



iRepertoire, Inc.

iRepertoire iR-Complete Dual Index Primer Kit User Manual for RNA

Constructing an Immune Repertoire Library for Next-Gen Sequencing
for RNA with Illumina Dual Indices, long or short amplicon



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Version Updates

Update Classification	Update Description	Version
Initiation	First version of manual for RNA primer sets inclusive of Illumina dual indices.	20220613
Minor	Added minor clarifications at processing steps.	20220713
Moderate	Indication that gel marker is not included in the final library and must be added.	20220819
Moderate	Fixed figure for PCR2 Mix tube label and fixed page numbers in the Sequencing and Data Analysis sections	20221011
Minor	Updated 96-reaction kit graphic on page 9 to accurately reflect the dual index pairs provided in the Sequencing section.	20221020
Minor	Changes to improve consistency and understanding. Clarified Data Submission section.	20230503

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Introduction

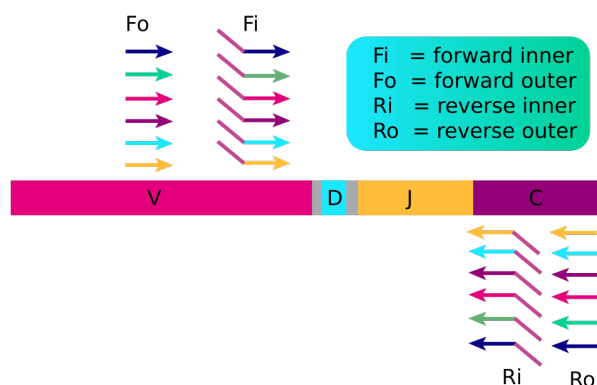
Please read this handbook carefully before beginning your experiment. The “Pooling Libraries,” “Template Selection,” and “General Information” sections are particularly important to read and understand before beginning your sample preparation. This manual provides instructions for amplifying RNA to create human or mouse immune repertoire libraries with iRepertoire’s dual indexed primer sets compatible with Illumina Next-Gen Sequencing Platforms (MiniSeq, iSeq, MiSeq, NextSeq, and NovaSeq). This manual specifically refers to the application of primer systems which are RNA compatible.

For gDNA only amplification, please refer to the gDNA-specific manual for separate instructions.

The arm-PCR Technology

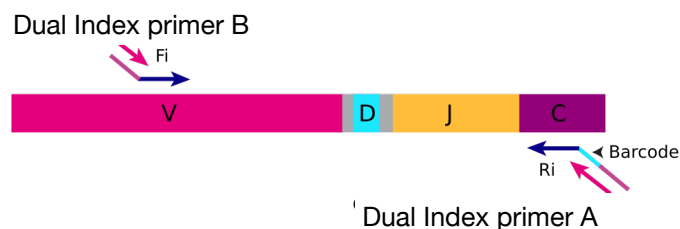
The arm-PCR (amplicon rescued multiplex PCR) technology is a patented multiplex amplification strategy (Patent No. 7,999,092) that utilizes a two-step reaction to amplify an immune repertoire, inclusively and semi-quantitatively.

First Round PCR



RT-PCR is performed with multiplex primers. Each of the unique V-genes is tagged and a universal priming site is added.

Second Round PCR



During PCR2, the dual-index primers are used for the exponential phase of amplification using the PCR1 products as a template.

During the first round of PCR, nested gene specific primers targeting each of the V and -C (RNA compatible) or V and -J (gDNA or RNA compatible) genes are used (primer system dependent). The forward primers Fo (forward-out) and Fi (forward-in) are located in the V genes. The reverse primers, Ro (reverse-out) and Ri (reverse-in), are located in each of the -C (RNA compatible) or -J genes (gDNA or RNA compatible). The Fi and Ri primers also include partial sequencing adaptors B and A, respectively, for the Illumina platforms. For the Ri primers, there are also barcodes in between the sequencing primer A and the -C or -J gene specific primers. The second round of PCR is carried out using dual index adaptor primers B and A, which represent a specific dual index pair. After magnetic bead clean-up or gel purification, the resulting product is ready for high throughput sequencing with Illumina next-generation sequencing platforms. No additional enzymatic steps are required. The first round of PCR introduces an internal sample barcode and partial sequencing primers into the PCR products. The exponential phase of the amplification is achieved by the dual index Illumina primers in the second round of PCR; therefore, the entire repertoire is amplified evenly and semi-quantitatively.

A note about this manual

This manual is separated into sections by the major steps necessary to produce an immune library. The major steps include the following: RT-PCR1 Set-up, RT-PCR1 Thermocycler Conditions, PCR2 Reaction Mix Set-up, RT-PCR1 Rescue and PCR2 Set-up, PCR2 Thermocycler Conditions, Library Clean-up, Assessing Library Generation, Pooling Libraries, NGS with the Illumina Platforms, and Data Analysis. Alternative protocols for sample processing are provided under each of these general categories. Prior to beginning the major steps, please read the “General Information” carefully.

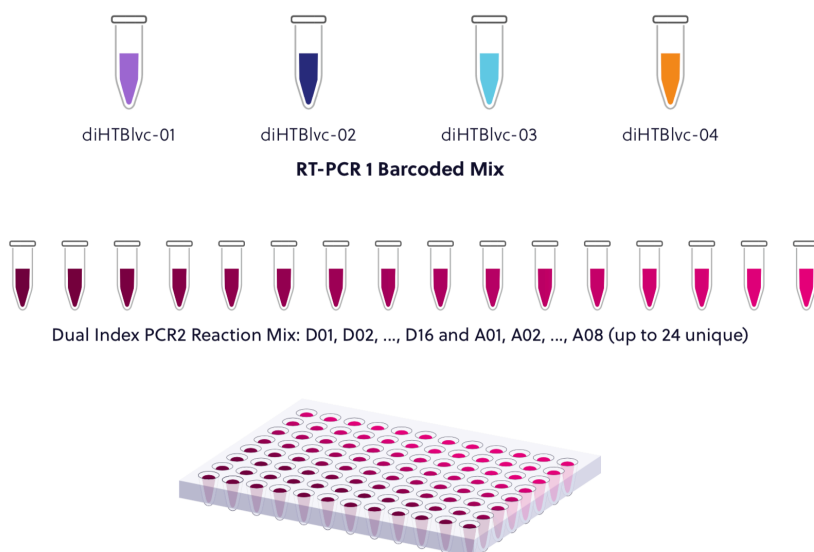
Understanding iRepertoire’s reagent system naming scheme

The end-product of amplification with iRepertoire’s reagent systems is a library of immune specific sequences that were produced using a gDNA, RNA, or total nucleic acid sample (depending upon the primer set ordered). We use the distinction between the term “sample” (the starting material) and “library” (end-product of amplification) in order to reduce confusion. This is useful since one sample can produce different libraries such as a TCR-beta, TCR-alpha, Ig-heavy, etc., depending upon the reagent system utilized during amplification. After PCR2, libraries are sequencing ready. For PCR2, the partial adaptors added in PCR1 are completed using the dual index barcoded PCR2 primers. These also contain a very specific label, which has shortened code that refers to specific dual index pairs.

Barcode multiplicity is achieved by making a unique combination of both internal and dual indexed series PCR2 primers. In order to demultiplex the data after analysis, **this combination of internal and external barcodes MUST BE unique.**

Dual indexed primer systems are offered in 4 sizes with the following configurations:

- 12 reaction kit, 1 internal barcode, 12 dual index adaptors
- 24 reaction kit, 2 internal barcodes, 12 dual index adaptors
- 48 reaction kit, 4 internal barcodes, 12 dual index adaptors
- 96 reaction kit, 4 internal barcodes, 24 dual index adaptors





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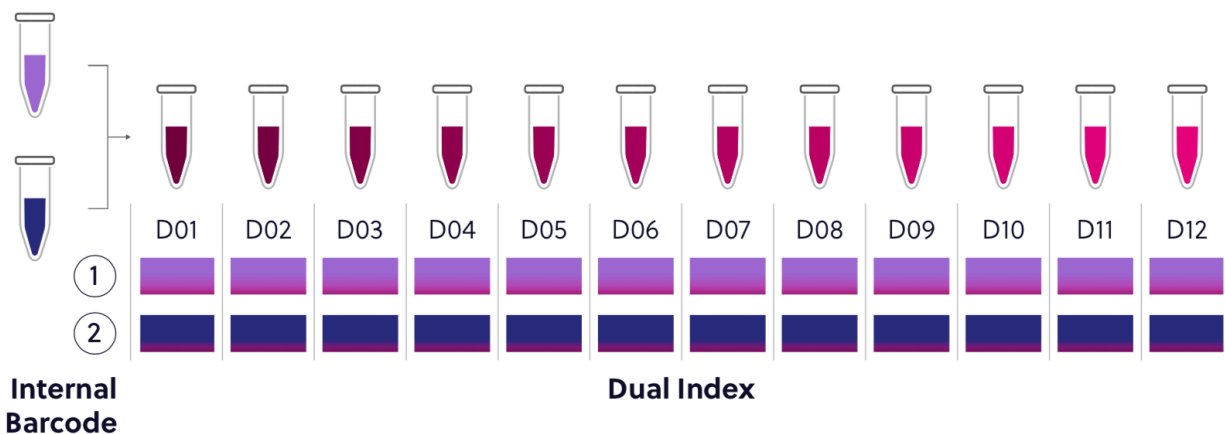
12 Reaction Kit Configuration

This kit contains 1 internal barcode and 12 dual index adaptors. This is the simplest configuration of the kit and can create 12 uniquely barcoded libraries.



