February 2020

QIAseq® Targeted RNAscan Panel Handbook

For constructing molecularly barcoded libraries from RNA for gene fusion analysis using digital NGS

Sample to Insight

QIAGEN

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Kit Contents

QIAseq Targeted RNAscan Panel (12) **QIAseq Targeted RNAscan HC Panel (12)**	**Catalog no. 333602** **Catalog no. 333612**
Box 1 of 2	
QIAseq Targeted RNAscan Panel	48 µl
RP Primer	12 µl
EZ Reverse Transcriptase	12 µl
BC3 Buffer, 5x	24 µl
RNase Inhibitor	12 µl
RH RNase	12 µl
dNTP	12 µl
XC Buffer, 10x	24 µl
BX Enzyme	12 µl
ERA Enzyme	120 µl
ERA Buffer, 10x	60 µl
DNA Ligase	120 µl
Ligation Buffer, 5x	240 µl
QIAseq RNA Buffer	300 µl
Nuclease-Free Water	1.5 ml
HotStarTaq® DNA Polymerase	22 µl
Handbook	1
Box 2 of 2	
QIAseq Beads	10 ml

QIAseq Targeted RNAscan Panel (96) **QIAseq Targeted RNAscan HC Panel (96)** **QIAseq Targeted RNAscan Custom Panel (96)** **QIAseq Targeted RNAscan Extended Panel (96)**	**Catalog no. 333605** **Catalog no. 333615** **Catalog no. 333625** **Catalog no. 333645**
Box 1 of 2	
QIAseq Targeted RNAscan Panel	384 µl
RP Primer	96 µl
EZ Reverse Transcriptase	96 µl
BC3 Buffer, 5x	192 µl
RNase Inhibitor	96 µl
RH RNase	96 µl
dNTP	96 µl
XC Buffer, 10x	192 µl
BX Enzyme	96 µl
ERA Enzyme	960 µl
ERA Buffer, 10x	480 µl
DNA Ligase	960 µl
Ligation Buffer, 5x	2 x 960 µl
QIAseq RNA Buffer	2 x 1200 µl
Nuclease-Free Water	2 x 1500 µl
HotStarTaq DNA Polymerase	180 µl
Handbook	1
Box 2 of 2	
QIAseq Beads	34 ml

QIAseq 12-Index I (48) **(12 sample index for 48 samples on Illumina® platform)**	**Catalog no. 333714**
IL-N7## Adapter	
Containing 12 tubes of molecularly barcoded adapters, each tube corresponding to one sample index; sufficient to process four samples	20 µl
Primers	
IL-S502 Index Primer	40 µl
IL-Forward Primer	40 µl
IL-Universal Primer	40 µl
QIAseq A Read 1 Primer I (100 µM)	24 µl

QIAseq 96-Index A, B, C or D set I (384) (96 sample index for 384 samples on Illumina platforms)	Catalog no. 333727 Catalog no. 333737 Catalog no. 333747 Catalog no. 333757
IL-N7 Adapter Pate A, B, C or D	
Four plates containing 12 molecularly barcoded adapters, each well corresponding to one sample index; kit is sufficient for 384 samples	One plate each (4 x 40 µl per adapter)
12-cap strip	48
IL-S5 Index Primer Plate A, B, C or D	
Containing four index primer arrays. Each array well contains one IL-S5## index primer and IL-Universal PCR primer pair for PCR amplification and sample indexing; kit is sufficient for 384 samples	4 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) (12 sample index for 48 samples on Ion Torrent® platform)	Catalog no. 333764
LT-BC# Adapter	
Containing 12 tubes of molecularly barcoded adapters, each tube corresponding to one 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) (96 sample index for 384 samples on Ion Torrent platform)	Catalog no. 333777
LT-BC Adapter Plate	
Four plates containing 96 molecularly barcoded adapters, each well corresponding to one sample index; 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 Targeted RNAscan Catalog Panel Information

Catalog no.	Product Name	No. total primers
FHS-001Z	Human Hematology Panel	156
FHS-002Z	Human Solid Tumor Panel	101
FHS-003Z	Human Lung Cancer Panel	137
FHS-3001Z	Human Oncology Panel	950

Shipping and Storage

The QIAseq Targeted RNAscan Panel Kit is shipped with dry ice (Box 1) and must be stored at –30 to –15°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

QIAseq Targeted RNAscan 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 QIAseq Targeted RNAscan 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 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 cause or contribute to disease. Next-generation sequencing of RNA (RNAseq) has been used to detect gene fusion, alternatively spliced transcripts, post-transcriptional modifications, mutations/SNPs and changes in gene expression. A fusion gene is a hybrid gene formed from two previously discrete genes. It can occur as a result of translocation, deletion, chromosomal inversion or transcription-generated chimeras. QIAseq Targeted RNAscan Panels use Single Primer Extension (SPE) and Molecular Barcode (MT) technologies in NGS to help identify and characterize fusion gene events at the RNA level with high efficiency, sensitivity and flexibility.

