



iRepertoire, Inc.

iRepertoire HTBI-vj User Manual for gDNA

Constructing an Immune Repertoire Library for Next-Gen Sequencing



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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
Minor	Corrected grammar and punctuation errors	V20210603
Minor	Updated table styling	V20210603
Moderate	Removed information about Roche and Ion Torrent (pg. 18) as platform compatible primers are no longer in production	V20210603
Major	PCR2 protocol corrected from 10 cycles to 30 cycles	V20211004

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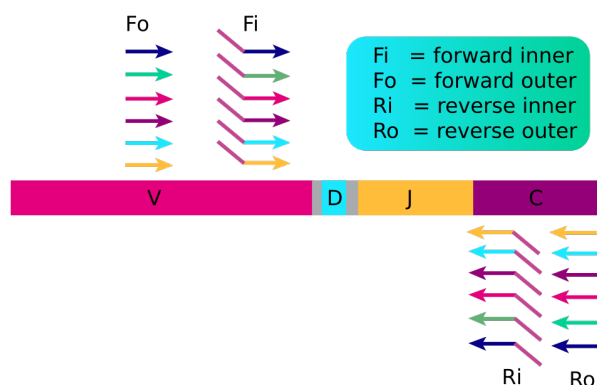
Introduction

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 gDNA to create a human TCR beta immunorepertoire library with HTBlvj primers compatible with Illumina Next-Gen Sequencing Platforms (GAIIx, HiSeq2000, and MiSeq). This manual specifically refers to the application of HTBlvj primer system to gDNA (not applicable to iR-cassettes, iR-Profile kits, or RNA-only iR-bulk primer reagent systems). For application of the HTBlvj primer system to RNA, please use the instructions (and suggested enzymes) provided in the “Universal Bulk Primer Manual.”

The arm-PCR Technology

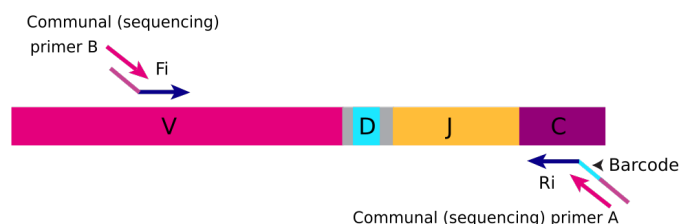
The arm-PCR (amplicon rescued multiplex PCR) technology is a patented multiplex amplification strategy (Patent No. 7,999,092) that utilizes a two-step reaction to amplify an immune repertoire, inclusively and semi-quantitatively.

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 J 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 J genes. The Fi and Ri primers also include sequencing adaptors B and A, respectively, for the Illumina platforms (HiSeq, MiSeq and GAIIx) for paired-end sequencing. For the Ri primers, there are also barcodes in between the sequencing primer A and the J 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 Illumina next-generation sequencing platforms. 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.

