iRepertoire, Inc.

User Manual for RepSeq and RepSeq+ iR-Cassettes with the iR-Flex System

Automated Construction of Human TCR/BCR Immune Repertoire Libraries on the iR-Flex System for Next-Gen Sequencing with Illumina NGS Systems

For Research Use Only. Not for use in diagnostic procedures except for any components, procedures, or assays explicitly labeled otherwise.

V20200810

iRepertoire, Inc.

iRepertoire® and the iR logo are registered trademarks of iRepertoire, Inc. iR-Flex System™, iR-Flex Processor™, iR-Processor™, iR-Flex Communication Module™, iR-Cassette™ are trademarks of iRepertoire, Inc. Illumina®, HiSeq®, NextSeq®, MiSeq®, and NovaSeq® are registered trademarks of Illumina, Inc. Qiagen®, QIA®, QIAquick®, RNeasy®, and RNAprotect® are registered trademarks of the Qiagen GmbH Corporation. All other brands and names contained herein are the property of their respective owner(s).

The iR-Flex System™, iR-Flex Processor™, iR-Processor™, and iR-Cassette™ components are covered by one or more US and worldwide patents pending. Arm-PCR is covered under US Patent 7,999,092 B2. Dam-PCR is patent pending under US Patent application WO/2018/165593.

For Research Use Only. NOT FOR USE IN DIAGNOSTIC PROCEDURES except for any components, procedures, or assays explicitly labeled otherwise.

iRepertoire, Inc. does not assume any liability, whether direct or indirect, arising out of the application or use of any products, component parts, or software described herein or from any information contained in this manual. Furthermore, sale of iRepertoire, Inc. products does not constitute a license to any patent, trademark, copyright, or common-law rights of iRepertoire or the similar rights of others. iRepertoire, Inc. reserves the right to make any changes to any processes, products, or parts thereof, described herein without notice. While every effort has been made to make this manual as complete and accurate as possible as of the publication date, iRepertoire assumes no responsibility that the goods described herein will be fit for any particular purpose for which you may be buying these goods.

iRepertoire, Inc.

Version Updates

Update Classification	Update Description	Version
Minor	Updated styling	V20200810

iRepertoire, Inc.

Table of Content

Introduction	5
General Information	6
iR-Flex System Set-up	8
iR-Flex Manager Runtime Software Features	18
iR-Cassette	25
Beginning An Experiment	26
Protocol: Running a RepSeq+ Cassette	27
Protocol: Running a RepSeq Cassette	33
Protocol: Elution of Library	37
Pooling Libraries and Pre-Seq QC	40
Sequencing and Data Analysis	42
Troubleshooting	47
Appendix A: Additional Terms and Conditions	50
Appendix B: Hardware Specifications	52

iRepertoire, Inc.

Introduction

The goal of the iR-Flex product line is to provide the end-user a solution to amplify immune repertoire libraries in an automated and closed format. The iR-Flex System includes the iR-Flex Processor, iR-Flex Communication Module, a computer, and a scanner. It works in conjunction with disposable single-use iR-Cassettes and performs all of the liquid-handling steps and PCR within the iR-Cassette. This enclosed system reduces the risk for sample cross-contamination during the many steps it takes to generate both RepSeq and RepSeq+ amplicon libraries.

This handbook describes how to set up an iR-Flex System, the features of the iR-Flex Manager software, and how to run RepSeq and RepSeq+ cassettes. This manual is applicable to iRepertoire RepSeq and RepSeq+ cassettes run on the iR-Flex System ONLY (not the iC-System® or standalone iR-Processor) and should not be applied to another cassette unless explicitly stated. Please read this manual completely before beginning the experiment.

The steps to produce an immune library are straightforward. Simply combine the template RNA, the RT-enzyme, and the RT-barcode mix (for RepSeq+) and add it to the iR-Cassette PCR well. Link the cassette to the computer, insert the iR-Cassette into the iR-Flex Processor, and all of the steps are performed automatically. After amplification and primer-dimer removal within the cassette, the cassette is removed from the iR-Flex 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 amplification 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 a compatible Illumina next-generation sequencing platform (NGS).

iRepertoire, Inc.

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 or pipette up and down. 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 RNA 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 (clean, no amplicon fridge), template addition, and the resulting amplified immune libraries. Do not use the same equipment for template processing as amplified library. For instance, do not use the same centrifuge for RNA extraction and amplified library. 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 until all processing and library retrieval is complete. **It is important not to handle cassettes of the same barcode at the same time.** Once the cassette has finished its run in the processor, open the black cap on top of the cassette to retrieve the product in a designated area for high concentration amplicon. After the library has been retrieved, lock the cassette and dispose in an appropriate biohazard container.

Elution of PCR product from Magnetic Beads

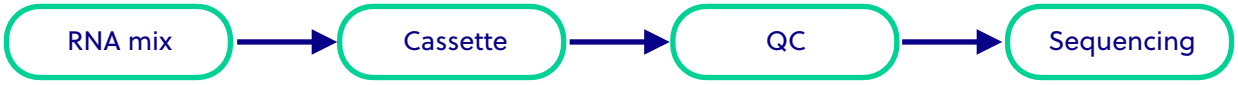
The final step prior to quantification is the elution of the barcoded library from the magnetic beads in the cassette. **Do not handle PCR products from different templates with the same barcode at the same time to avoid cross-contamination.**

iRepertoire, Inc.

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



Hardware Not Included:

- Recommended battery back-up

Labware and Materials Not Included:

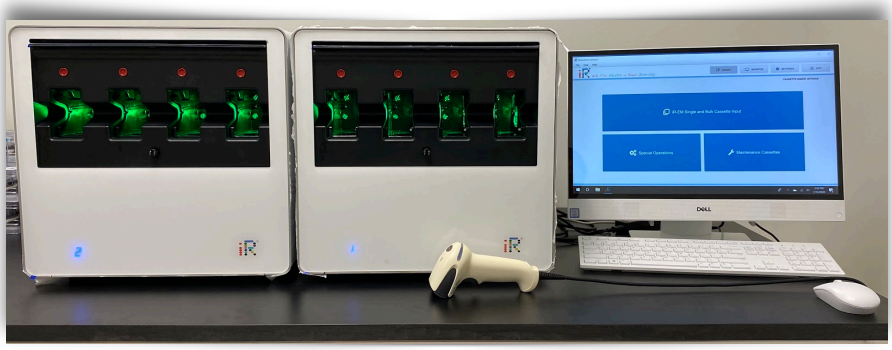
- Qiagen One-Step RT Enzyme (RepSeq kits only; available in kit Cat No. 210212, also by contacting customer service)
- NF water
- Vortex
- Cold block or ice
- Mini-centrifuge/Mini-spin
- Pipettes
- Filter tips
- PCR tubes
- Timer

iRepertoire, Inc.

iR-Flex System Set-up

Familiarizing Yourself with the iR-Flex System

The iR-Flex System consists of the iR-Flex Processor, iR-Flex Communication Module, a computer, and a barcode scanner. It also includes all required interconnect and AC power cables as further described in this manual. One system can include up to four iR-Flex Processors linked under the same operational control as described in this manual. **The computer model provided meets these minimal requirements for the iR-Flex Manager software; please note, brand and model may differ from system to system.**



Unpacking and Installation

Site Requirements: iRepertoire equipment must be located out of direct sunlight, away from heat sources and electrical interference. The site of installation should be free of excessive drafts, moisture, or dust and should not be subject to large temperature fluctuations. See the packing materials for a summary of the unpacking steps.

Use a level workbench that is large enough and strong enough to accommodate the computer, and iR-Flex Processors. Ensure the workbench is dry, clean, not subject to excessive vibration, and has additional space for accessories. For more information on the specifications refer to Appendix B.

The iR-Flex System must be placed within approximately 1.5m of a properly grounded AC power outlet. The power line to the instrument should be voltage regulated and surge protected. The iR-Flex Processor has an auto-switching power supply capable of operation at all standard line voltages (**115 VAC – 220 VAC,** 50-60 Hz, 460 W Single Phase).

Warning: To ensure that the iR-Flex Processor is disconnected from AC power, the units must be disconnected from the AC mains (power cords). Turning the power switch to the off position does not guarantee that the unit is electrically isolated from AC input power. To ensure proper ventilation and to have easy access to the iR-Flex Processor power switch, maintain a minimum clearance of 10 cm at the rear of the iR-Flex Processor unit. The iR-Flex Processor has additional ventilation at the unit bottom. Openings for ventilation must not be covered on either instrument.

iRepertoire Recommended Unpacking Procedures:

- The units are heavy and it is highly recommended that two people perform this procedure.
- Save all packing material for possible reshipment of the unit.

iRepertoire, Inc.

1) Place unit near final destination

2) Use scissors to cut banding

3) Remove packing tape. Do not slice with knife.

4) Open top of box

5) Locate all accessory boxes if applicable

6-7) Remove and save any accessory boxes if applicable

8-10) Slide outer carton up and off of the lower box floor. Save the carton.

11) Remove and save the top packing foam

12) Remove the packing tape without damaging the plastic protective

13) Slide the plastic bag down, exposing the unit.

14) iRepertoire™ recommends placing a piece of cardboard down on the work surface to help slide and position the unit once placed.

15) Tip unit back so as to get a front hand hold under the unit, then tip forward to get a rear hand hold

16) Using your legs, lift the unit straight up and out of the lower foam and box

17) Set the unit on the cardboard slide located on the work surface

18) Slide and position the unit to its final operating position and then remove the cardboard slide.

19) Save all packaging materials in case the unit needs to be returned to iRepertoire™

iRepertoire, Inc.

Diagrams of iR-Flex System Features

Figure 1: iR-Flex Processor: front view and major components

1. iR-Cassette Slot
2. iR-Cassette Slot abort push button
3. iR-Flex Processor front bezel
4. Magnetic door latch
5. Sliding dust cover door
6. iR-Flex Processor Address

