

iRepertoire **Bulk** Reagent Universal User Manual

Constructing an Immune Repertoire Library for Next-Gen Sequencing with Illumina platforms



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Version Updates

Update Classification	Update Description	Version
Major	Recommended DNA polymerase for PCR2 changed.	
Major	Incubation time of the beads was changed from 5 minutes to 3 minutes.	
Moderate	Updated PCR protocols to include hold at 4 °C after cycling.	
Minor	Updated protocols to indicate appropriate stopping points.	
Minor	Added "12 times" to the description of EtOH wash in selection protocol.	
Minor	Updated styling	V20200810
Minor	Updated graphics	V20200818

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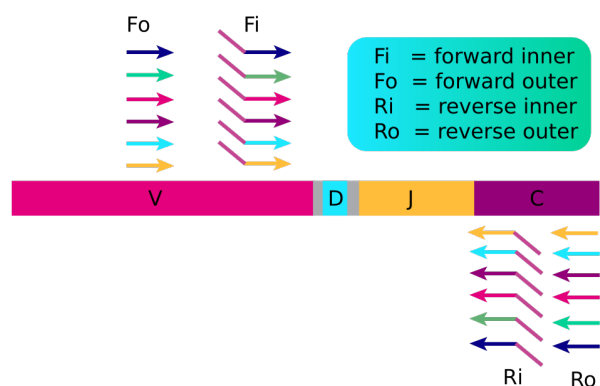
Introduction

Please read this handbook carefully before beginning your experiment. The “Pooling Samples,” “Template Selection,” and “General Information” sections are particularly important to read and understand before beginning your sample preparation. This manual provides instructions for amplifying a TCR and/or BCR immunorepertoire library compatible with Illumina Next-Gen Sequencing Platforms (HiSeq 2000, HiSeq 2500, MiniSeq and MiSeq). This manual is compatible with all iRepertoire Bulk Reagent Systems (not applicable to iR-cassettes or iR-Profile kits). Please see “Package Contents and Storage” for more information about the iRepertoire Bulk Reagent Systems.

The arm-PCR Technology

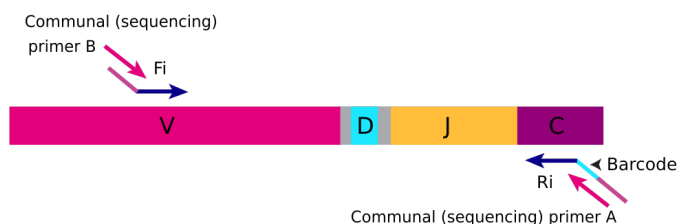
The arm-PCR (amplicon-rescued multiplex PCR) technology is a patented multiplex amplification strategy (Patent No. 7,999,092) that utilizes a two-step reaction to amplify an immune repertoire, inclusively and semi-quantitatively.

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 universal (sequencing) primers are used for the exponential phase of amplification using the PCR1 products as a template.

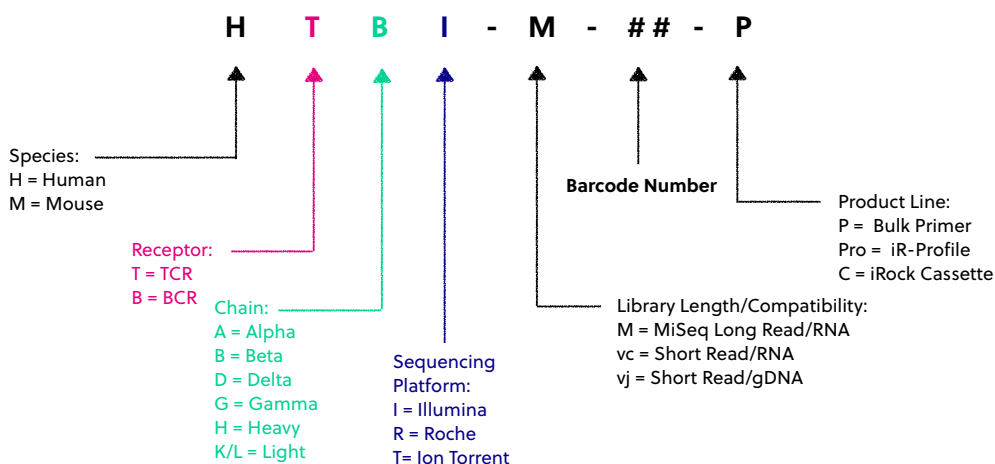
During the first round of PCR, nested gene-specific primers targeting each of the V and C genes are used. The forward primers Fo (forward-out) and Fi (forward-in) are located in the V genes. The reverse primers, Ro (reverse-out) and Ri (reverse-in), are located in each of the C genes. The Fi and Ri primers also include sequencing adaptors B and A, respectively, for the Illumina platforms (HiSeq and MiSeq) for paired-end sequencing. The Ri primers also include barcodes in between the sequencing primer A and the C gene specific primers. The second round of PCR is carried out using communal (sequencing) primers B and A. After gel purification or magnetic bead clean-up, the resulting product is ready for high-throughput sequencing with the Illumina MiSeq platform. No additional enzymatic steps are required. The first round of PCR introduces barcodes and sequencing primers into the PCR products. The exponential phase of the amplification is achieved by the communal primers in the second round of PCR; therefore, the entire repertoire is amplified evenly and semi-quantitatively, without introducing additional amplification bias.

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, PCR1 Rescue and PCR2 Set-up, PCR2 Thermocycler Conditions, Library Clean-up, Assessing Library Generation, Pooling Libraries, NGS with the Illumina MiSeq, and Data Analysis. Alternative protocols for sample processing are provided under each of these general categories. *Please note separate thermocycling conditions are provided for TCR and BCR amplification separately.* Prior to beginning the major steps, please read the “**General Information**” carefully.

Understanding iRepertoire’s reagent system naming scheme

For PCR1, the barcoded PCR1 bulk primers contain a very specific label, which conforms to iRepertoire’s naming scheme. These bulk reaction mixes contain a code similar to the one depicted above. This code contains important information such as the species, receptor, chain, sequencing platform, library length, the barcode number (MID), and the product line. The product line code for bulk primers is “P”. This is to distinguish this product line from our other products such as our iR-Profile kits or “Pro.” 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.



Multiplexing (pooling) libraries

This manual provides instructions for generating a library from ONE sample. The end-product of amplification with iRepertoire's reagent systems is a library of immune-T specific sequences that were produced using an RNA 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.

