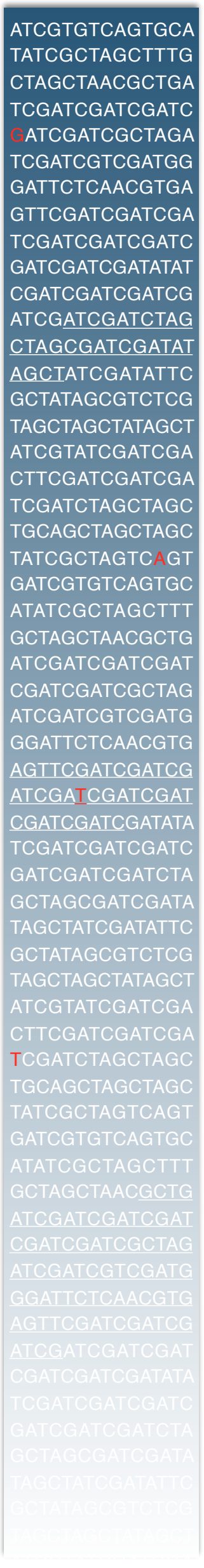


iRock 2.0

iRepertoire on cassette kit

User Manual for iRepertoire on Cassette Kit
iR-TCR and iR-BCR Reagent Systems 2.0

Automated Construction of Human TCR/BCR Immunorepertoire Library on the iR-Processor for Next-Gen Sequencing with the Illumina MiSeq

For research use only. Not to be used for clinical diagnostics.

v082016b

iRepertoire

We Find Health in Your Diversity.

Table of Contents

Introduction

This handbook is applicable to iRepertoire cassettes run on the iR-Processor™ ONLY (not the entire iC-System) and should not be applied to another cassette unless explicitly stated. Please read this manual completely before beginning the experiment. The "**Pooling Samples**" and "**General Information**" sections are particularly important to read and understand before beginning sample preparation.

The goal of the iRock product line is to provide the end-user a solution to amplify the immune repertoire in an automated and closed format. Furthermore, the amplified product, or library, is purified from primer-dimers in the cassette, removing the need for gel extraction of the primary amplification product. This goal is achieved by using the iR-Processor and the closed cassette. First, the user adds their template RNA with the RT-PCR1 enzyme mix. Two PCR reactions are completed by the instrument and residual primer-dimers are removed by magnetic selection of the primary product. After amplification and primer-dimer removal, the cassette is removed from the iR-Processor, and the amplified library is eluted from the magnetic beads in the cassette by the user. The cassette is placed on an iRep magnetic stand (provided with the kit), and the library is pipetted into a clean PCR tube. The amplified immune library is directly ready for quantification, pooling, QC, and sequencing using the Illumina MiSeq next generation sequencing platform.

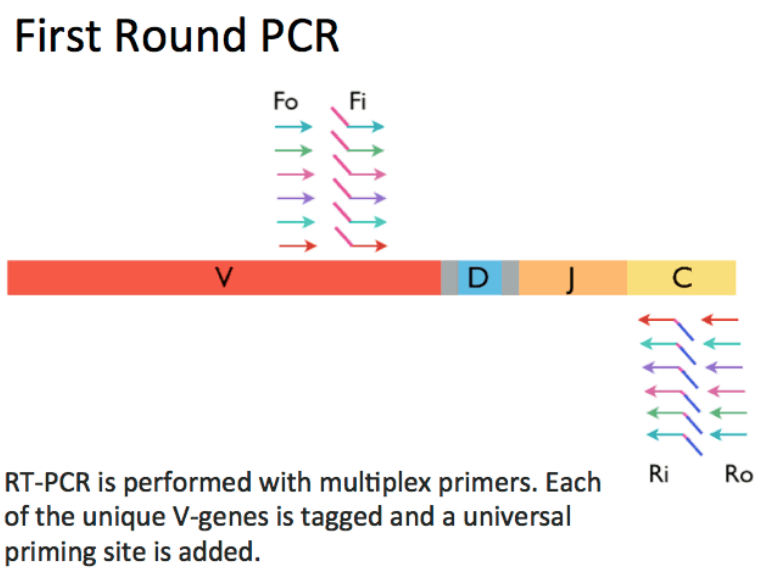
iRock 2.0 represents an updated version of the iRock product line, in which the amplicons after PCR1 are rescued by magnetic selection in the cassette. This additional selection of PCR1 represents several improvements to the first generation of the iRock product line and also has resulted in several major differences. These improvements and differences are summarized as follows and are reflected throughout the manual:

- ✦ Increased CDR3 discovery.
- ✦ Increased capacity for input RNA , resulting in a different sample set-up table.
- ✦ Improved sensitivity for low input RNA templates.
- ✦ Improved amplicon library quality, resulting in increased read throughput per sample.
- ✦ Decreased concentration of successfully amplified libraries due to additional selection within the cassette—therefore, parameters for determining successful amplification have changed.
- ✦ Changes to the reagent layout within the closed cassette. **Note**: if your iR-Processor was installed before January 2015, please contact customer support for the necessary files to update your iR-Processor. First generation cassettes will still be able to be used in the instrument, but an update will be required to run iRock 2.0 due to changes in reagent layout.