24 Reaction Kit Configuration

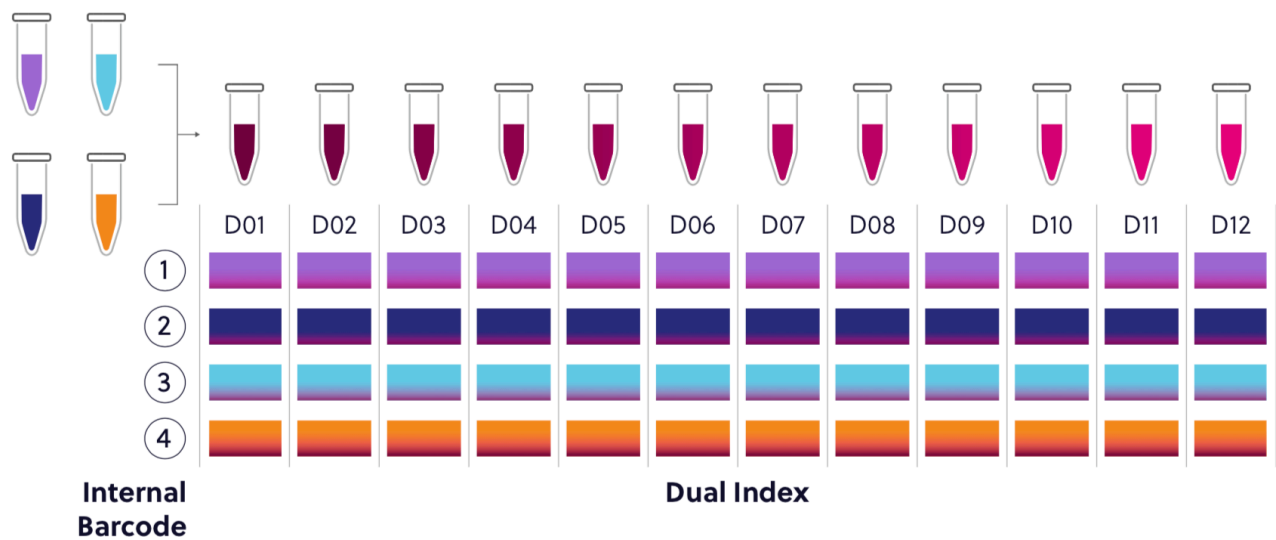
This kit contains 2 internal barcodes and 12 dual index adaptors. Ensure that a unique combination of internal and external barcodes are utilized to uniquely barcode each sample.





48 Reaction Kit Configuration

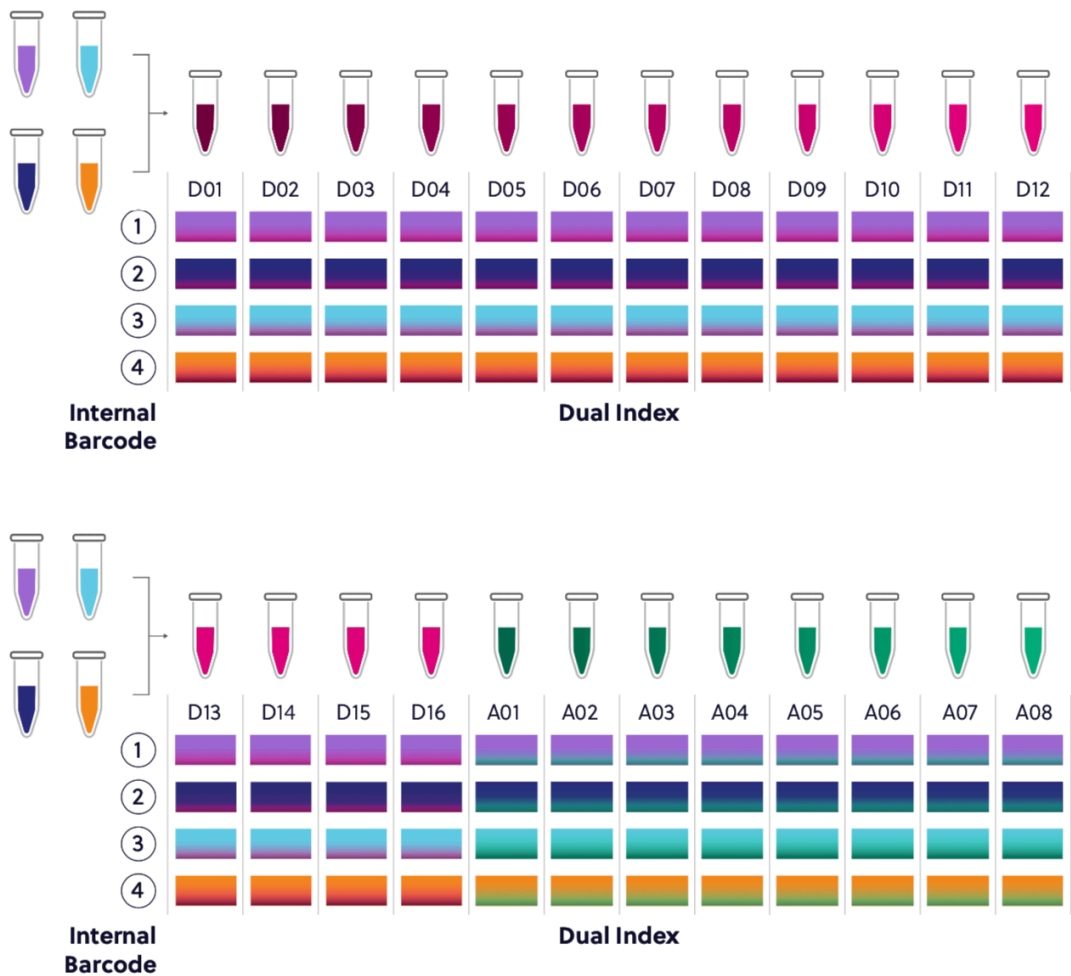
This kit contains 4 internal barcodes and 12 dual index adaptors. Ensure that a unique combination of internal and external barcodes are utilized to uniquely barcode each sample.





96 Reaction Kit Configuration

This kit contains 4 internal barcodes and 24 dual index adaptors. Ensure that a unique combination of internal and external barcodes are utilized to uniquely barcode each sample.

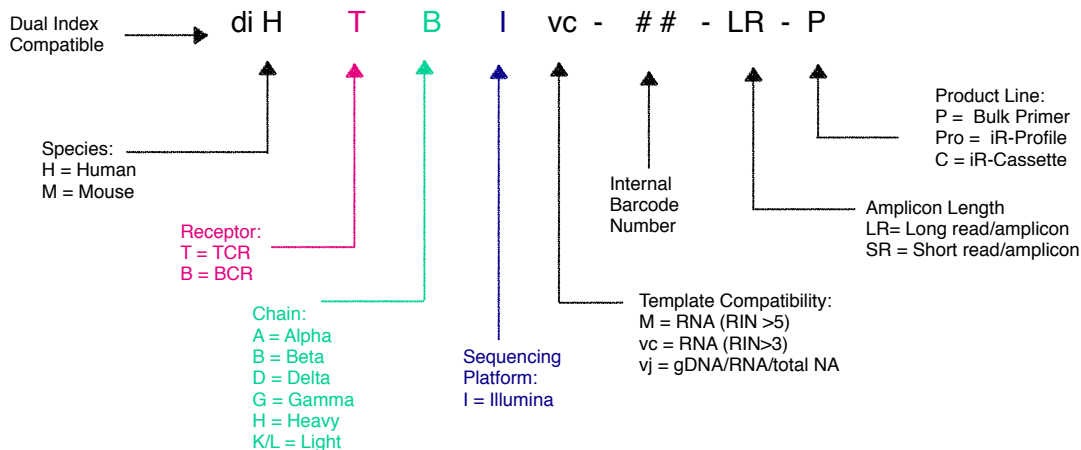




RT-PCR1 Labeling

For RT-PCR1, the barcoded RT-PCR1 bulk primers contain a very specific label, which conforms to iRepertoire’s naming scheme. These bulk reaction primer mixes contain a code similar to the one depicted below. This code contains important information such as the species, receptor, chain, sequencing platform, library length, the barcode number (molecular ID (MID)), and the product line. The product line code for bulk primer mixes is “P” and is the primer mix included in iR-Complete kits. Please be sure to record your sample name with the barcode information. A “Sample Designation Sheet” is provided for your convenience. The full reaction code enables downstream sequencing analysis in iRweb.

RT-PCR1 Primer Description



RT-PCR1 Barcoded Primer Tube Example Label

diHTB Ivc-04-SR-P
PCR1 Primer
4 µL/rxn 55 µL
Lot 20211216 Exp 2023.06.15

Dual Index PCR2 Primer Labeling

Labeling for PCR2 primer mixes are simplified to a single code, where X is the series A, D, D+, and EN refers to the series type. Each two digit number ## refers to a specific series diagonal pair of pre-mixed dual index primers. Therefore, D01 refers to Series D dual index pair 501-701. Please refer to the table in Section “Sequencing” for the key associating the dual index code with the dual index sequences.

Dual Index PCR2 Primer Description

PCR2 Dual Index Primer: X##

Dual Index PCR2 Primer Label Example for a 12-Reaction Kit

(label will change with kit size)

**PCR2 Illumina Dual
Index Adaptor: D01**

8 µL/rxn

Lot 20220512 Exp2025.05.11

Package Contents & Storage

Shipping & Storage

Upon receipt, store components as indicated in the table below:

- Primers and reagents should be stored in a clean area designated only for PCR Setup.
- Primers, iR-RT-PCR1 Buffer Mix, and iR-PCR2 Mix can also be stored at -20°C if desired.
- Ensure that caps are always secured tightly to reduce evaporation.
- Keep Selection Beads out of direct or UV light.

Package Contents

Package content differs depending on the size of the kit ordered. Refer to the following table for the total number of tubes depending on the kit size.

Storage Conditions		Number of Tubes Per Kit			
		12-Kit	24-Kit	48-Kit	96-Kit
iR-RT-PCR1 Enzyme Mix	-20°C	1	2	1	2
Control RNA	-20°C	1	2	1	2
iR-RT-PCR1 Buffer Mix	2-8°C	1	2	1	2
iR-PCR2 Mix	2-8°C	1	2	1	2
Selection Beads	2-8°C	1	2	1 bottle	2 bottles
iR-Wash Buffer-LR OR iR-Wash Buffer-SR	2-8°C	1	2	1 bottle	2 bottles
RT-PCR1 Barcoded Primers Tubes	2-8°C	1	2	4	4
PCR2 Dual Index Primers Tubes	2-8°C	12	12	12	24

Important note: All systems work with RNA samples. If genomic DNA samples are used, only the V-J primers should be used (diHTBlvj or diHBHlvj) because there is a large intron between the J and C genes.

Materials & Reagents Not Provided

Hardware and Disposables

Thermocycler
(recommended: ThermoScientific Veriti)

Vortex

Cold Block or Ice

Mini-centrifuge/ Mini-spin

Reagent reservoirs
(recommended: ThermoScientific 8096)

Agarose gel electrophoresis system and agarose
(optional)

Pipettes (Multi-channel pipette (200 μ L) optional)

*iR-Magnetic Strip Stand (Sold Separately)

Filter Tips

8-tube 0.2 mL PCR strips
(recommended: Eppendorf 951010022)

Microfuge tubes

Timer

Personal Protective Equipment:
(gloves, lab coats, etc)

*One 8-strip magnetic stand is provided with first order.

