June 2020

QIAseq® Multimodal Panel Handbook

Consolidated targeted next-generation sequencing of DNA and RNA

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq Multimodal Panel Catalog no. Number of samples	(12) 333932 12	HC (12) 333942 12	(96) 333935 96	HC (96) 333945 96	Custom (96) 333955 96
Multimodal DHS Panel (DNA)	120 µl	120 µl	960 µl	960 µl	960 µl
Multimodal VHS Panel (RNA)	96 µl	96 µl	768 µl	768 µl	768 µl
Fragmentation Buffer, 10x	40 µl	40 µl	192 µl	192 µl	192 µl
Fragmentation Enzyme Mix	90 µl	90 µl	384 µl	384 µl	384 µl
FERA Solution	15 µl	15 µl	60 µl	60 µl	60 µl
Side Reaction Reducer	48 µl	48 µl	192 µl	192 µl	192 µl
FG Solution	170 µl	170 µl	170 µl	170 µl	170 µl
Ligation Buffer, 5x	160 µl	160 µl	1250 µl	1250 µl	1250 µl
DNA Ligase	75 µl	75 µl	600 µl	600 µl	600 µl
Ligation Solution	125 µl	125 µl	970 µl	970 µl	970 µl
DNA Ligation Adapter	34 µl	34 µl	270 µl	270 µl	270 µl
ATP Solution	36 µl	36 µl	290 µl	290 µl	290 µl
PAP Enzyme	12 µl	12 µl	96 µl	96 µl	96 µl
PAP Dilution Buffer, 10x	24 µl	24 µl	192 µl	192 µl	192 µl
T4 Polynucleotide Kinase	12 µl	12 µl	125 µl	125 µl	125 µl
EZ Reverse Transcriptase	15 µl	15 µl	150 µl	150 µl	150 µl
RNase Inhibitor	12 µl	12 µl	96 µl	96 µl	96 µl
Multimodal RT Primer	12 µl	12 µl	96 µl	96 µl	96 µl
Multimodal RT Buffer, 5x	60 µl	60 µl	480 µl	480 µl	480 µl
Multimodal RT Enhancer	12 µl	12 µl	48 µl	48 µl	48 µl
TEPCR Buffer, 5x	60 µl x 2	60 µl x 2	500 µl x 2	500 µl x 2	500 µl x 2
HotStarTaq® DNA Polymerase (6 U/µl)	60 µl	60 µl	480 µl	480 µl	480 µl
Nuclease-free Water	1 tube	1 tube	10 ml	10 ml	10 ml
QIAseq Beads	10 ml	10 ml	38.4 ml x 2	38.4 ml x 2	38.4 ml x 2
QIAseq Bead Binding Buffer	10.2 ml	10.2 ml	10.2 ml x 2	10.2 ml x 2	10.2 ml x 2

Indexing for combined targeted DNA+RNA enrichment in a single tube

Note: Follow "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", page 43.

QIAseq Multimodal Index I **(12 DNA + 12 RNA sample indexes for 12 samples on Illumina® platform)** **Catalog no.** **Number of samples**	**(12)** **333962** **12**
Multimodal N7 Plate (12): Each plate allows N7 indexing of 12 samples: 12 for DNA and 12 for RNA. Each well in the plate is single use. **In each indicated well of the cuttable plate, there are dried N7 index primers for both DNA and RNA, in the same well.** The plates can be cut in columns to enable indexing of the desired number of samples.	1
Multimodal S5 Plate (12): Each plate allows S5 indexing of 12 samples: 12 for DNA and 12 for RNA. Each well in the plate is single use. SQDIB001 to SQDIB012 are mixed with universal DNA primer for DNA library amplification. SQDIB0049 to SQDIB060 are mixed with universal RNA primer for RNA library amplification. The plates can be cut in columns to enable indexing of the desired number of samples.	1
UPCR Buffer, 5x	60 µl x 3
DNA qPCR AMP Set	12 µl
RNA qPCR AMP Set	12 µl
QIAseq A Read 1 Primer I (100 µM)	24 µl
Multimodal Read 2 Primer (100 µM)	24 µl
Optical Thin-wall 8-cap Strips	24 strips

QIAseq Multimodal Index I Set A (Two identical sets of 48 DNA + 48 RNA sample indexes for Illumina platforms)	**(96)**
Catalog no.	**333965**
Number of samples	**96**
Multimodal N7 Plate Set A (48): Each plate allows N7 indexing of 48 samples: 48 for DNA and 48 for RNA. Each well in the plate is single use. **In each indicated well of the cuttable plate, there are dried N7 index primers for both DNA and RNA, in the same well.** The plates can be cut in columns to enable indexing of the desired number of samples.	2
Multimodal S5 Plate Set A (48): Each plate allows S5 indexing of 48 samples: 48 for DNA and 48 for RNA. Each well in the plate is single use. SQDIB001 to SQDIB048 are mixed with universal DNA primer for DNA library amplification. SQDIB0049 to SQDIB096 are mixed with universal RNA primer for RNA library amplification. The plates can be cut in columns to enable indexing of the desired number of samples.	2
UPCR Buffer, 5x	500 µl x 3
DNA qPCR AMP Set	96 µl
RNA qPCR AMP Set	96 µl
QIAseq A Read 1 Primer I (100 µM)	4 x 24 µl
Multimodal Read 2 Primer (100 µM)	4 x 24 µl
Optical Thin-wall 8-cap Strips	48 strips

QIAseq Multimodal Index I Set B **(Two identical sets of 48 DNA + 48 RNA sample indexes for Illumina platforms)** **Catalog no.** **Number of samples**	**(96)** **333975** **96**
Multimodal N7 Plate Set B (48): Each plate allows N7 indexing of 48 samples: 48 for DNA and 48 for RNA. Each well in the plate is single use. **In each indicated well of the cuttable plate, there are dried N7 index primers for both DNA and RNA, in the same well.** The plates can be cut in columns to enable indexing of the desired number of samples.	2
Multimodal S5 Plate Set B (48): Each plate allows S5 indexing of 48 samples: 48 for DNA and 48 for RNA. Each well in the plate is single use. SQDIB097 to SQDIB144 are mixed with universal DNA primer for DNA library amplification. SQDIB0145 to SQDIB192 are mixed with universal RNA primer for RNA library amplification. The plates can be cut in columns to enable indexing of the desired number of samples.	2
UPCR Buffer, 5x	500 µl x 3
DNA qPCR AMP Set	96 µl
RNA qPCR AMP Set	96 µl
QIAseq A Read 1 Primer I (100 µM)	4 x 24 µl
Multimodal Read 2 Primer (100 µM)	4 x 24 µl
Optical Thin-wall 8-cap Strips	48 strips

Indexing for separated targeted DNA and RNA enrichment in separate tubes

Note: Follow "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes", page 74.

QIAseq Multimodal Index I Set A SW **(Two identical sets of 48 DNA + 48 RNA sample indexes for Illumina platforms)** **Catalog no.** **Number of samples**	**(96)** **333985** **96**
Multimodal N7 Plate Set A SW (48): Each plate allows N7 indexing of 48 samples: 48 for DNA and 48 for RNA. Each well in the plate is single use. **In each indicated well of the cuttable plate, there are dried N7 index primers for DNA and RNA libraries, in separate wells.** The plates can be cut in columns to enable indexing of the desired number of samples.	2
Multimodal S5 Plate Set A (48): Each plate allows S5 indexing of 48 samples: 48 for DNA and 48 for RNA. Each well in the plate is single use. SQDIB001 to SQDIB048 are mixed with universal DNA primer for DNA library amplification. SQDIB0049 to SQDIB096 are mixed with universal RNA primer for RNA library amplification. The plates can be cut in columns to enable indexing of the desired number of samples.	2
UPCR Buffer, 5x	500 µl x 3
DNA qPCR AMP Set	96 µl
RNA qPCR AMP Set	96 µl
QIAseq A Read 1 Primer I (100 µM)	4 x 24 µl
Multimodal Read 2 Primer (100 µM)	4 x 24 µl
Optical Thin-wall 8-cap Strips	48 strips

QIAseq Multimodal Index I Set B SW **(Two identical sets of 48 DNA + 48 RNA sample indexes for Illumina platforms)** **Catalog no.** **Number of samples**	**(96)** **333995** **96**
Multimodal N7 Plate Set B SW (48): Each plate allows N7 indexing of 48 samples: 48 for DNA and 48 for RNA. Each well in the plate is single use. **In each indicated well of the cuttable plate, there are dried N7 index primers for DNA and RNA libraries, in separate wells.** The plates can be cut in columns to enable indexing of the desired number of samples.	2
Multimodal S5 Plate Set B (48): Each plate allows S5 indexing of 48 samples: 48 for DNA and 48 for RNA. Each well in the plate is single use. SQDIB097 to SQDIB144 are mixed with universal DNA primer for DNA library amplification. SQDIB0145 to SQDIB192 are mixed with universal RNA primer for RNA library amplification. The plates can be cut in columns to enable indexing of the desired number of samples.	2
UPCR Buffer, 5x	500 µl x 3
DNA qPCR AMP Set	96 µl
RNA qPCR AMP Set	96 µl
QIAseq A Read 1 Primer I (100 µM)	4 x 24 µl
Multimodal Read 2 Primer (100 µM)	4 x 24 µl
Optical Thin-wall 8-cap Strips	48 strips

Storage

QIAseq Multimodal Panels (except Ligation Solution, QIAseq Beads, and QIAseq Bead Binding Buffer) are shipped on dry ice and should be stored immediately upon receipt at –30 to –15°C in a constant-temperature freezer. The Ligation Solution, QIAseq Beads, and QIAseq Bead Binding Buffer are shipped on cold packs. Upon receipt, QIAseq Beads and Bead Binding Buffer should be stored at 2–8°C, while the Ligation Solution should be stored at –30 to –15°C in a constant-temperature freezer.

QIAseq Multimodal Index kits are shipped on dry ice and should be stored at –30 to –15°C upon arrival.

Intended Use

QIAseq Multimodal Panels and QIAseq Multimodal Index kits are intended 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 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 QIAseq Multimodal Panels is tested against predetermined specifications to ensure consistent product quality.

Introduction

Recent advancements in NGS have enabled the analysis of single nucleotide variants (SNVs), InDels, and copy number variants (CNVs) from DNA, and the analysis of fusions and gene expression levels from RNA. Existing solutions, however, only allow users to perform such DNA and RNA analyses using 2 separate workflows: one for DNA and one for RNA. Additionally, these solutions require separate inputs of DNA and RNA, making the sequencing of low-yielding samples very difficult.

To overcome the limitations of existing solutions, the QIAseq Multimodal Panels have been developed. QIAseq Multimodal Panels enable Sample to Insight®, simultaneous targeted next-generation sequencing (NGS) of DNA and RNA using total nucleic acids in a single-tube workflow. Resulting DNA and RNA libraries can be sequenced together for cost effectiveness. This highly optimized solution facilitates ultrasensitive DNA variant detection as well as fusions and gene expression detection from RNA using integrated unique molecular indices (UMIs) from cells, tissue, and biofluids. The starting material for QIAseq Multimodal can be total nucleic acid or separately isolated DNA and RNA.

The QIAseq Multimodal Panels use a targeted approach to sequencing by enriching specific genomic or transcriptomic regions. This enhances DNA and RNA NGS by enabling users to sequence specific regions of interest, which in turn effectively increases sequencing depth and sample throughput while minimizing cost. More importantly, QIAseq Multimodal Panels enable simultaneous enrichment for both DNA and RNA regions of interest using total nucleic acids as input, thereby saving precious biological material, maximizing library prep efficiency, and reducing handling errors. Using a robust chemistry with integrated UMIs, QIAseq Multimodal Panels enable sensitive detection of DNA and RNA analytes of interest. Furthermore, data analysis tools have been developed to perform all steps necessary to generate both a DNA sequence variant report as well as an RNA fusion and gene expression report from NGS data. Collectively, QIAseq Multimodal Panels are a Sample to Insight solution for consolidated targeted DNA and RNA analysis using NGS (Figure 1).

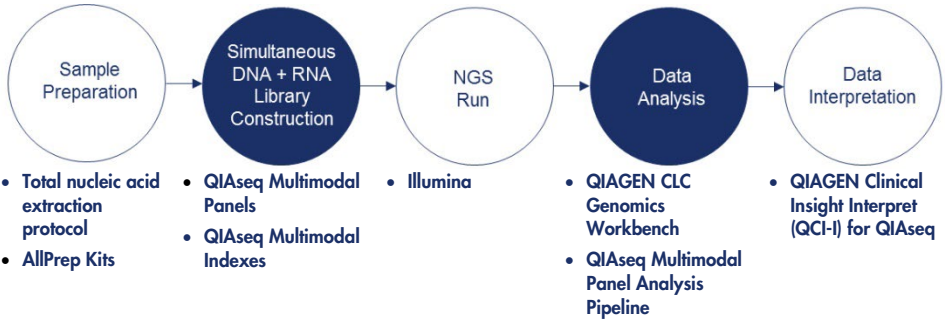


Figure 1. Overview of the Sample to Insight NGS workflow with QIAseq Multimodal Panels. The complete Sample to Insight procedure begins with total nucleic acid or AllPrep® (separate DNA and RNA) extractions. Next is library construction and target enrichment with QIAseq Multimodal Panels. Following NGS, data analysis is performed using the QIAseq Multimodal Panel Analysis Software pipeline in Genomics Workbench. Ultimately, detected variants can be interpreted with QIAGEN Clinical Insight Interpret (QCI™-I) for QIAseq.