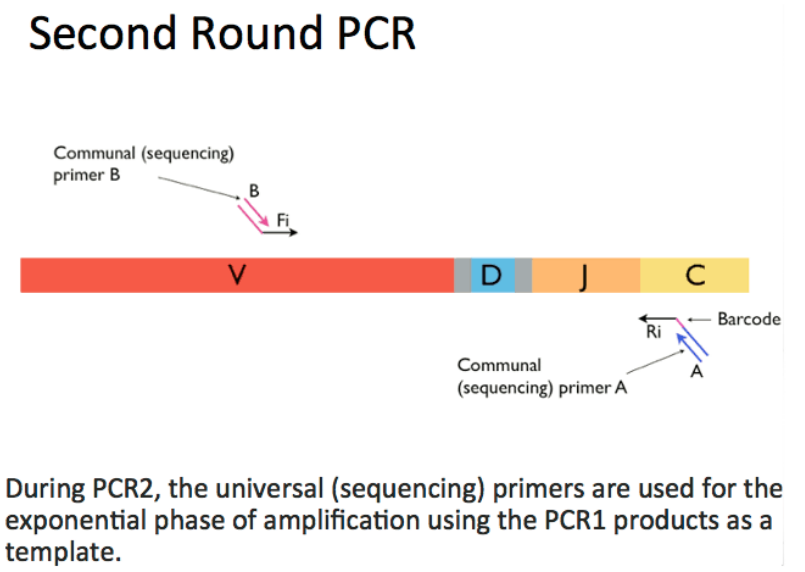
This manual provides instructions for amplifying a human TCR or BCR immunorepertoire library compatible with Illumina MiSeq next generation sequencing platform using the iRock product line. *Instructions for the sample set-up for the original iRock product line may be found in Appendix D.* This manual is compatible with the following iRepertoire Reagent Systems: HTBI-M, HTAI-M, HBHI-M, and HBKLI-M,. 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 (See Figure 1).



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



During PCR2, the universal (sequencing) primers are used for the exponential phase of amplification using the PCR1 products as a template.

During the first round of PCR, nested gene-specific primers targeting each of the V and C genes are used. The forward primers Fo (forward-out) and Fi (forward-in) are located in the V genes. The reverse primers, Ro (reverse-out) and Ri (reverse-in), are located the C genes. The Fi and Ri primers also include sequencing adaptors B and A (see Appendix 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. 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. After bead selection in the cassette, the resulting product is ready for high throughput sequencing with the Illumina platforms. No additional enzymatic steps are required, only quantification and QC prior to sequencing.

Pooling Samples

This manual provides instructions for generating a library from ONE sample in one cassette. One cassette includes enough reagents to produce 1 barcoded library [with the exception of the Qiagen One-Step reverse transcriptase (RT enzyme), which must be added to the cassette with the sample RNA]. However, to best utilize the Illumina sequencing capacity, libraries generated from up to 10 samples, each with a different barcode, can be pooled together in one single MiSeq flow cell. *Please note the cassettes are single-use only.*

To study multiple samples in the same sequencing run, you need to purchase multiple iRepertoire cassettes 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 human TCR beta repertoire of 20 samples, you should purchase 2 sets of 10 iRepertoire cassettes “HTBI-M-01-C” to “HTBI-M-10-C.” Then, use one set of 10 cassettes to amplify the first 10 samples. After arm-PCR on the iR-Processor platform, the 10 PCR products can be pooled for one single MiSeq run. The next set of barcodes (with the same barcode numbers as the first set) can be used again for the next set of 10 samples for the second MiSeq run, etc.

Template Selection: RNA vs. gDNA

The iRock system only accepts RNA as a template. gDNA will **NOT** work as there is an intron in between the C and rearranged V(D)J genes. For more information regarding RNA versus gDNA as a template, please visit our website at www.irepertoire.com.

Package Contents & Storage

Shipping & Storage

Upon receipt, store components as follows:

- Store iRock Cassettes (HTBI-M, HTAI-M, HBHI-M, or HBKLI-M) at 2 - 8ºC.
- Cassettes should be stored in a clean area designated for PCR setup.

Package Contents

Cassettes are sold in batches of 10 barcodes (molecular IDs). Each shipment of 5 sets (50 cassettes) comes with an iRepertoire magnetic stand.

We currently offer **four** reagent systems on the automated iR-Processor platform:

- HTBI-M for human TCR beta, Illumina MiSeq 250 PER, covers **V** and **C** genes;
- HTAI-M for human TCR alpha, Illumina MiSeq 250 PER, covers **V** and **C** genes;
- HBHI-M for human BCR heavy chain, Illumina MiSeq 250 PER, covers **V** and **C** genes;
- HBKLI-M for human BCR kappa/lambda light chain, Illumina MiSeq 250 PER, covers **V** and **C** genes;

IMPORTANT NOTE: All systems work with RNA samples. These systems are **NOT** compatible with gDNA.

Cassette Label

The cassette has five important pieces of information: 1) the reagent system name 2) the molecular identifier barcode, which is used by the iRweb software to demultiplex pooled samples 3) the physical barcode (CID number), which is interpreted by the iR-Processor 4) the lot number and 5) the cassette expiration date. Please be sure to record the molecular identifying barcode with the sample it is assigned to on a spreadsheet (as demonstrated in Table 2). This spreadsheet data will be necessary for submission of data through iRweb.

