or represent the product of an off-target amplification.

(continued)

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 TCRβ or TCRɣ 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).

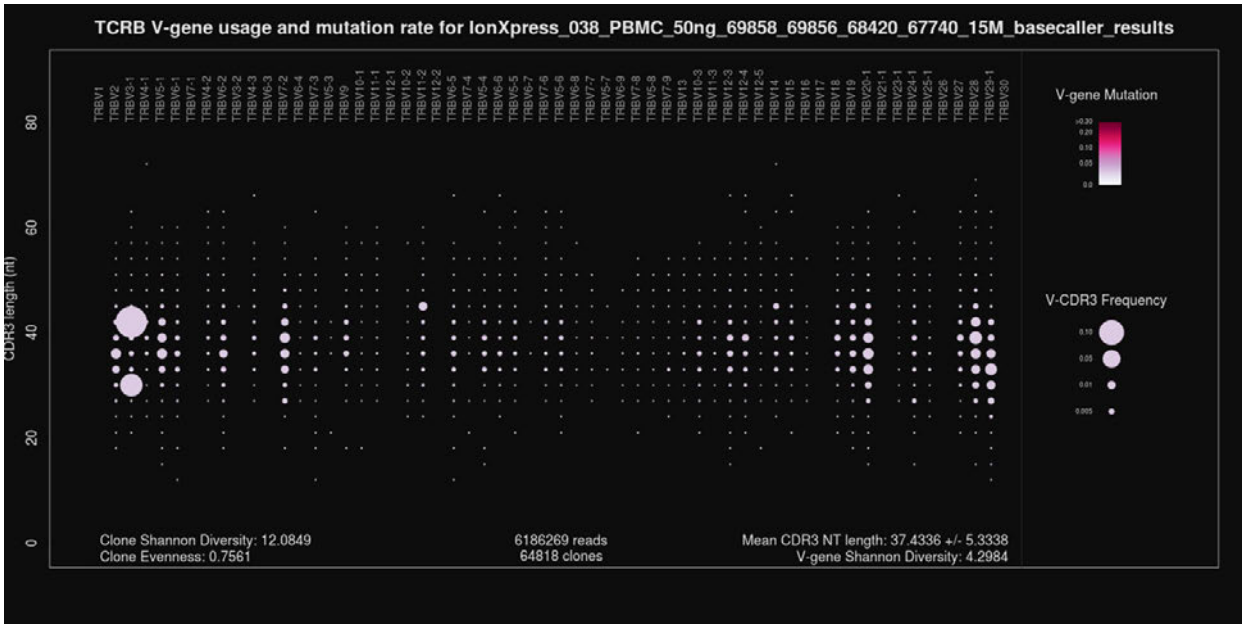


Figure 8 TCRB V-gene usage and mutation rate

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.

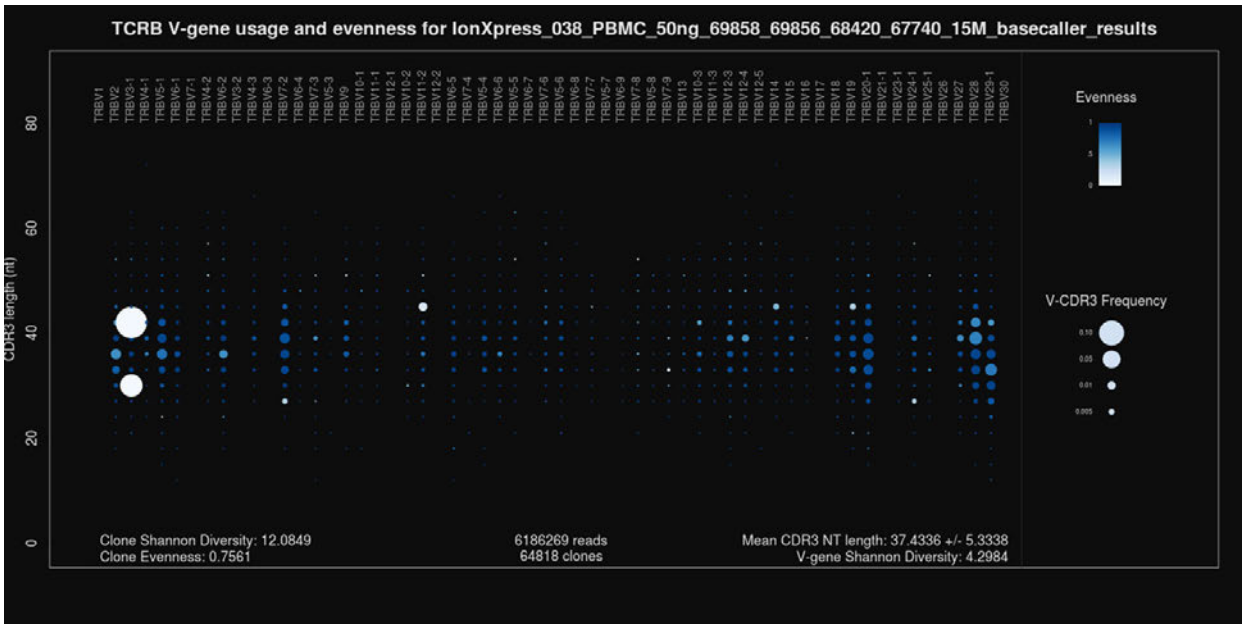


Figure 9 TCRB V-gene usage and evenness

Spectratyping plot highlighting evenness of identified clone sizes (Normalized Shannon Entropy). Circle color indicates the evenness of clone sizes for clones 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.

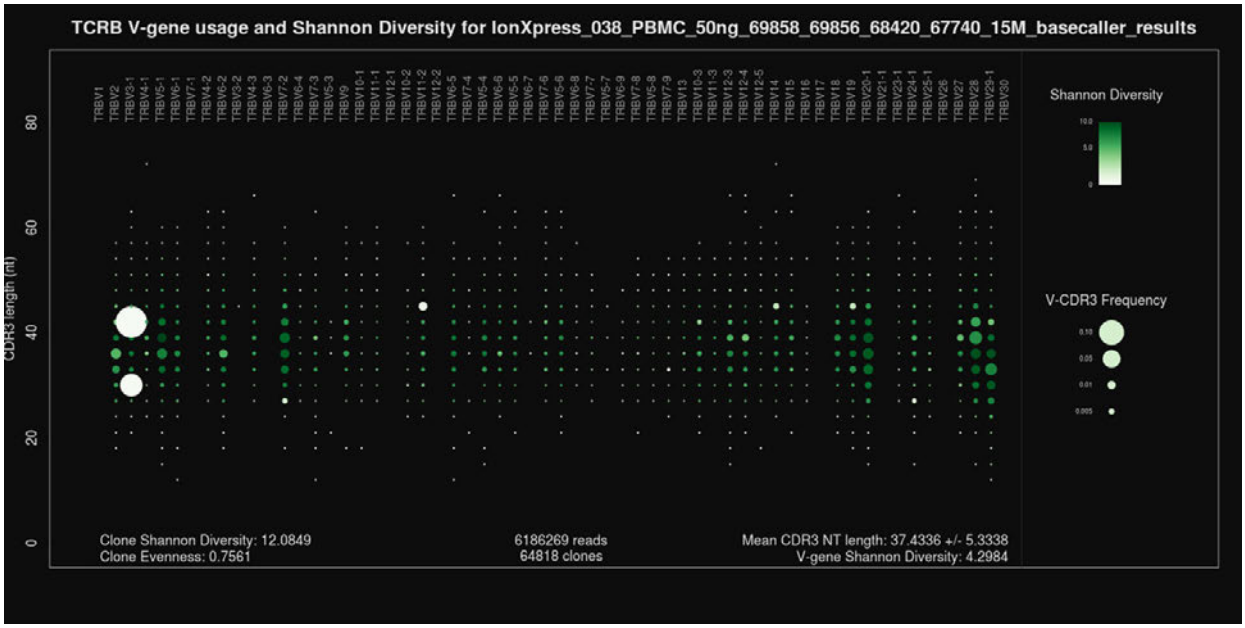


Figure 10 TCRB V-gene usage and Shannon Diversity

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.

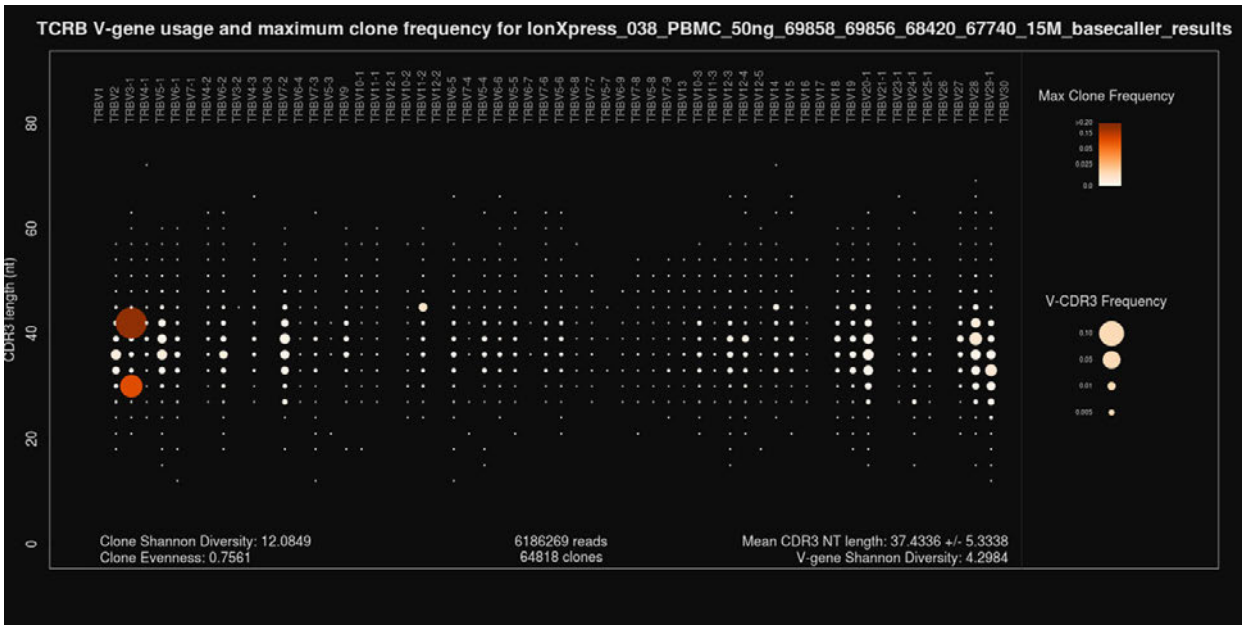


Figure 11 TCRB V-gene usage and largest clone frequency

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 but are also more commonly found in aged healthy individuals.