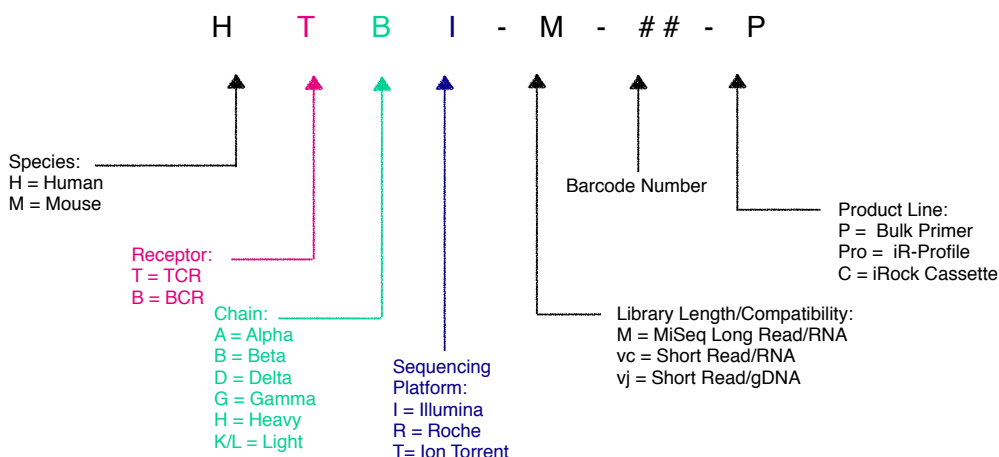


A note about this manual

This manual is separated into sections by the major steps necessary to produce an immune library. The major steps include the following: PCR1 Set-up, 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. 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 below. 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 specific sequences that were produced using a gDNA or 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 could 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. So, using 20 barcodes for one lane, all 200 samples can be sequenced in a single HiSeq run. However, 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 or flow cell. 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 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 main concern about using genomic DNA is the inclusiveness. There is only a certain amount of template DNA that one can add to a PCR reaction. Each human cell may have about 6.6 pg of genomic DNA of which the rearranged VDJ represents a very small portion. So, if 100 ng of genomic DNA is used in the reaction, this represents only about 16,000 cells, resulting in a restricted view of the repertoire. Although more genomic DNA 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 genomic DNA as a template to generate a sequencing library is the background. As Figure 2 demonstrates, after the VDJ rearrangement, those V and J segments not involved in the recombination will still be 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.

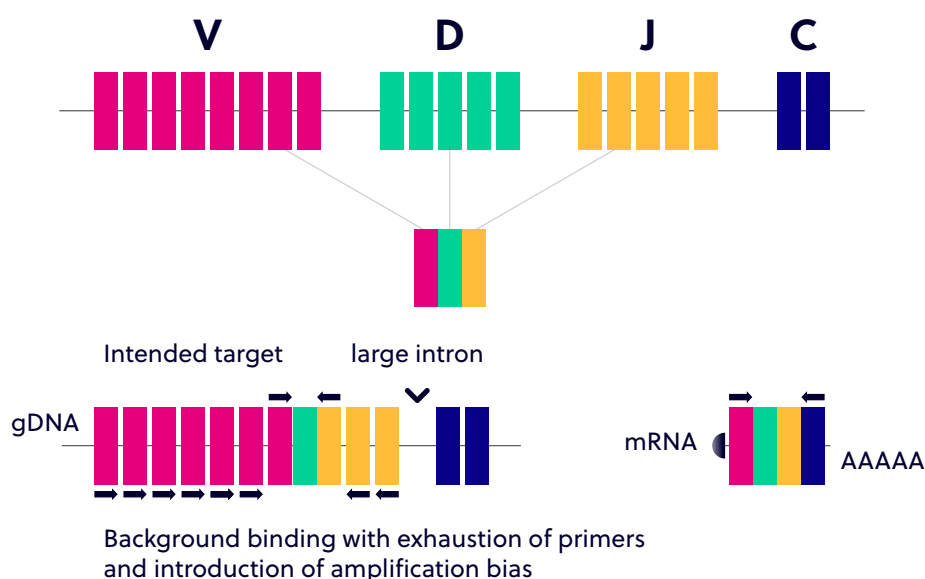


Figure 3. V(D)J rearrangement demonstrating the increased background that may occur due to the extraneous priming sites available within gDNA. This can generate background amplification, exhaust primers, and introduce bias.

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:

