July 2021

QIAseq® RNA Fusion XP Panel Handbook

For constructing molecularly barcoded libraries from RNA for gene fusion, gene expression, and RNA SNV/InDel analysis using digital NGS

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq RNA Fusion XP Panel	**(12)**
Catalog no.	**334602**
Box 1 of 2	
QIAseq RNA Fusion XP Panel	48 µl
RP Primer II	12 µl
EZ Reverse Transcriptase	15 µl
BC3 Buffer, 5x	24 µl
RNase Inhibitor	12 µl
RH RNase	12 µl
dNTP II	18 µl
BLU Buffer, 10x	24 µl
POL Enzyme	12 µl
ERA Enzyme	120 µl
ERA Buffer, 10x	60 µl
DNA Ligase	120 µl
Ligation Buffer, 5x	240 µl
TEPCR Buffer, 5x	60 µl
RNA Buffer II, 5x	48 µl
TaqIT Plus enzyme	15 µl
HotStarTaq® DNA Polymerase	30 µl
Box 2 of 2	
QIAseq Beads	10 ml

QIAseq RNA Fusion	XP Panel (96)	XP Custom Panel (96)	XP Extended Panel (96)
Catalog no.	**334605**	**334625**	**334645**
Box 1 of 2			
QIAseq RNA Fusion XP Panel		384 µl	
RP Primer II		96 µl	
EZ Reverse Transcriptase		96 µl	
BC3 Buffer, 5x		192 µl	
RNase Inhibitor		96 µl	
RH RNase		96 µl	
dNTP II		144 µl	
BLU Buffer, 10x		192 µl	
POL Enzyme		96 µl	
ERA Enzyme		960 µl	
ERA Buffer, 10x		480 µl	
DNA Ligase		960 µl	
Ligation Buffer, 5x		2 x 960 µl	
TEPCR Buffer		400 µl	
RNA Buffer II		2 x 384 µl	
TaqIT Plus enzyme		2 x 120 µl	
HotStarTaq DNA Polymerase		240 µl	
Box 2 of 2			
QIAseq Beads		55 ml	

QIAseq 8-Unique Dual Index Set A	**(48)**
Catalog no.	**333715**
(8 unique sample indices for reaction on Illumina® platform, order separately)	
UDIN-8AA	
Containing 1-strip tubes of molecularly barcoded adapters, each tube corresponding to 1 sample index; sufficient to process 6 samples	40 µl
UDIS-8AK	
Containing 1 plate of molecularly index primers, each assay well corresponds to 1 sample index primer and IL-Universal primer pair; sufficient to process total 8 x 6 samples	Dry plate
IL-Forward Primer	40 µl
QIAseq A Read 1 Primer I (100 µM)	24 µl

QIAseq 8-Unique Dual Index Set B	**(48)**
Catalog no.	**333716**
(8 unique sample indices for reaction on Illumina platform, order separately)	
UDIN-8BA	
Containing 1-strip tubes of molecularly barcoded adapters, each tube corresponding to 1 sample index; sufficient to process 6 samples	40 µl
UDIS-8BK	
Containing 1 plate of molecularly index primers, each assay well corresponds to one index primer and IL-Universal primer pair; sufficient to process total 8 x 6 samples	Dry plate
IL–Forward Primer	40 µl
QIAseq A Read 1 Primer I (100 µM)	24 µl

QIAseq 96—Unique Dual Index	Set A	Set B
Catalog no.	**333725**	**333735**
(96 sample indices for 384 samples on Illumina platforms, order separately)	**(384)**	
UDIN-96AA or UDIN-96BA		
Four plates, each containing 96 molecularly barcoded adapters, each well corresponding to 1 sample index; the kit is sufficient for 384 samples	Four plates each (4 x 10 µl per adapter)	
12-cap strip	48	
UDIS-96AK OR UDIS-96BK		
Containing 4-index primer arrays. Each array well contains 1 unique index primer and IL-Universal PCR primer for PCR amplification and sample indexing; the kit is sufficient for 384 samples	4 dry plates	
12-cap strip	48	
Primers		
IL-Forward Primer	310 µl	
QIAseq A Read 1 Primer I (100 µM)	4 × 24 µl	

QIAseq 12-Index L	(48)
Catalog no.	**333764**
(12 sample indices for 48 samples on Ion Torrent™ platform)	
LT-BC# Adapter	
Containing 12 tubes of molecularly barcoded adapters, each tube corresponding to 1 sample index; sufficient to process four samples	20 µl
Primers	
LT-P1 Primer	40 µl
LT-Forward Primer	40 µl
LT-Universal Primer	40 µl

QIAseq 96-Index L	**(384)**
Catalog no.	**333777**
(96 sample indices for 384 samples on Ion Torrent platform)	
LT-BC Adapter Plate	
Four plates containing 96 molecularly barcoded adapters, each well corresponding to 1 sample index; the kit is sufficient for 384 samples	4 plates (20 µl/well)
12-cap strip	48
Primers	
LT-P1 Primer	310 µl
LT-Forward Primer	310 µl
LT-Universal Primer	310 µl

QIAseq RNA Fusion XP Catalog Panel Information

Catalog no.	Product Name	Total Primers
JHS-001Z	Human Leukemia Panel	2010
JHS-002Z	Human Solid Tumor Panel	3161
JHS-003Z	Human Lung Cancer Panel	1210
JHS-004Z	Human Lymphoma Panel	1371
JHS-005Z	Human Sarcoma Panel	1238
JHS-3001Z	Human Oncology Research Panel	4366
JHS-3002Z	Human Pan Heme Panel	2393

Storage

The QIAseq RNA Fusion XP Panel Kit is shipped with dry ice (Box 1) and must be stored at –20°C upon arrival. Box 2 (QIAseq Beads) is shipped on cold packs and should be stored at 4°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.

Intended Use

The QIAseq RNA Fusion XP Panel Kits are for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of these 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 RNA Fusion XP Panel Kits is tested against predetermined specifications to ensure consistent product quality.

Introduction

Temporal and spatial misregulation of gene expression is a frequent cause or consequence of a disease. In addition, DNA-based changes in coding sequence, structural rearrangement, and post-transcriptional processing of RNA can alter the function of an mRNA and can cause or contribute to a disease. Next-generation sequencing (NGS) of RNA (RNAseq) has been used to detect gene fusion, alternatively spliced transcripts, post-transcriptional modifications, mutations/single nucleotide variants (SNVs), and changes in gene expression. A fusion gene is a hybrid gene formed from two previously discrete genes. It can occur because of translocation, deletion, chromosomal inversion, or transcription-generated chimeras. The QIAseq RNA Fusion XP Panels use single primer extension (SPE) and unique molecular index (UMI) technologies in NGS to help identify and characterize fusion gene events, gene expression, and SNV/InDel at the RNA level with high efficiency, sensitivity, and flexibility.

Principle and workflow

The QIAseq RNA Fusion XP Panels rely on highly efficient RNA conversion, gene-specific single-primer enrichment, and molecular barcoding for sensitive fusion, gene expression, and RNA SNV/InDel detection.

Unique molecular indices

The concept of UMIs or sometime called molecular barcoding is that, prior to any amplification, each original target molecule is "tagged by" a unique sequence "barcode". This is accomplished by the ligation of double-strand cDNA with a sample index adapter containing a 12-base random sequence. Statistically, this provides 4^{12} = 16,777,216 unique molecular tags for each adapter and each converted double-strand cDNA molecule in the sample receives a unique UMI sequence.

The barcoded cDNA molecules are then amplified by SPE for target enrichment and library amplification. Due to intrinsic noise and sequence-dependent bias, barcoded cDNA molecules may be amplified unevenly between different enriched targets. Therefore, target transcripts can be better evaluated by counting the number of unique molecular indices in the reads rather than counting the number of total reads for each transcript. Sequence reads having distinct UMIs represent different original molecules, while sequence reads having the same UMI are the results of PCR duplication from 1 original molecule and are counted together as 1 molecule.

Procedure

The QIAseq RNA Fusion XP Panels are provided as a single tube of primer mix, with up to 20,000 primers per tube (custom panel). The QIAseq RNA Fusion XP Panels can enrich selected transcripts using 10–250 ng of fresh total RNA or FFPE RNA. Although libraries can be constructed with as little as 1 ng fresh RNA, more RNA input will increase fusion detection sensitivity due to limited amount of original fusion RNA molecules present in low-input samples. Our general recommendation is to use 50–100 ng fresh total RNA or 100–200 ng FFPE RNA as the starting point if you have no prior experience with fusion analysis in your samples.

RNA samples are initially converted to first-strand cDNA. A separate, second-strand synthesis is used to generate double-stranded cDNA (ds-cDNA). This ds-cDNA is then end-repaired and A-tailed in a single-tube protocol. The prepared ds-cDNAs are then ligated at their 5′ ends to a sequencing platform-specific adapter containing UMI and sample index.

Adapter-ligated cDNA molecules are subject to limited target-barcode enrichment with SPE. This reaction ensures that intended targets are enriched sufficiently to be represented in the final library.

A universal PCR is then carried out with highly efficient, low error rate, fast processing Taq enzyme to amplify the library and add a second sample index (unique dual index, UDI, is recommended if available, platform specific) and other platform-specific required sequences.

The raw sequencing results should be analyzed using the QIAseq RNA Fusion XP Panel Analysis Software in GeneGlobe® at **geneglobe.qiagen.com**, which will automatically perform all steps necessary to generate a fusion, gene expression, and RNA SNV/InDel call report from your NGS data.

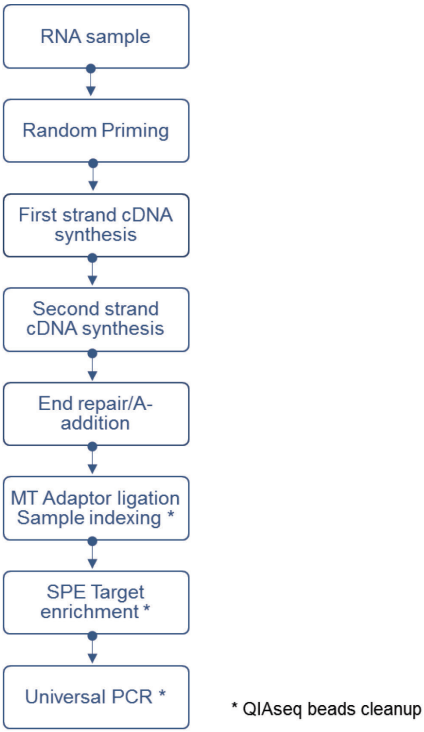


Figure 1. QIAseq RNA Fusion XP Panels workflow.

Description of protocols

This handbook contains 2 protocols. The first protocol details RNA Fusion XP library generation for the Illumina platform with the QIAseq Unique Dual Index Kit (page 21). The second protocol describes how to generate an NGS library for Thermo Fisher's Ion system platform with the QIAseq Index L Kit (page 36).

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.

In addition to the QIAseq RNA Fusion XP Panel Kits and QIAseq 8-UDI/96UDI, or 12/96 Index L Kits, the following supplies are required:

- High-quality nuclease-free water
- Microcentrifuge
- 1.5 ml or 2 ml LoBind® tubes (Eppendorf® AG)
- 0.2 ml PCR tubes and 0.2 ml 96-well PCR reaction plates (BIOplastics, cat. no. AB17500 or equivalent) or PCR strip tubes and caps
- Eppendorf twin.tec® PCR plate 96 LoBind (Eppendorf AG, cat. no. 0030129504 or equivalent) for handling with 96-well plate format in beads wash and library storage
- Thermal cycler
- Multichannel pipettor
- Single-channel pipettor
- Nuclease-free pipet tips and tubes
- Agilent® 4200 TapeStation®
- Agilent High Sensitivity D1000 ScreenTape® kit (cat. no. 5067-5584)
- 80% ethanol
- Magnet rack for 1.5 ml or 2 ml tubes (DynaMag™-2 Magnet, Thermo Fisher Scientific cat. no. 12321D, or equivalent)
- Magnetic separation rack for 96-well plates (DynaMag-96 Side Magnet, Thermo Fisher cat. no. 12331D, or equivalent)
- QIAseq Library Quant System: QIAseq Library Quant Array Kit (cat. no. 333304), QIAseq Library Quant Assay Kit (cat. no. 333314), or QIAxpert® Instrument (cat. no. 9002340; **www.qiagen.com/qiaxpertsystem-orderinginfo**)

- NGS sequencing platform (Illumina system or Thermo Fisher Ion Torrent system)
- Controls: Seraseq® Fusion RNA Mix v4 and Seraseq FFPE Tumor Fusion RNA v4 Reference Material from SeraCare Life Sciences; ALK RET ROS RNA fusion positive control from Horizon Discovery
- 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

Preparing RNA

High-quality RNA is essential for obtaining good sequencing results

The most important prerequisite for RNA sequence analysis is consistent, high-quality RNA from every experimental sample. Accordingly, sample handling and RNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts, or other contaminants may degrade RNA. Furthermore, contaminants can also decrease or completely block enzyme activities necessary for PCR performance.

