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QIAseq® Immune Repertoire RNA Library Handbook

Sample to Insight® solution for Immune Repertoire Sequencing from RNA

Sample to Insight

QIAGEN

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Kit Contents

Box 1

QIAseq® Immune Repertoire RNA Library Kit (including Human and Mouse)	**(12)**	**(96)**
Catalog no.	**333705**	**333705**
Number of samples	**12**	**96**
QIAseq TCR Panel	48 µl	384 µl
TCR RT Primer	12 µl	96 µl
EZ Reverse Transcriptase	15 µl	96 µl
BC3 Buffer, 5x	24 µl	192 µl
RNase Inhibitor	12 µl	96 µl
RH RNase	12 µl	96 µl
dNTP	12 µl	96 µl
XC Buffer, 10x	24 µl	192 µl
BX Enzyme	12 µl	96 µl
ERA Enzyme	120 µl	960 µl
ERA Buffer, 10x	60 µl	480 µl
DNA Ligase	120 µl	960 µl
Ligation Buffer, 5x	240 µl	2 x 1250 µl
Ligation Solution	2 x 125 µl	2 x 970 µl
QIAseq RNA Buffer	300 µl	1200 µl
Nuclease-free Water	1.5 ml	3 x 1.5 ml
HotStarTaq® DNA Polymerase	30 µl	240 µl
Quick-Start Protocol	1	1

Box 2

QIAseq Immune Repertoire RNA Library Kit (including Human and Mouse)	12	96
QIAseq Beads	10 ml	55 ml
Ligation Solution	2 x 125 µl	2 x 970 µl

GeneGlobe configuration no.	Product name	Total number of panel primers
IMHS-001Z-12	Human TCR sequencing kit (12 reactions)	76
IMHS-001Z-96	Human TCR sequencing kit (96 reactions)	76
IMMM-001Z-12	Mouse TCR sequencing kit (12 reactions)	72
IMMM-001Z-96	Mouse TCR sequencing kit (96 reactions)	72

QIAseq sample index kits (ordered separately)

QIAseq sample indexes are required for completion of the QIAseq immune repertoire RNA library protocol. Select the appropriate Index kits based on the total number of samples multiplexed per lane, and if unique dual indexing or combinatorial indexing is necessary.

QIAseq Unique Dual Indices with 10 base sample indexes (UDIs)

QIAseq 96-Unique Dual Index Set A or Set B (cat. nos. 333725, 333735). Each set of unique dual index sets allows for multiplexing 96 samples per flowcell lane. For indexing up to 192 samples, combine QIAseq 96-Unique Dual Index Set A and QIAseq 96-Unique Dual Index Set B.

QIAseq 96-Unique Dual Index Set*	QIAseq 96-Unique Dual Index Set A	QIAseq 96-Unique Dual Index Set B
Catalog no.	**333725**	**333735**
Number of samples	**384**	**384**
UDIN-96AA†	4 x 10 µl	N/A
UDIN-96BA†	N/A	4 x 10 µl
UDIS-96AK§	4 plates	N/A
UDIS-96BK§	N/A	4 plates
IL-Forward Primer (384)	310 µl	310 µl
QIAseq A Read1 Primer I (100 µM)	4 x 24 µl	4 x 24 µl
8-cap strips (24/bag)	4	4

* 10 bp unique dual indices

† Adapter Plate (UDIN-96AA/UDIN-96BA); each plate contains 96 molecularly indexed adapters. Each index can be used for up to 4 samples.

§ Dried Primer Plate (UDIS-96AK/UDIS-96BK); each well contains one sample index primer and IL-Universal PCR primer for sample indexing during PCR amplification. Each kit can process 384 samples (96 samples per plate).

QIAseq Combinatorial Indices with 8 base sample indexes (CDIs)

QIAseq 12-Index I (cat. no. 333714) is for multiplexing 12 samples per lane. A total of 48 samples can be indexed.

QIAseq 12-Index I* **(12 sample index for 48 samples on Illumina® platform)**	**(48)**
Catalog no.	**333714**
Number of samples	**48**
IL-N7## Adapter contains 12 tubes of molecularly indexed adapters, with each tube corresponding to one sample index; each index can be used for up to 4 samples	25 µl
IL-S502 Index Primer	40 µl
IL-Forward Primer	40 µl
IL-Universal Primer	40 µl
QIAseq A Read1 Primer I (100 µM)	24 µl

* 8 bp combinatorial dual indices

QIAseq 96-Index I Set A, B, C, or D (cat nos. 333727, 333737, 333747, 333757) is for multiplexing 96 samples per lane. To multiplex more than 96 samples, combine sample indexes from set A, B, C, or D. For example, to multiplex 384 samples, combine QIAseq 96-Index I Set A, QIAseq 96-Index I Set B, QIAseq 96-Index I Set C, and QIAseq 96-Index I Set D. QIAseq 96-Index I utilizes 8 base sample indexes.

QIAseq 96-Index I Set A, B, C, or D*	**QIAseq 96-Index I Set A**	**QIAseq 96-Index I Set B**	**QIAseq 96-Index I Set C**	**QIAseq 96-Index I Set D**
Catalog no.	**333727**	**333737**	**333747**	**333757**
Number of samples	**384**	**384**	**384**	**384**
IL-701NJ†	160 µl	N/A	160 µl	N/A
IL-716NJ†	N/A	160 µl	N/A	160 µl
IL-502SK§	4 plates	4 plates	N/A	N/A
IL-513SK§	N/A	N/A	4 plates	4 plates
IL-Forward Primer (384)	310 µl	310 µl	310 µl	310 µl
QIAseq A Read1 Primer I (100 µM)	4 x 24 µl	4 x 24 µl	4 x 24 µl	4 x 24 µl
12-cap strips	16	16	16	16

* 8 bp combinatorial dual indices

† Adapter Plate (IL-701NJ / IL-716NJ); contains 4 rows of 12 molecularly indexed adapters. Each well can be used on 8 samples.

§ Adapter plate (IL-502SK/IL-513SK) is supplied as a dried 96-well plate with 4 index plates per order. Each plate contains one IL-5## index primer and IL-Universal PCR primer pair for PCR amplification and sample indexing. Each kit can process up to 384 total samples.

Shipping and Storage

The QIAseq Immune Repertoire RNA Library Kit is shipped in 2 boxes.
[Skull and cross bones]

- Box 1 is shipped on dry ice and must be stored at –30 to –15°C in a constant-temperature freezer upon arrival.
- Box 2 is shipped on cold packs. Open immediately and store the QIAseq Beads and QIAseq Ligation solution at the appropriate temperatures.

  - QIAseq Beads (Box 2) should be stored at 2–8°C.
  - Ligation solution should be stored at –30 to –15°C.

When stored under these conditions and handled correctly, the product can be kept based on the expiration date on each component without reduction in performance.

QIAseq Index kits (ordered separately) are shipped on dry ice and should be stored at –30 to –15°C in a constant-temperature freezer upon arrival.

Intended Use

The QIAseq Immune Repertoire RNA Library Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the QIAseq Immune Repertoire RNA Library Kit is tested against predetermined specifications, to ensure consistent product quality.

Introduction

The QIAseq Immune Repertoire RNA Library Kit enables, targeted next-generation sequencing (NGS) of the T cell receptor (TCR) immune repertoire. This highly optimized solution incorporates unique molecular indices (UMIs) to facilitate ultrasensitive and accurate characterization of the immune repertoire in cells and tissues. The required input for a single QIAseq Immune Repertoire RNA Library ranges from 10 to 1000 ng of RNA.

The adaptive immune system is composed of T and B lymphocytes that bind antigens via highly specific TCRs and B cell receptors (BCRs) on their cell surfaces. To recognize a nearly infinite number of potential antigens, extensive sequence diversity of TCRs and BCRs is generated by somatic V(D)J recombination of the TCR and BCR loci, and by subsequent somatic hypermutation and class-switching recombination of the BCR upon antigen stimulation. Accurate characterization of the TCR and BCR repertoires is key to understanding adaptive immune responses and has many applications across different fields, including vaccine development, autoimmunity, monitoring treatment response in lymphoid malignancies, and immunotherapy.

Compared to traditional methods, NGS provides an unprecedented, high-resolution picture of the immune repertoire. However, many available immune repertoire sequencing methods involve multiplex PCR with primers targeting different V or J regions, which can introduce substantial amplification bias.

The QIAseq Immune Repertoire RNA Library Kit utilizes unique molecular indexing (UMIs) with targeted single primer extension (SPE) enrichment to robustly create targeted RNA-seq libraries for NGS instruments. This library construction approach greatly improves amplification uniformity compared to multiplexed PCR-based V and J primer pools. The incorporation of UMIs before the amplification step further reduces amplification bias and allows for accurate and sensitive TCR clonotype and repertoire diversity assessment.

Combined with a data analysis pipeline that has been developed to perform all necessary steps of NGS data processing including clonotype identification, the QIAseq Immune Repertoire RNA Library Kit is a complete sample-to-insight solution for precise characterization of the TCR immune repertoire using NGS.

Principle and procedure

The QIAseq Immune Repertoire RNA Library Kit relies on a highly efficient, TCR-specific cDNA synthesis, TCR gene-specific single primer enrichment (SPE) and unique molecular indexing (UMI) for accurate and sensitive TCR clonotype and diversity assessment. TCR reverse transcriptase and enrichment panel primers are provided, together with all of the necessary library construction reagents. The QIAseq Immune Repertoire RNA Library Kit is designed to enrich TCR α, β, γ and σ subunits using 10–1000 ng RNA (Figure 1, next page).