Principle and workflow

QIAseq Targeted RNAscan Panels rely on highly efficient RNA conversion, gene specific single primer enrichment, and molecular barcoding for sensitive fusion gene detection.

Molecular barcodes

The concept of 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 MT 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 barcodes in the reads rather than counting the number of total reads for each transcript. Sequence reads having

distinct MTs represent different original molecules, while sequence reads having the same MT are results of PCR duplication from one original molecule and are counted together as one molecule.

Procedure

QIAseq Targeted RNAscan Panels are provided as a single tube of primer mix, with up to 1000 primers per tube. QIAseq Targeted RNAscan Panels can enrich selected transcripts using 10–250 ng 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 double stranded cDNAs are then ligated at their 5′ ends to a sequencing platform-specific adapter containing MT 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 to amplify the library and add a second sample index (dual index if needed, platform specific) and other platform-specific required sequences.

The raw sequencing results should be analyzed using the QIAseq Targeted RNAscan Panel Analysis Software at **www.qiagen.com**, which will automatically perform all steps necessary to generate a fusion call report from your NGS data.

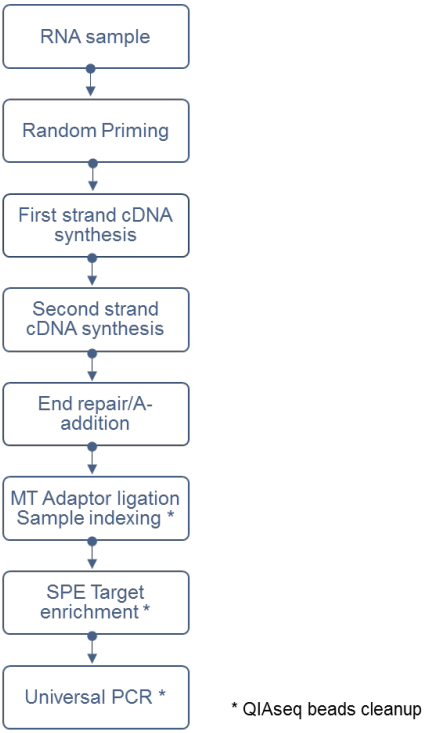


Figure 1. QIAseq Targeted RNAscan Panels workflow.

Description of protocols

This handbook contains 2 protocols. The first protocol details targeted RNAscan library generation for the Illumina platform with the QIAseq Index I Kit (page 19). The second protocol describes how to generate an NGS library for Thermo Fisher's Ion Torrent system platform with the QIAseq Index L Kit (page 35).

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 Targeted RNAscan Panel Kits and QIAseq 12/96 Index I or L Kits, the following supplies are required:

- High-quality nuclease-free water
- Microcentrifuge
- 1.5 ml or 2 ml LoBind tubes (Eppendorf)
- 0.2 ml PCR tubes, 0.2 ml 96-well PCR reaction plates (Bioplastics cat. No. AB17500 or equivalent) or PCR strip tubes and caps
- 0.3 ml 96-well PCR plate (ThermoScientific AB-1400 or equivalent) for handling with 96-well plate format in bead-wash stage
- Thermal cycler
- Multichannel pipettor
- Single-channel pipettor
- DNase-free pipette tips and tubes
- QIAxcel® or Agilent® 2100 Bioanalyzer
- Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)
- 80% ethanol
- Magnet rack for 1.5 ml or 2 ml tubes (DynaMag-2 ThermoFisher Scientific 12321D or equivalent)
- Magnetic separation rack for 96-well plates (DynaMag™-96 Side Magnet, ThermoFisher cat. no. 12331D or equivalent)

- QIAseq Library Quant system: QIAseq Library Quant Array Kit (cat. no. 333304) or QIAseq Library Quant Assay Kit (cat. no. 333314)
- NGS sequencing platform (Illumina system of ThermoFisher Ion Torrent system)
- Controls: ALK-RET-ROS1 Fusion FFPE RNA Reference Standard from Horizon Discovery
- For 96-well format handling, Axygen® silicone 96 round well compression flat mat for PCR microplates, Axygen Sealing Film Roller, 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. no. 74104, 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.

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®. Prepare dilutions and measure absorbance in

10 mM Tris.HCl, pH 7.5, not RNase-free water. The spectral properties of nucleic acids are highly dependent on pH.

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

Ribosomal RNA band integrity

Run an aliquot of each RNA sample on the Agilent Bioanalyzer using an RNA 6000 Nano LabChip® 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 2100 Bioanalyzer should be higher than 8.