Materials & Reagents Not Included

- Qiagen One-Step RT Enzyme (available in kit Cat No. 210212, also by contacting customer service)
- NF water
- Vortex
- Cold block or ice
- Mini-centrifuge/Mini-spin
- Pipettes
- Filter tips
- PCR 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 from sorted cells 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 from sorted cells. If using total RNA from whole blood, we recommend adding between 500-1000 ng (1600 ng maximum) of total RNA to the cassette.

RT-PCR / PCR Handling

With the use of the closed cassette, many of the contamination concerns associated with nucleic acid amplification set-up are alleviated. Nonetheless, it is important to put in place additional failsafes to both avoid cross contamination between samples and contamination of the laboratory environment with amplicons. In order to avoid contamination, we highly recommend that separate rooms and equipment be used for cassette storage, template addition, and the resulting amplified immune libraries. The use of a hood equipped with a UV light is recommended for template and enzyme addition step. **Once the template and enzyme have been added to the cassette, ensure that the cassette is closed by sealing the black cap prior to adding template and enzyme to another cassette.** *Do not lock the cassette.* **It is important not to handle cassettes of the same barcode at the same time.** Once the cassette has finished, open the cassette to retrieve the product in a separate room from PCR set-up. After the library has been retrieved, lock the cassette and dispose in an appropriate biohazard container.

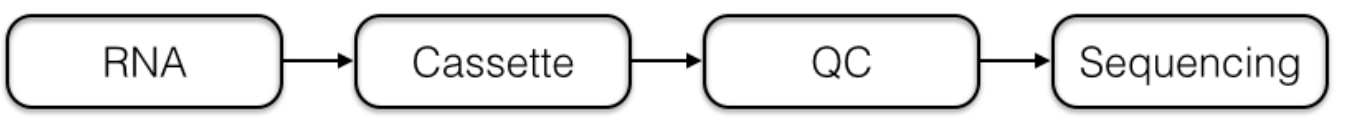
Elution of PCR product from Magnetic Beads

The final step prior to quantification is the elution of the barcoded library from the magnetic beads in the cassette. **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 (maximum for reaction: 1600 ng RNA).

Outline of the Procedure



The cassette performs the arm-PCR method, which includes two rounds of PCR reactions, PCR1 and PCR2. After PCR, the cassette will remove primer-dimers automatically. RNA should be used as a template. If gDNA is used, then there will not be any amplification due to a large intron gap between the J and C genes.

Outline of the Procedure continued

When preparing to begin the procedure, please follow the below outline.

- ☑ Read the "**iR-Processor: User Manual for Setting Up and Loading Script files on the iR-Processor™ v012016"** and complete set up of the Processor.
- ☑ Read the iRock manual prior to beginning.
- ☐ Fill out the sample spreadsheet, which can be found in Table 2.
- ☐ Follow the "**Cassette Preparation"** protocol.
- ☐ Add the template-water-enzyme mixture by following the "**Sample Preparation"** protocol.
- ☐ Load the cassette onto the processor by following the "**Running A Cassette"** protocol.
- ☐ Elute the amplified and purified library by following the "**Elution of Library"** protocol.
- ☐ Check the concentration of the amplified library.
- ☐ Pool libraries of different molecular IDs together for QC and sequencing. For a discussion on pooling of libraries, please visit www.irepertoire.com at "Document Center > Manuals" to download the Pooling Samples Guide.
- ☐ Submit pooled libraries for QC and sequencing on the Illumina MiSeq platform using 250 paired-end reads.
- ☐ Please fill out a data submission form, which can be found at www.irepertoire.com at Document Center > Forms > Data Submission Form. Instructions for data submission can be found on the data submission form.
- ☐ Submit the raw data from the Illumina MiSeq to iRepertoire. Data can be shared with iRepertoire using Illumina Basespace (share with info@irepertoire.com), via ftp, or by mailing a disk. Further instructions will be automatically e-mailed to you once the data form has been submitted.

I. Setting Up the iR-Processor

Before Beginning

Detailed instructions for setting up the iR-Processor may be found in the "**iR-Processor: User Manual for Setting Up and Loading Script files on the iR-Processor™ v012016**." These instructions should be adhered to, but a brief summary is provided below.

In addition to the iR-Processor, an Acer netbook (or similar), a Kvaser Leaflight cable, a CAN Bus Terminator, and appropriate power cords are included in a separate box in the shipment (see Figure 1 for a detailed diagram). These are actually not needed to run cassettes immediately. **All processor scripts are preloaded onto the iR-processor,** which reads the physical barcode i.d. on the cassette top. The iR-processor then runs the script instructed by the physical barcode. Therefore, after the iR-processor is powered on, it is ready to accept cassettes. The Kvaser Leaflight cable, the CAN Bus Terminator, and the netbook are required if an iR-Processor update is necessary. In the event of an update, please read and follow the instructions in the document entitled "Loading Script Files on the iR-Processor v012015".