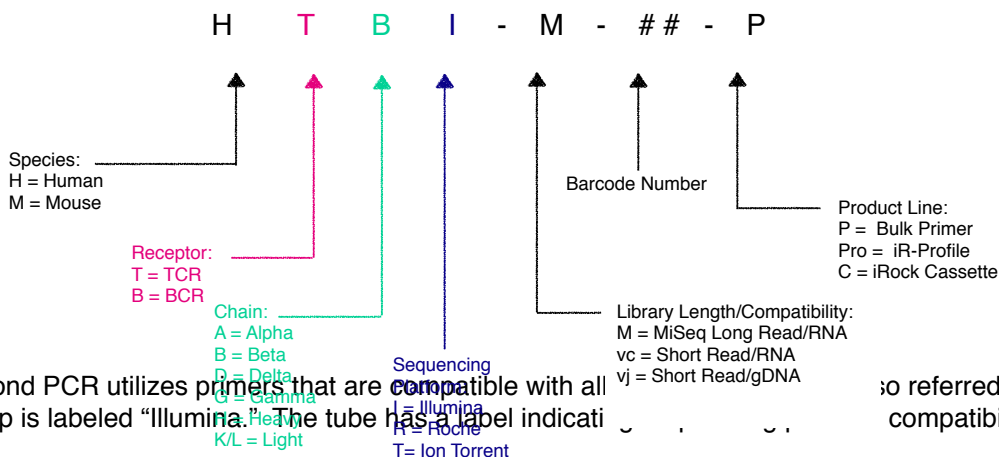
- Store iR Primers & Illumina Communal Primers at 2-8°C.
- Primers should be stored in a clean area designated only for PCR Setup.
- Optionally, primers can also be stored at -20°C.
- Ensure that the cap is secured tightly to reduce evaporation.

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 HTBIvc-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, HTBI-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 sequencing systems. The tube has a label indicating the sequencing system. This is referred to as Communal Primers.

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



Materials & Reagents Not Provided

- Qiagen TopTaq Master Mix Kit, Cat No. 200403 (preferred PCR1 kit)
- Qiagen Multiplex PCR Kit, Cat No. 206143 (preferred PCR2 kit)
- 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)
- Quickload Low Molecular Weight DNA Ladder, New England Biolabs, N0474S
- 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 μ L) 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.
- Ensure that gDNA is maintained at 4°C during template addition. Make sure the gDNA is well-mixed prior to adding to the PCR tube. Avoid excessive freeze-thawing of gDNA samples to prevent degradation.
- Between 100 ng (minimum) and 1000 ng (maximum) of template gDNA is recommended per 25 uL reaction. It is possible to achieve amplification with less than 100 ng gDNA. Determination of the appropriate protocol, low input or normal, must be determined empirically.

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 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 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 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 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

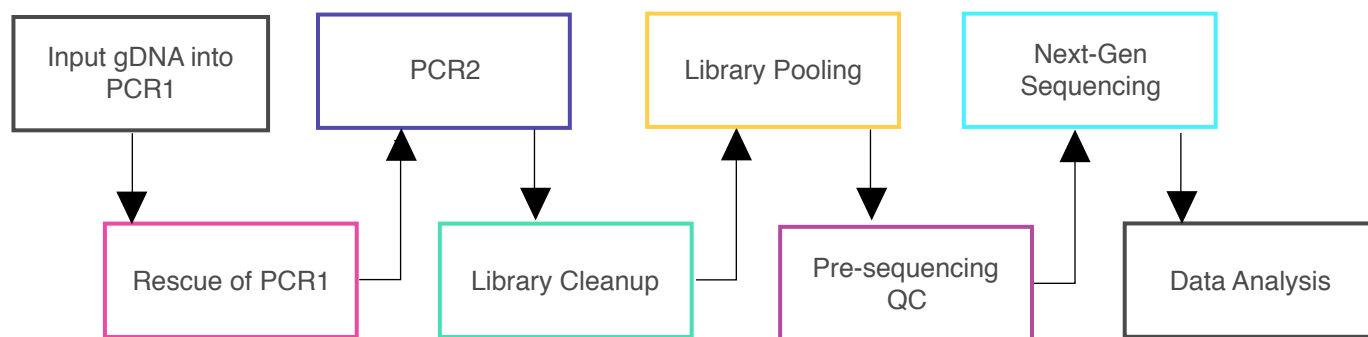
In order to reduce laboratory contamination, it is best to eliminate or reduce retrograde flow from contaminated areas to clean areas. To reduce retrograde laboratory flow, it is best to aliquot all “clean” reagents prior to handling any template or amplicon. This includes any SPRIselect beads required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

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, do not handle PCR products from different templates with the same barcode at the same time.

Outline of the Procedure

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.



PCR1 Set-up

Prior to Set-up

Ensure the PCR set-up area and pipettes are clean and free from contamination. It is recommended to program the thermocycler prior to beginning sample set-up.