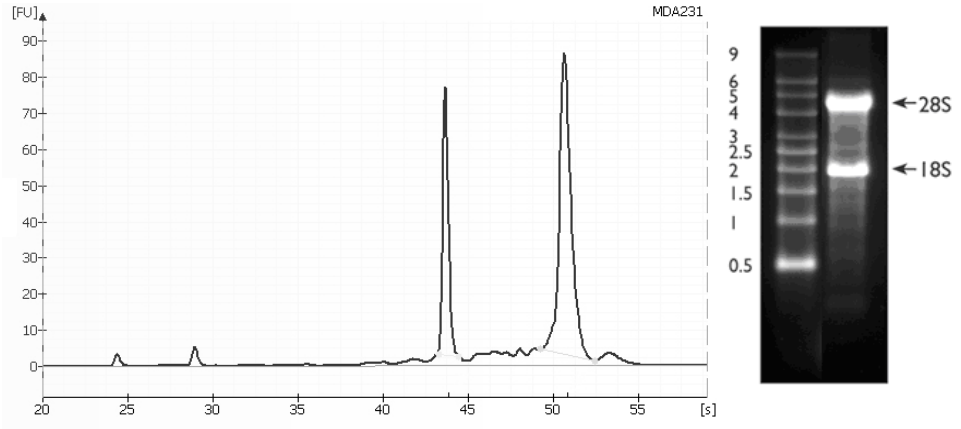


Figure 2. Ribosomal RNA integrity. Agilent Bioanalyzer electropherogram of high-quality total RNA showing sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). Agarose gel electrophoresis shows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

Starting RNA amounts

QIAseq Targeted RNAscan 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 with a low-input protocol based on research needs)

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 sufficient instrument read budget is allocated.

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

Read budget and sample-plex level

Sample multiplexing capacity is defined by the size of the gene fusion panel and sequencing platform read capacity. For the MiSeq®/NextSeq® platforms, sample indexing barcodes are available to multiplex up to 384 samples per run. 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. Below is a preliminary starting sample multiplex suggestion. Adjustments can be made as necessary.

Estimated number of multiplexed samples based on panel size

Instrument	Version	Capacity	100 primers	250 primers	500 primers	1000 primers
MiSeq	V2	15 M	10–25	4–8	2–4	1–2
MiSeq	V3	25 M	15–25	6–10	3–6	2–4
NextSeq 500	Mid Output	130 M	130–260	52–104	26–52	8–16
NextSeq 500	High Output	400 M	250–500	100–200	50–100	25–50
HiSeq® 2500 rapid run	V2 Dual Flowcell	600 M	360–750	150–300	75–150	36–75
Ion PGM®	318 Chip V2	5 M	3–5	1–2	1	0
Ion S5®	Ion 530 Chip	15 M	10–20	4–8	2–4	1
Ion S5	Ion 540 Chip	60 M	40–80	16–32	8–16	4–8

Plate format handling

QIAseq Targeted RNAscan Panels provide convenient workflow for handling 96 samples in 96-well plate format. Follow the special instructions to use 300 µl PCR plates in the QIAseq Beads cleanup stage for 96-well plate format handling.

Protocol: QIAseq Targeted RNAscan Panel for Illumina Instruments

First strand cDNA synthesis

Primer priming

1. Preheat the PCR cycler to 65°C with a heated lid (at 103°C).

2. Put a PCR strip or 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 to each tube, and then add water to reach total volume 6 µl if necessary.

Table 1. Primer priming

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

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.

Table 2. Cycler setting for priming

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

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 from the previous reaction. If handling more than 1 sample, prepare a first strand synthesis mix according to Table 3.

Table 3. Reverse transcription mix

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

* **Important note**: When working with low-input RNA (≤20 ng), please dilute the EZ Reverse Transcriptase with water (1 µl EZ+4 µl nuclease-free water) first, then use 1 µl for each reaction.

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 with a heated lid (103°C) and incubate as shown in Table 4.

Table 4. Cycler settings for reverse transcription

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

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

14. Add 10 µl second strand synthesis mix to each 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 with a heated lid (103°C) and incubate as show in Table 6.

Table 6. Cycler settings for second strand synthesis

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

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

Note: If using a non-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	Incubation time
1	4°C	1 min
2	20°C	30 min
3	65°C	30 min
4	4°C	Hold

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

20. It is important to follow the procedure described below in order 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 and nuclease-free water as indicated in Table 8.

Table 8. End repair/dA tailing mix

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

22. Add 20 µl reaction mix to each reaction.

23. Add 10 µl ERA enzyme 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.

24. Briefly spin down the sample tube/plate and immediately transfer to the prechilled thermal cycler (4°C). Resume the cycling program.

25. When the program is complete and sample block has returned to 4°C, remove samples from block and place on ice.

26. Immediately proceed to the next step.

Adapter ligation

27. If working with more than 1 sample, prepare a ligation mix according to Table 9.

28. Plan ahead by recording each dual index and its corresponding sample.

29. Transfer 5 µl of IL-N7## adapter with molecular tags into the PCR tube with 50 µl of A-tailed DNA from step 26. Mix gently by pipetting and cool on ice.

30. 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)
ER/AT sample from previous section	50
Adapter	5
5x Ligation Buffer	20
DNA Ligase	10
Nuclease-free water	15
Total	**50**

Note: Adapters in the QIAseq 96-Index A, B, C, D Set I (384) are in a 96-well plate. See format in Tables 10–11 below.

Table 10. IL-N7 Adapter Plates A and C

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

Table 11. IL-N7 Adapter Plates B and D

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

Layout of sample adapters in QIAseq 96-Index I Set A, B, C or D. Wells of the first four rows of each plate have adapters. Each well in each row contains one sample adapter, and the amount of adapter in each well is enough for 8 samples.