One **bulk** reagent catalog item includes enough reagents to produce 10 libraries (all of which will have the same barcode). However, to best utilize the Illumina sequencing capacity, multiple libraries, each with a different barcode, can be pooled together in one single HiSeq lane or MiSeq flow cell. The number of libraries added to the sequencing platform should be determined by the starting cell count and is limited by the number of barcodes offered for a specific catalog item.

To study multiple samples in the same sequencing run, you need to purchase **multiple** iRepertoire primer kits with **different** barcodes, amplify the samples **separately**, and pool the PCR products together before submitting the **pooled library** for sequencing. Our data analysis software will identify and differentiate the libraries based on the barcodes used during arm-PCR.

For example, if you want to study the human TCR beta repertoire of 200 samples, you can purchase iRepertoire products Catalog Numbers "HTBlvc-01-P" to "HTBlvc-20-P." Then, use each of the 20 kits to amplify the first 20 samples. After arm-PCR, the 20 PCR products can be pooled for one single HiSeq lane. The same 20 kits can be used again for the next 20 samples for the second HiSeq lane, etc. Currently, the Illumina HiSeq instrument can run two flow-cells in the same instrument run, totaling 16 lanes. Thus, if 20 barcodes are used for one lane, all 200 samples can be sequenced in a single HiSeq run. Because the libraries are considered low diversity, it is recommended that no more than 3 lanes of a flow cell be dedicated to iRepertoire libraries.

Of course, it is not necessary to pool as many as 20 libraries for one sequencing lane. The number of libraries pooled during a sequencing run depends upon the experimental design and the desired depth of sequencing coverage. In the previous example, one might decide to pool 10 libraries per lane to increase the sequencing depth. Please see the "Pooling Libraries" section for further information and suggestions regarding this topic.

Template Selection: RNA vs gDNA

Template Selection: RNA - the Preferred Template

About half of the papers published so far for immune repertoire sequencing use genomic DNA (gDNA) samples, while the other half use RNA. Although we have primers that work for both sample types (see below), we prefer RNA as a starting template, if possible.

The primary concern regarding the use of gDNA is inclusiveness. There is only a certain amount of template DNA that can be added to a PCR reaction. Each human cell may have approximately 6.6 pg of gDNA of which the rearranged VDJ represents a very small portion. If 100 ng of gDNA is used in the reaction, this represents roughly 16,000 cells only, resulting in a restricted view of the repertoire. Although more gDNA can be used, there is a limit to how much can be added. In contrast, the cDNA template created from iRepertoire's RNA reagent systems will be immune specific, maximizing the amount of desired template included in the reaction, and excluding unnecessary and potentially disruptive gDNA.

A second concern about using gDNA as a template for generating a sequencing library is background amplification. As the figure below demonstrates, after the VDJ rearrangement, the V and J segments not involved in the recombination will still be present in the genome and can serve as perfect binding sites for the primers. Binding at these sites will generate background amplifications, exhaust primers, and introduce bias.

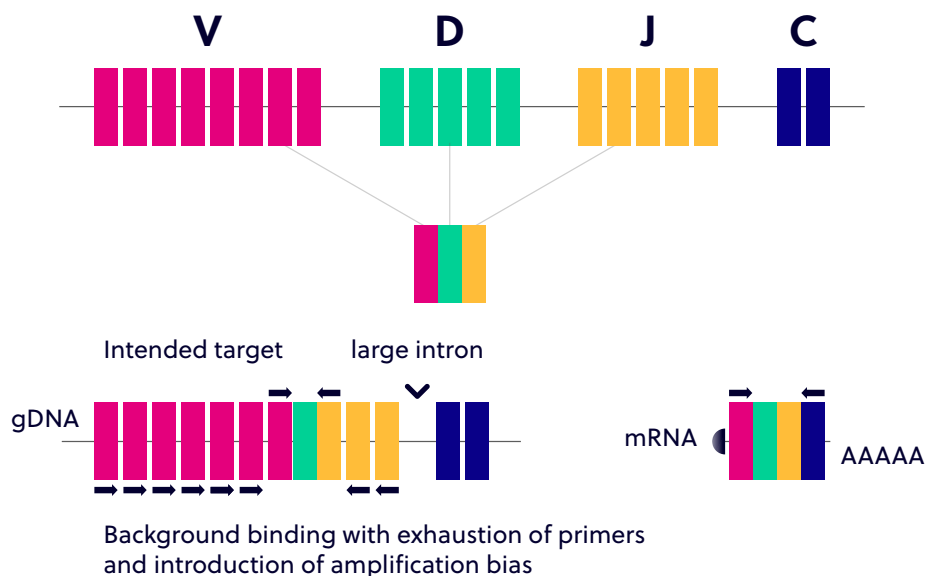


Figure 3. V(D)J rearrangement demonstrating the increased background that may occur due to the extraneous priming sites available within gDNA. This can generate background amplification, exhaust primers, and introduce bias.

Potential Reasons for Using gDNA

Those who prefer to use genomic DNA have a variety of valid reasons. Samples are much easier to obtain, and even biopsied samples from tissue or slides can be used. In addition, since each cell may only have one copy of the successfully rearranged VDJ, it may reflect the quantity of the repertoire better. In other words, identification of a successfully rearranged VDJ will not be skewed by expression levels as the relationship should be one cell to one VDJ rearrangement. However, one can also argue that the RNA expression level of the T and B cell receptors may reflect the functional status better.

So, gDNA or RNA? The answer really depends on your research goals. If the repertoire changes you are looking for will be represented by dominant clones, then, both gDNA and RNA will work. If, however, you need to see the broader diversity, RNA may be better. We are happy to offer primers for both types of template (see below).

Benefits of an Immune Specific cDNA Library Versus a Non-specific cDNA Library

We recommend using our primers to create cDNA rather than using poly dT primers or random hexamer primers for several reasons. By creating the cDNA using iRepertoire's Reagent Systems, the cDNA library will have an increased concentration of immune specific cDNA relative to a library produced from catch-all primers. These non-specific cDNA libraries will include cDNA sequences from any expressed protein in the cell, reducing the amount of immune specific cDNA template available for amplification. Furthermore, the creation of a robust cDNA library using non-specific primers requires a lot of input RNA. We can reduce the amount of input RNA down to a recommended 100 ng (however, less may also work) because the cDNA library produced will be immune specific.

Template Choice Directly Affects Primer Selection

All of our reagent systems work with RNA, including any products indicating "V-C" genes and the long read "-M" primer systems; however, due to an intron between J and C genes, only V-J primers should be used with genomic DNA (see the table below). We offer V-J primers covering the short read human TCR beta locus (HTB1vj) for use with the Illumina platforms (GA IIx, HiSeq2000, and MiSeq). For B-cell studies, we only offer long read primers "-M" (500+ cycles, 250 paired-end reads) in order to achieve more complete coverage of the V(D)J rearrangement.