Principle and procedure

QIAseq Multimodal Panels enable the simultaneous enrichment and library prep of DNA+RNA, with up to 28,000 primers per panel (20,000 DNA + 8,000 RNA). For DNA, the recommended input range is 10–40 ng for fresh samples or 40–250 ng for FFPE samples. For RNA, the recommended amount input range is 10–250 ng for fresh samples or ≤250 ng for FFPE samples (up to 500 ng for "severely" fragmented FFPE sample, with "severely" being defined as samples that have less than 40% of fragments >200 nt by smear analysis on the Bioanalyzer®). When working with total nucleic acid samples, input amounts should be based on DNA, because RNA is usually in vast excess to DNA. Lower input amounts are possible; however, this will lead to fewer sequenced UMIs and reduced variant detection sensitivity. The following reactions occur in a streamlined, single-tube workflow (Figure 2).

Nucleic acid fragmentation

RNA molecules are heat fragmented and DNA molecules are enzymatically fragmented, end repaired, and A-tailed within a single controlled multienzyme reaction.

RNA polyadenylation

Specific to RNA, synthetic polyadenylation is performed to create a binding site for subsequent reverse transcription.

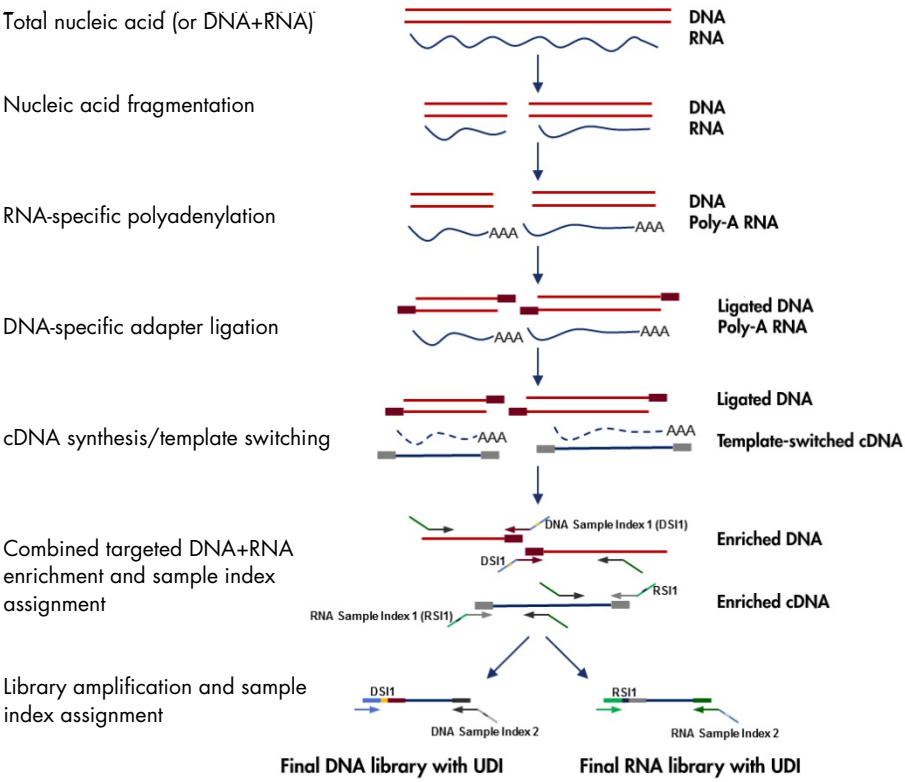


Figure 2. QIAseq Multimodal Panels workflow, using Combined Targeted DNA+RNA Enrichment. Alternatively, using Separated Targeted DNA and RNA Enrichment can be performed using "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes".

DNA ligation

Specific to DNA, UMI-containing adapters are ligated at the 3′ ends of the molecules. The UMI is a 12-base fully random sequence, which statistically provides 4^{12} possible sequences per adapter and ensures that each molecule receives a UMI sequence. In addition, this adapter contains a binding site for subsequent target enrichment.

RNA reverse transcription and template switching

Specific to RNA, reverse transcription and template switching are performed. For reverse transcription, the anchored oligo-dT primer contains a 10-base fully random UMI sequence, and the template switching oligonucleotide also contains a 10-base fully random UMI sequence. This allows each RNA molecule to be tagged with a unique UMI, regardless of which strand it was derived from. Lastly, the reverse transcription and template switching oligonucleotides each contain the same binding site for subsequent target enrichment.

Target enrichment

Two protocols are provided for target enrichment:

- "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", page 43.
- "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes", page 74.

IMPORTANT: **Table 1 outlines important points to consider when choosing between the 2 protocols.**

Table 1. Target enrichment options

Choose …	For …	Notes
"Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube"	**Maximal detection sensitivity of DNA and RNA variants**, because there is no splitting of samples	Points to note with the combined protocol are that RNA primers will amplify DNA, and DNA primers have the possibility of amplifying RNA. As a result, when considering sequencing read budgets, the panel size needs to be accounted for as "DNA+RNA" primers. For example, if you are working with a DNA panel of 10,000 primers and an RNA panel of 1,000 primers, the read budget ultimately needed for both the DNA and the RNA library must be based on 11,000 primers.
"Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes" **Note: Samples are split before targeted enrichment, so the input DNA recommendations would be doubled, compared to combined targeted enrichment workflow.**	**Maximal panel specificity** Flexibility for Custom Multimodal Primer Panel design Custom Multimodal Primer Panels with specific primer numbers **QIAseq Multimodal Panel UHS-5000Z**	With this protocol, when considering sequencing read budgets, the DNA panel and the RNA panel can be considered separately. For example, if you are working with a DNA panel of 10,000 primers and an RNA panel of 1,000 primers, the read budget ultimately needed for the DNA library must be based on 10,000 primers and the read budget ultimately needed for the RNA library must be based on 1,000 primers. Separated targeted DNA and RNA enrichment prevents the need to account for potential interactions (i.e. dimerization potential) between DNA and RNA primer pool When the number of DNA+RNA primers (assuming the number DNA primers is greater than the number of RNA primers) is ≥ 14,000 and at the same time the number of RNA primers is ≥ 2500, **"**Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes**"** workflow is highly recommended QIAseq Multimodal Pan Cancer Panel (UHS-5000Z) is designed exclusively for the "Separated Targeted DNA and RNA Enrichment in Separate Tubes" workflow

For both DNA and RNA, target enrichment is performed post-UMI assignment to ensure that molecules containing UMIs are sufficiently enriched in the sequenced library. For enrichment, ligated DNA molecules and reverse-transcribed/template-switched cDNA molecules are subject to several cycles of targeted PCR using a single primer extension (SPE) approach. This reaction includes highly optimized chemistry to amplify traditionally difficult regions using pools of DNA and RNA region-specific primers. Universal primers complementary to the DNA adapter-binding sequence and reverse-transcription/template-switching oligonucleotides ensure specificity for DNA and RNA molecules and assign the N7 index, which is one of the 2 sample unique dual indexes (UDIs). The N7 indexes are listed on **QIAGEN.com/QIAseqMultimodalPanels**.

Library amplification

A Universal PCR is ultimately carried out separately on DNA and RNA libraries to both optimally amplify each library as well as add the second UDI. Collectively, DNA and RNA libraries for a given sample have their own unique dual indexes. The S5 indexes are listed on **QIAGEN.com/QIAseqMultimodalPanels**.

Important: The sample recommendations for the UDIs should be maintained between the N7 and S5 indexes.Next-generation sequencing

QIAseq Multimodal Panels are compatible with Illumina NGS platforms including MiniSeq®, MiSeq®, NextSeq® 500/550, HiSeq® 2500, HiSeq 3000/4000, and NovaSeq™ 6000. The QIAseq Multimodal Panels cannot be used on Illumina's iSeq 100 platform. When using Illumina NGS systems, QIAseq Multimodal libraries require a custom sequencing primer for Read 1 (QIAseq A Read 1 Primer I), custom sequencing primer for Read 2 (Multimodal Read 2 Primer), and 149 bp paired-end reads.

Data analysis

Data from QIAseq Multimodal Panels can be analyzed using the QIAGEN CLC Genomics Workbench, which allows you to optimize analysis parameters for your specific panels. The parameters can then be locked for routine use. All detected variants can be further interpreted using QCI-I for QIAseq.

Alternatively, the QIAseq Multimodal Data Analysis pipeline is available at **ngsdataanalysis2.qiagen.com/MultiModal**. The pipeline automatically performs all steps necessary to generate a DNA sequence variant report, as well as an RNA fusion and differential expression analysis report from your raw NGS data. An explanation of the principles of UMI-directed variant detection and the features of the primary sequence analysis output can be found and downloaded from **doi.org/10.1093/bioinformatics/bty790**.* All detected variants can be further interpreted using QCI-I for QIAseq.

Principle of variant detection with UMIs

The principle of variant detection with UMIs is described in Figure 3. Due to intrinsic noise and sequence-dependent bias, indexed molecules may be amplified unevenly across the target regions. Target region coverage can be better achieved, however, by counting the number of UMIs rather than counting the number of total reads for each region. Sequence reads having different UMIs represent different original molecules, while sequence reads having the same UMIs are the result of PCR duplication from one original molecule. Errors from PCR amplification and from the sequencing process may also be present in final reads that lead to false positive variants in sequencing results. These artifactual variants can be

* Xu, C., Nezami Ranjbar, M.R., Wu, Z., DiCarlo, J., Wang, Y. (2017) Detecting very low allele fraction variants using targeted DNA sequencing and a novel molecular barcode-aware variant caller. BMC Genomics. **18**, 5.

greatly reduced by calling variants across all reads within a unique UMI instead of picking up variants at the original read level.

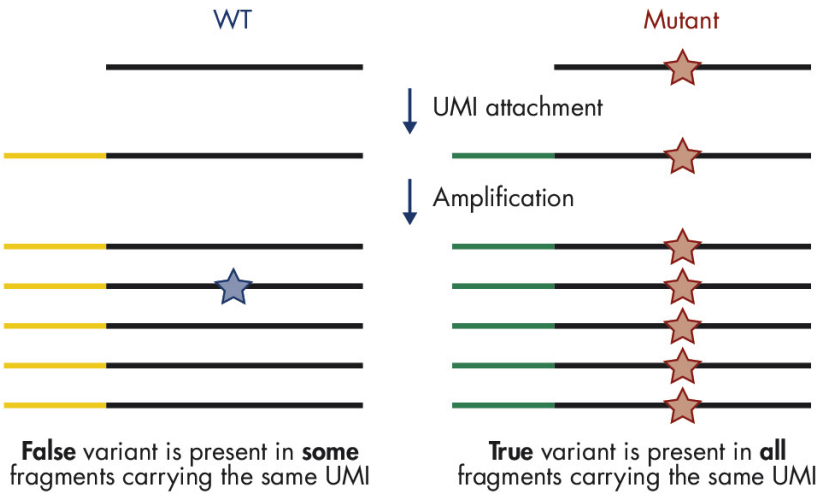


Figure 3. Principle of variant detection with UMIs. Each original molecule is tagged by a UMI. True variants are those mutations present in the majority of reads within a UMI, while false positives are mutations present in only one or a few reads within a UMI. Description of the variant calling algorithm can be found and downloaded from **doi.org/10.1186/s12864-016-3425-4**. *

* Xu, C., Nezami Ranjbar, M.R., Wu, Z., DiCarlo, J., Wang, Y. (2017) Detecting very low allele fraction variants using targeted DNA sequencing and a novel molecular barcode-aware variant caller. BMC Genomics. **18**, 5.

Important Notes

The most important prerequisite for sequence analysis is consistent, high-quality DNA and RNA from every experimental sample. Therefore, sample handling and isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts, or other contaminants may either degrade the nucleic acids or decrease the efficiency of – if not block completely – the enzymatic activity necessary for optimal targeted enrichment.

Total nucleic acid isolation

Supplementary protocols for the simultaneous isolation of total nucleic acid (DNA+RNA) from cells and tissue, blood, or FFPE samples are available at **www.qiagen.com/TotalNucleicAcid**.

Note: For quantification of DNA and RNA from total nucleic acid samples, we recommend the high-sensitivity Quant-iT™ dsDNA Assay Kit (Thermo Fisher Scientific, cat. no. Q33120) for DNA and the Quant-iT RNA Assay Kit (Thermo Fisher Scientific, cat. no. Q33140) for RNA. When working with total nucleic acid samples, input amounts should be based on DNA because RNA is usually in vast excess to DNA.

Simultaneous purification of DNA and RNA into separate eluates

The QIAGEN kits listed in Table 2 are recommended for the preparation of DNA and RNA samples from cells, tissues, and FFPE tissues. For whole blood, we recommend the PAXgene Blood DNA Kit (cat. no. 761133) and the PAXgene Blood RNA Kit (cat. no. 762174).