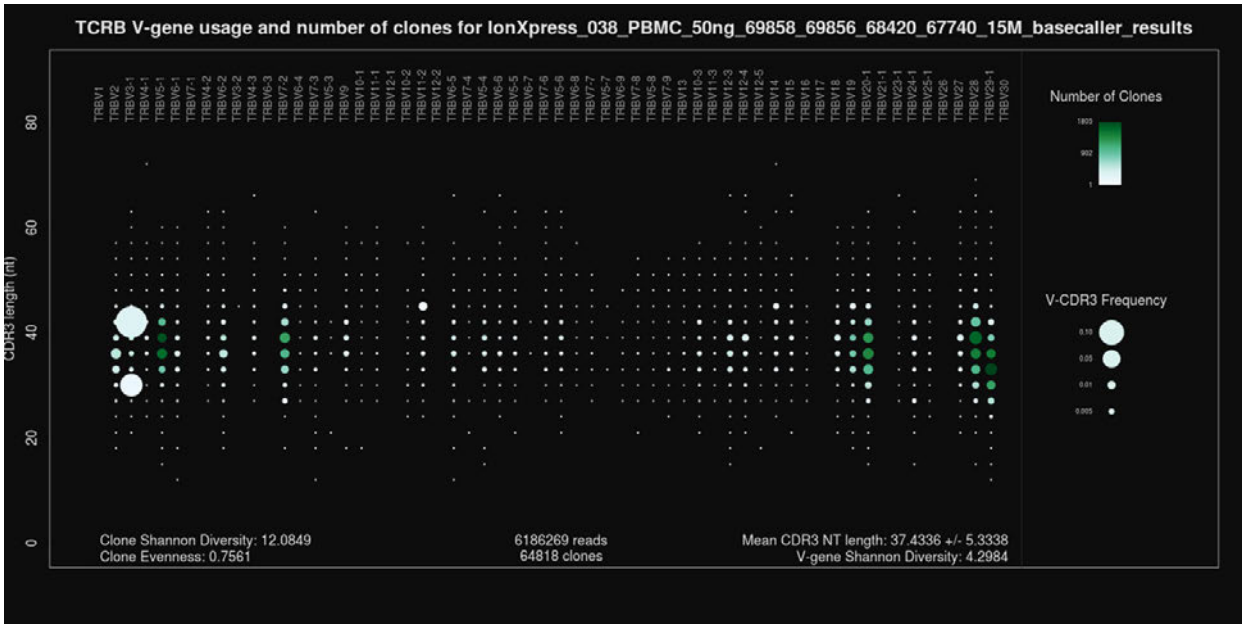


Figure 12 TCRB V-gene usage and number of clones

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 Oncomine™ TCR Pan-Clonality Assay (TCR beta and TCR gamma) analyses produce 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.

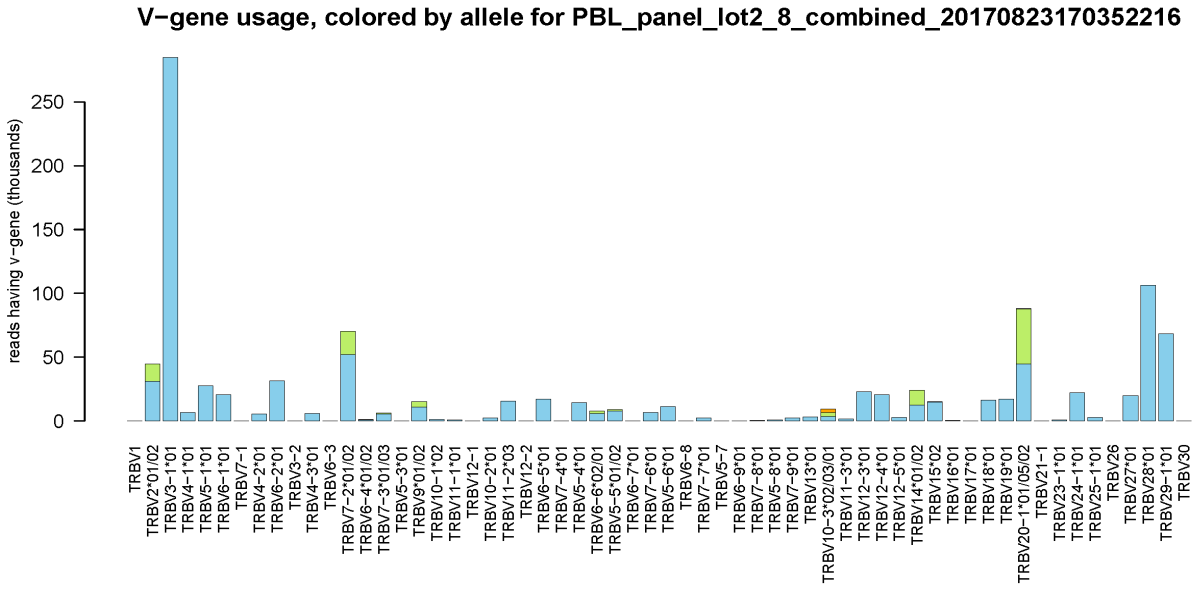


Figure 13 V-gene usage highlighting alleles

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

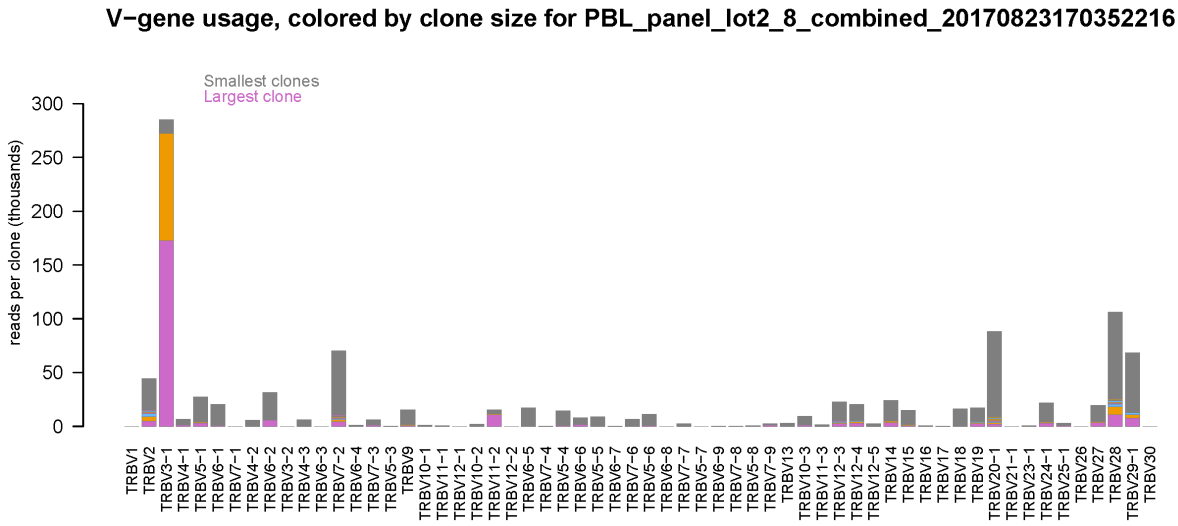


Figure 14 V-gene usage highlighting clone sizes

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

VJ-gene usage heatmap

The Oncomine™ TCR Pan-Clonality Assay (TCR beta and TCR gamma) analyses produce 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.

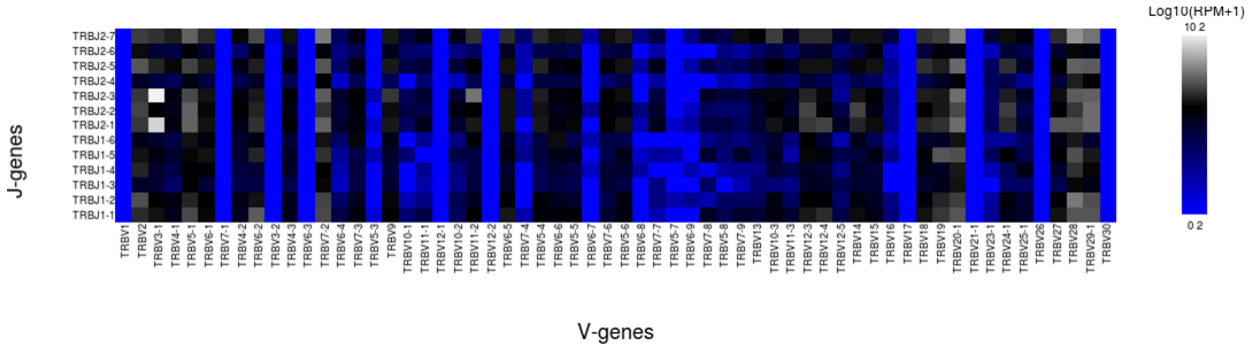


Figure 15 VJ-gene usage heatmap

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 TCRβ locus.

Clones detected per variable gene

The Oncomine™ TCR Pan-Clonality Assay (TCR beta and TCR gamma) analyses produce a graphical representation of the Variable genes usage among detected clones. Select **Clones detected per variable gene** from the **Views** list.

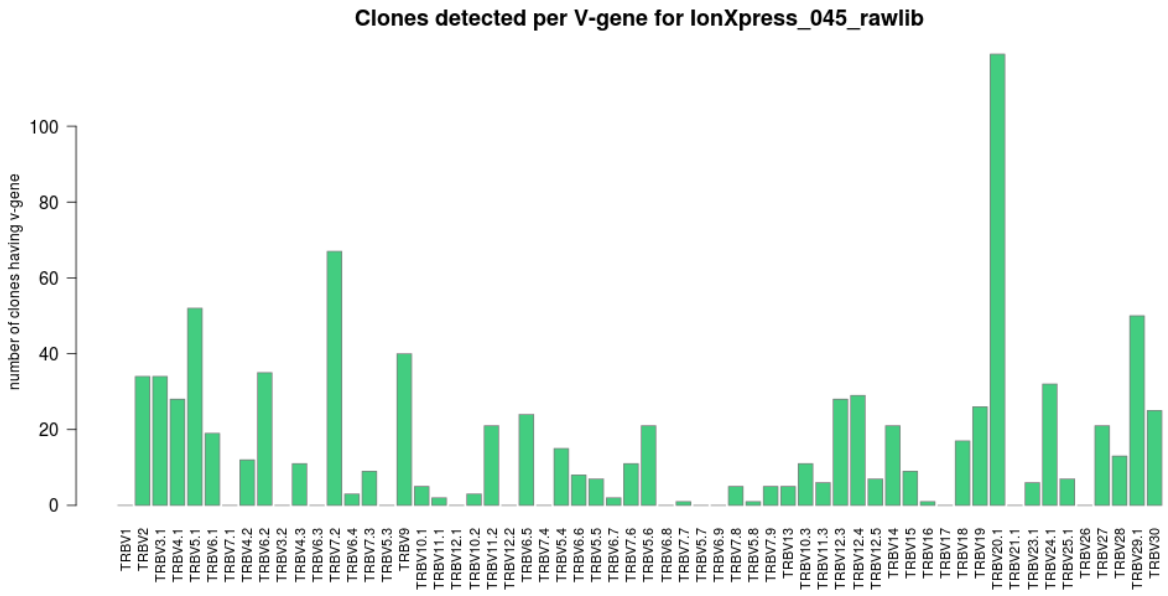


Figure 16 Clones detected per V-gene

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

CDR3 histogram

The Oncomine™ TCR Pan-Clonality Assay (TCR beta and TCR gamma) analyses produce a graphical representation of the CDR3 lengths of clones that are detected in a sample. Select **CDR3 histogram** from the **Views** list.

