February 2020

QIAseq® Targeted RNA Panels

For accurate molecular barcode-driven gene expression profiling by next-generation sequencing (NGS)

Contents

Kit Contents

QIAseq Targeted RNA Panel	(12)	Extended (12)	Custom (12)	(96)	Extended (96)	Custom (96)
Catalog no.	**333002**	**333012**	**333022**	**333005**	**333015**	**333025**
No. of reactions	**12**	**12**	**12**	**96**	**96**	**96**
RNase-free water	1000 µl	1000 µl	1000 µl	1000 µl x 2 tubes	1000 µl x 2 tubes	1000 µl x 2 tubes
Control P2	12 µl	12 µl	12 µl	96 µl	96 µl	96 µl
Buffer GE	24 µl	24 µl	24 µl	192 µl	192 µl	192 µl
RE3 Reverse Transcriptase Mix	24 µl	24 µl	24 µl	192 µl	192 µl	192 µl
5x Buffer BC3	48 µl	48 µl	48 µl	384 µl	384 µl	384 µl
QIAseq RNA 5x Buffer	300 µl	300 µl	300 µl	2400 µl	2400 µl	2400 µl
HotStarTaq® DNA Polymerase (6 U/µl)	60 µl	60 µl	60 µl	480 µl	480 µl	480 µl
RS2 Primer (10 µM)	20 µl	20 µl	20 µl	160 µl	160 µl	160 µl
BC Primer Mix	80 µl*	80 µl*	80 µl*	640 µl†	640 µl†	640 µl†
LA Primer Mix	80 µl‡	80 µl‡	80 µl‡	640 µl§	640 µl§	640 µl§
QIAseq Beads	10 ml	10 ml	10 ml	32 ml	32 ml	32 ml

* Contaning gene-specific primer mix for molecular barcoding; cataloged/extended/custom panel for 12 samples.

† Contaning gene-specific primer mix for molecular barcoding; cataloged/extended/custom panel for 96 samples.

‡ Containing gene-specific primer mix for amplification; cataloged/extended/custom panel for 12 samples.

§ Containing gene-specific primer mix for amplification; cataloged/extended/custom panel for 96 samples.

QIAseq Targeted RNA Custom Panel	**(384)**
Catalog no.	**333027**
No. of reactions	**384**
RNase-free water (2 tubes)	4 × 1000 µl
Control P2	4 × 96 µl
Buffer GE	4 × 192 µl
RE3 Reverse Transcriptase Mix	4 × 192 µl
5x Buffer BC3	4 × 384 µl
QIAseq RNA 5x Buffer	4 × 2400 µl
HotStarTaq DNA Polymerase (6 U/µl)	4 × 480 µl
RS2 Primer (10 µM)	4 × 160 µl
BC Primer Mix*	4 × 640 µl
LA Primer Mix†	4 × 640 µl
QIAseq Beads‡	4 × 32 ml

* Contaning gene-specific primer mix for molecular barcoding; cataloged/extended/custom panel for 384 samples.
† Containing gene-specific primer mix for amplification; cataloged/extended/custom panel for 384 samples.
‡ Sufficient for 96 samples.

QIAseq Targeted RNA 12- or 96-index for Illumina®*	**12 indexes**	**96 indexes**
Catalog no.	**333114**	**333117**
For no. of samples	**48**	**384**
RS-D# (4 µM)	36 µl	120 µl
FS-D# (4 µM)	48 µl	80 µl
QIAseq Read1 Primer I (100 µM)	24 µl	4 × 24 µl

* Ordered separately.

QIAseq Targeted RNA 96-index HT for Illumina	
Catalog no.	**333127**
Box containing 4 tubes of QIAseq Read1 Primer I (100 µM, 24 µl) and arrays. Each array well contains one universal PCR primer pair for PCR amplification and sample indexing – enough for a total of 384 samples (for indexing up to 96 samples per run) for targeted RNA sequencing on Illumina platforms	1

QIAseq Targeted RNA Read1 Primer I (for sequencing on Illumina platforms)	
Catalog no.	**333514**
QIAseq Read1 Primer I (100 µM)	24 µl

QIAseq Targeted RNA 12-index for Ion Torrent	
Catalog no.	**333214**
For no. of samples	**48**
FS-trP1 (4 µM)	144 µl
RS-ID# (4 µM)	12 µl

QIAseq Targeted RNA 96-index HT for Ion Torrent	
Catalog no.	**333217**
Box containing oligos in arays, each well of which containing one universal PCR primer pair for PCR amplification and sample indexing – enough for a total of 384 samples (for indexing up to 96 samples per run) for targeted RNA sequencing on Ion Torrent platforms	1

Shipping and Storage

QIAseq Targeted RNA Panels are shipped in 2 boxes. The first is a box of reagents, shipped on dry ice and should be stored immediately upon receipt at –30 to –15°C in a constant-temperature freezer. The other box is shipped on blue ice and should be stored immediately upon receipt in a refrigerator at 2–8°C.

Under these conditions, the components are stable for 6 months without showing any reduction in performance and quality, unless otherwise indicated on the label.

Intended Use

QIAseq Targeted RNA Panels are intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

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

Safety Information

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

Quality Control

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

Introduction

Targeted sequencing is particularly advantageous for achieving high coverage of regions/genes of interest while keeping the cost of sequencing and complexity of data interpretation manageable. In RNA analysis, a targeted approach can provide more evidence of low- abundance transcripts because, in whole-transcriptome sequencing, most sequence reads are consumed by mid- and high-abundance transcripts, often leaving inadequate coverage of low-abundance transcripts. Existing target enrichment, library preparation and sequencing steps all utilize DNA polymerase and amplification processes, introducing substantial bias and artifacts. PCR amplification bias significantly affects quantification accuracy, as final sequence read counts may not accurately represent the relative abundance of original fragments (1).

QIAseq Targeted RNA Panels integrate molecular barcode (or molecular tag, MT) technology into a highly multiplexed PCR-based target enrichment process, enabling unbiased and accurate quantification of a targeted panel of mRNA transcripts by next-generation sequencing (NGS). This system is platform agnostic and compatible with most medium- and high-throughput sequencers such as Life Technologies® Ion Personal Genome Machine® (Ion PGM®) and Ion Proton®, as well as Illumina®'s MiSeq® Personal Sequencer, NextSeq® 500, HiSeq® 1000, HiSeq 1500, HiSeq 2000, HiSeq 2500, and GAIIx.

