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User Manual for Long Read iR-Profile Reagent System

Constructing Human or Mouse TCR and BCR Immune Repertoire Libraries for
Next-Generation Sequencing with the Illumina MiSeq (500 Cycle+)

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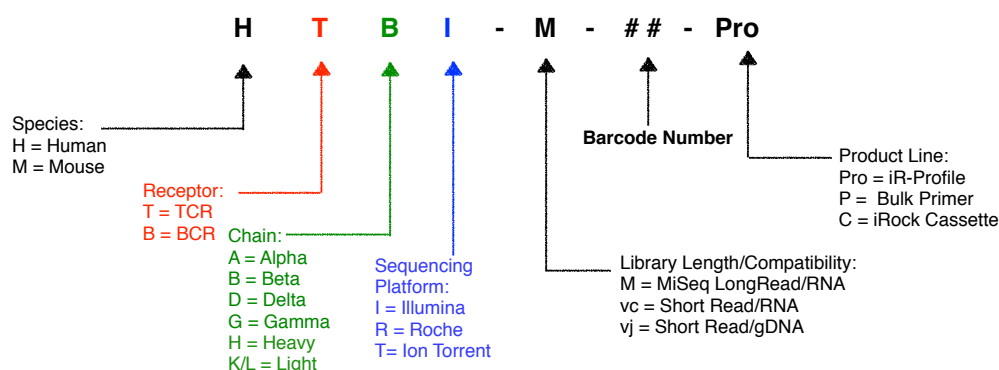
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Introduction

The goal of the iR-Profile product line is to provide an easy-to-use kit so that a user can go from sample RNA to immune sequence with ease. With the iR-profile system, researchers can choose the immune profile to fit their immune puzzle: T or B cell, multiple chains, and many barcoding options with all of the reagents necessary for library preparation. The kit is provided with pre-mixed reagents to use as a single tube, single reaction format for the preparation of 10 uniquely barcoded libraries. Since each library generated with the kit will have a different barcode or molecular ID (MID), multiple libraries can be multiplexed per Illumina MiSeq flow cell (see “Pooling Libraries”). This allows for better utilization of the sequencing flow cell, and thus, reduces the overall cost of a sequencing project.

During processing, two rounds of PCR will be performed. During the first round (PCR1), a reverse transcription and V-region tagging step is completed. After PCR1, the library is rescued with magnetic beads or template transfer, and PCR2 is performed using a PCR mix common to all PCR1 products (PCR2 Mix). For more information, please see “The arm-PCR Technology” section of this manual. After cleaning-up the library with the “PCR2 Clean-up Mix” (included) or gel extracting the band of interest, the amplified immune library is directly ready for quantification, pooling, QC, and sequencing using the Illumina MiSeq next generation sequencing platform. **Please read this handbook, particularly the “General Information” section, carefully before beginning your experiment.**

Understanding iRepertoire’s Reagent System Naming Scheme

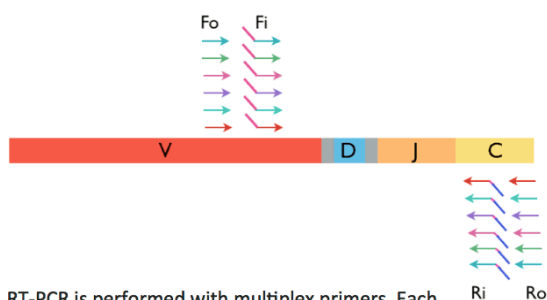


For PCR1, the barcoded PCR1 reaction mixes contain a very specific label, which conforms to iRepertoire’s naming scheme. These single reaction mixes contain a code similar to the one depicted above. This code contains important information such as the species, receptor, chain, sequencing platform, library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

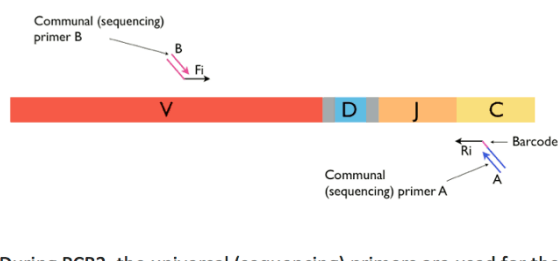
The arm-PCR Technology

The arm-PCR (amplicon rescued multiplex PCR) technology is a patented multiplex amplification strategy (Patent Nos. 7,999,092 and 9,012,148B2) that utilizes a two-step reaction to amplify an immune repertoire, inclusively and semi-quantitatively.