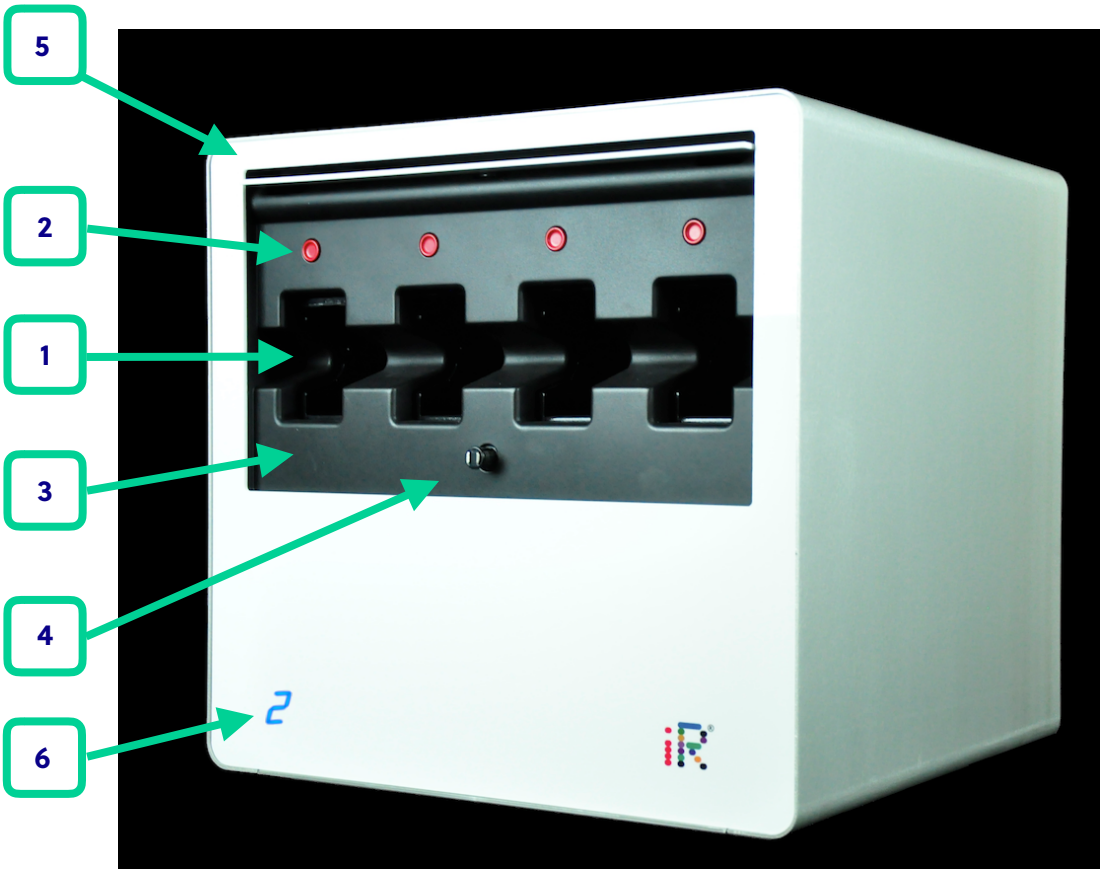
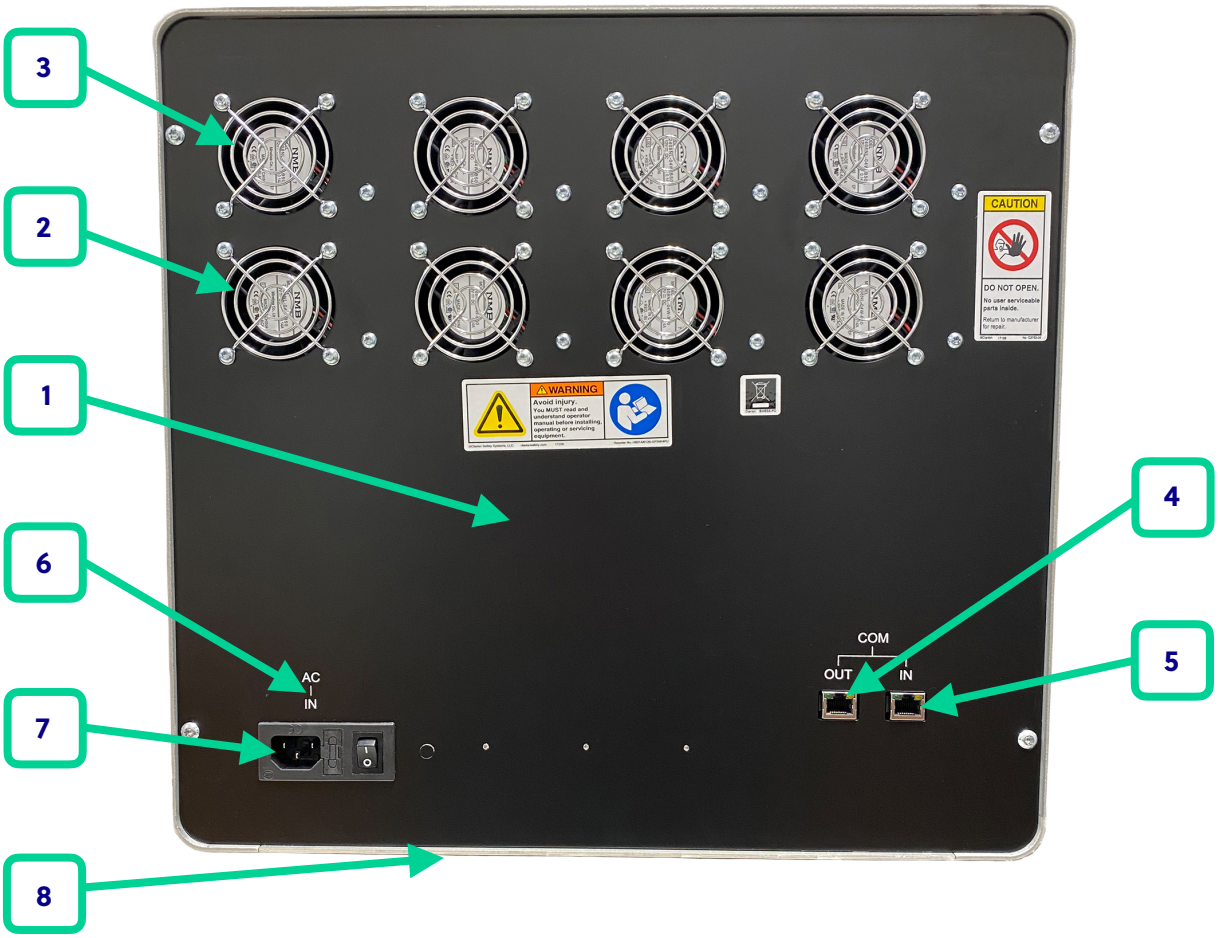


Figure 1: iR-Flex Processor: front view and major components

1. iR-Flex Processor back panel
2. iR-Cassette slot exhaust fan 2
3. iR-Cassette slot exhaust fan 1
4. Communications interface output connector port
5. Communications interface input connector port
6. AC power input connector label
7. AC power input connector
8. Bottom cooling air intake vents



Additional Features of iR-Flex Processor

Dust Cover Operation

The iR-Flex Processor includes a sliding dust cover door (**Figure 1, Item 5**) which can be retracted into the iR-Flex Processor front bezel (**Figure 1, Item 3**).

The iR-Flex Processor has a magnetic door latch (**Figure 1, Item 4**) that holds the sliding dust cover door closed when not in use and protects the iR-Flex Processor from dust and debris.

Located on the door is a red circle. This circle identifies the location of the magnetic latch hidden behind the door. To open the door, press the red circle until the latch releases. Remove pressure and, while holding the door at the edges, rotate the door up and slide it back into the iR-Flex Processor door storage slot. Once the door is slid back into the slot, you can release the door and it will be held in place by the metal disk and front bezel. The metal disk is located on the back side of the door just behind the red circle.

To close the door, lift the front edge of the door, which is located in the door storage slot so the metal disk clears the front bezel. Pull the door straight out until reaching its internal stops. Rotate the door down so it comes into contact with the magnetic latch. Press in on the red circle until you feel the latch engage. The door is now closed and will help protect the iR-Flex Processor from dust accumulation. During iR-Flex Processor operation, the door can remain open or closed.

Setting up the iR-Flex System

Method

1. Unbox the iR-Flex Processor and place it upright on a sturdy bench top as indicated in the "Quick Unpacking Guide" section.
2. Plug the provided 115 V AC cable into the back of the iR-Flex Processor (see **Figure 3** below) and into an appropriate power outlet.
3. Turn the iR-Flex Processor on by switching "I / O" power switch to the "on" position "I".
4. Once powered on, the blades will flash orange and then turn to green indicating that they are ready to accept cassettes.
5. The iR-Flex Processor is random access. This means that the blades run independently of one another and can be started at different times.
6. Find a flat surface next to the processors to start setting up the computer.
7. Locate the provided 65w-AC adaptor, this will be a black box with one end having one port for a three prong plug and the other end will have a cable already connected into the port on that end. Locate the provided three prong plug cable and connect it to the corresponding port on the 65w-AC adaptor. Plug this cable into an appropriate power outlet (see **Figure 4** below).
8. The other cable attached to the 65w-AC adaptor will plug into the back of the computer monitor in a circular port with a plug symbol under it (see **Figure 5** below).
9. Locate the provided handheld scanner and plug its USB cable into the USB port on the back of the computer monitor (see **Figure 6** below).
10. To turn on the computer, press the power button on the bottom right hand corner of the monitor.

Figure 3

Figure 4

Figure 5

Figure 6

11. To connect the processors to the computer, locate the gray USB cable and plug one end into an available USB port on the back of the computer monitor (see **Figure 7** below).
12. Locate the iR- Flex Communication Module. The iR- Flex Communication Module has two ports on one end, a round power port and a square USB port. On the other end it has a single interconnect port. Plug the opposite end of the gray USB cable from Step 11 in to the square USB port on the iR- Flex Communication Module (see **Figure 8** below).
13. Locate the black power cable and plug the power cord into the circular port next to the square USB port on the iR- Flex Communication Module (see **Figure 8** below) and plug the power cord into the proper outlet.
14. Locate the gray interconnect cable and plug one end into the interconnect port on the opposite end of the iR- Flex Communication Module (see **Figure 8** below). Plug the other end of the gray interconnect cable into the Communications interface input connector port (**IN**), which is located on the back panel of the processor (see **Figure 9** below).

Figure 7

Figure 8

Figure 9

15. Locate one of the blue interconnect cables and plug one end of it into the Communications interface output connector port (**OUT**), located on the back of the same processor that has the gray interconnect cable inserted into the Communications interface input connector (see **Figure 10** below).
16. Plug other end of the blue interconnect cable into the Communications interface input connector port (**IN**) of the next processor, which is located on the back panel of that processor shown in (see **Figure 11** below).
17. Repeat Steps 15 & 16 until all the processors are connected, and the last processor will have its Communications interface output connector port (**OUT**) not being used.
18. **Figure 12** (below) shows how the iR-Flex System should look fully set up and with all processors connected.

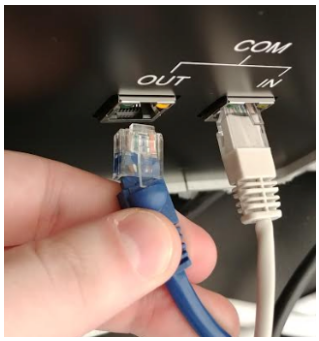


Figure 10

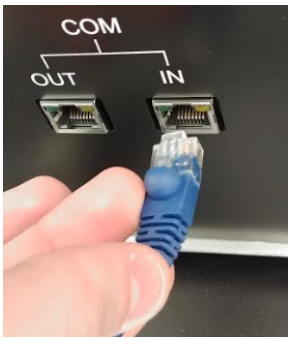


Figure 11

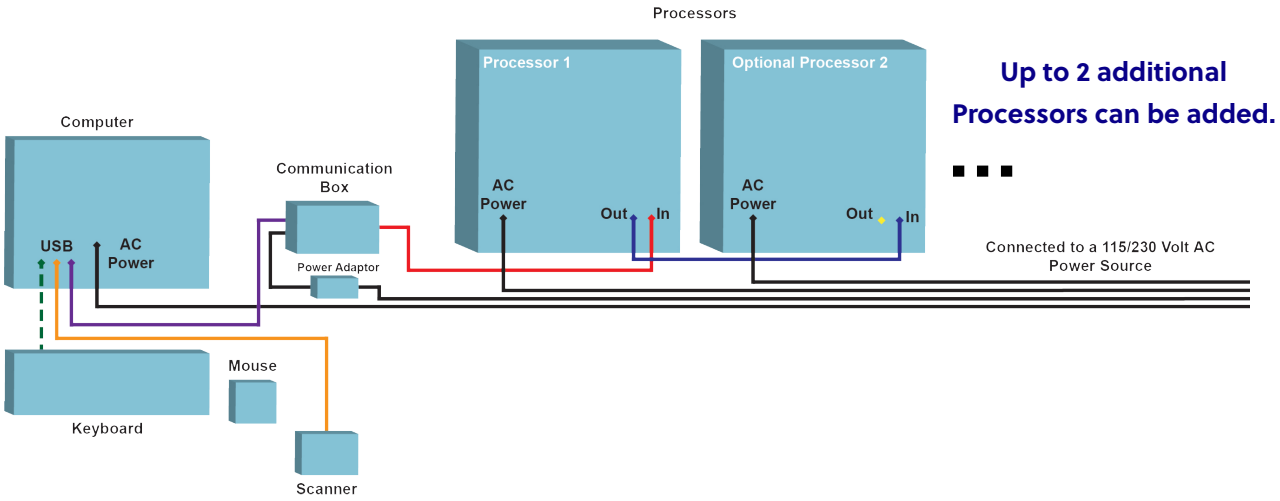


Figure 12

Installed System Self-Test Procedure

Running a Diagnostic Cassette

The iR-Flex System comes with 4 Diagnostic Cassettes. These iR-Cassettes do not contain liquid reagents. By running these Diagnostic Cassettes through the iR-Flex Processor described below, various aspects of the iR-Flex System can be tested and proper operation confirmed. If errors are detected during this test process, contact iRepertoire Technical Support at 1-256-327-0948 or by email at info@irepertoire.com.

