iRepertoire

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User Manual for iR-TCR Reagent System

Constructing Mouse TCR Immunorepertoire Library for Next-Gen Sequencing
with the 454 Platforms
(compatible with 454 and GS Junior)

For Research Use Only, Not to be Used for Clinical Diagnostics.

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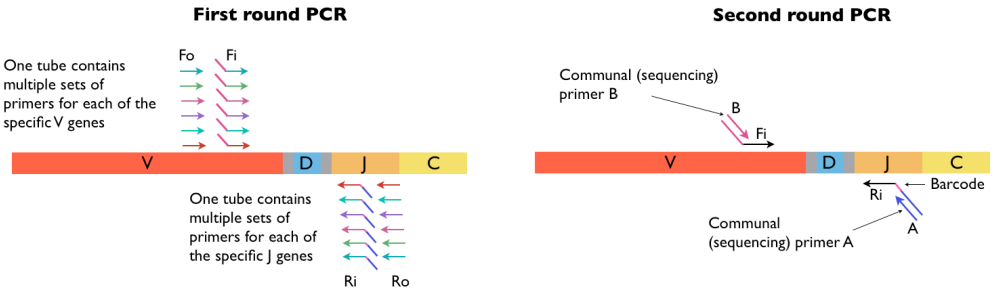
Introduction

Please read this handbook carefully before beginning your experiment. The "**Pooling Samples,**" "**Template Selection,**" and "**General Information**" sections are particularly important to read and understand before beginning your sample preparation. This manual provides instructions for amplifying a Mouse BCR Immunorepertoire library compatible with Roche 454 Sequencing Platforms (454 and GS Junior). This manual is compatible with the following reagent systems: MTBR, MTAR, and MTDGR . Please see "**Package Contents and Storage**" for more information about these iRepertoire Reagent Systems.

The arm-PCR Technology

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



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 454 platforms (454 and the GS Junior) 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 Roche 454 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.

Pooling Samples

This manual provides instructions for generating a library from ONE sample. One catalog item includes enough reagents to produce 10 libraries (all of which will have the same barcode). However, to best utilize the the Roche 454 sequencing capacity, libraries generated from up to 20 samples, each with a different barcode, can be pooled together in one sequencing run.

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

For example, if you want to study the mouse TCR beta chain repertoire of 200 samples, you could purchase iRepertoire products Catalog Numbers “MTBR-01-P” to “MTBR-20-P.” Then, use each of the 20 catalog items to amplify the first 20 samples. After arm-PCR, the 20 PCR products can be pooled for one sequencing run. Then, use these same primers again to amplify the next 20 samples for the second sequencing run, etc. This accounts for a total of 10 sequencing runs, where 20 different samples are studied in each run.

Of course, it is not necessary to pool as many as 20 samples for one sequencing run. In fact, due to the limited number of reads available on the Roche 454 system, we suggest pooling up to 5 samples. The number of samples pooled during a sequencing run depends upon the experimental design and the desired depth of sequencing coverage. It is also possible to sequence one sample at a time without pooling, especially for those studies where very deep coverage is desired.

Note: When samples are pooled for sequencing with Roche 454, sequencing is restricted to single-end reads from 454 primer-A.