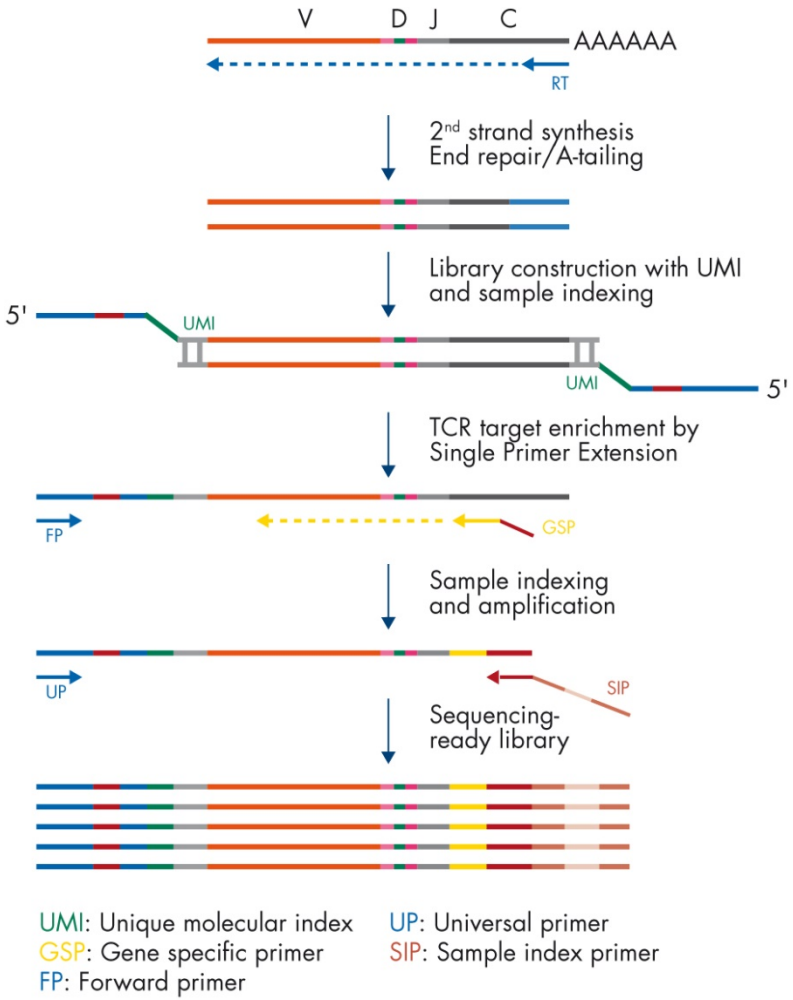


Figure 1. QIAseq Immune Repertoire RNA Library Kit workflow.

cDNA synthesis

RNA samples are first reverse transcribed into cDNA with TCR-specific RT primers. Subsequently, second-strand synthesis occurs, which generates double-stranded cDNA (ds-cDNA). This ds-cDNA is then end-repaired and A-tailed in a single-tube protocol.

UMI assignment

Prior to target enrichment and library amplification, each original cDNA molecule is assigned a UMI by ligating an adapter containing a 12-base fully random sequence (i.e., the UMI) to the ds-cDNA. Statistically, this process provides 4^{12} – 16,777,216 possible indices per adapter, with each DNA molecule in the sample receiving a unique UMI sequence. In addition, this ligated adapter also contains the first sample index.

Target enrichment and final library construction

Following UMI assignment, target enrichment is performed to ensure that TCR cDNA molecules are sufficiently enriched in the sequenced library. For enrichment, ligated cDNA molecules are subjected to targeted PCR using one TCR constant-region-specific primer and one universal primer complementary to the adapter. A universal PCR is ultimately carried out to amplify the library and introduce platform-specific adapter sequences, as well as additional sample indices.

NGS adapter and index technologies

The QIAseq Immune Repertoire RNA Library Kits can utilize either combinatorial dual-index adapters (CDI) or unique dual-index adapters (UDI) adapters. The UDI adapters significantly reduce the risk of index bleeding issues ("index hopping") associated with Illumina sequencing instruments that utilize patterned flow cells. With unique dual indexing (UDI), each sample will be assigned two unique sample indices to overcome the error introduced by image analysis, sequencing error, and demultiplexing, and to remove mis-assignment of sequencing data to the wrong samples.

Next-generation sequencing on Illumina NGS systems

The QIAseq Immune Repertoire RNA Library Kit is compatible with Illumina NGS systems (MiniSeq®, MiSeq®, NextSeq® 500, HiSeq® 2500, HiSeq 3000/4000, and NovaSeq™ 6000). When using Illumina NGS systems, QIAseq Immune Repertoire RNA libraries require a custom sequencing primer for Read 1 (QIAseq A Read1 Primer I). It is recommended to allocate between 2–10 million reads per sample.

Data analysis

The QIAseq Immune Repertoire pipeline automatically performs all steps necessary to generate TCR diversity and clonotype report from your raw NGS data.

The QIAseq Immune Repertoire RNA Library Kit Analysis pipeline is available through cloud, desktop, and server software packages. For cloud-based analysis, researchers can utilize the QIAseq section of GeneGlobe Analyze (https://geneglobe.qiagen.com/analyze) on **www.qiagen.com**. For desktop and server support, QIAGEN Genomics Workbench (**https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-clc-genomics-workbench/**) can be utilized.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- 80% ethanol (made fresh daily)
- Nuclease-free Water
- Nuclease-free pipette tips and tubes
- 1.5 ml LoBind® tubes (Eppendorf, cat. no. 022431021)
- 0.2 ml PCR tubes, 96-well PCR plates or PCR strips and caps
- Ice
- Microcentrifuge
- Thermal cycler
- Multichannel and single-channel pipettes
- **Library QC**: Agilent® 2100 Bioanalyzer® (Agilent, cat. no. G2939BA) or Agilent TapeStation® (Agilent, cat. no. G2991AA)

  Agilent High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626)

- Magnetic separation stands for 1.5 ml or 2 ml tubes (DynaMag™-2; Thermo Fisher Scientific, 12321D or equivalent)
- Magnetic separation stands for 96-well plates (DynaMag-96 Side Magnet; Thermo Fisher Scientific, cat. no. 12331D or equivalent)
- QIAseq Library Quant system: QIAseq Library Quant Array Kit (cat. no. 333304) or QIAseq Library Quant Assay Kit (cat. no. 333314)
- For 96-well format handling, Axygen® silicone 96 round well compression flat mat for PCR microplates, Axygen Sealing Film Roller and AlumaSeal® II sealing films are recommended

Important Notes

RNA quality

The most important prerequisite for RNA sequence analysis is consistent, high-quality RNA from every experimental sample. Therefore, sample handling and RNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts or other contaminants may either degrade the RNA or decrease the efficiency of, if not block completely, the enzymatic activity necessary for optimal targeted enrichment. Sample purity can be checked with the QIAxpert®. Due to the large intronic region between the TCR J and constant regions, it is recommended that RNA should be free of DNA to avoid wasting sequencing reads on intron region.

The QIAGEN® kits listed in Table 1 are recommended for the preparation of RNA samples from cells, tissues, and serum/plasma samples. For best results, all RNA samples should be resuspended in RNase-free water.

Important: Do not use DEPC-treated water.

Note: If RNA samples must be harvested from biological samples for which kits are not available, please contact Technical Support representatives for suggestions.

Table 1. Recommended kits for purification of total RNA

Kit	Starting material	Cat. no.
RNeasy® Mini Kit	Cells and tissue	74104, 74106
RNeasy Micro Kit	Small amount tissues and cells	74004
AllPrep® DNA/RNA Mini Kit (50)	Animal/human tissues and cells	80204
PAXgene® Blood RNA Kit (50)	Whole blood	762164

For best results, all RNA samples should also demonstrate consistent quality according to the following criteria:

RNA quantification

The concentration and purity should be determined by measuring the absorbance in a spectrophotometer such as a QIAxpert. As the spectral properties of nucleic acids are highly dependent on pH, we recommend preparing dilutions and measuring absorbance in 10 mM Tris·Cl, pH 7.5. The A_{260}/A_{280} ratio should be >1.8.

Ribosomal RNA band integrity

Run an aliquot of each RNA sample on the Agilent Bioanalyzer using an RNA 6000 Nano LabChip®. Verify that there are sharp bands/peaks present for both the 18S and 28S ribosomal RNAs (Figure 2). Any smearing of the RNA bands or shoulders on the RNA peaks indicates that degradation has occurred in the RNA sample.

For best results, the ribosomal bands should appear as sharp peaks. Ideally, the RIN number for non-FFPE RNA from the Agilent 2100 Bioanalyzer should be higher than 8.