Note: If samples must be harvested from biological samples for which kits are not available, please contact QIAGEN Technical Services (**support.qiagen.com**) for suggestions.

Table 2. Recommended AllPrep kits for simultaneous purification of DNA and RNA into separate eluates

Kit	Starting material	Cat. no.
AllPrep DNA/RNA Mini Kit	Cells and tissue	80204
AllPrep DNA/RNA FFPE Kit	FFPE samples	80234

Specific recommendations for FFPE samples (total nucleic acid or DNA)

If FFPE samples are used for QIAseq Multimodal Panels, the QIAseq DNA QuantiMIZE kits (cat. no. 333404 or 333414) are strongly recommended for determining the quality of each FFPE sample. Appendix A provides detailed information for FFPE DNA quality assessment and input amount.

Nucleic acid input amount and sequencing depth

The number of UMIs captured from the original DNA sample correlates with the DNA input amount and sequencing read depth. Adequate sequencing of captured UMIs requires relatively deep sequencing coverage. Table 3 provides guidance on variant detection with fresh DNA amounts at different depths of coverage. Additionally, the number of UMIs sequenced directly impacts the variant detection sensitivity. Therefore, low-frequency mutation detection usually requires more DNA input and sequencing at deeper coverage (i.e., more reads/UMI) to generate a sufficient amount of UMIs.

Table 3. Suggested fresh DNA input amount and read depth coverage for variant detection*

Variant frequency	Input (ng)	Read pairs/UMI	Mean read depth (coverage)
5%	10†	4	7200x
5%	20†	2	3640x
1%	40†	4	25,600x

* Variant detection is based on 90% sensitivity on the entire region of the QIAseq Multimodal DHS (DNA) Panel.

† If performing Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes, double the input amounts listed.

As RNA expression levels for each transcript varies broadly between different samples, there is no definitively calculation for a required number of reads. Based on previous testing with RNA libraries, allocation of 5000 reads per primer is a reasonable starting point. Table 4a (Combined Targeted DNA+RNA Enrichment) and Table 4b (Separated Targeted

DNA and RNA Enrichment) provide recommendations for the number of reads that should be allocated for the prepared QIAseq Multimodal DNA and RNA libraries. As a note, the QIAseq Multimodal Pan Cancer Panel (UHS-5000Z) is designed exclusively for the Separated Targeted DNA and RNA Enrichment workflow.

Table 4a. Read allocation for cataloged QIAseq Multimodal DNA and RNA libraries (Combined Targeted DNA+RNA Enrichment)

		Primer Number	UHS-005Z	UHS-009Z	UHS-006Z
		DNA primer #	**4149**	**6244**	**11243**
		RNA primer #	**487**	**1116**	**665**
Per DNA library	**Input**	**Coverage (X)**	**Reads allocated to each DNA library (M)***		
5% VAF	10 ng	7200	33	53	86
5% VAF	20 ng	3640	17	27	43
1% VAF	40 ng	25600	119	188	305
			Reads allocated to each RNA library (M)*		
Per RNA library		5000	23	37	60

* Reads allocated to each library (M) = Coverage X Primer number/10^6

Table 4b. Read allocation for cataloged QIAseq Multimodal DNA and RNA libraries (Separated Targeted DNA and RNA Enrichment)

		Primer Number	UHS-005Z	UHS-009Z	UHS-006Z	UHS-5000Z*
		DNA primer #	**4149**	**6244**	**11243**	**19995**
		RNA primer #	**487**	**1116**	**665**	**2571**
Per DNA library	**Input**	**Coverage (X)**	**Reads allocated to each DNA library (M)†**			
5% VAF	20 ng‡	7200	30	45	81	144
5% VAF	40 ng‡	3640	15	23	41	73
1% VAF	80 ng‡	25600	106	160	288	512
			Reads allocated to each RNA library (M)*			
Per RNA library		5000	2	6	3	13

* QIAseq Multimodal Pan Cancer Panel (UHS-5000Z) is designed exclusively for the "Separated Targeted DNA and RNA Enrichment" workflow.

† Reads allocated to each library (M) = Coverage X Primer number/10^6

‡ Since samples are split before targeted enrichment, the recommend DNA input is doubled, compared to combined targeted enrichment workflow.

Sample multiplexing recommendations for Illumina sequencing platforms

Sample multiplexing level is determined by the size of the panel, required depth of coverage, and sequencing platform total output. For the Illumina platforms, sample indexes are available to multiplex up to 96 samples, one targeted DNA, and one targeted RNA library per sample, per run. General guidelines are provided for the number of samples that can be multiplexed in different sequencing platforms, based on panel size and read depth calculated for QIAseq Multimodal DNA and RNA libraries prepared using the Combined Targeted DNA+RNA Enrichment workflow (Table 5a through Table 5d) or the Separated Targeted DNA and RNA Enrichment workflow (Table 6a through Table 6d). Fine-tuning the read depth is possible after the first run. See read allocation and sample multiplexing template for the custom panel on **www.qiagen.com/PROM-16466**.

Table 5a. Number of multiplexed samples (one DNA and one RNA library for each sample) based on a DNA+RNA panel size (Combined targeted DNA+RNA Enrichment workflow) with 500x mean coverage for the DNA library and 5000 reads/primer for the RNA library*

			UHS-005Z	UHS-009Z	UHS-006Z
		DNA primer #	**4149**	**6244**	**11243**
		RNA primer #	**487**	**1116**	**665**
Instrument	**Version**	**Capacity (paired-ends reads)**			
MiniSeq	Mid output	16M	N/A	N/A	N/A
MiniSeq	High output	50M	1	1	N/A
MiSeq	v2	30M	1	N/A	N/A
NextSeq 500	Mid output	260M	10	6	3
NextSeq 500	High output	800M	31	19	12
HiSeq 2500 rapid run	Dual Flowcell v2	1200M	47	29	18
HiSeq 3000	8 lanes per flow cell	5B	196	123	76
HiSeq 4000	8 lanes per flow cell	10B	392	247	152
NovaSeq 6000	SP (per flow cell)	1.6B	62	39	24

N/A: not applicable, no samples can be run.

* Based on 2 x 149 bp paired-end reads.

Table 5b. Number of multiplexed samples (one DNA and one RNA library for each sample) based on a DNA+RNA panel size (Combined targeted DNA+RNA Enrichment workflow) with 3640x mean coverage for the DNA library and 5000 reads/primer for the RNA library*

			UHS-005Z	UHS-009Z	UHS-006Z
		DNA primer #	**4149**	**6244**	**11243**
		RNA primer #	**487**	**1116**	**665**
Instrument	**Version**	**Capacity (paired-ends reads)**			
MiniSeq	Mid output	16M	N/A	N/A	N/A
MiniSeq	High output	50M	1	N/A	N/A
MiSeq	v2	30M	N/A	N/A	N/A
NextSeq 500	Mid output	260M	6	4	2
NextSeq 500	High output	800M	19	12	7
HiSeq 2500 rapid run	Dual Flowcell v2	1200M	29	18	11
HiSeq 3000	8 lanes per flow cell	5B	124	78	48
HiSeq 4000	8 lanes per flow cell	10B	249	157	97
NovaSeq 6000	SP (per flow cell)	1.6B	39	25	15

N/A: not applicable, no samples can be run.

* Based on 2 x 149 bp paired-end reads.

Table 5c. Number of multiplexed samples (one DNA and one RNA library for each sample) based on a DNA+RNA panel size (Combined targeted DNA+RNA Enrichment workflow) with 7200x mean coverage for the DNA library and 5000 reads/primer for the RNA library*

			UHS-005Z	UHS-009Z	UHS-006Z
		DNA primer #	**4149**	**6244**	**11243**
		RNA primer #	**487**	**1116**	**665**
Instrument	**Version**	**Capacity (paired-ends reads)**			
MiniSeq	Mid output	16M	N/A	N/A	N/A
MiniSeq	High output	50M	N/A	N/A	N/A
MiSeq	v2	30M	N/A	N/A	N/A
NextSeq 500	Mid output	260M	4	2	1
NextSeq 500	High output	800M	14	8	5
HiSeq 2500 rapid run	Dual Flowcell v2	1200M	21	13	8
HiSeq 3000	8 lanes per flow cell	5B	88	55	34
HiSeq 4000	8 lanes per flow cell	10B	176	111	68
NovaSeq 6000	SP (per flow cell)	1.6B	28	17	11

N/A: not applicable, no samples can be run.

* Based on 2 x 149 bp paired-end reads.

Table 5d. Number of multiplexed samples (one DNA and one RNA library for each sample) based on a DNA+RNA panel size (Combined targeted DNA+RNA Enrichment workflow) with 25,600x mean coverage for the DNA library and 5000 reads/primer for the RNA library*

			UHS-005Z	UHS-009Z	UHS-006Z
		DNA primer #	4149	6244	11243
		RNA primer #	487	1116	665
Instrument	**Version**	**Capacity (paired-ends reads)**			
MiniSeq	Mid output	16M	N/A	N/A	N/A
MiniSeq	High output	50M	N/A	N/A	N/A
MiSeq	v2	30M	N/A	N/A	N/A
NextSeq 500	Mid output	260M	1	1	N/A
NextSeq 500	High output	800M	5	3	2
HiSeq 2500 rapid run	Dual Flowcell v2	1200M	8	5	3
HiSeq 3000	8 lanes per flow cell	5B	35	22	13
HiSeq 4000	8 lanes per flow cell	10B	70	44	27
NovaSeq 6000	SP (per flow cell)	1.6B	11	7	4

N/A: not applicable, no samples can be run.

* Based on 2 x 149 bp paired-end reads.

Table 6a. Number of multiplexed samples (one DNA and one RNA library for each sample) based on DNA or RNA panel size (Separated targeted DNA and RNA Enrichment workflow) with 500x mean coverage for DNA library and 5000 reads/primer for the RNA library*

			UHS-005Z	UHS-009Z	UHS-006Z	UHS-5000Z
		DNA primer #	4149	6244	11243	19995
		RNA primer #	487	1116	665	2571
Instrument	**Version**	**Capacity (paired-ends reads)**				
MiniSeq	Mid output	16M	3	1	1	N/A
MiniSeq	High output	50M	11	5	5	2
MiSeq	v2	30M	6	3	3	1
NextSeq 500	Mid output	260M	57	29	29	11
NextSeq 500	High output	800M	177	91	89	35
HiSeq 2500 rapid run	Dual Flowcell v2	1200M	266	137	134	52
HiSeq 3000	8 lanes per flow cell	5B	1108	574	558	218
HiSeq 4000	8 lanes per flow cell	10B	2217	1149	1117	437
NovaSeq 6000	SP (per flow cell)	1.6B	354	183	178	70

N/A: not applicable, no samples can be run.

* Based on 2 x 149 bp paired-end reads.

Table 6b. Number of multiplexed samples (one DNA and one RNA library for each sample) based on DNA or RNA panel size (Separated targeted DNA and RNA Enrichment workflow) with 3640x mean coverage for DNA library and 5000 reads/primer for the RNA library*

			UHS-005Z	UHS-009Z	UHS-006Z	UHS-5000Z
		DNA primer #	**4149**	**6244**	**11243**	**19995**
		RNA primer #	**487**	**1116**	**665**	**2571**
Instrument	**Version**	**Capacity(paired-ends reads)**				
MiniSeq	Mid output	16M	N/A	N/A	N/A	N/A
MiniSeq	High output	50M	2	1	1	N/A
MiSeq	v2	30M	1	1	N/A	N/A
NextSeq 500	Mid output	260M	14	9	5	3
NextSeq 500	High output	800M	45	28	18	9
HiSeq 2500 rapid run	Dual Flowcell v2	1200M	68	42	27	14
HiSeq 3000	8 lanes per flow cell	5B	285	176	112	58
HiSeq 4000	8 lanes per flow cell	10B	570	353	225	116
NovaSeq 6000	SP (per flow cell)	1.6B	91	56	36	18

N/A: not applicable, no samples can be run.

* Based on 2 x 149 bp paired-end reads.

Table 6c. Number of multiplexed samples (one DNA and one RNA library for each sample) based on DNA or RNA panel size (Separated targeted DNA and RNA Enrichment workflow) with 7200x mean coverage for DNA library and 5000 reads/primer for the RNA library*

			UHS-005Z	UHS-009Z	UHS-006Z	UHS-5000Z
		DNA primer #	**4149**	**6244**	**11243**	**19995**
		RNA primer #	**487**	**1116**	**665**	**2571**
Instrument	**Version**	**Capacity (paired-ends reads)**				
MiniSeq	Mid output	16M	N/A	N/A	N/A	N/A
MiniSeq	High output	50M	1	N/A	N/A	N/A
MiSeq	v2	30M	N/A	N/A	N/A	N/A
NextSeq 500	Mid output	260M	8	5	3	1
NextSeq 500	High output	800M	24	15	9	5
HiSeq 2500 rapid run	Dual Flowcell v2	1200M	37	23	14	7
HiSeq 3000	8 lanes per flow cell	5B	154	98	59	31
HiSeq 4000	8 lanes per flow cell	10B	309	197	118	63
NovaSeq 6000	SP (per flow cell)	1.6B	49	31	18	10

N/A: not applicable, no samples can be run.