First Round PCR



Second Round PCR



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, MiSeq and GAIIx) for paired-end sequencing. For the Ri primers, there are also 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, 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.

An Introduction to 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, PCR1 Rescue and PCR2 Set-up, 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.

Package Contents & Storage

Shipping & Storage

Upon receipt, store components as follows:

- ◆ Store the larger box containing primer mixes at 2-8°C.
- ◆ Store any box containing enzyme or control RNA (unless specified otherwise) at -20°C
- ◆ Primers should be stored in a clean area designated only for PCR Setup.

Package Contents

- ◆ 10 individually barcoded PCR1 reaction mixes labelled “**PCR1 Rxn Mix**”: Each reaction mix contains a specific label as discussed in the Introduction section. Please be sure to record the associated iR-code with the sample name for downstream data analysis.
- ◆ 1 Positive Control reaction mix labelled “**Pos. Ctrl.**”: This reaction mix is the same as PCR1 Rxn Mix but specifically intended to serve as a positive control. The positive control is to assess reagent stability and provide a benchmark for comparison of the amplification of actual samples. It is not necessary to sequence the positive control.
- ◆ 1 Negative Control reaction mix labelled “**Neg. Ctrl.**”: This reaction mix is the same as PCR1 Rxn Mix but specifically intended to serve as a negative control. This sample should be treated like all other samples; however, NO template RNA should be added, only water. *Due to the sensitivity of the reaction, the negative control serves as an important indicator of sample handling and the guidelines outlined in the “General Information” section should be followed to mitigate cross-contamination of samples.*
- ◆ **PCR2 Mix**: This is the communal primer mix for PCR2 which can be applied to all PCR1 products.
- ◆ **PCR1 Rescue**: After PCR1, the PCR1 product will be rescued using this reagent.
- ◆ **PCR2 Clean-up**: After PCR2, the PCR2 product, or final library, will be cleaned in preparation for sequencing with this reagent.
- ◆ **Control RNA**: This template RNA should be added to the positive control reaction.
- ◆ **iR-PCR1 Enzyme mix**: This is the PCR1 RT-enzyme intended for the PCR1 reaction. It should be stored at -20°C.

IMPORTANT NOTE: All reagent systems work with RNA; however, due to an intron between J and C genes, only V-J primers should be used with genomic DNA.

Materials & Reagents Not Included

- ◆ Thermocycler (recommended: Biorad C1000)
- ◆ 85% Ethanol
- ◆ Nuclease-free water
- ◆ Reagent reservoirs (recommended: ThermoScientific 8096)
- ◆ Molecular Bioproducts DNA Away
- ◆ Molecular Bioproducts RNase Away
- ◆ Vortexer
- ◆ PCR cold block or ice
- ◆ Mini-centrifuge/ Mini-spin
- ◆ Pipettes
- ◆ Multi-Channel Pipette (200 μ L)
- ◆ Filter Tips
- ◆ 8-tube 0.2 mL PCR strips (recommended: Eppendorf 951010022)
- ◆ iR-Magnetic Strip Stand (Sold Separately)
- ◆ Microfuge tubes
- ◆ Timer
- ◆ Personal Protective Equipment (gloves, lab coats, etc.)
- ◆ E-Gel agarose gel electrophoresis system, Invitrogen by life technologies (optional)
- ◆ E-Gel 2% General Purpose Agarose 18 Pak, Invitrogen G5018-02 (optional)

General Information

Nucleic Acid Handling Precautions

- ◆ Maintain a sterile environment when handling nucleic acids (NA) to avoid any contamination from nucleases.
- ◆ Ensure that no nucleases are introduced into the solutions supplied with the panel.
- ◆ Make sure that all equipment that comes in contact with NA is sterile, including pipette tips and tubes.
- ◆ 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. **DO NOT VORTEX.** Avoid excessive freeze-thawing of RNA samples to prevent degradation.
- ◆ ***Between 50 ng (minimum) and 1800 ng (maximum) of template RNA is recommended.*** 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, the amplification of multiple targets simultaneously makes handling precautions even more important to avoid false positive results. **In order to avoid contamination, we highly recommend that separate rooms and equipment be used for RT-PCR/PCR setup, template addition, and gel extraction (if applicable).** *In addition, separate storage areas for primers, nucleic acids, and amplicons are highly recommended.*

The use of a hood equipped with a UV light is recommended for template addition 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.