Note: IL-N7 Adapter Plate A, B, C or D used in the ligation must be paired with IL-S5 Index Primer Plate A, B, C or D in the universal PCR step, respectively.

31. Add 45 µl of the ligation master mix to sample from step 26 and mix well by pipetting.

32. Incubate the ligation reaction at 20°C for exactly 15 min using a thermal cycler with the lid open.

    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.

33. Proceed immediately to adapter ligation cleanup.

    Note: Allow the QIAseq beads to sit at room temperature for ≥30 min before sample cleanup.

Sample cleanup 1

34. Transfer the 100 µl reaction product into a 1.5 ml DNA LoBind tube or transfer the samples into a 300 µl 96-well PCR plate for sample cleanup.

35. Beads wash:

    35a. For regular high-quality RNA with regular input (>20 ng), add 80 µl QIAseq beads to 100 µl reaction. Mix well by pipetting up and down at least 10 times.

    35b. For FFPE RNA and/or low RNA input (≤20ng), add 90 µl QIAseq beads to 100 µl reaction. Mix well by pipetting up and down at least 10 times.

36. Incubate for 5 min at room temperature.

37. Place the tube on a magnetic rack to separate beads from supernatant. After the solution is clear (10 min for 1.5 ml LoBind tube or about 15 min for 300 µl plate). Carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the cDNA target.

38. Completely remove residual supernatant (it is recommended using 10 µl tip to aspirate the trace amount of residual supernatant after the first aspiration).

39. Wash the beads with 260 µl 80% ethanol, rotate the tube three times. Wait 1 min with the tube on magnetic rack.

40. Remove the 80% ethanol completely.

41. Repeat steps 39 and 40 twice.

42. Dry beads by leaving the cap open for 10 min.

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

43. Elute DNA target from beads with 52 µl nuclease-free water. Mix well by pipetting.

44. Put the tube back into the magnetic rack to separate the beads.

45. Carefully transfer 50 µl sample to a new tube.

46. Beads wash:

    46a. For regular high-quality RNA with regular input (>20 ng), add 55 µl QIAseq Beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.

    46b. For FFPE RNA and/or low RNA input (≤20 ng), add 65 µl QIAseq beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.

47. Incubate for 5 min at room temperature.

48. Place the tube or PCR plate on the magnetic rack to separate beads from supernatant. After the solution is clear (about 5–10 min), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the cDNA target.

49. Add 200 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.

50. Repeat step 49 once more.

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

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

52. Elute beads by adding 12.4 µl nuclease-free water. Mix well by pipetting. Place tube or plate on the magnetic rack until solution is clear.

53. Transfer 10.4 µl supernatant to a clean PCR strip or regular 96-well PCR plate.

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

SPE target enrichment

54. Transfer the 10.4 µl eluted sample into PCR strip or plate.

55. Prepare SPE reaction mix for each sample according to Table 12 in PCR strips or a 96-well PCR plate. Mix gently by pipetting up and down.

Table 12. SPE reaction mix

	1 reaction (µl)
Purified sample from previous section	10.4
QIAseq RNA Buffer, 5x	4
QIAseq Targeted RNAscan panel	4
QIAseq IL-F	0.8
HotStarTaq DNA Polymerase	0.8
Total	**20**

56. Seal the wells with PCR tube caps. Place strips or plate in thermocycler and set up reaction parameters according to Table 13.

Table 13. Cycler setting for SPE

Step	Cycles	Incubation temperature	Incubation time
1	1	95°C	15 min
2	8	95°C 68°C	15 sec 10 min
3	1 1	72°C 4°C	5 min Hold

57. 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

58. 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.

59. Beads wash:

59a. For regular high-quality RNA with regular input (>20 ng), add 55 µl QIAseq beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.

59b. For FFPE RNA and/or low RNA input (≤20 ng), add 65 µl QIAseq beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.

60. Incubate for 5 min at room temperature.

61. Place the tube on a magnetic rack to separate beads from supernatant. After the solution is clear (about 5 min), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

62. Completely remove residual supernatant.

63. Add 200 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 two 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.

64. Repeat step 63 once more.

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

66. Elute 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.

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

Universal PCR amplification

68. Prepare universal PCR reaction mix for each sample according to Table 14 in PCR strips or a 96-well PCR plate. Mix gently by pipetting up and down.

Reaction components for universal PCR if using QIAseq 12-Index I:

Table 14. Universal PCR mix using 12-index kit

	1 reaction (µl)
Purified sample	13.4
QIAseq RNA Buffer, 5x	4
QIAseq IL-U Primer	0.8
QIAseq Index Primer (S5##)	0.8
HotStarTaq DNA Polymerase	1
Total	**20**

Reaction components for universal PCR if using QIAseq 96-Index I Set A, B, C or D:

Table 15. Universal PCR mix using 96-index kit

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

Note: Index I applies QIAseq IL-S5## 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 384-plex if using QIAseq 96-Index A, B, C, or D sets together.