Principle and procedure

The QIAseq targeted RNA panels rely on 2-stage PCR and MT for simple NGS library construction and PCR/sequencing bias reduction. Adapter ligation steps are eliminated and very low input of total RNA is required.

Molecular barcodes

The concept of molecular barcoding is that prior to any amplification, each original molecule is attached to a unique sequence barcode. This is accomplished by the BC primer mix which contains:

- MT consisting of 12 random bases, therefore providing 4^{12} = 16777216 unique molecular tags for each BC primer,
- gene-specific (GS) sequences, and
- common Universal primer sequence (RS2) (Figure 1).

Statistically, during the BC assignment step, each cDNA molecule in the sample receives a unique MT sequence.

Figure 1. Example of BC primer design.

The barcoded cDNA molecules are then amplified by PCR. Due to intrinsic noise and sequence-dependent bias, barcoded cDNA molecule may be amplified unevenly. Thus, target quantification can be better achieved by counting the number of unique molecular barcodes in the reads rather than counting the number of total reads for each gene.

Sequence reads having different MTs represent original molecules, while sequence reads having the same tag are results of PCR duplication from one original molecule (Figure 2).

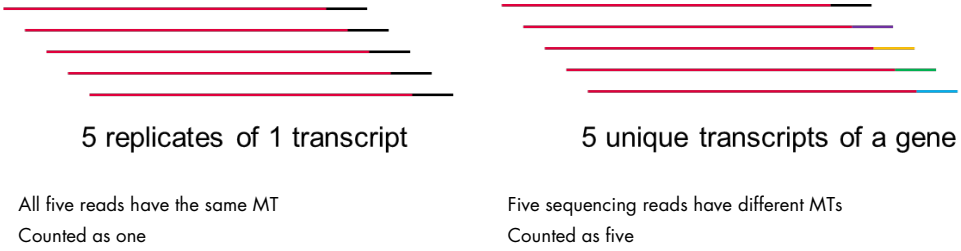


Figure 2. Principle of molecular barcoding. Each original molecule is tagged by a unique MT. Sequence reads having five different MTs are counted as five unique transcripts (right) and those having the same MT are counted as one unique transcript (left).

Procedure

The QIAseq Targeted RNA Panels workflow begins with converting total RNA into cDNA. This reaction is somewhat flexible with regard to input RNA, since 25 ng to 400 ng can be used for the reverse transcription reaction. Additionally, rRNA depletion, blocking or poly-A selection of mRNA is not necessary.

The MT assignment step makes use of a multiplex primer panel (targeting 12-1000 genes) and an input of 20 ng cDNA equivalent (cDNA made from 20 ng total RNA), although one can use 1.25–40 ng cDNA equivalent successfully.

After the molecular barcoding step with GSP1, the barcoded cDNA is purified over QIAseq Beads to remove residual primers/dimers, and a PCR reaction is set up with a second pool of gene-specific primers (GSP2) and the Common Uni1 primer (RS2), which anneals to a common sequence present on the GSP1 primers. This reaction ensures that intended targets are enriched sufficiently to be represented in the final library. A universal PCR is then run to amplify the products and add sample indexing as a final step. After a final QIAseq bead cleanup, the library construction is complete (Figure 3).

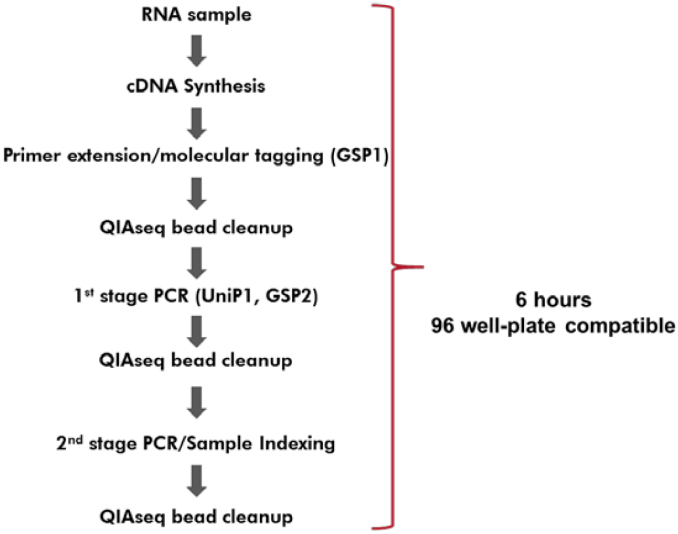


Figure 3. QIAseq Targeted RNA 2-stage PCR workflow.

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 RNA Kits, the following supplies are required: For NGS library construction on Illumina and Ion Torrent sequencers:

- High-quality nuclease-free water. **Important**: Do not use DEPC-treated water.
- Microcentrifuge
- 1.5 ml or 2 ml LoBind tubes
- 0.2 ml PCR tubes, 0.2 ml 96-well PCR reaction plates (Bioplastics cat. no. AB17500) or PCR strips and caps
- Thermal cycler
- Multichannel pipettor
- Single-channel pipettor
- DNase-free pipette tips and tubes
- 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™-96 Side Magnet (Thermo Fisher cat. no. 12331D) for 96-well plates

Important Notes

RNA preparation

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 RNeasy Plus Mini Kit (cat. no. 74134), AllPrep DNA/RNA Mini Kit (cat. no. 80204), AllPrep DNA/RNA FFPE Kit (cat. no. 80234) and RNease FFPE Kit (cat. no. 73504) are recommended for the preparation of RNA samples from fresh tissues and FFPE tissue samples. Ensure that samples have been treated for the removal of DNA, as DNA contamination will cause inaccuracies in RNA concentration measurements. **Do not omit the genomic DNA elimination step.** 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. Prepare dilutions and measure absorbance in 10 mM Tris·Cl, 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·Cl, pH 7.5. RNA integrity:

The integrity and size distribution of total RNA can be checked by the Agilent 2100 Bioanalyzer. For best results, the ribosomal bands should appear as sharp peaks (**Figure 4**, page 35). Ideally, the RIN number from the Agilent 2100 Bioanalyzer should be close to 10.

Due to effects of formalin fixation, embedding and storage, RNA from FFPE will usually show varying degrees of degradation (see Appendix B, page 34). For better results, at least 40% of the fragments for FFPE RNA should be greater than 200 nt in length.