Method

1. Unbox the iR-Processor and place it upright on a sturdy bench top following the instructions provided in the "**iR-Processor: User Manual for Setting Up and Loading Script files on the iR-Processor™ v012016**."
2. Plug the 115 V AC cable provided with the iR-Processor into the back of the iR-Processor and into an appropriate outlet.
3. Turn the iR-Processor on by switching "I / 0" power switch to the "on" position "I".
4. Once powered on, the blades will flash orange and then turn to green once they are ready to accept cassettes.
5. The iR-Processor is random access. This means that the blades run independently of one another and can be started at different times.
6. All cassette scripts are pre-loaded onto the iR-Processor. The scripts are pre-associated with the physical barcode adhered to the top of an iR-cassette.
7. After preparing the cassettes (see "Running A Cassette"), simply insert the cassette with the cassette label end facing the user into the iR-Processor until it clicks into place. **Do not try to force a cassette into the processor; it should slide in relatively easily.** If not, check the orientation.
8. The light inside that specific processor blade should turn to orange and the pipette inside the cassette will begin to move. This indicates that the cassette is running.
9. If for any reason you need to eject the cassette, turn off and on the processor by toggling the power switch (see Figure 2). **Warning:** all cassettes currently running will be ejected.
10. Close the dust cover after you have begun running your cassettes.
11. The approximate run time is about 7 hours. This includes two rounds of PCR and magnetic selection of both the PCR1 rescue and the primary product immune library.

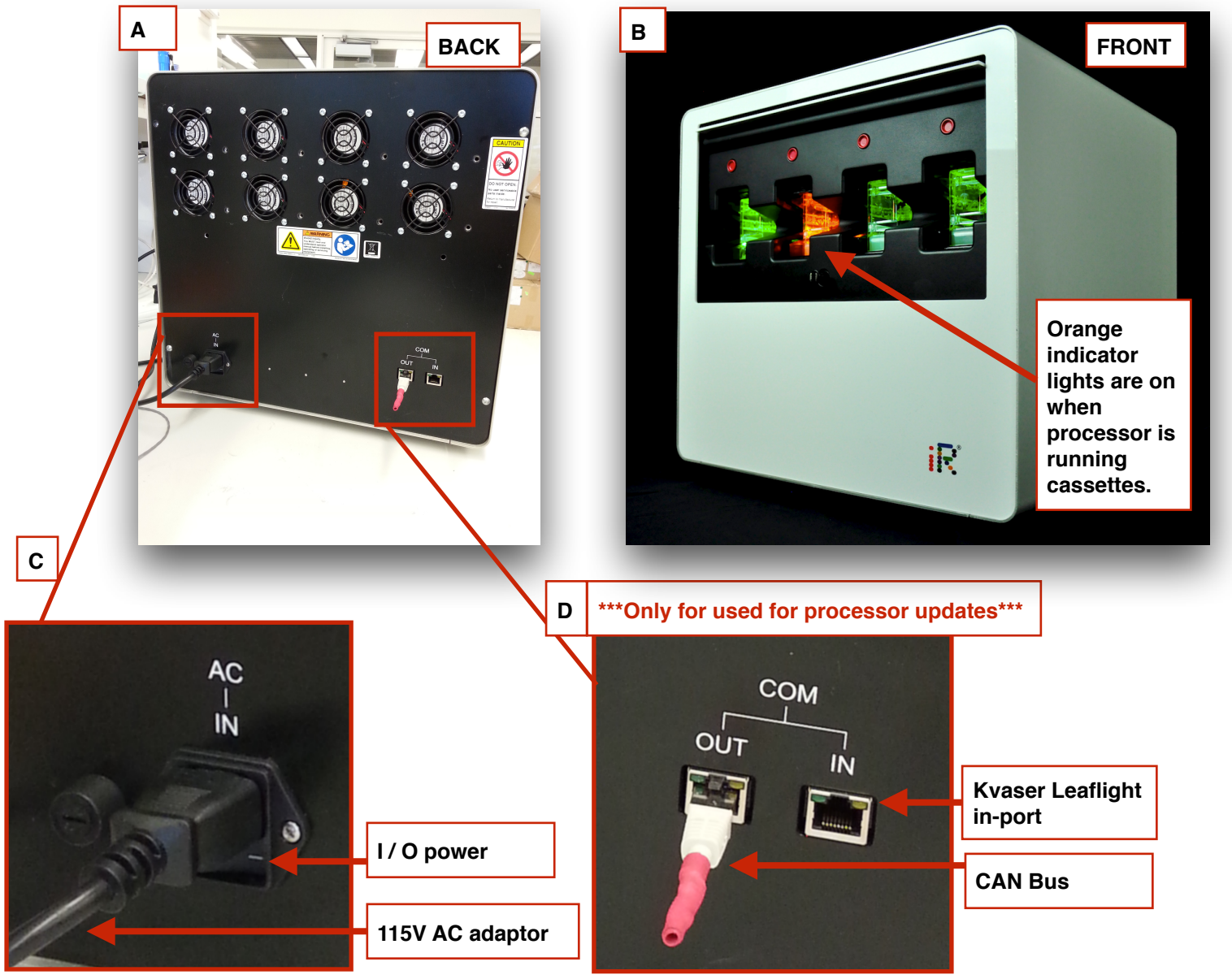


Figure 1. 1A. The back of the iR-processor ™. 1B. The front of the processor. Note: the orange indicator lights for operation. 1C. The 115V AC adaptor power cord and the I / O power switch. 1D. The Kvaser Leaflight in-port and CAN Bus Terminator port. THESE ARE ONLY NECESSARY FOR UPDATING SCRIPTS, and can do not need to be plugged in for regular operation of the instrument.

I. Sample Preparation

Before Beginning

Ensure the PCR setup area and pipettes are clean and free from contamination. Qiagen® One-Step RT-PCR reagents (particularly the reverse transcriptase) are temperature sensitive. Keep templates and enzyme on PCR cooler/ice until ready to load into the cassette.