Table 16. IL-S5 Index Primer Plate A or B

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

Table 17. IL-S5 Index Primer Plate C or D

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

Layout of IL-S5 Index Primer Plate in QIAseq 96-Index I Set A, B, C or D. Each well contains one predispensed sample index primer and universal primer pair for a single reaction. IL-N7 Adapter Plate A, B, C or D used in ligation must be paired with IL-S5 Index Primer Plate A, B, C or D in universal PCR step, respectively.

69. 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 thermocycler and set up reaction parameters according to Table 18.

Table 18. Cycler settings for universal PCR

Step	Cycles	Incubation temperature	Incubation time
1	1	95°C	15 min
2	25*	95°C	15 sec
		60°C	2 min
3	1	72°C	5 min
	1	4°C	Hold

* 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 25 cycles for regular input, fresh high-quality RNA samples (low plex panel could be 26), and using 28–30 cycles for low-input (≤20ng) or FFPE samples.

70. 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

71. Add 30 µl nuclease-free water to 20 µl reaction to bring the volume to 50 µl.

72. Transfer 50 µl PCR reactions to a 1.5 ml LoBind tube or leave it in 96-well PCR plate for purification.

73. Beads wash:

    73a. For regular high-quality RNA with regular input (>20 ng), add 55 µl QIAseq beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times

    73b. For FFPE RNA and/or low RNA input (≤20 ng), add 65 µl QIAseq beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times

74. Incubate for 5 min at room temperature.

75. Place the tube on a magnetic rack to separate beads from supernatant. After the solution is clear (about 2 min), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

76. Add 200 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 two 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 step 76 once more.

78. Place the tube or plate on the magnetic rack. Completely remove residual ethanol and dry beads for 5 min while the tube or plate is on the rack with the lid open.

    Note: Sufficient drying time is essential to ensure 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.

79. Elute DNA target beads into 25 µl sterile water. Mix well by pipetting. Place 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 an Agilent Bioanalyzer or QIAGEN's QIAseq Library Quant Array for Illumina.

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

Protocol: QIAseq Targeted RNAscan Panel for Ion Torrent (L)

First strand cDNA synthesis

Primer priming

1. Preheat 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 to each tube, and then add water to reach a total volume 6 µl if necessary.

Table 19. Primer priming

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

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.

Table 20. Cycler settings for priming

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

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.

Table 21. Reverse transcription mix

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

* **Important**: When working with low-input RNA (≤20 ng), please dilute the EZ Reverse Transcriptase with water (1 µl EZ+4 µl Nuclease-free water) first, then use 1 µl for each reaction.

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 PCR strip/plate into a cycler with a heated lid (103°C) and incubate as follows:

Table 22. Cycler settings for reverse transcription

Step	**Incubation temperature**	**Incubation time**
1	25°C	10 min
2	42°C	30 min
3	70°C	15 min
4	4°C	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 one sample based on Table 23.

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

Table 23. Second strand synthesis mix

	1 reaction (µl)
cDNA from previous section	10
BX Enzyme	1
XC Buffer	2
RH RNase	1
dNTP	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 with a heated lid and incubate as follows:

Table 24. Cycler settings for second strand synthesis

Step	Incubation temperature	Incubation time
1	37°C	7 min
2	65°C	10 min
3	80°C	10 min
4	4°C	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	Incubation time
1	4°C	1 min
2	20°C	30 min
3	65°C	30 min
4	4°C	Hold

19. Be certain to use the instrument's heated lid with the temperature set to ~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 in order 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 and nuclease-free water as indicated in the table.

23. Add 20 µl reaction mix to each reaction.

Table 26. End repair/dA tailing mix

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

24. Add 10 µl ERA enzyme 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.

25. Briefly spin down the sample tube/plate and immediately transfer to the prechilled thermal cycler (4°C). 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 one single barcoded adapter should be used per ligation reaction, open one 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 amount of adapters from the provided PCR plate. See Table 28 for layout of adapters in the PCR plate.

Table 27. Adapter ligation mix

	1 reaction (µl)
ER/AT sample from previous section	50
Adapter	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 one 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 thermocycler 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: Allow the QIAseq beads to sit at room temperature for ≥30 min before sample cleanup.

Sample cleanup 1

32. Transfer the 100 µl reaction product into a 1.5 ml DNA LoBind tube or transfer the samples into 300 µl 96-well PCR plate for sample cleanup.

33. Beads wash:

    33a. For regular high-quality RNA with regular input (>20 ng), add 80 µl QIAseq Beads to 100 µl reaction. Mix well by pipetting up and down at least 10 times.

    33b. For FFPE RNA and/or low RNA input (≤20 ng), add 90 µl QIAseq 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 beads from supernatant. After the solution is clear (10 min for 1.5ml LoBind tube or about 15 min for 300 µl plate). 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 260 µl 80% ethanol, rotate the tube three times. Wait 1 min with the tube on the magnetic rack.