Genomic DNA contamination

Most primers of QIAseq Targeted RNA Panels use an intraexon design which enables primer and multiplex panel optimization with genomic DNA. It is therefore critical to minimize or eliminate genomic DNA in your RNA samples. The QIAseq Targeted RNA First Strand Components contain a genomic DNA elimination mix. Moreover, 6 genomic DNA control assays are included in all cataloged and extended panels. These control assays are used to identify genomic DNA contamination and establish a background baseline in data analysis.

Read budget and sample plex level

Sample multiplexing capacity is defined by the size of the gene panel and sequencing platform read capacity. For the Ion Torrent and MiSeq/NextSeq 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. General guidelines are provided in Table 1, Table 2, and Table 3 for different read depths. Fine tuning for your read budget will be possible after your first test run.

Table 1. Number of multiplexed samples based on panel size and low read depth

			#Samples_Low Read depth Average 2000 reads/gene Average 1 read per MT			
Instrument	**Version**	**Capacity**	**100 genes**	**250 genes**	**500 genes**	**1000 genes**
MiSeq	V2	15 M	75	30	15	8
MiSeq	V3	25 M	125	50	25	13
NextSeq 500	High Output	400 M	2000	800	400	200
NextSeq 500	Mid Output	130 M	650	260	130	65
Ion Torrent PGM	Ion 314 Chip v2	400–550 K	2	1	0	0
Ion Torrent PGM	Ion 316 Chip v2	2–3 M	10	4	2	1
Ion Torrent PGM	Ion 318 Chip v2	4–5.5 M	20	8	4	2
Ion S5	Ion 520 Chip	3–5 M	15	6	3	2
Ion S5	Ion 530 Chip	15–20 M	75	30	15	8
Ion S5	Ion 540 Chip	60–80 M	300	120	60	30

Note: Dark gray wells indicate this is either limited by index number (>96) or by sequencing platform read budget (=0).

Table 2. Number of multiplexed samples based on panel size and moderate read depth

			#Samples_Moderate Read depth Average 5000 reads/gene Average 2–5 reads per MT			
Instrument	**Version**	**Capacity**	**100 genes**	**250 genes**	**500 genes**	**1000 genes**
MiSeq	V2	15 M	30	12	6	3
MiSeq	V3	25 M	50	20	10	5
NextSeq 500	High Output	400 M	800	320	160	80
NextSeq 500	Mid Output	130 M	260	104	52	26
Ion Torrent PGM	Ion 314 Chip v2	400–550 K	1	0	0	0
Ion Torrent PGM	Ion 316 Chip v2	2–3 M	4	2	1	0
Ion Torrent PGM	Ion 318 Chip v2	4–5.5 M	8	3	2	1
Ion S5	Ion 520 Chip	3–5 M	6	2	1	1
Ion S5	Ion 530 Chip	15–20 M	30	12	6	3
Ion S5	Ion 540 Chip	60–80 M	120	48	24	12

Note: Dark gray wells indicate this is either limited by index number (>96) or by sequencing platform read budget (=0).

Table 3. Number of multiplexed samples based on panel size and high read depth

			#Samples_High Read depth Average 15,000 reads/gene Average >5 reads per MT			
Instrument	**Version**	**Capacity**	**100 genes**	**250 genes**	**500 genes**	**1000 genes**
MiSeq	V2	15 M	10	4	2	1
MiSeq	V3	25 M	17	7	3	2
NextSeq 500	High Output	400 M	267	107	53	27
NextSeq 500	Mid Output	130 M	87	35	17	9
Ion Torrent PGM	Ion 314 Chip v2	400–550 K	0	0	0	0
Ion Torrent PGM	Ion 316 Chip v2	2–3 M	1	1	0	0
Ion Torrent PGM	Ion 318 Chip v2	4–5.5 M	3	1	1	0
Ion S5	Ion 520 Chip	3–5 M	2	1	0	0
Ion S5	Ion 530 Chip	15–20 M	10	4	2	1
Ion S5	Ion 540 Chip	60–80 M	40	16	8	4

Note: Dark gray wells indicate this is either limited by index number (>96) or by sequencing platform read budget (=0).

Sample indexing for QIAseq Targeted RNA libraries on Illumina and Ion Torrent platforms

For the Illumina platform, the indexes in RS-D501 to RS-D508 (for RS-D# primer) are adapted from the TruSeq HT index D501–D508. The indexes in FS-D701 to FS-D712 (for FS-D# primer) are adapted from the TruSeq HT index D701–D712.

For QIAseq Targeted RNA 96-index HT for Illumina (array), the Universal Index PCR reaction (uPCR) primer pairs (one RS-D# and one FS-D#) are printed in each well.

Unique dual indexes are the same as DAP or RAP Dual-Indexed Layout (Table 4).

Table 4. DAP or RAP Dual-Indexed Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	D701-D501	D702-D501	D703-D501	D704-D501	D705-D501	D706-D501	D707-D501	D708-D501	D709-D501	D710-D501	D711-D501	D712-D501
B	D701-D502	D702-D502	D703-D502	D704-D502	D705-D502	D706-D502	D707-D502	D708-D502	D709-D502	D710-D502	D711-D502	D712-D502
C	D701-D503	D702-D503	D703-D503	D704-D503	D705-D503	D706-D503	D707-D503	D708-D503	D709-D503	D710-D503	D711-D503	D712-D503
D	D701-D504	D702-D504	D703-D504	D704-D504	D705-D504	D706-D504	D707-D504	D708-D504	D709-D504	D710-D504	D711-D504	D712-D504
E	D701-D505	D702-D505	D703-D505	D704-D505	D705-D505	D706-D505	D707-D505	D708-D505	D709-D505	D710-D505	D711-D505	D712-D505
F	D701-D506	D702-D506	D703-D506	D704-D506	D705-D506	D706-D506	D707-D506	D708-D506	D709-D506	D710-D506	D711-D506	D712-D506
G	D701-D507	D702-D507	D703-D507	D704-D507	D705-D507	D706-D507	D707-D507	D708-D507	D709-D507	D710-D507	D711-D507	D712-D507
H	D701-D508	D702-D508	D703-D508	D704-D508	D705-D508	D706-D508	D707-D508	D708-D508	D709-D508	D710-D508	D711-D508	D712-D508

Please refer to Illumina **truseq_sample_pre_pooling_guide_15042173** for sample pooling guidelines (Dual-Indexed Sequencing).