Additional Reagents

Molecular biology grade, nuclease-free water

Molecular biology grade, Ethanol

Molecular Bioproducts DNA Away

Molecular Bioproducts RNase Away

Quickload 100 bp DNA Ladder, NEB (optional)

Qiagen QIAquick gel extraction kit,
Cat No. 28704 (optional)

General Information

Nucleic Acid Handling Precautions

- Maintain a sterile environment when handling nucleic acids (NA) to avoid any contamination from nucleases.
- Ensure that no nucleases are introduced into the solutions supplied with the panel.
- Make sure that all equipment that comes in contact with NA is sterile, including pipette tips and tubes.
- Ensure that RNA is maintained at 4°C during template addition. Make sure the RNA is well-mixed prior to adding to the PCR tube.
- The kit has been validated between 50 ng (minimum) and 1000 ng (maximum) of template RNA per 25 μ L reaction. It is possible to achieve amplification with less than 50 ng RNA. The “Normal” PCR protocol is capable of achieving amplification with as low as 20 pg input for high quality RNA. For RNA input less than this amount, alternative protocols are provided. Quality of RNA has the largest effect on amplification success. Determination of the appropriate protocol, low input or normal, must be determined empirically. FFPE RNA can be considered low quality, and an additional iR-Wash procedure is provided to improve amplification success rates of difficult to amplify samples.

PCR Handling

As with any amplification of nucleic acids, contamination is a concern. With arm-PCR, the amplification of multiple targets simultaneously makes handling precautions even more important to avoid false positive results. In order to avoid contamination, we highly recommend that separate rooms and equipment be used for RT-PCR1 setup, template addition, PCR2 setup, and gel extraction. In addition, separate storage areas for primers, PCR reagents, nucleic acids, and amplicons are highly recommended.

The use of hoods equipped with a UV light are recommended for RT-PCR1 setup, template addition, PCR2 set-up, and post-PCR processing. Separate pipettes and disposable materials, such as pipette tips and micro-centrifuge tubes, should be maintained in each room/area/hood. After RT-PCR1, all amplicon libraries will be associated with a unique barcode. However, steps should still be taken to avoid contamination of the laboratory environment with amplicons. Use separate hoods for template addition and post-PCR processing (including intermediate RT-PCR1 rescue and final library clean-up). Designating separate hoods and equipment for RT-PCR1 and PCR2 template addition provides an additional level of protection.

When handling tubes, change gloves frequently, particularly after touching any potentially contaminated surface such as a PCR instrument. Take care when opening tubes to avoid aerosol cross-contamination of samples. Clean all hoods and materials with DNA Away and RNase Away before and after each use. Reminders for RT-PCR/PCR handling are provided throughout this manual.

Reducing Retrograde Flow

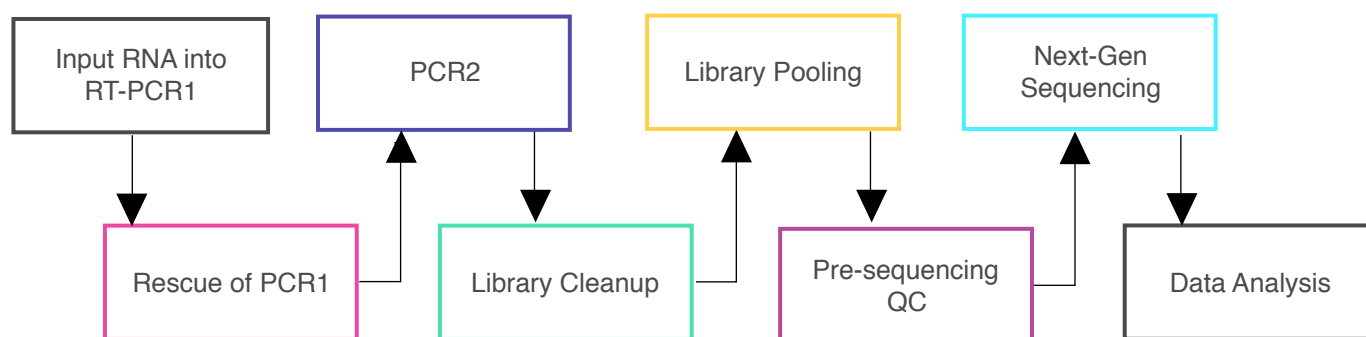
In order to reduce laboratory contamination, it is best to eliminate or reduce retrograde flow from contaminated areas to clean areas. To reduce retrograde laboratory flow, it is best to aliquot all “clean” reagents prior to handling any template or amplicon. This includes any Selection Beads required for downstream processing.

PCR2 Clean-up/Gel Extraction

For a better sequencing result, remove primer-dimers by gel extracting the target band of desired size from the agarose gel. To avoid cross-contamination, spin down tubes prior to opening in between steps and carefully handle PCR tube lids.

Outline of the Procedure

All of our reagent systems work with RNA, including any products indicating “V-C” genes and “-M” primer systems; however, due to an intron between J and C genes, only V-J primers should be used with genomic DNA. We offer V-J primers covering the human TCR beta chain (diHTB1vj) and human BCR IgH chain (diHBH1vj) for use with the Illumina platforms.



RT-PCR1 Set-up

Prior to Set-up

Ensure the PCR set-up area and pipettes are clean and free from contamination. One-Step RT-PCR reagents (particularly the reverse transcriptase) are temperature sensitive. Keep samples on PCR cold block/ice until the cyclers has reached 50 °C. It is recommended to program the thermocycler prior to beginning sample set-up. **There are different cycling conditions for BCRs and TCRs. Therefore, if amplifying both receptor types, two thermocyclers will be required.**

Experiment Design

1. Design your experiment and pre-assign barcodes to specific samples by completing Table 1, "Sample Designation Sheet." It is recommended to use a spreadsheet, e.g. Microsoft Excel to make the table.
2. Complete Table 2, "RT-PCR 1 Master Mix Preparation," to determine the amounts of each component to be added to the reaction for the number of reactions planned in the previous step.
3. It is highly recommended to include a negative control reaction in which the template addition is nuclease-free water (no RNA template) in order to evaluate proper sample handling technique, i.e., avoidance of sample cross-contamination.
4. It is highly recommended to include 1-2 positive control reactions with a known, high quality nucleic acid sample. Control RNA is provided with 13.75 μ L per every 12 reactions.
5. The final volume for the template and water mix is 13.75 μ L. The final reaction volume is 25 μ L and is composed of 11.25 μ L of the RT-PCR1 Master Mix (MM) and 13.75 μ L template-water mix.
6. The RT-PCR1 MM preparation should be performed in a separate area/room from the template addition.

Table 1: Sample Designation Sheet

Lab ID	Sample Name	Panel	Assigned Dual Index code	Internal Barcode	Input [RNA] ng/ μ L	Desired RNA Amount (ng)	RNA template amount (μ L)	H ₂ O (μ L)	RNA Template + H ₂ O (μ L)
Example	Sample_A	diHTBlvc	D01	10	126.2	500	3.96	9.79	13.75
Example	Sample_B	diHTBlvc	D02	10	300.5	500	1.66	12.09	13.75
1									13.75
2									13.75
3									13.75
4									13.75
5									13.75
6									13.75
7									13.75
8									13.75
9									13.75
10									13.75
11	Neg. Ctrl	diHTBlvc	D11		0	0	0	13.75	13.75
12	Pos. Ctrl	diHTBlvc	D12		—	—	13.75	0	13.75

Table 2: RT-PCR 1 Master Mix Preparation

NOTE: If performing amplification of more than one sample for the same sequencing run, prepare a master mix of the table components excluding the water and template in the master mix. *One internal primer is shared among 12 reactions. Ensure a unique combination of both internal barcode and dual index combination for each sample when designing the sequencing pool.* A RT-PCR1 MM can be prepared for all 12 reactions or less at once which includes the internal barcoded primer as part of the MM. **A new MM should be prepared for each unique internal barcode according to your design.** Add 10-15% extra of each component to the master mix to account for pipetting losses (RT-PCR1 Master Mix example is 15% additional (Table 2B)).

Table 2A. A Single Reaction Set-up Example

Reagent	Volume (μL) / 25 μL rxn	A Example Reaction
Nuclease-free H_2O^*	X μL	11.75
iR-RT-PCR1 Buffer Mix	6.25	6.25
iR-RT-PCR1 Enzyme Mix	1	1
iRepertoire RT-PCR1 Barcoded Primers	4	4
Template-Water Mix**	Y μL	2
Total	25.00	25

Table 2B. A 12 Reaction Master Mix Set-up Example

RT-PCR1 Master Mix (Example)	Amount (μL)	Master Mix (μL)	Number of Rxns
iR-RT-PCR1 Buffer Mix	6.25	86.25	12
iR-RT-PCR1 Enzyme Mix	1	13.8	12
iRepertoire RT-PCR1 Primers	4	55.2	12
Template-Water Mix	Add Separately (13.75)		
Total Rxn Vol. (μL)	25	Total Master (μL)	155.25
		For each tube (μL)	11.25

*Amount of H_2O is dependent on the template concentration and should be added to reach 25 μL as the final reaction volume (see next note).

**Template volume may vary from 1 to 13.75 μL for each 25 μL reaction. Between 50 and 1000 ng of template RNA is recommended. Adjust the volume of nuclease-free H_2O accordingly (see "Sample Designation Sheet"). The template amount must be determined empirically. To avoid contamination, apply the template in a separate designated area from the RT-PCR1 master mix preparation.

RT-PCR1 Master Mix Preparation Procedure

1. In a clean area, remove the iR-RT-PCR1 Enzyme Mix from the -20 °C storage and place on PCR cooler/ice. Gather the iRepertoire RT-PCR1 Barcoded Primers and the iR-RT-PCR1 Buffer Mix from the 2-8 °C. The RT-PCR1 primer mix is a tube labeled with an iR-code and a molecular ID with the designation “-P.” The dual index is assigned during PCR2. **Ensure a unique combination of both internal barcode and dual index combination for each sample when designing the sequencing pool.**
2. Vortex each reagent to ensure ample resuspension, and spin down the reagents in a MiniSpin in order to bring all reagents to the bottom of the tube.
3. Label a separate set of PCR strips appropriately to match Lab ID and Internal Barcode on the “Sample Designation Sheet,” and place PCR strips on a PCR cold block or ice to chill the tubes.
4. Prepare the RT-PCR1 Master Mix (MM) by pipetting the reagents in the order in which they appear in the example Table 2B. Mix well by vortexing followed by spin down in a MiniSpin. **The MM does NOT include the water or template. Make sure to adjust the calculations in the table for the number of reactions in your experimental design.**
5. There is 1 internal barcode for every 12 dual index primer pairs. For kits larger than 12 reactions, an additional uniquely barcoded internal MM will need to be made for every 12 reactions. The exception is the 96-reaction kit. In the 96-reaction kit, the internal barcodes repeat and can be made for 24 reactions. Twenty-four unique dual index primers are provided.
6. Add 11.25 μ L of the RT-PCR1 MM to each of the labelled PCR tubes.
7. Bring this “RT-PCR1 Barcoded MM” strip to the template addition area and set aside until further instruction.