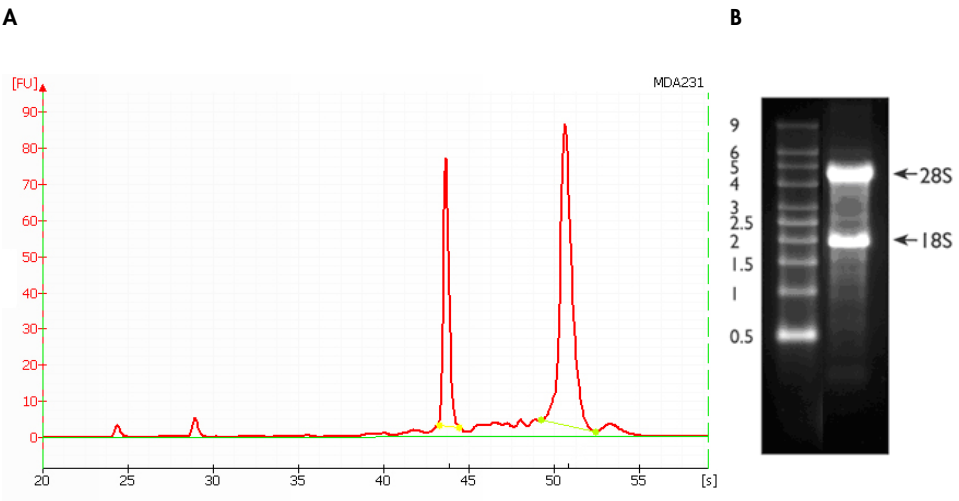


Figure 2. Ribosomal RNA integrity. A Agilent Bioanalyzer electropherogram of high-quality total RNA showing sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). **B** Agarose gel electrophoresis shows sharp bands (especially at the bottom of each band) for 28S and 18S ribosomal RNA.

RNA input amount

QIAseq Immune Repertoire RNA Library Kit provides results with as little as 10 ng or as much as 1000 ng total RNA per cDNA synthesis reaction.

The optimal amount of starting material depends on the relative TCR immune repertoire diversity and the abundance of the TCR clonotype. Detection of lower-abundance clonotypes requires more RNA; high-abundance clonotypes require less RNA. Greater amounts of input total RNA will provide better sensitivity for specific clonotype detection when sufficient instrument read budget is allocated.

For successful results, we recommend that first-time users start with 200 ng total RNA. We recommend using a consistent amount of total RNA for all reactions in a single experiment.

Sequencing capacity and sample multiplex level

Sample multiplexing is one of the most important NGS tools for increasing throughput and reducing costs. It works by combining multiple samples to be processed together in a single sequencing run; as a consequence, sequencing reads need to be demultiplexed by reassigning each single read to its original source library. This is facilitated by the integration of index sequences into the individual adapter molecules.

QIAseq Immune Repertoire RNA Library Kits include either a fully compatible CDI or UDI indexing solution. We recommend using the QIAseq 96-Unique Dual Index sets. Each QIAseq Immune Repertoire RNA Library index kit includes one of the following:

- QIAseq 96-Unique Dual Index Set A (384) (cat. no. 333725): UDIN-96AA paired with UDIS-96AK
- QIAseq 96-Unique Dual Index Set B (384) (cat. no. 333735): UDIN-96BA paired with UDIS-96BK
- QIAseq 96-Index I Set A (384) (cat. no. 333727): IL-701NJ paired with IL-502SK
- QIAseq 96-Index I Set B (384) (cat. no. 333737): IL-716NJ paired with IL-502SK
- QIAseq 96-Index I Set C (384) (cat. no. 333747): IL-701NJ paired with IL-513SK
- QIAseq 96-Index I Set D (384) (cat. no. 333757): IL-716NJ paired with IL-513SK
- QIAseq 12-Index I (48) (cat. no. 333714): IL-N701–IL-N707, IL-N710–IL-N712, IL-N714–IL-N715 all paired with IL-S502

CDI adapters use twelve i7 and eight i5 barcode motives that can be combined to form up to 96 combinatory dual indices. In contrast, QIAseq UDI kits use a fixed combination of 2 unique barcode motives per adapter molecule. Therefore, each single-index motive is only used once on any UDI adapter plate.

Usage of UDI adapters effectively mitigates the risk of read misassignment due to index hopping. This is enabled by filtering misassigned reads during the demultiplexing of individual samples, thus generating highly accurate output data.

To multiplex more than 96 libraries in a single sequencing run, combine indexing kits with different QIAseq 96-Index I (CDIs) or QIAseq 96-Unique Dual Index (UDIs) sets. For example, combining the combinatorial QIAseq 96-Index I Set A (or B, or C, or D) (96) kits will allow the generation of 384 libraries with different combinatorial sample indexes for 384-plex sequencing. Likewise, up to 192 samples can be run when using both the unique dual QIAseq 96-Unique Dual Index Set A and Set B (96) kits.

Sample multiplexing capacity is defined by the immune repertoire diversity and sequencing platform read capacity. General guidelines are provided for the number of samples that can be multiplexed in different sequencing platforms, based on 500–1000 ng PBMC RNA (Table 2). Fine-tuning the read depth and read budget will be possible after the first test run. Adjustments should be made for different sample type, input, and specific application, as necessary.

Table 2. Estimated number of multiplexed samples based on sequencing need*

Instrument	Version	Capacity (paired-ends reads)	Shallow	Deep
MiniSeq	Mid Output	16 M	8	1
MiniSeq	High Output	50 M	25	5
MiSeq	V2 Reagents	30 M	15	3
MiSeq	V3 Reagents	50 M	25	5
NextSeq 500	Mid Output	260 M	130	26
NextSeq 500	High Output	800 M	400	80
HiSeq 2500 rapid run	Dual Flowcell v2	1200 M	600	120

* Based on 2 x 251 bp paired-end reads for CDIs for complete variable regions; 2 x 249 bp paired-end reads for 96-UDIs for complete variable regions.

Next-generation sequencing read-length recommendations

When using Illumina NGS systems, QIAseq Immune Repertoire RNA libraries require a custom sequencing primer for Read 1 (QIAseq A Read1 Primer I). The QIAseq Immune Repertoire RNA libraries can be sequenced two ways depending on whether or not the complete variable region or mainly the CDR3 region require sequencing.

Complete variable region

QIAseq Immune Repertoire RNA CDI libraries require 251 bp paired-end reads and dual 8 bp indices, while QIAseq Immune Repertoire RNA UDI libraries require 249 bp paired-end reads and dual 10 bp indices.

CDR3 Region only

QIAseq Immune Repertoire RNA CDI libraries require asymmetrical 261 bp for Read 1 and 41 bp for Read 2 paired-end reads and dual 8 bp indices, while QIAseq Immune Repertoire RNA UDI libraries require 259 bp for Read 1 and 39 bp for Read 2 paired-end reads and dual 10 bp indices.

Protocol: QIAseq Immune Repertoire RNA Library Kit for Illumina Instruments

Important points before starting

- This protocol covers all procedures required for the preparation of libraries for Illumina sequencers from "standard RNA" (i.e., cells or tissues).
- Before setting up the reaction, it is critical to accurately determine the amount of the input RNA (10–1000 ng). We recommend using 200 ng total RNA. Lower input amounts are possible; however, this will lead to fewer sequenced UMIs and reduced detection of low-abundance clonotypes.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Protocol: RT primer hybridization

Procedure: RT primer hybridization

1. Pre-heat a thermal cycler to 65°C with a heated lid (set at 103°C).
2. Thaw the RNA samples on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
3. Prepare the reagents required for RT primer hybridization.
   - 3a. Thaw TCR RT primer if required at room temperature.
   - 3b. Mix by flicking the tube, and centrifuge briefly.
4. On ice, prepare the RT primer hybridization reactions as described in Table 3. Briefly centrifuge, mix by pipetting up and down 7 to 8 times (do not vortex), and briefly centrifuge again.

Table 3. Preparation of RT primer hybridization reactions

Component	1 reaction
RNA sample (10–1000 ng)	Variable
TCR RT Primer	1 µl
Nuclease-free Water	Variable
Total	**6 µl**

5. Transfer the tube from ice to the pre-heated thermal cycler, and incubate for 5 min at 65°C followed by incubating on ice for at least 2 min.
6. Upon completion, proceed immediately with "Protocol: Reverse transcription".

Protocol: Reverse transcription

Important points before starting

- The 6 µl product from "Protocol: RT primer hybridization", page 22, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure: Reverse Transcription

7. Prepare the reagents required for Reverse Transcription.
   7a. Thaw BC3 Buffer, 5x, if required at room temperature.
   7b. Mix by flicking the tube, and centrifuge briefly.
   Note: The RNase Inhibitor and EZ Reverse Transcriptase should be removed from the freezer just before use and placed on ice. After use, immediately return the enzymes to the freezer.
8. On ice, prepare the reverse transcription reactions as described in Table 4. Briefly centrifuge, mix by pipetting up and down 7 to 8 times (do not vortex), and briefly centrifuge again.

Table 4. Preparation of reverse transcription reactions

Component	1 reaction
RT primer hybridization reaction (already in tube)	6 µl
BC3 buffer, 5x	2 µl
RNase Inhibitor	1 µl
EZ Reverse Transcriptase*	1 µl
Total	**10 µl**

* When working with RNA amounts ≤20 ng, dilute 1 µl of the EZ Reverse Transcriptase to 5 µl using 4 µl of Nuclease-free Water. Pipet up and down 7 to 8 times to mix. Then, add 1 µl to the reaction.