Experiment Design

1. Design your experiment and pre-assign barcodes to specific samples by completing Table 1, "Sample Designation Sheet."
2. It is highly recommended to include a negative control reaction in which the template addition is nuclease-free water (no gDNA template) in order to evaluate proper sample handling technique, i.e., avoidance of sample cross-contamination.
3. The final volume for the template and water mix is 8.5 μL . The final reaction volume is 25 μL and is composed of 12.5 μL of the Qiagen 2x TopTaq Master Mix (MM), 4 μL barcoded PCR1 primers, and 8.5 μL template-water mix.
4. The Master Mix (MM) preparation should be performed in a separate area/room from the template addition.

Table 1

Sample Number	Sample Name	Barcode (MID)	Input [RNA] ng/ μL	Desired RNA Amount (ng)	RNA template amount (μL) max: 8 μL	H ₂ O (μL)	RNA Template + H ₂ O (μL)
Example	NEC-01	HTB1vj-01-P	126.2	500	3.96	4.04	8.5
Example	NEC-02	HTB1vj-02-P	300.5	500	1.66	6.34	8.5
1							8.5
2							8.5
3							8.5
4							8.5
5							8.5
6							8.5
7							8.5
8							8.5
9							8.5
10							8.5
11	Neg. Ctrl	N/A	0	0	0	8.5	8.5

PCR1 TopTaq Mix Preparation Procedure

1. In a clean area, remove the Qiagen TopTaq Master Mix kit from the 4°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. Vortex each reagent to ensure well re-suspended, 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 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. Aliquot 12.5 µL of the TopTaq Master Mix into each PCR tube.
5. 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, which reduces risk of sample cross-contamination.
6. Bring this “PCR1 Barcoded MM” strip to the template addition area and set aside until further instruction.

Reagent: Qiagen TopTaq 2x	Volume (µL) / 25 µL rxn	One Sample Reaction
Nuclease-free H ₂ O*	X µL	10.5
TopTaq 2x Master Mix	12.5	12.5
HTB1vj Primer	4	4
Template**	Y µL	2
Total	25.00	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 1-8.5 µL for each 25 µL reaction. Between 100-1000 ng of template 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.

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 18 for instructions regarding PCR2 Master Mix preparation.

PCR1 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 8.5 μL of template-water mix per 25 μL reaction.
2. Retrieve the “PCR1 Barcoded MM” strip and gently open each cap.
3. Add the appropriate amount of water corresponding to the correct sample position for each tube.
4. Next, add the appropriate amount of template to each PCR tube.
5. Cap each tube. Vortex and spin down all PCR reactions using a Mini-spin.
6. The final PCR1 reaction volume is 25 μL . Keep all reactions on ice.
7. Proceed to “PCR1 Thermocycler Conditions,” and add the PCR strips to the thermocycler once the temperature reaches 95 °C. Choose the correct PCR conditions based on the type of template input (Normal versus Low).

Template-Water Mix

Reagent	Volume (μL)	A Sample Reaction
Template**	X μL	3.50
Nuclease-free H ₂ O*	Y μL	5.00
Total	8.50	8.50

*Amount of H₂O is dependent on the template concentration and should be added to reach 8.5 μL as the final template-water addition volume (see next note).

**Template volume may vary from 1-8.5 μL . Between 100 ng (minimum) and 1000 ng (maximum) of template gDNA 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 that less than 100 ng of gDNA will yield successful library generation.

Note: Directly after PCR1 is a potential stopping point in the protocol. PCR1 products may be stored at -20 °C indefinitely.



PCR1 HTB1vj gDNA Thermocycler Conditions

There are two major categories of thermocycler PCR conditions: one for normal input and one for low input. The appropriate protocol should be determined empirically.

HTB1vj:
PCR 1 Conditions:
Normal Input

Temp °C	Time	# Cycles
95	3 min	1
95	30 sec	5
60	3 min	
72	60 sec	
94	30 sec	10
60	90 sec	
72	45 sec	
94	30 sec	10
72	2 min	
72	5 min	1
4	∞	Hold

HTB1vj:
PCR 1 Conditions:
Low Input

Temp °C	Time	# Cycles
95	3 min	1
95	30 sec	10
60	3 min	
72	60 sec	
94	30 sec	10
60	90 sec	
72	45 sec	
94	30 sec	10
72	2 min	
72	5 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.