Recommended RNA preparation method

QIAGEN's RNeasy® Mini Kit (cat. nos. 74104 and 74106), RNeasy Micro Kit (cat. no. 74004), AllPrep® DNA/RNA Mini Kit (cat. no. 80204), AllPrep DNA/RNA FFPE Kit (cat. no. 80234), and RNeasy FFPE Kit (cat. no. 73504) are recommended for the preparation of RNA samples from fresh tissues and FFPE tissue samples. If RNA samples need to be harvested from biological samples for which kits are not available, please contact Technical Support for suggestions. On-column DNase treatment is not recommended.

For best results, all RNA samples should be resuspended in RNase-free water. Do not use DEPC-treated water.

RNA quantification and quality control

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

The concentration and purity of RNA should be determined by measuring the absorbance in a spectrophotometer such as the QIAxpert.

Pure RNA has an A_{260}:A_{280} ratio of 1.9–2.1 in 10 mM Tris-HCl, pH 7.5.

Run an aliquot of each RNA sample on the Agilent 4200 TapeStation using RNA ScreenTape or the QIAxcel Advanced System using QIAxcel RNA QC Kit v2.0 (cat. no. 929104). 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 Bioanalyzer/TapeStation should be higher than 5. For low-quality RNA samples (like FFPE sample), RNA quality needs to be evaluated by the percentage of RNA fragments >200 nucleotides. It is better to be >30% though the QIAseq RNA Fusion XP may still generate library with fragmented samples when this number is as low as 20%.

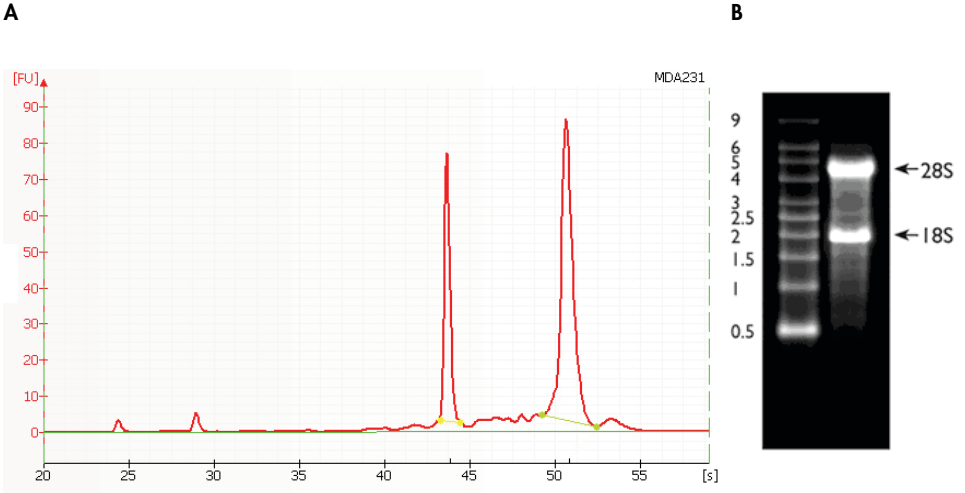


Figure 2. Ribosomal RNA integrity. A: Agilent Bioanalyzer electropherogram of high-quality total RNA showing sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). **B**: Agarose gel electrophoresis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

Starting RNA amounts

The QIAseq RNA Fusion XP Panels provide results with as little as 10 ng or as much as 250 ng total RNA per cDNA synthesis reaction (input lower than 10 ng could be evaluated based on research needs; also higher than 250 ng could be used under special situation with custom validation).

The optimal amount of starting material depends on the relative abundance of the transcripts of interest. Lower-abundance transcripts require more RNA; high-abundance transcripts require less RNA. Greater amounts of input total RNA will provide greater sensitivity for fusion gene call when enough read budget is allocated.

For successful results, we recommend that first-time users start with 50–100 ng total RNA or 100–200 ng FFPE RNA. We recommend using a consistent amount of total RNA for all reactions in a single experiment.

Sample index and sample plex

Using the QIAseq Unique Dual Index to work with the RNA Fusion XP is recommended to overcome the Illumina platform-related index bleed/hopping issue as RNA Fusion XP has high sensitivity, especially for fusion detection. The pairing of i7 and i5 needs to be maintained based on the setting of the QIAseq Unique Dual Index Kit for best performance.

The QIAseq RNA Fusion XP Panels are compatible with Illumina NGS platforms including MiSeq®, MiniSeq®, NextSeq® 500/550, HiSeq® 2500, HiSeq 3000/4000, and NovaSeq 6000. The QIAseq RNA Fusion XP Panel cannot be used on Illumina's iSeq® 100 platform due to primer setting. The QIAseq RNA Fusion XP sequencing requires QIAseq A Read 1 custom primer (included in QIAseq index kit); no custom Read 2 primer and custom index primer is needed as the standard Illumina primer is available in Illumina cartridges. Sequencing cartridges with 300 cycles or higher are needed.

The QIAseq RNA Fusion Panel is compatible with Thermo Fisher's Ion S5® System (including Ion GeneStudio® S5 System, Ion GeneStudio S5 Plus System, and Ion GeneStudio S5 Prime System). When using Thermo Fisher Ion NGS system, please refer to the user manual for more details.

Read budget and sample-plex level

Sample multiplexing capacity is defined by the size of the panel and sequencing platform read capacity. For the MiSeq/NextSeq platforms, sample indexing barcodes are available to multiplex up to 192 samples with UDI (please contact customer support for using UDI with more than 192 samples). For Ion Torrent platforms, sample indexing barcodes are available to multiplex up to 96 samples per run. The number of samples that can be sequenced together will depend on the size of your panel and the sequencing capacity of the intended instrument and kit. Fine tuning for your read budget will be possible after your first test run. Hereafter is a preliminary starting sample multiplex suggestion. Adjustments can be made as necessary.

Estimated sequencing depth for catalog panels

Catalog no.	Product Name	Reads
JHS-001Z	Human Leukemia Panel	3.0–7.0M
JHS-002Z	Human Solid Tumor Panel	4.0–7.0M
JHS-003Z	Human Lung Cancer Panel	2.0–4.0M
JHS-004Z	Human Lymphoma Panel	2.5–4.0M
JHS-005Z	Human Sarcoma Panel	2.0–4.0M
JHS-3001Z	Human Oncology Research Panel	5.5–10M
JHS-3002Z	Human Pan Heme Panel	4.0–7.0M

Note: The recommended reads number is for reference only; the best reads depth for each panel fully rely on the sample type, the input, and sensitivity requirement. User is recommended to optimize it with some control samples.

For the estimated sequencing depth and plex level under different instruments with different kits, please refer to QIAseq RNA Fusion XP All-in-One Tool (Excel file). It can be downloaded from **QIAGEN.com**. It is an estimation only as initial test setting. The best performance may need personally optimized setting base on instrument and kit's technical recommendations and user experience, as well as real sample validation.

Plate format handling

The QIAseq RNA Fusion XP Panels provide convenient workflow for handling 96 samples in 96-well plate format. Follow the special instructions to use 300 µl 96-well low-binding plates in the QIAseq Beads cleanup stage for 96-well plate format handling.

Automation processing needs to be optimized individually. Please contact QIAGEN's customer service for any special requirement and product customization needs.

Protocol: QIAseq RNA Fusion XP Panel for Illumina Instruments

First-strand cDNA synthesis

Primer priming

1. Pre-heat the PCR cycler to 65°C with a heated lid (at 103°C).
2. Put a PCR strip or a 96-well plate on ice.
3. Add 1–5 µl total RNA (10–250 ng total RNA) to the well, add 1 µl of RP Primer II to each tube, and then add water to reach the total volume of 6 µl if necessary.

Table 1. Primer priming

	1 reaction (µl)
RNA sample (x µl)	x (≤ 5)
RP Primer II	1
Nuclease-free water	5 – x
Total	6

Note: Please refer to Appendix G for the protocol of using FastSelect in RNA Fusion XP workflow to block unexpected rRNA and/or globin signal.

4. Mix by pipetting up and down 7 times and then spin down briefly. If using a 96-well plate, cover the plate with an aluminum foil seal, then briefly but gently vortex, and spin down briefly afterwards.
5. Transfer the strip/plate from ice to the cycler and incubate at 65°C for 5 min (silicone compression mat is recommended with plate).

Table 2. Cycler setting

Step	**Incubation temperature**	**Incubation time (min)**
1	65°C	5
2	Ice	≥2

6. Remove the strip/plate from the cycler and place on ice for at least 2 min.

7. Briefly centrifuge before next step.

Reverse transcription

8. Add each of the following reagents to the same tube/plate from the previous reaction. If handling more than 1 sample, prepare a first-strand synthesis mix according to Table 3.

Table 3. Reverse transcription

Step	Reaction volume (µl)
Random primed RNA from previous section	6
BC3 Buffer, 5x	2
RNase inhibitor	1
EZ Reverse Transcriptase	1
Total	**10**

9. Add 4 µl first-strand synthesis mix to each tube/well.
10. Mix by pipetting up and down 7 times and spin down briefly. If using a 96-well plate, cover the plate with an aluminum foil seal, then briefly but gently vortex, and spin down briefly afterwards.
11. Place the PCR strip/plate into a cycler (silicone compression mat is recommended with plate) with a heated lid (103°C) and incubate as follows:

Table 4. Cycler settings for reverse transcription

Step	Incubation temperature (°C)	Incubation time (min)
1	25	10
2	42	30
3	70	15
4	4	Hold

12. Remove the PCR strip/plate from the thermal cycler, briefly spin down, and place on ice.

    If reactions are to be stored after reverse transcription, transfer them to a –20°C freezer. Samples are stable overnight.

Second-strand synthesis

13. Add each of the following reagents to the same tube/well of the previous reaction. If handling more than 1 sample, prepare a second-strand synthesis mix based on Table 5.

Table 5. Second-strand synthesis

	1 reaction (µl)
cDNA from previous section	10
Nuclease-free water	5
BLU buffer	2
RH RNase	1
dNTP II	1
POL enzyme	1
Total	**20**

14. Add 10 µl second-strand synthesis mix to each tube/well.

15. Mix by pipetting up and down 7 times, and then spin down briefly. If using a 96-well plate, cover the plate with an aluminum foil seal, then briefly but gently vortex, and spin down briefly afterwards.

16. Place the PCR strip/plate into a cycler (silicone compression mat is recommended with plate) with a heated lid (103°C) and incubate as follows:

Table 6. Cycler settings for second-strand synthesis

Step	Incubation temperature (°C)	Incubation time (min)
1	37	7
2	65	10
3	80	10
4	4	Hold

17. Remove the PCR strip/plate from the thermal cycler, briefly spin down, and place on ice.

Note: It is okay for an overnight storage at –20°C if there is no time to process the following steps until eluting the DNA after first wash.

End repair/dA tailing

18. Enter the following program into a thermal cycler (Table 7).

    Note: Be certain to use the instrument's heated lid with the lid temperature setting being approximately 70°C if possible.

    Note: If there is no temperature-controlled lid, run with cycler lid open for step 2 and seal the strip or plate well. When the cycler reaches step 3, close the lid to avoid evaporation. Please spin down carefully after the run to remove any condensation.

Table 7. Cycler settings for end repair/dA tailing

Step	Incubation temperature (°C)	Incubation time (min)
1	4	1
2	20	30
3	65	30
4	4	Hold

19. When the cycler block reaches 4°C, pause the program.
20. It is important to follow the procedure described below to achieve optimal results. The final total reaction volume is 50 µl.
21. Prepare a reaction mix in a new LoBind tube on ice by combining ERA Buffer/Enzyme and nuclease-free water as indicated in Table 8 (10% or more extra volume should be added to compensate for the pipetting loss when preparing the master mix for multiple samples).

Table 8. End repair/dA tailing

	1 reaction (µl)
Second-strand product from previous section	20
ERA Buffer, 10x	5
Nuclease-free water	15
ERA Enzyme, 5x	10
Total	**50**

22. Add 30 µl reaction mix to each reaction and gently mix well by pipetting up and down 6–8 times. It is recommended to keep the PCR tube on ice for the whole time during reaction setup.

23. Briefly spin down the sample tube/plate and immediately transfer to the pre-chilled thermal cycler (4°C; silicone compression mat is recommended with plate). Resume the cycling program.
24. When the program is complete and sample block has returned to 4°C, remove samples from block and place on ice.
25. Immediately proceed to the next step.