9. Incubate the tube in a thermal cycler with a heated lid (103°C) according to Table 5.

Table 5. Thermal cycler settings for reverse transcription reactions

Step	Temperature	Time
1	42°C	30 min
2	70°C	15 min
3	4°C	Hold

10. Upon completion, proceed with "Protocol: Second-strand synthesis". Alternatively, the samples can be stored at –30 to –15°C in a constant temperature freezer.

Protocol: Second-strand synthesis

Important points before starting

- The 10 µl product from "Protocol: Reverse transcription", page 23, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure: Second-strand synthesis

11. Prepare the reagents required for second-strand synthesis.

    11a. Thaw XC Buffer and dNTP at room temperature.

    11b. Mix by flicking the tube, and then centrifuge briefly.

    Note: RH RNase and BX Enzyme should both be removed from the freezer just before use and placed on ice. After use, immediately return the enzymes to the freezer.

12. On ice, prepare the second-strand reactions as described in Table 6. Briefly centrifuge, mix by pipetting up and down 7 to 8 times (do not vortex), and briefly centrifuge again.

Table 6. Preparation of second-strand synthesis reactions

Component	1 reaction
Reverse-transcription reaction (already in tube)	10 µl
XC buffer	2 µl
RH RNase	1 µl
dNTP	1 µl
BX enzyme	1 µl
Nuclease-free Water	5 µl
Total	**20 µl**

13. Incubate the tube in a thermal cycler with a heated lid (103°C) according to Table 7.

Table 7. Thermal cycler settings for second-strand synthesis

Step	Temperature	Time
1	37°C	7 min
2	65°C	10 min
3	80°C	10 min
4	4°C	Hold

14. Upon completion, proceed with "Protocol: End-repair and A-addition".

Protocol: End-repair and A-addition

Important points before starting

- The entire 20 µl product from "Protocol: Second-strand synthesis", page 25, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure: End-repair and A-addition

15. Prepare the reagents required for end-repair and A-addition.

    15a. Thaw ERA Buffer, 10x, at room temperature.

    15b. Mix by flicking the tube, and then centrifuge briefly.

    Note: The ERA Enzyme should be removed from the freezer just before use and placed on ice. After use, immediately return the enzymes to the freezer.

16. On ice, prepare the end-repair and A-addition reactions as described in Table 8. Briefly centrifuge, mix by pipetting up and down 7 to 8 times (do not vortex), and briefly centrifuge again.

Table 8. Preparation of end-repair and A-addition reactions

Component	1 reaction
Second-strand product from previous section	20 µl
ERA Buffer, 10x	5 µl
Nuclease-free Water	15 µl
Total	**40 µl**

17. On ice, add 10 µl ERA Enzyme to each reaction. Briefly centrifuge, mix by pipetting up and down 7 to 8 times (do not vortex), and briefly centrifuge again.

    Important: Keep the reactions on ice during the entire reaction setup.

18. Program a thermal cycler according to Table 9. Set the heated lid to 70°C.

    Note: If using a non-temperature–controlled lid, run with cycler lid open for step 4 and seal the strip or plate well. When the cycler reaches step 5, close the lid to avoid evaporation. Centrifuge after the run to remove any condensation.

19. Prior to adding the tubes/plate to the thermal cycler, start the program. When the thermal cycler reaches 4°C, pause the program.

    Important: The thermal cycler must be pre-chilled and paused at 4°C.

20. Transfer the tubes/plate prepared in step 17 to the pre-chilled thermal cycler and resume the cycling program.

Table 9. Thermal cycler settings for end-repair and A-addition

Step	Temperature	Time
1	4°C	1 min
2	20°C	30 min
3	65°C	30 min
4	4°C	Hold

21. Upon completion, immediately proceed with “Protocol: Adapter ligation”.

Protocol: Adapter ligation

Important points before starting

- The 50 µl product from “Protocol: End-repair and A-addition”, page 26, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

QIAseq 96-Unique Dual Index Set Adapter Plate

- **Important**: The QIAseq 96-Unique Dual Index Set Adapter Plate (UDIN-96#A): A or B used in the adapter ligation reaction must be paired with the matching QIAseq 96-Unique Dual Index Set (UDIS-96#K) Primer Plate: A or B used in the Universal PCR amplification reaction.

  - The QIAseq 96-Unique Dual Index (UDIN-96#A) adapters are sealed in a 96-well plate with pierceable aluminum heat-sealing film.
  - The pierceable aluminum seal does not need to be removed; instead, puncture the film using standard 200 µl pipette tips to withdraw the appropriate adapter and adapter volume.
  - **Important**: Thaw the adapter plate on ice or store at 4°C before usage. After it is fully thawed, spin down the plate at 1000 g for 1 min.

QIAseq 12-Index I Adapters

- **Important**: The QIAseq 12-Index I Adapters (IL-N7##) are in individual tubes
- **Important**: Thaw the tubes on ice or store at 4°C before usage. After the tubes are fully thawed, spin down the tubes.

QIAseq 96-Index I Set Adapter Plate

- **Important**: The QIAseq 96-Index I Set Adapter Plate (IL-7##NJ): A, B, C, or D used in the adapter ligation reaction must be paired with the matching QIAseq 96-Index I (IL-5##SK) Primer Plate: A, B, C, or D used in the Universal PCR amplification reaction.

  - The QIAseq 96-Index I Set (IL-7##NJ) adapters are sealed in a 96-well plate that needs to have the seal removed.

- **Important**: Thaw the adapter plate on ice or store at 4°C before usage. After it is fully thawed, spin down the plate at 1000 g for 1 min. Carefully withdraw the appropriate adapter and adapter volume.

Procedure: Adapter Ligation

22. Prepare the reagents required for the Adapter Ligation.

    22a. Thaw DNA Ligation Adapter; Ligation Buffer, 5x; and Ligation Solution at room temperature.

    22b. Mix by flicking the tube, and then centrifuge briefly.

    Note: DNA Ligase should be removed from the freezer just before use and placed on ice. After use, immediately return the enzyme to the freezer.

23. On ice, prepare the adapter ligation reactions as described in Table 10. Briefly centrifuge, mix by pipetting up and down at least 10 to 12 times (do not vortex) after adding all reagents, and briefly centrifuge again.

    Important: Only one single-indexed adapter should be used per ligation reaction. Each sample will have a different IL-N7## adapter. Open one adapter tube at a time if using 12-index adapters and avoid cross-contamination. The QIAseq 96-Unique Dual Index set adapter layout is described in Figure 3, use a multichannel pipette to pierce the foil prior to using a multichannel pipette to pipet the appropriate amount of adapters.

For QIAseq 96-index I adapters (CDIs) supplied in a plate (layout described in Figure 6), use a multichannel pipette to pipet the appropriate amount of adapters.

Important: Pipet slowly to mix. The reaction mix is very viscous. Do not vortex.

Note: If setting up more than one reaction, prepare a volume of master mix 20% greater than what is required for the total number of reactions.

Table 10. Preparation of adapter ligation reactions

Component	1 reaction (µl)
End-repair and A-addition reaction (already in the tube)	50 µl
Ligation Buffer, 5x	20 µl
IL-N7## or IL-7##NJ or UDIN-96#A adapter*	2.5 µl
Ligation solution†	15 µl
DNA Ligase	10 µl
Nuclease-free Water	2.5 µl
Total	**100 µl**

* This IL-N7## component applies to the adapters with up to a 12-sample index for QIAseq 12-Index I or IL-7##NJ adapters with QIAseq 96-Index I Set A, B, C, or D set (CDIs), and UDIN-96#A for QIAseq 96-Unique Dual Index Set A or B.

† Ligation solution is very viscous. It should be added into each reaction individually and not pre-mixed with other components for a master mix. Do not coat the outside of the pipette tip with ligation solution or excess volume may be added.

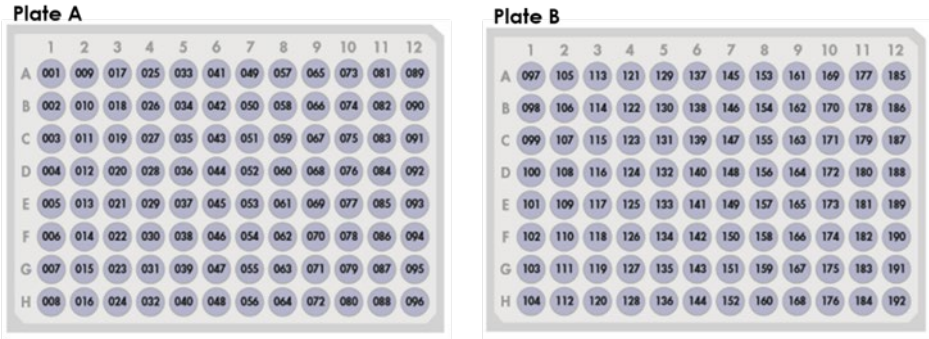


Figure 3. Layout of the pierceable QIAseq 96-Unique Dual Index Set Adapter Plates. QIAseq 96-Unique Dual Index Set A (96) layout (NQDIB001-NQDIB096) and QIAseq 96-Unique Dual Index Set B (96) layout (NQDIB097-NQDIB192).