NOTE: Preparation of the PCR2 Reaction Mix can also be completed in the clean area at the time the RT-PCR1 Master Mix is prepared. The PCR2 Master Mix can then be set aside at 4 °C until after RT-PCR1 completion. This reduces retrograde laboratory flow during downstream processing. See “PCR2 Reaction Mix Setup” for further instructions.

Template-Water Set-Up

1. Complete the Template-Water Mix calculation table below for each sample (also included in the “Sample Designation Sheet”). Please note there is a maximum of 13.75 μL of template-water mix per 25 μL reaction.
2. Label PCR strips matching the strip containing the barcoded primers and the MM. Place the PCR strip on a PCR cold block or ice to chill the tubes. This PCR strip will be the “Template-Water” strip.
3. Add the appropriate amount of nuclease-free water to each “Template-Water” tube according to the Sample Designation Sheet.
4. Next, add the appropriate amount of well mixed template RNA to the designated location in the “Template-Water” strip. Pipette up and down to mix. Repeat the process until all template-water mixes have been made. The final volume of each mix should be 13.75 μL .

Table 3: Template-Water Determination

Reagent	Volume (μL)	A Sample Reaction
Template**	X μL	8.75
Nuclease-free H ₂ O*	Y μL	5.00
Total	13.75	13.75

*Amount of H₂O is dependent on the template concentration and should be added to reach 13.75 μL as the final template-water addition volume (see next note).

**Template volume may vary from 1 to 13.75 μL . Between 50 ng (minimum) and 1000 ng (maximum) of template RNA is recommended per 25 μL reaction. Adjust the volume of nuclease-free H₂O accordingly. The template amount must be determined empirically. Apply the template in an area designated for template addition. It is possible to add less than 50 ng of RNA; however, an alternate RT-PCR1 protocol is suggested.



RT-PCR1 Set-Up

1. Retrieve the “RT-PCR1 Barcoded MM” strip and gently open each cap. Align the “Template-Water” strip with the “RT-PCR1 Barcoded MM” strip.
2. Using a multichannel pipette set to 14 μ L, transfer all of the “Template-Water” strip to the matching positions in the “RT-PCR1 Barcoded MM” strip, pipetting up and down to mix. Try to avoid generating bubbles.

NOTE: If a multichannel pipette is not available, open each position in the “RT-PCR1 Barcoded MM” strip one at a time. Transfer the corresponding template from the “Template-Water” strip into corresponding PCR1 tube, and close the PCR1 tube before proceeding to the next position. Discard the pipette tip between each addition.

3. Cap each tube. Vortex and spin down all PCR reactions using a MiniSpin.
4. The final PCR1 reaction volume is 25 μ L. Keep all reactions on ice.
5. Proceed to “RT-PCR1 Thermocycler Conditions,” and add the PCR strips to the thermocycler once the temperature reaches 50°C. Choose the correct PCR conditions based on the receptor type (BCR or TCR) and the amount of template input (Normal versus Low).

RT-PCR1 TCR Thermocycler Conditions

There are two major categories of thermocycler PCR conditions: one for T-cells (TCR) and one for B-cells (BCR). Furthermore, there is an additional protocol provided for low RNA template input (< 50 ng). Program the thermocycler with the appropriate protocol for the study design. The appropriate protocol should be determined empirically, since the “Normal Input” may work for high quality RNA amounts less than 50 ng. Short read and long read primer systems use the same protocol.


TCR:
RT-PCR 1 Conditions:
Normal Input

Temp °C	Time	# Cycles
50	60 min	1
95	15 min	1
94	30 sec	10
60	5 min	
72	45 sec	
94	30 sec	
72	3 min	10
72	15 min	1
4	∞	Hold

TCR:
RT-PCR 1 Conditions:
Low Input

Temp °C	Time	# Cycles
50	60 min	1
95	15 min	1
94	30 sec	5
60	5 min	
72	45 sec	
94	30 sec	
60	2 min	10
72	45 sec	10
94	30 sec	
72	3 min	
72	15 min	
4	∞	Hold

Please note, all thermocycler conditions are suggested based on results from a ThermoScientific Veriti thermocycler or a Biorad C1000 thermocycler. Optimization may be required for different brands of thermocyclers.

 **NOTE:** A potential stopping point is directly after RT-PCR1. Libraries can be placed at -20°C indefinitely. Amplicon rescue and PCR2 can be performed after the RT-PCR1 library is thawed. If performing RT-PCR1 on a different day than PCR2, it is recommended to make the PCR2 mix on the day of use.

RT-PCR1 BCR Thermocycler Conditions

There are two major categories of thermocycler PCR conditions: one for T-cells (TCR) and one for B-cells (BCR). Furthermore, there is an additional protocol provided for low RNA template input (< 50 ng). Program the thermocycler with the appropriate protocol for the study design. The appropriate protocol should be determined empirically, since the “Normal Input” may work for high quality RNA amounts less than 50 ng.

BCR: RT-PCR 1 Conditions: Normal Input

Temp °C	Time	# Cycles
50	60 min	1
95	15 min	1
94	30 sec	10
63	5 min	
72	45 sec	
94	30 sec	
72	3 min	10
72	15 min	1
4	∞	Hold

BCR: RT-PCR 1 Conditions: Low Input

Temp °C	Time	# Cycles
50	60 min	1
95	15 min	1
94	30 sec	5
63	5 min	
72	45 sec	
94	30 sec	
63	2 min	10
72	45 sec	10
94	30 sec	
72	3 min	
72	15 min	1
4	∞	Hold

Please note, all thermocycler conditions are suggested based on results from a ThermoScientific Veriti thermocycler or a Biorad C1000 thermocycler. Optimization may be required for different brands of thermocyclers.

NOTE: A potential stopping point is directly after RT-PCR1. Libraries can be placed at -20°C indefinitely. Amplicon rescue and PCR2 can be performed after the RT-PCR1 library is thawed. If performing RT-PCR1 on a different day than PCR2, it is recommended to make the PCR2 mix on the day of use.

PCR2 Reaction Mix Set-up

Note: Preparation of the PCR2 Reaction Mix can be completed in the clean area at the time the RT-PCR1 Master Mix is prepared or while RT-PCR1 is in the thermocycler. The PCR2 Master Mix can then be set aside at 4°C until after RT-PCR1 completion.

1. Complete the PCR2 Master Mix calculations for the number of PCR2 reactions required. Please include 10-15% extra of each component to account for pipetting losses. An example table is provided (Table 4).

NOTE: The Dual Index Primers are Illumina-specific and are compatible with the RT-PCR1 molecular IDs provided by iRepertoire (unless stated otherwise). Each sample needs to be barcoded with a unique combination of internal and dual index primer. Using spatial layout to make the PCR2 assignment according to the “Sample Designation Sheet” is highly recommended.

2. In a clean area, place the iR-PCR2 Mix and iRepertoire PCR2 Dual Index Primers in PCR cooler block/ice.

There are 12 Illumina dual index primer pairs for every internal barcode. The exception is the 96-reaction kit. In the 96-reaction kit, the internal barcodes repeat and can be made for 24 reactions. Twenty-four unique dual index primers are provided.

3. Once thawed, vortex each reagent to ensure it is well re-suspended, and spin down the reagents in a MiniSpin in order to bring all reagents to the bottom of the tube. Place in cooler block/ice.
4. Label a separate set of PCR strips appropriately to match Lab ID and dual index barcode on the “Sample Designation Sheet,” and place PCR strips on a PCR cold block or ice to chill the tubes.
5. Mix each reagent in the order it appears in the table. Once the PCR2 MM strip is prepared, set aside at 4 °C until further instruction.

Table 4: PCR Master Mix Preparation

PCR2 Single Reaction Example

PCR2 Master Mix Example For 12 Reactions (with 15% additional)

PCR2 MM Single Rxn	Volume (μL) / 50 μL rxn	Promega Multiplex Mix Master Mix	Amount (μL)	Master Mix (μL)	Total Number Rxns
Nuclease-free H ₂ O	17	Nuclease-free H ₂ O	17	234.6	12
iR-PCR2 Mix	25	iR-PCR2 Mix	25	345	12
iR- PCR2 Dual Index Primers	Individually Assigned (8 μL for each DI)	Total V	42	Total Master Vol. (μL)	579.6
PCR 1 product**	0				
Total	42			Vol. per rxn (μL)	42

** RT-PCR1 product serves as the template for PCR2. RT-PCR1 rescue will be performed with bead selection, and therefore, the RT-PCR1 bead pellet is not included in the calculation. To avoid contamination, add the RT-PCR1 amplicon template in a separate designated area, not in the clean room.



PCR2 Reaction Mix Set-up

Preparation of the PCR2 Reaction Mix can be completed in the clean area at the time the RT-PCR1 Master Mix is prepared or while RT-PCR1 is in the thermocycler. The PCR2 Master Mix can then be set aside at 4°C until after RT-PCR1 completion.

1. Aliquot 8 μ L of each unique dual index barcode for each sample into the PCR strips according to the “Sample Designation Sheet” changing pipette tips in between each addition.
2. Aliquot 42 μ L of the PCR2 Master Mix or “PCR2 MM” to each PCR reaction tube changing pipette tips in between each addition.
3. Cap each tube. Vortex and spin down all PCR reactions using a MiniSpin.
4. Bring this “PCR2 MM” strip to the PCR-dedicated space for PCR selection and processing.
5. The final PCR2 reaction volume is 50 μ L. Keep all reactions on ice.
6. Proceed to “PCR1 Rescue with Magnetic Beads and PCR2 Set-up.”

PCR1 Rescue & PCR2 Set-up

Prior to Set-up

Ensure the setup area and pipettes are clean and free from contamination. It is recommended to perform the rescue in a hood or area designated for intermediate or post-PCR processing. Do not use the hood dedicated for template addition!

1. Prepare adequate amount of fresh 85% ethanol (for example, mix 8.5 mL of 100% ethanol with 1.5 mL of molecular biology grade water in a 15 mL conical vial to make 10 mL of 85% ethanol). Cap the vial and mix well by inverting 10 times. Set aside until given further instructions.