Adapter ligation

26. If working with more than 1 sample, prepare a ligation mix according to Table 9.
27. Record each dual index and its corresponding sample as planned.
28. Transfer 5 µl of i7 adapter with molecular tags into the PCR tube/plate with 50 µl of A-tailed DNA from step 26. Mix gently by pipetting and cool on ice.
29. Prepare the following ligation reaction master mix in a separate tube on ice and mix well by pipetting. It can be scaled as needed for the desired number of samples (10% extra volume added to compensate for the pipetting loss when preparing the master mix for multiple samples).

Table 9. Ligation mix

	1 reaction (µl)
Ligation Buffer, 5x	20
DNA Ligase	10
Nuclease-free water	15
Total	**45**

Note: It is recommended to use QIAseq Unique Dual Index for RNA Fusion XP. Adapters in the QIAseq 8-Unique Dual Index Sets A and B or QIAseq 96-Unique Dual Index Sets A and B are in 96-well plate. Please refer to QIAseq Fusion XP All-in-One Tool for more details (Excel file; download from **QIAGEN.com**)

Table 10-A. 96-UDI adapter plate A

	1	2	3	4	5	6	7	8	9	10	11	12
A	NQDI B001	NQDI B009	NQDI B017	NQDI B025	NQDI B033	NQDI B041	NQDI B049	NQDI B057	NQDI B065	NQDI B073	NQDI B081	NQDI B089
B	NQDI B002	NQDI B010	NQDI B018	NQDI B026	NQDI B034	NQDI B042	NQDI B050	NQDI B058	NQDI B066	NQDI B074	NQDI B082	NQDI B090
C	NQDI B003	NQDI B011	NQDI B019	NQDI B027	NQDI B035	NQDI B043	NQDI B051	NQDI B059	NQDI B067	NQDI B075	NQDI B083	NQDI B091
D	NQDI B004	NQDI B012	NQDI B020	NQDI B028	NQDI B036	NQDI B044	NQDI B052	NQDI B060	NQDI B068	NQDI B076	NQDI B084	NQDI B092
E	NQDI B005	NQDI B013	NQDI B021	NQDI B029	NQDI B037	NQDI B045	NQDI B053	NQDI B061	NQDI B069	NQDI B077	NQDI B085	NQDI B093
F	NQDI B006	NQDI B014	NQDI B022	NQDI B030	NQDI B038	NQDI B046	NQDI B054	NQDI B062	NQDI B070	NQDI B078	NQDI B086	NQDI B094
G	NQDI B007	NQDI B015	NQDI B023	NQDI B031	NQDI B039	NQDI B047	NQDI B055	NQDI B063	NQDI B071	NQDI B079	NQDI B087	NQDI B095
H	NQDI B008	NQDI B016	NQDI B024	NQDI B032	NQDI B040	NQDI B048	NQDI B056	NQDI B064	NQDI B072	NQDI B080	NQDI B088	NQDI B096

Table 10-B. 96-UDI adapter plate B

	1	2	3	4	5	6	7	8	9	10	11	12
A	NQDI B097	NQDI B105	NQDI B113	NQDI B121	NQDI B129	NQDI B137	NQDI B145	NQDI B153	NQDI B161	NQDI B169	NQDI B177	NQDI B185
B	NQDI B098	NQDI B106	NQDI B114	NQDI B122	NQDI B130	NQDI B138	NQDI B146	NQDI B154	NQDI B162	NQDI B170	NQDI B178	NQDI B186
C	NQDI B099	NQDI B107	NQDI B115	NQDI B123	NQDI B131	NQDI B139	NQDI B147	NQDI B155	NQDI B163	NQDI B171	NQDI B179	NQDI B187
D	NQDI B100	NQDI B108	NQDI B116	NQDI B124	NQDI B132	NQDI B140	NQDI B148	NQDI B156	NQDI B164	NQDI B172	NQDI B180	NQDI B188
E	NQDI B101	NQDI B109	NQDI B117	NQDI B125	NQDI B133	NQDI B141	NQDI B149	NQDI B157	NQDI B165	NQDI B173	NQDI B181	NQDI B189
F	NQDI B102	NQDI B110	NQDI B118	NQDI B126	NQDI B134	NQDI B142	NQDI B150	NQDI B158	NQDI B166	NQDI B174	NQDI B182	NQDI B190
G	NQDI B103	NQDI B111	NQDI B119	NQDI B127	NQDI B135	NQDI B143	NQDI B151	NQDI B159	NQDI B167	NQDI B175	NQDI B183	NQDI B191
H	NQDI B104	NQDI B112	NQDI B120	NQDI B128	NQDI B136	NQDI B144	NQDI B152	NQDI B160	NQDI B168	NQDI B176	NQDI B184	NQDI B192

Layout of sample adapters in QIAseq 96-Unique Dual Index Sets A and B. Each well of the 96-well plate has adapters for single usage purpose. It is heat sealed with pierceable aluminum film. Please use spin down and use pipette tip or puncture tool to pierce the film. Please make sure taking exactly 5 µl for each reaction (extra volume is for handling convenience only).

Note: QIAseq 96-UDI adapter plates A and B used in the ligation must be paired with QIAseq 96-UDI index primer plate A and B in the universal PCR step, respectively. Each index in 96-UDI is 10 nt in length.

Table 11. 8-UDI adapter sets A and B

	Set A	Set B
A	N701	N716
B	N702	N720
C	N703	N721
D	N704	N722
E	N706	N726
F	N707	N727
G	N712	N728
H	N714	N729

Layout of sample adapters in QIAseq 8-Unique Dual Index Sets A and B. Each well of the 8-well strip has adapters in solution. Please spin down before usage. Please make sure taking exactly 5 µl for each reaction (each adapter is good for 6 reactions, a total of 48 reaction for a kit).

Note: QIAseq 8-UDI adapter sets A and B used in the ligation must be paired with QIAseq 8-UDI index primer plate sets A and B in the universal PCR step, respectively. QIAseq 8-UDI uses 8 nt Illumina index. It can be selected from Illumina Nextera® XT v2 index for easy sample sheet generation.

Note: For index sequence, please refer to QIAseq RNA Fusion XP All-in-One Tool as Excel file from **QIAGEN.com**.

30. Add 45 µl of the ligation reaction mix to the sample from step 28 and mix well by pipetting.

31. Incubate the ligation reaction as in Table 12 using a thermal cycler with the lid open.

    Important: Do not use a heated lid. Pause the cycler during the first step before adding the samples.

Table 12. Cycler settings for ligation

Step	Incubation temperature (°C)	Incubation time (min)
1	4	1 (hold till sample is ready)
2	20	15
3	4	Hold

32. Proceed immediately to adapter ligation cleanup.

    Note: Mix the QIAseq Beads well before usage.

Sample cleanup 1

33. Transfer the 100 µl reaction product into a 1.5 ml DNA LoBind tube or transfer the samples into a 300 µl 96-well low-binding plate for sample cleanup.
34. Add 80 µl QIAseq Beads to 100 µl reaction. Mix well by pipetting up and down at least 10 times.
35. Incubate for 5 min at room temperature.
36. Place the tube on a magnetic rack to separate the beads from supernatant. After the solution is clear (10 min usually), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the cDNA target.
37. Completely remove residual supernatant (using 10 µl tip to aspirate the trace amount of residual supernatant after the first aspiration is recommended; spin down is helpful if any residual is on side wall).
38. Wash the beads with 220 µl 80% ethanol; rotate the tube 3 times. Wait 1 min with the tube on magnetic rack.
39. Remove the 80% ethanol completely.
40. Repeat the above wash step once. Carefully aspirate the trace amount of residual ethanol.
41. Dry beads by leaving the cap open for 10 min (no more than 15 min is needed).

    Note: Enough drying time is essential to ensure that all traces of ethanol are removed. Cracks will be observed in the bead pellet when drying is complete. Do not over dry the beads as this will significantly decrease elution efficiency, especially for larger fragments.
42. Elute DNA target from beads with 50 µl nuclease-free water. Mix well by pipetting. No bead separation is needed (this is for faster and easy handling; traditional method by adding 52 µl water to elute 50 µl supernatant is still fine).
43. Add 55 µl QIAseq Beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.
44. Incubate for 5 min at room temperature.
45. Place the tube or low-binding plate on the magnetic rack to separate the beads from supernatant. After the solution is clear (approx. 5–10 min), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the cDNA target.

46. Add 220 µl freshly made 80% ethanol to the tube or well while it is on the magnetic rack. Rotate the tube once on the magnet to wash the beads, and then carefully remove and discard the supernatant.
47. Repeat the above wash step once more.
48. Completely remove residual ethanol and dry the beads for 5 min while the tube or plate is on the rack with the lid open.

    Note: Enough drying time is essential to ensure that all traces of ethanol are removed. Cracks will be observed in the bead pellet when drying is complete. Do not over dry the beads as this will significantly decrease elution efficiency, especially for larger fragments.
49. Elute the beads by adding 12.4 µl nuclease-free water. Mix well by pipetting. Place the tube or plate on the magnetic rack until solution is clear.
50. Transfer 10.4 µl supernatant to a clean PCR strip or 96-well PCR plate.

    If the reactions are to be stored after bead-based cleanup, transfer them to a –20°C freezer. Samples are stable overnight.

SPE target enrichment

51. Transfer the 10.4 µl eluted sample into PCR strip or plate.
52. Prepare SPE reaction mix for each sample according to Table 13; add 9.6µl mix in PCR strips or a 96-well PCR plate. Mix gently by pipetting up and down.

    Note: Extra volume of 10% or more should be added to compensate for the pipetting loss when preparing the master mix for multiple samples.

Table 13. SPE reaction

	1 reaction (µl)
Purified sample from previous section	10.4
TEPCR Buffer, 5x	4
QIAseq RNA Fusion XP Panel	4
IL-Forward Primer	0.8
HotStarTaq DNA Polymerase	0.8
Total	**20**

53. Seal the wells with PCR tube caps. Place strips or plate in thermal cycle r(silicone compression mat is recommended with plate) and set up reaction parameters according to Table 14.

Table 14. Cycler setting for SPE

Step	Cycles	Incubation temperature (°C)	Incubation time
1	1	95	13 min
		98	2 min
2	8	98	15 s
		68	10 min
3	1	72	2 min
	1	4	Hold

54. After the reaction is complete, place the reactions on ice and proceed to the next step.

    Optional: SPE reaction may be run overnight and left in the thermal cycler at 4°C.

Sample cleanup 2

55. Add 30 µl nuclease-free water to 20 µl reaction to bring the volume to 50 µl and transfer into a 1.5 ml DNA LoBind tube or keep in the 96-well PCR plate for purification.
56. Mix the QIAseq Beads well before usage. Add 55 µl QIAseq Beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.
57. Incubate for 5 min at room temperature.
58. Place the tube on a magnetic rack to separate the beads from supernatant. After the solution is clear (approx. 5 min), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.
59. Completely remove residual supernatant.
60. Add 220 µl freshly made 80% ethanol to the tube or well while it is on the magnetic rack. Rotate the tube or move the plate side to side in the 2 positions of the magnet to wash the beads. Wait 1 min with the tube on magnetic rack, and then carefully remove and discard the supernatant.
61. Repeat the above wash step once more.

62. Completely remove residual ethanol and dry the beads for 5–10 min while the tube or plate is on the rack with the lid open.

    Note: Enough drying time is essential to ensure that all traces of ethanol are removed. Cracks will be observed in the bead pellet when drying is complete. Do not over dry the beads as this will significantly decrease elution efficiency, especially for larger fragments.

63. Elute the beads into 15.4 µl sterile water. Mix well by pipetting. Place the tube or plate on the magnetic rack until the solution is clear.

64. Transfer 13.4 µl supernatant to a clean PCR strip or 96-well PCR plate.

Universal PCR amplification

65. Prepare universal PCR reaction mix for each sample according to Table 15 in PCR strips or a 96-well PCR plate. Mix gently by pipetting up and down.

    Note: QIAseq UDI index primer plate is in aluminum porch without sealing. There is no need to worry about the contamination. It is recommended to seal the remained wells after the first time usage.

    Note: UDI index primer plate has universal PCR primer and index primer immobilized in each well. Mix the purified sample and reaction mix inside each well to resuspend the primer. Please spin down the reaction solution well and make sure no bubble there.

Table 15. Universal PCR mix using UDI index kit

	1 reaction (µl)
Purified sample	13.4
RNA Buffer II, 5x	4
Nuclease-free water	1.8
TaqIt Plus enzyme	0.8
Total	**20**

Note: This step applies different QIAseq UDI index to the other side of the target (opposite to the adapter side) for dual sample index. Total sample index level can be up to 192-plex if using QIAseq 96-Unique Dual Index Sets A and B sets together (For more UDIs, please contact QIAGEN customer support).