* Based on 2 x 149 bp paired-end reads.

Table 6d. Number of multiplexed samples (one DNA and one RNA library for each sample) based on DNA or RNA panel size (Separated targeted DNA and RNA Enrichment workflow) with 25,600x mean coverage for DNA library and 5000 reads/primer for the RNA library*

			UHS-005Z	UHS-009Z	UHS-006Z	UHS-5000Z
		DNA primer #	**4149**	**6244**	**11243**	**19995**
		RNA primer #	**487**	**1116**	**665**	**2571**
Instrument	**Version**	**Capacity (paired-ends reads)**				
MiniSeq	Mid output	16M	N/A	N/A	N/A	N/A
MiniSeq	High output	50M	N/A	N/A	N/A	N/A
MiSeq	v2	30M	N/A	N/A	N/A	N/A
NextSeq 500	Mid output	260M	2	1	N/A	N/A
NextSeq 500	High output	800M	7	4	2	1
HiSeq 2500 rapid run	Dual Flowcell v2	1200M	11	7	4	2
HiSeq 3000	8 lanes per flow cell	5B	46	30	17	9
HiSeq 4000	8 lanes per flow cell	10B	92	60	34	19
NovaSeq 6000	SP (per flow cell)	1.6B	14	9	5	3

N/A: not applicable, no samples can be run.

* Based on 2 x 149 bp paired-end reads.

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 Multimodal Panels and the QIAseq Multimodal Index kit, the following are required:

- EvaGreen®, 20x in water (Biotium, cat. no. 31000-T or 31000)
- 80% ethanol (made fresh daily) *
- Nuclease-free pipette tips and tubes
- 1.5 ml LoBind® tubes (Eppendorf, cat. no. 022431021)
- PCR tubes (0.2 ml individual tubes [VWR, cat. no. 20170-012] or tube strips [VWR, cat. no. 93001-118]) or plates
- Ice
- Microcentrifuge
- Thermal cycler
- Magnet for bead cleanups:
  - **Tubes**: MagneSphere® Technology Magnetic Separation Stand (Promega, cat. no. Z5342)
  - **Plates**: DynaMag™-96 Side Magnet (Thermo Fisher Scientific Inc., cat. no. 12331D)
- 2100 Bioanalyzer (Agilent, cat. no. G2939BA)
- Agilent® High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626)

* Do not use denatured alcohol, which contains other substances, such as methanol or methylethylketone.

Protocol: Nucleic Acid Fragmentation, Standard Samples

Important points before starting

- This protocol describes fragmentation of nucleic acids from "standard samples" (i.e., cells or tissue). For fragmentation of FFPE samples, please refer to "Protocol: Nucleic Acid Fragmentation, FFPE Samples", page 32.
- This protocol is designed to work with either total nucleic acid eluates (those containing DNA+RNA) or separate DNA and RNA eluates.
- When performing "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", the recommended amount of DNA is 10–40 ng.
- When performing "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes", the recommended amount of DNA is 20-80 ng.
- The recommended amount of RNA is 10 ng to 250 ng total RNA. When working with total nucleic acid samples, we recommend basing the input on the amount of quantified DNA.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure

1. Thaw nucleic acid samples on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
2. Prepare the reagents required for the fragmentation.
   2a. Thaw Fragmentation Buffer, 10x, and FERA Solution at room temperature (15–25°C).
   2b. Mix by flicking the tube, and then centrifuge briefly.

   Note: Side Reaction Reducer and Fragmentation Enzyme Mix should be removed from the freezer just before use and placed on ice. After use, immediately return the enzymes to the freezer.

3. On ice, prepare the fragmentation mix according to Table 7. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 7. Reaction mix for nucleic acid fragmentation

Component	Volume/reaction
DNA (see input recommendation in the "Important points before starting" section)*	Variable A
DNA (see input recommendation in the "Important points before starting" section)*	Variable B
Fragmentation Buffer, 10x	2 µl
FERA Solution	0.6 µl
Side Reaction Reducer	1.6 µl
Fragmentation Enzyme Mix	4 µl
Nuclease-free Water	11.8 µl – variable A (DNA) – variable B (RNA)
Total	**20 µl**

* Instead of adding DNA and RNA separately, total nucleic acid containing both DNA and RNA can be added. If adding total nucleic acid, base the input amount on DNA.

4. Program the thermal cycler according to Table 8. Use the instrument's heated lid.

Table 8. Incubation conditions for nucleic acid fragmentation

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	32°C	24 min
3	72°C	30 min
4	4°C	Hold

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

   Important: The thermal cycler must be prechilled and paused at 4°C.

6. Transfer the tubes/plate prepared in step 3 to the prechilled thermal cycler and resume the program.
7. Upon completion, allow the thermal cycler to return to 4°C.
8. Place the samples on ice and immediately proceed to “Protocol: RNA Polyadenylation”, page 35.

Protocol: Nucleic Acid Fragmentation, FFPE Samples

Important points before starting

- This protocol describes fragmentation of nucleic acids from FFPE samples. For fragmentation of "standard samples" (i.e., cells or tissue), please refer to "Protocol: Nucleic Acid Fragmentation, Standard Samples", page 29.
- This protocol is designed to work with either total nucleic acid eluates (those containing DNA+RNA) or separate DNA and RNA eluates.
- The recommended amount of FFPE DNA is up to 250 ng DNA if QIAseq QuantiMIZE kits have been used (See "Appendix A: FFPE Sample Quality and Quantity", page 72). If an alternative method was used to determine the concentration of FFPE DNA, then up to 100 ng DNA can be used. The recommended amount of FFPE RNA is 250 ng total RNA (up to 500 ng for "severely" fragmented FFPE sample, with "severely" being defined as samples that have less than 40% of fragments >200 nt by smear analysis on the Bioanalyzer). When working with total nucleic acid samples, we recommend basing the input on the amount of quantified DNA.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure

1. Thaw nucleic acid samples on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
2. Prepare the reagents required for the fragmentation.
   2a. Thaw Fragmentation Buffer, 10x, and FERA Solution at room temperature.
   2b. Mix by flicking the tube, and centrifuge briefly.

   Note: Side Reaction Reducer and Fragmentation Enzyme Mix should be removed from the freezer just before use and placed on ice. After use, immediately return the enzymes to the freezer.

3. On ice, prepare the fragmentation mix according to Table 9. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.
   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 9. Reaction mix for nucleic acid fragmentation

Component	Volume/reaction
DNA (see input recommendation in the "Important points before starting" section)*	Variable A
RNA (see input recommendation in the "Important points before starting" section)*	Variable B
Fragmentation Buffer, 10x	2 µl
FERA Solution	0.6 µl
Side Reaction Reducer	1.6 µl
Nuclease-free Water	11.8 µl – variable A (DNA) – variable B (RNA)
Total	**16 µl**

* Instead of adding DNA and RNA separately, total nucleic acid containing both DNA and RNA can be added. If adding total nucleic acid, base the input amount on DNA.

4. Incubate for 15 min at 37°C and then place on ice.
5. Add 4 µl of Fragmentation Enzyme Mix to each reaction. Briefly centrifuge, mix by pipetting up and down 10–12 times (do not vortex), and then briefly centrifuge again.
   Important: Keep the reaction tubes/plate on ice during the entire reaction setup.
6. Program the thermal cycler according to Table 10. Use the instrument's heated lid.

Table 10. Incubation conditions for nucleic acid fragmentation

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	32°C	14 min
3	72°C	30 min
4	4°C	Hold

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

   Important: The thermal cycler must be prechilled and paused at 4°C.

8. Transfer the tubes/plate prepared in step 5 to the prechilled thermal cycler and resume the program.
9. Upon completion, allow the thermal cycler to return to 4°C.
10. Place the samples on ice, and immediately proceed to “Protocol: RNA Polyadenylation”, page 35.

Protocol: RNA Polyadenylation

Important points before starting

- The product from "Protocol: Nucleic Acid Fragmentation, Standard Samples", page 29, or "Protocol: Nucleic Acid Fragmentation, FFPE Samples", page 32, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure

1. Prepare the reagents required for the polyadenylation.
   1a. Thaw PAP Dilution Buffer, 10x, and ATP Solution at room temperature.
   1b. Mix by flicking the tube, and then centrifuge briefly.
   Note: T4 Polynucleotide Kinase and PAP Enzyme should be removed from the freezer just before use and placed on ice. After use, immediately return the enzymes to the freezer.
2. Prepare 1x PAP Dilution Buffer by diluting 2 µl of the 10x PAP Dilution Buffer with 18 µl Nuclease-free Water.
3. Use the 1x PAP Dilution Buffer to dilute an aliquot of the PAP Enzyme from 5 U/µl to 2 U/µl. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.
4. Prepare the RNA polyadenylation mix according to Table 11. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.
   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 11. Reaction mix for RNA polyadenylation

Component	Volume/reaction
Fragmentation reaction (already in tube)	20 µl
ATP Solution	1.25 µl
T4 Polynucleotide Kinase	1 µl
Diluted PAP Enzyme (2 U/µl)*	1 µl
Nuclease-free Water	1.75 µl
Total	**25 µl**

* Ensure PAP Enzyme has been diluted from its stock 5U/µl concentration to 2U/µl using 1x PAP Dilution Buffer.

5. Incubate the reactions in a thermal cycler according to Table 12. Use the instrument's heated lid.

Table 12. Incubation conditions for RNA polyadenylation

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	30°C	10 min
3	4°C	Hold

6. Upon completion, place the reactions on ice and proceed to "Protocol: DNA Ligation", page 37.

Protocol: DNA Ligation

Important points before starting

- The product from "Protocol: RNA Polyadenylation", page 35, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- QIAseq Beads are used for all reaction cleanups.
- **Important**: Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure

1. Prepare the reagents required for the DNA ligation.
   1a. Thaw DNA Ligation Adapter; Ligation Buffer, 5x; and Ligation Solution at room temperature.
   1b. Mix by flicking the tube, and then centrifuge briefly.

   Note: DNA Ligase should be removed from the freezer just before use and placed on ice. After use, immediately return the enzyme to the freezer.
2. Prepare the DNA ligation mix according to Table 13. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.

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

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 13. Reaction mix for DNA ligation

Component	Volume/reaction
RNA polyadenylation reaction (already in tube)	25 µl
Ligation Buffer, 5x	10 µl
DNA Ligation Adapter	2.8 µl
DNA Ligase	5 µl
Ligation Solution*	7.2 µl
Total	**50 µl**

* Ligation Solution is very viscous. It should be added into each reaction individually and not premixed with other components for a master mix. Do not coat the outside of the pipette tip with Ligation Solution, because in doing so, excess volume may be added.

3. Incubate the reactions in a thermal cycler according to Table 14.

   Important: Do not use the heated lid.

Table 14. Incubation conditions for DNA ligation

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	20°C	15 min
3	4°C	Hold

4. Add 50 µl of Nuclease-free Water to bring each sample to 100 µl.
5. Add 130 µl QIAseq Beads, and then mix by vortexing.
6. Incubate for 5 min at room temperature.
7. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates).
8. After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Important: Do not discard the beads, because they contain the DNA of interest.
9. Add 80 µl of Nuclease-free Water to resuspend the beads and then 128 µl of QIAseq NGS Bead Binding Buffer. Mix by vortexing and incubate for 5 min at room temperature.

10. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates). Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.
11. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
12. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol after the second wash. Remove the ethanol with a 200 µl pipette tip first, spin down briefly and then use a 10 µl pipette tip to remove any residual ethanol.
13. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.
14. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 19 µl Nuclease-free Water. Mix well by pipetting.
15. Return the tube/plate to the magnetic rack until the solution has cleared.
16. Transfer 16.62 µl of the supernatant to clean tubes/plate.

    Proceed to “Protocol: Reverse Transcription”, page 40. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: Reverse Transcription

Important points before starting

- The 16.62 µl product from "Protocol: DNA Ligation", page 37, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- QIAseq Beads are used for all reaction cleanups.
- **Important**: Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure

1. Prepare the reagents required for the reverse transcription.
   1a. Thaw the Multimodal RT Primer; Multimodal RT Buffer, 5x; and Multimodal RT Enhancer at room temperature.
   1b. Mix by flicking the tube, and then centrifuge briefly.