Primer and Template Compatibility

Template	V-J Primers	V-C Primers
RNA	✓	✓
gDNA	✓	Do not use

Package Contents & Storage

Shipping & Storage

Upon receipt, store components as follows:

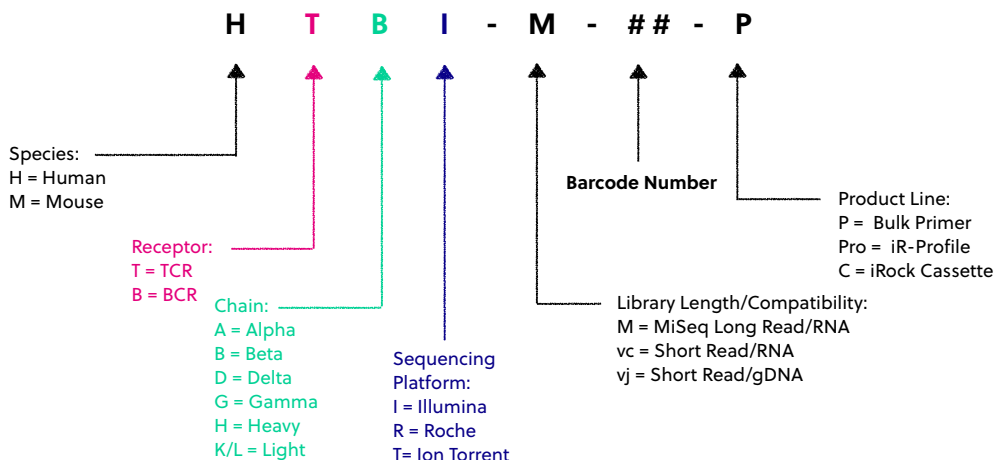
- Store iR Primers & Illumina Communal Primers at 2-8°C.
- Primers should be stored in a clean area designated only for PCR Setup.
- Optionally, primers can also be stored at -20°C.
- Ensure that the cap is secured tightly to reduce evaporation.

Package Contents

Each bulk primer catalog item (each barcode) includes two primer tubes, each for one round of PCR (PCR1 and PCR2). The first PCR reaction (PCR1) will utilize the tube labeled with a molecular ID (also referred to as a barcode) such as HTBlvc-X-P or HBHI-M-X-P (depending on the reagent system purchased). The "X" indicates the molecular ID. The second PCR reaction (PCR2) is referred to as the "Communal Primers." Each catalog item can generate libraries for 10 samples with the same barcode.

Tube Labels

PCR1: The primer mix used in PCR1 has a cap that is labeled with the reagent system as depicted below. On the tube label, three important pieces of information are included: 1) the iR-code (for example, HTBl-M) ; 2) vc or vj (if you do not see vc or vj, then the primer kit is a vc system), and 3) the barcode number. Please remember to record the barcode number together with the sample identity if working with more than one sample (see "The Sample Designation Sheet").



PCR2: The second PCR utilizes primers that are compatible with all barcodes. They are also referred to as Communal Primers. The cap is labeled "Illumina." The tube has a label indicating sequencing platform compatibility.

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



Materials & Reagents Not Provided

- Qiagen One-Step RT-PCR Kit, Cat No. 210212
- Promega GoTaq G2 Hot Start Colorless Master Mix, Cat. No. M7432 or M7433
- Qiagen QIAquick gel extraction kit, Cat No. 28704
- Beckman Coulter SPRIselect Magnetic Beads, Cat No. B23317
- RNase Inhibitor, Promega Cat No. N2115
- Thermocycler (recommended: Biorad C1000)
- Vortex
- Cold Block or Ice
- Mini-centrifuge/Mini-spin
- Molecular biology grade Ethanol
- Molecular biology grade nuclease-free water
- Reagent reservoirs (recommended: ThermoScientific 8096)
- E-Gel agarose gel electrophoresis system, Invitrogen by Life Technologies
- E-Gel 2% General Purpose Agarose 18 Pak, Invitrogen G5018-02
- Pipettes (Multi-channel pipette (200 µL) optional)
- iR-Magnetic Strip Stand (Sold Separately)
- Filter Tips
- 8-tube 0.2 mL PCR strips (recommended: Eppendorf 951010022)
- Microfuge tubes
- Timer
- Molecular Bioproducts DNA Away
- Molecular Bioproducts RNA Away
- Personal Protective Equipment (gloves, lab coats, etc)

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.
- RNA is extremely sensitive to degradation. Ensure that RNA is stored at -80 °C prior to use and maintained at 4 °C during template addition. Once thawed, gently flick RNA sample and agitate by pipetting to mix. Make sure RNA is well mixed prior to adding to the PCR tube. Avoid excessive freeze-thawing of RNA samples to prevent degradation.
- ***Between 50 ng (minimum) and 1000 ng (maximum) of template RNA is recommended per 25 uL 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 10 ng 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.

RT-PCR / PCR Handling

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

The use of a hood equipped with a UV light is recommended for 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. After PCR1, all cDNA 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 PCR1 rescue and final library clean-up). Designating separate hoods and equipment for 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 SPRIselect beads required for downstream processing.

Mixed-Receptor Amplification

Please note that there are separate PCR1 protocols for T-cell and B-cell receptor amplifications. This should be considered when designing and setting up experiments, since two separate thermocycler conditions may be required depending upon the reactions ordered. In this case, use of separate PCR strips for each receptor type (BCR or TCR) during set up is necessary. PCR2 protocols are the same for each receptor type. It is recommended to program the thermocycler prior to beginning sample set-up.