IL-N701NJ Adapter Plate in QIAseq 96-Index I Set A or C

	1	2	3	4	5	6	7	8	9	10	11	12
A	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
B	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
C	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
D	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
E												
F												
G												
H												

IL-N716NJ Adapter Plate in QIAseq 96-Index I Set B or D

	1	2	3	4	5	6	7	8	9	10	11	12
A	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
B	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
C	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
D	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
E												
F												
G												
H												

Figure 4. Layout of sample adapters (IL-7##NJ) in QIAseq 96-Index I Set A, B, C, or D. Rows A through D of each plate have adapters. Rows E through H are empty. Each well in each row contains one sample adapter, and the amount of adapter in each well is enough for eight samples. IL-N7 Adapter Plate of A, B, C, or D set used in the ligation must be paired with IL-S5 Index Primer Plate of A, B, C, or D set in the Universal PCR step, respectively.

24. Incubate the reactions in a thermal cycler, with the lid open, according to Table 11.
25. **Important**: Do not use heated lid.

Table 11. Incubation conditions for DNA ligation

Step	Temperature	Time
1	4°C	1 min
2	20°C	15 min
3	4°C	Hold

26. Upon completion, place the reactions on ice and proceed with "Protocol: Cleanup of Adapter-Ligated DNA". Alternatively, the samples can be stored at –30 to –15°C in a constant temperature freezer for up to 3 days.

Protocol: Cleanup of adapter-ligated DNA

Important points before starting

- The entire 100 µl adapter ligated DNA from "Procedure: Adapter Ligation" is the starting material for the cleanup of adapter-ligated DNA.
- Equilibrate the QIAseq Beads to room temperature (15–25°C) for 20–30 min before use.
- Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color. If a delay in the protocol occurs, simply vortex the beads.
- **Important**: Prepare fresh 80% ethanol daily.

Procedure: Cleanup of adapter-ligated DNA

27. Transfer the 100 µl adapter ligation product into a 1.5 ml DNA LoBind tube or a 300 µl 96-well PCR plate.
28. Add 80 µl QIAseq beads. Mix well by pipetting up and down 10 times or vortexing.
29. Incubate for 5 min at room temperature.
30. Place the tubes/plate on a magnetic rack for 10 min (for 1.5 ml LoBind tubes) or ~15 min (for 300 µl plates) to separate the beads from the supernatant. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Important: Do not discard the beads as they contain the DNA of interest.
31. With the beads still on the magnetic stand, completely remove residual supernatant.
32. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Carefully remove and discard the wash.
33. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol wash after this second wash. Briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol.
34. With the beads still on the magnetic stand, air-dry at room temperature for 10 min.

    Note: Visually inspect that the pellet is completely dry, and that all residual ethanol is evaporated.
35. Remove the tube from the magnetic stand, and elute the DNA from the beads by adding 52 µl Nuclease-free Water. Mix well by pipetting.
36. Return the tubes/plate to the magnetic rack until the solution has cleared.
37. Transfer 50 µl supernatant to clean tubes/plate.
38. Add 35 µl QIAseq Beads to the 50 µl supernatant. Mix well by pipetting up and down 10 times or vortexing.
39. Incubate for 5 min at room temperature.

40. Place the tubes/plate on a magnetic rack for 5 min (for tubes) or 10 min (for plates) to separate the beads from the supernatant. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Important: Do not discard the beads as they contain the DNA of interest.

41. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Carefully remove and discard the wash.
42. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol wash after this second wash. Briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol.

43. With the beads still on the magnetic stand, air-dry at room temperature for 10 min.

    Note: Visually inspect that the pellet is completely dry, and that all residual ethanol is evaporated. Ethanol carryover to the target enrichment PCR step will affect PCR efficiency.

44. Remove the beads from the magnetic stand, and elute cDNA from beads by adding 12.4 µl Nuclease-free Water. Mix well by pipetting.
45. Return the tubes/plate to the magnetic rack until the solution has cleared.
46. Transfer 10.4 µl of the supernatant to clean PCR tubes or plate.
47. Proceed with "Protocol: Target enrichment". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer for up to 3 days.

Protocol: Target enrichment

Important points before starting

- The entire 10.4 µl product from "Protocol: Cleanup of adapter-ligated DNA", page 33, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure: Target enrichment

48. Prepare the reagents required for the target enrichment.
    48a. Thaw QIAseq RNA Buffer, 5x; QIAseq TCR Panel; and IL-Forward Primer at room temperature.
    48b. Mix by flicking the tube, and then centrifuge briefly.
    Note: HotStarTaq DNA Polymerase should be removed from the freezer just before use and placed on ice. After use, immediately return the enzyme to the freezer.
49. Prepare the target enrichment reactions as described in Table 12, on ice. Briefly centrifuge, mix gently by pipetting up and down 7 to 8 times, and briefly centrifuge again.

Table 12. Preparation of target enrichment reactions

Component	1 reaction
Adapter-ligated DNA (from "Protocol: Cleanup of adapter-ligated DNA")	10.4 µl
QIAseq RNA Buffer, 5x	4 µl
QIAseq TCR panel	4 µl
IL-Forward primer	0.8 µl
HotStarTaq DNA Polymerase	0.8 µl
Total	**20 µl**

50. Program a thermal cycler using the cycling conditions in Table 13.

Table 13. Cycler setting for target enrichment

Step	Cycles	Temperature	Time
1	1	95°C	15 min
2	8	95°C 68°C	15 s 10 min
3	1 1	72°C 4°C	5 min Hold

51. Place the target enrichment reaction in the thermal cycler and start the run.
52. After the reaction is complete, place the reactions on ice and proceed with "Protocol: Cleanup of target enrichment". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer for up to 3 days.

Protocol: Cleanup of target enrichment

Important points before starting

- The entire 20 µl Target Enriched DNA reaction from the "Procedure: Target Enrichment" is the starting material for the cleanup of target enrichment.
- Equilibrate the QIAseq Beads to room temperature (15–25°C) for 20–30 min before use.
- Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color. If a delay in the protocol occurs, simply vortex the beads.
- **Important**: Prepare fresh 80% ethanol daily.

Procedure: Cleanup of target enrichment

53. Transfer the completed target enrichment reaction into a 1.5 ml DNA LoBind tube or a 96-well PCR plate.

54. Add 80 µl Nuclease-free Water to bring each sample to 100 µl.
55. Add 70 µl QIAseq beads. Mix well by pipetting up and down at 10 times or vortexing.
56. Incubate for 5 min at room temperature.
57. Place the tubes/plate on a magnetic rack for 5 min (for tubes) or 10 min (for plates) to separate the beads from the supernatant. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Important: Do not discard the beads as they contain the DNA of interest.
58. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Carefully remove and discard the wash.
59. Repeat the ethanol wash.
60. **Important**: Completely remove all traces of the ethanol wash after this second wash. Briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol.
61. With the beads still on the magnetic stand, air-dry at room temperature for 10 min.

    Note: Visually inspect that the pellet is completely dry, and that all residual ethanol is evaporated. Ethanol carryover to the next step will affect PCR efficiency.
62. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 15.4 µl Nuclease-free Water. Mix well by pipetting.
63. Return the tubes/plate to the magnetic rack until the solution has cleared.
64. Transfer 13.4 µl of the supernatant to clean PCR tubes or plate.
65. Proceed with "Protocol: Universal PCR". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer for up to 3 days.

Protocol: Universal PCR

Important points before starting

- The 13.4 µl product from "Protocol: Cleanup of target enrichment", page 36, is the starting material for this protocol.

- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- **Important**: The QIAseq 96-Unique Dual Index Set (UDIS-96#K) Index Primer Plate must be paired with the matching QIAseq 96-Unique Dual Index Set (UDIN-96#A) Adapter Plate: A or B used in the Adapter Ligation reaction.

    - The UDIS-96#K Index Primer Plate A or B contains pre-dispensed, dried index primers and the Universal PCR primer in a 96-well plate.
    - Use standard 200 µl pipet tips to add components directly in the UDIS-96#K Index Primer plates for the universal PCR reaction. See Figure 5 for the layout of the index primers in the plate.

- **Important**: The QIAseq 12-Index I Adapter (IL-S502) is in an individual tube and must be paired with the IL-N7## tubes.

    - **Important**: Thaw the tubes on ice or store at 4°C before usage. After the tubes are fully thawed spin down the tubes.

- **Important**: The QIAseq 96-Index I Set A, B, C, or D (IL-5##SK) Plate must be paired with the matching QIAseq 96-Index I Set (IL-7##NJ) Adapter Plate: A, B, C, or D used in the Adapter Ligation reaction.

    - The QIAseq 96-Index I Set (IL-5##SK) Plates contain pre-dispensed, dried index primers, and the Universal PCR primer and are sealed in a 96-well plate that needs to have the seal removed.
    - Add the universal PCR reaction components directly in the appropriate IL-5##SK Index Primer plates. See Figure 6 for the layout of the index primers in the plate.

Procedure: Universal PCR

66. Prepare the reagents required for the universal PCR.

66a. Thaw QIAseq RNA Buffer, 5x, at room temperature and bring the appropriate IL-S502 tube, IL-5##SK, or UDIS-96#K plates to room temperature.

66b. Mix by flicking the tube, and then centrifuge briefly.

Note: HotStarTaq DNA Polymerase should be removed from the freezer just before use and placed on ice. After use, immediately return the enzyme to the freezer.

67. Prepare the Universal PCR according to Table 14 or Table 15, depending on index set. Briefly centrifuge, mix by pipetting up and down 7 to 8 times, and briefly centrifuge again.