Mixed-Receptor iR-Profile kits

Since it is possible to mix-and-match reactions with iR-profile, 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.

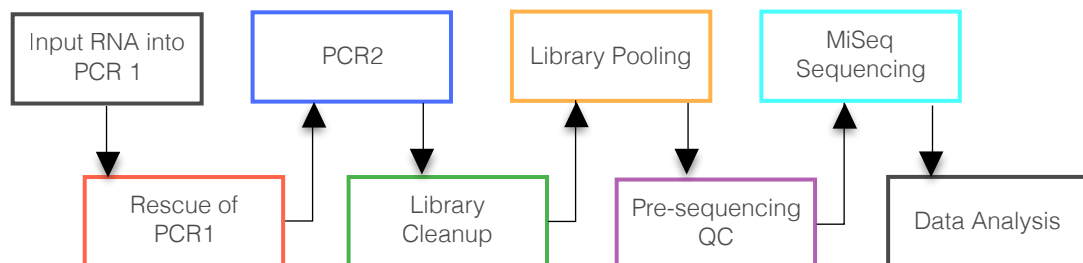
PCR2 Clean-up/Gel Extraction

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. **To avoid cross-contamination, do not handle PCR products from different templates with the same barcode at the same time.** iR-Profile kits should contain all unique barcodes, but it is possible to have redundant barcodes if multiple kits are purchased.

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, 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 smaller volume and use the entire RNA elution as the template for the PCR1 protocol provided for low sample input.

Outline of the Procedure



PCR1 Set-up

Before Beginning

Ensure the PCR setup 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.

Template-Water-Enzyme Mix Set-up

1. Design your experiment and pre-assign barcodes to specific samples by completing the “Sample Designation Sheet” section.
2. Complete the Template-Water-Enzyme Mix calculation table below for each sample (also included in the “Sample Designation Sheet”). Please note there is a maximum of 23.2 μL of template RNA addition allowed. Each reaction will also receive 1.8 μL of iR-PCR1 enzyme mix per reaction. Nuclease-free water should be added to adjust the final volume of the Template-Water-Enzyme Mix to 25 μL .

Reagent	Volume (μL)	A Sample Reaction
Nuclease-free H_2O *	X μL	8.75
Template**	Y μL	14.45
iR-PCR1 Enzyme Mix	1.80	1.80
Total	25.00	25.00

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

Template volume may vary from 1-23.2 μL . Between 50 ng (minimum**) and 1800 ng (**maximum**) of template RNA is recommended. 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.

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. Make sure each tube cap is closed.

NOTE 1: When preparing the full 12 libraries, it is required to use a minimum of two 8-tube PCR strips. We recommend you leave the first position of the strip empty for easier strip handling, which reduces the possibility of cross-contamination.

NOTE 2: There are separate PCR1 protocols for T-cell and B-cell receptor amplifications. Use of separate PCR strips for each receptor type (BCR or TCR) during set-up is necessary.

4. Take the “iR-PCR1 Enzyme Mix” and “Control RNA” from the -20 °C storage and place on ice.
5. Add the appropriate amount of template RNA, water, and “iR-PCR1 Enzyme Mix” into the appropriate labelled position on the PCR1 strip. Close each tube once all components are added. Remember to keep everything on ice or a PCR cold block during set-up.
6. For positive and negative controls, please refer to the “Sample Designation Sheet” for the amounts of Control RNA or nuclease-free water, which should be added to the reaction.
7. At this point, each sample tube should contain 25 μ L of RNA template, water, and “iR-PCR1 Enzyme Mix” on ice.
8. Continue to the PCR1 set-up.