Note: the Diagnostic Cassette must be stored at room temperature. Do not refrigerate.

1. Click on the iRepertoire icon on the desktop to open the iR-Flex Manager software.
2. Click on the iR-EM Single and Bulk Cassette Input button.
3. Click on the Single Input button.
4. With the Handheld Scanner, scan the QR Code on the top of the Diagnostic Cassette and fill in the Sample Name and ID boxes on the screen (any name will work).
5. Click Save.
6. Click OK when finished saving.
7. Insert all Diagnostic Cassettes into all iR-Cassette Slots. The iR-Flex Processor indicator light should be showing solid green.
8. Upon successful insertion and recognition of the Diagnostic Cassette by the iR-Flex Processor, the iR-Cassette Slot indicator light will change to an amber color. This indicates that the execution of the iR-Flex Processor Diagnostic Sequence will commence. The expected run time will be approximately 20 minutes. The progress of the run can be observed on the appropriate iR-Flex Processor Slot progress bar shown on the system monitor.
9. Upon successful completion of the iR-Flex Processor Sequence, the Diagnostic Cassette will eject and the iR-Cassette Slot will blink green. The Diagnostic Cassette status on the system monitor will display "Completed".
10. Remove the Diagnostic Cassette from the iR-Cassette Slot. Once the Diagnostic Cassette is removed, the iR-Cassette Slot indicator light will change from blinking green light back to solid green.
11. If an error occurs, the light on the iR-Flex Processor blade will flash red. Please refer to the "Troubleshooting" section of this manual for further instructions.
12. The Diagnostic Cassette can be reused an unlimited amount of times and does not need to be rescanned after the first use. Just insert the Diagnostic Cassette, and the Diagnostic Sequence will commence.

General Maintenance

Cleaning and Decontamination Instructions

The iR-Flex System is designed in such a way that decontamination by the user will not be necessary. If a contamination event occurs please stop all activity on the affected unit immediately, seal the affected unit in a biohazard bag and contact iRepertoire Technical Support at 1-256-327-0948 or by email at info@irepertoire.com for further instructions.

To clean the exterior of the iR-Flex Processor, use a mild detergent mixed with water and wipe the exterior with a soft cloth. Avoid the use of bleach or ethanol as these substances may cause harm or destruction of the iR-Flex System components. Before cleaning any unit of the iR-Flex System, ensure that the unit is powered off and that all cords have been removed.

Note: Do not clean the back panel as damage could occur.
Note: Do not clean the iR-Cassette slots on the iR-Flex Processor.
Note: Ensure that the unit is completely dry before plugging in any cords and powering the unit back on.

Maintenance Procedures

The iR-Flex System is designed in such a way that maintenance by the end user will not be necessary. If any of the components of the system do not operate as expected, contact iRepertoire Technical Support at 1-256-327-0948 or by email at info@irepertoire.com.

Disposal and Recycling Information

This symbol indicates that the product must be disposed of properly according to local laws and regulations. When the product reaches the end of its useful life, contact local authorities to learn about recycling options.

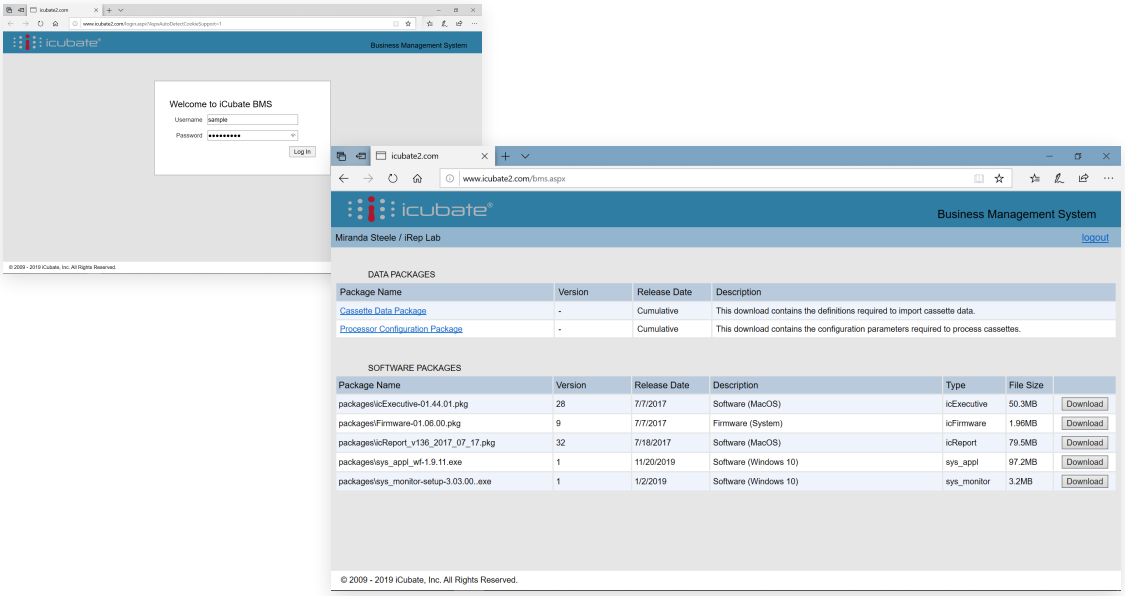
iRepertoire, Inc.

Familiarizing Yourself with the iR-Flex Manager Runtime Software Features

Downloading Firmware Updates and Scripts

The Business Management System (BMS)

The BMS allows for the download of the most recent script files and licenses necessary for operating the iR-Flex System and running an iR-Cassette. Licenses for each iR-Cassette will be released into your BMS account at the time of purchase. This is typically the first step taken prior to running real (non-Diagnostic) cassettes.



Script Downloading Guide From the Internet

Note: Do not alter the software package. Download and use directly.

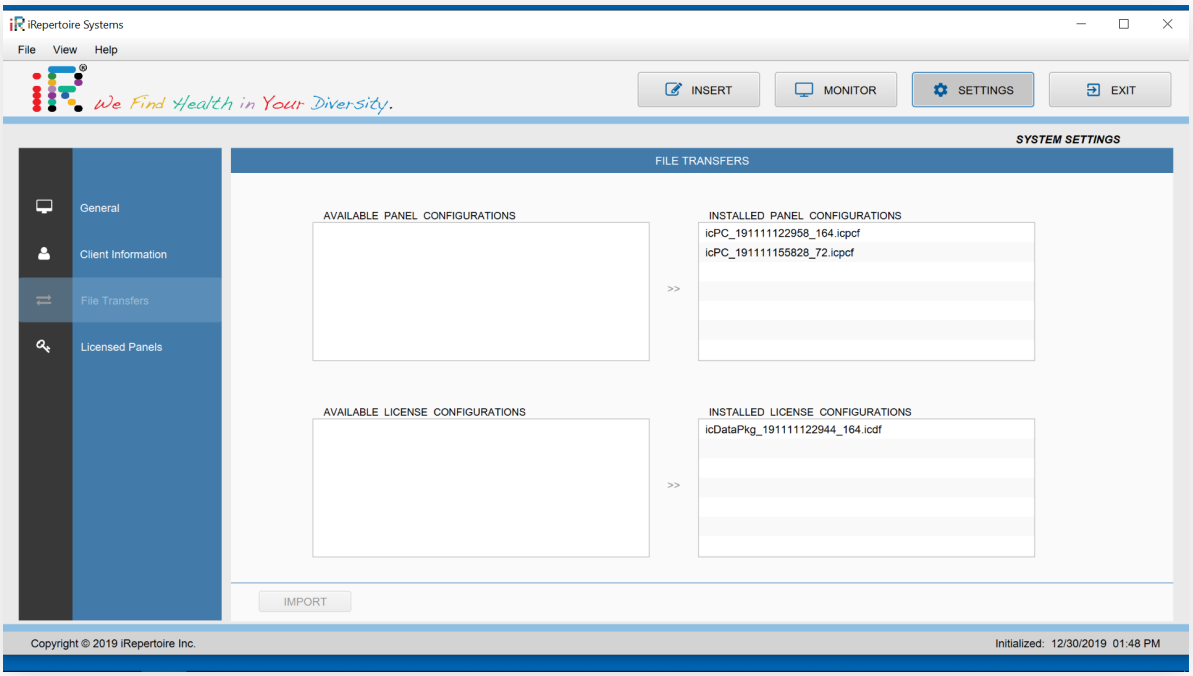
1. Click on the system application desktop icon.
2. Press the F5 key to go directly to the BMS page.
3. Type in your username and password that was issued to login.
4. Click on the cassette data package.
5. Click Save, wait a moment for it to save, once it's finished saving it will have **open**, **open folder**, and **view download** just click the [**X**].
6. Click processor configuration package.
7. Click save and wait until you have **open, open folder**, and **view downloads** just click [**X**].
8. Logout of your account and Exit the BMS page.

iRepertoire, Inc.

Installing Scripts, Licenses, and Firmware

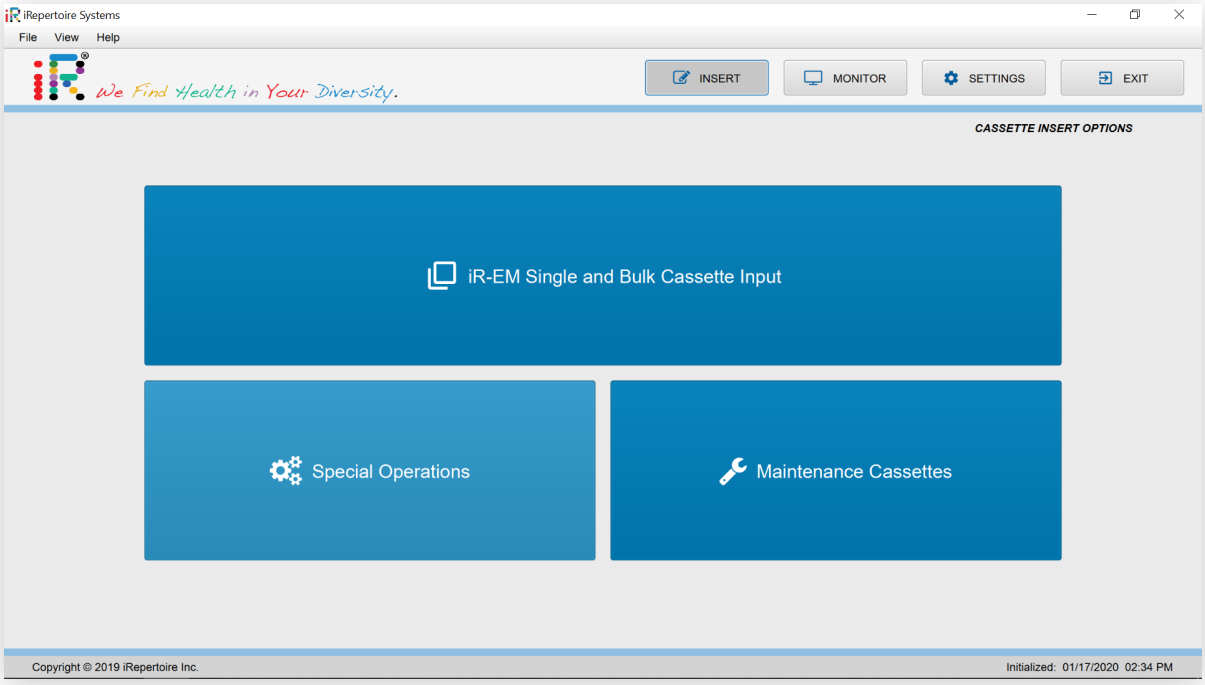
Installing Scripts, Licenses, And Firmware in the iR-Flex System

1. In the iR-Flex Manager software, click on Settings, then click on File Transfers.
2. Click on the Data package shown in panel A under available panel configurations, then click on the arrows between the two boxes.
3. Wait for the panel to install, once the panel is updated click OK.
4. Click on the license package in panel B under available license configuration, then click the arrows between the two boxes.
5. Wait for the license to be installed, once installed click OK.
6. Click Licensed Panel on the left to view the panels that were downloaded.
7. To double check the license total number go to the General tab under Settings.