For Ion Torrent platform, the index ID1–ID96 for RS-ID# primer is adapted from IonXpress_1–IonXpress_96.

For QIAseq Targeted RNA 96-index HT L For Ion Torrent (array), the Universal Index PCR reaction (uPCR) primer pairs (1–96: FS-trP1 and one of RS-ID1 toRS_ID96) are printed in each well.

Table 5. QIAseq Targeted RNA 96-index HT L for Ion Torrent (array)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

Custom Sequencing Primer for Illumina platforms

For QIAseq Targeted RNA library construction, a common Uni1 primer (RS2) is designed for amplifying all molecules in both 2nd stage PCR reaction and final universal PCR steps. If an Illumina Read1 Sequence Primer is used, all reads will start with RS2. This may result in low diversity and sequencing quality issues. Accordingly, the QIAseq Read1 Primer I should be used as Custom Sequencing Read1 Primer on all Illumina platforms.

Refer to Appendix E, page 38, for instructions for MiSeq and NextSeq instruments.

For further information, please refer to the Illumina website for **miseq_using_custom_primers_15041638_b** or **nextseq_using_custom_primers_15057456** or **hiseq-custom-primers-reference-guide-15061846-b** for MiSeq, NextSeq, or HiSeq run setup.

Protocol: First Strand Synthesis

Important points before starting

- **Important**: Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- The QIAseq Targeted RNA First Strand Synthesis Components are not compatible with the chemicals in DNA-free kits from Ambion. If your RNA sample has been treated with DNA-free reagents, contact QIAGEN Technical Service.
- **Important**: Do not ignore the genomic DNA elimination step.

Procedure

1. Thaw QIAseq Targeted RNA First Strand Synthesis Components reagents. Briefly centrifuge (10–15 s) to bring the contents to the bottoms of the tubes.

2. Prepare the genomic DNA elimination mix for each RNA sample according to Table 6. Mix gently by pipetting up and down and then centrifuge briefly.

Table 6. Genomic DNA elimination mix

Component	Per sample (µl)
RNA	x (400 ng*)
Buffer GE	2
RNase-free water	8–x
Total volume	**10**

* Default amount for standard protocol is 400 ng. RNA amount can range from 25 ng – 5 µg. Modifications are recommended for low-input or FFPE samples (Appendix A, page 31).

3. Incubate the genomic DNA elimination mix for 5 min at 42°C, then immediately place on ice for at least 1 min.

4. Prepare the reverse-transcription mix according to Table 7.

Table 7. Reverse-transcription mix

Component	Per sample (µl)	Per *n* samples (µl)
5x Buffer BC3	4	4.4 x *n*
Control P2	1	1.1 x *n*
RE3 Reverse Transcriptase Mix	2	2.2 x *n*
RNase-free water	3	3.3 x *n*
Total volume	**10**	**11 x n**

5. Add 10 µl reverse-transcription mix to each tube containing 10 µl genomic DNA elimination mix. Mix gently by pipetting up and down.

6. Incubate at 42°C for exactly 15 min, then immediately stop the reaction by incubating at 95°C for 5 min.

7. Place the reactions on ice and proceed to the next protocol.

Note: If you wish to store the reactions prior starting the next protocol, transfer them to a –15 to –30°C freezer.

Protocol: Molecular Barcode Assignment

1. Prepare BC assignment reaction mix for each sample according to Table 8 in PCR strips or a 96-well PCR plate. Mix gently by pipetting up and down.

Table 8. Prepare BC assignment reaction mix for each sample

Component	Per sample (µl)
QIAseq RNA 5x Buffer	2
BC Primer Mix (100 nM each)	2
HotStarTaq DNA Polymerase (6 U/µl)	0.4
DNase-free water	4.6
cDNA	1*
Total volume	**10**

* Default amount for standard protocol is 1 µl. Modifications are recommended for low-input or FFPE samples (Appendix A, page 31).

2. Seal the wells with caps or film. Place the strips or plate in the thermal cycler and set up reaction parameters according to Table 9.

Table 9. Thermal cycler program

Number of cycles	Temperature (°C)	Time
1	95	15 min
1	55	15 min
1	65	15 min
1	72	7 min
1	4	∞

3. After the program is complete, place the reactions on ice and proceed to the next protocol.

Note: If the samples are to be stored prior to the next protocol, transfer them to a –15 to –30°C freezer.

Protocol: Cleanup of BC Assignment Reaction with 2 Rounds of QIAseq Beads Purification

Default amounts for standard protocol are shown below. Modifications are recommended for low-input or FFPE samples (Appendix A, page 31).

Bring QIAseq Beads to RT for 30 min and mix well before use.

1. Add 40 µl water to the 10 µl reaction mix, bringing the total volume to 50 µl, and transfer into a 1.5 ml DNA LoBind tube or 96-well PCR plate for purification.
2. Add 65 µl (1.3x volume) QIAseq Beads to the 50 µl reaction mix. Mix well by pipetting up and down at least 10 times.
3. Incubate for 5 min at room temperature.
4. Place the tube or 96-well PCR plate on a magnetic rack to separate beads from the supernatant. After the solution is clear (~2 min), carefully remove and discard the supernatant. Be careful not to disturb the beads, because they contain the DNA target.
5. Quick-spin the tube or tap the plate to settle any residual liquid to the bottom, and then completely remove residual supernatant.
6. Elute the DNA target beads into 26 µl sterile water. Mix well by pipetting. Place the tube or plate on the magnetic rack until the solution is clear.
7. Transfer 25 µl supernatant to a clean DNA LoBind tube or a new 96-well PCR plate and proceed to the second round of purification.
8. Add 32.5 µl (1.3x volume) QIAseq Beads to 25 µl supernatant. Mix well by pipetting up and down at least 10 times.

9. Incubate for 5 min at room temperature.

10. Place the tube or PCR plate on a magnetic rack to separate beads from the supernatant. After the solution is clear (~2 min), carefully remove and discard the supernatant. Be careful not to disturb the beads, because they contain the DNA target.

11. Quick-spin the tube or tap the plate to settle any residual liquid to the bottom then completely remove residual supernatant.

12. Add 200 µl freshly made 80% ethanol to the tube or wells while on the magnetic rack. Rotate the tube or move the plate side-to-side between the 2 positions of the magnet to wash the beads, and then carefully remove and discard the supernatant.