2. Label a new set of PCR strips with Lab IDs matching those on the RT-PCR1 strip. These will be your rescue strips.

NOTE: In order to facilitate use of a multi-channel pipette, the rescue strips should mimic the positioning of the samples in the RT-PCR1 reaction strips.

3. Pre-load the new set of PCR strips (the rescue strips) with 25 μ L of nuclease-free water.

4. Invert the Selection Beads and vortex for at least 20 seconds. Ensure the beads are fully resuspended. These beads will be used to rescue RT-PCR1 products.

NOTE: For storing beads, keep the beads out of direct and UV light.

5. Pipette appropriate amount of the beads into the appropriately labeled positions on the rescue strips based on Table 5 below. The bead amount needed depends upon the length of the desired amplicon (long read and short read). Ensure the beads remain well resuspended by vortexing every fourth addition. Mix by pipetting with the water pre-loaded in the tube.

6. When RT-PCR1 is complete, remove the RT-PCR1 strips from the thermocycler.

7. Wipe down the outside of the tubes with DNA Away prior to placing the strips on a PCR rack in the hood.

8. Change gloves prior to beginning the transfer.

9. PROCESS ONE STRIP AT A TIME.

10. First, carefully open the completed RT-PCR1 reaction strip.

11. Transfer 25 μ L of the RT-PCR1 product using a multi-channel pipette to the rescue strip pre-loaded with nuclease-free water and Selection Beads and mix by pipetting up and down until there is uniform color throughout. DO NOT CAP the rescue strips. (The empty RT-PCR1 strip can be capped and discarded once transfer is complete.)

12. Incubate the beads with the RT-PCR1 product for 3 minutes at room temperature with the caps open.

Table 5: Selection Bead Volume

Amplicon Length	Selection Beads Volume (μ L)/sample
Human Short Read (SR)	45
Human Long Read (LR)	35
Mouse Short Read (SR)	45
Mouse Long Read (LR)	40

For difficult samples (low input and FFPE) or samples that have experienced amplification issues, an additional wash step with iR-Wash Buffer is recommended. To add the wash step before PCR2, see the next optional 'iR-Wash Buffer'. If skipping the 'iR-Wash Buffer', go directly to 'Traditional Bead Clean-up'.

iR-Wash Buffer (optional)

For difficult samples (low input and FFPE) or samples that have experienced amplification issues, an additional wash step with iR-Wash Buffer is recommended. If skipping the 'iR-Wash Buffer', go directly to 'Traditional Bead Clean-up'.

Bead Clean-up with iR-Wash Buffer (optional)

1. After incubation, place the strip on the iR-Magnetic Strip Stand and allow the beads to collect. This may take 2-3 minutes. Allow the beads to collect for additional time if the supernatant is not clear. During this time, the RT-PCR1 library is collecting on the beads.
2. With the PCR strip on the magnetic stand, discard the liquid in the tubes by pipetting. The bead is associated with the library.
3. Remove the PCR strip from the magnetic stand, resuspend the bead pellet in 50 μ L of appropriate iR-Wash Buffer. There are two types available, one for long read (LR) and one for short read (SR) amplicons.
4. Incubate for 3 min with the lids open (off the magnet).
5. Place back onto the magnetic stand. Allow the beads to collect. Discard the supernatant. The beads are associated with the library. (This process is repeated in the next 3 steps).
6. Remove the PCR strip from the magnetic stand, resuspend the bead pellet in 50 μ L of appropriate iR-Wash Buffer.
7. Incubate for 3 min with the lids open (off the magnet).
8. Place back onto the magnetic stand. Allow the beads to collect. Discard the supernatant. The beads are associated with the library.
9. Pour the fresh 85% ethanol into a sterile reagent reservoir. (Optionally, the 85% ethanol can be added one step at a time to each PCR tube.)
10. WHILE KEEPING THE PCR STRIP ON THE MAGNET, wash the bead pellet with 160 μ L of the fresh 85% ethanol by pipetting up and down.
11. Discard the ethanol by pipetting.
12. Remove any residual ethanol by pipetting. Remove the strip from the stand and allow the bead pellet to air dry in a ventilated hood for 3-5 minutes. It is important that the bead pellet be completely dried as residual ethanol can inhibit PCR.
13. While the first strip is drying, repeat the process for the next unprocessed strip.
14. **Once dry, proceed to "PCR2 Set-up." (Skip the 'Traditional Bead Clean-up'.)**

Amplicon Length	iR-Wash Buffer Selection	Volume per Reaction (μ L)
Long Read (LR)	iR-Wash Buffer-LR	100 (50 x 2)
Short Read (SR)	iR-Wash Buffer-SR	100 (50 x 2)



Traditional Bead Clean-up

Bead Clean-up

1. After incubation, place the strip on the iR-Magnetic Strip Stand and allow the beads to collect. This may take 2-3 minutes. Allow the beads to collect for additional time if the supernatant is not clear. During this time, the RT-PCR1 library is collecting on the beads.
2. Set the multi-channel pipette to 160 μ L. With the strip on the stand, use the multi-channel pipette to aspirate and discard the liquid in the strip.
3. Pour the fresh 85% ethanol into a sterile reagent reservoir. (Optionally, the 85% ethanol can be added one step at a time to each PCR tube.)
4. WHILE KEEPING THE PCR STRIP ON THE MAGNET, wash the bead pellet with 160 μ L of the fresh 85% ethanol by pipetting up and down.
5. Discard the ethanol by pipetting.
6. Remove any residual ethanol by pipetting. Remove the strip from the stand and allow the bead pellet to air dry in a ventilated hood with lids open for 3-5 minutes. It is important that the bead pellet be completely dried as residual ethanol can inhibit PCR.
7. While the first strip is drying, repeat the process for the next unprocessed strip.
8. **Once dry, proceed to “PCR2 Set-up.”**

PCR2 Set-up

PCR2 Set-up

1. Retrieve the “PCR2 MM strip” from the 4 °C storage.
2. Briefly, vortex and spin down (in a MiniSpin) the “PCR2 MM” tube.
3. To the dried bead pellet, add 50 μ L of “PCR2 MM” to each tube and resuspend the beads. Ensure the correct dual index barcode is matching the assigned internal barcode on the “Sample Designation Sheet.” DISCARD the used pipette tip and use a new tip for the next tube if using a single channel pipette. Ensure that the beads are well suspended in the mix.
4. When all transfers are complete, ensure the PCR2 strips are tightly capped, vortex briefly, and spin down.
5. Proceed to “PCR2 Thermocycler Conditions,” and add the PCR2 strips to the thermocycler once ready.

Note: PCR2 is performed with the beads in the reaction mix. The resulting barcoded cDNA library will be in the reaction supernatant.

After RT-PCR1, all cDNA libraries contain communal binding sites engineered into RT-PCR1 primers. Program the thermocycler according to the parameters listed below for PCR2 (Table 6). The same PCR2 protocol can be used for all library types: TCR, BCR, normal, and low input libraries.

Table 6. PCR 2 Conditions for Dual Index Library Amplification

Temp °C	Time	# Cycles
95	15 min	1
94	30 sec	30
72	2 min	
72	5 min	1

NOTE: Directly after PCR2 is a potential stopping point in the protocol. PCR2 products may be stored at -20 °C indefinitely prior to library clean-up.

Library Clean-up

Once PCR2 is complete, the libraries need to be cleaned prior to sequencing. There are two options for library clean-up: the “PCR2 Bead Clean-up Method” or the “Gel Extraction Method.” The selection of method is largely dependent upon user preference. However, in cases of low RNA input or low quality RNA input, a large amount of primer-dimers may be produced. There is a limit to the amount of primer-dimers which can be removed by the “PCR2 Bead Clean-up Method.” In this case, gel extraction may be the best option for library clean-up. For high quality or normal RNA input, the bead clean-up should be sufficient. Determination of the appropriate library clean-up must be determined empirically and is dependent upon sample input and quality.

Before Beginning

Ensure the setup area and pipettes are clean and free from contamination. It is recommended to perform library clean-up in a hood or area designated for post-PCR processing. Do not use the hood dedicated for template addition. Do not use equipment that is used for clean processing.

Option 1: PCR2 Bead Clean-up Method

PCR2 Clean-up Mix Strip Preparation

1. Label a new set of PCR strips with Lab IDs matching the PCR2 strip.
2. Invert the Selection Beads three times and vortex for at least 10 seconds. Ensure the Selection Beads are fully resuspended.
3. Pipette the volume of beads indicated in the table below depending upon the primer system into the sample positions of the freshly labeled PCR strips which are appropriately labelled. Ensure the Selection Beads remain well resuspended by vortexing every fourth addition. Cap each position on the strip after adding the beads.
4. Set aside and wait for PCR2 to complete.

Amplicon Length	PCR2 Clean-up Selection Beads Volume (μL)
Human Short Read (SR)	45
Human Long Read (LR)	35
Mouse Short Read (SR)	45
Mouse Long Read (LR)	40

Library Clean-up

Option 1 Continued: PCR2 Transfer to the Clean-up Mix Strip

1. When PCR2 is complete, open the freshly labelled strip containing the Selection Beads.
2. Remove the PCR2 strips from the thermocycler. After PCR2, the barcoded cDNA library is in the reaction supernatant.
3. Wipe down the outside of the PCR2 strips with DNA Away prior to placing the strips on the magnetic stand.
4. Change gloves prior to beginning the transfer.
5. PROCESS ONE STRIP AT A TIME.
6. Place the PCR2 strip on the iR-Magnetic Strip Stand and allow the beads to collect. This may take 2-3 minutes. Allow the beads to collect for additional time if the supernatant is not clear. **DO NOT DISCARD THE SUPERNATANT.** The supernatant contains the amplified library.
7. Using a multi-channel pipette set to 50 μ L, transfer the libraries from the PCR2 strip to the strip containing the Selection Beads.
8. Mix by pipetting up and down until uniform color throughout. DO NOT CAP THE Bead Selection strips. (The used PCR2 strip with the old bead pellet can be capped and discarded.)
9. Let the Bead Selection strip incubate on a PCR rack (at room temperature) for 5 minutes.
10. After incubation, place the strip on the iR-Magnetic Strip Stand and allow the beads to collect. This may take 2-3 minutes. Allow the beads to collect for additional time if the supernatant is not clear. Leave the strip on the magnet until instructed otherwise.

Note: During this step, the library is being recaptured onto the bead. The next step involves washing the bead pellet.