Single Input versus Bulk Input

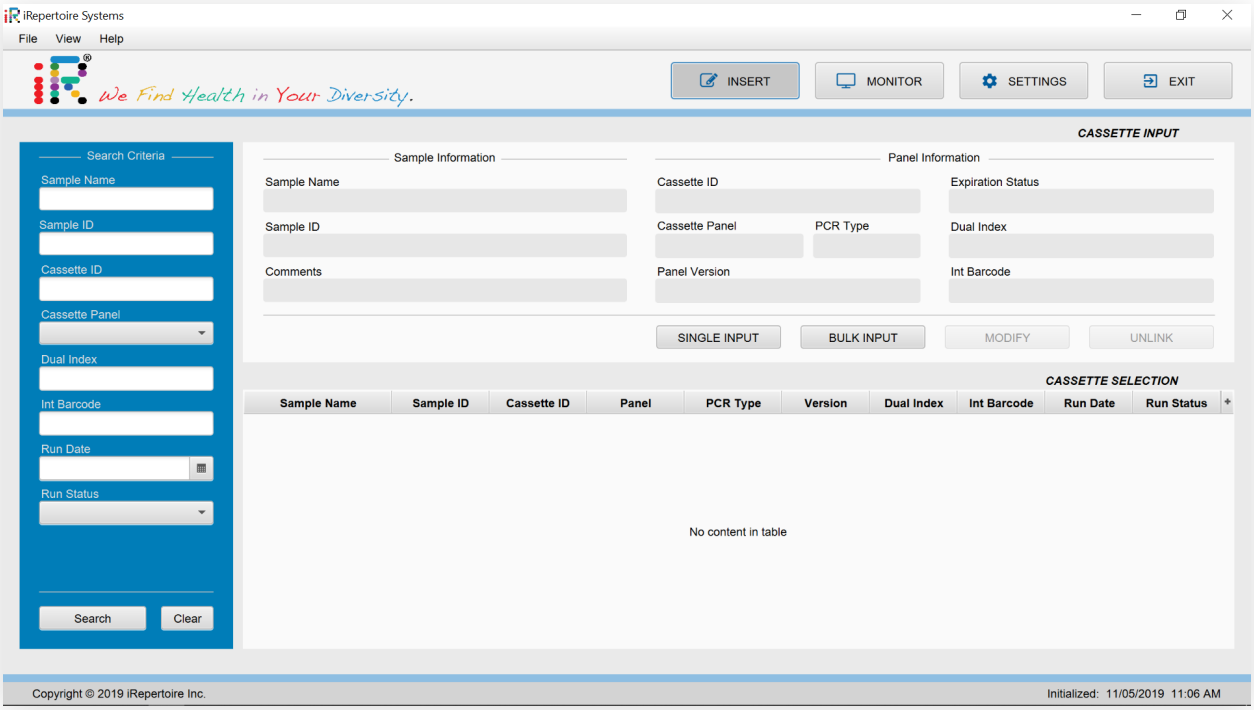
The purpose of single input is to allow input of a small number of cassettes into the system. Bulk input can be used when you are planning to run many cassettes at one time. Data entry into the system under these circumstances can be lengthy. The bulk input option allows you to avoid long wait times for loaded cassettes (ie, sample and RT-enzyme are loaded) by bypassing the data entry step. An additional benefit of bulk input is that the process is faster than loading them one by one like when using single input. For bulk input, an excel template and/or iR-Experiment Manager software compatible with the iR-Flex Manager will be provided by iRepertoire. The generated sheet will have all the information needed to scan in the cassettes.



iRepertoire, Inc.

Single Input Cassette Instructions

1. To get started with Single Input cassette processing, click the iR-EM Single and Bulk Cassette Input button.
2. Click the Single Input button in the center.
3. Once the Single Cassette Input panel pops up, enter the sample name, sample ID and select the PCR Type from the dropdown menu (if available). The sample ID is the lab assigned ID that is used in place of using the full sample name when processing (for instance, 01, 02, 03...might be placed on the top of the tube. This can be recorded here.)
4. Scan the QR code on the cassette. The sections in gray should auto-populate on their own. Then click Save.
5. Additional cassettes with different Cassette ID's can be added the same way.
6. Once all of the desired cassettes have been entered within the iR-Flex Manager, they can be pushed into one of the blades for processing.



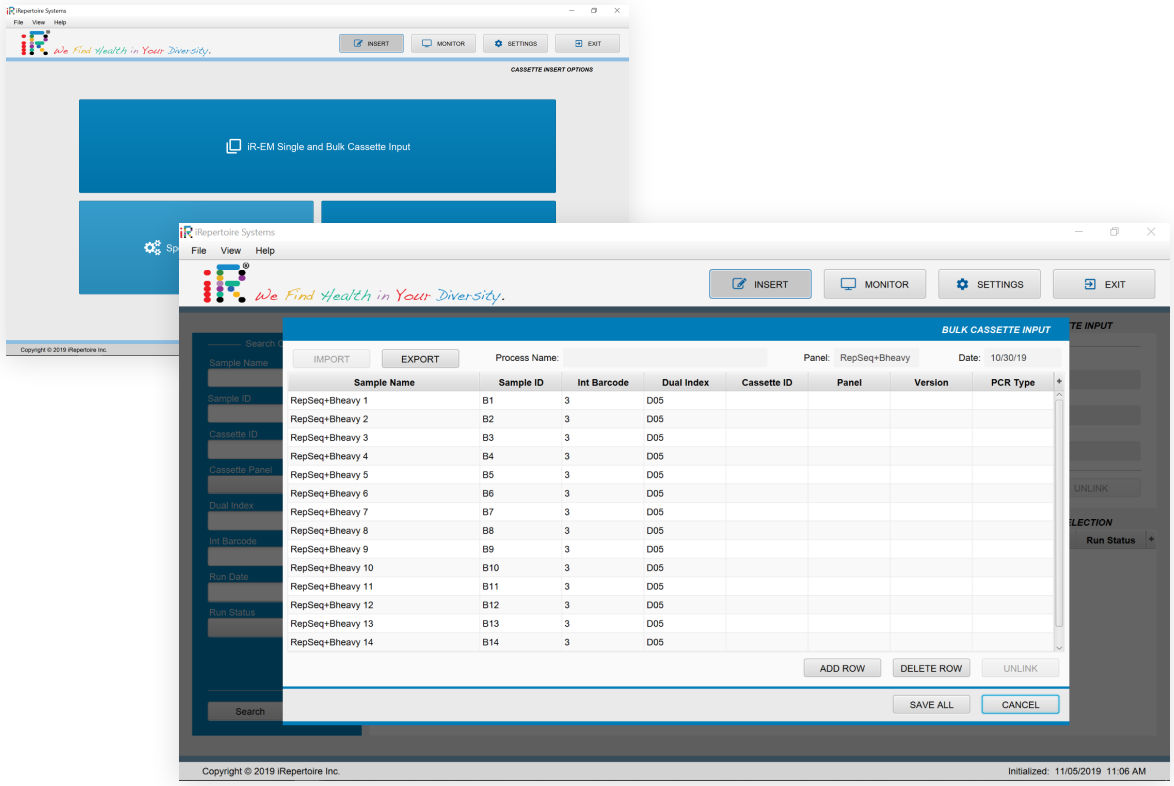
Bulk Input Experiment Manager Sheet

Prior to running a bulk experiment, the iR-Experiment Manager (iR-EM) can be used for Bulk Cassette input. The iR-EM is a separate, standalone software which can be used on your local computer during the experiment design. It associates sample names with dual index adaptor sequences. Alternatively, a spreadsheet may be designed on a computer with a compatible spreadsheet editor. Any spreadsheet editor may be used to create and edit an Experiment Manager spreadsheet but the iR-Flex Manager software only works with .cvs and .xlsx file types. Fill out the blank spots corresponding to the Investigator Name, Experiment Name, Date, Panel and Description under the Header section. Under the Data section, fill out the Sample ID, Sample Name, Internal Barcode, and Dual Index for each individual cassette that will be run during processing. Once finished, place the file into the import folder of the iR-Flex Manager software.

[Header]			
FileVersion	1.0		
Investigator Name			
Experiment Name			
Date			
Panel			
Description			
[Data]			
Sample_ID	Sample_Name	Internal_Barcode	Dual_Index

Bulk Input with iR-Experiment Manager Sheet

1. To get started with Bulk Input cassette processing, click the iR-EM Single and Bulk Cassette Input button
2. Click the Bulk Input button in the center.
3. Once the Bulk Cassette Input panel pops up, click Import, and then click on the iR-Experiment Manager file that corresponds to your bulk input plan.
4. The Bulk Input screen will pop up and will now allow you to scan the QR code on multiple cassettes corresponding to the samples entered in the iR-Experiment Manager file. If needed, more cassettes can be added by clicking on the Add Row button.
5. When finished, click Save All on the Bulk Input screen. At this point, the cassettes can now be pushed into the blades for processing.



Viewing Previously Run Cassettes

A list of previous cassettes is available in the cassette selection panel. The sidebar allows for the user to search for specific types of cassettes using a wide range of search parameters including the following: Sample Name, Sample ID, Cassette ID, Cassette Panel, Dual Index, Int Barcode , Run Date, and Run Status.

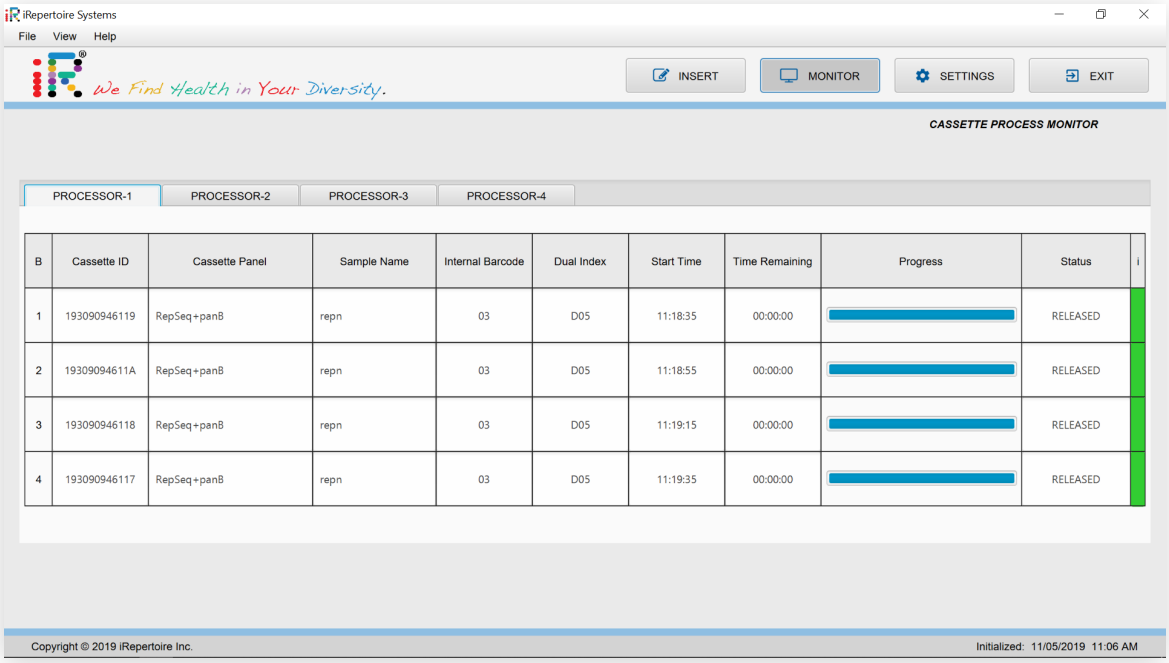
Additional Features

Modifying an Input

It is possible to modify a cassette input before it starts running. To do this, click on the desired input, click modify and change the desired values. Click Save once you are finished editing.