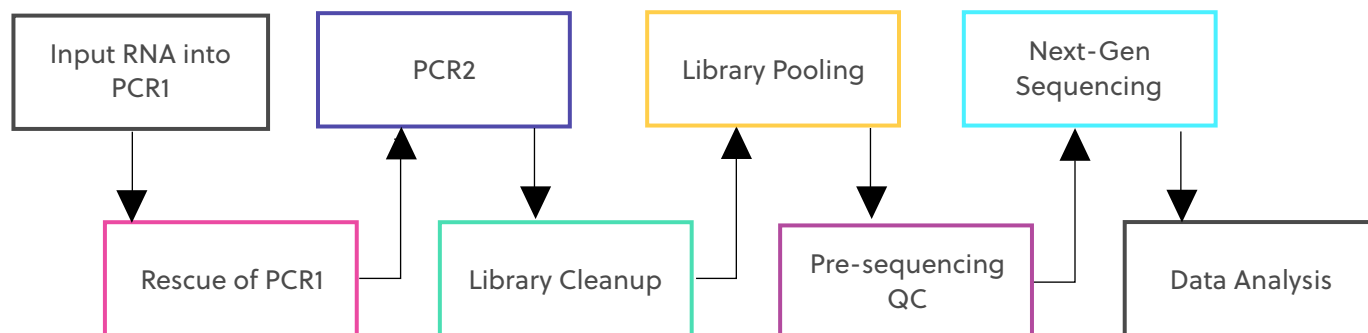
PCR2 Clean-up/Gel Extraction

For a better sequencing result, remove primer-dimers by using the protocol provided for library clean-up, or alternatively, gel extracting the target band of desired size from the agarose gel. Gel extraction is the preferred method if the input is from a low cell count sample. In all cases, it is recommended to run a portion of the library on either an agarose gel or Bioanalyzer in order to assess the amplification of the primary band relative to residual primer-dimers. **To avoid cross-contamination, do not handle PCR products from different templates with the same barcode at the same time.**

Minimum Cell Counts

The library can be made from as few as 100 sorted cells. However, to prevent RNA degradation and loss during extraction, carrier RNA (such as yeast tRNA) should be added into the sample **before** RNA extraction. If carrier RNA is used (typically with 500 cells or less), an accurate RNA concentration of the original sample will be difficult to assess. Therefore, for samples with low cell counts, we recommend that you elute the RNA in a lower volume in order to increase the concentration prior to adding it to the PCR1 reaction. A PCR protocol for low abundant samples is provided.

Outline of the Procedure





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 cycler 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 and PCR1 Master Mix Preparation

1. Design your experiment and pre-assign barcodes to specific samples by completing Table 1, "Sample Designation Sheet."
2. Complete Table 2, "Qiagen OneStep 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. 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 7.25 μ L of the Master Mix (MM), 4 μ L barcoded PCR1 primers, and 13.75 μ L template-water mix.
5. The Master Mix (MM) preparation should be performed in a separate area/room from the template addition.

Table 1 Sample Designation Sheet

Sample Number	Sample Name	Barcode (MID)	Input [RNA] ng/μL	Desired RNA Amount (ng)	RNA template amount (μL) max: 13.75 μL		RNA Template + H ₂ O (μL)
						H ₂ O (μL)	
Example	NEC-01	HTBI-M-01-P	126.2	500	3.96	9.79	13.75
Example	NEC-02	HTBI-M-02-P	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	N/A	0	0	0	13.75	13.75

Table 2 Qiagen OneStep 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 primers, water, and template in the master mix*. Uniquely barcoded primers will need to be added to each sample reaction separately if samples are to be pooled for the same sequencing run (see “Multiplexing or Pooling Libraries” for more information). Add 10-15% extra of each component to the master mix to account for pipetting losses (“PCR1 Master Mix” example is 15% additional).

Reagent	Volume (μL) / 25 μL rxn	A Sample Reaction
Nuclease-free H ₂ O*	X μL	11.75
5X Buffer	5	5
dNTP Mix	1	1
RNasin (40U/μL)	0.25	0.25
Enzyme Mix	1	1
iRepertoire Primers	4	4
Template**	Y μL	2
Total	25.00	25

PCR1 Master Mix (Example)	Amount (μL)	Master Mix (μL)	No. Rxns
5X Buffer	5	69	12
dNTP Mix	1	13.8	12
RNasin (40U/μL)	0.25	3.45	12
Enzyme Mix	1	13.8	12
Primer Mix	Add Separately		
Template	Add Separately		
Total Rxn Vol. (μL)	7.25	Total Master (μL)	100.05
		For each tube (μL)	7.25

*Amount of H₂O 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 1-13.75 μL for each 25 μL reaction. Between 50-1000 ng of template RNA or gDNA (V-J primer systems only) is recommended. Adjust the volume of nuclease-free H₂O 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 PCR1 master mix preparation.



RT-PCR1 Master Mix Preparation Procedure

1. In a clean area, remove the Qiagen OneStep RT-PCR Kit from the -20 °C storage and place on PCR cooler/ice. Gather the iRepertoire Primers for PCR1 from the 2-8 °C. This is a tube labeled with an iR-code and a molecular ID with the designation "-P."
2. **Once thawed, 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 the PCR strips appropriately to match sample ID and barcode on the "Sample Designation Sheet," and place PCR strips on a PCR cold block or ice to chill the tubes.
4. Prepare the PCR1 Master Mix (MM) by mixing the reagents in the order in which they appear in the example table. The MM does NOT include the water, primers, or template. *Make sure to adjust the calculations in the table for the number of reactions in your experimental design.*
5. Add 7.25 µL of the PCR1 MM to each of the labelled PCR tubes.
6. Carefully, add 4 µL of the iRepertoire PCR1 barcoded primers according to the Sample Designation Sheet. After each addition, gently cap the tube. **DO NOT** vortex or mix vigorously. ***The goal is to avoid barcoded primer on the lid of the PCR tube and thus reduce risk of sample cross-contamination.***
7. Bring this "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 PCR1 Master Mix is prepared. The PCR2 Master Mix can then be set aside at 4 °C until after PCR1 completion. This reduces retrograde laboratory flow during downstream processing. See page 21 for instructions regarding PCR2 Master Mix preparation.

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 NF-water to each PCR1 MM 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 .

Reagent	Volume (μL)	A Sample Reaction
Template**	X μL	8.75
Nuclease-free H_2O *	Y μL	5.00
Total	13.75	13.75

*Amount of H_2O 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 1-13.75 μL . Between 50 ng (minimum**) and 1000 ng (**maximum**) of template RNA is recommended per 25 μL reaction. Adjust the volume of nuclease-free H_2O 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 PCR1 protocol is suggested.