   Note: The RNase Inhibitor and EZ Reverse Transcriptase should be removed from the freezer just before use and placed on ice. After use, immediately return the enzymes to the freezer.
2. Prepare the reverse transcription mix according to Table 15. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 15. Reaction mix for reverse transcription

Component	Volume/reaction
Sample (from "Protocol: DNA Ligation", page 37)	16.62 µl
Multimodal RT Primer	1 µl
Multimodal RT Buffer, 5x	5 µl
Multimodal RT Enhancer	0.5 µl
RNase Inhibitor	0.63 µl
EZ Reverse Transcriptase	1.25 µl
Total	**25 µl**

3. Incubate the reactions in a thermal cycler according to Table 16. Use the instrument's heated lid.

Table 16. Incubation conditions for reverse transcription

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	25°C	10 min
3	42°C	45 min
4	70°C	15 min
5	4°C	Hold

4. Add 75 µl of Nuclease-free Water to bring each sample to 100 µl.
5. Add 130 µl QIAseq Beads and mix by vortexing or by pipetting up and down several times.
6. Incubate for 5 min at room temperature.
7. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates).
8. After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

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

9. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
10. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, spin down briefly, and then use a 10 µl pipette tip to remove any residual ethanol.
11. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required. Ethanol carryover to the next universal PCR step will affect PCR efficiency.
12. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 15 µl Nuclease-free Water.

    Important: If performing separated DNA and RNA target enrichment (Appendix B, page 74), elute by adding 22.4 µl Nuclease-free Water.
13. Return the tube/plate to the magnetic rack until solution the solution has cleared.
14. Transfer 12.4 µl of the supernatant to clean tubes/plate.

    Important: If performing separated DNA and RNA target enrichment (Appendix B), transfer 10.2 µl of the eluate to each of 2 tubes and proceed to the Appendix B protocol.
15. Proceed to "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", page 43. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube

Important points before starting

- The 12.4 µl product from “Protocol: Reverse Transcription”, page 40, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- The final library dual sample index is determined by the combination of the QIAseq Multimodal N7 Plate and the QIAseq Multimodal S5 Plate. QIAseq Beads are used for all reaction cleanups.

IMPORTANT: To use this protocol, one of the following is required:

- **QIAseq Multimodal Index I (12)** (cat. no. 333962)
- **QIAseq Multimodal Index I Set A (96)** (cat. no. 333965)
- **QIAseq Multimodal Index I Set B (96)** (cat. no. 333975)

These plates come in either 12- or 48-reaction formats allowing the indexing of 12 DNA and RNA samples or 48 DNA and RNA samples (using one plate of Set A or Set B). In each indicated well of the cuttable plate, there are dried N7 index primers for both DNA and RNA. The plates can be cut in columns to enable indexing of the desired number of samples.

Two plates of Set A or Set B are included for each index kit for making a total of 96 DNA and 96 RNA libraries. By combining Set A and Set B, up to 96 DNA and 96 RNA libraries can be multiplexed. Each well in the plate is single use.

- **Important**: The required combinations of indexes are described in the sequencing sample setup sheets:
  - Sample Sheet Multimodal UDI Set A: **www.qiagen.com/PROM-15281**
  - Sample Sheet Multimodal UDI Set B: **www.qiagen.com/PROM-15282**
  - Sample Sheet Multimodal UDI Set A and Set B: **www.qiagen.com/PROM-15283**
- **Important**: Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure

1. Prepare the reagents required for target enrichment.

   1a. Thaw TEPCR Buffer, 5x; Multimodal DHS Panel (DNA); and Multimodal VHS Panel (RNA); and bring QIAseq Multimodal N7 Plate to room temperature.

   1b. Mix by flicking the tube, and then centrifuge briefly.

   Note: QIAseq Multimodal N7 Plate only needs to be centrifuged, not mixed.

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

2. Prepare the target enrichment mix according to Table 17. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 17. Reaction mix for target enrichment

Component	Volume/reaction
Sample (from "Protocol: Reverse Transcription", page 40)	12.4 µl
TEPCR Buffer, 5x	8 µl
Multimodal DHS Panel (DNA)	10 µl
Multimodal VHS Panel (RNA)	8 µl
HotStarTaq DNA Polymerase (6 U/µl)	1.6 µl
Total	**40 µl**

3. Add the 40 µl target enrichment reaction mix into a well of a QIAseq Multimodal N7 Plate (Table 18, Table 19, and Table 20), which are cuttable plates that contain predispensed, dried index primer pairs for both DNA and RNA samples in the same well.
   Note: The plates can be cut in columns to enable indexing of the desired number of samples.

Table 18. Layout of QIAseq Multimodal N7 Plates, 12 reactions

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1 DNAp-M001 RNAp-M049	S9 DNAp-M009 RNAp-M057	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
B	S2 DNAp-M002 RNAp-M050	S10 DNAp-M010 RNAp-M058	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
C	S3 DNAp-M003 RNAp-M051	S11 DNAp-M011 RNAp-M059	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
D	S4 DNAp-M004 RNAp-M052	S12 DNAp-M012 RNAp-M060	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	S5 DNAp-M005 RNAp-M053	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
F	S6 DNAp-M006 RNAp-M054	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
G	S7 DNAp-M007 RNAp-M055	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
H	S8 DNAp-M008 RNAp-M056	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

Table 19. Layout of QIAseq Multimodal N7 Plates, 48 reactions Set A

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1 DNAp-M001 RNAp-M049	S9 DNAp-M008 RNAp-M057	S17 DNAp-M017 RNAp-M065	S25 DNAp-M025 RNAp-M073	S33 DNAp-M033 RNAp-M081	S41 DNAp-M041 RNAp-M089	Empty	Empty	Empty	Empty	Empty	Empty
B	S2 DNAp-M002 RNAp-M050	S10 DNAp-M010 RNAp-M058	S18 DNAp-M018 RNAp-M066	S26 DNAp-M026 RNAp-M074	S34 DNAp-M034 RNAp-M082	S42 DNAp-M042 RNAp-M090	Empty	Empty	Empty	Empty	Empty	Empty
C	S3 DNAp-M003 RNAp-M051	S11 DNAp-M011 RNAp-M059	S19 DNAp-M019 RNAp-M067	S27 DNAp-M027 RNAp-M075	S35 DNAp-M035 RNAp-M083	S43 DNAp-M043 RNAp-M091	Empty	Empty	Empty	Empty	Empty	Empty
D	S4 DNAp-M004 RNAp-M052	S12 DNAp-M012 RNAp-M060	S20 DNAp-M020 RNAp-M068	S28 DNAp-M028 RNAp-M076	S36 DNAp-M036 RNAp-M084	S44 DNAp-M044 RNAp-M092	Empty	Empty	Empty	Empty	Empty	Empty
E	S5 DNAp-M005 RNAp-M053	S13 DNAp-M013 RNAp-M061	S21 DNAp-M021 RNAp-M069	S29 DNAp-M029 RNAp-M077	S37 DNAp-M037 RNAp-M085	S45 DNAp-M045 RNAp-M093	Empty	Empty	Empty	Empty	Empty	Empty
F	S6 DNAp-M006 RNAp-M054	S14 DNAp-M014 RNAp-M062	S22 DNAp-M022 RNAp-M070	S30 DNAp-M030 RNAp-M078	S38 DNAp-M038 RNAp-M086	S46 DNAp-M046 RNAp-M094	Empty	Empty	Empty	Empty	Empty	Empty
G	S7 DNAp-M007 RNAp-M055	S15 DNAp-M015 RNAp-M063	S23 DNAp-M023 RNAp-M071	S31 DNAp-M031 RNAp-M079	S39 DNAp-M039 RNAp-M087	S47 DNAp-M047 RNAp-M095	Empty	Empty	Empty	Empty	Empty	Empty
H	S8 DNAp-M008 RNAp-M056	S16 DNAp-M016 RNAp-M064	S24 DNAp-M024 RNAp-M072	S32 DNAp-M032 RNAp-M080	S40 DNAp-M040 RNAp-M088	S48 DNAp-M048 RNAp-M096	Empty	Empty	Empty	Empty	Empty	Empty

Table 20. Layout of QIAseq Multimodal N7 Plates, 48 reactions Set B

	1	2	3	4	5	6	7	8	9	10	11	12
A	S49 DNAp-M097 RNAp-M145	S57 DNAp-M105 RNAp-M153	S65 DNAp-M113 RNAp-M161	S73 DNAp-M121 RNAp-M169	S81 DNAp-M129 RNAp-M177	S89 DNAp-M137 RNAp-M185	Empty	Empty	Empty	Empty	Empty	Empty
B	S50 DNAp-M098 RNAp-M146	S58 DNAp-M106 RNAp-M154	S66 DNAp-M114 RNAp-M162	S74 DNAp-M122 RNAp-M170	S82 DNAp-M130 RNAp-M178	S90 DNAp-M138 RNAp-M186	Empty	Empty	Empty	Empty	Empty	Empty
C	S51 DNAp-M099 RNAp-M147	S59 DNAp-M107 RNAp-M155	S67 DNAp-M115 RNAp-M163	S75 DNAp-M123 RNAp-M171	S83 DNAp-M131 RNAp-M179	S91 DNAp-M139 RNAp-M187	Empty	Empty	Empty	Empty	Empty	Empty
D	S52 DNAp-M100 RNAp-M148	S60 DNAp-M108 RNAp-M156	S68 DNAp-M116 RNAp-M164	S76 DNAp-M124 RNAp-M172	S84 DNAp-M132 RNAp-M180	S92 DNAp-M140 RNAp-M188	Empty	Empty	Empty	Empty	Empty	Empty
E	S53 DNAp-M101 RNAp-M149	S61 DNAp-M109 RNAp-M157	S69 DNAp-M117 RNAp-M165	S77 DNAp-M125 RNAp-M173	S85 DNAp-M133 RNAp-M181	S93 DNAp-M141 RNAp-M189	Empty	Empty	Empty	Empty	Empty	Empty
F	S54 DNAp-M102 RNAp-M150	S62 DNAp-M110 RNAp-M158	S70 DNAp-M118 RNAp-M166	S78 DNAp-M126 RNAp-M174	S86 DNAp-M134 RNAp-M182	S94 DNAp-M142 RNAp-M190	Empty	Empty	Empty	Empty	Empty	Empty
G	S55 DNAp-M103 RNAp-M151	S63 DNAp-M111 RNAp-M159	S71 DNAp-M119 RNAp-M167	S79 DNAp-M127 RNAp-M175	S87 DNAp-M135 RNAp-M183	S95 DNAp-M143 RNAp-M191	Empty	Empty	Empty	Empty	Empty	Empty
H	S56 DNAp-M104 RNAp-M152	S64 DNAp-M112 RNAp-M160	S72 DNAp-M120 RNAp-M168	S80 DNAp-M128 RNAp-M176	S88 DNAp-M136 RNAp-M184	S96 DNAp-M144 RNAp-M192	Empty	Empty	Empty	Empty	Empty	Empty

4. Briefly centrifuge, mix by pipetting up and down 8 times, and then briefly centrifuge again.

   Note: If only a column is used, cut that column from the cuttable plate and proceed to the next step.

5. Program a thermal cycler using the cycling conditions in Table 21 (DNA+RNA primers <1500) or Table 22 (DNA+RNA primers ≥1500).

Table 21. Cycling conditions for target enrichment if DNA+RNA primers <1500

Step	Time	Temperature
Initial denaturation	13 min	95°C
	2 min	98°C
8 cycles	15 sec	98°C
	10 min	68°C
Hold	5 min	72°C
	∞	4°C

Table 22. Cycling conditions for target enrichment if number of primers ≥1500/tube

Step	Time (1500–12,000 primers/tube)	Time (>12,000 primers/tube)	Temperature
Initial denaturation	13 min 2 min	13 min 2 min	95°C 98°C
6 cycles	15 s 15 min	15 s 30 min	98°C 65°C
1 cycle	5 min	5 min	72°C
Hold	5 min	5 min	4°C
Hold	∞	∞	4°C

6. Place the target enrichment reaction in the thermal cycler and start the run.
7. Once the run has finished, add 60 µl of Nuclease-free Water to bring each sample to 100 µl.
8. Add 100 µl QIAseq Beads and mix by vortexing or by pipetting up and down several times.
9. Incubate for 5 min at room temperature.

10. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates).
11. After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

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

    Important: When DNA+RNA primer ≥12000, add 75 µl of Nuclease-free Water to resuspend beads, and then add 75 µl of QIAseq Bead Binding Buffer. Mix by vortexing or pipetting up and down. Repeat step 9 to 11.
12. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
13. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, spin down briefly, and then use a 10 µl pipette tip to remove any residual ethanol.
14. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min or longer.

    Note: Visually inspect the pellet to confirm that it is completely dry. Ethanol carryover to the next universal PCR step will affect PCR efficiency.
15. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 25 µl Nuclease-free Water. Mix well by pipetting.
16. Return the tube/plate to the magnetic rack until the solution has cleared.
17. Transfer 24 µl of the supernatant to clean tubes/plate. This will be used in the next 2 protocols.
18. Proceed to "Protocol: qPCR Determination of Universal PCR Cycles", page 51. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: qPCR Determination of Universal PCR Cycles

Important points before starting

- Two microliters of the product from "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", page 43, or "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes", page 74, is the starting material for each of the reaction mixes.
- **Important**: EvaGreen, 20x in water, is required for this procedure and must be purchased from Biotium (cat. no. 31000-T, 31000)
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure

1. Prepare the reagents required for the qPCR.
   1a. Thaw UPCR Buffer, 5x; DNA qPCR AMP Set; and RNA qPCR AMP. Set at room temperature.
   1b. Mix by flicking the tube, and then centrifuge briefly.
   Note: HotStarTaq DNA Polymerase should be removed from the freezer just before use and placed on ice. After use, immediately return the enzyme to the freezer.
2. Prepare the qPCR reactions according to Table 23 for DNA library or Table 24 for RNA library. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.
   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 23. Reaction mix for qPCR of DNA library

Component	Volume/reaction
Sample (from "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", page 43) or DNA sample (from "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes", page 74)	2 µl
UPCR Buffer, 5x	2 µl
Nuclease-free Water	4.1 µl
DNA qPCR AMP Set	1 µl
HotStarTaq DNA Polymerase (6 U/µl)	0.4 µl
EvaGreen, 20x in water*	0.5 µl
Total	**10 µl**

* Must be purchased from Biotium (cat. no. 31000-T, 31000).