Monitoring a Run

Progress of a cassette can be monitored within the software and using visual cues from the iR-Flex Processor. If you wish to monitor a run, the iR-Flex Manager software gives you a basic overview of every cassette running within the processors blades. The monitor screen shows the Cassette ID, Cassette Panel, Sample Name, Internal Barcode, Dual Index, Start Time, Time Remaining, and Status for each individual blade. A progress bar is also included for blades with actively running cassettes. Prior to insertion of cassettes, the iR-Flex Processor blades should all be green. Once the run has begun, the blade color will turn orange. Once compete, the blade will turn back to flashing green. If a blade has a red light, see the Troubleshooting Guide.



Post-Cassette Run

After successful cassette completion, the blade will flash green. A successful cassette may also exhibit condensation in its interior. At this point the cassette can be eluted, this process is covered in the Elution of a Library in Cassette section of the manual. If the blade is flashing red, consult the troubleshooting guide in the troubleshooting section.

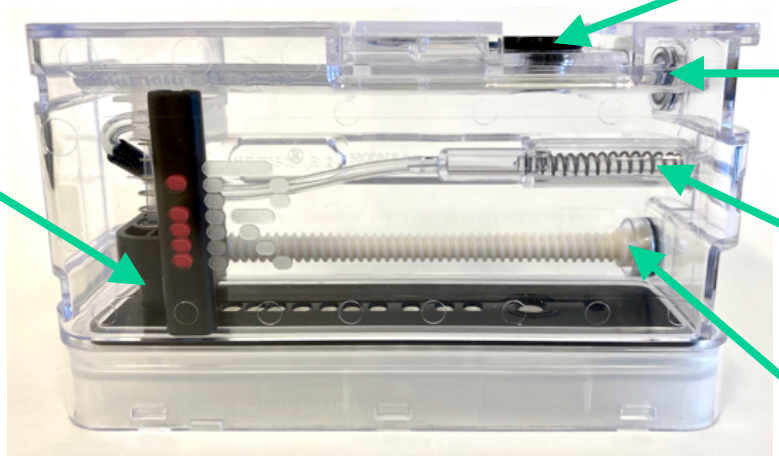
Special Operations

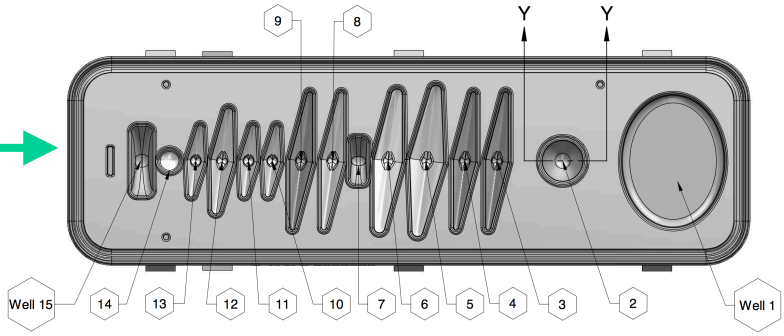
Special operations will be coming in the near future.

iRepertoire, Inc.

iR-Cassette

Cassette Layout / Guide





iRepertoire, Inc.

Beginning An Experiment

Outline of the Procedure

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

- [x] Read this manual prior to beginning.
- [ ] For first time use, set-up the iR-Flex system and run Diagnostic Cassette.
- [ ] Choose the appropriate section of this manual for the type of cassette which is to be run: RepSeq+ or RepSeq.
- [ ] Design the experiment prior to beginning. The iR-Experiment Manager can be utilized to design, track, and export iR-Flex Manager compatible files for bulk input of iR-Cassettes. Excel or similar spreadsheet software can also be used.
- [ ] Follow the "**Cassette Preparation"** protocol for the appropriate iR-Cassettes type.
- [ ] Add the template-water-RTmix-enzyme mixture by following the "**Sample Preparation"** protocol.
- [ ] Load the iR-Cassettes onto the iR-Flex 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.
- [ ] Submit pooled libraries for QC and sequencing on an Illumina platform compatible with the read length.
- [ ] Please fill out a data submission form, which iRepertoire Customer Support will provide.
- [ ] Submit the raw data from the Illumina sequencer to iRepertoire. Data can be shared with iRepertoire using Illumina Basespace (share with info@irepertoire.com), via ftp, or by mailing a disk.

iRepertoire, Inc.

Running a RepSeq+ Cassette

RepSeq+ Amplification

RepSeq+ Amplification Based on dam-PCR Technology
The dimer avoided multiplex PCR (dam-PCR) technology is a multiplex amplification strategy that allows for extensive clean-up and control of dimers introduced in multi-stage, multiplexed PCR methods (patent pending, WO2018165593A1). This multiplex amplification method greatly improves amplification signal by removing the potential for dimer formation during PCR. It also reduces bias by performing single-cycle V-tagging and extension and provides the option for superior error removal through the use of unique molecular indices or UMI. With UMI, each strand of RNA is tagged with a unique nucleotide-based label. After amplification, it is possible to collapse the effects of amplification and count individual, unique RNA species. In terms of the immune repertoire, this novel method allows for multiple immune receptor chains to be amplified in the same reaction, revealing the identity and relative clonotype frequency amongst combinations of immune receptor chains.

Template Selection: RNA vs. gDNA
The RepSeq+ cassette 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.

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.

iRepertoire, Inc.

Package Contents and Storage

Shipping & Storage

Upon receipt, store components as follows:

- Store RepSeq+ cassettes (more information in Cassette Label section) at 2 - 8°C.
- Cassettes should be stored in a clean area designated for PCR setup.
- Store the white box at -20°C upon receipt. This box includes the RT-Mix, Human RNA (positive control), and Enzyme Mix. The RT-Mix may be stored at 2 - 8°C upon receipt for convenience, if desired.

Package Contents

RepSeq+ cassettes are sold in batches of 10 barcodes (10 unique dual index molecular IDs). For every 10 RepSeq+ cassettes ordered, 11 are provided. The 11th cassette may be used as a positive control with the provided RNA. Each shipment also comes with an iRepertoire magnetic stand.

For every set of 10 unique dual index cassettes:

- 1 additional Positive Control Cassette
- 1 tube of Positive Control RNA
- 1 tube of Enzyme Mix
- 1 tube of RT-Mix

There are six different RepSeq+ cassette systems available on the automated iR-Flex system platform available: pan T, pan B, HDB (IgH, delta, beta), Tbeta (1 chain), Bheavy, and 7chain (TCR abdg, BCR IgH/KL). If you are interested in another combination of chains, please contact customer support. Presently, the cassettes are only available for human immune library generation in long read format (250+ PER/500+ cycle kit compatible); however, a short read format (150+PER/300+ cycle kit compatible) is expected to release soon. Please see the next section, Cassette Label, for more information about your specific RepSeq+ products.

Important note: All systems work with RNA samples. These systems are **NOT** compatible with gDNA.

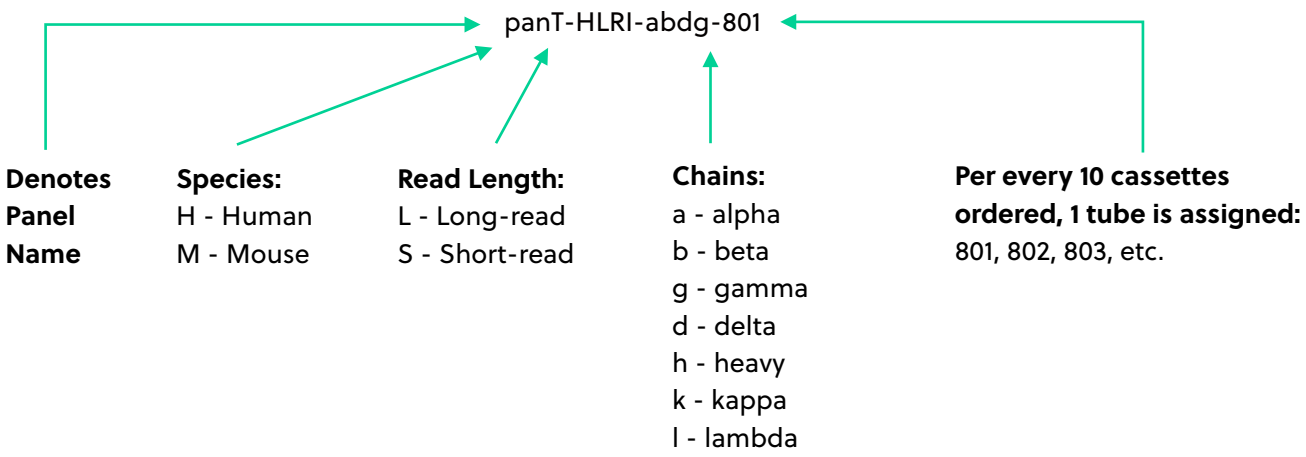
Tube Labels

There are three tubes that come with each set of 10 cassettes:

- Human (or Mouse) Ctrl RNA (10 ng)
- Enzyme Mix (25 µL)
- RT-Mix tube: 1 barcoded tube for every 11 cassettes (labeling varies dependent on the system(s) ordered, but is always 250 µL; includes positive control cassette. Always note this internal barcode sample association when running an experiment.)

iRepertoire, Inc.

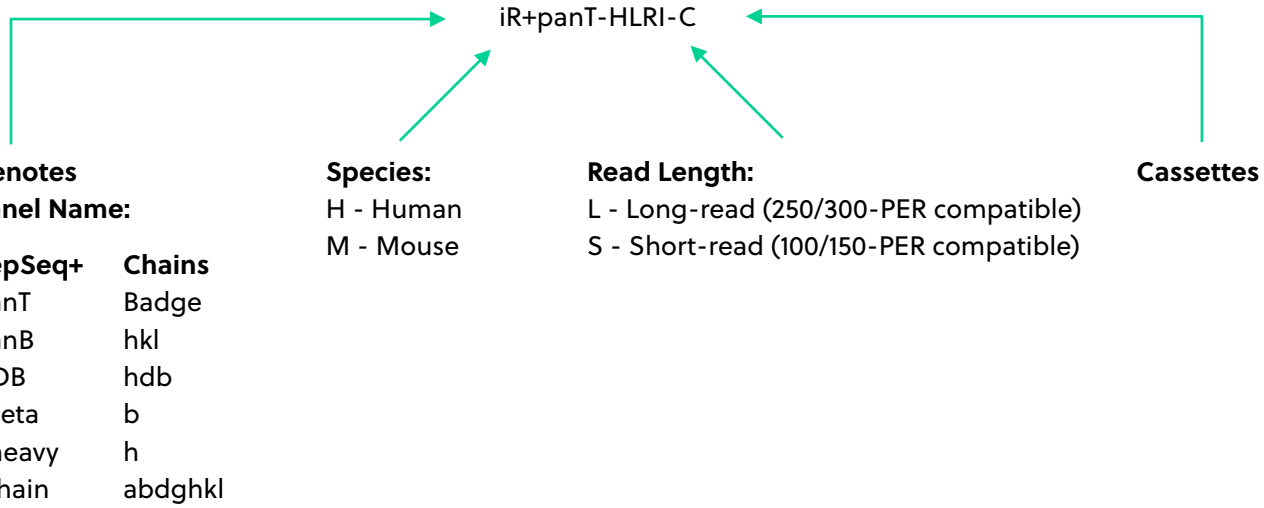
Example labeling for RT-PCR Mix:



Cassette Label

The cassette has five important pieces of information: 1) the reagent system name 2) the dual index identifier barcode, or D# or A#, which is used during Illumina dual-index sequencing 3) the physical 2D-barcode, which is interpreted by the iR-Flex Processor 4) the lot number and 5) the cassette expiration date. Please be sure to record the molecular identifying barcode (D# or A#) and the RT-Mix barcode with the sample it is assigned to on a spreadsheet (as demonstrated in Table 2). This spreadsheet data will be necessary for setting up the sequencing run and submission of data through iRweb.