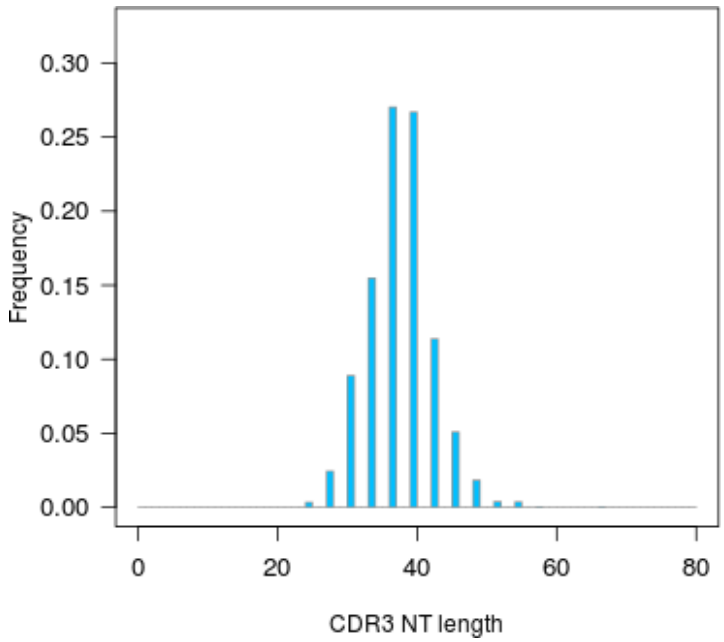
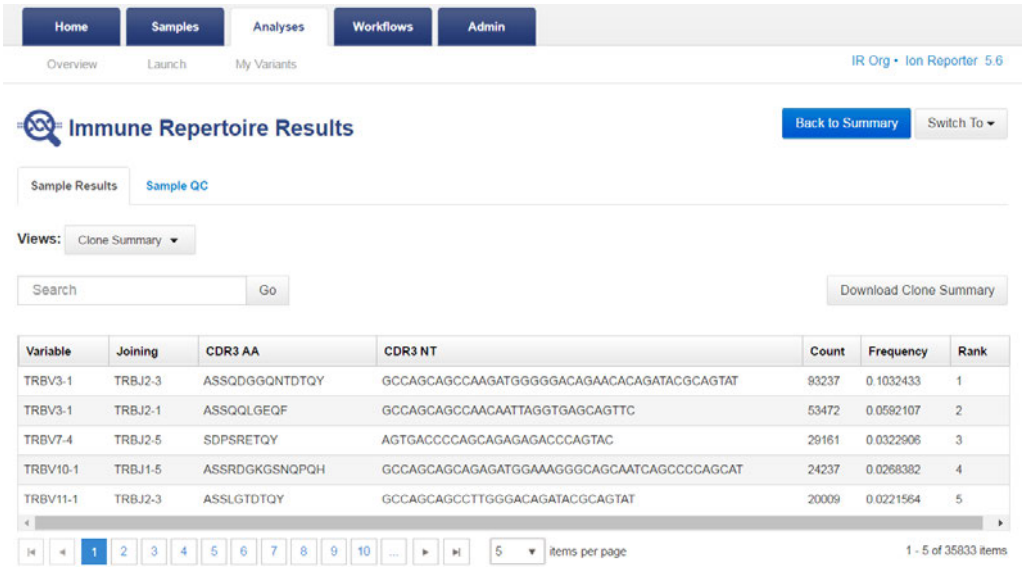


Figure 17 Relative frequency vs CDR3 nucleotide length of identified clones

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. Both productive and unproductive rearrangements are reported in the clone summary table. 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.



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 short read assay results may include multiple variable gene assignments.

① Sample 1 ② Sample 2

The following information is provided in a comparison **Clone Summary** table.

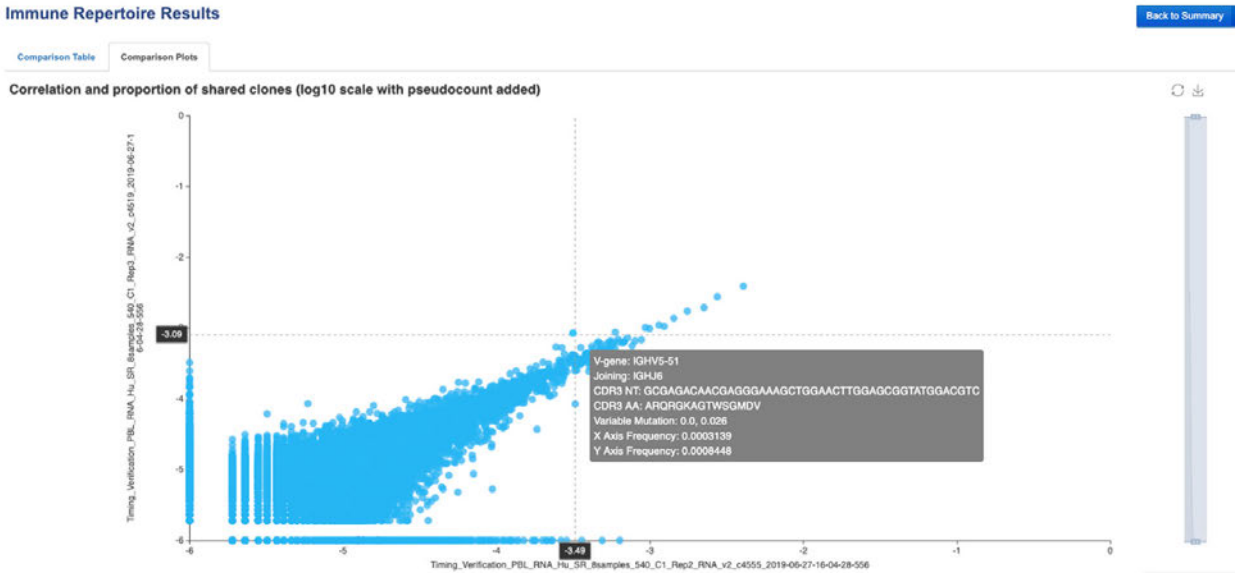
Column name	Description
Variable	The best matching IMGT variable gene of the rearrangement.
Joining	The best matching IMGT joining gene of the rearrangement.
CDR3 AA	The CDR3 amino acid sequence of the rearrangement, denoted using the IMGT definition of the CDR3 region.
CDR3 NT	The CDR3 nucleotide sequence 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

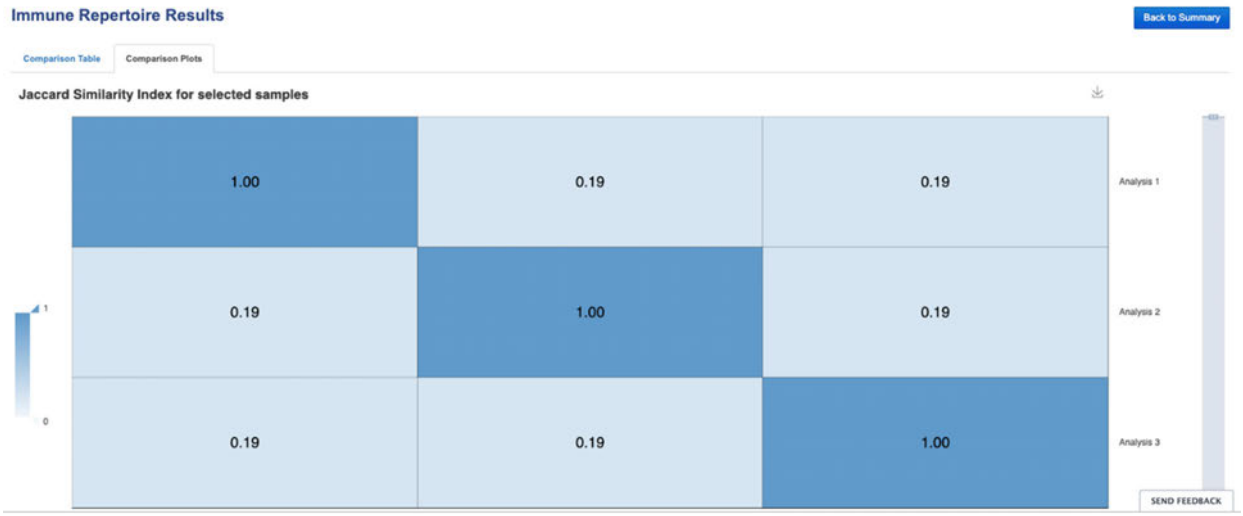
The results of comparing of 2 samples include a scatterplot indicating the frequency of clones across both samples.



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 155 for column header descriptions.

Note: The Convergent TCR Frequency metric was introduced in Ion Reporter™ Software 5.10 . 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.

13 Results – Oncomine™ BCR asssays

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 98. 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.

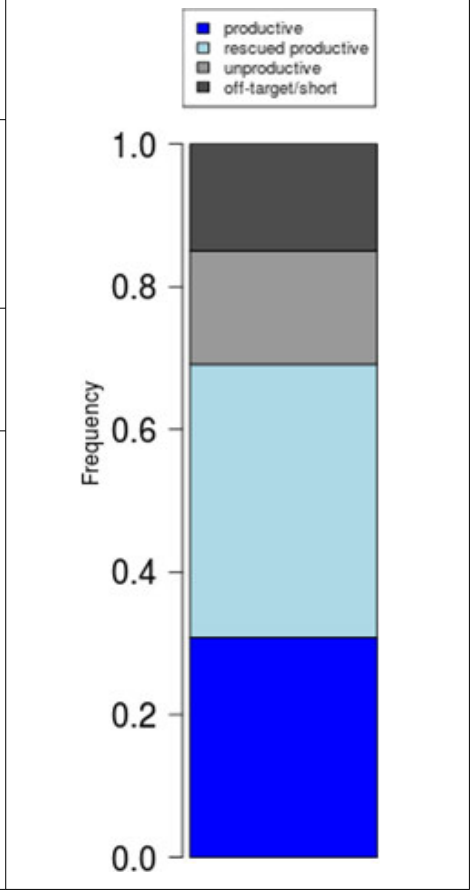
   Note: See “Multi-sample analysis” on page 99 to perform a multi-sample analysis.
6. In the **Immune Repertoire Results** summary screen, click the link in the **Sample** column to open the **Immune Repertoire Results** summary for that sample.
7. Click either the **Sample Results** or **Sample QC** tab, then select the graphical representation of the data from the **Views** dropdown list.