PCR1 Set-up

1. Remove the larger iR-Profile box from 4 °C storage.
2. Take out the barcoded “PCR1 Rxn Mix,” “Pos. Ctrl.,” “Neg. Ctrl.,” “PCR1 Rescue,” and “PCR2 Cleanup” tubes.
3. **Note: One “PCR1 Rxn Mix” tube is a single *barcoded* reaction!!!**
4. Set-aside the “PCR1 Rescue” and “PCR2 Clean-up” tubes. *This allows the reagents to equilibrate to room temperature prior to selection and PCR2.*
5. Place the larger iR-Profile box back into the 4 °C storage until PCR2.
6. **Briefly, vortex and spin down (in a Mini-spin) the “PCR1 Rxn Mix” tubes, including both “Pos. Ctrl.” and “Neg. Ctrl.” tubes.**
7. Opening one PCR tube cap containing your Template-Enzyme-Water mix and one “PCR1 Rxn Mix” at a time, pipette 20 μ L of each “PCR1 Rxn Mix” into the corresponding labelled PCR tube. Gently mix by pipetting up and down. DISCARD used pipette tip and use a new tip for the next tube.
8. Cap each PCR tube after the addition of the “PCR1 Rxn Mix.” Cap the used “PCR1 Rxn Mix” 1.5 mL tube and discard. Continue this process until all of the reactions have received “PCR1 Rxn Mix” including controls.
9. The final PCR1 reaction volume is 45 μ L. Remember to keep all reactions on ice.
10. Vortex and spin down PCR strips using a Mini-spin.
11. Proceed to “PCR1 Thermocycler Conditions,” and add the PCR strips to the thermocycler once the temperature reaches 50 °C.

Sample Designation Sheet

An Excel version of this form is available for download in the document section of iRepertoire's website (<http://www.irepertoire.com/#!manuals/c222i>) or by contacting customer service. This form can also be used as a guide during the submission of data to iRepertoire for downstream data analysis.

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* are necessary during data analysis (please see example below).

Sample Number	Sample Name	Barcode (MID)	Input [RNA] ng/ μ L	Desired RNA Amount (ng)	RNA template amount (μ L) max: 23.2 μ L	H ₂ O (μ L)	iR-PCR1 Enzyme Mix	PCR1 Rxn Mix (μ L)	Total PCR1 Reaction Volume (μ L)
Example	NEC-01	HTBI-M-01-Pro	126.2	500	3.96	19.24	1.8	20	45
Example	NEC-01	HTAI-M-02-Pro	126.2	500	3.96	19.24	1.8	20	45
1							1.8	20	45
2							1.8	20	45
3							1.8	20	45
4							1.8	20	45
5							1.8	20	45
6							1.8	20	45
7							1.8	20	45
8							1.8	20	45
9							1.8	20	45
10							1.8	20	45
11	Neg. Ctrl	N/A	0	0	0	23.2	1.8	20	45
12	Pos. Ctrl	N/A	2.2	50	23.2	0	1.8	20	45

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.

TCR: PCR 1 Conditions: Normal Input

Tem °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: PCR 1 Conditions: Low Input

Tem °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	
94	30 sec	10
72	3 min	
72	15 min	1
4	∞	Hold

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: PCR 1 Conditions: Normal Input

Tem °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: PCR 1 Conditions: Low Input

Tem °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	
94	30 sec	10
72	3 min	
72	15 min	1
4	∞	Hold

PCR1 Rescue and PCR2 Set-up


Before Beginning

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.**

There are two options for PCR1 rescue. The first option is to rescue the entire PCR1 product using the “PCR1 Rescue Mix” provided. 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.**

Option 1: PCR1 Rescue Method with 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 further instructions.
2. Retrieve the “PCR1 Rescue Mix” which should be at room temperature. If not, let sit for 15 minutes in order to equilibrate to room temperature prior to set-up.
3. Label a new set of PCR strips with sample ids matching the 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 PCR1 reaction strips.
4. Invert the “PCR1 Rescue Mix” 3 times and vortex for at least 10 seconds. Ensure the “PCR1 Rescue Mix” is fully resuspended.
5. Pipette 36 μ L of the “PCR1 Rescue Mix” into the positions on the freshly labeled PCR strips (*rescue strips*) which are appropriately labelled. Ensure the “PCR1 Rescue Mix” remains well resuspended by vortexing every fourth addition. Cap each position on the rescue strip after adding the “PCR1 Rescue Mix.”
6. When PCR1 is complete, remove the PCR1 strips from the thermocycler.
7. Wipe down the outside of the tubes with DNA Away prior to placing the strips on a PCR rack in the hood.
8. Change gloves prior to beginning the transfer.
9. **PROCESS ONE STRIP AT A TIME.**
10. First, open the *rescue strip* containing the “PCR1 Rescue Mix” (the beads).
11. Next, open the completed PCR1 reaction strip.