Library Clean-up

Option 1 Continued: Washing the Bead Pellet and Eluting the Library

1. Set a multi-channel pipette to 160 μ L. With the strip on the stand, use the multi-channel pipette to aspirate and discard the liquid in the strip.
2. Pour the remaining fresh 85% ethanol into a sterile reagent reservoir. (Optionally, the 85% ethanol can be added one step at a time to each PCR tube.)
3. WHILE KEEPING THE PCR STRIP ON THE MAGNET, wash the bead pellet with 160 μ L of the fresh 85% ethanol by pipetting up and down.
4. Discard the ethanol by pipetting.
5. Remove any residual ethanol by pipetting. Remove the strip from the magnetic stand and allow the bead pellet to air dry in a ventilated hood for 3-5 minutes.
6. While the first strip is drying, repeat this process for the next unprocessed strip.

Option 1 Continued: Final Library Elution

1. Once all strips are dry, the library can be eluted from the bead. Label a final set of strips in preparation for this step.
2. Add 37 μ L of nuclease-free water to each bead pellet and resuspend the beads by pipetting up and down. A well-suspended bead pellet is rusty colored.
3. Cap each strip and vortex to make sure fully resuspended and spin down.
4. Incubate the beads with the nuclease-free water for 2 minutes.
5. Place the strip on the iR-Magnetic Strip Stand and allow the beads to collect. This may take 2-3 minutes. Allow the beads to collect for additional time if the supernatant is not clear. DO NOT DISCARD THE SUPERNATANT. The supernatant contains the amplified library
6. Transfer the cleaned and sequencing ready library to the final set of labelled PCR strips.
7. Proceed to “Assessing Library Generation.”

 **NOTE:** After Bead Selection library clean-up is a potential stopping point in the protocol. Libraries may be stored at -20 °C indefinitely prior to library pooling. It is recommended to measure the concentration of each library in the pool at the same time directly prior to pooling to achieve evenly pooled libraries.


Library Clean-up

Option 2: Gel Electrophoresis and Extraction

If option 1 was selected, continue to “Assessing Library Generation.” A hand-cast 2-4% agarose gel can be used for DNA sample screening and library gel purification of targets. Each amplified repertoire represents a large number of sequences distributed over a size range, and the gel should be excised to cover the major product band as demonstrated in the section, “Assessing Library Generation.” Due to variation in gel apparatus and gel-casting systems, please refer to the user manual of the device on-hand for further guidance on setting-up and running an agarose gel. Provided below are recommendations.

- The PCR2 reaction mix after PCR2 contains magnetic beads; it should be placed on the iR-magnetic Strip Stand until the supernatant is clear prior to loading the gel.
- PCR2 can be added directly to an agarose gel with the use of additional dye markers and a DNA ladder.
- Hand cast 2.5% gels can be made with large tooth combs capable of holding 50 μ L per lane. This reduces the amount of agarose in the gel slice and improves the gel extraction efficiency.
- Ethidium bromide can be mixed directly with the agarose and TAE buffer after heating for downstream imaging.
- Always use a gel ladder/marker to assess the library size. 100 bp ladder is recommended.
- When performing gel extraction, we recommend using Qiagen QIAquick Gel Extraction kit, Cat No. 28704 per the manufacturer’s instructions.
- For each individual sample, it is beneficial to use one QIAquick column per sample. This will require repeated additions of the sample to the same column.
- The goal is to extract the band of interest in a lower volume, typically 37 μ L, in order to increase the concentration of each library prior to pooling. This will make the pooling portion easier.
- If primer-dimers are present, they typically appear around the 150-180 bp marker.

Caution: Wear personal protective equipment such as gloves and a lab coat when handling ethidium bromide. UV light can cause serious burns to the eyes and skin; take precautions to protect your eyes and skin by using an appropriate UV apparatus.

 **NOTE:** After agarose gel extraction is a potential stopping point in the protocol. Libraries may be stored at -20 °C indefinitely prior to library pooling. It is recommended to measure the concentration of each library in the pool at the same time directly prior to pooling to achieve evenly pooled libraries.

Assessing Library Generation

Check and Record Library Concentration

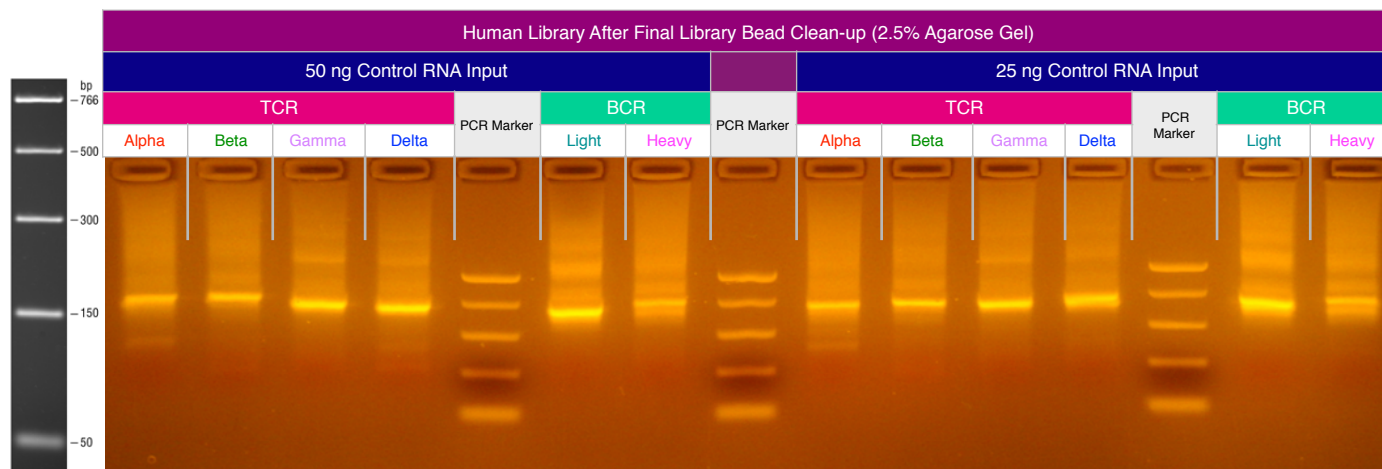
1. In all cases, it is advisable to check the amplification of a small portion of each library on an agarose gel or a Bioanalyzer. To determine if library generation was successful, use a spectrophotometer such as the Nanodrop, to measure the cDNA concentration. The average concentration of an amplified library is 30-50 ng/ μ L with an input of 100 ng of total RNA and an elution of 37 μ L of nuclease-free water when measured with a Nanodrop. The average concentration ranges in the table below may not apply if using another method of concentration determination such as a Bioanalyzer.
2. If the concentration is greater or equal to 10 ng/ μ L when eluted in 37 μ L of nuclease-free water, then the library generation is considered a success.
3. If the concentration is between 4 and 10 ng/ μ L, then check for a major product by running some or all of the amplicons on a 2.5% agarose gel to see if amplification was achieved. If all product was run, gel purify the major band.
4. If the concentration is less than 4 ng/ μ L, then typically the run is not considered a success. However, it is still advisable to check the amplification on an agarose gel as a major product may be present.
5. Smearing above the major band product is expected and is library. Smearing or non-specific amplification below the expected primary product could indicate poor library generation.

Concentration (ng/ μ L) from Nanodrop	Result
≥ 10	Success
4-10	Check a Portion on Gel
≤ 4	Unsuccessful

Amplicon Library Size: Long Read Systems (250/300) PER

Each amplified repertoire represents a large number of sequences distributed over a size range. The gel images and tables below can be used as a guide indicating the range of potential product sizes.

Human



Mouse

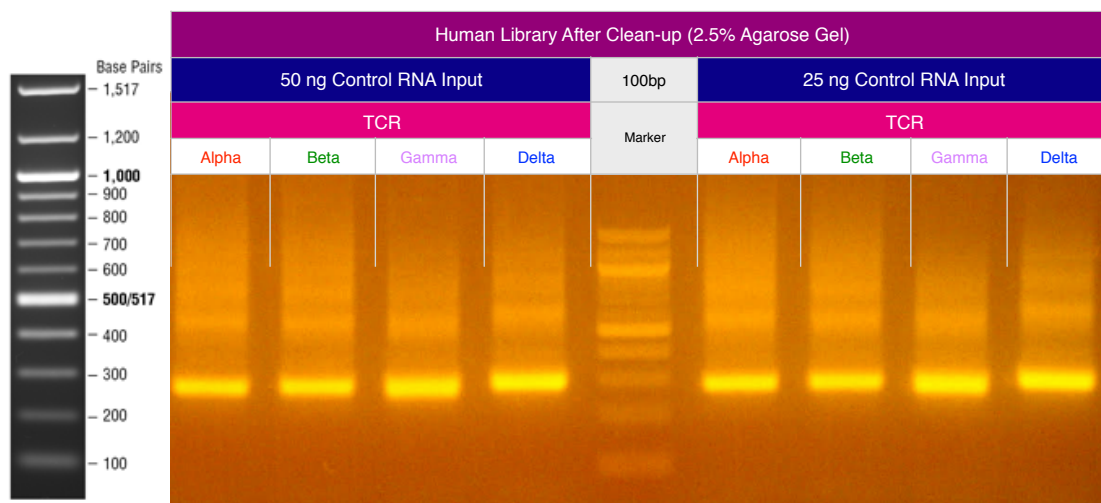


Human Long Read	Amplicon Size Range (bp)	Mouse Long Read	Amplicon Size Range (bp)
HTAI-M	475-522	MTAI-M	380-550
HTBI-M	500-540	MTBI-M	380-550
HTDI-M	470-545	MTDI-M	380-550
HTGI-M	470-545	MTGI-M	380-550
HBHI-M	490-570	MBHI-M	380-550
HBKLI-M	470-520	MBKLI-M	380-550

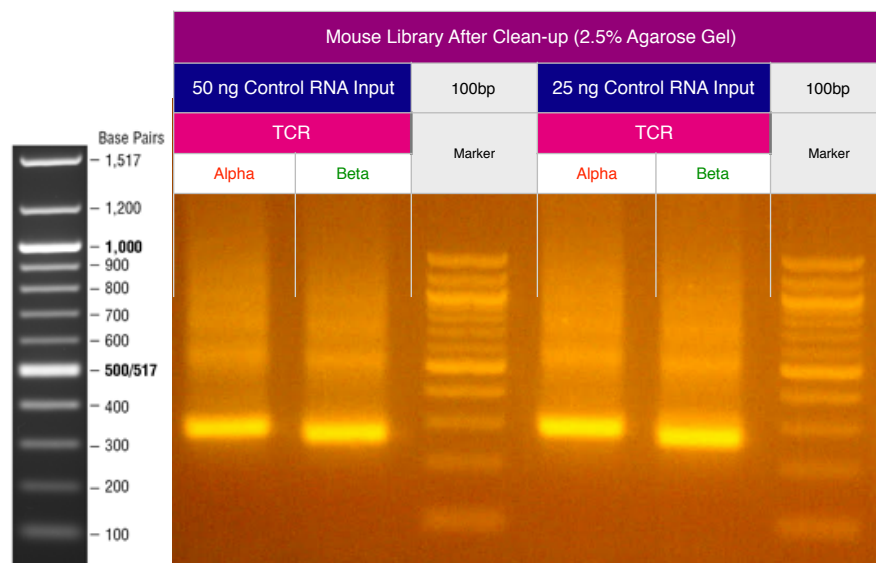
Amplicon Library Size: Short Read Systems (100/150) PER

Each amplified repertoire represents a large number of sequences distributed over a size range. The gel images and tables below can be used as a guide indicating the range of potential product sizes.