Sample QC tab

The Oncomine™ BCR IGH, BCR IGKL, and IGHV Leader-J analysis results are represented graphically. Select the QC metric to view from the **Views** list.

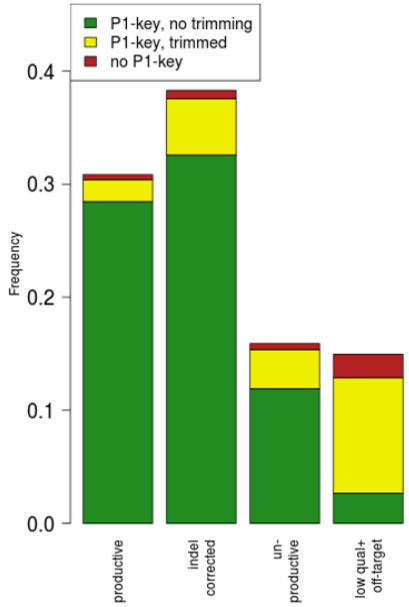
Read classification

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 an out-of-frame variable or joining gene or a premature stop codon arising naturally (in gDNA samples) or from uncorrectable sequencing or PCR errors.	
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.	

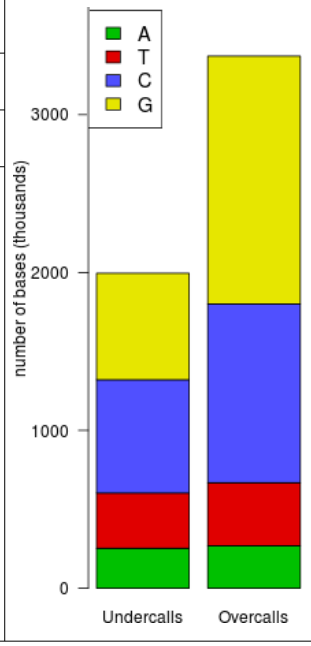
Proportion of full length, quality trimmed and reads lacking P1-key, by read classification

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.

Color	Description	Example
Red	Quality trimmed reads lacking an identifiable P1-key.	
Yellow	Quality trimmed reads having an identified P1-key.	
Green	Full length reads having an identified P1-key, and no quality trimming.	

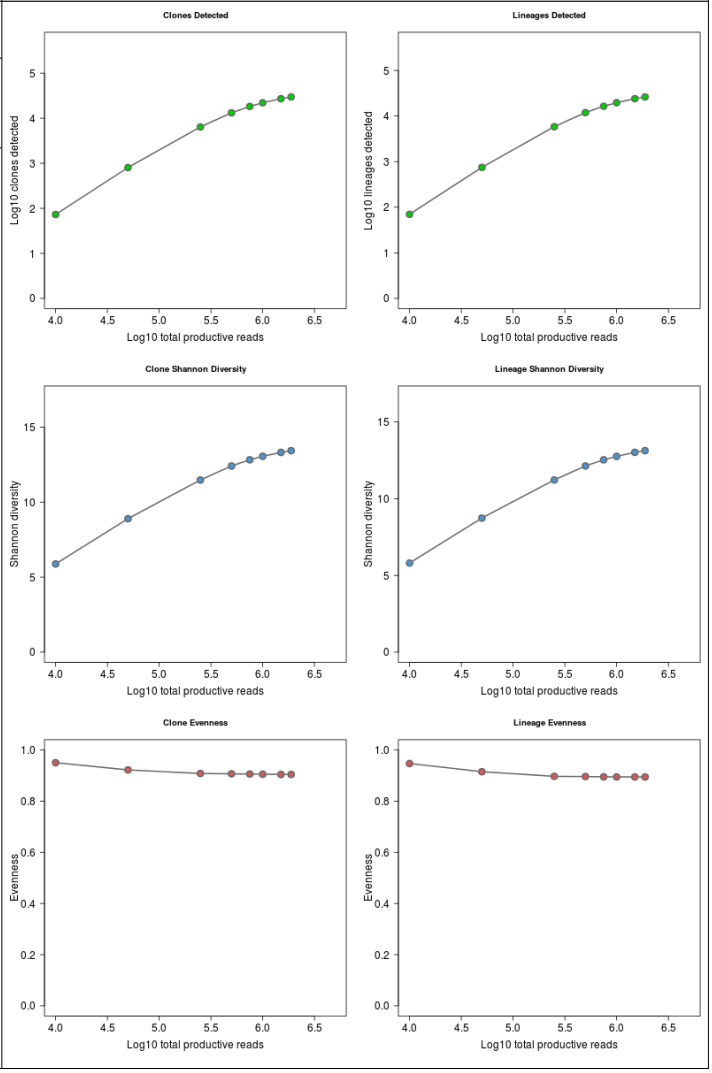
Base composition of overcalled and undercalled homopolymers

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.

Color	Description	Example
Yellow	Guanidine (G)	
Blue	Cytosine (C)	
Ted	Thymidine (T)	
Green	Adenosine (A)	

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.

Color	Description	Example
Green	Clones & Lineages detected	
Blue	Clone & Lineage Shannon diversity	
Orange	Clone & Lineage Evenness	

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 an out-of-frame variable or joining gene or a premature stop codon arising naturally (in gDNA samples) or from uncorrectable sequencing or PCR errors.
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, IGK, or IGL 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). Spectratyping plots can be further partitioned by kappa or lambda light chain using buttons above the plot in the Oncomine BCR IGKL-SR analysis workflow. 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).

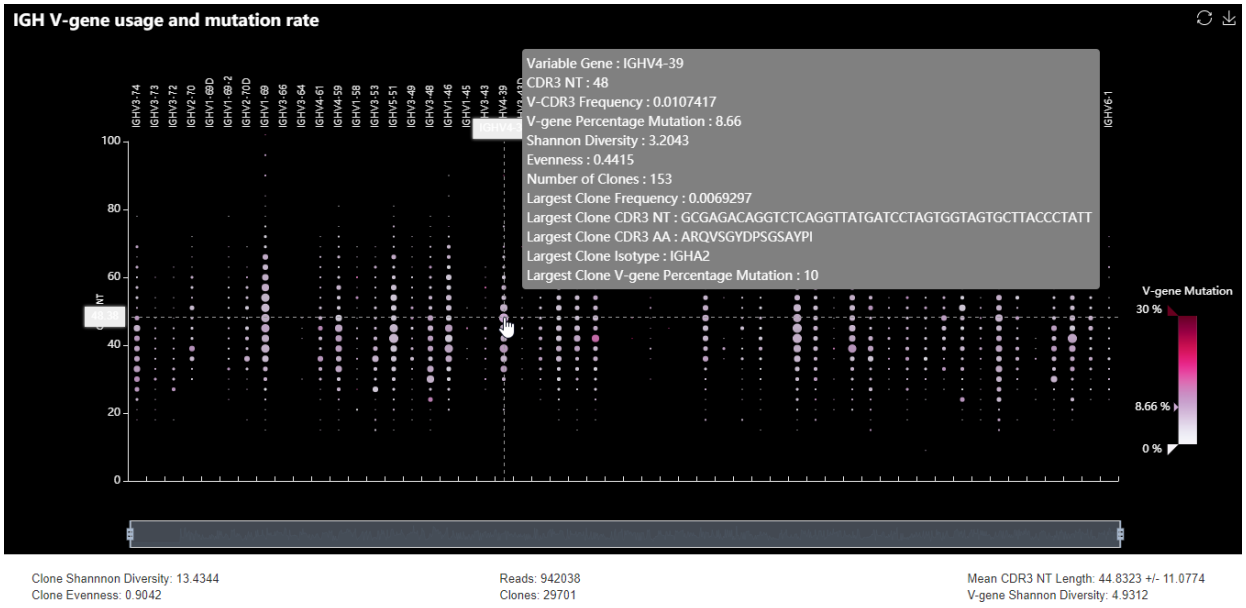


Figure 18 IGH V-gene usage and mutation rate

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.

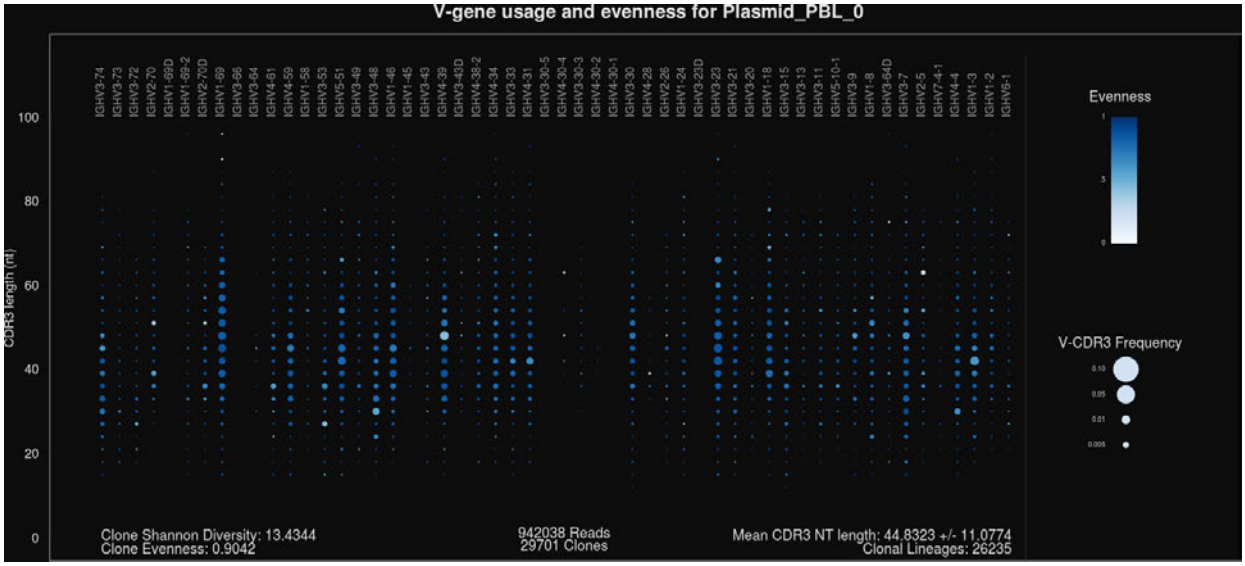


Figure 19 IGH V-gene usage and evenness

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.