13. Repeat step 12 once more.

14. Briefly spin the tube or plate, or gently (avoid splashing) tap the plate against the table to settle any residual liquid to the bottoms of the wells (visually confirm this). Place on the magnetic rack, completely remove residual liquid, and dry beads for 5–10 min while the tube or plate is on the rack with the lid open.

15. Elute DNA target beads into 12 µl sterile water. Mix well by pipetting. Place the tube or plate on the magnetic rack until the solution is clear.

16. Transfer 10 µl supernatant to a clean PCR strip or 96-well PCR plate.

    Note: If the samples are to be stored prior to the next protocol, transfer them to a –15 to –30°C freezer.

Protocol: 1st Stage PCR Setup

1. Prepare 1st stage PCR mix for each sample according to Table 10 in a PCR strip or 96-well PCR plate. Mix gently by pipetting up and down.

Table 10. Prepare 1st stage PCR mix for each sample

Component	Per sample (µl)
QIAseq RNA 5x Buffer	5
LA Primer Mix (100 nM each)	5
RS2 primer (10 µM)	1.5
Purified product from previous protocol	10
HotStarTaq DNA Polymerase (6 U/µl)	1
DNase-free water	2.5
Total volume	**25**

2. Seal the wells with caps or film. Place the strip or plate in a thermal cycler, and set up reaction parameters according to Table 11.

Table 11. Thermal cycler program

Number of cycles	Temperature (°C)	Time
1	95	15 min
8	95 60	15 sec 5 min
1	4	∞

3. After the program is complete, place the reactions on ice and proceed with sample purification using QIAseq Beads.

Note: If the samples are to be stored prior to the next protocol, transfer them to a –15 to –30°C freezer.

Protocol: Cleanup of 1st Stage PCR with One Round of QIAseq Beads Purification

Bring QIAseq Beads to RT for 30 min, and mix well before use.

1. Transfer 25 µl PCR reaction to a 1.5 ml LoBind tube, or leave it in the 96-well PCR plate for purification.

2. Add 40 µl (1.6x volume) QIAseq Beads to 25 µl PCR reaction. Mix well by pipetting up and down at least 10 times.

3. Incubate for 5 min at room temperature.

4. Place the tube or 96-well PCR plate on a magnetic rack to separate beads from the supernatant. After the solution is clear (~2 min), carefully remove and discard the supernatant. Be careful not to disturb the beads, because they contain the DNA target.

5. Quick-spin the tube or tap the plate to settle any residual liquid to the bottom of the plate, and then completely remove residual supernatant.

6. Add 200 µl freshly made 80% ethanol to the tube or plate while on the magnetic rack. Rotate the tube or move the plate side-to-side between the 2 positions of the magnet to wash the beads, and then carefully remove and discard the supernatant.

7. Repeat step 6 once more.

8. Briefly spin the tube or plate, or gently (avoid splashing) tap the plate against the table to settle any residual liquid to the bottoms of the wells (visually confirm this). Place on the magnetic rack, completely remove residual liquid and dry beads for 5–10 min while the tube or plate is on the rack with the lid open.

9. Elute DNA target beads into 27 µl sterile water. Mix well by pipetting.

10. Place tube or plate on the rack until solution is clear.

11. Transfer 25 µl supernatant to a clear PCR strip or 96-well plate.

    Note: If the samples are to be stored prior to the next protocol, transfer them to a –15 to –30°C freezer.

Protocol: 2nd Stage Universal Index PCR

1. Prepare universal sample index PCR (uPCR) mix for each sample according to Table 12 (Illumina platforms) or Table 13 (Ion Torrent platforms) in a PCR strip or plate. Mix gently by pipetting up and down.

Table 12. Prepare universal index PCR for Illumina platforms

Component	Per sample (µl)	HT array (µl)
QIAseq RNA 5x Buffer	10	10
RS-D# (4 µM)*	2.5	–
FS-D# (4 µM)*	2.5	–
Purified product from previous protocol	25	25
HotStarTaq DNA Polymerase (6 U/µl)	2	2
DNase-free water	8	13
Total volume	**50**	**50**

* When QIAseq Targeted RNA 96-index HT Array is used, add DNase-free water instead of index primers.

Table 13. Prepare universal index PCR for Ion Torrent platforms

Component	Per sample (µl)	HT array (µl)
QIAseq RNA 5x Buffer	10	10
FS-trP1 (4 µM)*	2.5	–
RS-ID# (4 µM)*	2.5	–
Purified product from previous protocol	25	25
HotStarTaq DNA Polymerase (6 U/µl)	2	2
DNase-free water	8	13
Total volume	**50**	**50**

* When QIAseq Targeted RNA 96-index HT Array is used, add DNase-free water instead of index primers.

2. Seal the wells with caps or film. Place the strip or plate in a thermal cycler and set up reaction parameters according to Table 14.

Table 14. Thermal cycler program

Number of cycles	Temperature (°C)	Time
1	95	15 min
n (refer to Table 15)	95 60	15 sec 2 min
1	4	∞

Table 15. Universal index PCR cycle number for x-plex level

X-plex level*	*n* cycles
x ≤ 50	24
50 < x ≤ 100	22
100 < x ≤ 400	20
400 < x ≤ 1000	18

* Refer to panel-specific spec sheets or **qiagen.com** to determine x-plex levels.

3. After the program is complete, place the reactions on ice and proceed with sample purification using QIAseq Beads.

   Note: If the samples are to be stored prior to the next protocol, transfer them to a –15 to –30°C freezer.

Protocol: Cleanup of Universal PCR (uPCR) with One Round of QIAseq Beads Purification

Bring QIAseq Beads to RT for 30 min, and mix well before use.

1. Transfer 50 µl PCR reaction to a 1.5 ml LoBind tube, or leave it in the 96-well PCR plate for purification.

2. Add 55 µl (1.1x volume) QIAseq Beads to 50 µl reaction. Mix well by pipetting up and down at least 10 times.

3. Incubate for 5 min at room temperature.

4. Place the tube or plate on a magnetic rack to separate beads from the supernatant. After the solution is clear (~2 min), carefully remove and discard the supernatant. Be careful not to disturb the beads, as they contain the DNA target.

5. Quick-spin the tube or tap the plate to settle any residual liquid to the bottom of the plate then completely remove residual supernatant.