Method

1. Review Table 1 and make sure that the sample sheet (Table 2) is complete.
2. Take out only cassette barcodes associated with the completed sample sheet of the 4ºC storage.
3. Flick the RNA template tube and the RT enzyme and quick spin.
4. Prepare the template, water, and enzyme mix just before ready to load into the cassette. For best results, keep the template-water-enzyme mix on ice/cold block until ready to load.
5. Proceed to the "**Running A Cassette**" portion of the manual in the next section.

Table 1: Sample Set-up example for iRock 2.0.

Reagent	Volume (µL)	A Sample Reaction
Nuclease-free H_2O*	X µL	8.75
Template**	Y µL	9.65
Enzyme	**1.60**	**1.60**
Total	20.00	20.00

**Amount of H_2O is dependent on the template concentration and should be added to reach 18.4 µL as the final template volume (see note below). The final reaction volume added to iRock 2.0 cassettes is 20 µL.*

***Template volume may vary from 1 - 18.4 µl. 100 ng **minimum** of template RNA is recommended when using extracted nucleic acid from **sorted** cells. If using total RNA from whole blood or tissue, then 1000 ng is recommended (maximum of 1600 ng). Adjust the volume with nuclease-free H_20 accordingly. The template amount must be determined empirically. To avoid contamination, apply the template in a separate designated area.*

Table 2: Sample Set-up Sheet

Sample Number	Sample Name	Barcode (Molecular ID)	Input RNA Concentration (ng/µL)	Desired RNA Amount (ng)	RNA template amount (µL) max: 18.4 µL	H_2O (µL)	Qiagen® One-Step RT Enzyme (µL)	Total Reaction Volume (µL)†
Ex	NEC-01	HTBI-M-01	126.2	500	3.96	14.44	1.6	20
1							1.6	20.00
2							1.6	20.00
3							1.6	20.00
4							1.6	20.00

†The total reaction volume (µL) should be equal to 20.0 µL for all reactions.

II. Running a Cassette

Method

1. Ensure the iR-Processor is connected to an appropriate power outlet and all blades are back-lit with a green light.
2. Remove the cassette from the 2 - 8ºC.
3. Vortex the cassette for 3 pulses of 4 seconds each by holding the cassette over the vortexer in the center.
4. *Gently* tap the cassette on the bench top 10 times to remove air bubbles from the bottom of the wells.
5. Bring the reagents to the bottom of the cassette by gentling applying a quick flick of the wrist to the cassette with a sudden stop. This is effective for bringing the volume down and removing air bubbles. Without turning the cassette upside down, look underneath through the clear plastic to make sure the fluid is at the bottom of the wells without any air bubbles between the bottom of the well and the fluid.
6. If air bubbles are present or reagent is stuck at the top of the well, repeat steps 3-5.
7. Using a separate pipette tip for each sample, add 20.0 µL of template-water-enzyme mix to the cassette PCR well. Dispense the loading template-water-enzyme mixture into the PCR well to the first stop of the pipette. Do not go to the second stop. Doing so could introduce an air bubble into the bottom of the PCR well.
8. Seal the cassette by closing the black cassette cap. *Do not slide the clear cap sealer over the black cassette cap to the locked position.* If the cassette is locked, then it will be difficult to retrieve the final product for sequencing.
9. Slide the cassette into the processor with the barcode side closest to the user. The cassette will lock into place and the light color will change from green to orange. The cassette is running.
10. The cassette run time is approximately 7 hours. This time is approximate, therefore, the cassette may finish slightly before this time.
11. When the cassette processing is complete, it will eject from the iR-Processor slot. Each cassette ejects when its processing is complete.
12. It is recommended to remove the PCR product from the bead as soon as possible, but it can be left on the bead for up to 12 hours. Please note that leaving the PCR product on the bead can make it more difficult to retrieve the purified product.

III. Elution of Library

Method

1. Carefully remove the cassette from the iR-Processor and place on a flat, steady surface.
2. Carefully, and without inverting the cassette, look underneath the cassette to see if there is a bead pellet (See Figure 2). If there is not, please refer to Appendix C and contact iRepertoire at info@irepertoire.com.
3. Open the black cassette cap.
4. Aspirate (pipette up) 37 μL of nuclease-free water using a sterile pipette tip but do not dispense into the PCR well containing the bead pellet of the cassette immediately.
5. Without inverting the cassette, look underneath the cassette and into the PCR well. Attempt to push the bead down to the bottom of the well using the pipette tip and then dispense the water from the pipette tip into the PCR well on top of the bead.
6. Gently pipette up and down 30 times to fully incorporate the beads into the elution water. The elution water should be an even rusty color throughout. There may be a few clumps, but the bead should be mostly resuspended.
7. Incubate the cassette at room temperature for 2 minutes.
8. Place the cassette on the iRep magnetic stand. Ensure that the PCR well is fitted onto the slot with the magnet before proceeding (see Figure 3).
9. Slowly aspirate the supernatant from the PCR well without removing the cassette from the magnetic stand and place into an appropriately labeled PCR tube (not provided). Ensure that the beads remain in the cassette's PCR well and do not transfer to the PCR tube.
10. Lock the cap when finished retrieving product. Discard the cassette in an appropriate biohazard container.
11. Proceed to "**Determining if Library Generation was Successful**"