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12. Transfer **40 μ L** of PCR1 product into the “PCR1 Rescue Mix” strip using a multi-channel pipette and mix by pipetting up and down until uniform color throughout. **DO NOT CAP THE “PCR1 Rescue Mix” 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 product can be used as a template for transfer into PCR2 if necessary in the future.)
 13. Let incubate on a PCR rack (at room temperature) for **5 minutes** with the caps open.
 14. 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.
 15. 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.
 16. 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.)
 17. **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.
 18. Discard the ethanol by pipetting.
 19. 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.*
 20. While the first strip is drying, repeat the process beginning at step 11 through step 20 for the second strip.
 21. Retrieve the “PCR2 mix” (pink solution) from the iR-Profile box at 4 °C.
 22. **Briefly, vortex and spin down (in a Mini-spin) the “PCR2 Mix” tube.**
 23. To the dried bead pellet, add 50 μ L of “PCR2 Mix” 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.
 24. When all transfers are complete, ensure the PCR2 strips are tightly capped, vortex briefly, and spin down.
 25. 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.

Option 2: Transfer Rescue Method with 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 PCR1 strip.
3. **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.
4. Retrieve the “PCR2 mix” (pink solution) from the iR-Profile box at 4 °C.
5. **Briefly, vortex the “PCR2 Mix” tube and spin down (in a Mini-spin).**
6. To the new set of freshly labelled PCR strips, add 48 μ L of “PCR2 Mix” to each tube which will contain a sample.
7. When PCR1 is complete, remove the PCR1 strips from the thermocycler.
8. Wipe down the outside of the tubes with DNA Away prior to placing the strips on a PCR rack in the hood.
9. Change gloves prior to beginning the transfer.
10. First, open the freshly labeled strip containing the “PCR2 Mix”.
11. Next, open the completed PCR1 reaction strip.
12. Transfer 2 μ L 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.
13. **When the transfer is complete, ensure the PCR2 strips are tightly capped, vortexed briefly, and spun down.**
14. The final PCR2 reaction volume is 50 μ L.
15. 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.

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), normal, and low input samples.

PCR 2 Conditions:

PCR 2: Normal Input	Tem °C	Time
1	95	3 min
30	94	30 sec
	72	90 sec
1	72	15 min

Library Clean-up

Before Beginning

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

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

Option 1: PCR2 Clean-up Mix Method

PCR2 Clean-up Mix Strip Preparation

1. Label a new set of PCR strips with sample ids matching the PCR2 strip.
2. Invert the “PCR2 Clean-up Mix” three times and vortex for at least 10 seconds. Ensure the “PCR2 Clean-up Mix” is fully resuspended.
3. Pipette 36 μ L of the “PCR2 Clean-up Mix” into the sample positions on the freshly labeled PCR strips which are appropriately labelled. Ensure the “PCR2 Clean-up Mix” remains well resuspended by vortexing every fourth addition. Cap each position on the strip after adding the “PCR2 Clean-up Mix.”
4. Set aside and wait for PCR2 to complete.

PCR2 Transfer to the Clean-up Mix Strip

1. When PCR2 is complete, open the freshly labelled strip containing the “PCR2 Clean-up Mix.”
2. Remove the PCR2 strips from the thermocycler. **After PCR2, the barcoded cDNA library is in the reaction supernatant.**
3. Wipe down the outside of the PCR2 strips with DNA Away prior to placing the strips on the magnetic stand.
4. Change gloves prior to beginning the transfer.
5. **PROCESS ONE STRIP AT A TIME.**