38. Remove the 80% ethanol completely.

39. Repeat steps 37 and 38 twice.

40. Dry the beads by leaving the cap open for 10 min.

    Note: Sufficient drying time is essential to ensure 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 beads with 52 µl nuclease-free water. Mix well by pipetting.

42. Put tube back on the magnetic rack to separate the beads.

43. Carefully transfer 50 µl sample to a new tube.

44. Beads wash:

    44a. For regular high-quality RNA with regular input (>20 ng), add 55 µl QIAseq beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.

    44b. For FFPE RNA and/or low RNA input (≤20 ng), adding 65 µl QIAseq beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.

45. Incubate for 5 min at room temperature.

46. Place the tube or PCR plate on the magnetic rack to separate beads from supernatant. After the solution is clear (about 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.

47. 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.

48. Repeat step 47 once more.

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

    Note: Sufficient drying time is essential to ensure 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.

50. 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.

51. Transfer 10.4 µl supernatant to a clean PCR strip or regular 96-well PCR plate.

SPE target enrichment

52. Transfer the 10.4 µl eluted sample into PCR strip or plate.

53. Prepare SPE reaction mix for each sample according to Table 29 in PCR strips or a 96-well PCR plate. Mix gently by pipetting up and down.

Table 29. SPE reaction mix

	1 reaction (µl)
Purified sample	10.4
QIAseq RNA Buffer, 5x	4
QIAseq Targeted RNAscan Panel	4
LT-Forward primer	0.8
HotStarTaq DNA Polymerase	0.8
Total	**20**

54. Seal the wells with PCR tube caps. Place strips or plate in thermocycler and set up reaction parameters according to Table 30.

Table 30. Cycler settings for SPE target enrichment

Step	Cycles	Incubation temperature	Incubation time
1	1	95°C	15 min
2	8	95°C 68°C	15 sec 10 min
3	1 1	72°C 4°C	5 min Hold

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

Sample cleanup 2

56. 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.

57. Beads wash:

    57a. For regular high-quality RNA with regular input (>20 ng), add 55 µl QIAseq beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.

    57b. For FFPE RNA and/or low RNA input (≤20ng), add 65 µl QIAseq Beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.

58. Incubate for 5 min at room temperature.

59. Place the tube on a magnetic rack to separate beads from supernatant. After the solution is clear (about 5 min), carefully remove and discard supernatant. Be careful not to disturb the beads, which contain the DNA target.

60. Completely remove residual supernatant.

61. Add 200 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 two 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.

62. Repeat step 61 once more.

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

    Note: Sufficient drying time is essential to ensure 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.

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

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

Universal PCR amplification

66. 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.

Table 31. Universal PCR reaction mix

	1 reaction (µl)
Purified sample	13.4
QIAseq RNA buffer, 5x	4
LT-Universal primer	0.8
LT-P1 primer	0.8
HotStarTaq DNA Polymerase	1
Total	**20**

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

Table 32. Cycler setting for universal PCR

Step	Cycles	Incubation temperature	Incubation time
1	1	95°C	15 min
2	25*	95°C 60°C	15 sec 2 min
3	1 1	72°C 4°C	5 min Hold

* 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 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 (<20ng) or FFPE samples.

68. 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

69. Add 30 µl nuclease-free water to a 20 µl reaction to bring the volume to 50 µl.

70. Transfer 50 µl PCR reactions to a 1.5 ml LoBind tube or leave it in 96-well PCR plate for purification.

71. Add 37 µl QIAseq beads to 50 µl PCR reaction. Mix well by pipetting up and down at least 10 times.

72. Incubate for 5 min at room temperature.

73. Place the tube on a magnetic rack to separate beads from supernatant.

74. After the solution is clear (about 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.

75. Add 20 µl QIAseq beads to 85 µl sample and mix well by pipetting up and down at least 10 times.

76. Incubate for 5 min at room temperature.

77. Place the tube on the magnetic rack to separate beads from supernatant. Carefully remove all the supernatant.

78. 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 two 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.

79. Repeat step 78 once more.

80. Place the tube or plate on the magnetic rack. Completely remove residual ethanol and dry beads for 5 min while the tube or plate is on the rack with the lid open.

    Note: Sufficient drying time is essential to ensure 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.

81. 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.

82. Transfer 21 µl supernatant to a clean PCR strip or 96-well PCR plate.

83. Proceed to library quantification. The concentration of the library can be determined using an Agilent Bioanalyzer or QIAGEN's QIAseq Library Quant Array for Ion Torrent.