Important: The IL-N7 Adapter Plate of A, B, C, or D set used in the adapter ligation reaction must be paired with the matching IL-S5 Index Primer Plate of A, B, C, or D set used in the Universal PCR reaction.

Important: If using QIAseq 96-index I set A, B, C, or D, mix components directly in IL-S5 Index Primer Plate in A, B, C, or D set, which contains pre-dispensed, dried IL-S5 index primer, and Universal PCR primers. See Figure 6 for layout of index primers in the plate.

Table 14. Setup of Universal PCR reaction if using QIAseq 12-index I (48)

Component	1 reaction
Purified sample (from "Procedure: Target enrichment")	13.4 µl
QIAseq RNA Buffer, 5x	4 µl
IL-Universal Primer	0.8 µl
IL-S502 Index Primer	0.8 µl
HotStarTaq DNA Polymerase	1 µl
Total	**20 µl**

Table 15. Setup of Universal PCR if using QIAseq 96-index I Set A, B, C, or D* or QIAseq 96-Unique

Dual Index Set A or B†

Component	1 reaction
Purified sample	13.4 µl
QIAseq RNA Buffer, 5x	4 µl
HotStarTaq DNA Polymerase	1 µl
Nuclease-free Water	1.6 µl
Total	**20 µl**

* Applies to QIAseq IL-S5##SK Index Primer Plate in A, B, C, or D set. The final library dual sample index is determined by the combination of the IL-N7##NJ Adapter Plate and the QIAseq IL-S5##SK Index Primer Plate. Total sample index level can be up to 384-plex if using QIAseq 96-index A, B, C, and D sets together.

† Applies to QIAseq UDIS-96#K Index Primer Plate in A or B set. The final library dual sample index is determined by the unique UDIN-96#A Adapter Plate and the QIAseq UDIS-96#K Index Primer Plate. Total sample index level can be up to 192-plex if using QIAseq 96-Unique Dual Index Sets A and B together.

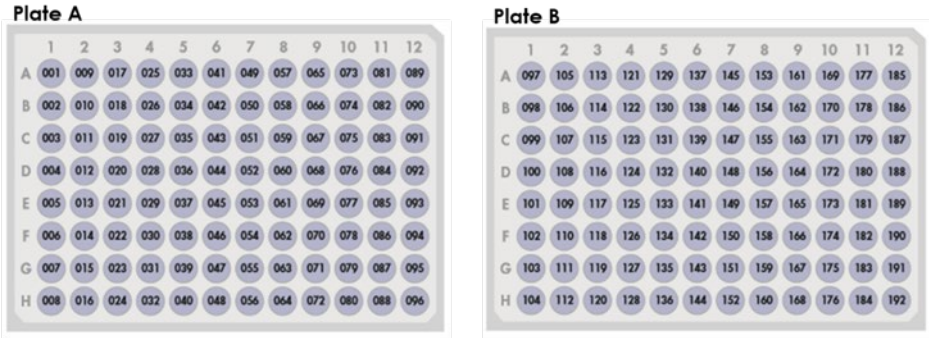


Figure 5. Layout of the pierceable QIAseq 96-Unique Dual Index Set Adapter Plates. QIAseq 96-Unique Dual Index Set A (96) layout (SQDIB001- SQDIB096) and QIAseq 96-Unique Dual Index Set B (96) layout (SQDIB097- SQDIB192).

IL-S502SK Index Primer Plate in QIAseq 96-index I Set A or B

	1	2	3	4	5	6	7	8	9	10	11	12
A	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502
B	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503
C	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505
D	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506
E	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507
F	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508
G	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510
H	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511

IL-S513SK Index Primer Plate in QIAseq 96-index I Set C or D

	1	2	3	4	5	6	7	8	9	10	11	12
A	S513	S513	S513	S513	S513	S513	S513	S513	S513	S513	S513	S513
B	S515	S515	S515	S515	S515	S515	S515	S515	S515	S515	S515	S515
C	S516	S516	S516	S516	S516	S516	S516	S516	S516	S516	S516	S516
D	S517	S517	S517	S517	S517	S517	S517	S517	S517	S517	S517	S517
E	S518	S518	S518	S518	S518	S518	S518	S518	S518	S518	S518	S518
F	S520	S520	S520	S520	S520	S520	S520	S520	S520	S520	S520	S520
G	S521	S521	S521	S521	S521	S521	S521	S521	S521	S521	S521	S521
H	S522	S522	S522	S522	S522	S522	S522	S522	S522	S522	S522	S522

Figure 6. Layout of IL-S5##SK Index Primer Plate in QIAseq 96-Index I Set A, B, C, or D. Each well contains one pre-dispensed, dried sample index primer and universal primer pair for a single reaction. In the Universal PCR step, IL-N7##NJ Adapter Plate in A, B, C, or D set used in ligation must be paired with IL-S5##SK Index Primer Plate in A, B, C, or D set, respectively.

68. Program a thermal cycler using the cycling conditions in Table 16

Table 16. Cycling conditions for universal PCR

Step	Cycles	Temperature	Time
1	1	95°C	15 min
2	25–30*	95°C 60°C	15 s 2 min
3	1 1	72°C 4°C	5 min Hold

* Cycle numbers can be adjusted based on sample type and user experience. Library yield is related to input amount and sample type. For normal PBMC RNA, it is recommended to use 30 cycles for 10–50 ng, 29 cycles for 50–100 ng, 27 cycles for 100–500 ng and 25 cycles for 500–1000 ng.

69. After the reaction is complete, place the reactions on ice and proceed with "Protocol: Cleanup of Universal PCR". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer for up to 3 days.

Protocol: Cleanup of Universal PCR

Important points before starting

- The entire 20 µl Universal PCR reaction from the "Procedure: Universal PCR" is the starting material for the cleanup of Universal PCR.
- Equilibrate the QIAseq Beads to room temperature (15–25°C) for 20–30 min before use.
- Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color. If a delay in the protocol occurs, simply vortex the beads.
- **Important**: Prepare fresh 80% ethanol daily.

Procedure: Cleanup of Universal PCR

70. Transfer the completed Universal PCR reaction into a 1.5 ml DNA LoBind tube or a 96-well PCR plate.
71. Add 80 µl Nuclease-free Water to bring each sample to 100 µl.
72. Add 70 µl QIAseq beads. Mix well by pipetting up and down at 10 times or vortexing.
73. Incubate for 5 min at room temperature.
74. Place the tubes/plate on a magnetic rack for 5 min (for tubes) or 10 min (for plates) to separate the beads from the supernatant. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Important: Do not discard the beads as they contain the DNA of interest.
75. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Carefully remove and discard the wash.
76. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol wash after this second wash. Briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol.
77. With the beads still on the magnetic stand, air-dry at room temperature for 10 min.

    Note: Visually inspect that the pellet is completely dry, and that all residual ethanol is evaporated, but be careful not to overdry the beads as this will significantly decrease elution efficiency.
78. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 30 µl Nuclease-free Water. Mix well by pipetting.
79. Return the tubes/plate to the magnetic rack until the solution has cleared.
80. Transfer 28 µl supernatant to clean PCR tubes or plate.

81. Proceed with “Appendix A: Library Quantification Using the QIAseq Library Quant System”, page 54. Alternatively, the library can be stored in a –30 to –15°C in a constant-temperature freezer for up to 3 months prior to quantification using the QIAseq Library Quant System. Once quantification is performed, proceed with the “Protocol: Sequencing Setup on Illumina MiSeq and NextSeq”, next page.

    Note: The Agilent Bioanalyzer can be used to check the fragment size and concentration with the High Sensitivity DNA Kit. For details, please refer to Appendix C: Library .

Protocol: Sequencing Setup on Illumina MiSeq and NextSeq

Important notes before starting:

- Accurate library quantification is essential for accurate flow cell clustering and instrument loading. Libraries quantitated by qPCR is the most accurate. We highly recommend to use the QIAseq Library Quant System (cat. no. 333304 or 333314).
- Due to the library size, the extension time should be 4 min at 72°C instead of 2 min indicated in the QIAseq Library Quant System protocol.
- Recommendations for library dilution concentrations and library loading concentrations are based on the QIAseq Library Quant System (see Appendix A: Library Quantification Using the QIAseq Library Quant System, page 54).
- QIAseq A Read1 Primer I (Custom Read 1 Sequencing Primer) MUST be used when performing sequencing on Illumina platforms.
- QIAseq A Read1 Primer I (the Custom Read 1 Sequencing Primer) goes into the following specific reagent cartridge positions:

  - MiniSeq Position #15
  - MiSeq Position #18
  - NextSeq Position #7
- Download Illumina compatible sample sheets for different sequencing instruments at **www.qiagen.com**, from the "Resources" tab of the QIAseq Immune Repertoire RNA libraries.
- Paired-end sequencing is required for the QIAseq Immune Repertoire RNA libraries on Illumina platforms.

- When using 10 bp Indexing kits such as the QIAseq 96-Unique Dual Index Set A (cat. no. 333725) or QIAseq 96-Unique Dual Index Set B (cat. no. 333735)

  - Index 1 and Index 2 are 10 bp cycles each
  - 249 bp paired-end sequencing is recommended for complete variable regions sequencing
  - 259/39 bp asymmetric paired-end sequencing is recommended for CDR3 region sequencing

- When using 8 bp index kits such as QIAseq 12-Index I (cat. no. 333714) or QIAseq 96-Index I Set A, B, C, or D (cat. nos. 333727, 333737, 333747, 333757)

  - Index 1 and Index 2 are 8 bp cycles each
  - 251 bp paired-end sequencing is recommended for complete variable region sequencing.
  - 261/41 bp asymmetrical paired-end sequencing is recommended for CDR3 region sequencing

- For complete instructions on how to denature sequencing libraries, prepare custom read 1 sequencing primers, and set up a sequencing run, please refer to the system-specific Illumina documentation.
- Instrument-specific imagery is included to aid in sequencing preparations.