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. Note: Qiagen Multiplex PCR kit (cat. no. 206143) is the recommended PCR2 kit.

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 (i.e., Illumina) 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 Qiagen Multiplex Mix Kit 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.

PCR2 Single Reaction Example

Qiagen Multiplex Mix Single Rxn	Volume (μL) / 50 μL rxn
Nuclease-free H ₂ O	20
2x Qiagen Multiplex MM	25
Communal Primers	5
PCR 1 product**	0
Total	50

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

Qiagen Multiplex Mix Master Mix	Amount (μL)	Master Mix (μL)	Total Number Rxns
Nuclease-free H ₂ O	20	276	12
2x Qiagen Multiplex MM	25	345	12
Communal Primers	5	69	12
PCR1 product**	0		
Total V	50	Total Master Vol. (μL)	690
		Vol. per rxn (μL)	50

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

PCR1 Rescue with Magnetic Beads & 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 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 the PCR1 strip, which is currently in the thermocycler.

NOTE: In order to facilitate use of a multi-channel pipette, set-up and label a fresh set of PCR strips that mimic the 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 and aliquot only what is required for a specific experiment and bring the aliquot to the processing location.

4. Pipette 36 μ L of the beads into the positions on the freshly labeled PCR strips (rescue strips) which are appropriately labelled. Ensure the beads remain well resuspended by vortexing every fourth addition. Cap each position on the rescue strip after adding the beads.
5. When PCR1 is complete, remove the 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 PCR1 reaction strip.
10. Add 10 μ L of nuclease-free water to each reaction of the PCR1 reaction strip, bringing the volume of each reaction from 25 μ L to 35 μ L. Mix by pipetting up and down.
11. Open the rescue strip containing the beads.
12. Transfer 30 μ L of the PCR1 product using a multi-channel pipette to the rescue strip and mix by pipetting up and down until uniform color throughout (1.2x bead selection). DO NOT CAP the rescue strips. (The PCR1 strip can be capped and discarded once transfer is complete. Alternatively, the 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 PCR1 product for 5 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 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 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 50 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.

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 using agarose gel extraction.

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 normal and low input samples.

PCR 2 Conditions for

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

Library Clean-up

Once PCR2 is complete, it is necessary to clean the library prior to pooling and sequencing to remove residual primer-dimers and the remaining enzyme in the mix. For the HTB1vj reagent system, gel purification is the recommended method for library clean-up prior to pooling. 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.

- PCR product without pre-loaded dye will require a dye marker. If Coral Load dye (pink dye marker available with TopTaq MM) or other loading dye was added to the PCR2 MM, the PCR2 product can be added directly to an agarose gel without the use of additional dye markers.
- 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. Low molecular weight ladder (NEB cat. no. N0474S) 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.