6. Place the PCR2 strip on the iR-Magnetic Strip Stand and allow the beads to collect. This may take 2-3 minutes. Allow the beads to collect for additional time if the supernatant is not clear. **DO NOT DISCARD THE SUPERNATANT.** The supernatant contains the amplified library.
7. Using a multi-channel pipette **set to 50 μ L**, transfer the libraries from the PCR2 strip to the strip containing the “PCR2 Clean-up Mix”.
8. Mix by pipetting up and down until uniform color throughout. **DO NOT CAP THE “PCR2 Clean-up Mix” strips.** (The used PCR2 strip with the bead pellet can be capped and discarded.)
9. Let the “PCR2 Clean-up Mix” strip incubate on a PCR rack (at room temperature) for 5 minutes.
10. After incubation, place the strip on the iR-Magnetic Strip Stand and allow the beads to collect. This may take 2-3 minutes. Allow the beads to collect for additional time if the supernatant is not clear. *Leave the strip on the magnet until instructed otherwise.*
11. ***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 into a sterile reagent reservoir. (Optionally, the 85% ethanol can be added one step at a time to each PCR tube.)
3. ***WHILE KEEPING THE PCR STRIP ON THE MAGNET***, wash the bead pellet with 160 μ L of the *fresh* 85% ethanol by pipetting up and down.
4. Discard the ethanol by pipetting.
5. Remove any residual ethanol by pipetting. Remove the strip from the magnetic stand and allow the bead pellet to air dry in a ventilated hood for 3-5 minutes.
6. While the first strip is drying, repeat this process for the next unprocessed strip.

Final Library Elution

1. Once all strips are dry, the library can be eluted from the bead. *Label a final set of strips in preparation for this step.*
2. Add 37 μ L of nuclease-free water to each bead pellet and resuspend the beads by pipetting up and down. A well-suspended bead pellet is rusty colored.
3. Cap each strip and vortex to make sure fully resuspended and spin down.
4. Incubate the beads with the nuclease-free water for 2 minutes.
5. Place the strip on the iR-Magnetic Strip Stand and allow the beads to collect. This

may take 2-3 minutes. Allow the beads to collect for additional time if the supernatant is not clear. **DO NOT DISCARD THE SUPERNATANT.** The supernatant contains the amplified library.

6. Transfer the cleaned and sequencing ready library to the final set of labelled PCR strips.
7. Proceed to “Assessing Library Generation.”

Option 2: Gel Electrophoresis and Extraction

If option 1 was selected, 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 “PCR1 Rescue Mix” was used, the PCR2 reaction should be placed on the iR-magnetic Strip Stand until the supernatant is clear prior to loading the gel.
- ◆ PCR2 can be added directly to an agarose gel *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. 100 bp ladder is recommended.
- ◆ When performing gel extraction, we recommend using Qiagen QIAquick gel extraction kit, Cat No. 28704 per the manufacturer’s instructions.
- ◆ For each individual sample, it is beneficial to use one QIAquick column per sample. This will require repeated additions of the sample to the same column.
- ◆ The goal is to extract the band of interest in a lower volume, typically 37 μ L, in order to increase the concentration of each library prior to pooling. This will make the pooling portion easier.
- ◆ If primer-dimers are present, they typically appear around the 150 bp marker.

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

Assessing Library Generation

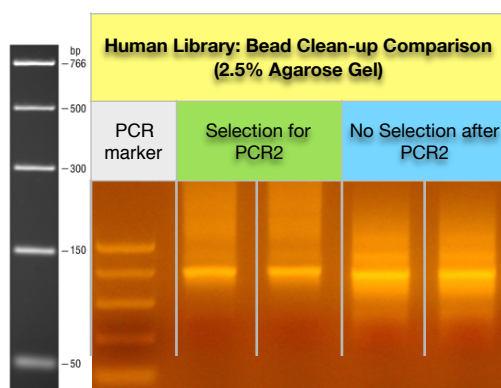
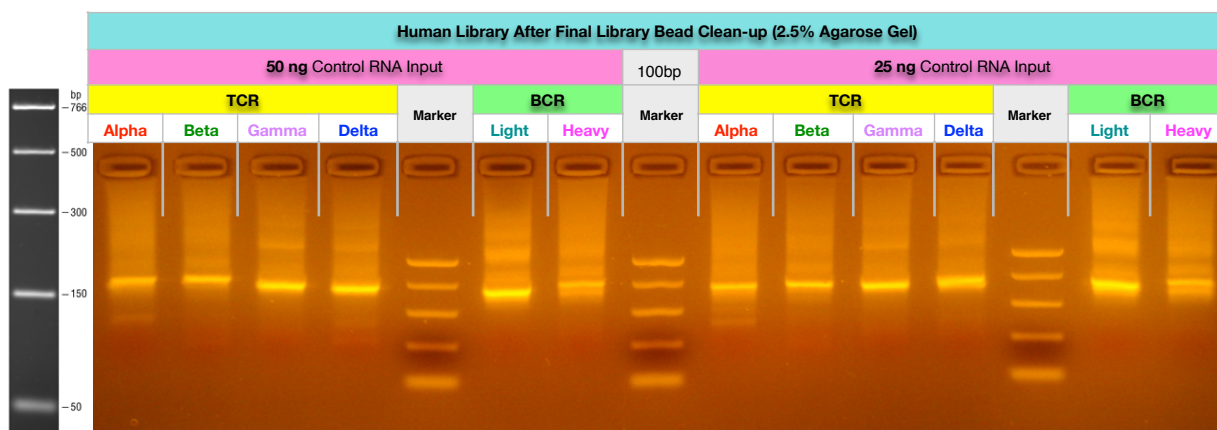
Check and Record Library Concentration