Troubleshooting Guide

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

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

Prepare library for sequencing

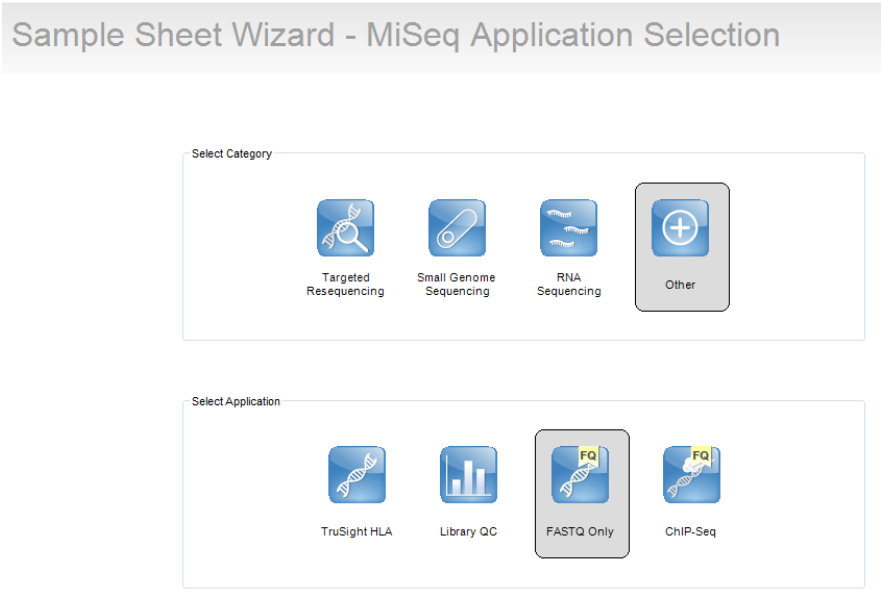
After library quantification, dilute library to 2 nM for MiSeq and 0.5 nM for NextSeq, then combine libraries with different sample indices in equimolar amounts if similar sequencing depth is needed for each library. If combining libraries with same number of primers, pool equal volume of individual library at 2 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 6–8 pM on MiSeq and 0.8–1.0 pM on NextSeq. If using an Agilent Bioanalyzer to determine concentration, use 10 pM on MiSeq and 1.2 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 Targeted DNA Panel is compatible with Illumina Nextera XT V2 adapter sample index system.

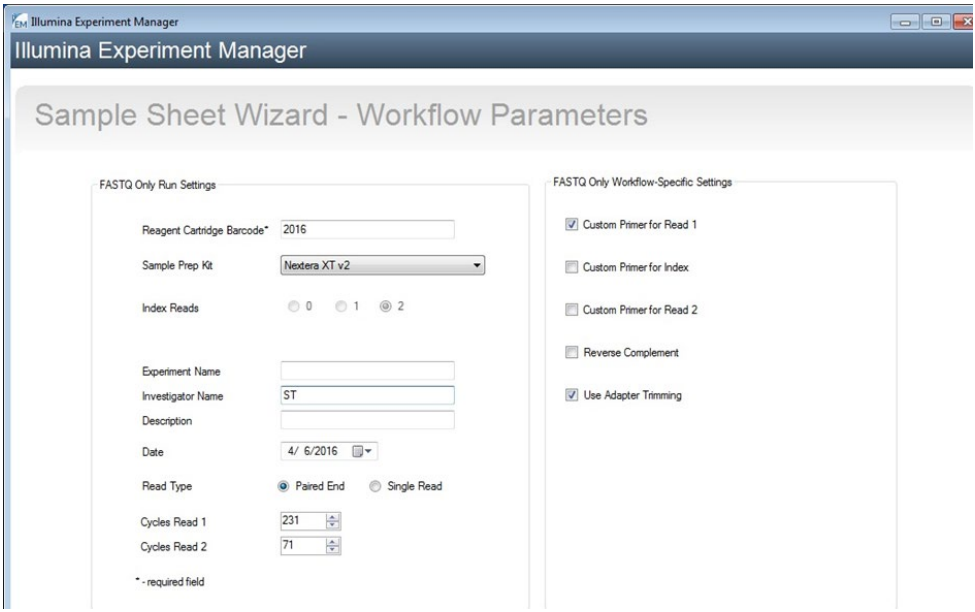
Sample sheet setup on MiSeq

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



For **Category**, select **Other**.

For **Select Application**, check **FASTQ Only**.



For **Sample Prep Kit**, select **Nextera v2**.

Index Reads, select **2**.

For **Read Type**, select **Paired End** read.

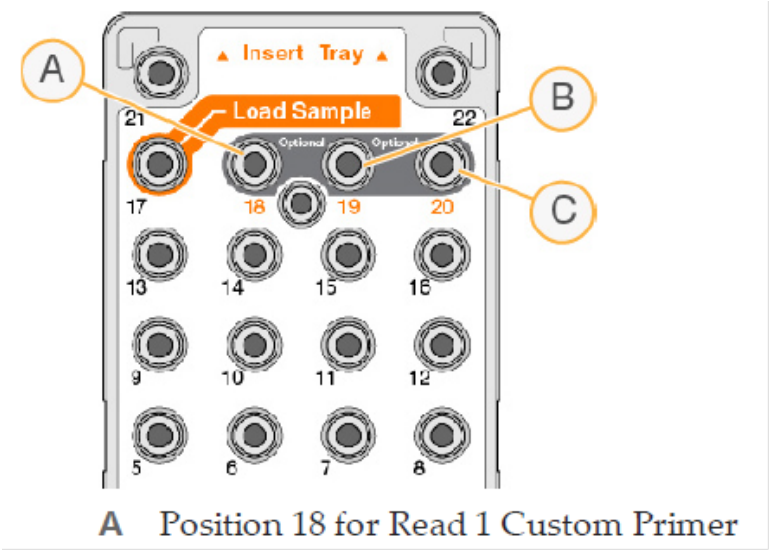
Cycles for Read 1: **231** and Read 2: **71**.