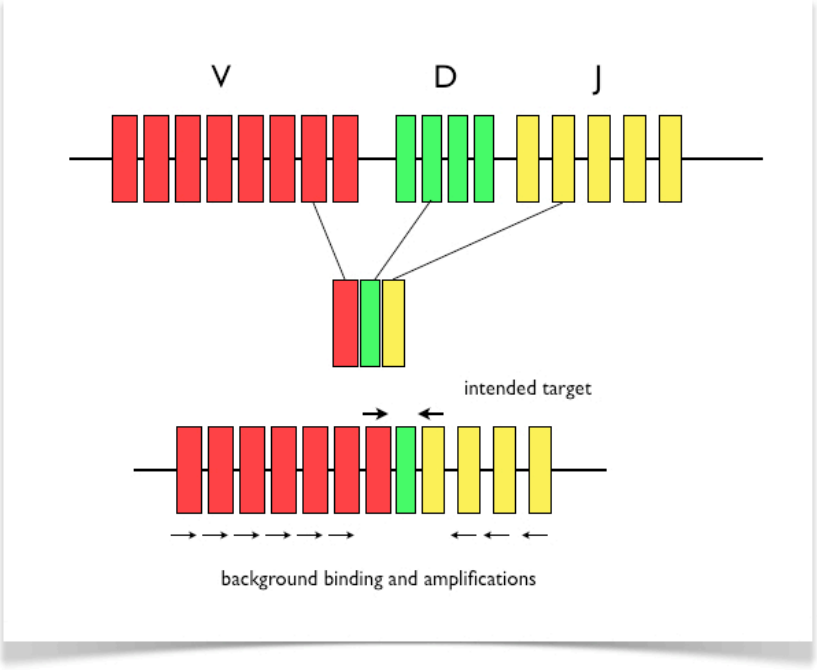
Template Selection: RNA versus gDNA

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 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

Package Contents & Storage

Shipping & Storage

Upon receipt, store components as follows:

- Store iR Primers (MTBR, MTAR, and MTDGR) & Roche 454 Communal Primers at 2-8˚C.
- Primers should be stored in a clean area designated only for PCR Setup.

Package Contents

Each catalog item includes two primer tubes, each for one round of PCR (PCR1 and PCR2). The first PCR reaction (PCR1) will utilize either the MTBR, MTAR, or MTDGR primers (depending on the reagent system purchased; see below). The second PCR reaction (PCR2) uses the "Communal Primers." Each catalog item can generate libraries for 10 samples **with the same barcode**.

For Mouse T cell repertoire amplification, we currently offer **three** Roche reagent systems:

- MTAR for mouse TCR alpha chain, Roche platforms, covers **V** and **C** genes;
- MTBR for mouse TCR beta chain, Roche platforms, covers **V** and **C** genes;
- MTDGR for mouse TCR delta gamma chains, Roche platforms, covers **V** and **C** genes;

IMPORTANT NOTE: All systems work with RNA samples. DNA samples are not recommended with the Mouse BCR Roche reagent systems (MTBR, MTAR, and MTDGR)because there is a large intron between the J and C genes.

Tube Labels

PCR1: The primer mix used in PCR1 has a cap that is labeled either "MTBR," "MTAR," or "MTDGR," corresponding to the catalog number of the order placed. On the tube label, two important pieces of information are included: 1) MTBR, MTAR, and MTDGR and 2) the barcode number. **Please remember to record the barcode number together with the sample identity if working with more than one sample (see "Pooling Samples" for more information).**

PCR2: The second PCR utilizes primers that are compatible with all 20 different barcodes. They are also referred to as Communal Primers. The cap is labeled "454 Lib-A." The tube has a label indicating sequencing platform compatibility.

Materials & Reagents Not Included

- Qiagen One-Step RT-PCR Kit, Cat No. 210212
- Qiagen Multiplex PCR Kit, Cat No. 206143
- Qiagen QIAquick gel extraction kit, Cat No. 28704
- RNase Inhibitor
- Thermo cycler
- Vortex
- Cold Block or Ice
- Mini-centrifuge/ Mini-spin
- E-Gel agarose gel electrophoresis system, Invitrogen by life technologies
- E-Gel 2% General Purpose Agarose 18 Pak, Invitrogen G5018-02
- E-Gel 4% General Purpose Agarose 18 Pak, Invitrogen G5018-04
- Pipettes
- Filter Tips
- PCR Tubes
- Microfuge tubes
- Timer

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. Gently flick RNA sample to mix once thawed, **DO NOT VORTEX**. Avoid excessive freeze-thawing of RNA samples to prevent degradation.
- **A minimum of 100 ng of RNA with a 260/280 of 1.8 or greater is recommended as the starting material to obtain the best diversity of the library.** It may be possible to achieve amplification with less than 100 ng NA.