Table 16-A. 96-UDI index primer plate A

	1	2	3	4	5	6	7	8	9	10	11	12
A	SQDI B001	SQDI B009	SQDI B017	SQDI B025	SQDI B033	SQDI B041	SQDI B049	SQDI B057	SQDI B065	SQDI B073	SQDI B081	SQDI B089
B	SQDI B002	SQDI B010	SQDI B018	SQDI B026	SQDI B034	SQDI B042	SQDI B050	SQDI B058	SQDI B066	SQDI B074	SQDI B082	SQDI B090
C	SQDI B003	SQDI B011	SQDI B019	SQDI B027	SQDI B035	SQDI B043	SQDI B051	SQDI B059	SQDI B067	SQDI B075	SQDI B083	SQDI B091
D	SQDI B004	SQDI B012	SQDI B020	SQDI B028	SQDI B036	SQDI B044	SQDI B052	SQDI B060	SQDI B068	SQDI B076	SQDI B084	SQDI B092
E	SQDI B005	SQDI B013	SQDI B021	SQDI B029	SQDI B037	SQDI B045	SQDI B053	SQDI B061	SQDI B069	SQDI B077	SQDI B085	SQDI B093
F	SQDI B006	SQDI B014	SQDI B022	SQDI B030	SQDI B038	SQDI B046	SQDI B054	SQDI B062	SQDI B070	SQDI B078	SQDI B086	SQDI B094
G	SQDI B007	SQDI B015	SQDI B023	SQDI B031	SQDI B039	SQDI B047	SQDI B055	SQDI B063	SQDI B071	SQDI B079	SQDI B087	SQDI B095
H	SQDI B008	SQDI B016	SQDI B024	SQDI B032	SQDI B040	SQDI B048	SQDI B056	SQDI B064	SQDI B072	SQDI B080	SQDI B088	SQDI B096

Table 16-B. 96-UDI index primer plate B

	1	2	3	4	5	6	7	8	9	10	11	12
A	SQDI B097	SQDI B105	SQDI B113	SQDI B121	SQDI B129	SQDI B137	SQDI B145	SQDI B153	SQDI B161	SQDI B169	SQDI B177	SQDI B185
B	SQDI B098	SQDI B106	SQDI B114	SQDI B122	SQDI B130	SQDI B138	SQDI B146	SQDI B154	SQDI B162	SQDI B170	SQDI B178	SQDI B186
C	SQDI B099	SQDI B107	SQDI B115	SQDI B123	SQDI B131	SQDI B139	SQDI B147	SQDI B155	SQDI B163	SQDI B171	SQDI B179	SQDI B187
D	SQDI B100	SQDI B108	SQDI B116	SQDI B124	SQDI B132	SQDI B140	SQDI B148	SQDI B156	SQDI B164	SQDI B172	SQDI B180	SQDI B188
E	SQDI B101	SQDI B109	SQDI B117	SQDI B125	SQDI B133	SQDI B141	SQDI B149	SQDI B157	SQDI B165	SQDI B173	SQDI B181	SQDI B189
F	SQDI B102	SQDI B110	SQDI B118	SQDI B126	SQDI B134	SQDI B142	SQDI B150	SQDI B158	SQDI B166	SQDI B174	SQDI B182	SQDI B190
G	SQDI B103	SQDI B111	SQDI B119	SQDI B127	SQDI B135	SQDI B143	SQDI B151	SQDI B159	SQDI B167	SQDI B175	SQDI B183	SQDI B191
H	SQDI B104	SQDI B112	SQDI B120	SQDI B128	SQDI B136	SQDI B144	SQDI B152	SQDI B160	SQDI B168	SQDI B176	SQDI B184	SQDI B192

Layout of 96-UDI index primer plate in QIAseq 96-Unique Dual Index Sets A and B. Each well contains 1 pre-dispensed sample index primer and universal primer pair for a single reaction. The QIAseq 96-UDI adapter plates A and B used in ligation must be paired with the 96-UDI index primer plates A and B in universal PCR step, respectively. Each sample index in QIAseq 96-UDI is 10 nt in length.

Table 17-A. 8-UDI index primer plate A

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

Layout of 8-UDI Index Primer Plate in QIAseq 8-Unique Dual Index Set A. Each well contains 1 pre-dispensed sample index primer and universal primer pair for a single reaction. The QIAseq 8-UDI adapter set A used in ligation must be paired with the 8-UDI index primer plate A in universal PCR step. Each sample index in 96-UDI is 8 nt in length (Illumina Nextera XT V2).

Table 17-B. 8-UDI index primer plate set B

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

Layout of 8-UDI Index Primer Plate in QIAseq 8-Unique Dual Index Set B. Each well contains 1 pre-dispensed sample index primer and universal primer pair for a single reaction. QIAseq 8-UDI adapter set B used in ligation must be paired with the 8-UDI index primer plate B in universal PCR step. Each sample index in 96-UDI is 8 nt in length (Illumina Nextera XT V2).

66. Seal the wells with PCR tube caps or seal the 96-well PCR plate with sealing film. Place strips or plate (with compression pad) in thermal cycler and set up reaction parameters according to Table 18.

Table 18. Cycler settings for universal PCR

Step	Cycles	Incubation temperature (°C)	Incubation time
1	1	95	2 min
		98	1 min
2	25*	95	15 s
		60	1 min
3	1	72	1 min
	1	4	hold

* **Note**: Cycle numbers can be adjusted based on library generation experience as the target expression level could vary significantly between different experiments. Library yield is also related with input and sample type, as well as panel primer number. It is recommended using 18–25 cycles for regular input, fresh high-quality RNA samples (low-plex panel could be 26) and using 28–30 cycles for low-input (≤20 ng) or FFPE samples.

67. After the reaction is complete, place the reactions on ice and proceed to the next step.

    If reactions are to be stored after bead-based clean up, transfer them to a –20°C freezer. Samples are stable overnight.

Sample cleanup 3

68. Add 30 µl nuclease-free water to 20 µl reaction to bring the volume to 50 µl.
69. Transfer 50 µl PCR reactions to a 1.5 ml LoBind tube or leave it in 96-well PCR plate for purification.
70. Mix the QIAseq Beads well before usage. Add 55 µl QIAseq Beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.
71. Incubate for 5 min at room temperature.
72. Place the tube on a magnetic rack to separate the beads from supernatant. After the solution is clear (approx. 5 min), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

73. Add 220 µl freshly made 80% ethanol to the tube or plate while it is on the magnetic rack. Rotate the tube or move the plate side to side in the 2 positions of the magnet to wash the beads. Wait 1 min with the tube on magnetic rack, and then carefully remove and discard the supernatant.
74. Repeat the above wash step once more.
75. Place the tube or plate on the magnetic rack. Completely remove residual ethanol and dry the beads for 5–10 min while the tube or plate is on the rack with the lid open.

    Note: Enough drying time is essential to ensure that all traces of ethanol are removed. Cracks will be observed in the bead pellet when drying is complete. Do not over dry the bead as this will significantly decrease elution efficiency, especially for larger fragments.
76. Elute DNA target beads into 25 µl sterile water. Mix well by pipetting. Place the tube or plate on the rack until solution is clear.
77. Transfer 21 µl supernatant to a clean PCR strip or 96-well PCR plate.
78. Proceed to library quantification. The concentration of the library can be determined using QIAGEN's QIAseq Library Quant Array for Illumina or QIAxpert (see Appendix F: Library Quantification). Library quality can be checked with TapeStation HSD1000.

    If libraries are to be stored after bead-based clean up, transfer them to a –20°C freezer. Individual stored samples are stable overnight or longer (re-do quantification and quality check after long time storage).

Protocol: QIAseq RNA Fusion XP Panel for Ion Torrent (L)

Refer to the QIAseq Fusion XP quick reference protocol and/or QIAseq Fusion XP setting tool (Excel) to have a quick library generation protocol and refer to the following protocol for more details.

First-strand cDNA synthesis

Primer priming

1. Pre-heat the PCR cycler to 65°C with a heated lid (103°C).
2. Put a PCR strip or 96-well PCR plate on ice.
3. Add 1–5 µl total RNA (10–250 ng total RNA) to the well, add 1 µl of RP Primer II to each tube, and then add nuclease-free water to reach a total volume of 6 µl if necessary.

Table 19. Primer priming

	1 reaction (µl)
RNA sample (x µl)	x (≤ 5)
RP Primer II	1
Nuclease-free water	5 – x
Total	**6**

Please refer to Appendix G for the protocol of using FastSelect™ in RNA Fusion XP workflow to block unexpected rRNA and/or globin signal.

4. Mix by pipetting up and down 7 times, and then spin down briefly. If using a 96-well plate, cover the plate with an aluminum foil seal, then briefly but gently vortex and spin down briefly afterwards.
5. Transfer the strip/plate from ice to the cycler (silicone compression mat is recommended with plate) and incubate at 65°C for 5 min.

Table 20. Cycler settings for priming

Step	Incubation temperature	Incubation time (min)
1	65°C	5
2	Ice	≥2

6. Remove the strip/plate from the cycler and place on ice for at least 2 min.
7. Briefly centrifuge.

Reverse transcription

8. Prepare a first-strand synthesis mix first if handling more than 1 sample based on Table 21.

   Note: Extra volume of 10% or more should be added to compensate for the pipetting loss when preparing the master mix for multiple samples.

Table 21. Reverse transcription

	1 reaction (µl)
Random primed RNA	6
BC3 Buffer, 5x	2
RNase Inhibitor	1
EZ Reverse Transcriptase	1
Total	**10**

9. Add 4 µl first-strand synthesis mix to each well.
10. Mix by pipetting up and down 7 times and spin down briefly. If using a 96-well plate, cover the plate with an aluminum foil seal, then briefly but gently vortex and spin down briefly afterwards.

11. Place the PCR strip/plate into a cycler (silicone compression mat is recommended with plate) with a heated lid (103°C) and incubate as follows:

Table 22. Cycler settings for reverse transcription

Step	Incubation temperature (°C)	Incubation time (min)
1	25	10
2	42	30
3	70	15
4	4	Hold

12. Remove the PCR strip/plate from the thermal cycler, briefly spin down and place on ice.

Second-strand synthesis

13. Add each of the following reagents to the same tube of the previous reaction. Prepare a second-strand synthesis mix first if handling more than 1 sample based on Table 23.

    Note: Extra volume of 10% or more should be added to compensate for the pipetting loss when preparing the master mix for multiple samples.

14. Add 10 µl second-strand synthesis mix to each well.

Table 23. Second-strand synthesis

	1 reaction (µl)
cDNA from previous section	10
POL Enzyme	1
BLU Buffer	2
RH RNase	1
dNTP II	1
Nuclease-free water	5
Total	**20**

15. Mix by pipetting up and down 7 times, and then spin down briefly. If using a 96-well plate, cover the plate with an aluminum foil seal, then briefly but gently vortex and spin down briefly afterwards.

16. Place the PCR strip/plate into a cycler (silicone compression mat is recommended with plate) with a heated lid and incubate as follows:

Table 24. Cycler settings for second-strand synthesis

Step	Incubation temperature (°C)	Incubation time (min)
1	37	7
2	65	10
3	80	10
4	4	Hold

17. Remove the PCR strip/plate from the thermal cycler, briefly spin down and place on ice.

End repair/dA tailing

18. Enter the following program into a thermal cycler:

Table 25. Cycler settings for end repair/dA tailing

Step	Incubation temperature (°C)	Incubation time (min)
1	4	1
2	20	30
3	65	30
4	4	Hold

19. Be certain to use the instrument's heated lid with the temperature set to approximately 70°C.

    Note: If there is no temperature-controlled lid, run the cycler with the lid open and seal the strip or plate well. At cycle step 3, close the lid to avoid evaporation. Spin down carefully after the run to remove any condensation.

20. When the thermal cycler block reaches 4°C, pause the program.

21. It is important to follow the procedure described below to achieve optimal results. The final total reaction volume is 50 µl.

22. Prepare a reaction mix in a new LoBind tube on ice by combining ERA Buffer, ERA enzyme, and nuclease-free water as indicated in the Table 26.

23. Add 30 µl reaction mix to each reaction.

Table 26. End repair/dA tailing reaction

	1 reaction (µl)
Second-strand product from previous section	20
ERA Buffer, 10x	5
Nuclease-free water	15
ERA enzyme, 5x	10
Total	**50**

24. Gently mix well by pipetting up and down 6–8 times. It is recommended to keep the PCR tube on ice for the whole time during reaction setup.
25. Briefly spin down the sample tube/plate and immediately transfer to the pre-chilled thermal cycler (4°C; silicone compression mat is recommended with plate). Resume the cycling program.
26. When the program is complete and sample block has returned to 4°C, remove samples from the block and place on ice.
27. Immediately proceed to the next step.