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. The average concentration of an amplified library is 50 ng/ μ L with an input of 100 ng of total RNA and an elution of 37 μ L of nuclease-free water when measured with a Nanodrop. The average concentration ranges in the table below may not apply if using another method of concentration determination such as a Bioanalyzer.
2. If the concentration is greater or equal to 10 ng/ μ L when eluted in 37 μ L of nuclease-free water, then the library generation is considered a success.
3. If the concentration is between 4 and 10 ng/ μ L, then check for a major product by running some or all of the amplicons on a 2.5% agarose gel to see if amplification was achieved. If all product was run, gel purify the major band.
4. If the concentration is less than 4 ng/ μ L, then typically the run is not considered a success. However, it is still advisable to check the amplification on an agarose gel as a major product may be present.

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

Human Library Sizes for Long Read Systems

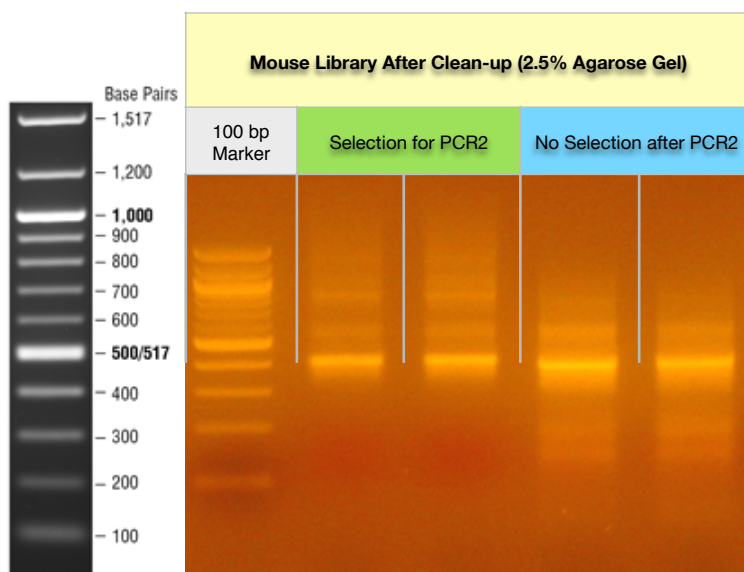
Each amplified repertoire represents a large number of sequences distributed over a size range. The gel images and table below can be used as a guide indicating the range of potential product sizes. Provided are libraries generated from control RNA covering all **human** TCR and BCR chains with both 50 ng and 25 ng of RNA input. Also provided are gel images of cleaned libraries versus libraries with no clean-up.



Primer Type	Amplicon Size Range (bp)
HTAI-M	475-522
HTBI-M	500-540
HTDI-M	470-545
HTGI-M	470-545
HBHI-M	490-570
HBKLI-M	470-520

Mouse Library Sizes for Long Read Systems

Each amplified repertoire represents a large number of sequences distributed over a size range. The gel images and table below can be used as a guide indicating the range of potential product sizes. Provided are libraries generated from control RNA covering all **mouse** TCR and BCR chains with both 50 ng and 25 ng of RNA input. Also provided are gel images of cleaned libraries versus libraries with no clean-up.



Mouse Long Read	Amplicon Size Range (bp)
MTAI-M	380-550
MTBI-M	380-550
MTDI-M	380-550
MTGI-M	380-550
MBHI-M	380-550
MBKLI-M	380-550

Example Pooling Libraries Table

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

Sample Name	Barcode	Sample input	Chain	[cDNA] ng/μL	A260/280	Volume Pooled (μL)	Total cDNA pooled (ng)

Total ng (10 Libraries)	
Total Volume	
Theoretical Concentration	
Actual Concentration	
A260/280	

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 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 samples 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 precipitated and reconstituted in a lower volume.