Human



Mouse



Human Short Read	Amplicon Size Range (bp)
HTAlvc	222-276
HTBlvc	241-280
HTGlvc	212-280
HTDlvc	212-280

Mouse Short Read	Amplicon Size Range (bp)
MTAlvc	200-310
MTBlvc	200-290

Pooling Libraries

The end-product of amplification with iRepertoire's reagent systems is a library of immune specific sequences that were produced using a RNA/gDNA sample. We use the distinction between the term "sample" (the starting material) and "library" (end-product of amplification) in order to reduce confusion. This is useful since one sample can produce different libraries such as a TCR-beta, TCR-alpha, Ig-heavy, etc., depending upon the reagent system utilized during amplification.

Pooling libraries into one Illumina flow cell reduces the overall per library sequencing cost. Our software pipeline, iRweb, recognizes the barcodes and decodes them automatically during analysis. Please remember that in order to pool libraries for a sequencing flow cell, each sample must be amplified with a unique barcode combination prior to pooling so that the software, iRweb, can detect and distinguish the libraries during data analysis. An exception to this rule is when different chains are pooled together. In this case, the same barcode can be utilized, and the software will de-multiplex by chain.

The decision on how to pool the samples is completely yours to make. Consider the input cell count of the sample prior to deciding a pooling strategy. You can add less library for those samples with lower cell counts and pool more of libraries corresponding to higher cell counts in order to adjust the read throughput to your needs. You may also want each sample to be evenly represented. If so, pool equal amounts of mass from each library into a single tube to meet the pooled library requirements of your sequencing vendor. (If amplifying the same chain, the amplicon size will be almost equivalent from library to library. Pooling by mass in this case is the same as pooling equimolar amounts. If there is a significant difference in amplicon size from library to library, you should pool equimolar amounts to achieve even read depth.) An example pooling sample sheet for pooling by mass is provided on the following page.

iRepertoire libraries are considered low diversity. It is highly recommended to perform a Phi-X spike-in as discussed in the section "NGS with Illumina Platforms." Furthermore, it is recommended to pool at least 4 different molecular IDs or barcodes per flow cell. This can improve run metrics and per library read throughput.

If you decide to perform sequencing with iRepertoire, we require 1 µg of pooled library at a concentration of 20 ng/µL. Less is acceptable, but the additional requested amount is advisable in case a repeat is necessary. Libraries should be submitted as a single pool for each flow cell with the outer box appropriately labeled indicating amplicon library. Additional fees apply for pooling individual libraries and sequencing services. Please refer to your sequencing vendor for their requirements. If the resulting pooled library does not meet concentration minimums, the pooled library can be concentrated by re-purifying the library over a single PCR purification column and eluting in a smaller volume. Alternatively, the pooled library can be concentrated by bead selection and re-elution in a lower volume, or precipitated and reconstituted in a lower volume.

Example Pooling Libraries Table for diHTBlvc

Sample Name	Panel	External Barcode	Internal Barcode	Sample Type	[cDNA] ng/ μ L	A260/280	Volume Pooled (μ L)	Total cDNA pooled (ng)
Sample 1	diHTBlvc	D01	1	Total RNA	28.33	1.88	5.29	150.00
Sample 2	diHTBlvc	D02	1	Total RNA	27.37	1.75	5.48	150.00
Sample 3	diHTBlvc	D03	1	Total RNA	29.09	1.88	5.16	150.00
Sample 4	diHTBlvc	D04	1	Total RNA	30.72	1.83	4.88	150.00
Sample 5	diHTBlvc	D05	1	Total RNA	29.12	1.86	5.15	150.00
Sample 6	diHTBlvc	D06	1	Total RNA	30.07	1.83	4.99	150.00
Sample 7	diHTBlvc	D07	1	Total RNA	33.29	1.93	4.51	150.00
Sample 8	diHTBlvc	D08	1	Total RNA	31.11	1.8	4.82	150.00
Sample 9	diHTBlvc	D09	1	Total RNA	39.38	2.02	3.81	150.00
Sample 10	diHTBlvc	D10	1	Total RNA	26.39	1.88	5.68	150.00

Total ng	1,500.00
Total Volume	49.77
Theoretical Concentration	30.14
Actual Concentration	29.60
A260/280	2.03

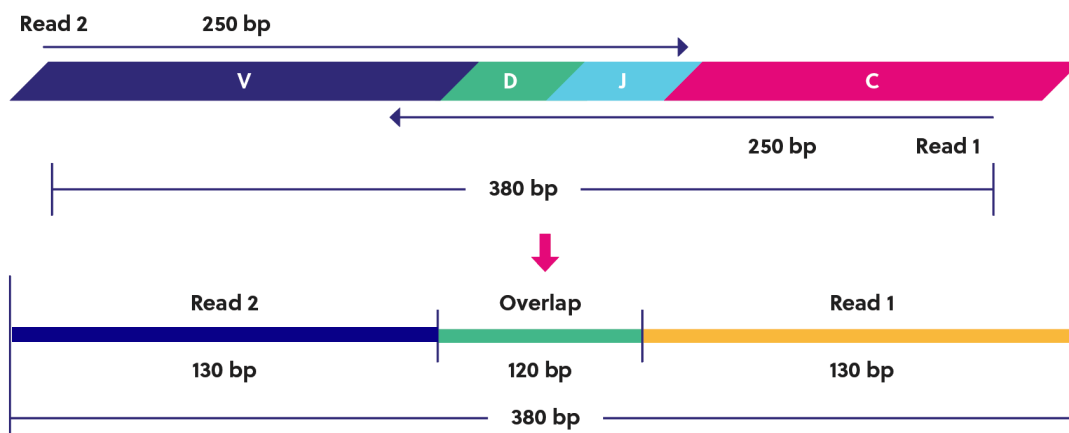
Blank Pooling Libraries Table

Sample Name	Panel	External Barcode	Internal Barcode	Sample Type	[cDNA] ng/μL	A260/280	Volume Pooled (μL)	Total cDNA pooled (ng)
								150.00
								150.00
								150.00
								150.00
								150.00
								150.00
								150.00
								150.00
								150.00
Total ng (10 Libraries)								1,500.00
Total Volume								
Theoretical Concentration								
Actual Concentration								
A260/280								

Data Structure

iRepertoire offers two different types of library preparations: RepSeq, based on **amplicon rescue multiplex PCR (arm-PCR)**, and RepSeq+, based on **dimer avoided multiplex PCR (dam-PCR)**. RepSeq+ has the option of including **universal molecular identifiers (UMI)**. Primer designs for both systems are built around the paired-end or single-end reads available with the Illumina iSeq, MiniSeq, MiSeq, NextSeq, and NovaSeq platforms. The term “paired-end read” or PER refers to the reading of both the forward and reverse template strands of the same receptor sequence during sequencing. The overall read length of the sequence can be increased by using the sequence read from both strands (with some overlap between both reads to increase confidence in the paired-read). We call this process **read stitching**. Data can also be prepared as single-end read. In this case, the data for a strand of DNA is read from Read 1 uni-directionally from the C-region into the V-region. Data for FR1 and CDR1 are lost but coverage of the CDR3 and into CDR2 is provided. Since Read 1 starts on the anti-sense strand, the read includes the UMI portion (if applicable-RepSeq+ only), the sample barcode, a portion of the C-gene, J, D, and V. If the sequencer fails to sequence Read 2, typically CDR3 information can still be obtained from Read 1.

In PER mode, the software pipeline first demultiplexes sequencing data based on molecular barcodes and then stitches Read 1 and Read 2 in order to extend the sequencing coverage of the receptor sequence. The image below demonstrates read stitching for our human long-read primers. The stitching process is similar on the short read systems; however, the insert is approximately 150 bp, not 380 bp. For human, the average MiSeq amplicon length is 500 bp including adaptors. Subtract approximately 120 bp for the adaptors, and the insert is on average 380 bp. Remember that this value varies depending upon receptor editing.



NGS with Illumina Platforms

Sequencing Recommendations

1. Although this manual is written with instructions specifically for the Illumina MiSeq or NextSeq, libraries generated are compatible with all Illumina platforms, including iSeq, MiniSeq, MiSeq, NextSeq and NovaSeq. The sequencing kit recommended is determined based on the amplicon read length of the system, either long or short, and the desired coverage of the targeted sequence.
2. After the size-selection procedure, the purified product should go through cluster generation directly without further amplification as the full-length Illumina pair-end sequencing adaptors are already associated with the VDJ sequences. We do recommend the QC steps listed below prior to sequencing.
3. Libraries can be sequenced with paired-end reads (PER; for example, 250 cycles x 250 cycles) to cover the entire VDJ, or optionally, single end read (SER) from read 1 (for example, 500 or 600 cycles for long amplicon products), which will cover a portion of the VDJ including the CDR3 region. Please note, sequencing quality does drop towards the ends of the reads. *For brevity, this manual demonstrates single end read 300 cycles and paired-end read 250 x 250.* For short read systems, a 300 cycle kit may be used with paired end reads of 150 x 150 or as single end read 300 cycles.
4. **For SER, we have found that 300-cycle sequencing kits can also be used to sequence the longer amplicon products on the NextSeq utilizing 300-cycles from Read 1.** This approach does not capture the entire targeted region but does capture from the end of FR2, CDR2, FR3, CDR3, FR4 and the beginning of the C-region providing coverage of the CDR3 region and a large enough portion of the V-gene for accurate assignment.
5. As for coverage, we recommend 5 reads for each cell so that, theoretically, every cell will be sequenced according to the Poisson model. For instance, if your sample contains about 1 million T cells, we recommend you allocate about 5 million reads for this sample. Alternatively, libraries can be sequenced at survey level by pooling more libraries per flow cell. For lower cell count samples, it is often possible to “fit” more libraries in a flow cell since the read depth needs are less. For higher cell count samples, it is also possible to select a fixed depth per library and chain. We recommend at least 1 million reads *each* for TCR alpha, beta, and IgH chains and at least 500K reads *each* for TCR delta, gamma, IgK and IgL (assuming the source of the cells is whole blood and not enriched for a specific cell type). If incorporating replicates, we recommend that they be treated as independent libraries and sequenced at the aforementioned recommended depths.
6. To obtain as estimated final mapped read output per flow cell, please see the sequencing table on the next page. The flow cell read output can be divided by the planned number of libraries to estimate the reads each library will achieve post-analysis. For instance, pooling 10 libraries on a v2-500 cycle kit will yield approximately 800K-1 million mapped sequences per sample.
7. **Prior to processing the samples on the Illumina platform, it is recommended to spike in 10-15% of PhiX (sold by Illumina) per flow cell to improve the efficiency of the run.** iRepertoire libraries are considered low diversity. If PhiX is not available, or you would like another option, then it is possible to spike in another library that is a whole genome or exome that has high diversity. It is not recommended to use any RNAseq products.
8. Our software pipeline recognizes the barcodes and decodes them automatically during analysis. Barcode information is available under the Guides tab in the online [Learning Center at https://irepertoire.com/learning-center/](https://irepertoire.com/learning-center/).