Sequencing preparations for MiSeq when using 8 base CDI indexes: QIAseq 12-Index I (cat. no. 333714) or QIAseq 96-Index I Set A, B, C, or D (cat. nos. 333727, 333737, 333747, 333757)

1. **Sample sheet setup**: Set up the sample sheet with Custom Sequencing Read 1 primer using Illumina Experiment Manager v1.2, or later (Figure 7).
2. The 8 bp sample CDI indices of QIAseq Immune Repertoire libraries are compatible with Illumina Nextera XT v2 adapter sample index system. Select and check the parameters as follows:

   Category: Select Other

   Select Application: Check FASTQ Only

   Sample Prep Kit: Select Nextera XT v2

   Index Reads: Select 2

   Read Type: Select Paired-End Read

   For complete variable region:

   Cycles for Read 1 and 2: Select 251

   For CDR3 region sequencing only

   Cycles for Read 1: Select 261

   Cycles for Read 2: Select 41

   Important: Check Custom Primer for Read 1

   Important: Check Use Adapter Trimming

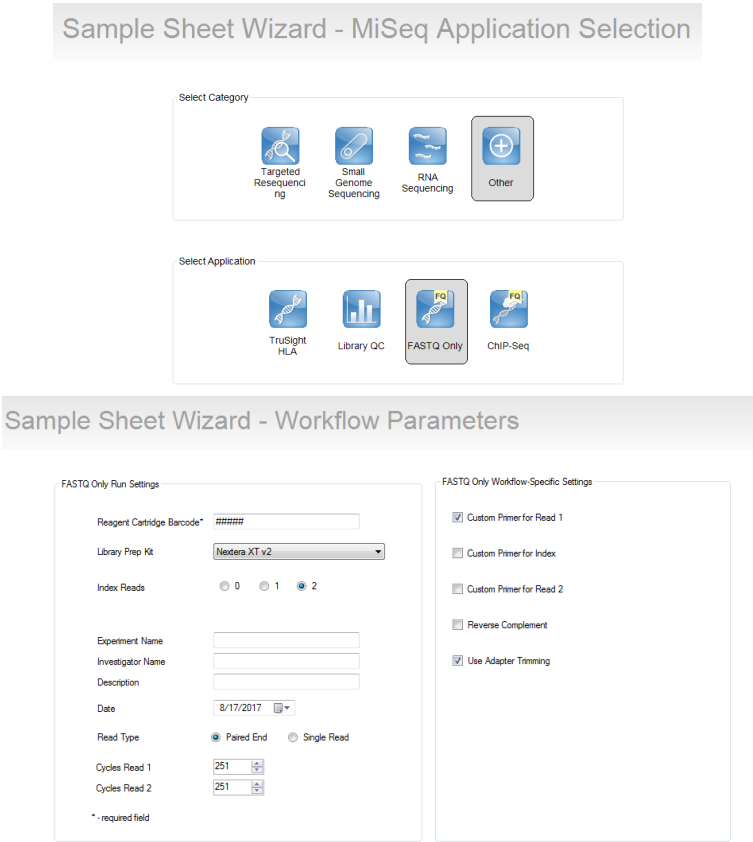


Figure 7. Sample sheet using Illumina Experiment Manager.

3. **Sample dilution and pooling**: Dilute libraries to 2 or 4 nM for MiSeq. Then, combine libraries with different sample indexes in equimolar amounts if similar sequencing depth is needed for each library.

   Note: Recommendations for library dilution concentrations are based on QIAseq Library Quant System.

4. **Library preparation and loading**: Prepare and load the library onto a MiSeq according to the MiSeq System Denature and Dilute Libraries Guide. The final library concentration is 10–12 pM on the MiSeq.

   Note: Recommendations for library loading concentrations are based on QIAseq Library Quant System.

5. **Custom Sequencing Primer for Read 1 preparation and loading**: Use 597 µl HT1 (Hybridization Buffer) to dilute 3 µl QIAseq A Read 1 Custom Primer I (provided) to obtain a final concentration of 0.5 µM. Load 600 µl of the diluted QIAseq A Read 1 Primer I to position 18 of the MiSeq reagent cartridge (Figure 8). For more details, please refer to the Illumina protocol: miseq_using_custom_primers_15041638_b.pdf for the MiSeq.

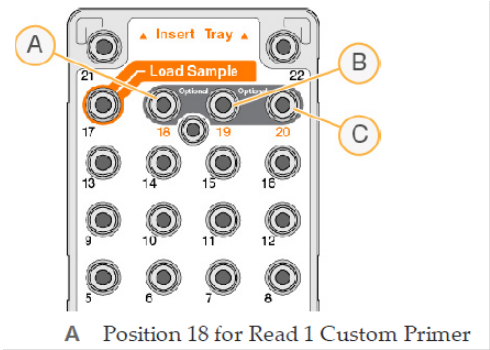


Figure 8. MiSeq Reagent Cartridge. A: Position 18 for Read 1 Custom Primer. B and C are not relevant.

6. Upon completion of the sequencing run, proceed to "Appendix B: Data Analysis Using QIAGEN's QIAseq Immune Repertoire RNA Library Kit Data Analysis Portal".

Sequencing preparations for MiSeq with 10 base UDI Sample Indexes: QIAseq 96-Unique Dual Index Set A (cat. no. 333725) or QIAseq 96-Unique Dual Index Set B (cat. no. 333735)

7. When working with the QIAseq Immune Repertoire RNA Panel custom QIAseq 96-UDIs, use Local Run Manager (LRM) v2 on the instrument to upload a sample sheet (see the "Resource" tab of the QIAseq Immune Repertoire RNA Panel and download the

appropriate template) and proceed with sequencing: Read 1 is 249 bp, Read 2 is 249 bp, and each Index Read is 10 bp.

8. Follow steps 3–5 in "Sequencing preparations for MiSeq when using 8 base CDI indexes: QIAseq 12-Index I (cat. no. 333714) or QIAseq 96-Index I Set A, B, C, or D (cat. nos. 333727, 333737, 333747, 333757)".

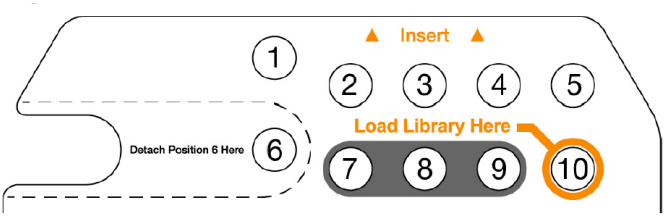
Sequencing preparations for NextSeq when using 8 base CDI indexes: QIAseq 12-Index I (cat. no. 333714) or QIAseq 96-Index I Set A, B, C, or D (cat. nos. 333727, 333737, 333747, 333757)

9. **Read setup**: Use asymmetrical paired-end reads (261 cycles for read 1 and 41 cycles for read 2) with NextSeq 300 cycles kit and it is mainly for CDR3 region sequencing.
10. **Indexing setup**: Use dual indexes (8 bp cycles of each).
11. **Sample dilution and pooling**: Dilute libraries to 0.5, 1, 2, or 4 nM for NextSeq. Then, combine libraries with different sample indexes in equimolar amounts if similar sequencing depth is needed for each library.

    Note: Recommendations for library dilution concentrations are based on the QIAseq Library Quant System.
12. **Library preparation and loading**: Prepare and load the library onto a NextSeq according to the NextSeq System Denature and Dilute Libraries Guide. The final library concentration is 1.0–1.2 pM on the NextSeq.

    Note: Recommendations for library loading concentrations are based on the QIAseq Library Quant System.
13. **Custom Sequencing Primer for Read 1 preparation and loading**: Use 1994 µl HT1 (Hybridization Buffer) to dilute 6 µl QIAseq A Read 1 Primer I (provided) to obtain a final concentration of 0.3 µM. Load 2 ml of the diluted QIAseq A Read 1 Primer I to position 7 of the NextSeq reagent cartridge (Figure 9).

    Note: All other steps, refer to run setup workflow as described in the NextSeq 500 System Guide (part #15046563) or NextSeq 550 System Guide (part #15069765-02).



Position #	Custom Primer
7	Custom Read 1 primer

Figure 9. NextSeq reagent cartridge.

14. Upon completion of the sequencing run, proceed to with "Appendix B: Data Analysis Using QIAGEN's QIAseq Immune Repertoire RNA Library Kit Data Analysis Portal".