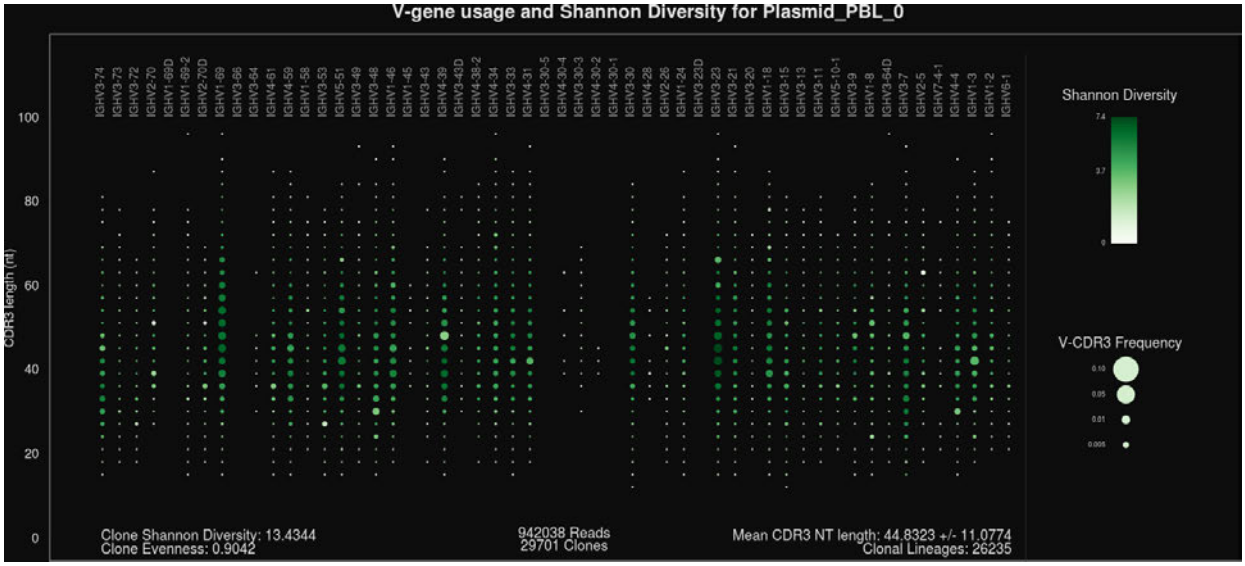


Figure 20 IGH V-gene usage and Shannon Diversity

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.

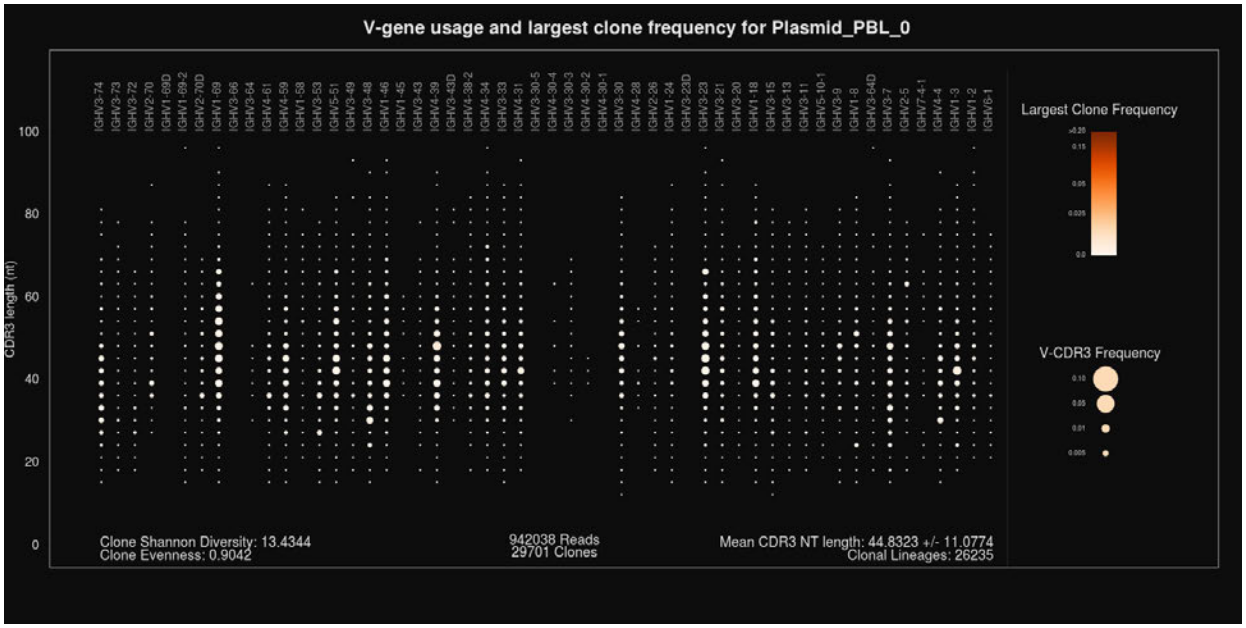


Figure 21 IGH V-gene usage and largest clone frequency

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.

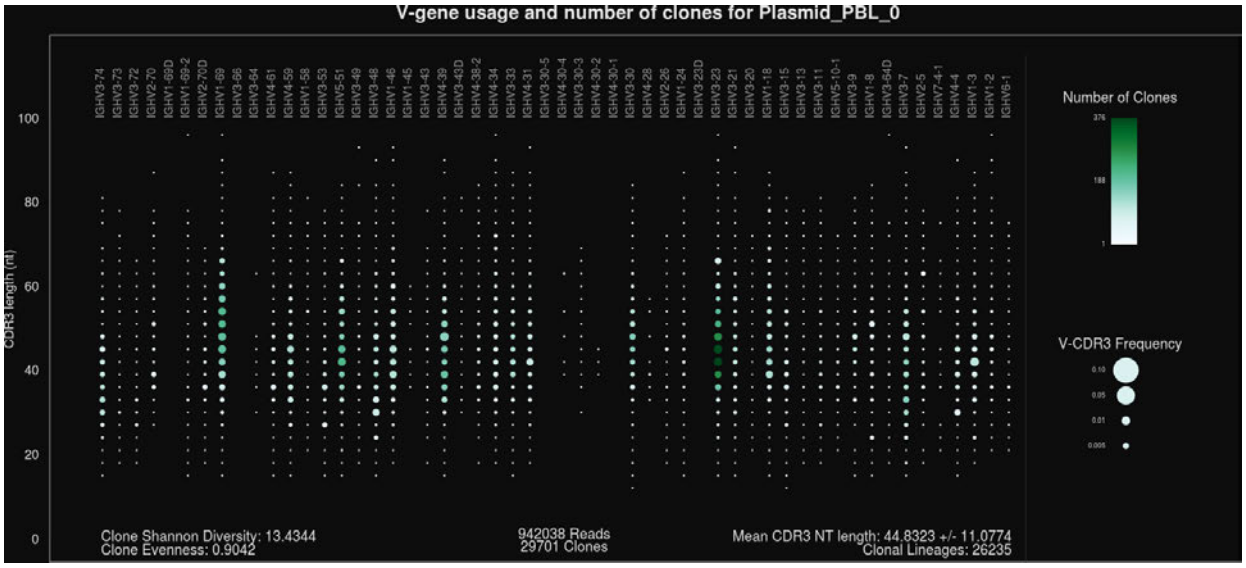


Figure 22 IGH V-gene usage and number of clones

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 Oncomine™ BCR analyses produce 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, IGK, or IGL loci.

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

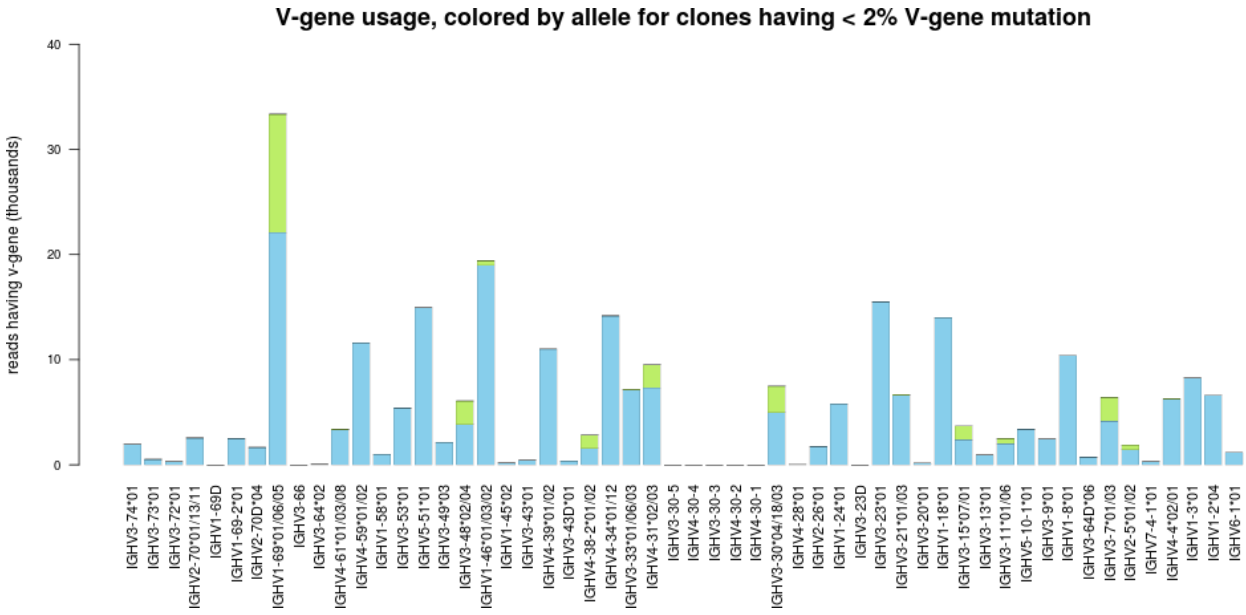


Figure 23 V-gene usage highlighting alleles

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.

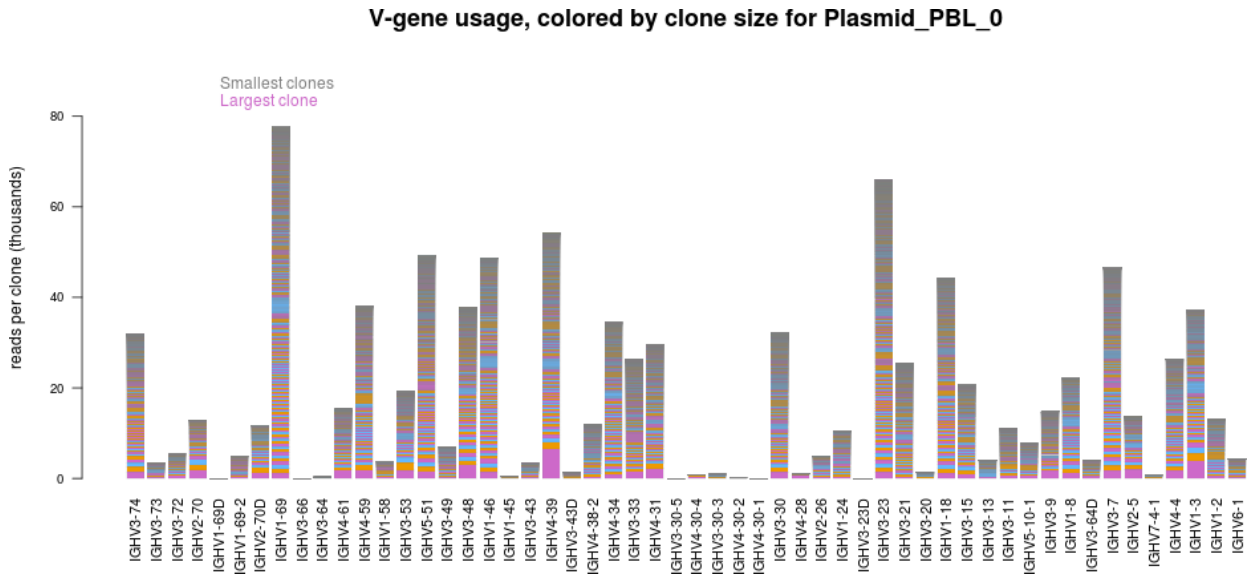


Figure 24 V-gene usage highlighting clone sizes

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 Oncomine™ BCR analyses produce 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.