Adapter ligation

28. Prepare a reaction mix for adapter ligation according to Table 27, adding the components to the PCR tube or plate containing cDNA that has undergone end-repair and A-addition. Keep on ice and mix well by pipetting. It can be scaled as needed for the desired number of samples (10% extra volume added to compensate for the pipetting loss when preparing the master mix for multiple samples).

    Note: Only 1 single barcoded adapter should be used per ligation reaction; open 1 adapter tube at a time if using 12-index adapters and avoid cross-contamination. For 96-index adapters, use a multichannel pipet to take the appropriate volume of adapters from the provided PCR plate. See Table 28 for layout of adapters in the PCR plate.

Table 27. Adapter ligation

	1 reaction (µl)
ER/AT sample from previous section	50
Adapter LT-BC	5
Ligation Buffer, 5x	20
DNA Ligase	10
Nuclease-free water	15
Total	**100**

Table 28. LT-BC adapter plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC8	BC9	BC10	BC11	BC12
B	BC13	BC14	BC15	BC16	BC17	BC18	BC19	BC20	BC21	BC22	BC23	BC24
C	BC25	BC26	BC27	BC28	BC29	BC30	BC31	BC32	BC33	BC34	BC35	BC36
D	BC37	BC38	BC39	BC40	BC41	BC42	BC43	BC44	BC45	BC46	BC47	BC48
E	BC49	BC50	BC51	BC52	BC53	BC54	BC55	BC56	BC57	BC58	BC59	BC60
F	BC61	BC62	BC63	BC64	BC65	BC66	BC67	BC68	BC69	BC70	BC71	BC72
G	BC73	BC74	BC75	BC76	BC77	BC78	BC79	BC80	BC81	BC82	BC83	BC84
H	BC85	BC86	BC87	BC88	BC89	BC90	BC91	BC92	BC93	BC94	BC95	BC96

Layout of sample indexed molecule barcode adapters in QIAseq 96-Index L. Each well contains 1 sample indexed molecule barcode adapter. The amount of adapter in each well is good for 4 samples.

29. Mix the components well by pipetting up and down 7–8 times.
30. Program a thermal cycler to incubate at 20°C for exactly 15 min.

    Important: Do not use a heated lid. Recommended cycler settings are as follows: 4°C for 1 min, 20°C for 15 min, followed by a 4°C hold. Pause the cycler during the first step before adding the samples.
31. After the reaction is complete, place the reactions on ice and proceed with cleanup using QIAseq Beads (**Note**: QIAseq Beads can be used directly from fridge after fully mixed).

Sample cleanup 1

32. Transfer the 100 µl reaction product into a 1.5 ml DNA LoBind tube or transfer the samples into a 300 µl 96-well low-binding plate for sample cleanup.
33. Mix QIAseq Beads and add 80 µl beads to 100 µl reaction. Mix well by pipetting up and down at least 10 times.
34. Incubate for 5 min at room temperature.
35. Place the tube on a magnetic rack to separate the beads from supernatant. After the solution is clear (10 min usually), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the cDNA target.
36. Completely remove residual supernatant (it is recommended using a 10 µl tip to aspirate the trace amount of residual supernatant after the first aspiration).
37. Wash beads with 220 µl 80% ethanol, rotate the tube 3 times. Wait 1 min with the tube on the magnetic rack.
38. Remove the 80% ethanol completely.
39. Repeat the above wash steps once. Remove trace amount of ethanol carefully.
40. Dry the beads by leaving the cap open for 10 min.

    Note: Enough drying time is essential to ensure that all traces of ethanol are removed. Cracks will be observed in the bead pellet when drying is complete. Do not over dry the bead as this will significantly decrease elution efficiency, especially for larger fragments.
41. Elute DNA target from the beads with 50 µl nuclease-free water. Mix well by pipetting. No beads separation is needed.
42. Add 55 µl QIAseq Beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.
43. Incubate for 5 min at room temperature.
44. Place the tube or PCR plate on the magnetic rack to separate the beads from supernatant. After the solution is clear (approx. 5–10 min), wait 1 min with the tube on the magnetic rack, then carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the cDNA target.

45. Add 220 µl freshly made 80% ethanol to the tube or well while it is on the magnetic rack. Rotate the tube on the magnet to wash the beads, and then carefully remove and discard the supernatant.
46. Repeat the above wash step once more.
47. Completely remove residual ethanol and dry the beads for 5–10 min while the tube or plate is on the rack with the lid open.

    Note: Enough drying time is essential to ensure that all traces of ethanol are removed. Cracks will be observed in the bead pellet when drying is complete. Do not over dry the bead as this will significantly decrease elution efficiency, especially for larger fragments.
48. Elute beads by adding 12.4 µl nuclease-free water. Mix well by pipetting. Place the tube or plate on the magnetic rack until solution is clear.
49. Transfer 10.4 µl supernatant to a clean PCR strip or regular 96-well PCR plate.

SPE target enrichment

50. Transfer the 10.4 µl eluted sample into PCR strip or plate.
51. Prepare SPE reaction mix for each sample according to Table 29 in PCR strips or a 96-well PCR plate. Add 9.6 µl mix to each sample then mix gently by pipetting up and down.

Table 29. SPE reaction mix

	1 reaction (µl)
Purified sample	10.4
TEPCR Buffer, 5x	4
QIAseq RNA Fusion XP Panel	4
LT-Forward Primer	0.8
HotStarTaq DNA Polymerase	0.8
Total	**20**

52. Seal the wells with PCR tube caps. Place strips or plate (silicone compression mat is recommended with plate) in thermal cycler and set up reaction parameters according to Table 30.

Table 30. Cycler settings for SPE target enrichment

Step	Cycles	Incubation temperature (°C)	Incubation time
1	1	95	13 min
		98	2 min
2	8	98	15 s
		68	10 min
3	1	72	2 min
	1	4	Hold

53. After the reaction is complete, place on ice and proceed to next step.

Sample cleanup 2

54. Add 30 µl nuclease-free water to 20 µl reaction to bring the volume to 50 µl and transfer into a 1.5 ml DNA LoBind tube or keep in the plate for purification.
55. Mix QIAseq Beads and add 55 µl beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.
56. Incubate for 5 min at room temperature.
57. Place the tube on a magnetic rack to separate the beads from supernatant. After the solution is clear (approx. 5 min), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.
58. Completely remove residual supernatant.
59. Add 220 µl freshly made 80% ethanol to the tube or well while it is on the magnetic rack. Rotate the tube or move the plate side to side in the 2 positions of the magnet to wash the beads. Wait 1 min with the tube on magnetic rack, and then carefully remove and discard the supernatant.
60. Repeat the above wash step once more.

61. Completely remove residual ethanol and dry the beads for 5–10 min while the tube or plate is on the rack with the lid open.

    Note: Enough drying time is essential to ensure that all traces of ethanol are removed. Cracks will be observed in the bead pellet when drying is complete. Do not over dry the beads as this will significantly decrease elution efficiency, especially for larger fragments.

62. Elute beads into 15.6 µl sterile water. Mix well by pipetting. Place tube or plate on the magnetic rack until solution is clear.

63. Transfer 13.6 µl supernatant to a clean PCR strip or 96-well PCR plate.

Universal PCR amplification

64. Prepare the universal PCR reaction mix for each sample according to Table 31 in PCR strips or a 96-well PCR plate. Mix gently by pipetting up and down.

    Note: Extra volume of 10% or more should be added to compensate for the pipetting loss when preparing the master mix for multiple samples.

Table 31. Universal PCR reaction

	1 reaction (µl)
Purified sample	13.6
QIAseq RNA Buffer II, 5x	4
LT-Universal Primer	0.8
LT-P1 Primer	0.8
TaqIT Plus	0.8
Total	**20**

65. Seal the strips with PCR tube caps or seal the 96-well PCR plate with sealing film. Place the strips or plate (with compression pad) in thermal cycler and set up reaction parameters according to Table 32.

Table 32. Cycler settings for universal PCR

Step	Cycles	Incubation temperature (°C)	Incubation time
1	1	95	2 min
		98	1 min
2	25*	95	15 s
		60	1 min
3	1	72	1 min
	1	4	Hold

* **Note**: Cycle numbers can be adjusted based on library generation experience as the target expression level could vary significantly between different experiments. Library yield is also related with input and sample type, as well as panel primer number. It is recommended to use 18–25 cycles for regular input fresh high-quality RNA samples (low-plex panel could be 26) and to use 28–30 cycles for low input (<20 ng) or FFPE samples.

66. After the reaction is complete, place the reactions on ice and proceed to next step.

    If reactions are to be stored after bead-based clean up, transfer them to a –20°C freezer. Samples are stable overnight.

Sample size selection

67. Add 30 µl nuclease-free water to a 20 µl reaction to bring the volume to 50 µl.
68. Transfer 50 µl PCR reactions to a 1.5 ml LoBind tube or leave it in 96-well PCR plate for purification.
69. Mix QIAseq Beads and add 35 µl beads to 50 µl PCR reaction. Mix well by pipetting up and down at least 10 times.
70. Incubate for 5 min at room temperature.
71. Place the tube on a magnetic rack to separate the beads from supernatant.
72. After the solution is clear (approx. 5 min), carefully aspirate the supernatant and put 85 µl supernatant into a new 1.5 ml LoBind tube or a new PCR plate. Be careful not to take the beads, which contain large DNA that is not of interest.

73. Add 29 µl QIAseq Beads to 85 µl sample and mix well by pipetting up and down at least 10 times.
74. Incubate for 5 min at room temperature.
75. Place the tube on the magnetic rack to separate the beads from supernatant. Carefully remove all the supernatant.
76. Add 220 µl freshly made 80% ethanol to the tube or plate while it is on the magnetic rack. Rotate the tube or move the plate side to side in the 2 positions of the magnet to wash the beads. Wait 1 min with the tube on magnetic rack, and then carefully remove and discard the supernatant.
77. Repeat the above wash step once more.
78. Place the tube or plate on the magnetic rack. Completely remove residual ethanol and dry the beads for 5–10 min while the tube or plate is on the rack with the lid open.

    Note: Enough drying time is essential to ensure that all traces of ethanol are removed. Cracks will be observed in the bead pellet when drying is complete. Do not over dry the beads as this will significantly decrease elution efficiency, especially for larger fragments.
79. Elute DNA target beads into 25 µl sterile water. Mix well by pipetting. Place the tube or plate on the rack until solution is clear.
80. Transfer 21 µl supernatant to a clean PCR strip or 96-well PCR plate.
81. Proceed to library quantification. The concentration of the library can be determined using QIAGEN's QIAseq Library Quant Array for Ion Torrent or QIAxpert (see Appendix F: Library Quantification). Library quality can be checked with TapeStation HSD1000.

    If libraries are to be stored after bead-based clean up, transfer them to a –20°C freezer. Individual stored samples are stable overnight or longer (re-do quantification and quality check after long time storage).

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 or protocols in this handbook (for contact information, visit **support.qiagen.com**).

	Comments and suggestions
Low library yield	
Low quality sample	High quality RNA samples are always recommended for optimal performance of our well-optimized chemistry. DV200 ≥ 30% is needed.
Less efficient target enrichment and/or universal PCR	Target enrichment (SPE) and universal PCR step is critical for library generation, especially for low input and/or low quality samples. Right volume of enzyme and buffer, enough cycles, and right temperature for PCR need to be carefully controlled.
Loss of sample during beads cleanup	The QIAseq Beads cleanup step is critical for high recovery efficiency. Carefully handle the bead without any beads lost. Ethanol carryover will affect elution efficiency and following reactions
Wrong library size peak	
Large size peak and/or flat peak in large size region	RNA Fusion XP-enriched molecule size varied in nature, and during universal PCR, the overamplification may happen that generate single-strand amplicon and they can be partially bind and can mimic the large size molecule. Overamplified library will not affect the sequencing.
Small size peak (<200bp)	The primer and/or primer dimmer need to be removed with each bead's cleanup step. Low input and/or low quality sample may challenge the cleanup and adjust the sample, and/or cleanup step may help for that.
Poor sequencing	
High cluster density and/or low passing filter rate	Library quantification is important; underestimation of library concentration or wrong dilution calculation will have higher than expected density and low passing filter rate.
Low cluster density	Library quantification error could be the reason; overestimation of library concentration or wrong dilution calculation will be the reason; high NaOH will also make the cluster density lower.