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 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

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. Our software pipeline recognizes the barcodes and decodes them automatically during analysis. Barcode information is available at <http://www.irepertoire.com/#/other/c9z>.

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'-AATGATACGGCGACCAACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT-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 iR-Profile 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 this kit, 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.
3. 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.
4. 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 iR-Profile 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:

1. **Remove all check marks** (i.e., deselect “Use Adapter Trimming”).

Illumina Experiment Manager

Sample Sheet Wizard - Workflow Parameters

FASTQ Only Run Settings

Reagent Cartridge Barcode*

Sample Prep Kit

Index Reads ☒ 0 ☐ 1 ☐ 2

Experiment Name

Investigator Name

Description

Date

Read Type ☒ Paired End ☐ Single Read

Cycles Read 1

Cycles Read 2

* - 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

Cancel Back Next

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 <http://www.irepertoire.com/#!data-submission/cc44>. 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* are necessary during data analysis (please see example in the "Sample Designation Sheet"). If submitted this way, all chains indicated will be provided on iRweb under one sample name header.

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
601 Genome Way Suite 3005
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)

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 [Document Center](#) on www.irepertoire.com and download the [Data Analysis Guide](#).

Contact

iRepertoire, Inc. 601 Genome Way, Huntsville, AL 35806. (256)-327-0948

E: info@irepertoire.com; W: www.iRepertoire.com

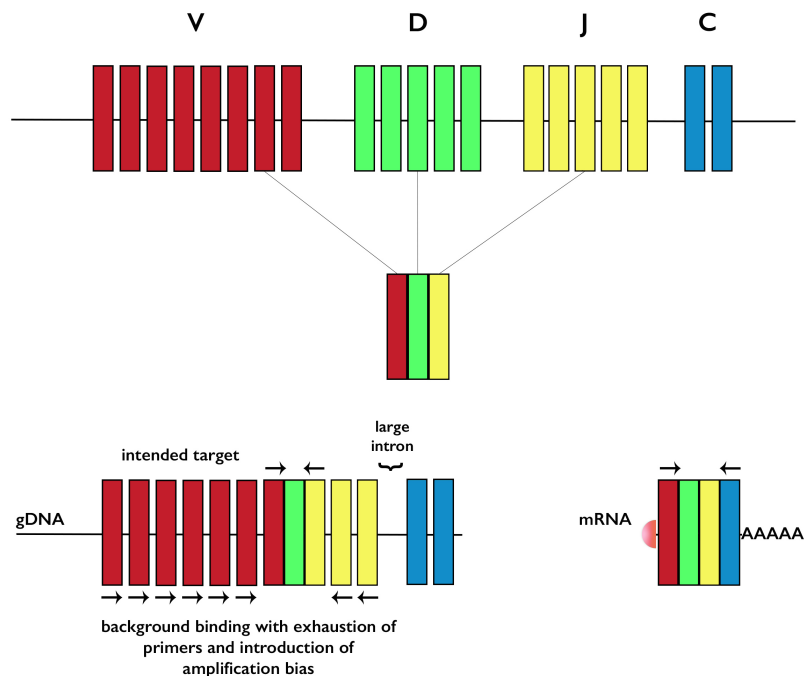
Appendix A: Template Selection- RNA versus gDNA

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 the figure below 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.



VDJ 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 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; however, due to an intron between J and C genes, only V-J primers should be used with genomic DNA (see the table below).

For T cell studies, we have both V-C and V-J primers for the Illumina platforms (GA IIx, HiSeq2000, and MiSeq) and V-C primers for the Roche 454 system (please see our Catalog for more information). The V-J primers amplify about 100 bp sequences around the CDR3 region and are suitable for both genomic DNA and RNA samples; the V-C primers amplify about 150 bp sequences around the CDR3 region and are good for RNA samples only because there is a large intron in between the C and rearranged VDJ genes. For B cell studies, we have V-C gene primers for both the Illumina and Roche 454 platforms. Please contact us if there is a set of V-J primers for the Illumina or Roche platform that you would like to use but not currently listed in our catalog.

Primer and Template Compatibility

Template	V-J Primers	V-C Primers
RNA	√	√
gDNA	√	DO NOT USE

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