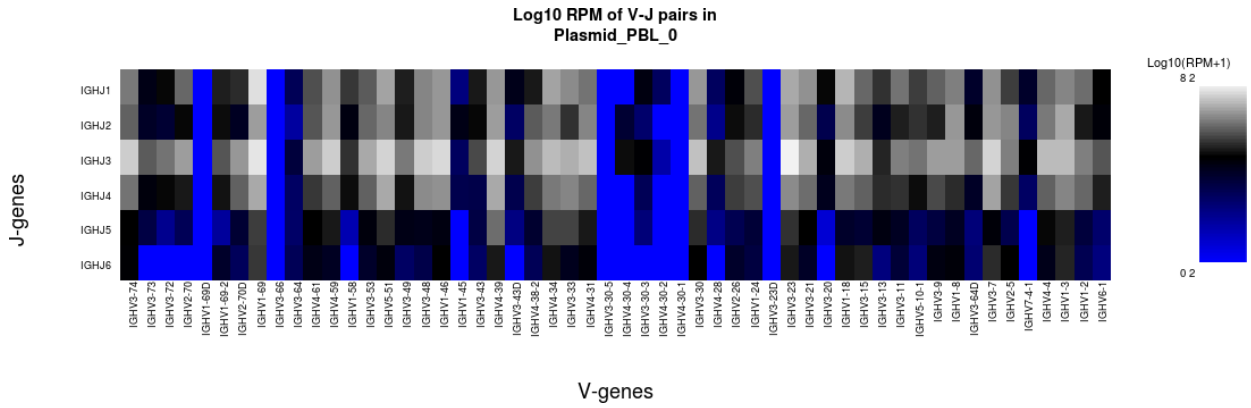
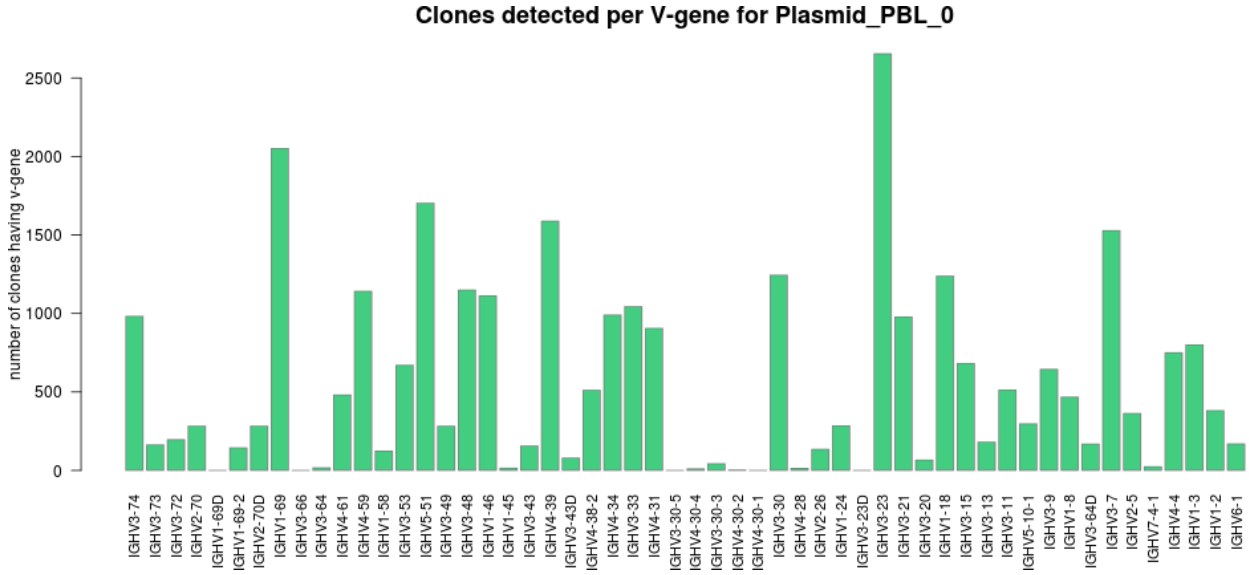


Figure 25 VJ-gene usage heatmap

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, IGK, or IGL loci.

Clones detected per variable gene

The Oncomine™ BCR analyses produce 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, IGK, or IGL loci.

CDR3 histogram

The Oncomine™ BCR IGH 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.

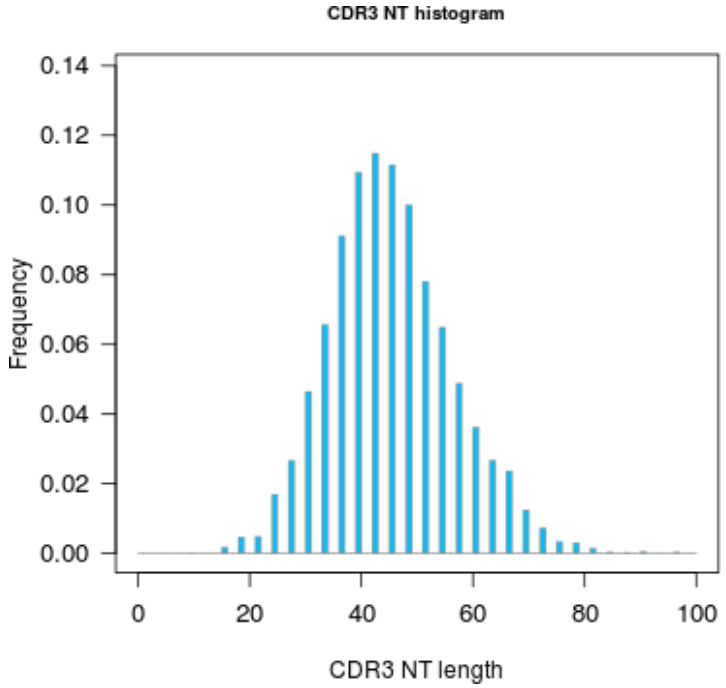


Figure 26 Relative frequency (Y-axis) of identified clones with a given CDR3 nucleotide length (X-axis)

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, IGK, or IGL 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:

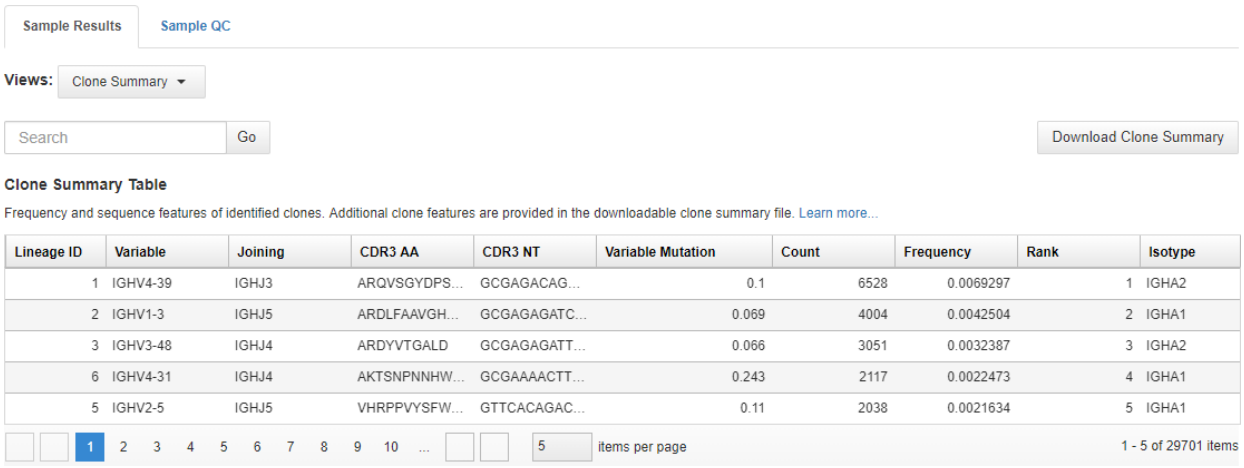
- Have the same variable and joining genes, excluding allele information.
- Have CDR3 regions of the same nucleotide sequence length.
- Have ≥85% CDR3 region sequence homology with one another.

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.

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

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 variable gene mutation (somatic hypermutation rate), and the clonal lineage assignment (Lineage ID) are also listed.



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. When comparing across repertoire workflows, in some instances results may indicate multiple variable genes that are associated with a single CDR3NT sequence. This can arise from differences in the coverage of the variable gene provided by assays that produce amplicons of varying length.

① Sample 1 ② Sample 2

Table 5 Clone summary information fields

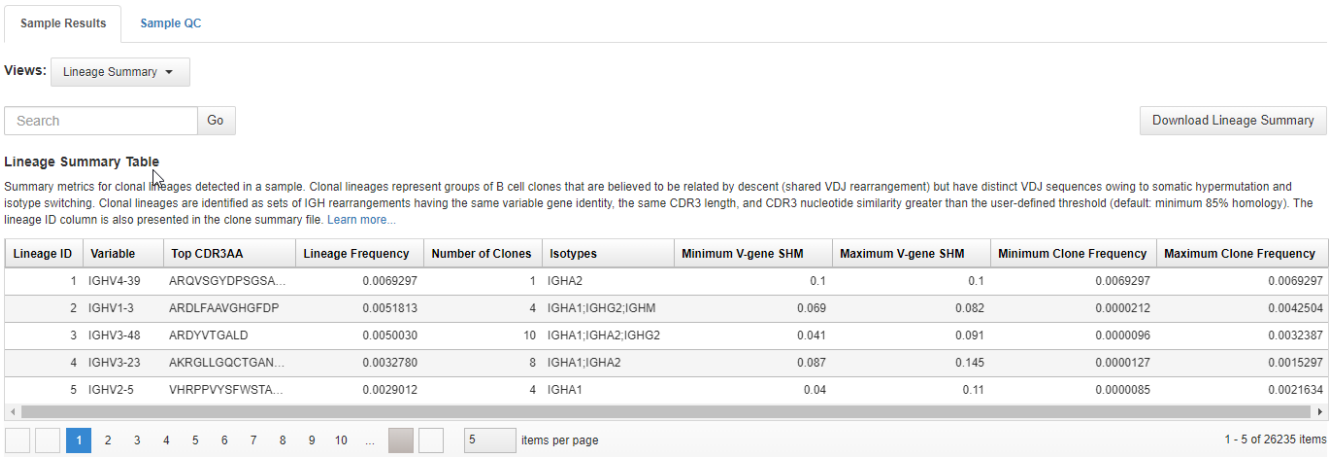
Coloumn header	Description
Lineage ID	Lineage ID represents the rank order of the clonal lineage abundance. Calculated as the sum of the frequencies of all members of the clonal lineage. Lineage 1 corresponds to the most abundant lineage, followed by Lineage 2, until the least abundant lineage is reached.
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.
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.
Isotype	The isotype identified for the clone[1].