Detection issue	Comments and suggestions
Fusion detection	Sample quality and tumor/normal cell ratio are important factors for the fusion detection sensitivity. Input level and/or sequencing depth will also affect it.
RNA SNV/InDel	Expression level and allele express frequency and tumor/normal cell ratio as well as sequencing depth all contribute to the detection sensitivity and accuracy.
Gene expression	Over high or over low expression level and rare transcript may challenge the gene expression detection, optimization of target list and/or design, as well as deep sequencing may help

Appendix A: QIAseq UDI Run Setting with Illumina Platform

Prep tab in BaseSpace Sequence Hub

For the NextSeq and MiniSeq Sequencing Systems, Illumina offers the **Prep** tab in BaseSpace Sequence Hub.

QIAseq 8-UDI

The QIAseq 8-Unique Dual Index Kit uses the Illumina Nextera XT v2 index (partial), so user can pick the right pair (see Illumina protocol and/or RNA Fusion XP All-in-One Tool for the index pairing information) after selecting Nextera XT v2 from Illumina's list.

QIAseq 96-UDI

The QIAseq 96-UDI uses QIAGEN's 10 nt index design. As the **Prep** tab in BaseSpace Sequence Hub only supports 8 x 12 custom kit, that means QIAseq 96-Unique Dual Index Kit can only be used with limited plex level ≤ 8 within the **Prep** tab. Using Illumina's Local Run Manager (LRM) for setting more than 8 samples in a run is recommended. For setting custom index kit with BaseSpace Sequence Hub, please download the RNA Fusion XP All-in-One Tool Excel file from **QIAGEN.com**. Following the instruction of how to use Illumina **Prep** tab in BaseSpace Sequence Hub, custom kit input requires to copy any pair for total ≤8 pairs of the necessary QIAseq UDI information to the file and upload it. Then follow the standard BaseSpace Sequence Hub handling workflow to finalize the run setting. For plex level of more than 8 with NextSeq, please either run the sequencing without sample sheet first (just define each read cycle number and custom read 1 primer) then use LRM to do demultiplexing with sample sheet later (copy from All-in-One Tool), or directly use LRM to do run setting before sequencing.

Illumina Experiment Manager

Illumina Experiment Manager (IEM) is compatible with the HiSeq, MiSeq, and NovaSeq Sequencing Systems.

QIAseq 8-UDI

The QIAseq 8-Unique Dual Index Kit uses the Illumina Nextera XT v2 index (partial), so user can pick the right pair (see Illumina protocol and/or RNA Fusion XP All-in-One Tool for the pairing information) after selecting Nextera XT v2 from IEM's list.

QIAseq 96-UDI

IEM is a user interface for assembling input CSV (comma-separated variables) data that the sequencer software can read directly. The information included has reagent barcode reference, run name, investigator name, sample names, read adapter sequences, indices used, and other tracking information for the pooled samples. Since QIAseq indices are not included by default with IEM, specific index information needs to be added to the IEM data source.

Directly modify the resulting CSV file with corrected indices. All sample sheet generated by IEM can be opened in any text editor or Microsoft Excel after generation. At that point the user can directly modify indices (or any other element of the file) prior to loading into the Illumina sequencing software. While this may be the quickest option for a one-off sequencing run, we highly recommend setting up sample sheet with QIAseq UDI indices copied from All-in-one tool Excel file so human input errors are minimized.

Local Run Manager

LRM software is an integrated solution designed to create sequencing runs, monitor run status, analyze sequencing data, and view results. LRM software integrates with the instrument control software and can be directly accessed on the instrument through a web browser. An off-instrument version of the same easy-to-use LRM software is available and compatible with the MiniSeq System, the MiSeq Series, and the NextSeq Series.

Please be aware that the format for LRM sample sheet may not be the same as sample sheet from IEM. The best way to generate the right LRM usable sample sheet is opening a sample sheet that had been be used successfully to copy all the text except the index information to a new file in Notepad; modify the run name, user name, run date, and setting of read 1 and 2 length (standard setting is 231/71 or 229/69 for some instrument with QIAseq 96-UDI; run with QIAseq DNA need 151/151 or 149/149 for some instruments with QIAseq 96-UDI); add CustomRead1PrimerMix C1 under [setting]; copy the right QIAseq 96-UDI index information from QIAseq Fusion XP All-in-One Tool to the index information part; then save as csv file so LRM can load the sample sheet for run setting.

LRM also provides **run the sequencing without sample sheet** option. Just input the right cycles for the run, for example, run MiSeq with QIAseq 96-UDI and set as Read1: 231 cycles, Index 1: 10 cycles, Index 2: 10 cycles, Read 2: 71 cycles. After running, the data can be analyzed with LRM to do demultiplexing with sample sheet later (copy from All-in-One Tool), or directly use LRM to do run setting before sequencing.

Appendix B: Sequencing Setup on Illumina MiSeq and NextSeq with QIAseq A Read 1 Primer I as Custom Sequencing Primer

Note: Illumina custom primer guide may ask to use custom index prime together with custom read 1 primer, but as RNA Fusion XP read structure use the standard Illumina index primer for index reading, there is no need to select and use "custom" index primer. For any extreme case that the software blocks the sequencing setting moving forward, customer still can take Illumina standard index primer to the custom index primer well and select the custom index primer in sequencing setting. Please refer to Illumina Instrument user manual for the location of standard and custom primer well location.

Note: Please refer to the QIAseq Fusion XP All-in-One Tool (**QIAGEN.com**) for quick working sheet generation and sequencing setting.

Prepare library for sequencing

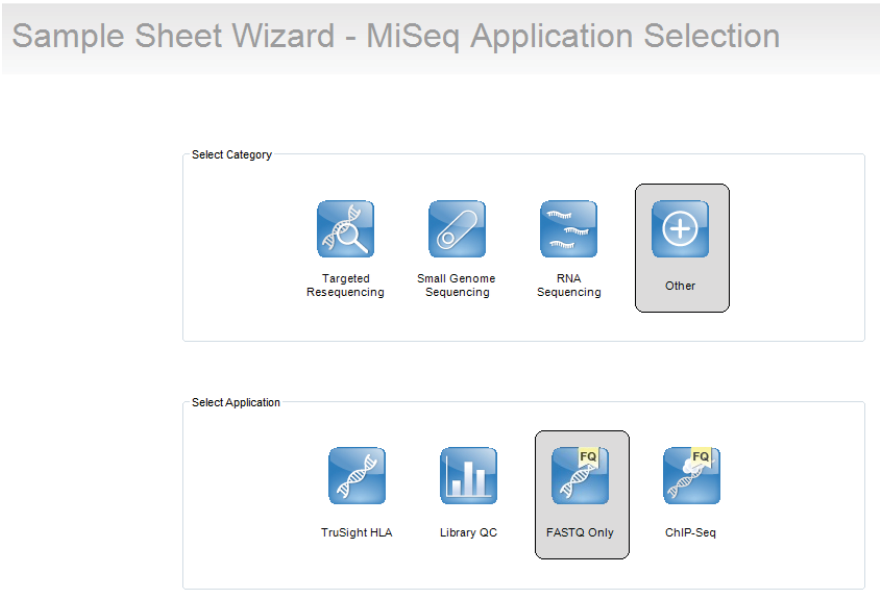
After library quantification, dilute library to 2 or 4 nM for MiSeq and 0.5 nM for NextSeq, then combine libraries with different sample indices in equimolar amounts if similar sequencing depth is needed for each library. If combining libraries with same number of primers, pool equal volume of individual library at 2 or 4 nM (or 0.5 nM for NextSeq) together.

Prepare library to load on the MiSeq or NextSeq according to Illumina's protocol. If using the QIAseq Library Quant Array to determine concentration, the final total library concentration is 8–10 pM on MiSeq and 0.8–1.0 pM on NextSeq. If using a QIAxpert and 3.5 converting factor to determine concentration, use 10–12 pM on MiSeq and 1.2–1.5 pM on NextSeq.

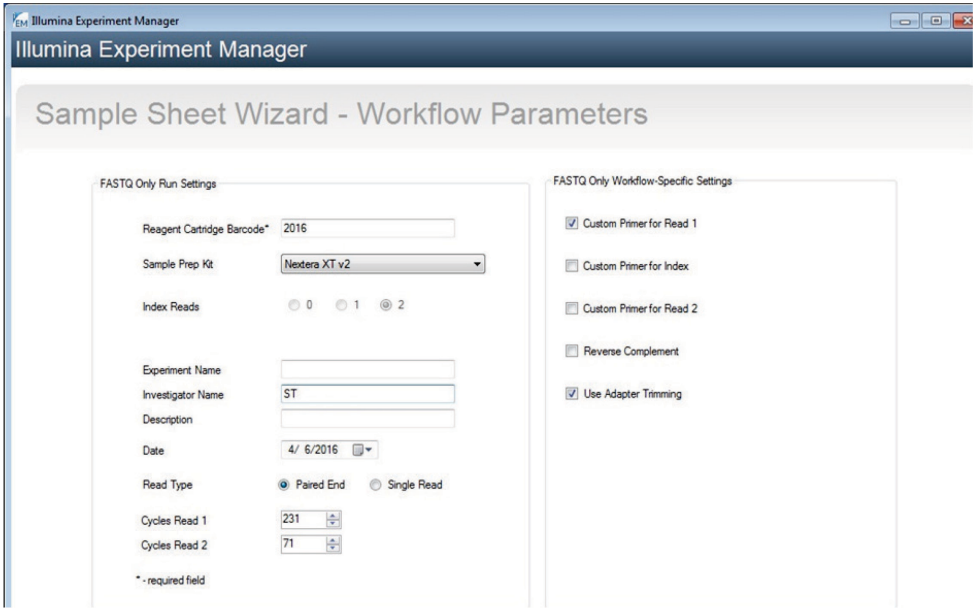
Use diluted QIAseq A Read 1 Primer custom sequencing primer (provided at 100 µM) when setting up sequencing run. Sample index of QIAseq 8-UDI is compatible with Illumina Nextera XT v2 adapter sample index system. QIAseq 96-UDI is a 10 nt index UDI system design by QIAGEN. Please refer to the QIAseq RNA Fusion XP All-in-One Tool to check the recommendation of cycle number setting with different kits.

Sample sheet setup on MiSeq with IEM

Set up sample sheet with custom sequencing read 1 primer using IEM v1.2, or later.



For **Category**, select **Other**.
For **Select Application**, check **FASTQ Only**.



For Sample Prep Kit, select **Nextera XT v2** when using QIAseq 8-UDI index kit.
Index Reads, select **2**.
For **Read Type**, select **Paired End**.
Reads setting:

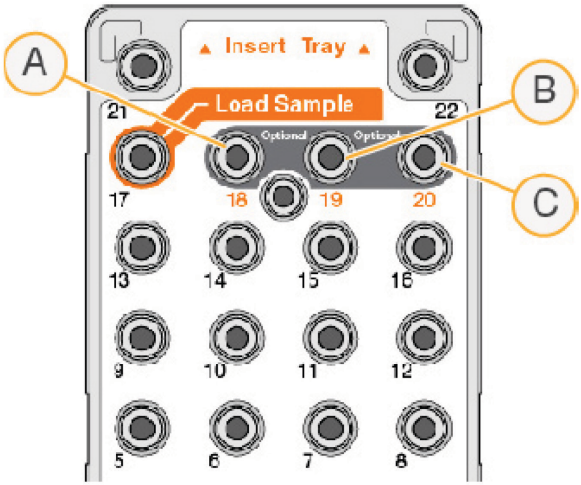
- Cycles for Read 1:231 and Read 2:71: this setting is for high sensitivity fusion detection, especially for libraries generated with high quality RNA samples.
- Alternative setting when running RNA Fusion XP with QIAseq DNAseq is Read 1:151 and Read 2:151 especially for low quality FFPE sample-generated libraries that the advantage of asymmetry reads setting for fusion detection is limited.

Make sure to check Custom Primer for Read 1 as well as Use Adapter Trimming.

Prepare and load custom primer on MiSeq

Use 597 µl HT1 (hybridization buffer) to dilute 3 µl of QIAseq A Read 1 Primer I (provided) to a final concentration of 0.5 µM.

Load 600 µl diluted QIAseq A Read 1 Primer I to Position 18 of MiSeq reagent cartridge.



A Position 18 for Read 1 Custom Primer

For more details, please refer to Illumina protocol:

MiSeq System Custom Primers Guide (15041638 01) for MiSeq. Please note that there is no need for custom primer 2 for RNA Fusion XP product.

Sequencing setup on NextSeq

Use QIAseq A Read 1 Primer I as custom read 1 primer (provided) when setting up sequencing run. Please refer to Illumina protocol for NextSeq run with more details.

Run setting selection: Paired end read.