RT-PCR1 Set-Up

1. Retrieve the "PCR1 Barcoded MM" strip and **gently** open each cap. Align the "Template-Water" strip with the "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 "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 "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 Mini-spin.***
4. The final PCR1 reaction volume is 25 μ L. Keep all reactions on ice.
5. Proceed to "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 type 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). Please 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	10
72	3 min	
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	10
60	2 min	
72	45 sec	10
94	30 sec	
72	3 min	10
72	15 min	1
4	∞	Hold

Please note, all thermocycler conditions are suggested based on results from 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). Please 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	10
72	3 min	
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	10
63	2 min	
72	45 sec	10
94	30 sec	
72	3 min	10
72	15 min	1
4	∞	Hold

Please note, all thermocycler conditions are suggested based on results from 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

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

Recommended PCR2 Set-up

PCR2 Single Reaction Example

Multiplex Mix Single Rxn	Volume (μL) / 50 μL rxn
Nuclease-free H ₂ O	18
Promega G2 HotStart Colorless MM	25
Communal Primers	5
PCR 1 product**	2
Total	50

PCR2 Master Mix Example (with 15% additional)

Multiplex Mix Master Mix	Amount (μL)	Master Mix (μL)	Total Number Rxns
Nuclease-free H ₂ O	18	248.4	12
Promega G2 HotStart Colorless MM	25	345	12
Communal Primers	5	69	12
PCR1 product**	2		
Total V	50	Total Master Vol. (μL)	662.4
		Vol. per rxn (μL)	48

** PCR1 serves as the template for PCR2. PCR1 rescue may be performed with bead selection or transfer of PCR1 into PCR2. In either case, 2 μL may be dedicated in the master mix for this template addition. *To avoid contamination, apply the PCR1 template in a separate designated area.*



PCR2 Reaction Mix Set-up

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.

Note: The Communal Primers are NGS-platform specific (eg. Illumina or Ion Torrent) and are compatible with the PCR1 molecular IDs provided by iRepertoire (unless stated otherwise). Therefore, the PCR2 MM for more than one sample type (i.e., samples with different barcodes) can include the same Communal Primers, and the mix can be used for all of the PCR2 reactions.

2. In a clean area, remove the Promega GoTaq G2 Hot Start Master Mix from the -20 °C storage and place on PCR cooler/ice. Gather the iRepertoire Communal Primers for PCR2 from the 2-8 °C.
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.**
4. Mix each reagent in the order it appears in the table. Once the PCR2 MM is prepared, set aside at 4 °C until further instruction.

PCR1 Rescue & PCR2 Set-up

There are two options for PCR1 rescue. The first option is to rescue the entire PCR1 product using SPRIselect beads. This option is preferable since it improves both unique CDR3 discovery and overall library quality. The second method is PCR1 transfer. This method is faster and easier to perform, while still providing good unique CDR3 discovery. **For low input samples, "Option 1" is the recommended method.**

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.

PCR1 Rescue & PCR2 Set-up

Option 1: PCR1 Rescue Method using Beads & PCR2 Set-up

1. Prepare 10-mL of fresh 85% ethanol by mixing 8.5 mL of 100% ethanol with 1.5 mL of molecular biology grade water in a 15 mL conical vial. 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 sample 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. Invert the SPRIselect beads and vortex for at least 20 seconds. Ensure the beads are fully resuspended. These beads will be used to rescue PCR1.
NOTE: It is best to keep a stock bottle of SPRIselect beads in a clean area. Aliquot only what is required for a specific experiment, and bring the aliquot to the processing location.
4. Pipette 36 μL of the beads into the appropriately labeled positions on the *rescue strips*. Ensure the beads remain well resuspended by vortexing every fourth addition. Cap each position on the rescue strip after adding the beads.
5. When RT-PCR1 is complete, remove the RT-PCR1 strips from the thermocycler.
6. Wipe down the outside of the tubes with DNA Away prior to placing the strips on a PCR rack in the hood.
7. Change gloves prior to beginning the transfer.
8. **PROCESS ONE STRIP AT A TIME.**
9. First, carefully open the completed RT-PCR1 reaction strip.
10. Add **25 μL** of nuclease-free water to each reaction of the RT-PCR1 strip, bringing the volume of each reaction from 25 μL to 50 μL .
11. Open the *rescue strip* containing the beads.
12. Transfer **40 μL** of the RT-PCR1 product using a multi-channel pipette to the rescue strip and mix by pipetting up and down until there is uniform color throughout. **DO NOT CAP** the rescue strips. (The RT-PCR1 strip can be capped and discarded once transfer is complete. Alternatively, the RT-PCR1 strip can be capped and stored at -20°C as a precaution until all processing has been successfully completed. This remaining RT-PCR1 strip can be used as a template for transfer into PCR2 if necessary in the future.)
13. Incubate the beads with the RT-PCR1 product for 3 minutes at room temperature **with the caps open**.

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 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 **half (5 mL)** of 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 85% ethanol by pipetting up and down approximately 12 times.
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 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 strip.
8. Once dry, proceed to "PCR2 Set-up"

PCR2 Set-up

1. Retrieve the "PCR2 MM" from the 4 °C storage.
2. **Briefly, vortex and spin down (in a Mini-spin) the "PCR2 MM" tube.**
3. To the dried bead pellet, add 48 μ L of "PCR2 MM" to each tube and resuspend the beads. DISCARD the used pipette tip and use a new tip for the next tube. 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. The conditions for PCR2 are the same regardless the receptor-type but are dependent upon the type of master mix utilized during PCR2 set-up.



Option 2: Transfer Rescue Method & PCR2 Set-up

1. **Skip this option if option 1 was selected and proceed to PCR2 Thermocycler Conditions.**

2. Label a new set of PCR strips with sample IDs matching the RT-PCR1 strip.

NOTE: In order to facilitate use of a multi-channel pipette, set-up and label a fresh set of PCR strips that mimic the RT-PCR1 reaction strips.

3. Retrieve the "PCR2 MM" from the 4 °C.

4. **Briefly, vortex the "PCR2 MM" tube and spin down in a Mini-spin.**

5. To the new set of freshly labelled PCR strips, add 48 µL of "PCR2 Mix" to each tube, which will contain a sample.

6. When PCR1 is complete, remove the PCR1 strips from the thermocycler. **Briefly, vortex the PCR1 strips tube and spin down (in a Mini-spin) to ensure they are well mixed.**

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. First, open the freshly labeled PCR strip containing the "PCR2 MM".

10. Next, open the completed PCR1 reaction strip.

11. Transfer 2 µL of PCR1 product into the "PCR2 Mix" strip using a multi-channel pipette and mix by pipetting up and down. Cap the strip containing the PCR2 reaction mix.

12. **When the transfer is complete, ensure the PCR2 strips are tightly capped, vortexed, and spun down.**

13. The final PCR2 reaction volume is 50 µL.

14. Proceed to "PCR2 Thermocycler Conditions," and add the PCR2 strips to the thermocycler.

NOTE: The conditions for PCR2 are the same regardless the receptor-type but are dependent upon the type of master mix utilized during PCR2 set-up.

PCR2 Thermocycler Conditions

After PCR1, all cDNA libraries are associated with a unique barcode and contain a communal binding site. Program the thermocycler according to the parameters listed below for PCR2. The same PCR2 protocol can be used for both receptor types (BCR and TCR) and for both normal and low input samples.