[1] Isotype identification with the Oncomine™ BCR IGH-LR Assay assay only.

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.



Click the column header cells to sort the table.

Table 6 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.
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.

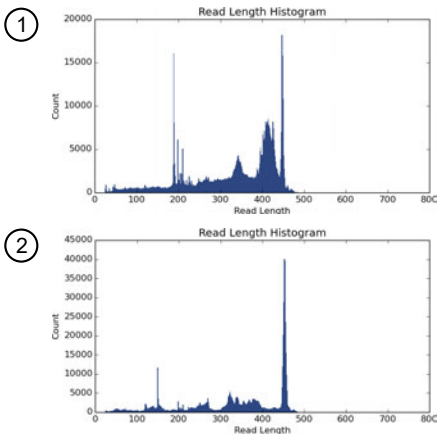
  c. Open the `.lineage_summary` CSV file to view the additional information that is included in the spreadsheet.

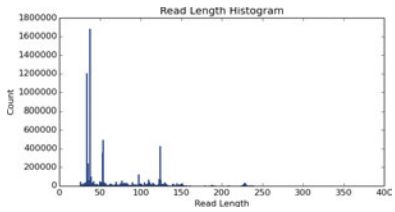
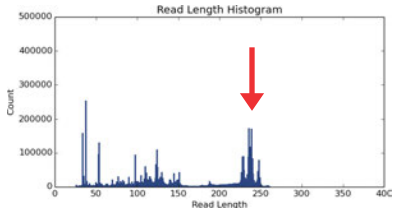
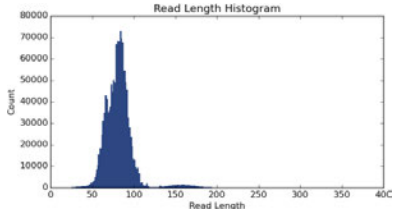
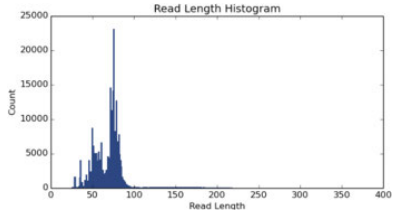
See the **Glossary** on page 155 for column header descriptions.

A

Troubleshooting

Library yield and quantification

Observation	Possible cause	Recommended action
Off target peak at short read length (<200 bp) in sequencing results **Details:** Example Oncomine™ IGH FR2-J Assay read length histograms.  ① Before re-purification ② After re-purification	Short off-target products (<200 bp), or possibly primer- or adapter-dimers (<70 bp)	Perform additional library amplification and re-purify the library with Agencourt™ AMPure™ XP Reagent (1X sample volume) twice, then elute in 50 µL Low TE. For more information, see “Library clean up (off-target products)” on page 145.
Starting library concentration is low and qPCR yields are <25 pM **Details:** Example Oncomine™ IGHV Leader-J Assay read length histograms.  ① Before addition of more DNA and/or re-purification ② After addition of more DNA and/or re-purification	Sample DNA quality was low	Increase DNA input or the number of target amplification cycles during target amplification. 1. Perform additional library amplification immediately following adapter ligation and purification. 2. Re-purify the library with Agencourt™ AMPure™ XP Reagent (1X sample volume) twice, then elute in 50 µL Low TE.

Observation	Possible cause	Recommended action
FFPE, low quality, or otherwise degraded sample types (for example, BMMC, BMA, PBMC) **Details:** Example Oncomine™ IGH FR2-J Assay read length histograms. ①  ②  ① Before re-purification ② After re-purification	Sample DNA input or quality waslow	Perform additional library amplification and re-purify the library with Agencourt™ AMPure™ XP Reagent (1X sample volume) twice, then elute in 50 µL Low TE.
Assay read length histogram does not match expected profile **Details:** See "Review sequencing run results" on page 94 for examples of expected read length histogram profiles. The example below is of Oncomine™ TCR Pan-Clonality Assay read length histograms from normal PBL gDNA samples. ①  ②  ① Expected Oncomine™ TCR Pan-Clonality Assay read length histogram ② Abherent Oncomine™ TCR Pan-Clonality Assay read length histogram	Reagent issue causes abherent read length histogram for polyclonal samples.	Contact your field service representative.

Observation	Possible cause	Recommended action
Library concentration is low **Details:** (Library concentration is NOT indicative of quality.)	Sample nucleic acid was mis-quantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit or Qubit™ Fluorometer.
	Residual ethanol in sample DNA inhibited target amplification.	Incubate uncapped tube in hood for 1 hour.
		Speed-vac tube at room temperature for 5 minutes.
	Residual ethanol from AMPure™ purification inhibited library amplification.	Carefully remove all drops of ethanol before library amplification, then centrifuge plate, if necessary.
	PCR, digestion, or ligation was inefficient.	Ensure proper dispensing and mixing of viscous components at each step.
	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.
Library concentration is too high	Sample nucleic acid was mis-quantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit.
	More sample was used than recommended.	Add less sample, or decrease target amplification cycles.
	High level of short off-target amplification product.	Perform library clean up purification, see"Library clean up (off-target products)" on page 145.

Other

Observation	Possible cause	Recommended action
(DNA only) Library concentration is low or high, with low % productive reads and high % off-target reads	dNTP concentration is not optimal.	Repeat dilution of the 25 mM dNTP stock solution. If the problem persists, perform a titration experiment, adding 2 µL of dNTP solution ranging from 0.5 to 1 mM. The 7.5 mM dNTP solution should have the lowest % of off-target reads and the highest % of productive reads.
The number of on-target reads is lower than expected	Unknown.	Increase the number of target amplification cycles by 2.

Observation	Possible cause	Recommended action
The number of on-target reads is lower than expected *(continued)*	Sample ID Panel targets were counted as off-target reads.	Add back the on-target reads from the Sample ID Panel.
Barcode representation is uneven (Equalizer™ kit not used)	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 50 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.

B Supplemental information

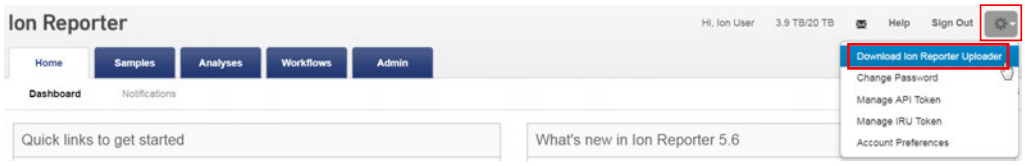
Install the IonReporterUploader plugin in Ion Reporter™ Software

The IonReporterUploader 5.6 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 5.6 plugin from http://iru.ionreporter.thermofisher.com/. To reinstall or update IonReporterUploader 5.6 plugin in Torrent Suite™ Software 5.2 or later.

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

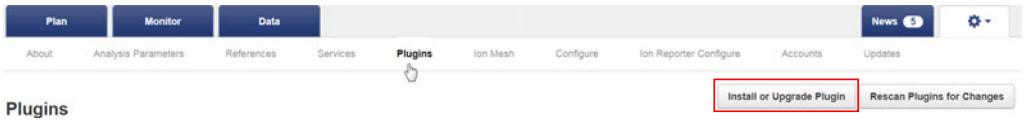
1. Sign in to Ion Reporter™ Software, then click ⚙ **(Settings)** ▸ **Download Ion Reporter Uploader**.



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 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 5.18 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.

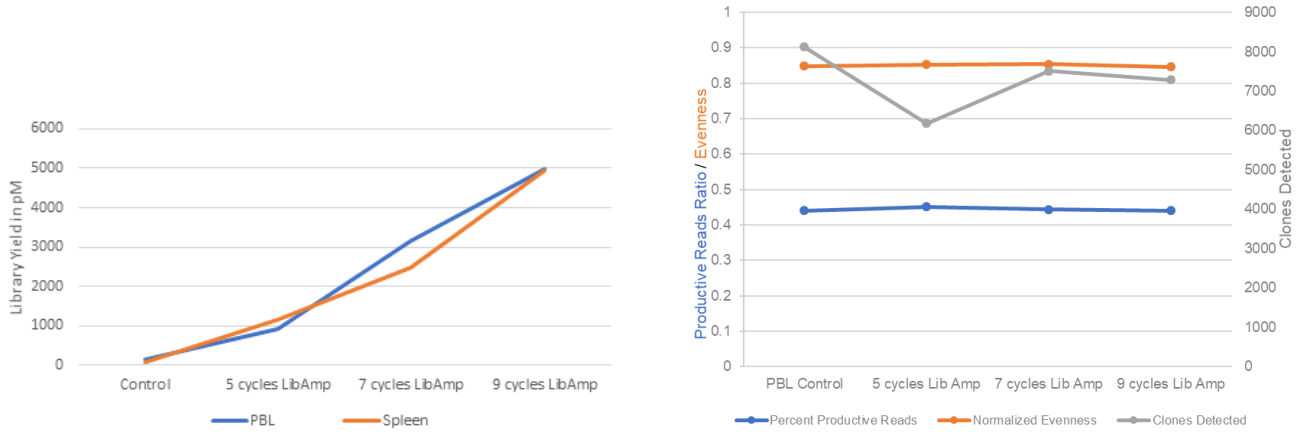


Figure 27 Effect of library amplification on yield

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

3. 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.

4. 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 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.

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 above, **add 48 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.**
7. Remove the plate with purified libraries from the plate magnet, then **add 50 µL 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.

For quantification, prepare a 10,000-fold dilution of each purified library by performing serial dilutions. Combine 2 µL of supernatant with 198 µL Nuclease-free Water to prepare a 100-fold dilution. Then, combine 2 µL of 100-fold dilution with 198 µL Nuclease-free Water to prepare a 10,000-fold dilution of each purified library.

Quantify the amplified 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 undergone amplification typically have yields of 2,000–20,000 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
1:10,000 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 PCR instrument.