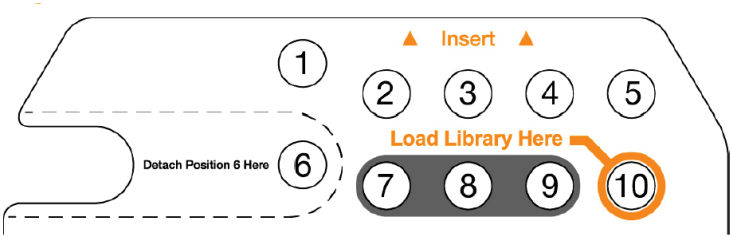
Cycles for Read 1:231 and cycles for Read 2:71 when QIAseq 8-UDI is used (or 229/69 for NextSeq with QIAseq 96-Unique Dual Index Kit, setting with LRM is recommended).

For runs connected to BaseSpace, use of custom primers is specified on the Planned Runs screen of the **Prep** tab. For runs using the standalone configuration, use of custom primers is specified on the NGS Run Setup screen.

Prepare and load custom primer on NextSeq

Use 1994 µl HT1 (Hybridization Buffer) to dilute 6 µl of QIAseq A Read1 Primer (provided) to final 0.3 µM.

Load 2 ml diluted QIAseq A Read1 Primer to Position 7 of NextSeq reagent cartridge.



Position #	Custom Primer
7	Custom Read 1 primer

For all other steps, refer to run setup workflow as described in the NextSeq 500/550 System Guide or NextSeq System Custom Primers Guide.

Appendix C: Sequencing Setup on the Ion System

After the library is constructed, follow the Ion Chef and Ion Chip Kit user manual to determine the final library concentration that need to be used.

After combining libraries with different indices, proceed to template preparation and sequencing according to the manufacturer's instructions. The sample index of QIAseq RNA Fusion XP Panel for Ion System is compatible with the Ion Xpress adapter sample index system. Sequencing read length of 200 bases or longer is recommended for QIAseq RNA Fusion XP Panels on the Ion system.

If different RNA Fusion XP Panels are used, dilute the library to the right concentration, then mix them equally if same level of reads is preferred, or adjust the volume from different library to get different reads budget (large panel may need more reads).

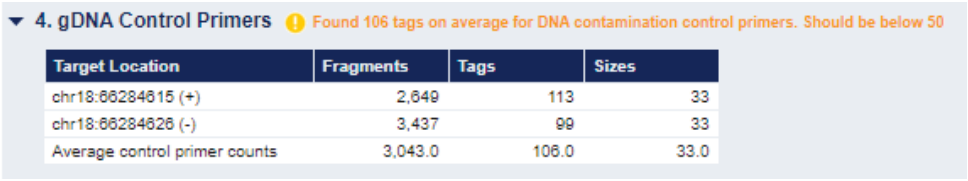
Appendix D: FFPE RNA Quality and Quantity

Total RNA present in FFPE archives is usually damaged and fragmented to an uncertain extent. Commonly used RNA quantification methods including spectrometers or fluorometers do not differentiate between amplifiable and non-amplifiable RNA. Therefore, they cannot reliably measure the amplifiable amounts of RNA that are able to participate in the targeted enrichment step in the NGS workflow such as QIAseq RNA Fusion XP Panel.

The performance of the QIAGEN QIAseq RNA Fusion XP Panels is optimized for high-quality FFPE RNA samples. We recommend using the QIAxcel Advanced or an Agilent Bioanalyzer/TapeStation to check the RNA quality first.

In TapeStation Analysis Software, select **Region view**, then go to **Region settings** to define the region from 200 to 20,000, then apply the change to the data file. Then in the **Region table**, it will have **% of Total shown**. This number can be used to judge the FFPE sample quality. It is better to use the sample with DV2000 >30% (Samples with DV200 between 20% and 30% could be tested as it still has the opportunity to generate library with highly efficient and highly sensitive QIAseq RNA Fusion XP system).

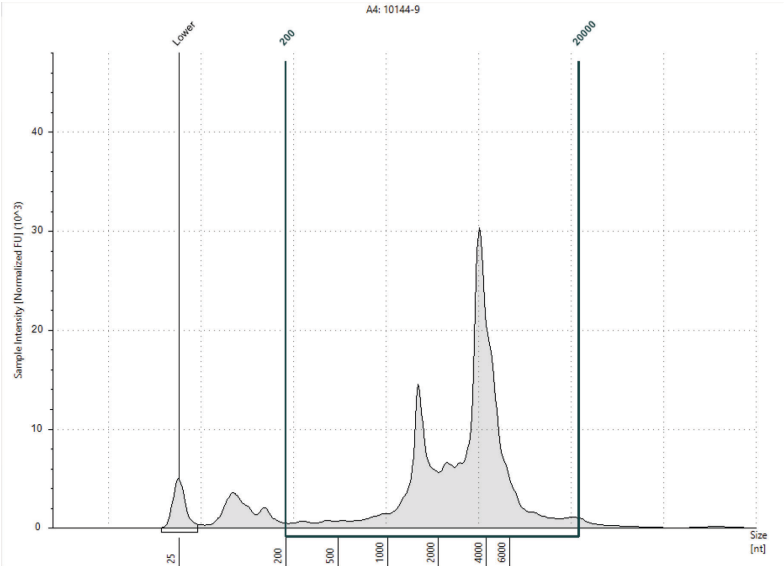
Low quality FFPE RNA will have (1) low sensitivity for fusion call and poor RNA SNV/Indel call, (2) low yield or even failed on library generation, and (3) higher background (higher noise level fusion call). (4) Internal QC will report warning for gDNA signal level and low RNA reference UMI level.



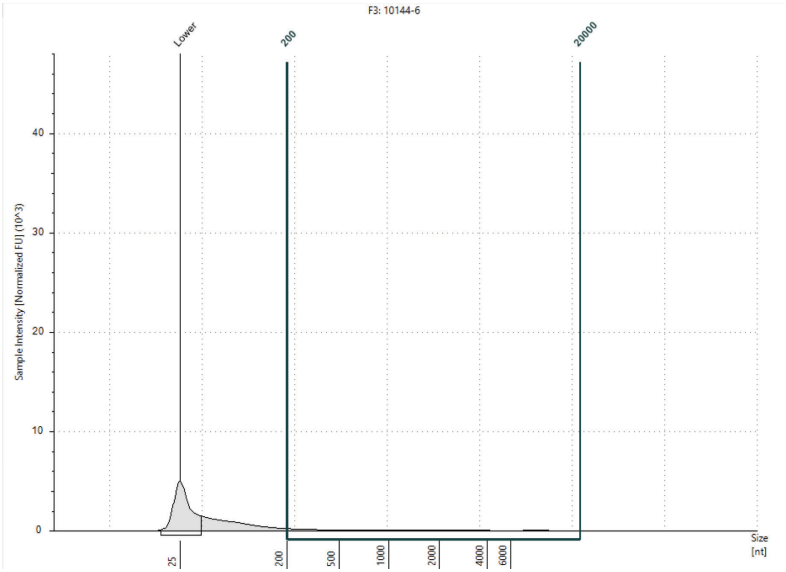
4. gDNA Control Primers Found 106 tags on average for DNA contamination control primers. Should be below 50

Target Location	Fragments	Tags	Sizes
chr18:66284615 (+)	2,649	113	33
chr18:66284626 (-)	3,437	99	33
Average control primer counts	3,043.0	106.0	33.0

Low quality FFPE samples may generate higher gDNA signal and a warning will be shown. It does not affect the strong fusion call but provides information for troubleshooting of false negative or high background noise (multiple fusion calls with weak signal).



Good quality of FFPE RNA, DV200 > 70%



Region Table | Sample Table ⚠

From [nt]	To [nt]	Average Size [nt]	Conc. [ng/µl]	Region Molarity [nmol/l]	% of Total
200	20000	6262	3.41	1.60	18.34

Bad quality FFPE RNA, DV200 < 30%

Appendix E: Fast SPE Enrichment

The RNA Fusion XP workflow has an option for fast SPE enrichment reaction if the short library generation time is critical. Please be aware that the efficiency could be lower with the fast protocol; please verify protocol based on user's sample type, sample quality and sample input, as well as sensitivity and specificity requirement.

Continued from the standard protocol after first beads cleanup and elution

82. Transfer the 10.4 µl eluted sample into PCR strip or plate.
83. Prepare SPE reaction mix for each sample according to Table 33 in PCR strips or a 96-well PCR plate. Mix gently by pipetting up and down.

Table 33. SPE reaction mix

	1 reaction (µl)
Purified sample	10.4
TEPCR Buffer, 5x	4
QIAseq RNA Fusion XP Panel	4
Forward primer	0.8
TaqIT Plus	0.8
Total	**20**

84. Seal the wells with PCR tube caps. Place strips or plate in thermal cycler and set up reaction parameters according to Table 34.

Table 34. Cycler settings for SPE target enrichment

Step	Cycles	Incubation temperature (°C)	Incubation time
1	1	95	2 min
		98	1 min
2	8	95	15 s
		68	1 min
3	1	72	2 min
	1	4	Hold

85. After the reaction is complete, place on ice and proceed to next step.

Appendix F: Library Quantification

Library concentration of the QIAseq RNA Fusion XP Panels can be determined by using QIAGEN's QIAseq Library Quant System. With this system, the correct dilution of the library can be determined for sequencing. Please refer to the QIAseq Library Quant user manual for library quantification.

Please note that the concentration measured with QIAseq Library Quant System with standard settings could be 1.5–2 times less than its actual concentration, so if using the measured concentration directly, use 6–8 pM for MiSeq and 0.8–1.0 pM for NextSeq.

Library concentration of the QIAseq RNA Fusion XP Panels also can be determined by using the QIAGEN's QIAxpert system. With this system, the concentration can be determined as ng/µl; it can be converted to nM by using 3.5 as converting factor. For example, 80ng/µl x 3.5 = 280 nM, then it can be diluted to 4 nM or 2 nM for further Illumina sequencing processing. The final input will be 10–12 pM for Miseq, HiSeq, and NovaSeq and 1.0–1.5pM for NextSeq/MiniSeq. Best loading concentration may need to be optimized based on the real sample loading test.

Appendix G: Blocking Unexpected rRNA and/or Globin Signal with FastSelect in RNA Fusion XP Workflow

The RNA Fusion XP catalog panel and custom panels are designed with high specificity for the primer pool, so most of the time there is very limited non-specific signal from rRNA and/or globin molecules (depending on the sample type). Due to the sample variation and or specific design request challenge, occasionally a large portion of reads may go with rRNA and/or globin under very limited number of primers. It is recommended to optimizing the final primer pool by removing those primers and/or primer re-design. For a quick improvement on initial test or for a quick solution for the unexpected non-specific signal, a modified step of primer priming is provided as below.

It is recommended to dilute the FastSelect with water first. Dilution of 1:10–20 is recommended as initial test. It may be adjusted base on input and rRNA signal strength. Mix the diluted FastSelect as below with RNA sample and RP Primer II, then heat at 65°C for 5 min and cool down with ice for 2 min.

Table 35. Alternative primer priming

	1 reaction (µl)
RNA sample (x µl)	x (≤4)
Diluted FastSelect	1
RP Primer II	1
Nuclease-free water	4 – x
Total	**6**

It is fine to adjust the FastSelect concentration with less volume so more RNA can be used or if 5 µl RNA is needed to maintain the enough input, mixing 30 µl RNA with 3 µl FastSelect first, then take 5 µl for primer reaction.

Go with the standard first-strand synthesis after the primer step.

Appendix H: Data Analysis using QIAGEN's QIAseq RNA Fusion XP Data Analysis Software

After sequencing, results can be analyzed using QIAGEN's Cloud-Based QIAseq RNA Fusion XP Panel Data Analysis Software. It can be approached within **QIAGEN.com** webpage under GeneGlobe Data Analysis Center. Our data analysis software will perform read trimming (removing adapter sequences), mapping, UMI counting, and fusion identification and classification. Please refer to the corresponding document for data analysis.