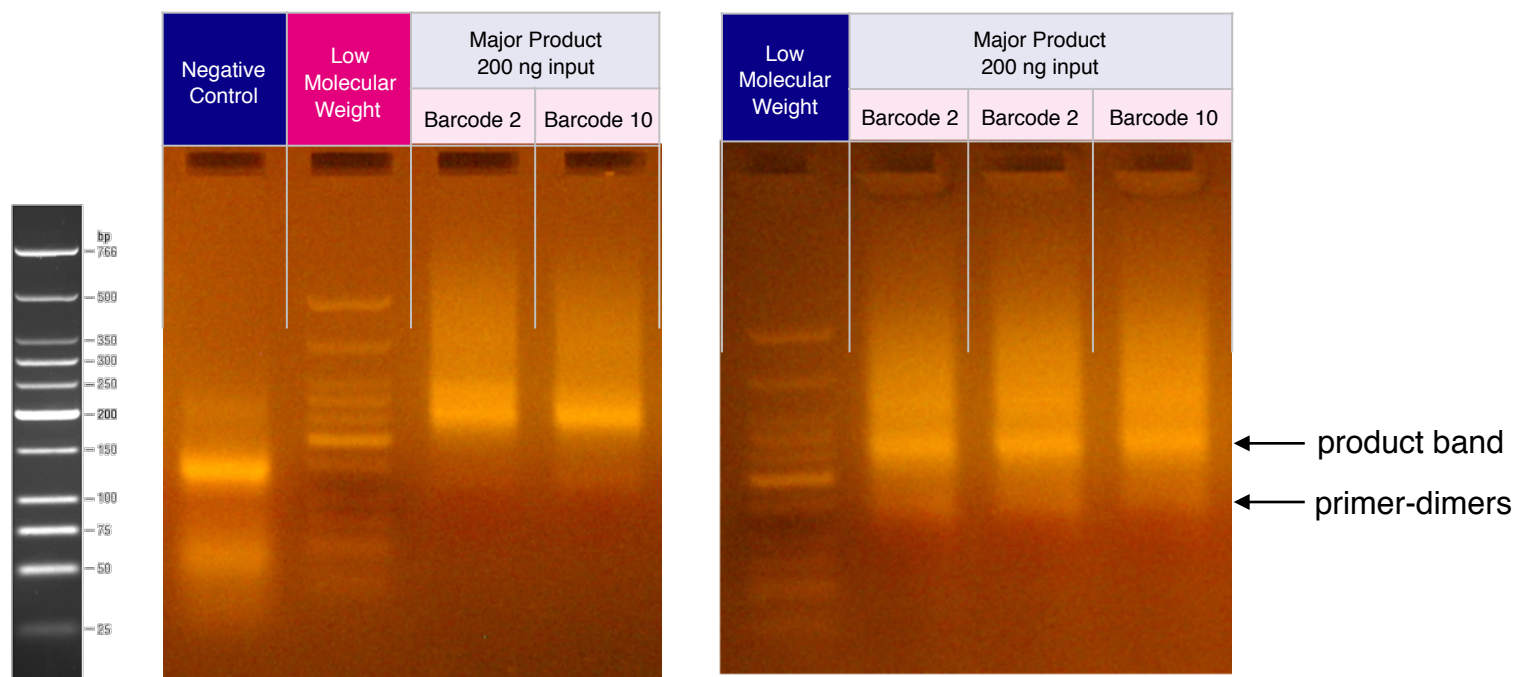
Caution: Wear personal protective equipment such as gloves and a lab coat when handling ethidium bromide. UV light can cause serious burns to the eyes and skin; take precautions to protect your eyes and skin by using appropriate UV protection equipment.

Note: 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. The presence of a primary product band (225-270 bp) on an agarose gel indicates that library generation was successful. 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.
2. During gel purification, select the primary product band when excising from the agarose gel. Primer-dimers are evident from 150-180 bp, which is near the primary product band for the HTB1vj products of 225-270 bp. Care should be taken to exclude the primer-dimers when selecting the band of interest.
3. Once gel purified, measure and record the concentration of the library using an apparatus such as a Nanodrop. Be sure to blank the apparatus with buffer or water used during elution from the column.



Product sizes may differ depending on the sample.

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 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 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.) 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 HTB1vj

Sample Name	Barcode	Sample input	Chain	[cDNA] ng/ μ L	A260/280	Volume Pooled (μ L)	Total cDNA pooled (ng)
Sample 1	5	Total RNA	HTB1vj	28.33	1.88	5.29	150.00
Sample 2	6	Total RNA	HTB1vj	27.37	1.75	5.48	150.00
Sample 3	8	Total RNA	HTB1vj	29.09	1.88	5.16	150.00
Sample 4	12	Total RNA	HTB1vj	30.72	1.83	4.88	150.00
Sample 5	15	Total RNA	HTB1vj	29.12	1.86	5.15	150.00
Sample 6	18	Total RNA	HTB1vj	30.07	1.83	4.99	150.00
Sample 7	20	Total RNA	HTB1vj	33.29	1.93	4.51	150.00
Sample 8	21	Total RNA	HTB1vj	31.11	1.8	4.82	150.00
Sample 9	23	Total RNA	HTB1vj	39.38	2.02	3.81	150.00
Sample 10	24	Total RNA	HTB1vj	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	Barcode	Sample input	Chain	[cDNA] ng/ μ L	A260/280	Volume Pooled (μ L)	Total cDNA pooled (ng)
							150.00
							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							