6. Add 200 µl freshly made 80% ethanol to the tube or plate while on the magnetic rack. Rotate the tube or move the plate side-to-side between the two positions of the magnet to wash the beads, then carefully remove and discard the supernatant.

7. Repeat step 6 once more.

8. Briefly spin the tube or plate, or gently (avoid splashing) tap the plate against the table to settle any residual liquid to the bottoms of the wells (visually confirm this). Place on the magnetic rack, completely remove residual liquid and dry beads for 5–10 min while the tube or plate is on the rack with the lid open.

9. Elute DNA target beads into 27 µl sterile water. Mix well by pipetting.

10. Place tube or plate on the rack until the solution is clear.

11. Transfer 25 µl supernatant to a clean LoBind tube or plate and proceed to QIAseq Library Quant or Bioanalyzer for measurement.

    Note: If the samples are to be stored prior to the next protocol, transfer them to a –15 to –30°C freezer.

Appendix A: Considerations for Different RNA Input Amounts

- RNA amounts can range from 25 ng to 5 µg for the First Strand Synthesis step.
- If RNA input is more than 400 ng, dilute cDNA and use the amount equivalent to 20 ng RNA for BC assignment reaction.
- If RNA input is 100–400 ng, use the standard protocol (see Table 16 for examples).
- If low RNA input or FFPE samples (variable quality/quantity) are used, refer to Table 16 for volume modification in first strand cDNA synthesis, BC assignment reaction, and 1st round beads purification steps.

Table 16. Recommendations for starting with different RNA inputs

	Standard protocol		
RNA (ng)	400	200	100
RT reaction (µl)	20	20	20
Genomic DNA elimination mix setup	**Volume (µl)**		
RNA	x	x	x
Buffer GE	2	2	2
RNase-free water	8–x	8–x	8–x
Total volume	**10**	**10**	**10**
Reverse-transcription mix setup	**Volume (µl)**		
5x Buffer BC3	4	4	4
Control P2	1	1	1
RE3 Reverse Transcriptase Mix	2	2	2
RNase-free water	3	3	3
Total volume	**10**	**10**	**10**

Barcode assignment reaction setup	**Volume (µl)**		
QIAseq RNA 5x Buffer	2	2	2
BC Primer Mix (100 nM each)	2	2	2
HotStarTaq DNA Polymerase (6 U/µl)	0.4	0.4	0.4
DNase-free water	4.6	3.6	1.6
cDNA	1	2	4
Total volume	**10**	**10**	**10**

1st round beads purification	**Volume (µl)**
BC reaction volume	10
Water	40
QIAseq Beads	65

	Low-input		**FFPE**
RNA (ng)	50	25	400
RT reaction (µl)	20	10	20

Genomic DNA elimination mix setup	**Volume (µl)**		
RNA	x	x	x
Buffer GE	2	1	2
RNase-free water	8–x	4–x	8–x
Total volume	**10**	**5**	**10**

Reverse-transcription mix setup	**Volume (µl)**		
5x Buffer BC3	4	2	4
Control P2	1	0.5	1
RE3 Reverse Transcriptase Mix	2	1	2
RNase-free water	3	1.5	3
Total volume	**10**	**5**	**10**

Barcode assignment reaction setup	**Volume (µl)**		
QIAseq RNA 5x Buffer	4	4	4
BC Primer Mix (100 nM each)	4	4	4
HotStarTaq DNA Polymerase (6 U/µl)	0.8	0.8	0.8
DNase-free water	3.2	3.2	3.2
cDNA	8	8	8
Total volume	**20**	**20**	**20**

1st round beads purification	**Volume (µl)**
BC reaction volume	20
Water	80
QIAseq Beads	130

Appendix B: RNA Quality

The integrity and size distribution of total RNA can be checked by the Agilent 2100 Bioanalyzer. For best results, the ribosomal bands should appear as sharp peaks (Figure 4).

RNA from FFPE will usually show varying degrees of degradation (Figure 5). For better results, at least 40% of the fragments should be greater than 200 nt in length.

For individual or global smear analysis

The percentage of fragments within a given range for a specific sample or all samples in a run can be obtained by performing a local or global smear analysis on Bioanalyzer electropherograms. Fragment range typically spans 150–200 nt to 10,000 nt.

Select **local** for a select sample or **global** for smear analysis of all samples. You will need to switch from **Normal analysis** to **Advanced analysis** and check the box for **Perform Smear analysis**. Double-clicking on regions will bring up the prompt to select nucleotide ranges. Input your desired range; if an error occurs with the upper range, input the Bioanalyzer recommended upper range and select **OK**. Select the region tabs for data.

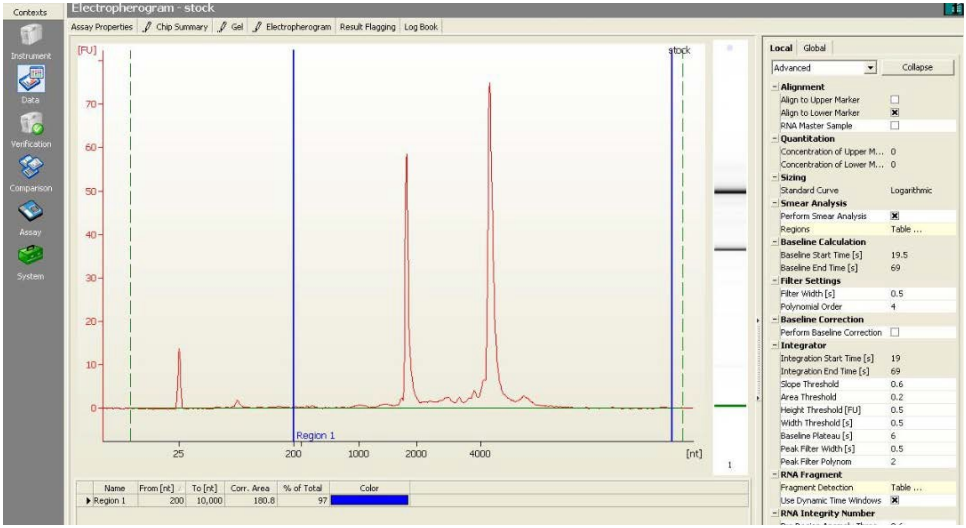


Figure 4. Local smear analysis: sample showing MCF7 RNA. A clear separation of 18S and 28S RNA from MCF7 cells observed with no smear of degraded RNA. Ninety-seven percent of fragments are >200 nt.