Figure 1: Example of a iR-Cassette label



Sample Preparation

Method

1. Review Table 1 and make sure that the sample sheet (Table 2) is complete. Alternatively, instead of completing Table 2, use the iR-Experiment Manager (iR-EM) to setup your experiment. Files exported from this application are compatible with the bulk input feature of the iR-Flex Manager System.
2. Pre-assign the cassette Illumina dual index barcode (D#) and the RT-Mix barcode to each sample. Ensure your inventory does contain these cassette dual index barcodes prior to assigning them.
3. Thaw the RT-Mix and the sample RNA template. Vortex the RT-Mix and the RT enzyme and flick (do not vortex) the RNA template tube followed by a quick spin.
4. Prepare the template, water, RT-Mix, and enzyme mix directly before you are ready to load into the cassette. It is highly recommended that this preparation is performed in a PCR strip, per sample, to avoid any bubbles via pipetting directly into the cassette during set up. For best results, keep the template-water-RT-Mix-Enzyme Mix on ice/cold block until ready to load.
5. Proceed to the "**Running A Cassette**" portion of the manual on the next page.

Reagent	Volume (µL)	An Example Reaction
Nuclease-free H2O*	X µL	9.3
Template **	Y µL	8.7
RT-Mix	20.0	20.0
Enzyme Mix	2.0	2.0
Total	**40.00**	**40.00**

Table 1: Sample Set-up example for an iR-Harmony cassette

**Amount of* H_2O is dependent on the template concentration and should be added to reach 18.2 µL as the final template-water mix volume (see note below). This is combined with the Enzyme Mix and the RT-Mix. The final reaction volume added to iR-Harmony cassettes is 40 µL.

***Template volume may vary from 1 - 18 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.

Sample Number	Sample Name	Di number on RepSeq+ Cassette)	RT-Mix Barcode (ID#)	Input RNA [ ] (ng/µL)	Desired RNA Amount (ng)	RNA template amount (µL) max: 18.2 µL	H_2O (µL)	Enzyme Mix (µL)	RT-Mix (µL)	Total Reaction Volume (µL)†
Ex	NEC-01	D01	801	126.2	500	3.96	14.04	2.0	20	40.00
1								2.0		40
2								2.0		40
3								2.0		40
4								2.0		40

†The total reaction volume (µL) should be equal to 40.0 µL for all reactions.

Running a Cassette

Method

1. Ensure the iR-Flex System set-up process is complete and that cassette licenses and firmware updates have been downloaded to the computer.
2. Remove the cassette from the 2 - 8°C.
3. Scan the cassettes prior to adding any sample. Select the appropriate input, single or bulk input, best suited for the experiment. See "Single Versus Bulk Input" under the section "Familiarizing Yourself with the iR-Flex Manager Runtime Software Features" for more information.
4. Vortex the cassette for two pulses at least 30 seconds each pulse by holding the cassette at a 45 degree angle to the vortexer head and sliding back and forth. This step is to ensure any settled beads and reagents are well mixed prior to beginning.
5. Gently tap the cassette on the bench top 10 times to remove air bubbles from the bottom of the wells.
6. 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.
7. If air bubbles are present or reagent is stuck at the top of the well, repeat steps 3-5.
8. Using a separate pipette tip for each sample, add 40.0 µL of template-water-RT Mix-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.
9. 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.
10. 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 now running.
11. The cassette run time is panel dependent and will be displayed on the iR-Flex Manager System run time software under the "Monitor" tab .
12. When the cassette processing is complete, it will eject from the iR-Flex Processor slot. Each cassette ejects when its processing is complete, and the lights will flash green.
13. 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.
14. Proceed to "**Elution of a Library**" section of this manual.

iRepertoire, Inc.

Running a RepSeq Cassette

RepSeq Amplification

RepSeq Amplification Based on arm-PCR Technology (formerly iRock cassettes)
Amplicon rescued multiplex PCR (arm-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. Unlike RepSeq+, only one receptor chain is amplified per cassette, and umi is not included on the final product. 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, respectively, for the Illumina sequencing platforms (MiSeq, NextSeq, HiSeq, and NovaSeq) 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.

Template Selection: RNA vs. gDNA
The RepSeq cassette 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.

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.

iRepertoire, Inc.

Package Contents and Storage

Shipping & Storage

Upon receipt, store components as follows:

- Store RepSeq Cassettes (HTBI-M, HTAI-M, HBHI-M, or HBKLI-M) at 2 - 8°C.
- Cassettes should be stored in a clean area designated for PCR setup.
- Qiagen One-step RT-PCR enzyme mix or iR-Enzyme mix (must be purchased separately).

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 **six** reagent systems on the automated iR-Flex 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;
- HTBIvc for human TCR beta, Illumina MiSeq 150 PER, covers **V** and **C** genes;
- HTAIvc for human TCR alpha, Illumina MiSeq 150 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 four 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 lot number and 4) 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.

Sample Preparation

Method

1. Review Table 1 and make sure that the sample sheet (Table 2) is complete. Alternatively, instead of completing Table 2, use the iR-Experiment Manager (iR-EM) to setup your experiment. Files exported from this application are compatible with the bulk input feature on the iR-Flex Manager System.
2. Pre-assign the cassette molecular barcode to each sample. Ensure your inventory does contain these molecular barcodes prior to assigning them.
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 RepSeq Cassette**" portion of the manual in the next section.

Reagent	Volume (µL)	An Example Reaction
Nuclease-free H2O*	X µL	8.75
Template **	Y µL	9.65
Enzyme Mix	1.60	1.60
Total	**20.00**	**20.00**

Table 1: Sample Set-up example for iRock 2.0

*Amount of H2O 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 H20 accordingly. The template amount must be determined empirically. To avoid contamination, apply the template in a separate designated area.

Sample Number	Sample Name	Barcode (Molecular ID)	Input RNA Concentration (ng/µL)	Desired RNA Amount	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
2							1.6	20
3							1.6	20
4							1.6	20

Table 2: Sample Set-up sheet

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

Running a RepSeq Cassette

Method

1. Ensure the iR-Flex System set-up process is complete and that cassette licenses and firmware updates have been downloaded to the computer.
2. Remove the cassette from the 2 - 8°C.
3. Scan the cassettes prior to adding any sample. Select the appropriate input (single or bulk input) best suited for the experiment. See "Single Versus Bulk Input" under the section "Familiarizing Yourself with the iR-Flex Manager System Runtime Software Features" for more information.
4. Vortex the cassette for two pulses at least 30 seconds each pulse by holding the cassette at a 45 degree angle to the vortexer head and sliding back and forth. This step is to ensure any settled beads and reagents are well mixed prior to beginning.
5. Gently tap the cassette on the bench top 10 times to remove air bubbles from the bottom of the wells.
6. 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.
7. If air bubbles are present or reagent is stuck at the top of the well, repeat steps 3-5.
8. 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.
9. 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.
10. 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 now running.
11. The cassette run time is panel dependent and will be displayed on the iR-Flex Manager System run time software under the "Monitor" tab.
12. When the cassette processing is complete, it will eject from the iR-Flex Processor slot. Each cassette ejects when its processing is complete, and the lights will flash green.
13. 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.
14. Proceed to "**Elution of a Library**" section of this manual.

iRepertoire, Inc.

Elution of a Library in Cassette and Determining if Library Generation Was Successful

Elution of a Library in Cassette

1. Carefully remove the cassette from the iR-Flex 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 1** below). If there is not, please refer to Troubleshooting 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 2** below).
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**" section of this manual.

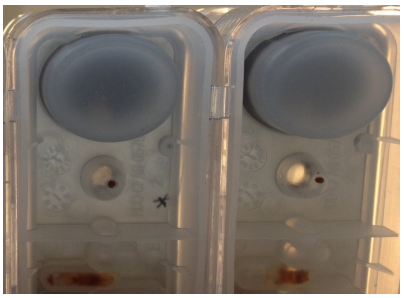


Figure 1: Picture of bead pellet in PCR well after cassette processing has completed.

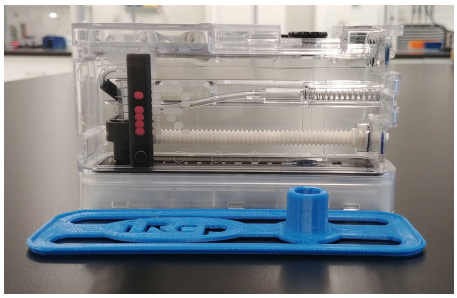


Figure 2: Orientation of iRep magnetic stand and the cassette.

Determining if Library Generation is 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 RepSeq/RepSeq+ amplified library will depend on the chain(s) amplified but is typically greater than 15 ng/μL with an input of 800 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.

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

Table 1: Concentration results to determine success

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. In general, for long read PCR products, the library size is ~500 bp, and for short read PCR products, it is ~300 bp. 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 3 shows a 2% agarose gel where the amplification was performed both manually and on the iR-Flex Processor with primer-dimer removal.

The situation depicted in Figure 3 is the most likely scenario if amplifying a library that produces primer-dimers. In this case, the cassette can efficiently remove these artifacts. Additional procedures are included within RepSeq+ cassettes to mitigate dimer and non-specific amplicon formation. 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.

	Amplicon Size Range (bp)
HBHI-M	490 - 570
HBTI-M	500 - 540
≤ 4	Unsuccessful

Table 2: Size ranges (bp) of the PCR product of a BCR and TCR library for Illumina MiSeq

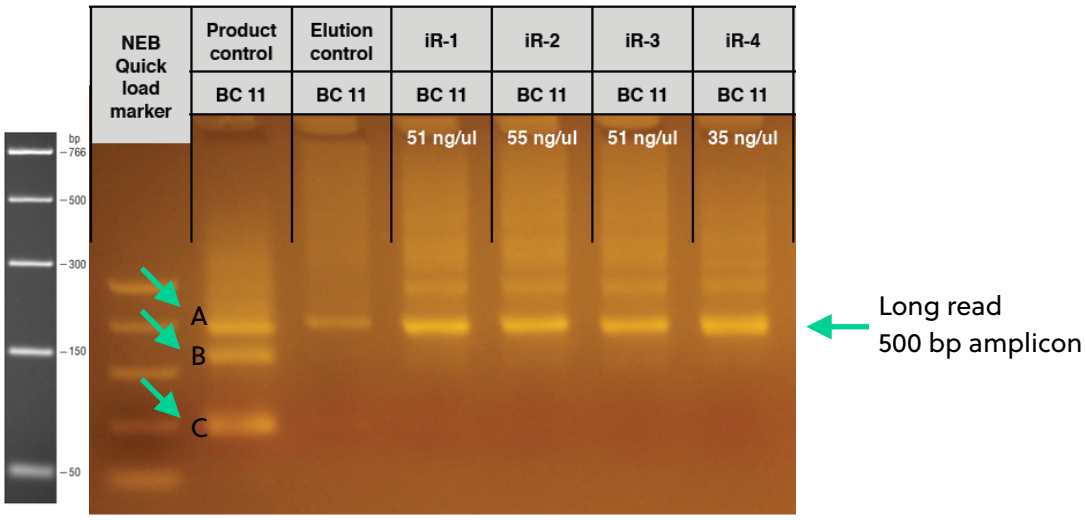


Figure 3: Sample agarose gel output for amplicons (9 μL loaded on the gel) from both manual amplification (product control and manual elution control) and automated library preparation with primer-dimer removal on the iR-System (blades iR-1, iR-2, iR-3, and iR-4). A: Major product. B: Non-specific band. C: Primer dimers. Gel ladder is not to scale.

Pooling Libraries and Pre-Seq QC

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 selection of chains utilized during amplification. This manual provides instructions for generating from one to seven libraries from ONE sample in ONE cassette including TCR alpha, beta, delta, gamma, and Ig heavy, Ig kappa, and Ig lambda. For RepSeq+, libraries all come from the same sample in one cassette, the resulting libraries will all have the same combination of barcodes. These barcodes include an internal "C-primer" barcode and a dual index PCR2 barcode. The internal barcode (800 series) is introduced during reverse transcription when the RT-mix is added to the sample RNA outside of the cassette. The second barcode pair is an Illumina dual-index adaptor pair which is introduced inside of the cassette. Each cassette is denoted with a specific "D" number, which corresponds to a specific dual-index combination. For RepSeq libraries, an internal 6 nucleotide barcode is included in each cassette. There is no dual index assignment.