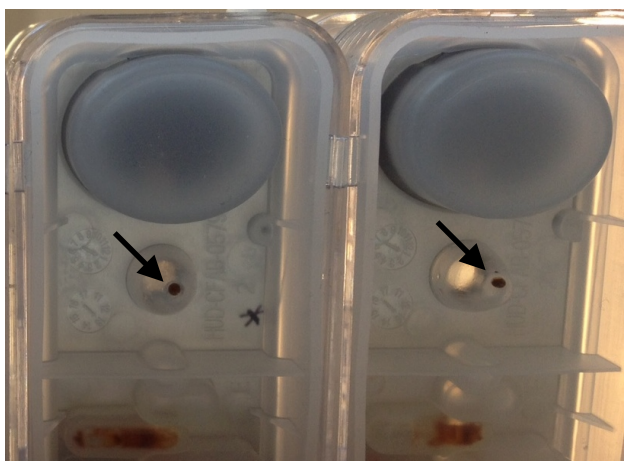


Figure 2: Picture of bead pellet in PCR well after cassette processing has completed.

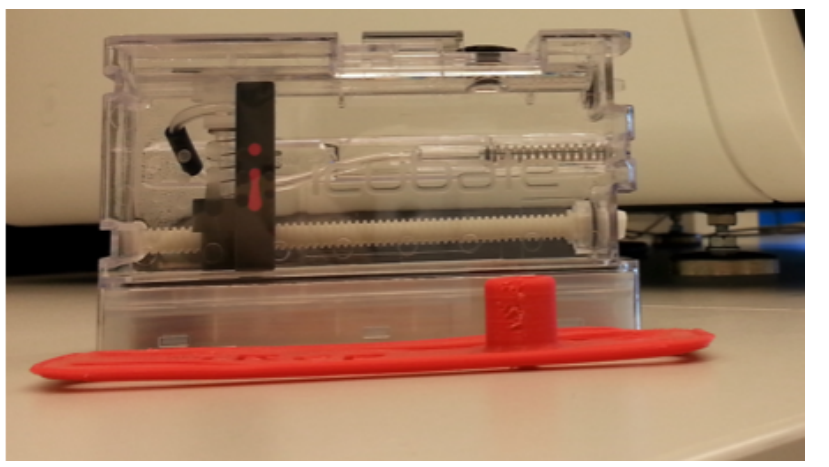


Figure 3: Orientation of iRep magnetic stand and the cassette.

IV. Determining if Library Generation was Successful

1. In all cases, it is advisable to check the amplification on 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 using iRock 2.0 is 20 ng/μL with an input of 1000 ng of total RNA and an elution of 37 μL of nuclease-free water when measured with a Nanodrop. The ranges in Table 3 may not apply if using another method of concentration determination such as a Bioanalyzer.
2. If the concentration is greater or equal to 10 ng/μL when eluted in 37 μL of nuclease-free water, then the run is considered a success.
3. If the concentration is between 4 and 10 ng/μL, then check for a major product by running some or all of the amplicons on a 2% agarose gel to see if amplification was achieved. If all product was run, gel purify the major band.
4. If the concentration is less than 4 ng/μL, then typically the run is not considered a success (See Table 3). However, it is still advisable to check the amplification on an agarose gel as a major product may be present.

Table 3: Concentration results to determine success

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

Amplicon Size Range and Primer-dimer Removal

Each amplified repertoire represents a large number of sequences distributed over a size range. The size range is dependent on the BCR or TCR type and the primers used in the amplification. The PCR product of the Illumina heavy chain BCR library (HBHI-M) is around 530 bp, ranging from 490 - 570 bp. The PCR product of the Illumina TCR beta library (HTBI-M) is around 520 bp, ranging from 500 - 540 bp. On a 2% agarose gel, the major product of these libraries appear at the 500 bp mark as demonstrated in Figure 4.

One of the benefits of using the iR-Cassette™ for immune repertoire amplification is that primer-dimers are removed automatically and gel purification is not required for this step. If the concentration of the amplified library is between 4 and 10 ng/μL, then gel purification might be necessary to ensure that the major product is present. If primer-dimers are present, they typically appear around the 150 bp marker. Figure 4 shows a 2% agarose gel where the amplification was performed both manually and on the iR-Processor with primer-dimer removal.

The situation depicted in Figure 4 is the most likely scenario if amplifying a library that produces primer-dimers. In this case, the cassette can efficiently remove these artifacts. However, there is a limit to the amount of primer-dimers which can be removed by the magnetic beads in the cassette. A run with no RNA template results in primer-dimer production only. After bead selection, the concentration of the product (or "primer-dimers") is on average 3 ng/μL. Therefore, this run would be considered unsuccessful.

Table 4: Size ranges (bp) of the PCR product of a BCR and TCR library for Illumina MiSeq.