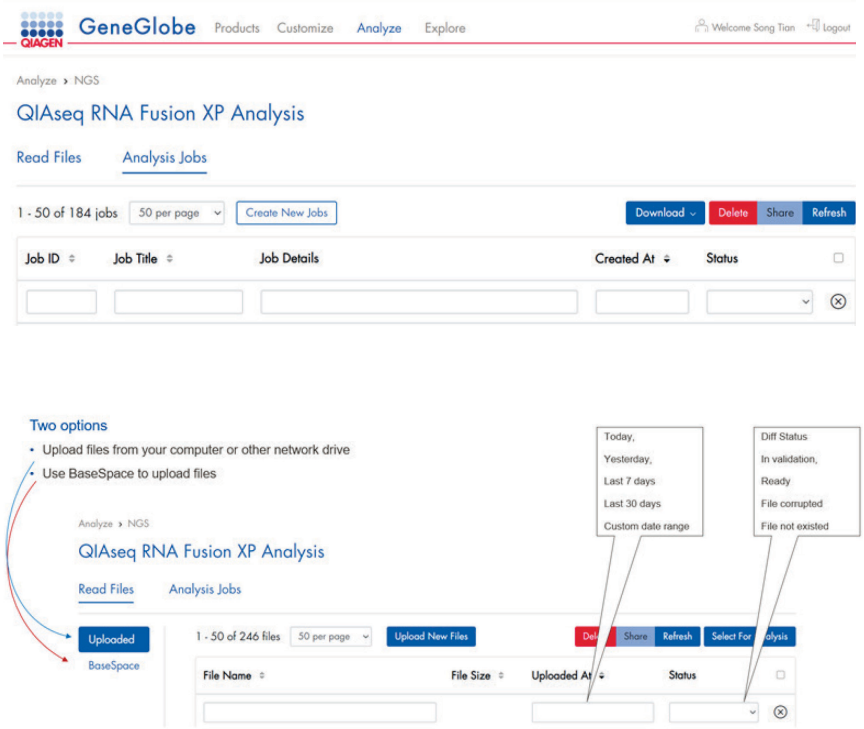
It also can be analyzed by QIAGEN's Genomic Workbench (**https://digitalinsights.qiagen.com**). Please contact QIAGEN's Technical Support team for more details.

Currently, GeneGlobe data analysis tool acceptable file extensions are ".fastq" or ".fastq.gz" for Illumina reads and ".basecaller.bam" for Ion reads. Please submit only unaligned base-caller BAM files generated by Torrent Server 3.4.1 or higher. Please do not submit aligned BAM files.

Important: Please do not refresh the browser or navigate to other pages while uploading files.

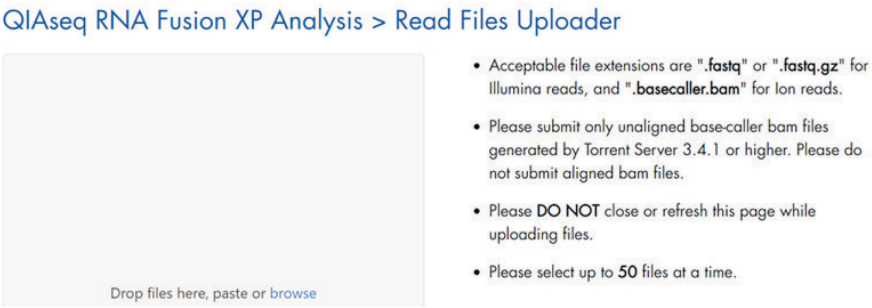
Please go to the GeneGlobe in **QIAGEN.com** webpage, then go to the **Analyze Data**. Please select the NGS (log in if you already registered; otherwise please register at **QIAGEN.com** first).

Find the QIAseq RNA Fusion XP Analysis to access the free online analysis tool. In **Read Files** tab, customer can sort the reads by uploaded date or file name. Files can be selected as Preselect Files for analysis or as Share Files for data sharing. Currently the database will keep the reads file for at least 3 months after uploading is finished. Please save you raw reads in a safe drive for any needs of additional analysis.



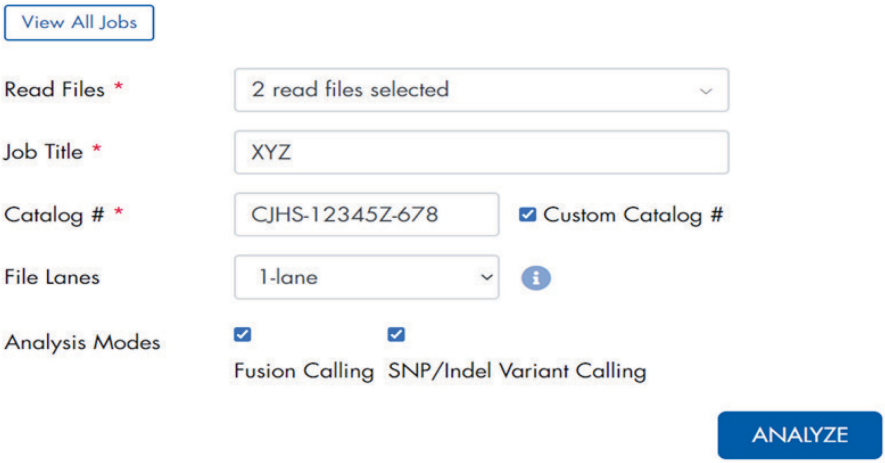
Select direct upload FASTQ files (will be kept for 3 months) or link your BaseSpace account files with the analysis tool.

Direct upload FASTQ files: Drop files to the uploader or browser file for upload.

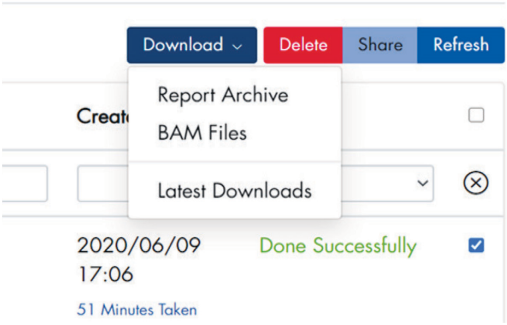


BaseSpace files link: log in your BaseSpace account, and find the files from **Runs/Projects**, grant download and then select the files for analysis.

After selecting the files for analysis, input the job title. For catalog panel, select from dropdown list; if using custom panel, highlight the **Custom Catalog #** on the right, then input the number. Select lane number and select the analysis you want. Fusion analysis is selected by default; if you want SNV/InDel and gene expression analysis, please also select the next option (we currently cannot do transcript variants calling), then click **ANALYZE**.



Getting result: Select the finished jobs. Click **Download**, then select **Report Archive** for regular analysis report; if more details need to be checked, BAM files can be downloaded by selecting **BAM Files**. The report generation will need some time; it will be shown as "queued" first; it can be checked by clicking the **Latest Downloads** (may need refresh page). It will have a green "ready" sign when it is done. The top 1 will be latest combined analysis report. Please download the result in time by clicking the **Download** under **Status**, as it will be removed automatically after 7 days. The result will be downloaded as a zip file. Please unzip it first. The jobs will be retained for 7 days from its created date.



Result folder 1: Summary

~Combined.enrichment-metrics: The summary of the run, like reads number, trimmed reads number, on-target percentage, etc.

~Combined.fusion.xlsx: The same one as RNAscan summary file. It has fusion summary and more details.

Combined.gene-expression: It reports the average UMI for all the genes that are targeted as GEX (gene expression); if no GEX targets, then it will only report the reference assay result.

~Combined.gene-expression-extra: It will report other genes that are not defined as GEX targets (it may be from fusion and SNV/InDel primers).

~Combined.snv-indel: It report RNA SNV/InDel similar as DNA SNP/InDel.

Result folder 2: Tables

- ~Fusion: HTML report as RNAscan and report other details in per_primer file, filtered fusion, passed fusion, and VCF file for fusion report.
- ~Gene-expression: More reports by using different way for calculation like min max, for reads or UMI count.
- ~Snv-indel: More details for SNV/InDel call.

Ordering Information

Product	Contents	Cat. no.
QIAseq RNA Fusion XP Panel (12)*	Kit containing reagents for first-strand synthesis, second-strand synthesis, end-repair/A-addition, gene-specific amplification, and QIAseq Beads for RNA Fusion XP sequencing, fixed panel for 12 samples	334605
QIAseq RNA Fusion XP Panel (96)*	Kit containing reagents for first-strand synthesis, second-strand synthesis, end-repair/A-addition, gene-specific amplification, and QIAseq Beads for RNA Fusion XP sequencing, fixed panel for 96 samples	334605
QIAseq RNA Fusion XP Custom Panel*	Kit containing reagents for first-strand synthesis, second-strand synthesis, end-repair/A-addition, gene-specific amplification, and QIAseq Beads for RNA Fusion XP sequencing, custom panel for 96 samples	334625
QIAseq RNA Fusion XP Extended Panel*	Kit containing reagents for first-strand synthesis, second-strand synthesis, end-repair/A-addition, gene specific amplification, and QIAseq Beads for RNA Fusion XP sequencing, extended panel for 96 samples	334645

Product	Contents	Cat. no.
QIAseq 8-Unique Dual Index Set A (48)*	Box containing unique dual-indexed adapters, for indexing up to 8 samples for QIAseq Targeted Panel sequencing on Illumina platforms; enough to process a total of 48 samples; the first of 2 sets required for multiplexing up to 16 samples	333715
QIAseq 8-Unique Dual Index Set B (48)*	Box containing unique dual-indexed adapters, for indexing up to 8 samples for QIAseq Targeted Panel sequencing on Illumina platforms; enough to process a total of 48 samples; the second of 2 sets required for multiplexing up to 16 samples	333716
QIAseq 96-Unique Dual Index Set A (384)*	Box containing unique dual-indexed adapters, for indexing up to 96 samples for QIAseq Targeted Panel sequencing on Illumina platforms; enough to process a total of 384 samples; the first of 2 sets required for multiplexing up to 192 samples	333725
QIAseq 96-Unique Dual Index Set B (384)*	Box containing unique dual-indexed adapters, for indexing up to 96 samples for QIAseq Targeted Panel sequencing on Illumina platforms; enough to process a total of 384 samples; the second of 2 sets required for multiplexing up to 192 samples	333735

Product	Contents	Cat. no.
QIAseq 12-Index I (48)*	Box containing oligos, 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 oligos, enough for a total of 384 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; 1 of 4 sets	333727
QIAseq 96-Index I Set B (384)*	Box containing oligos, enough for a total of 384 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; 2 of 4 sets	333737
QIAseq 96-Index I Set C (384)* (pending)	Box containing oligos, enough for a total of 384 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; 3 of 4 sets required for multiplexing 384 samples	333747
QIAseq 96-Index I Set D (384)* (pending)	Box containing oligos, enough for a total of 384 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; 4 of 4 sets required for multiplexing 384 samples	333757
QIAseq 12-Index L (48)*	Box containing oligos, enough for a total of 48 samples, for indexing up to 12 samples for targeted panel sequencing on Ion Torrent platforms	333764

Product	Contents	Cat. no.
QIAseq 96-Index L (384)*	Box containing oligos in arrays, enough for a total of 384 samples, for indexing up to 96 samples for targeted panel sequencing on Ion Torrent platforms	333777
Human XpressRef Universal Total RNA	2 tubes each containing 100 µg human RNA at 1 mg/ml	338112
RNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents, and buffers	74104
RNeasy FFPE Kit (50)	50 RNeasy MinElute® Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-free buffers, and Nuclease-Free Water	73504
PAXgene® Blood RNA Kit (50)	50 PAXgene Spin Columns, 50 PAXgene Shredder Spin Columns, Processing Tubes, RNase-Free DNase I, RNase-free reagents, and buffers; to be used in conjunction with PAXgene Blood RNA Tubes	762174
RNeasy Micro Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free DNase I, Carrier RNA, RNase-free reagents, and buffers	74004
QIAamp® RNA Blood Mini Kit (50)	50 QIAamp Mini Spin Columns, 50 QIAshredder Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents, and buffers	52304

Product	Contents	Cat. no.
QIAseq FastSelect – rRNA HMR Kit (24)	Includes 3 tubes of QIAseq FastSelect reagent for rRNA removal; sufficient for 24 reactions from human, mouse, and rat samples	334386
QIAseq FastSelect – rRNA/Globin Kit (24)	Includes 1 tube of QIAseq FastSelect reagent for rRNA removal and 1 tube of QIAseq FastSelect reagent for globin mRNA removal	335376

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

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Document Revision History

Date	Changes
11/2020	Initial revision
07/2021	Changed "SNP" to "SNV" for RNA throughout the document. Included the number (5x) of TEPCR Buffer and RNA Buffer II in the first table of the Kit Contents section. Updated the second table of the Kit Contents section. Updated the QIAseq 8-Unique Dual Index tables of the Kit Contents table. Updated Tables 2, 3, 5, 8, 13, 21, 23, 26, 27, 31 titles; the Equipment and Reagents to Be Supplied by User section; the Important Notes section; and the incubation time on Sample Cleanup 1 sections. Changed "QIAseq IL-F" to "IL-Forward Primer" in Table 13. Changed the buffer in Tables 13, 29, and 33 from "QIAseq RNA Buffer II, 5x" to "TEPCR Buffer, 5x". Updated the content of Tables 9 and 15. Updated the number of recommended cycles in Tables 18 and 32 footnotes. Corrected some measurements under Sample size selection section. Updated protocol step 56 under Sample cleanup 2 section. Updated Appendices A and B. Changed the number of retention days of the reports in Appendix H.

Notes

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Limited License Agreement for QIAseq RNA Fusion XP Panel Kit

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