Pooling libraries from multiple samples into one Illumina MiSeq, HiSeq, NextSeq, or NovaSeq flow cell reduces the overall per-library sequencing cost. However, read depth considerations must be taken into account before deciding a pooling strategy. Illumina software will automatically demultiplex the data based on the dual index information provided during the sequencing set-up (if applicable). This will produce a pair of Read 1 and Read 2 fastq files for each dual-index adaptor pair in the run. For RepSeq libraries, the run will be setup without dual index, and the libraries will be in the undetermined file if pooled with Illumina indexed libraries. Then, our software pipeline will recognize the internal barcodes on the C-primers and further decode them during analysis automatically. Please remember that in order to pool libraries for a sequencing flow cell, each sample must be amplified with a unique barcode combination (internal RT-Mix barcode plus dual index pair or unique internal barcode) prior to pooling so that the software pipeline can detect and distinguish the libraries during data analysis.

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. Determine the estimated read throughput on the sequencing platform you plan to use. Divide the expected number of reads on that platform by the number of libraries in the pool (considering each chain as its own unique library) to get an estimate of the reads per library. It is likely that upwards of 30% of raw reads will be filtered due to sequencing errors, quality scores, mapping issues, etc. We generally recommend allocating 5-10 reads per cell, so knowing the initial cell count is beneficial. 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.)

iRepertoire libraries are considered low diversity. It is highly recommended to perform a Phi-X spike-in. **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.

Pre-Sequencing QC Steps

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. We do recommend the QC steps listed below prior to sequencing.
2. Libraries can be sequenced with paired-end reads (for example, 250 cycles x 250 cycles) to cover the entire VDJ or single end read from read 1, which will cover a portion of the VDJ including the CDR3 region. This manual describes paired-end read 250 x 250. For short read systems, a 300 cycle kit may be used with paired end reads of 150 x 150.
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 flow cell need to be labeled with different barcodes, while samples sequenced in different lanes or runs can be labeled with the same barcode (see "Pooling Libraries"). The clonality of the sample is another factor which should be considered when deciding depth.
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 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. These steps can be performed on the multiplex pool of libraries. Since the qPCR step mimics the PCR on the flow cell, it is the gold standard for determining the library concentration and making the final library dilution. 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.

iRepertoire, Inc.

Sequencing and Data Analysis

Sequencing RepSeq+ on the Illumina MiSeq

MiSeq Sample Sheet Wizard Workflow Parameters: REPSEQ+
NOTE: THESE INSTRUCTIONS APPLY TO LIBRARIES WITH ILLUMINA DUAL INDEX ADAPTORS ON THE END LIBRARY (REPSEQ+). 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 RepSeq+ Cassettes **do use** Illumina dual indexing (unlike RepSeq cassettes which do not), and it is important to set up the sample sheet appropriately. On the Illumina Experiment Manager (v 1.12 or earlier) 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 HT
3. Index Reads: Select the "2" 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").

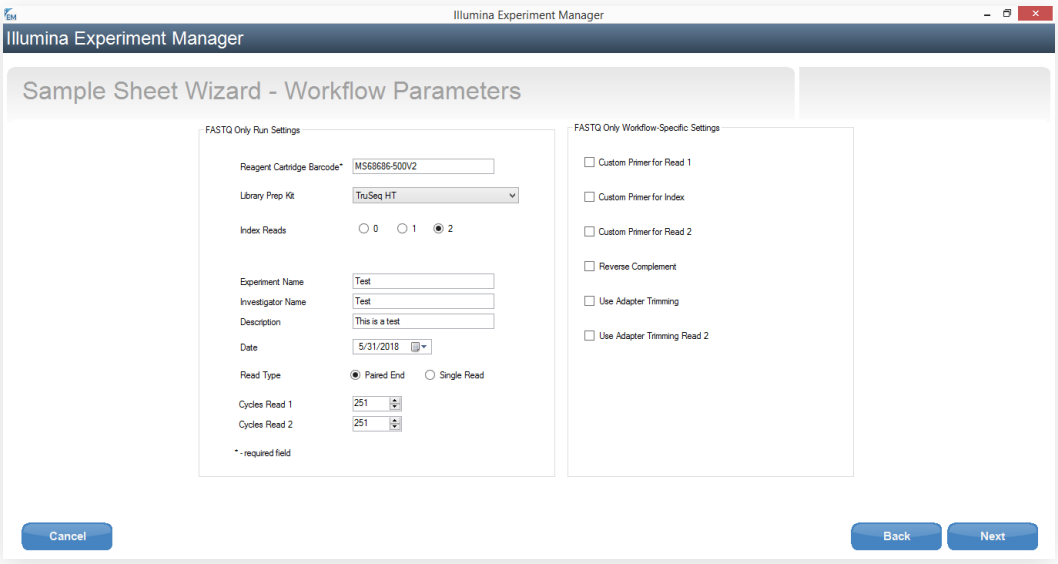


Figure 1: Illumina MiSeq Sample Sheet Wizard workflow parameter settings for running RepSeq+ cassette series on the Illumina MiSeq or libraries with Illumina dual index adaptors incorporated.

iRepertoire, Inc.

Sequencing RepSeq+ on the Illumina MiSeq

MiSeq Sample Sheet Wizard Workflow Parameters: REPSEQ+

Under the Sample Selection Sheet (after clicking next)

1. Select "Add Blank Row" and add rows to match the number of samples in your library pool.
2. Fill in Sample ID, Sample Name, and leave the fields for "Plate" and "Well" blank.
3. Select the correct index combination of i7 and i5 from the pull down matching the sample (see Table 4 below). **Note:** The index sequence will autofill.
4. Leave sample project blank and under description add the RT-Mix barcode (eg. 801).
5. Click finish and save the sample sheet. This sheet may also be used to submit the data to iRepertoire.
6. Proceed to running the instrument.

D#/A# Cassette Label	Index 1 (i7)	Index 1(i7) Sequence	Index 2 (i5)	Index 1(i5) Sequence
D01	D701	ATTACTCG	D501	TATAGCCT
D02	D702	TCCGGAGA	D502	ATAGAGGC
D03	D703	CGCTCATT	D503	CCTATCCT
D04	D704	GAGATTCC	D504	GGCTCTGA
D05	D705	ATTCAGAA	D505	AGGCGAAG
D06	D706	GAATTCGT	D506	TAATCTTA
D07	D707	CTGAAGCT	D507	CAGGACGT
D08	D708	TAATGCGC	D508	GTACTGAC
A01	A701	ATCACGAC	A501	TGAACCTT
A02	A702	ACAGTGGT	A502	TGCTAAGT
A03	A703	CAGATCCA	A503	TGTTCTCT
A04	A704	ACAAACGG	A504	TAAGACAC
A05	A705	ACCCAGCA	A505	CTAATCGA
A06	A706	AACCCCTC	A506	CTAGAACA
A07	A707	CCCAACCT	A507	TAAGTTCC
A08	A708	CACCACAC	A508	TAGACCTA

Table 1: Cassette D or A number and Corresponding Illumina Dual Index Pair

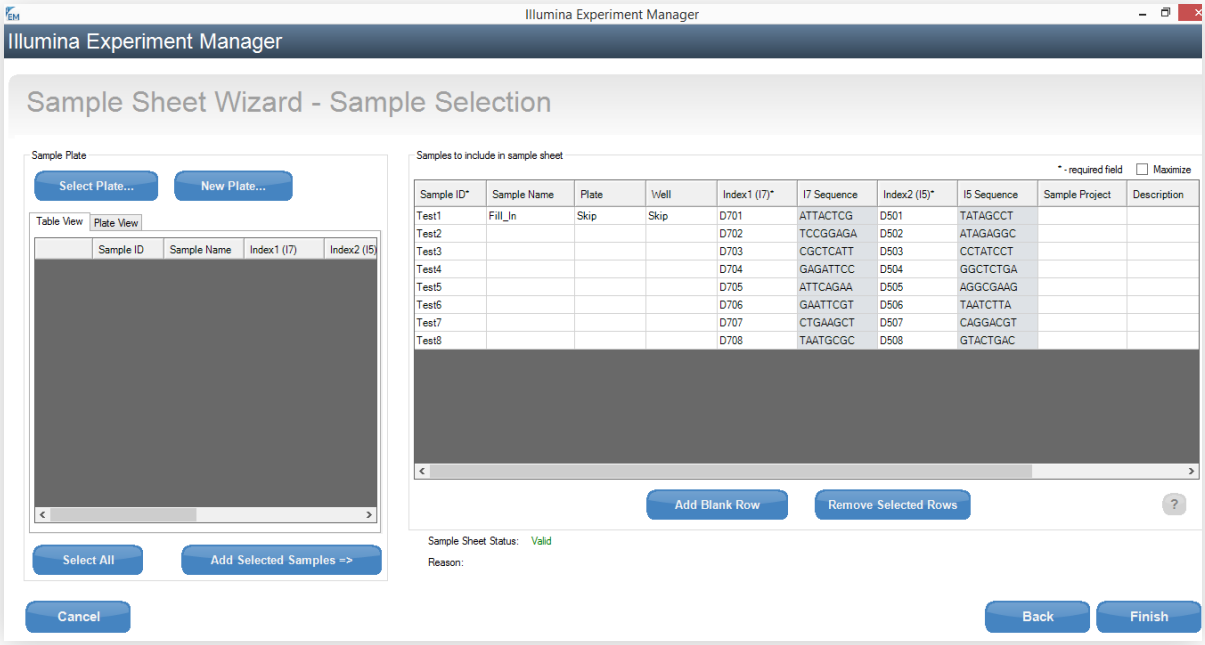


Figure 2: Illumina MiSeq Sample Selection window after clicking "Next." The i7 and i5 sequences corresponding to the D number on the cassette and shown in Table 3 should be selected.

iRepertoire, Inc.

Sequencing RepSeq on the Illumina MiSeq

MiSeq Sample Sheet Wizard Workflow Parameters: REPSEQ

Note: THESE INSTRUCTIONS APPLY TO LIBRARIES WITHOUT DUAL INDEX ADAPTORS ON THE END LIBRARY (REPSEQ). 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 on RepSeq cassettes**; we use our own molecular identifiers to de-multiplex the data during analysis.

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").

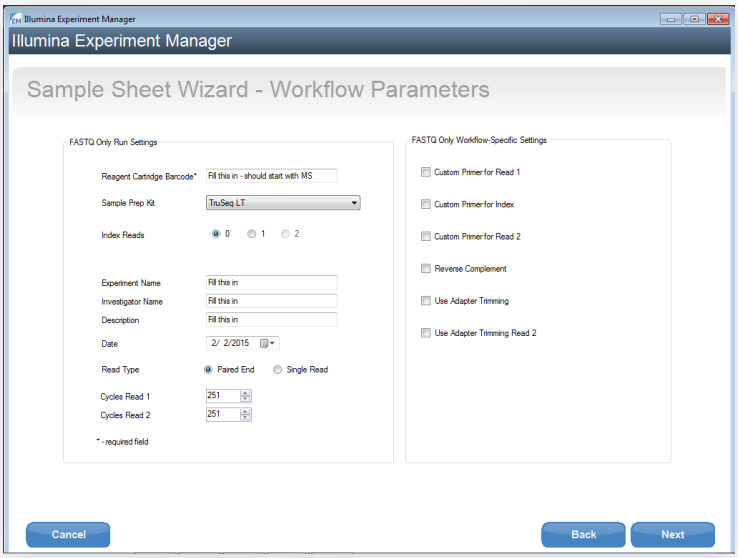


Figure 3: Illumina MiSeq Sample Sheet Wizard workflow parameter settings for running RepSeq reagent systems on the Illumina MiSeq.

Data Analysis

After sequencing, our software pipeline recognizes the dual index adaptors (if applicable) and internal barcodes including immune receptor chain. The pipeline automatically decodes them during analysis. Check the Product Manuals in the Learning Center of the website or 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 if sequencing on an Illumina MiSeq or Illumina iSeq. Please note for larger output platforms, such as NextSeq, HiSeq, and NovaSeq, data processing fees apply as detailed in our Data Management Policy (provided separately). 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 Services > Submission 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:
    (1) N-addition (normalized and non-normalized)
    (2) CDR3 length (normalized and non-normalized)
    (3) V, J nibbling (normalized and non-normalized)
    (4) 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.

iRepertoire, Inc.