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_using_custom_primers_15041638_b xd for MiSeq.

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, **nextseq_using_custom_primers_15057456** for NextSeq run, for more details.

Run setting selection: **Paired end** read.

Cycles for Read 1: **231**.

Cycles for Read 2: **71**.

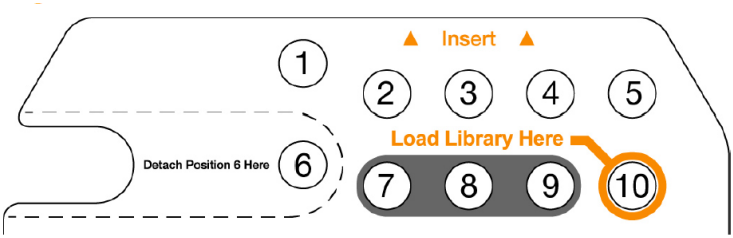
Dual indexes (8 cycles of each)

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 are 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

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

Appendix B: Sequencing Setup on the Ion System

After the library is constructed, follow Appendix D, page 56, to determine the library dilution factor (which dilutes libraries to 4 pM) and dilute each individual library according to this factor.

Libraries with different sample indices can be combined in equimolar amounts if similar sequencing depth is needed for each library. If combining libraries with the same number of primers, pool equal volumes of individual libraries at 4 pM together. It is not recommended to combine libraries with different primer numbers, as the expression of different genes in different panels is not only dependent on primer number, but also on the target expression level. It is difficult to estimate the correct mix ratio between different libraries.

After combining libraries with different indices, proceed to template preparation and sequencing according to the manufacturer's instructions. Sample index of QIAseq Targeted RNAscan 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 Targeted RNAscan Panels on the Ion system.

Appendix C: 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 Targeted RNAscan Panel.

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

Appendix D: Library Quantification

Library concentration of QIAseq Targeted RNAscan 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 5–6 pM for MiSeq and 0.6–0.75 pM for NextSeq.

Appendix E: Data Analysis using QIAGEN's QIAseq Targeted RNAscan Data Analysis Software

After sequencing, results can be analyzed using QIAGEN's Cloud-Based QIAseq Targeted RNAscan Panel Data Analysis Software. Our data analysis software will perform mapping to the reference transcriptome, MT counting, read trimming (removing primer sequences) and fusion identification and classification. Please refer to the corresponding document for data analysis.

Ordering Information

Product	Contents	Cat. no.
QIAseq Targeted RNAscan Panel (12)	Kit containing reagents for first strand synthesis, second strand synthesis, end-repair/A-addition, gene specific amplification and QIAseq Beads for targeted RNAscan sequencing, fixed panel for 12 samples	333602
QIAseq Targeted RNAscan HC Panel (12)	Kit containing reagents for first strand synthesis, second strand synthesis, end-repair/A-addition, gene specific amplification and QIAseq Beads for targeted RNAscan sequencing, fixed panel for 12 samples	333612
QIAseq Targeted RNAscan Panel (96)	Kit containing reagents for first strand synthesis, second strand synthesis, end-repair/A-addition, gene specific amplification and QIAseq Beads for targeted RNAscan sequencing, fixed panel for 96 samples	333605
QIAseq Targeted RNAscan HC Panel (96)	Kit containing reagents for first strand synthesis, second strand synthesis, end-repair/A-addition, gene specific amplification and QIAseq Beads for targeted RNAscan sequencing, fixed panel for 96 samples	333615
QIAseq Targeted RNAscan Custom Panel	Kit containing reagents for first strand synthesis, second strand synthesis, end-repair/A-addition, gene specific amplification and QIAseq Beads for targeted RNAscan sequencing, custom panel for 96 samples	333625
QIAseq Targeted RNAscan Extended Panel	Kit containing reagents for first strand synthesis, second strand synthesis, end-repair/A-addition, gene specific amplification and QIAseq Beads for targeted RNAscan sequencing, extended panel for 96 samples	333645

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; one of four 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; two of four sets	333737
QIAseq 96-Index I Set C (384)	Box containing oligos, enough for a total of 384 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; three of four sets required for multiplexing 384 samples	333747
QIAseq 96-Index I Set D (384)	Box containing oligos, enough for a total of 384 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; four of four sets required for multiplexing 384 samples	333757
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
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

Product	Contents	Cat. no.
Related products		
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, 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 Microarray Tissue Mini Kit (50)	RNeasy Mini Spin Columns, Collection Tubes, QIAzol® Lysis Reagent, RNase-free reagents and buffers	73304
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

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Document Revision History

Date	Changes
02/2020	QIAseq bead component size changed from 4.2 ml to 10 ml.

Notes

Limited License Agreement for the QIAseq Targeted RNAscan Panel Handbook

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