Sequencing preparations for NextSeq with 10 base UDI Sample Indexes: QIAseq 96-Unique Dual Index Set A (cat. no. 333725) or QIAseq 96-Unique Dual Index Set B (cat. no. 333735)

15. When working with the QIAseq Immune Repertoire RNA libraries custom QIAseq 96-UDIs, use Local Run Manager (LRM) v2 on the instrument to upload a sample sheet (see the "Resource" tab of the QIAseq Immune Repertoire RNA Panel and download the appropriate template) and proceed with sequencing: Read 1 is 259 bp, Read 2 is 39 bp, and each Index Read is 10 bp.
16. Follow steps 11–13 in "Sequencing preparations for NextSeq when using 8 base CDI indexes: QIAseq 12-Index I (cat. no. 333714) or QIAseq 96-Index I Set A, B, C, or D (cat. nos. 333727, 333737, 333747, 333757)".

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
Low library yield	
a) Suboptimal reaction conditions due to low RNA quality	Make sure to use high-quality RNA to ensure optimal activity of the library enzymes.
b) Inefficient PCR	QIAseq Beads need to be completely dried before elution. Ethanol carryover to target enrichment and universal PCR will affect reaction efficiency.
c) Insufficient universal PCR cycles	Increase universal PCR cycle numbers to achieve sufficient library amount for sequencing.
Unexpected signal peaks	
a) Larger library fragments after universal PCR	After the universal PCR, library fragments are larger than the intended peak and can be a PCR artifact due to over-amplification of the library. Over-amplification of the library won't affect the QIAseq Immune Repertoire RNA Library Kit sequencing performance. Decreasing the universal PCR cycle numbers can reduce over-amplification.
b) Small fragments in final library	Make sure the correct bead amount is used. PCR tube or plate must be sealed well to prevent evaporation during PCR reaction.
Sequencing issues	
a) Too low or too high cluster density	Accurate library quantification is the key for optimal cluster density on any sequencing instrument. The QIAseq Library Quant System method, which involves PCR-based quantification, is recommended. Other methods may lead to the incorrect quantification of the library especially when there is over-amplification or the library concentration is very high.

		Comments and suggestions
b)	Very low clusters passing filter	Make sure the library is quantified accurately and that the correct amount is loaded onto the sequencing instrument. In addition, the QIAseq A Read1 Primer I (100 µM) Custom Read 1 Sequencing primer MUST be used when sequencing on any Illumina platform.
Clonotype detection issues		
a)	Known clonotype not detected	Clonotype and immune repertoire diversity detection sensitivity is directly related to the input RNA and read depth. See Table 2 for suggested sequencing capacity needed.

Appendix A: Library Quantification Using the QIAseq Library Quant System

The library yield measurements from the Bioanalyzer or TapeStation rely on fluorescence dyes that intercalate into DNA. These dyes cannot discriminate between molecules with or without adapter sequences, yet only complete QIAseq Immune Repertoire RNA libraries with full adapter sequences will be sequenced. Due to the superior sensitivity of qPCR, we recommend quantifying the libraries using QIAGEN's QIAseq Library Quant Array Kit (cat. no. 333304) or QIAseq Library Quant Assay Kit (cat. no. 333314), which contains laboratory-verified forward and reverse primers, together with a DNA standard. With this system, the correct dilution of the library can be determined for sequencing. Please refer to the relevant handbook(s) (available at **www.qiagen.com**) for library quantification.

Important: The extension time should be 4 minutes at 72°C instead of the 2 minutes indicated in the standard protocol of the QIAseq Library Quant System.

Appendix B: Data Analysis Using QIAGEN's QIAseq Immune Repertoire RNA Library Kit Data Analysis Portal

After sequencing, the results can be analyzed using QIAGEN's QIAseq Immune Repertoire RNA Library Kit data analysis portal. Our data analysis software will perform mapping to the reference sequence, UMI counting, read trimming (removing primer sequences) as well as immune repertoire diversity assessment and clonotype classification. For the analysis, use Google Chrome™ or Mozilla® Firefox®, as the portal is not compatible with Internet Explorer™ (IE). Log in or create a QIAGEN account at **www.qiagen.com**. From there, log in to the GeneGlobe Data Analysis Center at: **www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page/**.

To start the analysis, select:

1. Next-generation sequencing
2. mRNA/lncRNA
3. QIAseq Immune Repertoire RNA Library Kit

Appendix C: Library QC

NGS Library QC

After the library is constructed and purified, QC can be performed with the QIAxcel, or Agilent's Bioanalyzer or TapeStation can be used to check for the correct fragment size distribution of library fragments and for the absence of adapters or adapter-dimers (~<200 bp) and concentration with the High Sensitivity DNA Kit. Libraries prepared with QIAseq Immune Repertoire RNA Library Kits demonstrate a size distribution between 600–2000 bp (Figure 10). Amounts of DNA under the appropriate peaks can be used to quantify the libraries. However, due to the superior sensitivity of qPCR, we recommend quantifying the libraries using the QIAseq Library Quant System (see "Appendix A: Library Quantification Using the QIAseq Library Quant System", page 54).

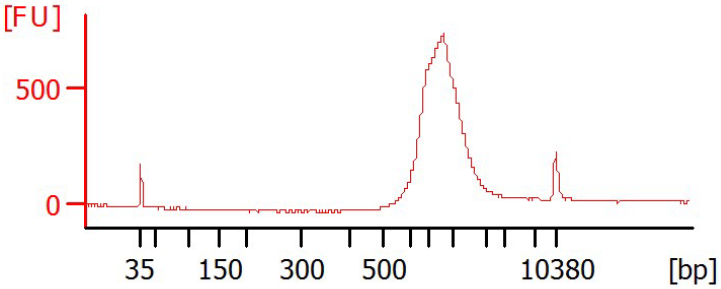


Figure 10. Bioanalyzer image of QIAseq Immune Repertoire RNA Library Kit library for Illumina instruments. A library was prepared using the QIAseq Immune Repertoire RNA Library Kit and assessed using an Agilent 2100 Bioanalyzer. The size of the majority of the library fragments are between 600–2000 bp.

Ordering Information

Product	Contents	Cat. no.
QIAseq Human TCR Sequencing kit (12)*	All reagents (except indexes) for human TCR immune repertoire sequencing; fixed panel for 12 samples	333705
QIAseq Human TCR Sequencing kit (96)*	All reagents (except indexes) for human TCR immune repertoire sequencing; fixed panel for 96 samples	333705
QIAseq Mouse TCR Sequencing kit (12)*	All reagents (except indexes) for mouse TCR immune repertoire sequencing; fixed panel for 12 samples	333705
QIAseq Mouse TCR Sequencing kit (96)*	All reagents (except indexes) for mouse TCR immune repertoire sequencing; fixed panel for 96 samples	333705
QIAseq Unique Dual Indices		
QIAseq 96-Unique Dual Index Set A (384)	Box containing unique molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples uniquely for targeted panel sequencing on Illumina platforms; Set A (of A and B) required for multiplexing 192 samples in one run	333725

Product	Contents	Cat. no.
QIAseq 96-Unique Dual Index Set B (384)	Box containing unique molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples uniquely for targeted panel sequencing on Illumina platforms; Set B (of A and B) required for multiplexing 192 samples in one run	333735
QIAseq Combinatorial Dual Indices		
QIAseq 12-Index I (48)	Box containing molecularly-indexed adapters and primers, enough for a total of 48 samples – for indexing up to 12 samples for targeted panel sequencing on Illumina platforms	333714
QIAseq 96-Index I Set A (384)	Box containing molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; one of four sets required for multiplexing 384 samples	333727
QIAseq 96-Index I Set B (384)	Box containing molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; two of four sets required for multiplexing 384 samples	333737

Product	Contents	Cat. no.
QIAseq 96-Index I Set C (384)	Box containing molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; three of four sets required for multiplexing 384 samples	333747
QIAseq 96-Index I Set D (384)	Box containing molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; four of four sets required for multiplexing 384 samples	333757
Related products		
QIAseq Library Quant Array Kit	Reagents for quantification of libraries prepared for Illumina or Ion Torrent platforms; array format	333304
QIAseq Library Quant Assay Kit	Reagents for quantification of libraries prepared for Illumina or Ion Torrent platforms; assay format	333314
RNeasy Mini Kit (50)	Reagents for purification of high-quality RNA from cells, tissues, and yeast, 100 µg RNA capacity	74104
RNeasy Micro Kit (50)	Reagents for purification of high-quality RNA from cells, tissues, and yeast, 45 µg RNA capacity	74004
AllPrep DNA / RNA Mini Kit (50)	Reagents for simultaneous purification of genomic DNA and total RNA from the same biological sample	80204

Product	Contents	Cat. no.
PAXgene Blood RNA Kit (50)	Reagents for isolation and purification of intracellular RNA from blood stabilized in PAXgene Blood RNA Tubes	762174
QIAamp® RNA Blood Mini Kit (50)	Reagents for purification of cellular RNA from fresh whole blood	52304

* Visit www.qiagen.com/GeneGlobe to search for and order these products.

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Document Revision History

Date	Changes
11/2017	Initial release.
07/2019	New label quantity for EZ Reverse Transcriptase from 12 µl to 15 µl; Layout updates
03/2020	Change in the volume of QIAseq Beads in Box 2 from 7 ml to 10 ml.
07/2021	Implemented changes for QIAseq Unique Dual Indices (UDI). Revised the "Shipping and Storage" section. Updated the "Sequencing capacity and sample multiplex level" section. Updated the "Protocol: QIAseq Immune Repertoire RNA Library Kit for Illumina Instruments". Updated the "Protocol: Sequencing Setup on Illumina MiSeq and NextSeq". Added the UDIs in the “Ordering Information” section.

Notes

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