Table 24. Reaction mix for qPCR of RNA library

Component	Volume/reaction
Sample (from "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", page 43) or RNA sample (from "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes", page 74)	2 µl
UPCR Buffer, 5x	2 µl
Nuclease-free Water	4.1 µl
RNA qPCR AMP Set	1 µl
HotStarTaq DNA Polymerase (6 U/µl)	0.4 µl
EvaGreen, 20x in water*	0.5 µl
Total	**10 µl**

* Must be purchased from Biotium (cat. no. 31000-T, 31000).

3. Program a qPCR instrument using the cycling conditions in Table 25.

   Note: No melting curve is required.

Table 25. Reaction mix for qPCR of RNA library

Step	Time	Temperature
Hold	13 min	95°C
	2 min	98°C
2-step cycling		
Denaturation	15 s	98°C
Annealing/Extension*	2 min	62°C
Cycle number	**30 cycles**	
Hold	∞	4°C

* Perform fluorescence data collection.

4. Following the reaction, determine the C_T values. Based on the C_T values, the number of universal PCR cycles is defined as $C_T^{(qPCR)}$+3, for both the DNA and RNA libraries.

   For example, if the DNA qPCR is C_T=19, then perform 22 cycles for DNA universal PCR. If the RNA qPCR is C_T=15, then perform 18 cycles for RNA universal PCR.

   Alternative method:

   When the run has finished, observe the amplification plot in "Log View" and define the baseline using "auto baseline". Using the "Log View" of the amplification plot, determine the cycle in which the amplification curve reaches its Plateau Phase, and use 2 cycles fewer. For example, if the plateau phase is reached when the C_T is 18, then 16 is the required number of universal PCR amplification cycles.

5. Once the amplification cycles for universal PCR have been determined, proceed to "Protocol: Universal PCR", page 54.

Protocol: Universal PCR

Important points before starting

- Nine microliters of the product from "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", page 43, or "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes", page 74, is the starting material for each of the reaction mixes.
- The number of cycles required for amplification are determined in "Protocol: qPCR Determination of Universal PCR Cycles", page 51.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- QIAseq Multimodal S5 Plates are used and come in either 12- or 48-reaction formats allowing the indexing of 12 DNA and RNA samples or 48 DNA and RNA samples, respectively. Each well in a QIAseq Multimodal S5 Plates is single use. SQDIB001 to SQDIB048 and SQDIB097 to SQDIB144 are mixed with universal DNA primer for DNA library amplification. SQDIB0049 to SQDIB096 and SQDIB0145 to SQDIB192 are mixed with universal RNA primer for RNA library amplification. The S5 primers are expected to be used in pairs with N7 primers, with SQDIB001 being paired with DNAp-M001, SQDIB002 being paired with DNAp-M002, etc.; and SQDIB049 being paired with RNAp-M049 and SQDIB050 being paired with RNAp-M050, etc. The plates can be cut in columns to enable indexing of the desired number of samples.
- The final library dual sample index is determined by the combination of the QIAseq Multimodal N7 Plate and the QIAseq Multimodal S5 Plate.
- **Important**: The required combinations of indexes are described in the sequencing sample setup sheets:
  - Sample Sheet Multimodal UDI Set A: **www.qiagen.com/PROM-15281**
  - Sample Sheet Multimodal UDI Set B: **www.qiagen.com/PROM-15282**
  - Sample Sheet Multimodal UDI Set A and Set B: **www.qiagen.com/PROM-15283**

- QIAseq Beads are used for all reaction cleanups.
- **Important**: Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure

1. Prepare the reagents required for universal PCR.
   1a. Thaw UPCR Buffer, 5x, and bring QIAseq Multimodal S5 Plate to room temperature.
   1b. Mix by flicking the tube, and then centrifuge briefly.

   Note: HotStarTaq DNA Polymerase should be removed from the freezer just before use and placed on ice. After use, immediately return the enzyme to the freezer.
2. Prepare the universal PCR according to Table 26 for DNA library or Table 27 for RNA library. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.
   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 26. Reaction mix for universal PCR of DNA library

Component	Volume/reaction
Sample (from "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", page 43) or DNA sample (from "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes", page 74)	9 µl
UPCR Buffer, 5x	5 µl
Nuclease-free Water	10 µl
HotStarTaq DNA Polymerase (6 U/µl)	1 µl
Total	**25 µl**

Table 27. Reaction mix for universal PCR of RNA library

Component	Volume/reaction
Sample (from "Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube", page 43) or RNA sample (from "Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes", page 74)	9 µl
UPCR Buffer, 5x	5 µl
Nuclease-free Water	10 µl
HotStarTaq DNA Polymerase (6U/µl)	1 µl
Total	**25 µl**

3. To the QIAseq Multimodal S5 Plate (Table 28, Table 29, or Table 30), add the 25 µl reaction mix for universal PCR of DNA library to the DNA wells (columns 1–6), and add the 25 µl reaction mix for universal PCR of RNA library to the RNA wells (columns 7–12) of a QIAseq Multimodal S5 Plate.

   Note: The QIAseq Multimodal S5 plates are cuttable plates that contain predispensed, dried index primers for both DNA and RNA samples in separate wells. The DNA and RNA primers are expected to be used in pairs: for example, sample 1 should use DNA primer SQDIB001 and RNA primer SQDIB049, sample 2 should use DNA primer SQDIB002 and RNA primer SQDIB050, etc.

   Note: If only a column is used, cut that column from the cuttable plate and proceed to next step.

Table 28. Layout of QIAseq Multimodal S5 Plates, 12 reactions

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1 SQDIB001 uDNA Pr	S9 SQDIB009 uDNA Pr	Empty	Empty	Empty	Empty	S1 SQDIB049 uRNA Pr	S9 SQDIB057 uRNA Pr	Empty	Empty	Empty	Empty
B	S2 SQDIB002 uDNA Pr	S10 SQDIB010 uDNA Pr	Empty	Empty	Empty	Empty	S2 SQDIB050 uRNA Pr	S10 SQDIB058 uRNA Pr	Empty	Empty	Empty	Empty
C	S3 SQDIB003 uDNA Pr	S11 SQDIB011 uDNA Pr	Empty	Empty	Empty	Empty	S3 SQDIB051 uRNA Pr	S11 SQDIB059 uRNA Pr	Empty	Empty	Empty	Empty
D	S4 SQDIB004 uDNA Pr	S12 SQDIB012 uDNA Pr	Empty	Empty	Empty	Empty	S4 SQDIB052 uRNA Pr	S12 SQDIB060 uRNA Pr	Empty	Empty	Empty	Empty
E	S5 SQDIB005 uDNA Pr	Empty	Empty	Empty	Empty	Empty	S5 SQDIB053 uRNA Pr	Empty	Empty	Empty	Empty	Empty
F	S6 SQDIB006 uDNA Pr	Empty	Empty	Empty	Empty	Empty	S6 SQDIB054 uRNA Pr	Empty	Empty	Empty	Empty	Empty
G	S7 SQDIB007 uDNA Pr	Empty	Empty	Empty	Empty	Empty	S7 SQDIB055 uRNA Pr	Empty	Empty	Empty	Empty	Empty
H	S8 SQDIB008 uDNA Pr	Empty	Empty	Empty	Empty	Empty	S8 SQDIB056 uRNA Pr	Empty	Empty	Empty	Empty	Empty

Table 29. Layout of QIAseq Multimodal S5 Plates, 48 reactions Set A

	1		2		3		4		5		6		7		8		9		10		11		12	
A	S1	SQDIB001 uDNA Pr	S9	SQDIB009 uDNA Pr	S17	SQDIB017 uDNA Pr	S25	SQDIB025 uDNA Pr	S33	SQDIB033 uDNA Pr	S41	SQDIB041 uDNA Pr	S1	SQDIB049 uRNA Pr	S9	SQDIB057 uRNA Pr	S17	SQDIB065 uRNA Pr	S25	SQDIB073 uRNA Pr	S33	SQDIB081 uRNA Pr	S41	SQDIB089 uRNA Pr
B	S2	SQDIB002 uDNA Pr	S10	SQDIB010 uDNA Pr	S18	SQDIB018 uDNA Pr	S26	SQDIB026 uDNA Pr	S34	SQDIB034 uDNA Pr	S42	SQDIB042 uDNA Pr	S2	SQDIB050 uRNA Pr	S10	SQDIB058 uRNA Pr	S18	SQDIB066 uRNA Pr	S26	SQDIB074 uRNA Pr	S34	SQDIB082 uRNA Pr	S42	SQDIB090 uRNA Pr
C	S3	SQDIB003 uDNA Pr	S11	SQDIB011 uDNA Pr	S19	SQDIB019 uDNA Pr	S27	SQDIB027 uDNA Pr	S35	SQDIB035 uDNA Pr	S43	SQDIB043 uDNA Pr	S3	SQDIB051 uRNA Pr	S11	SQDIB059 uRNA Pr	S19	SQDIB067 uRNA Pr	S27	SQDIB075 uRNA Pr	S35	SQDIB083 uRNA Pr	S43	SQDIB091 uRNA Pr
D	S4	SQDIB004 uDNA Pr	S12	SQDIB012 uDNA Pr	S20	SQDIB020 uDNA Pr	S28	SQDIB028 uDNA Pr	S36	SQDIB036 uDNA Pr	S44	SQDIB044 uDNA Pr	S4	SQDIB052 uRNA Pr	S12	SQDIB060 uRNA Pr	S20	SQDIB068 uRNA Pr	S28	SQDIB076 uRNA Pr	S36	SQDIB084 uRNA Pr	S44	SQDIB092 uRNA Pr
E	S5	SQDIB005 uDNA Pr	S13	SQDIB013 uDNA Pr	S21	SQDIB021 uDNA Pr	S29	SQDIB029 uDNA Pr	S37	SQDIB037 uDNA Pr	S45	SQDIB045 uDNA Pr	S5	SQDIB053 uRNA Pr	S13	SQDIB061 uRNA Pr	S21	SQDIB069 uRNA Pr	S29	SQDIB077 uRNA Pr	S37	SQDIB085 uRNA Pr	S45	SQDIB093 uRNA Pr
F	S6	SQDIB006 uDNA Pr	S14	SQDIB014 uDNA Pr	S22	SQDIB022 uDNA Pr	S30	SQDIB030 uDNA Pr	S38	SQDIB038 uDNA Pr	S46	SQDIB046 uDNA Pr	S6	SQDIB054 uRNA Pr	S14	SQDIB062 uRNA Pr	S22	SQDIB070 uRNA Pr	S30	SQDIB078 uRNA Pr	S38	SQDIB086 uRNA Pr	S46	SQDIB094 uRNA Pr
G	S7	SQDIB007 uDNA Pr	S15	SQDIB015 uDNA Pr	S23	SQDIB023 uDNA Pr	S31	SQDIB031 uDNA Pr	S39	SQDIB039 uDNA Pr	S47	SQDIB047 uDNA Pr	S7	SQDIB055 uRNA Pr	S15	SQDIB063 uRNA Pr	S23	SQDIB071 uRNA Pr	S31	SQDIB079 uRNA Pr	S39	SQDIB087 uRNA Pr	S47	SQDIB095 uRNA Pr
H	S8	SQDIB008 uDNA Pr	S16	SQDIB016 uDNA Pr	S24	SQDIB024 uDNA Pr	S32	SQDIB032 uDNA Pr	S40	SQDIB040 uDNA Pr	S48	SQDIB048 uDNA Pr	S8	SQDIB056 uRNA Pr	S16	SQDIB064 uRNA Pr	S24	SQDIB072 uRNA Pr	S32	SQDIB080 uRNA Pr	S40	SQDIB088 uRNA Pr	S48	SQDIB096 uRNA Pr