Troubleshooting

Issue	Solution
No bead pellet	a) Take picture of top, bottom, and sides. Be careful not to turn cassette upside down. Place cassette in a refrigerator (for amplicon). Contact iRepertoire support and provide description and images. In some cases, a rescue well may be available and instructions can be provided to retrieve PCR product. b) Hardware initialization issues may result in a cassette not being run. Are wells punctured? If not, follow steps in (a) but also provide the iR-Flex Manager system logs and indicate the serial number of the Processor and blade position.
Small bead pellet	a) Reagents in cassette not mixed well enough - Before loading samples, ensure that the cassette is mixed well by using a vortex for at least 30 seconds. Using centrifugal force, flick the cassette down to ensure that the reagents are in the bottom of the wells. b) Small bead pellet typically has retrievable PCR product of significant concentration. Since this is a post-PCR processing event, retrieved library should be unaffected in terms of immune repertoire information and can be sequenced. If not enough library is obtained from the pellet, heat the pellet at 50℃ in the elution water for 10 minutes to improve yield.
Cassette pop-out	a) The communication between the hardware and software timed out during the initialization of the cassette. After the cassette pops out, wait until it is flashing green, remove it from the blade and push it back in again. The cassette should not require unlinking in this situation. b) Unlink the cassette in the software. Rescan the cassette using single cassette input and try the process again. c) It is also possible the QR code on cassette label is not aligned. Carefully remove and recenter if it is noticeably off center.
Cassette in processor not running	a) Press the red eject button above the blade to retrieve the cassette. Then push the cassette back in. b) If (a) is not successful, repeat, unlink the cassette, relink the cassette, and try again. c) If (a) and (b) fails, turn the processor off using the toggle switch at the back and that will eject the cassette when the processor is rebooting. The cassette should work upon rebooting and reinsertion. **Warning: other cassettes will be ejected if the Processor is turned off.**

Issue	Solution
Bent pipette tip	a) This is a cassette issue. Contact iRepertoire following the instructions below. Pictures are very important. Place the cassette in a bag and hold in the refrigerator until further instructions. We may ask that the cassette be sent to us.
Leaking internal reagents	a) After running a cassette, this may be condensation in the cassette and is no cause for concern. b) Before running a cassette, likely, this is a cassette manufacturing issue. Contact iRepertoire following the instructions below. Pictures are very important. Place the cassette in a bag and hold in the refrigerator until further instructions. We may ask that the cassette be sent to us.
Cassette ID not found	a) This means that the cassette is not scanned into the system. Link the cassette.
Processor blade red light	a) This can happen for a variety of reasons but is usually a hardware-software connection issue or a hardware fault. It indicates a detected error on the blade. If the system red-lights on cassette initialization, hit the eject button. If the cassette will not eject and no other blades are in operation, reboot the system (see b). If other blades are running, allow the other cassettes to complete and reinitialize the entire system after the run is complete following steps (b) and (c) in order. b) A copy of the iR-Flex Manager system logs can be found by clicking on the files tab in the upper left hand corner. Click on the export system logs to zip this file, and the log files will be available on the desktop. Email the log files with a description of your experience to iRepertoire. c) Reboot the iR-Flex Manager system by power cycling the processors. Wait for all the blades to turn green. Exit out of the iR-Flex Manager software and restart the computer. Wait a few minutes for the programs to activate and start the iR-Flex Manager software. Upon rebooting, run the Diagnostic cassette on that blade to ensure a major hardware fault is not present prior to using again. If red-light persists, follow step (b).
Frozen System Monitor screen	a) If the screen is frozen on the system monitor tab, then bring up the task manger by holding down Ctrl+Alt+Delete. Alternately, right click the task bar that runs across the bottom of the screen. Click on java(TM) Platform SE binary, click end task, and restart the computer. b) Turning off the computer while processors are running will NOT turn off the processors. They will still continue to run and the cassettes should complete normally. However, the software may not register that the cassettes are running. The flashing green light should be used to indicate successful cassette completion.

Troubleshooting

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 iRepertoire branded side).
6. Pictures of the bottom wells (be careful not to turn the cassette upside down).
7. iR-Cassette Lot #:
8. iR-Flex Processor Serial # and blade position.
9. A copy of the iR-Flex Manager system logs which can be found by clicking on the files tab in the upper left hand corner and then click on the export system logs to zip file and that will transfer the log files on to the desktop ready to be emailed to iRepertoire.

iRepertoire, Inc.

Appendix A: Additional Terms and Conditions

Standard Terms and Conditions for Use of Product

By opening the packaging containing this product ("Product") or by using such Product in any manner, you are consenting and agreeing to be bound by the following terms and conditions. You are also agreeing that the following terms and conditions constitute a legally valid and binding contract that is enforceable against you. If you do not agree to all of the terms and conditions set forth below, you must promptly return the Product for a full refund prior to using them in any manner.

Acceptance

ALL SALES ARE SUBJECT TO AND EXPRESSLY CONDITIONED UPON THE TERMS AND CONDITIONS CONTAINED HEREIN, AND UPON RECIPIENT'S ASSENT THERETO. NO VARIATION OF THESE TERMS AND CONDITIONS SHALL BE BINDING UPON IREPERTOIRE ("PROVIDER") UNLESS AGREED TO IN WRITING AND SIGNED BY AN AUTHORIZED REPRESENTATIVE OF PROVIDER. For purposes of this agreement, "RECIPIENT" shall be the party to whom Provider sells or provides Product. Recipient, by accepting the Product shall be deemed to have assented to the terms and conditions set forth herein, notwithstanding any terms contained in any prior or later communications from Recipient and whether or not Provider shall specifically or expressly object to any such terms.

Warranties

Any warranty obligations for the Product shall be exclusively provided in writing to Recipient directly by Provider. EXCEPT FOR A ONE-YEAR WARRANTY ON ALL PARTS AND LABOR FOR PRODUCT, PROVIDER MAKES NO WARRANTY WHATSOEVER REGARDING THE PRODUCT AND PROVIDER SPECIFICALLY DISCLAIMS ALL WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. PROVIDER SHALL NOT, IN ANY EVENT, BE LIABLE FOR INCIDENTAL, CONSEQUENTIAL, OR SPECIAL DAMAGES OF ANY KIND RESULTING FROM ANY USE OR FAILURE OF THE PRODUCT, EVEN IF PROVIDER HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGE INCLUDING, WITHOUT LIMITATION, LIABILITY FOR LOSS OF WORK IN PROGRESS, DOWN TIME, LOSS OF REVENUE OR PROFITS, FAILURE TO REALIZE SAVINGS, LOSS OF PRODUCTS OF BUYER OR OTHER USE OF ANY LIABILITY OF BUYER TO A THIRD PARTY ON ACCOUNT OF SUCH LOSS, OR FOR ANY LABOR OR ANY OTHER EXPENSE, DAMAGE OR LOSS OCCASIONED BY SUCH PRODUCT INCLUDING PERSONAL INJURY OR PROPERTY DAMAGE CAUSED BY PROVIDER'S GROSS NEGLIGENCE.

Recipient's Use of Product

Recipient agrees that no rights or licenses under Provider's patents, patents pending, or any patent applications shall be implied from the sale of the Product, except as expressly provided herein, and Recipient does not receive any right under Provider's patent rights hereunder. Recipient acknowledges and agrees that Product is sold and licensed only for use with Provider's standard iR-Cassette™or iC-Cassette™. Recipient further acknowledges that the Product has not received approval from the United States Food and Drug Administration or other federal, state, or local regulatory agencies and has not been tested by Provider for safety or efficacy in food, drug, medical device, cosmetic, commercial, or any other use, unless otherwise stated in Provider's technical specifications or material data sheets furnished to Recipient.

Recipient expressly represents and warrants to Provider that Recipient will properly test and use any Product in accordance with the practices of a reasonable person who is an expert in the field and in strict compliance with the United States Food and Drug Administration and all applicable domestic and international laws and regulations, now and hereinafter enacted.

RECIPIENT HEREBY GRANTS TO PROVIDER A NONEXCLUSIVE, WORLDWIDE, UNRESTRICTED, ROYALTY- FREE, FULLY PAID-UP LICENSE, WITH THE RIGHT TO GRANT AND AUTHORIZE SUBLICENSES, UNDER ANY AND ALL PATENT RIGHTS IN INVENTIONS COMPRISING MODIFICATIONS, EXTENSIONS, OR ENHANCEMENTS MADE BY RECIPIENT TO THE PRODUCT OR TO THE MANUFACTURE OR USE OF THE PRODUCT ("IMPROVEMENT PATENTS"), TO MAKE, HAVE MADE, USE, IMPORT, OFFER FOR SALE OR SELL ANY AND ALL PRODUCT; EXPLOIT ANY AND ALL METHODS OR PROCESSES; AND OTHERWISE EXPLOIT IMPROVEMENT PATENTS FOR ALL PURPOSES. NOTWITHSTANDING THE FOREGOING, "IMPROVEMENT PATENTS" SPECIFICALLY EXCLUDES PATENT CLAIMS CONCEIVED AND REDUCED TO PRACTICE BY BUYER CONSISTING OF METHODS OF SAMPLE PREPARATION, METHODS OF CONJUGATING PRODUCT TO ANALYTES, THE COMPOSITION OF MATTER OF THE SPECIFIC CHEMISTRIES OF THE ASSAYS DEVEL- OPED BY RECIPIENT AND METHODS OF PERFORMING THE ASSAYS (I.E., THE PROTOCOL FOR THE ASSAY). Recipient has the responsibility and herby expressly assumes the risk to verify the hazards and to conduct any further research necessary to learn the hazards involved in using the Product. Recipient also has the duty to warn Recipient's customers, employees, agents, assigns, officers, successors and any auxiliary or third party personnel (such as freight handlers, etc.) of any and all risks involved in using or handling the Product. Recipient agrees to comply with instructions, if any, furnished by Provider relating to the use of the Product and not misuse the Product in any manner. Recipient shall not reverse engineer, decompile, disassemble or modify the Product. Recipient acknowledges that Provider retains ownership of all patents, trademarks, trade secrets, and other proprietary rights relating to or residing in the Product.

Recipient's Representations, Release, and Indemnity

Recipient represents and warrants that it shall use the Product in accordance with the above paragraph, "Recipient's Use of Product," and that any such use of Product will not violate any law, regulation, judicial order, or injunction. Recipient agrees to release, discharge, disclaim and renounce any and all claims, demands, actions, causes of action and/or suits in law or equity, now existing or hereafter arising, whether known or unknown, against Provider, and their respective officers, directors, employees, agents, successors and assigns (collectively the "Released Parties"), with respect to the use of the Product.

iRepertoire, Inc.

Appendix B: Hardware Specifications

iR-System™ Hardware Specifications:

iR-Processor™
Processor weight approx. 102 lbs.
* * Contains processing slots
AC input requirements: 100-240 VAC, fused @ 5 amps, 50-60Hz Single Phase
Physical dimensions: 17 inches W x 17 inches D x 17 inches H
Heater operating range: 35°C to 95°C nominal

iRepertoire Operating System Environment
Indoor use only
Operating temperature: 15°C to 30°C (59°F to 86°F)
Humidity: 20%–80%, noncondensing
Altitude: Operation up to 2400 m (7874 ft.) above mean sea level
Shipping and storage: The allowable shipping and storage temperature and humidity ranges are 0°C to + 50°C and 20 -80% noncondensing, respectively.

iRepertoire Inc.

800 Hudson Way, Suite 2304,
Huntsville, AL 35806 USA

iR-Flex System™: User Manual
Rev. February 2020

iRepertoire Inc. reserves the right to modify its products and services at any time. This guide is subject to change without notice. Although prepared to ensure accuracy, iRepertoire assumes no liability for errors or omissions, or for any damages resulting from the application or use of this information.

All other trademarks are trademarks of their respective companies. The contents of this manual are the property of iRepertoire and are copyrighted. Except as specified in the End User License Agreement, any reproduction in whole or in part is strictly prohibited.

info@irepertoire.com
iRepertoire.com