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

   a. Enter the concentrations of the control library standards.

   b. Select ROX™ Reference Dye as the passive reference dye.

   c. Select a reaction volume of 20 µL.

d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

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 × the dilution factor.

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 83, then perform template preparation, without further dilution.
- 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 pM/25 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 83, 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, transfer the supernatant to a new well and seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

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-µL pipette to measure the reaction volume in each well, then add nuclease-free water to bring the volume in each well to 30 µL.
2. To 30 µL purified library, add 30 µL (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 µL 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 µL 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 “Combine libraries” on page 83. For quantification, prepare a 100-fold dilution of each library by combining 2 µL of supernatant with 198 µL Nuclease-free Water.

C Guidelines for common immune repertoire sequencing applications

Guidelines for clonality testing

Table 7 Input and multiplexing guidelines for clonality testing

Assay	Sample input, gDNA (peripheral blood or bone marrow, Lymphoma FFPE)[1]	Multiplexing level (# of libraries per chip)		
		Ion 530™ Chip	Ion 540™ Chip	Ion 550™ Chip
Oncomine™ BCR Pan-Clonality Assay	100–200 ng	8	8–16	12–24
Oncomine™ TCR Pan-Clonality Assay				
Oncomine™ IGH FR3(d)-J Assay				
Oncomine™ IGH FR2-J Assay	100–200 ng	8	8–16	Not recommended

[1] For known clonal or cell line samples we recommend 5–10 ng gDNA input. During assay validation known clonal bone marrow samples were used at 50 ng gDNA input.

Table 8 General guidelines for NGS-based clonality determination

Number of dominant sequences				
1–2	3–4		>4	0
Category 1: Optimal results >100,000 total raw reads per sample (>50,000 in select cases)[1]				
If dominant sequences are >2.5% of the total reads and >10X the polyclonal background	Same VJ use: Align and merge if related. If 1–2 dominant clones (after additional merging) >2.5% of total reads and > 10X the polyclonal background	Different VJ uses >2.5% of total reads and >10X the polyclonal background	Different VJ uses >2.5% of total reads and >10X the polyclonal background	All merged sequences <2.5%
Monoclonal (monoallelic/biallelic) or biclonal		Oligoclonal		Not clonal

Table 8 General guidelines for NGS-based clonality determination *(continued)*

Number of dominant sequences				
1–2	**3–4**		**>4**	**0**
Category 2: Qualified results (30,000 to 50,000 raw reads per sample)				
If dominant sequences >5% of total reads and >20X polyclonal background	Same VJ use Align and merge if related 1–2 dominant (after additional merging) >5% of total reads and > 20X the polyclonal background	Different VJ uses >5% of total reads and >20X polyclonal background	Different VJ uses >5% of total reads and >20X polyclonal background	All merged sequences <2.5%
Monoclonal (monoallelic/biallelic) or biclonal (confirm with other primer sets)		Oligoclonal (confirm with other primer sets)	Oligoclonal (confirm with other primer sets)	Not clonal
Category 3: Qualified results (<30,000 raw reads per sample)				

[1] Total of 50–100K reads suitable for analysis of a clonal sample provided that the polyclonal background is highly variable.

Table 8 adapted from: Arcila, Maria E. et.al. *Establishment of Immunoglobulin Heavy (IGH) Chain Clonality Testing by Next-Generation Sequencing for Routine Characterization of B-Cell and Plasma Cell Neoplasms*. **Journal of Molecular Diagnostics**, Vol. 21, No. 2, March 2019; with permission from Elsevier (lic. # 5107240389068).

Guidelines for SHM analysis

Table 9 Input and multiplexing guidelines for IGH variable gene somatic hypermutation (SHM) analysis

Application	Sample input, gDNA (peripheral blood or bone marrow)[1]	Multiplexing level (# of libraries per Ion 530™ Chip)
Oncomine™ IGHV Leader-J Assay	200 ng	8[2]
Oncomine™ IGH FR1-J Assay		

[1] For known clonal or cell line samples we recommend 5–10 ng gDNA input. During assay validation known clonal bone marrow samples were used at 50 ng gDNA input.

[2] Higher multiplexing levels (up to 12 samples per Ion 530™ Chip) may be possible. Consult the 'downsampling' analysis in the QC tab of Ion Reporter analysis results to evaluate the effect of sequencing depth on SHM analysis.

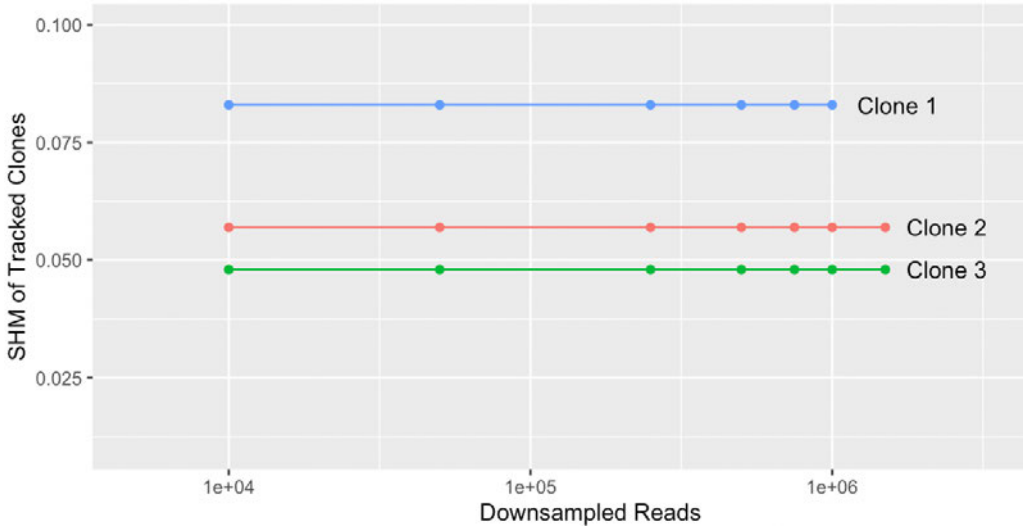


Figure 28 Effect of read count downsampling on SHM determination by the Oncomine™ IGHV Leader-J Assay

- 10 ng input
- 500 ng input
- 1000 ng input

Peripheral blood leukocyte gDNA input at 10 ng, 500 ng, and 1,000 ng and the somatic hypermutation rate tracked via three distinct index clones as reads are downsampled from 1E+6 to 1E+4, showing consistency in SHM rate over this read count range.

Guidelines for quantification of clone cell number by cell line control addition to a peripheral blood sample.

A control cell line at a known concentration (cell equivalents) can be added to a sample to estimate the number of cell equivalents corresponding to a particular clone of interest in a sample. The frequency of this control cell line, once determined, can be used to back-calculate the number of cell equivalents of any clone of interest detected in the sample.

Table 10 is a demonstration of the accuracy of a control cell line frequency determination using the Oncomine™ BCR Pan-Clonality Assay using a cell line which expresses both IGH and IgK rearrangements added to a background peripheral blood sample.

- The control cell line is added to pools of 24 µg of gDNA per sample at 100 cell equivalents. In this test one sample was polyclonal, and three samples were clonal.
- Each sample contains approximately 4 million cell equivalents (that is, 6 pg DNA per cell, 24 µg DNA per library) in total.
- The cell line control should be detected at a frequency of ~10^{-4}.
- Once known, the frequency determined for the control cell line can be applied to estimate the cell equivalents of any detected clone in the sample based on the frequency of the detected clone.

Table 10

	Polyclonal sample	Clonal sample 1	Clonal sample 2	Clonal sample 3
Oncomine™ BCR Pan-Clonality Assay (IGH analysis)	0.79×10^{-4}	0.78×10^{-4}	0.83×10^{-4}	0.87×10^{-4}
Oncomine™ BCR Pan-Clonality Assay (IgK analysis)	1.14×10^{-4}	03.47×10^{-4}	4.88×10^{-4}	2.35×10^{-4}

Guidelines for rare clone detection

Table 11 General guidelines for rare clone detection using the Oncomine™ TCR Pan-Clonality Assay, Oncomine™ BCR Pan-Clonality Assay, and Oncomine™ IGH FR3(d)-J Assay.

Limit of Detection (LOD)	Confidence interval (CI)	Total DNA input	# of libraries per sample	# of raw reads per library	Estimated libraires per Ion 540™ Chip/ Ion 550™ Chip
10^{-4}	>95%	500 ng	1	2 M	32/48
10^{-5}		4 µg (2 µg per library)	2	4M	16/24
10^{-6}		24 µg (2 µg per library)	12		

Table 12 General guidelines for rare clone detection using the Oncomine™ IGH FR2-J Assay

Limit of Detection (LOD)	Confidence interval (CI)	Total DNA input	# of libraries	# of raw reads per library	Estimated libraires per Ion 540™ Chip
10^{-4}	>95%	500 ng	1	2 M	32
10^{-5}		4 µg (2 µg per library)	2	4M	16
10^{-6}		24 µg (2 µg per library)	12		

D

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)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

Documentation and Support

Related documentation

Document	Pub. No.
Oncomine™ Human Immune Repertoire User Guide	MAN0017438
Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide	MAN0013432
Ion AmpliSeq™ Library Kit Plus User Guide	MAN0017003
Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide	MAN0016854
Ion 540™ Kit – Chef User Guide	MAN0010851
Ion 550™ Kit – Chef User Guide	MAN0017275
Ion Library TaqMan™ Quantitation Kit User Guide	MAN0015802
Demonstrated Protocol: Sample Quantification for Ion AmpliSeq™ Library Preparation Using the TaqMan™ RNAse P Detection Reagents Kit	MAN0007732

Note: For additional documentation, see “Customer and technical support” on page 154.

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:

$$-\sum_{i=1}^{R} p_i \log_2(p_i)$$

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.

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 *(new in Ion Reporter™ Software 5.10)*

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

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:

- Number of clones
- Number of lineages
- Clone evenness
- Lineage evenness
- Clone Shannon Diversity
- Lineage Shannon Diversity
- Convergent BCR Frequency
- Convergent BCR Frequency- all CDRs

In cases where insufficient reads are available for a particular downsampling depth the corresponding fields will assume an 'NA' value.

Evenness

Also known as the normalized Shannon Diversity. Measurement of the similarity of clone sizes. Calculated as:

$$\frac{-\sum_{i=1}^{R} p_i \log_2(p_i)}{\log_2(R)}$$

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.

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.

Isotype specific statistics

For each isotype, the clone summary file provides an indication of:

- The frequency of reads corresponding to each isotype (e.g., IGHM_frequency)
- The number of reads corresponding to each isotype (e.g., IGHM_counts)
- The number of clones possessing each isotype (e.g., IGHM_clones)
- The number of lineages possessing each isotype (e.g., IGHM_lineages)
- The aggregate frequency of clones having >10% variable gene mutation for each isotype (e.g., IGHM_frequency_high_SHM_clones)

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.