	Amplicon Size Range (bp)
HBHI-M	490 - 570
HBTI-M	500 - 540

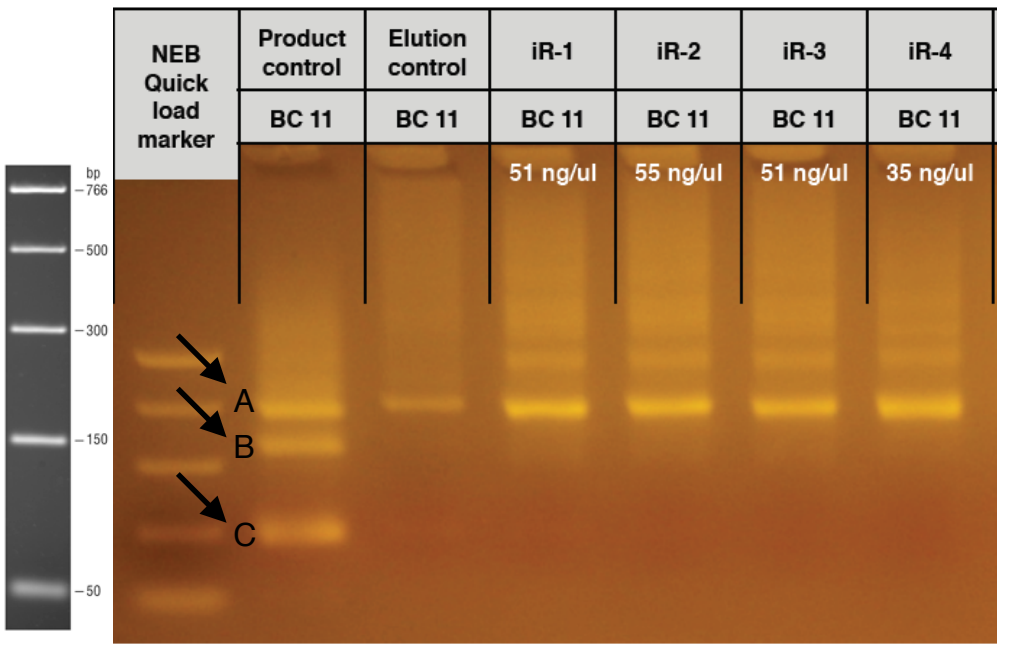


Figure 4: Sample agarose gel output for amplicons from both manual amplification (product control and manual elution control) and automated library preparation with primer-dimer removal on the iC-System (blades iR-1, iR-2, iR-3, and iR-4).

A: Major product. B: Non-specific band. C: Primer dimers.
Gel ladder is not to scale.

High Throughput Sequencing

After going through QC, the Illumina MiSeq Reagent v2 500 cycle sequencing kit (MS-102-2003) can be applied directly to the library constructed by the iRock cassette due to the built-in adaptor sequence in the library (see “Appendix A” for more information). Up to 10 libraries can be pooled together to reduce sequencing cost on the Illumina MiSeq if library construction kits with different barcodes were applied to different 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 cassettes in their experiment will be provided with complimentary basic data analysis service. Sequence data can be shipped with a portable hard disk or an online file sharing method such as Dropbox or Illumina’s Basespace. **We suggest users backup their data before sending it to us.** Submit the raw data from the Illumina MiSeq to iRepertoire directly without any manipulation. Please fill out a data submission form, which can be found at www.irepertoire.com at Document Center > Forms. Instructions for data submission can be found on the data submission form.

Data Analysis

1. Barcode sequencing decoding
2. V, D, J, & C mapping
3. CDR 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. 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/nir/, 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.

Appendix A

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. 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 (see above). We do recommend the QC steps listed below prior to sequencing.
2. We recommend paired-end sequencing for product amplified with primers listed in this manual. 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. Note that samples sequenced in the same lane need to be labeled with different barcodes, while samples sequenced in different lanes or runs can be labeled with the same barcode (see "**Pooling Samples**").
4. Prior to processing the samples on the Illumina platform, it is recommended to spike in 15 - 30% of PhiX (sold by Illumina) per flow cell to improve the efficiency of the run. 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.

QC Prior to Sequencing

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. From this information, you can perform the PhiX spike-in, dilution, and load onto the Illumina Miseq platform for 250 paired-end reads as per the instructions provided by Illumina. If sequencing with a vendor, the vendor typically handles the pre-sequencing QC steps. As stated in the sequencing suggestions, the immune repertoire libraries are considered low diversity libraries, and a PhiX spike-in is highly recommended.

Appendix B

MiSeq Sample Sheet Wizard Workflow Parameters

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.

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”).