NGS with Illumina Platforms

Sequencing Kits and Read Depths

	ITEM	MAX PAIRED END READ (PER)	MAX SINGLE END READ (SER)	ESTIMATED READS POST-FILTER	NOTES
Illumina MiSeq	Nano kit	250	500	1 million	Generally used with iPair / iPair+ projects, cell line sequencing, or for bulk samples that have very few cells
	V2-300 Cycle kit	150	300	10 million	For use with long read primers as SER, or short read primers as SER or PER
	V2-500 Cycle kit	250	500	10 million	For use with long or short read primer systems
	V2-600 Cycle kit	300	600	13 million	For use with long or short read primer systems
Illumina NextSeq	Mid-throughput	150	300	100 million	For use with long or short read primers as SER, or short read primers as SER or PER
	550 High Output	150	300	300 million	For use with long or short read primers as SER, or short read primers as SER or PER
Illumina NovaSeq	SP-500 Cycle Kit	250	500	500 million	For use with long or short read primer systems

Pre-Sequencing QC

Once the libraries have been pooled into one tube, the final pooled library is ready for pre-sequencing QC. We highly recommend qPCR using the Illumina P5 and P7 partial adaptors on a 10 nM dilution to independently validate concentration and determine the final concentration for sequencing. There are several commercial kits available with DNA standards to achieve this such as the KAPA qPCR library quantification kit or Meridian Biosciences JetSeq NGS kit. This step provides accurate concentration information in order to achieve an ideal cluster density on the sequencing flow cell since it mimics how the library will interact with the flow cell. From this information, you can perform the PhiX spike-in, dilution, and load onto the Illumina flow cell. Refer to the Illumina manual associated with your instrument for detailed information regarding sequencing library preparation and recommended loading concentrations as these vary depending upon the flow cell utilized.

Sequencing

Sequencing with MiSeq/NextSeq Control Software

Local Run Manager Wizard Workflow Parameters:

NOTE: THESE INSTRUCTIONS APPLY TO LIBRARIES WITH ILLUMINA DUAL INDEX ADAPTORS ON THE END LIBRARY (iR-COMPLETE LIBRARIES ONLY). These instructions do NOT apply to legacy iR-Profile kits or legacy bulk primer kits. For detailed instructions on running the MiSeq or NextSeq, please see the Illumina manual associated with the device. There will be minor differences between different sequencer control software and software versions.

To run an iRepertoire generated library on the MiSeq or NextSeq, follow the set-up detailed below. In the MiSeq Control Software or the NextSeq Control Software Welcome Page, select experiment followed by manual run setup. See Figure 1 and Figure 2 for example set ups.

Under Run Setup:

1. Enter the Run Name and Library ID. It is recommended that unique and identifiable values are given.
2. For Read Type, choose either Single Read or Paired End
3. If Read Type Single Read was chosen:
 1. The Read Length for Read 1 is 301
 2. The Read Length for Read 2 is 0
4. If Read Type Paired End was chosen:
 1. The Read Length for Read 1 is 251
 2. The Read Length for Read 2 is 251
5. The Read Length for both Index 1 and Index 2 is 8
6. Ensure an Output Folder is selected to save the run data to.
7. Optionally add a Sample Sheet. A Sample Sheet can be created from either Illumina Experiment Manager or Local Run Manager.

Sequencing

Figure 1: Example of how to set up a single-end read run in NextSeq Control Software

Run Name *	Example			Library ID	XXXX-ID-XXXX		
Read Type	<input checked="" type="radio"/> Single Read			<input type="radio"/> Paired End			
Read Length *	Read 1	Index 1	Index 2	Read 2			
	301	8	8	0			
Custom Primers	<input type="checkbox"/> Read 1	<input type="checkbox"/> Index 1	<input type="checkbox"/> Index 2	<input type="checkbox"/> Read 2			
Output Folder *	D:\Output\						Browse
Sample Sheet							Browse
	<input checked="" type="checkbox"/> Purge consumables for this run						

Figure 2: Example of how to set up a paired-end read run in NextSeq Control Software

Run Name *	Example			Library ID	XXXX-ID-XXXX		
Read Type	<input type="radio"/> Single Read			<input checked="" type="radio"/> Paired End			
Read Length *	Read 1	Index 1	Index 2	Read 2			
	251	8	8	251			
Custom Primers	<input type="checkbox"/> Read 1	<input type="checkbox"/> Index 1	<input type="checkbox"/> Index 2	<input type="checkbox"/> Read 2			
Output Folder *	D:\Output\						Browse
Sample Sheet							Browse
	<input checked="" type="checkbox"/> Purge consumables for this run						



Table: "D" or "A" PCR2 Mix Label and Corresponding Illumina Dual Index Pair

D#/A# Dual Index Label	Index 1 (i7)	Index 1 (i7) Sequence	Index 2 (i5)	MiSeq Index 2 (i5) Sequence	NextSeq Index 2 (i5) Sequence
D01	D701	ATTACTCG	D501	TATAGCCT	AGGCTATA
D02	D702	TCCGGAGA	D502	ATAGAGGC	GCCTCTAT
D03	D703	CGCTCATT	D503	CCTATCCT	AGGATAGG
D04	D704	GAGATTCC	D504	GGCTCTGA	TCAGAGCC
D05	D705	ATTCAGAA	D505	AGGCGAAG	CTTCGCCT
D06	D706	GAATTCGT	D506	TAATCTTA	TAAGATTA
D07	D707	CTGAAGCT	D507	CAGGACGT	ACGTCCTG
D08	D708	TAATGCGC	D508	GTA CTGAC	GTCAGTAC
D09	D709	CGGCTATG	D509	TTCGGATG	CATCCGAA
D10	D710	TCCGCGAA	D510	ACTCATAA	TTATGAGT
D11	D711	TCTCGCGC	D511	GCGCCTCT	AGAGGCGC
D12	D712	AGCGATAG	D512	CGCGGCTA	TAGCCGCG
D13	D713	GAATAATC	D513	TTATTCGT	ACGAATAA
D14	D714	ATGCGGCT	D514	CCTACGAA	TTCGTAGG
D15	D715	TTAATCAG	D515	AGCAGATC	GATCTGCT
D16	D716	ACTGCTTA	D516	GCGGAGCG	CGCTCCGC
A01	A701	ATCACGAC	A501	TGAACCTT	AAGGTTCA
A02	A702	ACAGTGGT	A502	TGCTAAGT	ACTTAGCA
A03	A703	CAGATCCA	A503	TGTTCTCT	AGAGAACA
A04	A704	ACAAACGG	A504	TAAGACAC	GTGTCTTA
A05	A705	ACCCAGCA	A505	CTAATCGA	TCGATTAG
A06	A706	AACCCCTC	A506	CTAGAACA	TGTTCTAG
A07	A707	CCCAACCT	A507	TAAGTTCC	GGAACCTA
A08	A708	CACCACAC	A508	TAGACCTA	TAGGTCTA



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Data Analysis

Data Submission Form

Customers using our primers in their experiment will be provided with complimentary data analysis service if sequencing on an Illumina iSeq, MiniSeq, or MiSeq. Please note for larger output platforms, such as NextSeq, HiSeq, and NovaSeq, data processing fees apply as detailed in our Data Management Policy (provided separately). A completed data submission form is required for data processing. A data submission form can be submitted through our website at <https://irepertoire.com/data-submissions/>. One data submission form is required for each flow cell of data.

Please note, if multiple libraries with different chains (TRB, TRA, IgH, etc.) are generated for the same sample, multiple entries of the same sample name (with the chain id) should be placed on the submission form so that the data for the different libraries will be displayed under one sample name on iRweb.

Data Submission Methods

There are multiple methods to submit data to iRepertoire for analysis. The preferred method is to share the Project with info@irepertoire.com through Illumina's BaseSpace website. BaseSpace is a cloud-based solution provided by Illumina, which facilitates the rapid sharing of project NGS data with ease. Alternatively, sequence data, in the form of fastq files, can be sent to iRepertoire via secure ftp on our company-managed server. For a sftp account, please contact customer support at info@irepertoire.com. Sequence data can also be shipped with a portable hard disk* or an online file sharing method such as Google Drive or Dropbox. We suggest users backup their data before sending it to us.

If shipping a hard drive, please ship to:

iRepertoire Inc.
Attn: Data Team
800 Hudson Way, Suite 2304
Huntsville, AL 35806

*Shipping fees apply if return shipment is required.

Basic Data Analysis

Barcode de-multiplexing and filtering
V, D, J, C mapping
CDRs identification
Analysis results rendered through web platform
V-J combination distribution (2-D, 3-D map)
Listing CDR3 peptides
CDR3 algebra, compare repertoires and identify shared CDR3s
Diversity index calculation (D50)
Displaying V, D, J and C mapping
Distributions including:
 N-addition (normalized and non-normalized)
 CDR3 length (normalized and non-normalized)
 V, J nibbling (normalized and non-normalized)
 V, J usage (normalized and non-normalized)



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Please visit the demo site to experience our software pipeline.

Visit this link: <https://irweb.irepertoire.com/nir/> then, use “demo” for user ID, and “12345” as the password. Follow the navigation on the left to see the features of the iRweb software. For a walkthrough, please visit the [Learning Center](#) on www.irepertoire.com and download the [iRweb: Data Analysis Guide](#).

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