PCR 2 Conditions

PCR2	Temp °C	Time
1	94	3 min
30	94	30 sec
	72	90 sec
1	72	15 min
1	4	∞

NOTE: A potential stopping point in the protocol is directly after the completion of PCR2. Libraries can be placed at -20 °C indefinitely. Final library clean-up can be performed once libraries are thawed.

Library Clean-up

Once PCR2 is complete, it is highly recommended to clean the library prior to pooling and sequencing in order to remove residual primer-dimers and the remaining enzyme in the mix. Gel purification or SPRIselect bead purification are two recommended methods for library clean-up prior to pooling. The choice of method is largely dependent upon the amplicon length, the template type, and user preference. The following are suggested guidelines:

- For short-read primer systems (~300 bp amplicon length), gel purification is the recommended method since it is difficult to select the primary band from the primer-dimer band (~160-180 bp). It is possible in some cases to perform sufficient clean-up by bead selection on short-read products, particularly when the input RNA template is of high quality and the RNA input is sufficient.
- For low input RNA samples or sample RNA from FFPE (for either the long-read or short-read systems), gel purification is the preferred method.
- For the long-read and high quality or normal RNA input, the bead clean-up method should be sufficient.
- In all cases, determination of the appropriate library clean-up must be determined empirically and is dependent upon sample input and quality.

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.***

Option 1: Magnetic Bead Library Clean-up

PCR2 Clean-up Mix Strip Preparation

1. Label a new set of PCR strips with sample IDs matching the PCR2 strips.
2. Invert the SPRIselect beads and vortex for at least 10-20 seconds. Ensure the beads are fully resuspended.
Important: The amount of beads used for clean-up depends upon the expected amplicon size— 41 μ L for Short Read (100/150 PER) or 33 μ L for Long Read (250/300 PER).
3. Pipette 41 μ L (for Short Read products) **OR** 33 μ L (for Long Read products) of the magnetic beads into the sample positions on the freshly labeled PCR strips. Ensure the beads remain well resuspended by vortexing every fourth addition. Cap each position on the strip after adding the beads. This strip is referred to as the “PCR2 Clean-up Mix Strip.”
4. Set aside and wait for PCR2 completion.



PCR2 Transfer to the Clean-up Mix Strip

1. When PCR2 is complete, open the freshly and appropriately labelled "PCR2 Clean-up Mix Strip."
2. Remove the PCR2 strips from the thermocycler. **After PCR2, the barcoded cDNA library is in the reaction supernatant if beads were used for PCR1 rescue.**
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.**

NOTE: If RT-PCR1 rescue was performed using the transfer method, skip this step and proceed to the next step.

7. Using a multi-channel pipette **set to 45 μ L**, transfer the libraries from the PCR2 strip to the "PCR2 Clean-up Mix Strip."
8. Mix by pipetting up and down until uniform color throughout. **DO NOT CAP** the "PCR2 Clean-up Mix Strip." (The used PCR2 strip with the bead pellet can be capped and discarded.)
9. Allow the "PCR2 Clean-up Mix" strip to incubate on a PCR rack at room temperature for 3 minutes.
10. After incubation, place the "PCR2 Clean-up Mix 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.

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 prepared in the the RT-PCR1 Rescue step 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 85% ethanol by pipetting up and down.
4. Discard the ethanol by pipetting.
5. Repeat this step by pipetting 160 μ L of the fresh, 85% ethanol to the PCR2 bead pellet on the magnet. Wash the pellet by pipetting up and down.
6. Discard the ethanol by pipetting.
7. 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.
8. While the first strip is drying, repeat this process for the next unprocessed strip.

Final Library Elution

1. Once all strips are dry, the library can be eluted from the beads. Label a final set of strips in preparation for this step.
2. Ensure that the "PCR2 Clean-up Mix Strip" is no longer on the magnetic stand.
3. 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 mixture has a uniform, rust color.
4. Cap each strip and vortex to make sure they are fully resuspended and spin down.
5. Incubate the beads with the nuclease-free water for 2 minutes.
6. 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.
7. Transfer the cleaned and sequencing ready library to the final set of labelled PCR strips.
8. Proceed to "Assessing Library Generation."

Option 2: Gel Electrophoresis and Extraction

If Option 1 “Magnetic Bead Library Clean-up” was selected, it is still advisable to run a small portion of the purified library on an agarose gel or Bioanalyzer (continue to “Assessing Library Generation”). A hand-cast 2-4% agarose gel or 2% E-gel agarose gel from Invitrogen 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.

- **If beads were used for PCR2 library clean-up, the PCR2 reaction should be placed on the iR-magnetic Strip Stand until the supernatant is clear prior to loading the gel.**
- PCR product without pre-loaded dye will require a dye marker.
- 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. Using a lower elution volume facilitates easier library pooling.
- 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 appropriate UV protection equipment.

NOTE: A potential stopping point in the protocol is directly after the agarose gel extraction. Libraries can be placed at -20 °C indefinitely until pooling. It is recommended that the concentration of each library in the pool is measured at the same time directly prior to pooling to achieve evenly pooled libraries.

Assessing Library Generation

Check and Record Library Concentration

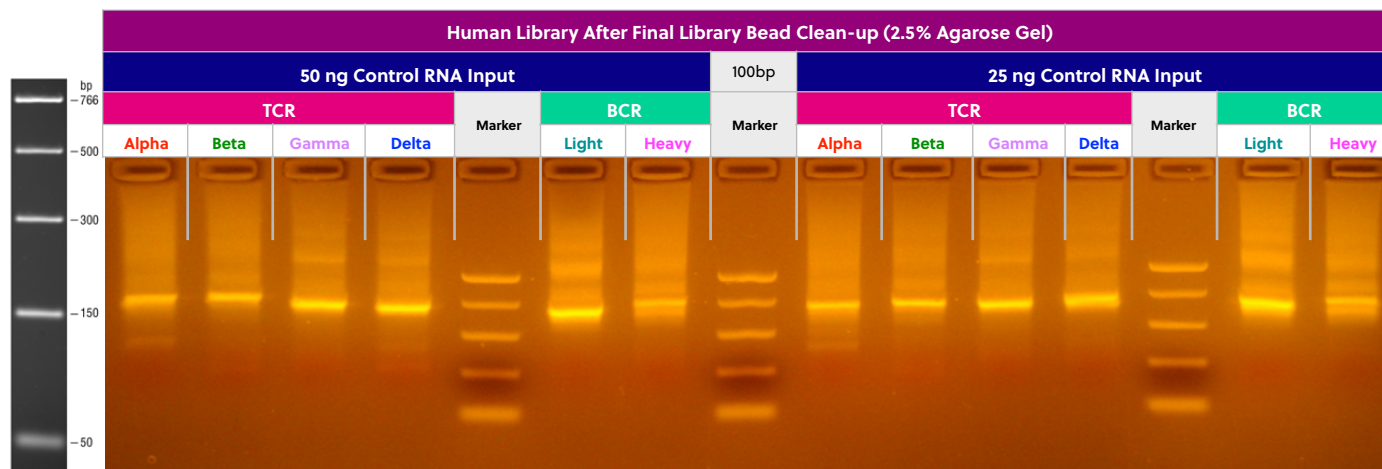
1. **In all cases, it is advisable to check the amplification of a small portion of each library on an agarose gel.** To determine if library generation was successful, use a spectrophotometer such as the Nanodrop, to measure the DNA concentration. *Be sure to blank the device with the water or EB used during the final elution step.* The average concentration of an amplified library is 50 ng/μL, with an input of 100 ng of total RNA and an elution of 37 μL 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/μL when eluted in 37 μL of nuclease-free water, then the library generation is considered a success.
3. If the concentration is between 4 and 10 ng/μL, 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/μL, 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. For amplicon size information, please refer to the gel image examples on the next page.