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

Although this manual is written with instructions specifically for the Illumina MiSeq, libraries generated with the HTBlvj system are also compatible with HiSeq and GAIIx. The sequencing kit recommended is determined based on the amplicon read length of the system, either long or short. For the short read systems, the MiSeq Reagent Kit v2 300 cycle (150 paired-end read; cat. no. MS-102-2002) is recommended. A minimum of 250 cycles (125 paired-end read) is required to sequence HTBlvj amplicons. 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/).

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'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

Adaptor B:

5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-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.
5. 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.
7. 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.
9. 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.

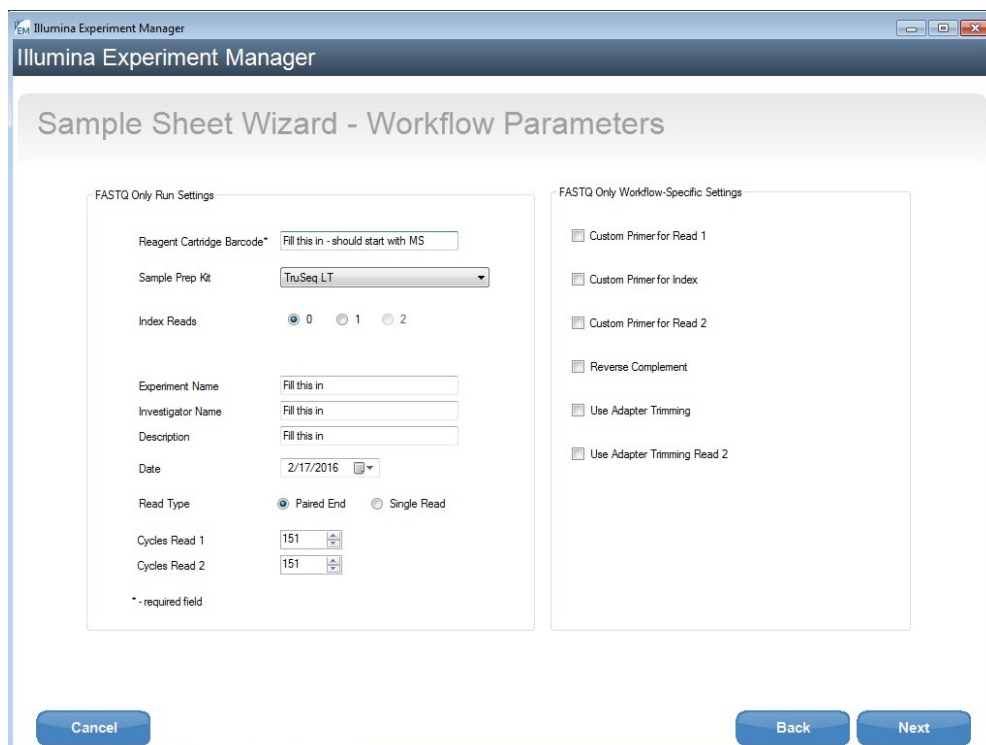
On the Experiment Manager Welcome Screen, select “Sequence.” After logging in, select “Other” and “FASTQ Only.”

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: 151
7. Cycles Read 2: 151

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*: Fill this in - should start with MS
- Sample Prep Kit: TruSeq LT (dropdown menu)
- Index Reads: 0 (selected), 1, 2 (radio buttons)
- Experiment Name: Fill this in (text field)
- Investigator Name: Fill this in (text field)
- Description: Fill this in (text field)
- Date: 2/17/2016 (calendar icon)
- Read Type: Paired End (selected), Single Read (radio buttons)
- Cycles Read 1: 151 (spin box)
- Cycles Read 2: 151 (spin box)
- * - 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 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 a 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 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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