Table 30. Layout of QIAseq Multimodal S5 Plates, 48 reactions Set B

	1	2	3	4	5	6	7	8	9	10	11	12
A	S49 SQDIB097 uDNA Pr	S57 SQDIB105 uDNA Pr	S65 SQDIB113 uDNA Pr	S73 SQDIB121 uDNA Pr	S81 SQDIB129 uDNA Pr	S89 SQDIB137 uDNA Pr	S49 SQDIB145 uRNA Pr	S57 SQDIB153 uRNA Pr	S65 SQDIB161 uRNA Pr	S73 SQDIB169 uRNA Pr	S81 SQDIB177 uRNA Pr	S89 SQDIB185 uRNA Pr
B	S50 SQDIB098 uDNA Pr	S58 SQDIB106 uDNA Pr	S66 SQDIB114 uDNA Pr	S74 SQDIB122 uDNA Pr	S82 SQDIB130 uDNA Pr	S90 SQDIB138 uDNA Pr	S50 SQDIB146 uRNA Pr	S58 SQDIB154 uRNA Pr	S66 SQDIB162 uRNA Pr	S74 SQDIB170 uRNA Pr	S82 SQDIB178 uRNA Pr	S90 SQDIB186 uRNA Pr
C	S51 SQDIB099 uDNA Pr	S59 SQDIB107 uDNA Pr	S67 SQDIB115 uDNA Pr	S75 SQDIB123 uDNA Pr	S83 SQDIB131 uDNA Pr	S91 SQDIB139 uDNA Pr	S51 SQDIB147 uRNA Pr	S59 SQDIB155 uRNA Pr	S67 SQDIB163 uRNA Pr	S75 SQDIB171 uRNA Pr	S83 SQDIB179 uRNA Pr	S91 SQDIB187 uRNA Pr
D	S52 SQDIB100 uDNA Pr	S60 SQDIB108 uDNA Pr	S68 SQDIB116 uDNA Pr	S76 SQDIB124 uDNA Pr	S84 SQDIB132 uDNA Pr	S92 SQDIB140 uDNA Pr	S52 SQDIB148 uRNA Pr	S60 SQDIB156 uRNA Pr	S68 SQDIB164 uRNA Pr	S76 SQDIB172 uRNA Pr	S84 SQDIB180 uRNA Pr	S92 SQDIB188 uRNA Pr
E	S53 SQDIB101 uDNA Pr	S61 SQDIB109 uDNA Pr	S69 SQDIB117 uDNA Pr	S77 SQDIB125 uDNA Pr	S85 SQDIB133 uDNA Pr	S93 SQDIB141 uDNA Pr	S53 SQDIB149 uRNA Pr	S61 SQDIB157 uRNA Pr	S69 SQDIB165 uRNA Pr	S77 SQDIB173 uRNA Pr	S85 SQDIB181 uRNA Pr	S93 SQDIB189 uRNA Pr
F	S54 SQDIB102 uDNA Pr	S62 SQDIB110 uDNA Pr	S70 SQDIB118 uDNA Pr	S78 SQDIB126 uDNA Pr	S86 SQDIB134 uDNA Pr	S94 SQDIB142 uDNA Pr	S54 SQDIB150 uRNA Pr	S62 SQDIB158 uRNA Pr	S70 SQDIB166 uRNA Pr	S78 SQDIB174 uRNA Pr	S86 SQDIB182 uRNA Pr	S94 SQDIB190 uRNA Pr
G	S55 SQDIB103 uDNA Pr	S63 SQDIB111 uDNA Pr	S71 SQDIB119 uDNA Pr	S79 SQDIB127 uDNA Pr	S87 SQDIB135 uDNA Pr	S95 SQDIB143 uDNA Pr	S55 SQDIB151 uRNA Pr	S63 SQDIB159 uRNA Pr	S71 SQDIB167 uRNA Pr	S79 SQDIB175 uRNA Pr	S87 SQDIB183 uRNA Pr	S95 SQDIB191 uRNA Pr
H	S56 SQDIB104 uDNA Pr	S64 SQDIB112 uDNA Pr	S72 SQDIB120 uDNA Pr	S80 SQDIB128 uDNA Pr	S88 SQDIB136 uDNA Pr	S96 SQDIB144 uDNA Pr	S56 SQDIB152 uRNA Pr	S64 SQDIB160 uRNA Pr	S72 SQDIB168 uRNA Pr	S80 SQDIB176 uRNA Pr	S88 SQDIB184 uRNA Pr	S96 SQDIB192 uRNA Pr

4. Program a thermal cycler using the cycling conditions in Table 31.

Table 31. Cycling conditions for universal PCR

Step	Time	Temperature
Hold	13 min	95°C
	2 min	98°C
2-step cycling		
Denaturation	15 s	98°C
Annealing/Extension	2 min	62°C
Cycle number	**Based on "Protocol: qPCR Determination of Universal PCR Cycles", page 51**	
Hold	∞	4°C

5. After the reaction is complete, add 75 µl of Nuclease-free Water to bring each sample to 100 µl.
6. Add 100 µl QIAseq Beads, and then mix by vortexing or pipetting up and down several times.
7. Incubate for 5 min at room temperature.
8. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates) to separate the beads from the supernatant.
9. After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Important: Do not discard the beads, because they contain the DNA of interest.
10. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
11. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, spin down briefly, and then use a 10 µl pipette tip to remove any residual ethanol.

12. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.

13. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 20 µl Nuclease-free Water. Mix well by pipetting.
14. Return the tube/plate to the magnetic rack until the solution has cleared.
15. Transfer 18 µl of the supernatant to clean tubes/plate.
16. Proceed to "Recommendations: Library QC and Quantification", page 62. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Recommendations: Library QC and Quantification

NGS library QC

QC can be performed with the Agilent Bioanalyzer or TapeStation. Check for the correct size distribution of library fragments (~400–500 bp median size) and for the absence of adapters or adapter–dimers (~<200 bp) (Figure 4).

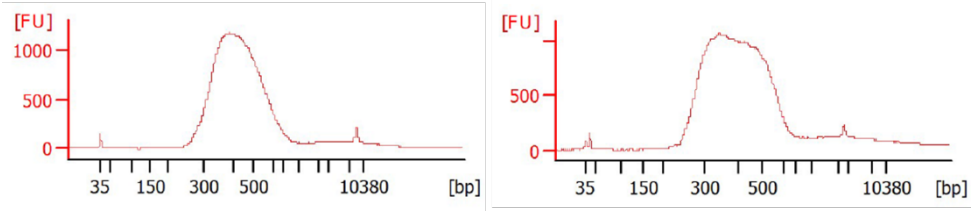


Figure 4. QIAseq Multimodal Targeted DNA (left) and Targeted RNA (right) libraries.

Preferred library quantification method

The library yield measurements from the Bioanalyzer or TapeStation® rely on fluorescence dyes that intercalate into DNA or RNA. These dyes cannot discriminate between molecules with or without adapter sequences, yet only complete QIAseq Multimodal libraries with full adapter sequences will be sequenced. As a result, QIAGEN's QIAseq Library Quant Array Kit (cat. no. 333304) or QIAseq Library Quant Assay Kit (cat. no. 333314), which contains laboratory-verified forward and reverse primers, together with a DNA standard, is highly recommended for accurate quantification of the prepared QIAseq Multimodal library.

Protocol: Sequencing Setup on Illumina MiSeq and NextSeq

Important points before starting

- **Important**: Recommendations for library dilution concentrations and library loading concentrations are based on QIAseq Library Quant System.
- **Important**: QIAseq A Read 1 Primer I (Custom Read 1 Sequencing Primer) and Multimodal Read 2 Primer (Custom Read 2 Sequencing Primer) **must** be used when performing sequencing on Illumina platform.
- **Important**: QIAseq A Read 1 Primer I (Custom Read 1 Sequencing Primer) goes into the following specific reagent cartridge positions:
  - MiSeq position #18
  - NextSeq position #7
  - **Important**: Multimodal Read 2 Primer (the Custom Read 2 Sequencing Primer) goes into the following specific reagent cartridge positions:
    MiSeq position #20
  - NextSeq position # 8
- **Important**: Paired-end sequencing **should** be used for QIAseq Multimodal libraries on Illumina platforms.
  - Read 1: 149 bp
  - Read 2: 149 bp
  - Custom Index 1: 10 bp
  - Custom Index 2: 10 bp
- For complete instructions on how to denature sequencing libraries, prepare custom index primers, and set up a sequencing run, please refer to the system-specific Illumina documents.
- Instrument-specific imagery is included to aid in sequencing preparations.

Sequencing preparations for MiSeq

1. Download the appropriate template from the "Resource" tab of the QIAseq Multimodal Panel:
   - Sample Sheet Multimodal UDI Set A (also used for Index 1–12): **www.qiagen.com/PROM-15281**
   - Sample Sheet Multimodal UDI Set B: **www.qiagen.com/PROM-15282**
   - Sample Sheet Multimodal UDI Set A and Set B: **www.qiagen.com/PROM-15283**
2. On the template:
   2a. Modify **Investigator Name**, **Date**, **Sample_ID**, and **Sample Name**.
   Important: We recommend adding **-DNA** in the Sample name of a DNA library and **-RNA** for an RNA library, to allow automatic parsing of the DNA and RNA libraries during data analysis. If the libraries are not labeled, they must be manually parsed into either the DNA or RNA box.
   2b. Delete any unused index pairs and save the sample sheet for uploading.
   2c. Read 1 is **149 bp**, Read 2 is **149 bp**, and each Index Read is **10 bp**.
3. **Sample dilution and pooling**: Dilute libraries to 2 or 4 nM for MiSeq. Then combine libraries with different sample indexes in equimolar amounts if similar sequencing depth is needed for each library.
   Note: If combining libraries with the same number of primers, pool equal volumes of the individual libraries at 4 nM together. If the libraries have different primer numbers, then combine the libraries at volume ratios according to their number of primers. For example, Library A has 5000 primers at 4 nM, and Library B has 600 primers at 4 nM; combining 50 µl Library A with 6 µl Library B will result in similar coverage depth for both libraries A and B in the same sequencing run.
4. **Library preparation and loading**: Prepare and load library on a MiSeq according to the *MiSeq System Denature and Dilute Libraries Guide*. The final library concentration is 10–12 pM on MiSeq.
   Note: Recommendations for library loading concentrations are based on the QIAseq Library Quant System.

5. **Custom sequencing primer for Read 1 preparation and loading**: Use 597 µl HT1 (hybridization buffer) from the Illumina Sequencing Kit to dilute 3 µl of QIAseq A Read 1 Primer I (provided) to obtain a final concentration of 0.5 µM. Load 600 µl of the diluted QIAseq A Read 1 Primer I to position 18 of the MiSeq reagent cartridge (Figure 5). For more details, please refer to Illumina's *MiSeq System: Custom Primers Guide*.
6. **Custom sequencing primer for Read 2 preparation and loading**: Use 597 µl HT1 (hybridization buffer) from the Illumina Sequencing Kit to dilute 3 µl of Multimodal Read 2 Primer (provided) to obtain a final concentration of 0.5 µM. Load 600 µl of the diluted QIAseq Read 2 Primer to position 20 of the MiSeq reagent cartridge (Figure 5). For more details, please refer to Illumina's *MiSeq System: Custom Primers Guide*.

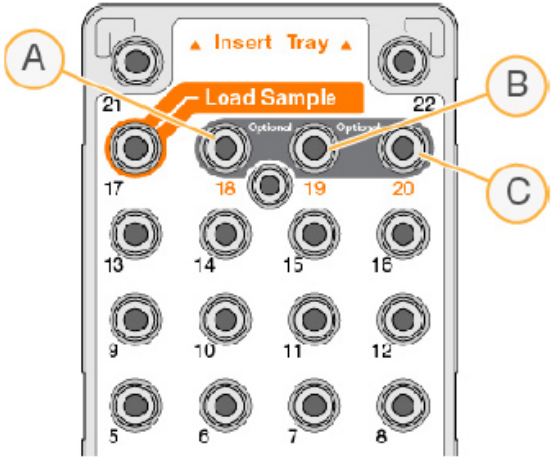


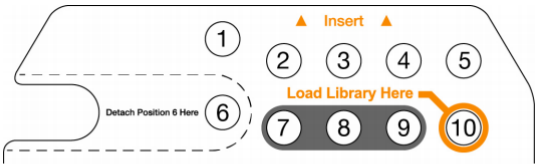
Figure 5. MiSeq reagent cartridge. A: position 18 for Read 1 Custom Primer; **C**: position 20 for Read 2 Custom Primer.

7. Upon completion of the sequencing run, proceed to "Protocol: Data Analysis Using the QIAseq Multimodal Data Analysis Portal", page 68.

Sequencing preparations for NextSeq

1. **Sample dilution and pooling**: Dilute libraries to 0.5, 1, 2, or 4 nM for NextSeq. Then combine libraries with different sample indexes in equimolar amounts if similar sequencing depth is needed for each library.
   Note: Recommendations for library dilution concentrations are based on the QIAseq Library Quant System.
   Note: If combining libraries with the same number of primers, pool equal volumes of the individual libraries at 4 nM together. If the libraries have different primer numbers, then combine the libraries at volume ratios according to their number of primers. For example, Library A has 5000 primers at 4 nM, and Library B has 600 primers at 4 nM; combining 50 µl Library A with 6 µl Library B will result in similar coverage depth for both libraries A and B in the same sequencing run.
2. **Library preparation and loading**: Prepare and load library onto a NextSeq according to the *NextSeq System Denature and Dilute Libraries Guide*. The final library concentration is 1.2–1.5 pM on NextSeq.
   Note: Recommendations for library loading concentrations are based on the QIAseq Library Quant System.
3. **Custom sequencing primer for Read 1 preparation and loading**: Use 1994 µl HT1 (hybridization buffer) from the Illumina Sequencing Kit to dilute 6 µl of QIAseq A Read 1 Primer I (provided) to obtain a final concentration of 0.3 µM. Load 2 ml of the diluted QIAseq A Read 1 Primer I to position 7 of the NextSeq reagent cartridge (Figure 6).
   Note: All other steps refer to run setup workflow as described in the NextSeq 500 System Guide (part # 15046563) or NextSeq 550 System Guide (part # 15069765-02).