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. 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 PCR setup and template addition. Separate pipettes and disposable materials, such as pipette tips and micro-centrifuge tubes, should be maintained in each room/area. When performing PCR2, keep PCR1 products closed in the template addition area until ready to be added. Immediately recap the PCR tube once complete. Designating separate hoods and equipment for PCR1 and PCR2 template addition provides an additional level of protection.

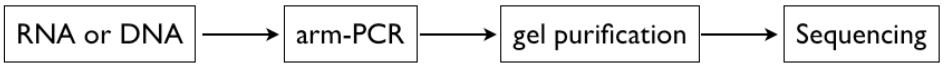
Gel Extraction

For a better sequencing result, remove non-specific PCR products and primer-dimers by extracting the target band of desired size from agarose gel. Extra attention is required during the gel extraction step. **Do not handle PCR products from different templates with the same barcode at the same time to avoid cross-contamination.**

Minimum Cell Counts

The library can be made from as few as 3000 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 use the entire RNA in the reaction by eluting the RNA in a smaller volume.

Outline of the Procedure



The arm-PCR method includes two rounds of PCR reactions, PCR1 and PCR2 (see below for both protocols). Genomic DNA should only be used with the V-J primer systems; RNA can be used as a template for both V-J and V-C primer systems.

Protocol

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 cooler/ice until the cycler has reached 50˚C.

PCR1

1. Remove the Qiagen OneStep RT-PCR Kit from the -20˚C storage and place on PCR cooler/ice. Gather the iRepertoire Primers for PCR1 from the 2-8˚C. This is a tube labeled MTBR, MTAR, or MTDGR, depending on the catalog item ordered.
2. Complete Table 1 to determine the amounts of each component to be added to the reaction.

Note: If performing amplification of more than one sample for the same sequencing run, prepare a master mix of the table components ***excluding*** the primers and template in the mix. **Unique barcoded primers will need to be added to each sample reaction separately if samples are to be pooled for the same sequencing run (see "Pooling Samples").** Add 10% extra of each component to the master mix to account for pipetting losses.

Table 1. Qiagen OneStep RT-PCR

Reagent	Volume (µL) / 25 µL rxn	A Sample Reaction
Nuclease-free H_2O*	X µL	11.75
5X Buffer	5.0	5.0
dNTP Mix	1.0	1.0
RNasin (40U/µL)	0.25	0.25
Enzyme Mix	1	1
iRepertoire Primers	4	4
Template**	Y µL	2
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 reaction volume (see next note).*

***Template volume may vary from 1-13.75 µL. 100 ng minimum of template RNA or DNA (V-J primer systems only) is recommended. Adjust the volume of nuclease-free H_2O accordingly. The template amount must be determined empirically. To avoid contamination, apply the template in a separate designated area.*

3. Add the reagents in the order listed in Table 1 to a PCR tube situated in a PCR cooler/ice.

Note: If handling multiple samples to be studied in the same sequencing run, aliquot the master mix into the appropriate number of sample tubes, add 4 µl of iRepertoire PCR1 primers (using a unique barcode primer for each separate sample reaction), add corresponding sample template (100 ng minimum recommended), and **be sure to record the sample identity and the associated primer barcode number**.

4. Mix by flicking tube and spin.

5. Transfer reactions to a thermal cycler programmed with the cycling conditions in Table 2. Approximate run time is 150 minutes.

Table 2. PCR 1 Cycling Conditions

˚C	Time	# Cycles
50	40 min	1
95	15 min	1
94	30 sec	15
60	2 min	
72	30 sec	
94	30 sec	10
72	2 min	
72	10 min	1
4	∞	Hold

PCR2

1. Remove the Qiagen Multiplex PCR kit from the -20˚C and let thaw at room temperature. Gather the tube labeled “454 Lib-A Primers” from the 2-8˚C storage.