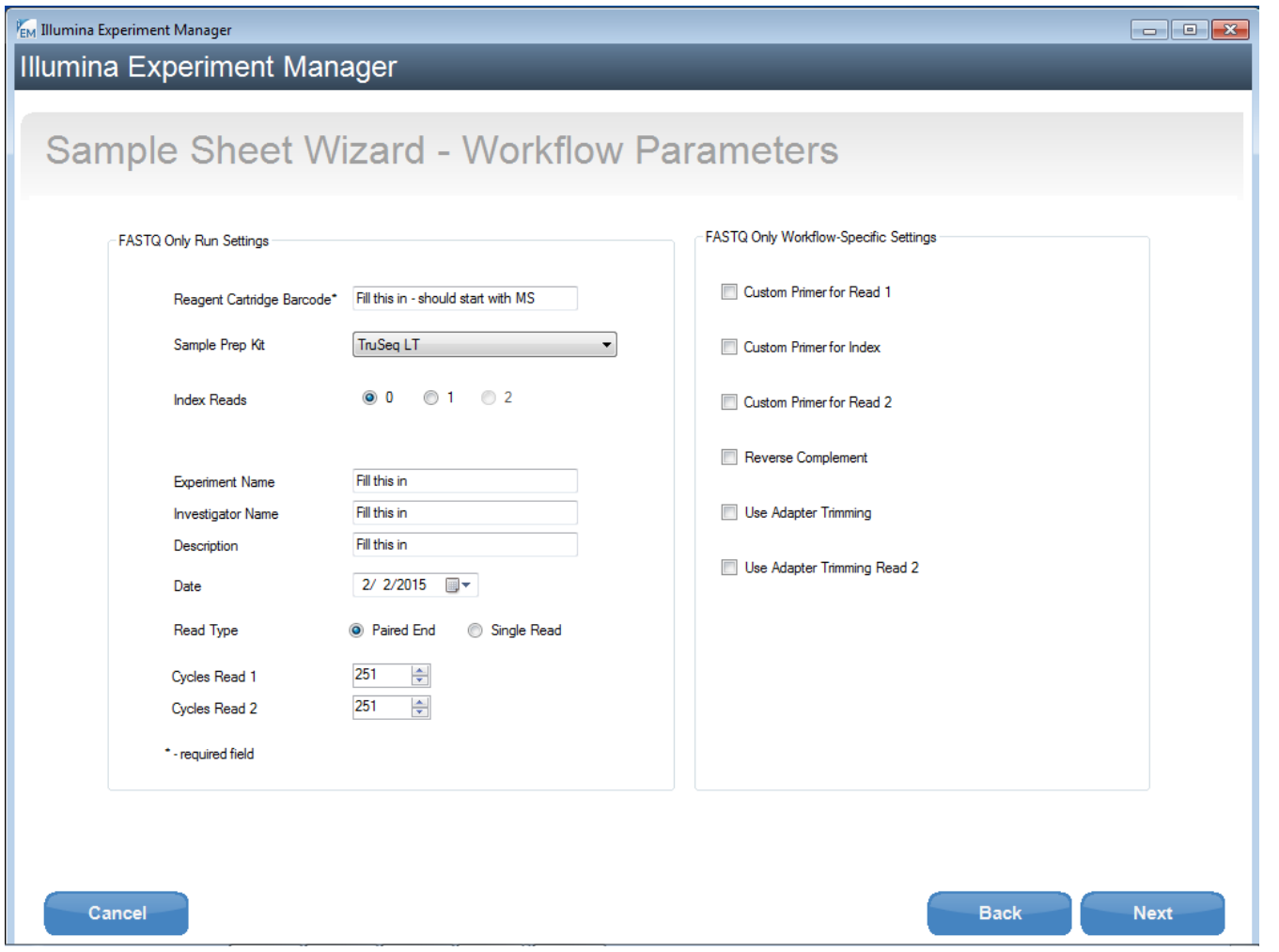


Figure 5: Illumina MiSeq Sample Sheet Wizard workflow parameter settings for running iRock reagent systems on the Illumina MiSeq.

Appendix C

Troubleshooting Issues

If you run into issues with a cassette and suspect that it is not sample related, please forward the following information to info@irepertoire.com so that we may help trouble shoot the issue to the best of our ability.

1. Were beads in the PCR loading well when the cassette finished?
2. What was the concentration of the eluted library?
3. Did you run agarose gel electrophoresis? If so, please send us the image with the gel marker labeled.
4. Please also send us a picture of the top of the cassette.
5. A picture of the side of the cassette (showing foil puncturing if possible from the iCubate branded side).
6. Pictures of the bottom wells.
7. iR-Cassette Lot #:
8. iR-Processor Blade#:
9. A copy of the master log (applies to full iC-system only).

Appendix D

iRock 1.0 Sample Set-Up

Method

Review Table 1 and make sure that the sample sheet (Table 2) is complete.

Take out only cassette barcodes associated with the completed sample sheet of the 4ºC storage.

Flick the RNA template tube and the RT enzyme and quick spin.

Prepare the template, water, and enzyme mix just before ready to load into the cassette. For best results, keep the template-water-enzyme mix on ice/cold block until ready to load.

Proceed to the “Running A Cassette” portion of the manual in the next section.

Table 1: Sample Set-up example.

Reagent	Volume (µL)	A Sample Reaction
Template*	X µL	8.75
Nuclease-free H2O**	Y µL	7.25
Enzyme	**1.60**	**1.60**
Total	17.60	17.60

*Template volume may vary from 1 - 16.0 µl. 100 ng minimum of template RNA is recommended when using extracted nucleic acid from sorted cells. If using total RNA from whole blood or tissue, then 1000 ng is recommended (maximum of 1600 ng). Adjust the volume with nuclease-free H20 accordingly. The template amount must be determined empirically. To avoid contamination, apply the template in a separate designated area.

**Amount of H2O is dependent on the template concentration and should be added to reach 16.00 µL as the final reaction volume (see above note).

Table 2: Sample Set-up Sheet

Sample Number	**Sample Name**	**Barcode (Molecular ID)**	**Input RNA Concentration (ng/µL)**	**Desired RNA Amount (ng)**	**RNA template amount (µL) max: 16.00 µL**	H2O (µL)	**Qiagen® One-Step RT Enzyme (µL)**	Total Reaction Volume (µL)†
Ex	NEC-01	HTBI-M-01	126.2	500	3.96	12.04	1.6	17.6
1							1.6	17.6
2							1.6	17.6
3							1.6	17.6
4							1.6	17.6

†The total reaction volume (µL) should be equal to 17.6 µL for all reactions.

We Find Health in Your Diversity.

WWW.IREPERTOIRE.COM

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