Concentration (ng/μL)	Result
≥10	Success
4 - 10	Check a Portion on Gel
≤4	Likely unsuccessful

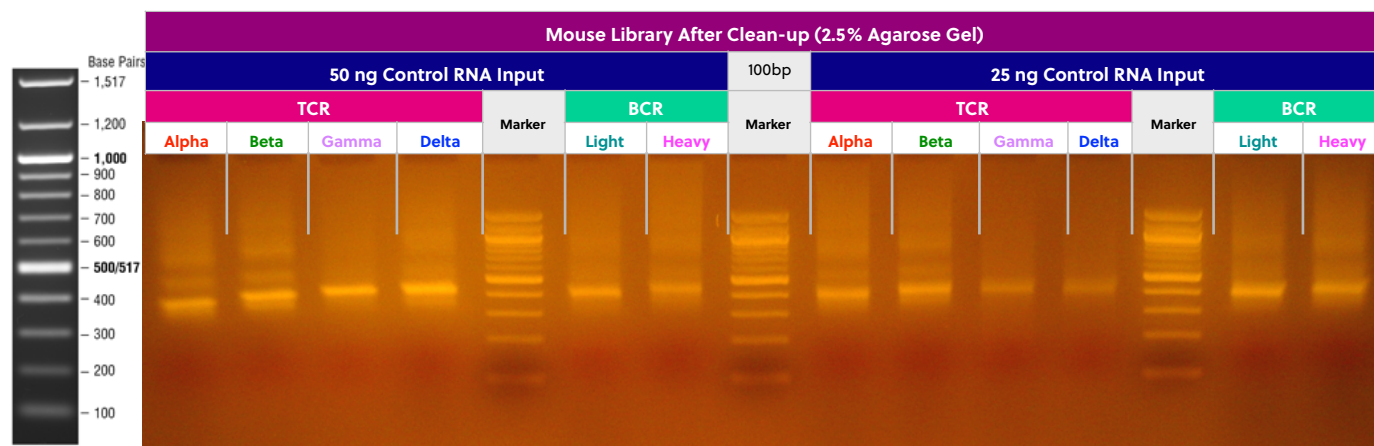
Amplicon Library Size: Long Read Systems (250/300) PER

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

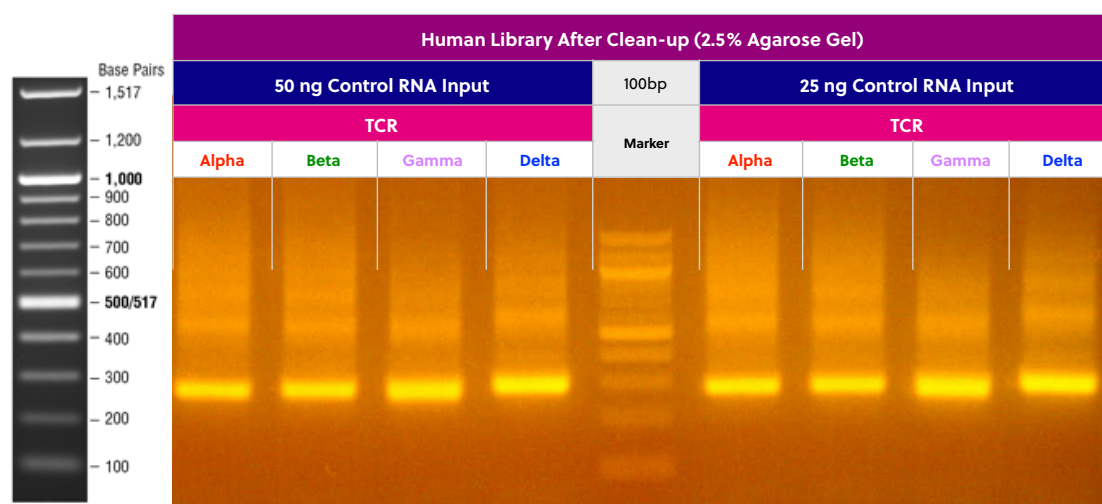


Human Long Read	Amplicon Size Range (bp)	Mouse Long Read	Amplicon Size Range (bp)
HTAI-M	475-522	MTAI-M	380-550
HTBI-M	500-540	MTBI-M	380-550
HTDI-M	470-545	MTDI-M	380-550
HTGI-M	470-545	MTGI-M	380-550
HBHI-M	490-570	MBHI-M	380-550
HBKLI-M	470-520	MBKLI-M	380-550

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 *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 MiSeq flow cell or one HiSeq lane 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 prior to pooling so that 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.) 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 the Illumina MiSeq." **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. Additional fees apply for 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 MTBI-M

Sample Name	Barcode	Sample input	Chain	[cDNA] ng/μL	A260/280	Volume Pooled (μL)	Total cDNA pooled (ng)
Sample 1	5	Total RNA	MTBI-M	28.33	1.88	5.29	150.00
Sample 2	6	Total RNA	MTBI-M	27.37	1.75	5.48	150.00
Sample 3	8	Total RNA	MTBI-M	29.09	1.88	5.16	150.00
Sample 4	12	Total RNA	MTBI-M	30.72	1.83	4.88	150.00
Sample 5	15	Total RNA	MTBI-M	29.12	1.86	5.15	150.00
Sample 6	18	Total RNA	MTBI-M	30.07	1.83	4.99	150.00
Sample 7	20	Total RNA	MTBI-M	33.29	1.93	4.51	150.00
Sample 8	21	Total RNA	MTBI-M	31.11	1.8	4.82	150.00
Sample 9	23	Total RNA	MTBI-M	39.38	2.02	3.81	150.00
Sample 10	24	Total RNA	MTBI-M	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