Figure 5. Local smear analysis: sample showing melanoma FFPE RNA. RNA from FFPE samples will be degraded; percentages will be returned based on the severity of degradation. In this sample, 30% of fragments are >200 nt.

Appendix C: Library Quantification and Quality Control

Library concentration can be determined by the QIAseq Library Quant Assay/Array Kit (cat. no. 333304 or 333314) or the Agilent 2100 Bioanalyzer. Library size can be checked by the Agilent 2100 Bioanalyzer. Please refer to the corresponding user manual for library quantification and quality control.

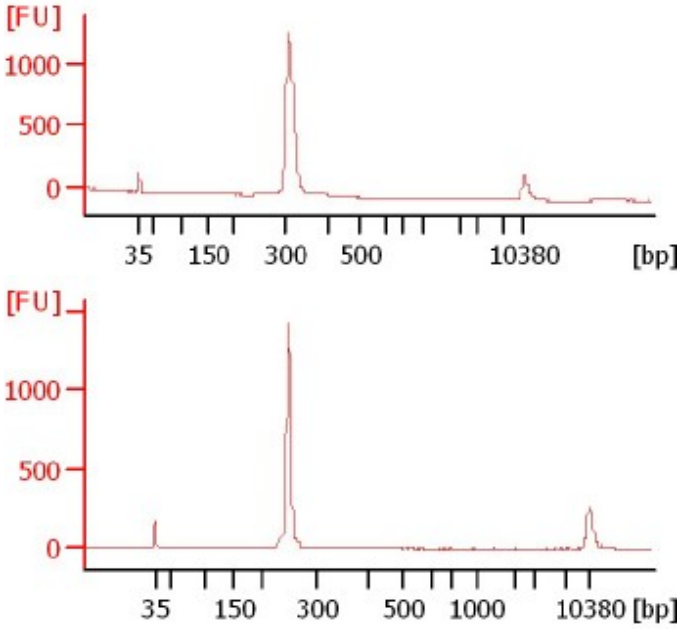


Figure 6. Bioanalyzer image of a QIAseq Targeted RNA Library prepared for sequencing on Illumina (above) or Ion Torrent (below) platforms. Peaks of approximately 300 bp or 250 bp are observed, respectively.

Appendix D: Combine Libraries for Multiplex Sequencing

Libraries can be combined into one sequencing run as long as each library uses a different sample barcode.

QIAseq Targeted RNA Libraries on Ion Torrent platforms

After the library is constructed, refer to Appendix C, page 36, to determine the library concentration, and then dilute each individual library to 4–8 pM. Combine libraries in equimolar amounts and mix well. At least 25 µl of the mixture is required. Proceed to template preparation using the mixture.

QIAseq Targeted RNA Libraries on Illumina platforms

After the library is constructed, refer to Appendix C to determine the library concentration. Dilute individual libraries to 4 nM (or 2 nM or 500 pM), and then combine libraries in equimolar amounts, and mix well. Proceed to denature libraries using fresh NaOH and generate clusters using this mixture. Please refer to the corresponding machine manual for details.

Please refer to Illumina **truseq_sample_pre_pooling_guide_15042173-d** for sample pooling guidelines (Dual-Indexed Sequencing).

Appendix E: Use Custom Sequencing Primer for Illumina Library on MiSeq

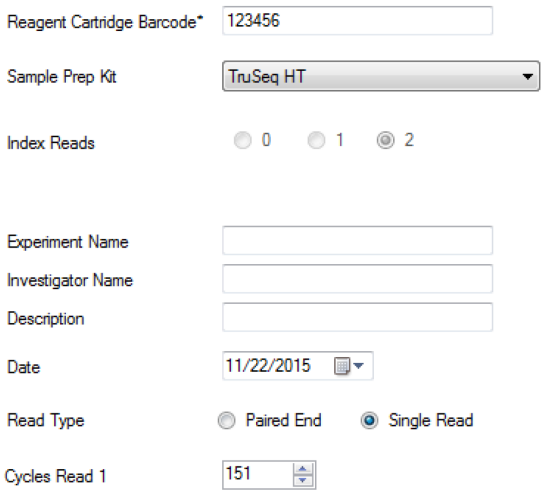
QIAseq Read1 Primer I must be used as Custom Sequencing Read1 Primer on the Illumina MiSeq. To do so, specify the use of custom primers in the sample sheet then load the prepared custom primers into the empty reservoirs on the MiSeq reagent cartridge.

Sample sheet setup

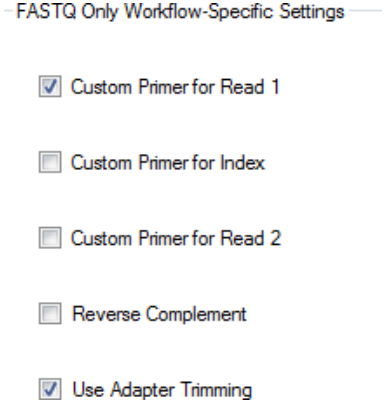
You can set up your sample sheet for custom primers using Illumina Experiment Manager v1.2 (or later versions).

Select the following options:

- Sample Prep Kit: **TruSeq HT**
- Index Reads: **2**
- Read Type: **Single Read**
- Cycles Read 1: **151**

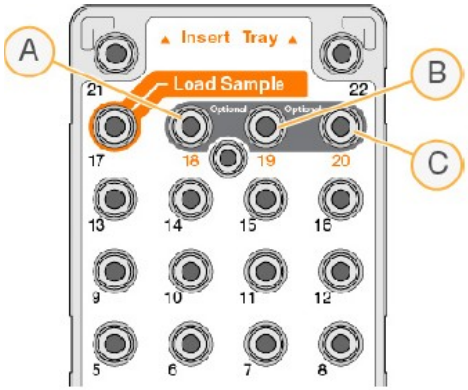


Make sure to select the check boxes for **Custom Primer for Read1** and **Use Adapter Trimming**.



Preparing and loading custom primers

Use 597 µl HT1 (hybridization buffer) to dilute 3 µl QIAseq Read1 Primer I to 0.5 µM. Load 600 µl diluted QIAseq Read1 Primer I to Position 18.