2. Prepare a Master Mix (MM) using Table 3 as a guide. Prepare enough for the number of reactions plus 10% to account for pipetting losses. When preparing a MM for multiple samples, **DO NOT** include the PCR1 product yet.

   Note: The 454 Lib-A Primers, also referred to as the Communal Primers, are compatible with each of the 20 different barcodes. Therefore, the MM for more than one sample type (ie. samples with different barcodes) can include the same Communal Primers, and the mix can be used for all of the PCR2 reactions.

   Table 3. Example Qiagen Multiplex Master Mix Preparation for **ONE** sample

Reagent	Volume (µL) / 50 µL rxn
Nuclease-free H_2O	18
Multiplex MM	25
Communal Primers	5
PCR 1 product*	2
Total	50

   *PCR1 serves as the template for PCR2. *To avoid contamination, apply the PCR1 template in a separate designated area.*

3. Vortex the MM and place 48 µL in each PCR tube. Cap the tubes and transfer to the template addition area.

4. Obtain products from PCR1. Vortex, spin down and place products in a clean rack.

5. Add 2 µL PCR1 product to the corresponding PCR tube. Mix and spin.

6. Transfer reactions to a thermal cycler programmed with the cycling conditions in Table 4. Approximate running time is 120 minutes.

   Table 4. PCR2 Cycling Conditions

˚C	Time	# Cycles
95	15 min	1
94	30 sec	40
55	30 sec	
72	30 sec	
72	5 min	1
4	∞	Hold

Gel Electrophoresis & Size Selection

Hand-cast 2-4% agarose gel or 2% and 4% E-gel agarose gel from Invitrogen can be used for DNA sample screening and gel purification of interesting targets. For better sequencing results, gel purification is recommended to remove primer-dimers (recommended kit: Qiagen QIAquick gel extraction kit, Cat No. 28704). The primer-dimers are located around the 50 bp marker. Each amplified repertoire represents a large number of sequences distributed over a size range. The PCR product of the Mouse Roche 454 libraries range from 280-450 bp. **It is recommended that the gel be cut to cover the major product within this range.**

High Throughput Sequencing

Roche 454 GS FLX Titanium Lib-A sequencing kit can be applied directly to the library constructed by the iRepertoire kit due to the built-in adaptor sequence in the library. **Sequencing is restricted to single-end reads from primer A only.** The read length should be 300 bp or more. Up to 20 libraries can be pooled together to reduce the sequencing cost if library construction kits with different barcodes were added to different samples. However, due to the number of available reads, we suggest pooling up to 5 samples. Our software pipeline recognizes the barcodes and decodes them automatically during analysis. Contact us for the barcode sequence, if necessary.

Data Upload

Customers using our primers in their experiment will be provided with complimentary data analysis service. Sequence data can be shipped with a portable hard disk or an online file sharing method such as Dropbox. **We suggest users backup their data before sending it to us.**

Data Analysis

1. Barcode sequencing decoding
2. V, D, J, C mapping
3. CDRs identification
4. Analysis results rendered through web pages
5. V-J combination distribution (2-D, 3-D map)
6. Listing CDR3
7. CDR3 algebra, compare repertoires and identify shared CDR3
8. Diversity index calculation
9. Displaying V, D, J and C mapping
10. Web access to distributions including:
    a. N-addition (normalized and non-normalized)
    b. CDR3 length (normalized and non-normalized)
    c. V, J nibbling (normalized and non-normalized)
    d. V, J usage (normalized and non-normalized)

Please visit the demo site to experience our software pipeline.

Visit this link: https://irweb.irepertoire.com/ir/ then, use “demo” for user ID, and “12345” as the password. Follow the navigation on the left to see the features of the iR software.

Contact

iRepertoire, Inc. 601 Genome Way, Suite 3005, Huntsville, AL 35806. (256)-327-0948
E: info@irepertoire.com; W: www.iRepertoire.com

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