[illegible]

NGS with the Illumina MiSeq

Pre-Sequencing QC

Once the libraries have been pooled, the final pooled library is ready for pre-sequencing QC. There are certain quality control checks that should be adhered to prior to sequencing. We recommend that Qubit fluorescent-based quantification be performed, then bioanalysis with Agilent's Bioanalyzer, followed by KAPPA analysis (qPCR) on a 10 nM dilution to independently validate concentration and determine the final concentration for sequencing. The most important QC step is the KAPPA qPCR analysis as this provides accurate concentration information in order to achieve an ideal cluster density on the sequencing flow cell. From this information, you can perform the PhiX spike-in, dilution, and load onto the Illumina MiSeq platform for 250/300 paired-end reads (long read) or 150 paired-end reads (short read) as per the instructions provided by Illumina. *The immune repertoire libraries are considered low diversity libraries, and a PhiX spike-in is highly recommended (see sequencing recommendations below).*

Sequencing Kit Recommendation

The sequencing kit recommended is determined based on the amplicon read length of the system, either long or short. For long read systems, the Illumina MiSeq Reagent Kit v2 500-cycle (250 paired-end read; cat. no. MS-102-2003) or v3 600-cycle (300 paired-end read; cat. no. MS-102-3003) can be applied directly to the library constructed by the iRepertoire kit due to the built-in adaptor sequence in the library. For the short read systems, the MiSeq Reagent Kit v2 300 cycle (150 paired-end read; cat. no. MS-102-2002) can be used. Our software pipeline recognizes the barcodes and decodes them automatically during analysis. Barcode information is available under the Guides tab in the [Learning Center at https://irepertoire.com/learning-center/](https://irepertoire.com/learning-center/).

Sequencing Adaptors

After the second round of amplification, the full-length Illumina sequencing adaptor A (below) is associated with an amplicon at the C or J region while adaptor B (below) is associated with an amplicon at the V region.

Adaptor A:

5'-AATGATACGGCGACCAACGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT-3'

Adaptor B:

5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3'

Sequencing Suggestions

1. The barcodes or molecular IDs provided in the kit are specific to iRepertoire products and are NOT the same as Illumina indices (unless otherwise stated). Please refer to “MiSeq Set-up” for further information.
2. If pooling iRepertoire libraries with libraries containing Illumina indices on the same flow cell, the sequencing results for the iRepertoire libraries will be in the undetermined fastq file.
3. 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 have been attached to the VDJ sequences already.
4. Paired-end sequencing is required for libraries generated with iRepertoire Illumina primers, and the paired-read data are used in downstream data processing on iRweb. Paired-end reads are from one DNA fragment. This information is useful for extending sequencing reads to cover the entire CDR3 region and for identifying V and J germline segments reliably. In addition, we also use the paired-end information to calibrate whether a CDR3 fragment is authentic.
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. The Illumina MiSeq Reagent Kit v2 500-cycle produces approximately 17 million reads pre-data filtering. Please refer to Illumina’s website for updated information regarding their kit’s read-throughput.
6. 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. If adding 5 or less barcodes per flow cell, it may be necessary to increase the percentage of PhiX to 25-30%. 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.



MiSeq Set-up

For detailed instructions on running the MiSeq, please see the Illumina manual associated with the device. To run an iRepertoire generated library on the MiSeq, follow the Sample Sheet Wizard set-up detailed below. Please note that we do not use Illumina indexing; we use our own molecular identifiers to de-multiplex the data during analysis. *If pooling iRepertoire libraries with libraries containing Illumina indices on the same flow cell, the set up referenced here should **NOT** be followed.* In this case, the sequencing results for the iRepertoire libraries will be in the undetermined fastq file.

On the Experiment Manager Welcome Screen, select "Sequence." After logging in, select "Other" and "FASTQ Only."

Under FASTQ Only Run Settings:

1. Reagent Cartridge Barcode: **Fill this in**
2. Sample Prep Kit: **TruSeq LT**
3. Index Reads: **Select the "0" dial**
4. Fill in the fields from "Project Name" through the "Date"
5. Read Type: **Select Paired-end dial**
6. Cycles Read 1: **251**
7. Cycles Read 2: **251**

Under FASTQ Only Workflow Specific Settings:

Remove all check marks (i.e., deselect "Use Adapter Trimming").

The screenshot shows the 'Illumina Experiment Manager' window with the 'Sample Sheet Wizard - Workflow Parameters' tab selected. The window is divided into two main sections: 'FASTQ Only Run Settings' and 'FASTQ Only Workflow-Specific Settings'.

FASTQ Only Run Settings:

- Reagent Cartridge Barcode*: A text input field with a placeholder 'Fill this in - should start with MS'.
- Sample Prep Kit: A dropdown menu showing 'TruSeq LT'.
- Index Reads: Radio buttons for 0 (selected), 1, and 2.
- Experiment Name: A text input field with a placeholder 'Fill this in'.
- Investigator Name: A text input field with a placeholder 'Fill this in'.
- Description: A text input field with a placeholder 'Fill this in'.
- Date: A date picker showing '2/17/2016'.
- Read Type: Radio buttons for 'Paired End' (selected) and 'Single Read'.
- Cycles Read 1: A numeric input field showing '251'.
- Cycles Read 2: A numeric input field showing '251'.
- * - required field

FASTQ Only Workflow-Specific Settings:

- ☐ Custom Primer for Read 1
- ☐ Custom Primer for Index
- ☐ Custom Primer for Read 2
- ☐ Reverse Complement
- ☐ Use Adapter Trimming
- ☐ Use Adapter Trimming Read 2

At the bottom of the window, there are three buttons: 'Cancel', 'Back', and 'Next'.



iRepertoire, Inc.

Data Analysis

Data Submission Form

Customers using our primers in their experiment will be provided with complimentary data analysis service. **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 data 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 the Illumina's BaseSpace website. BaseSpace is a cloud-based solution, which facilitates the rapid sharing of project NGS data with ease. Alternatively, sequence data can be sent to iRepertoire via ftp. For an ftp 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 back up 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 Guides section of the [Learning Center](#) on www.irepertoire.com and download the [Data Analysis Guide](#).

Contact

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