A Position 18 for Read 1 Custom Primer

For more details, refer to **Illumina miseq_using_custom_primers_15041638_b** for MiSeq.

Appendix F: Use Custom Sequencing Primer for Illumina Library on NextSeq

QIAseq Read1 Primer I must be used as **Custom Sequencing Read1 Primer** on the Illumina NextSeq.

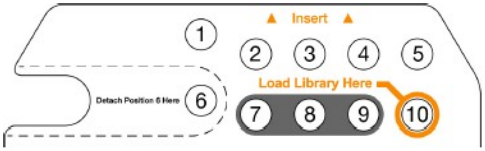
Please refer to **nextseq_using_custom_primers_15057456** for NextSeq run.

Single read (150 cycle, v2 Reagent) and dual indexes (eight cycles of each) are the default selections.

Using custom sequencing primers for a run on the Illumina NextSeq system requires 2 additional steps during the run setup:

1. Specify that custom primers are part of the run. For runs connected to BaseSpace, use of custom primers is specified on the **Planned Runs** screen of the **Prep** tab. For runs using standalone configuration, use of custom primers is specified on the **NCS Run Setup** screen.
2. Prepare and add 2 ml of each custom primer to the custom primer positions on the NextSeq reagent cartridge.

Use 1994 µl HT1 (hybridization buffer) to dilute 6 µl QIAseq Read1 Primer I to 0.3 µM. Load 2 ml diluted QIAseq Read1 Primer I to Position 7.



Position #	Custom Primer
7	Custom Read 1 primer

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

Appendix G: Data Analysis Using QIAGEN's QIAseq Targeted RNA Analysis Software

After sequencing, results can be analyzed using QIAGEN's cloud-based QIAseq Targeted RNA Analysis Portals. The QIAseq Targeted RNA Primary Data Analysis Tool will perform mapping of reads to the reference genome (GRCh38), read trimming and molecular barcode counting. The processed primary data can then be input to the QIAseq RNA Secondary Analysis Tool where other normalization methods can be applied and differential gene expression can be evaluated.

Troubleshooting Guide

For more information, see the Frequently Asked Questions page 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**).

References

1. Peng, Q., Satya, R.V., L., Marcus, L., Randad, P., and Wang, Y. (2015) Reducing amplification artifacts in high multiplex amplicon sequencing by using molecular barcodes. BMC Genomics **16**, 589.

Ordering Information

Product	Contents	Cat. no.
QIAseq Targeted RNA Panel (12)	Kit containing reagents for first strand synthesis, molecular barcoding, gene-specific amplification and QIAseq Beads for targeted RNA sequencing; fixed panel for 12 samples	333002
QIAseq Targeted RNA Panel (96)	Kit containing reagents for first strand synthesis, molecular barcoding, gene-specific amplification and QIAseq Beads for targeted RNA sequencing; fixed panel for 96 samples	333005
QIAseq Targeted RNA Extended Panel (12)	Kit containing reagents for first strand synthesis, molecular barcoding, gene-specific amplification and QIAseq Beads for targeted RNA sequencing; extended panel for 12 samples	333012
QIAseq Targeted RNA Extended Panel (96)	Kit containing reagents for first strand synthesis, molecular barcoding, gene-specific amplification and QIAseq Beads for targeted RNA sequencing; extended panel for 96 samples	333015
QIAseq Targeted RNA Custom Panel (12)	Kit containing reagents for first strand synthesis, molecular barcoding, gene-specific amplification and QIAseq Beads for targeted RNA sequencing; custom panel for 12 samples	333022
QIAseq Targeted RNA Custom Panel (96)	Kit containing reagents for first strand synthesis, molecular barcoding, gene-specific amplification and QIAseq Beads for targeted RNA sequencing; custom panel for 96 samples	333025

Product	Contents	Cat. no.
QIAseq Targeted RNA Custom Panel (384)	Kit containing reagents for first strand synthesis, molecular barcoding, gene-specific amplification and QIAseq Beads for targeted RNA sequencing; custom panel for 384 samples	333027
QIAseq Targeted RNA 12-index I (48)	Box containing oligos – enough for a total of 48 samples (for indexing up to 12 samples per run) and Custom Sequencing Primer for targeted RNA sequencing on Illumina platforms	333114
QIAseq Targeted RNA 96-index I (384)	Box containing oligos – enough for a total of 384 samples (for indexing up to 96 samples per run) and Custom Sequencing Primer for targeted RNA sequencing on Illumina platforms	333117
QIAseq Targeted RNA 96-index HT I (384)	Box containing oligos in arrays – enough for a total of 384 samples (for indexing up to 96 samples per run) and Custom Sequencing Primer for targeted RNA sequencing on Illumina platforms	333127
QIAseq Targeted RNA 12-index L (48)	Box containing oligos – enough for a total of 48 samples (for indexing up to 12 samples per run) for targeted RNA sequencing on Ion Torrent platforms	333214
QIAseq Targeted RNA 96-index HT L (384)	Box containing oligos – enough for a total of 384 samples (for indexing up to 96 samples per run) for targeted RNA sequencing on Ion Torrent platforms	333217

Product	Contents	Cat. no.
Related Products		
QIAseq Library Quant Array Kit	Kit containing arrays and master mix for DNA and RNA library quantification prior to NGS	333304
QIAseq Library Quant Assay Kit	Kit containing assays and master mix for DNA and RNA library quantification prior to NGS	333314
RNeasy® Plus Mini Kit (50)	Fifty RNeasy Mini Spin Columns, gDNA Eliminator Spin Columns, collection tubes, RNase-free water and buffers	74134
AllPrep® DNA/RNA Mini Kit (50)	Fifty minipreps: AllPrep DNA Spin Columns, RNeasy Mini Spin Columns, collection tubes, RNase-free water and buffers	80204
AllPrep DNA/RNA FFPE Kit (50)	Fifty RNeasy MiniElute Spin Columns, 50 QIAamp MiniElute Spin Columns, collection tubes, RNase-free reagents and buffers	80234
Human XpressRef Universal Total RNA	Two tubes each containing 100 µg human RNA at 1 mg/ml	338112
RNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-free buffers, RNase-free water	73504

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Document Revision History

Date	Changes
02/2020	QIAseq bead component size changed from 4 ml to 10 ml. Deleted reference to ioncommunity.lifetechnologies.com, because the link no longer works. Removed handbooks from “Kit Contents”. Shipping and storage information added.

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