4. **Custom sequencing primer for Read 2 preparation and loading**: Use 1994 µl HT1 (hybridization buffer) from the Illumina Sequencing Kit to dilute 6 µl of Multimodal Read 2 Primer to obtain a final concentration of 0.3 µM. Load 2 ml of the diluted Multimodal Read 2 Primer to position 8 of the NextSeq reagent cartridge (Figure 6).



Position #	Custom Primer
7	Custom Read 1 primer
8	Custom Read 2 primer

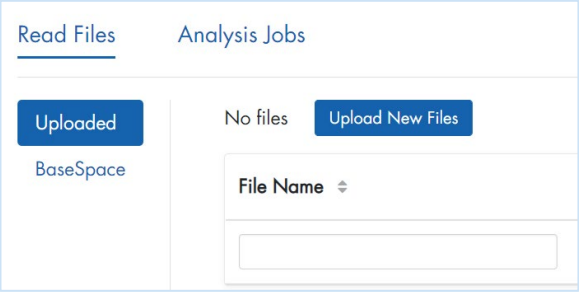
Figure 6. NextSeq reagent cartridge.

5. When working with the QIAseq Multimodal custom UDIs, use Local Run Manager (LRM) V2 on the instrument to upload sample sheet (see page 66 for downloading the appropriate template and modifying the template) and proceed with sequencing: Read 1 is 149 bp, Read 2 is 149 bp, and each Index Read is 10 bp.
6. Upon completion, proceed to "Protocol: Data Analysis Using the QIAseq Multimodal Data Analysis Portal", page 68.

Protocol: Data Analysis Using the QIAseq Multimodal Data Analysis Portal

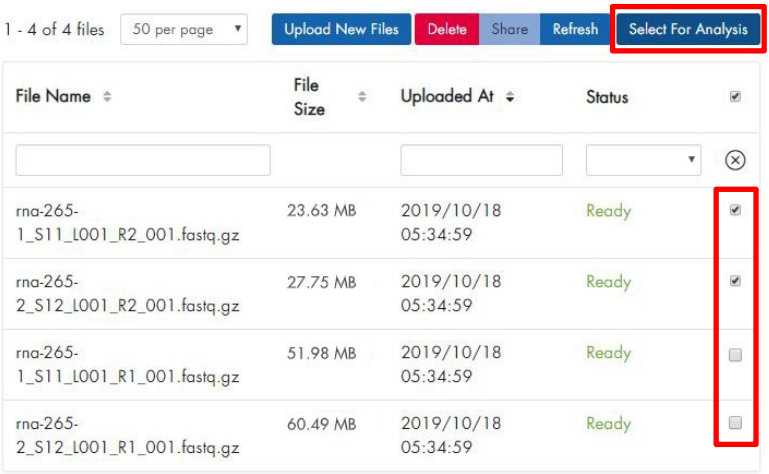
After sequencing, the results can be analyzed using QIAGEN's QIAseq Multimodal Data Analysis Portal. Our data analysis pipeline will perform mapping to the reference genome, UMI counting, read trimming (removing primer sequences), and variant identification. All detected variants can be further interpreted using QCI-I.

1. Go to the QIAseq Multimodal Data Analysis Portal, **ngsdataanalysis2.qiagen.com/MultiModal/**
2. Log in to the portal.
3. In the **Read Files** tab, select **BaseSpace** to upload files from BaseSpace, or select **Uploaded** > **Upload New Files** to upload files from your local hard drive.

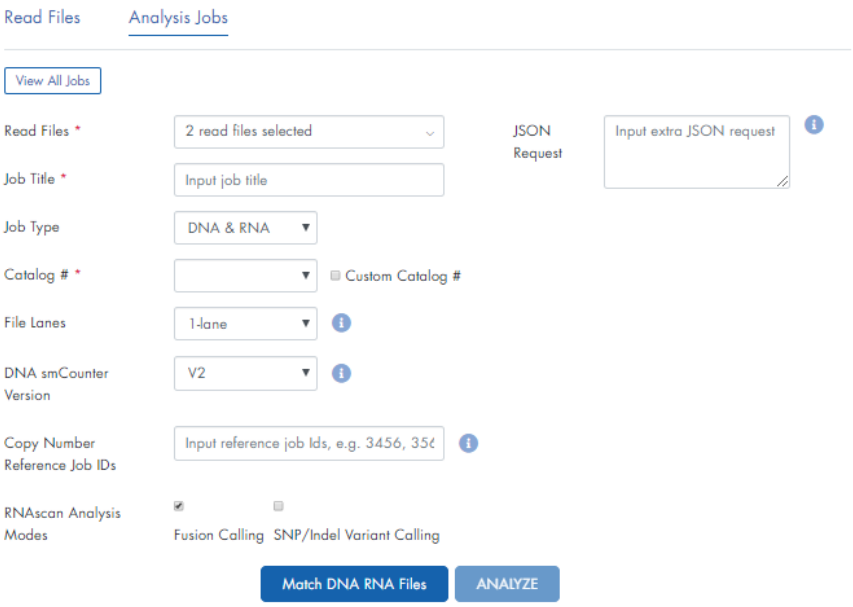


Note: All files that have been uploaded to the portal are listed under the **Read Files** tab. Here it is possible to delete files that are no longer needed and share files with collaborators.

4. Select the boxes next to the files that will be analyzed, and then click **Select For Analysis**.



5. Under the **Analysis Jobs** tab, configure the analysis per the drop-down menus and check boxes as described below:



- **Read Files**: Verify that the correct read files have been selected.
- **Job Title**: Enter a title for the analysis job.
- **Job Type**: Select **DNA & RNA**, **DNA Only**, or **RNA only**.
- **Catalog #**: If using a catalog panel, select the number from the dropdown menu. If using a custom panel, enter the custom catalog number manually.
- **File Lanes**: Choose **1-lane** if MiSeq/HiSeq/NovaSeq was used or if NextSeq was used with concatenated files. Choose **4-lane** if NextSeq was used without ultimately having the files concatenated.
- **DNA smCounter Version**: Select the appropriate version, based on the experimental needs. For guidance, select **Information** .
- **Copy Number Reference Job IDs**: For copy number analysis, normal sample(s) need to be analyzed with the portal before case samples are set up. Enter the job ID corresponding to your control samples for copy number analysis.
- **RNAscan Analysis Modes**: Select **Fusion Calling** and/or **SNP/Indel Variant Calling**. By default, gene expression analysis is provided with each selection.

6. Click **Match DNA RNA Files** to manually drag the selected read files into either the **DNA Files** box or the **RNA Files** box.

   Note: We recommend that you include **-DNA** at the end of the sample name for a DNA library and **-RNA** for an RNA library, to allow automatic parsing of the DNA and RNA libraries during data analysis.

7. Click **ANALYZE**. The analysis job status changes from "Queued" to "In progress", and then to "Done successfully".

8. Once the analysis is completed, output files can be downloaded by clicking **Download**.

   Note: Ultimately, detected variants can be interpreted with QCI-I.

Troubleshooting Guide

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

	Comments and suggestions
Low library yield	
a) Suboptimal reaction conditions due to low sample quality	Make sure to use high-quality samples to ensure optimal activity of the library enzymes.
b) Inefficient targeted enrichment or universal PCR	QIAseq beads need to be completely dried before elution. Ethanol carryover to targeted enrichment and universal PCR will affect PCR reaction efficiency.
Unexpected signal peaks	
a) Short peaks <200 bp	These are primer–dimers from targeted enrichment or universal PCR (<200 bp). The presence of primer–dimers indicates either not enough DNA/RNA input or inefficient PCR reactions or handling issues with bead purifications.
b) Larger DNA fragments after universal PCR	After the universal PCR, library fragments are larger than the intended peak and can be a PCR artifact due to overamplification of the DNA library. Overamplification of the library won't affect the sequencing performance. Decreasing the number of universal PCR cycle numbers can reduce overamplification.
Sequencing issues	
a) Too low or too high cluster density	Accurate library quantification is the key for optimal cluster density on any sequencing instrument. PCR-based quantification method is recommended. Other methods may lead to incorrect quantification of the library, especially when there is overamplification.
b) Very low clusters passing filter	Make sure the library is accurately quantified and that the correct amount is loaded onto the sequencing instrument. In addition, the QIAseq A Read 1 Primer I (100 µM) Custom Read 1 Sequencing Primer and Custom Multimodal Read 2 Primer (100uM) **must** be used when sequencing on any Illumina platform.
Variant detection issues	
Known variants not detected	Variant detection sensitivity is directly related to the input DNA and read depth. Check Table 3 (page 21), Table 4 (page 22), and Table 5 (page 23) to determine if the required input DNA, UMI numbers, and read depth are met for the specific variant detection application.

Appendix A: FFPE Sample Quality and Quantity

High-sensitivity Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific, cat. no. Q33120) is recommended for quantification of DNA from FFPE total nucleic acid samples. This kit or other methods like Nanodrop® or QIAxpert® can be used for quantification of FFPE DNA (in separate elutes with RNA).

Genomic DNA present in FFPE archives is usually damaged and fragmented to an uncertain extent. The QIAseq DNA QuantiMIZE System is a qPCR-based approach that determines the quantity and quality of the DNA amenable to PCR-based targeted enrichment prior to NGS. The system provides a cost-effective approach to qualify and quantify the DNA isolated from biological samples – mainly for FFPE samples. Please refer to the corresponding handbook for determining FFPE DNA quantity and quality with the QIAseq DNA QuantiMIZE System.

FFPE DNA input can be determined by the following: If FFPE DNA is defined as high quality (quality control [QC] score ≤0.04) by QuantiMIZE, then up to 100 ng of DNA can be used. If the DNA is determined as low quality (QC score >0.04) then up to 250 ng of DNA can be used. The QC score of QuantiMIZE reflects the amount of amplifiable DNA present in the sample, therefore correlating with the number of UMIs that can be sequenced in the library (Figure 7).

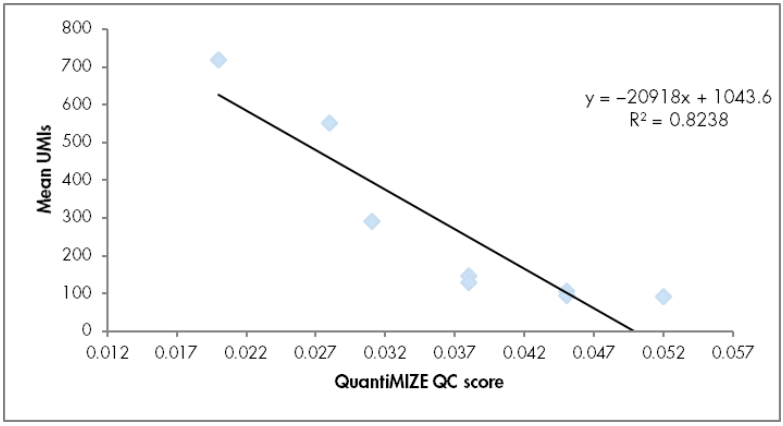


Figure 7. Correlation between QIAseq QuantiMIZE QC Score and the number of UMIs.

Compared to the same amount of fresh DNA, only 10–50% of UMIs can be captured from FFPE DNA, depending on the quality. This is due to a lower amplifiable DNA amount present in the FFPE samples. Therefore, a higher input amount is recommended for FFPE DNA samples to ensure that enough UMIs can be sequenced for variant detection.

However, if the quality of the FFPE DNA is not assessed by QIAseq QuantiMIZE kits, up to 100 ng can be used. If the FFPE DNA quality is high, an input of more than 100 ng will potentially overload the QIAseq Multimodal Panels system.

Appendix B: Separated Targeted DNA and RNA Enrichment in Separate Tubes

Important points before starting

- Two 10.2 µl aliquots of the product from "Protocol: Reverse Transcription", page 40, is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- The final library dual sample index is determined by the combination of the QIAseq Multimodal N7 Plate SW and the QIAseq Multimodal S5 Plate. QIAseq Beads are used for all reaction cleanups.

  Important: The required combinations of indexes are described in the sequencing sample setup sheets:

  - Sample Sheet Multimodal UDI Set A: **www.qiagen.com/PROM-15281**
  - Sample Sheet Multimodal UDI Set B: **www.qiagen.com/PROM-15282**
  - Sample Sheet Multimodal UDI Set A and Set B: **www.qiagen.com/PROM-15283**
- **Important**: Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

IMPORTANT: To use this protocol, one of the following is required:

- **QIAseq Multimodal Index I Set A SW (96)** (cat. no. 333985)
- **QIAseq Multimodal Index I Set B SW (96)** (cat. no. 333995)

These plates come in 48-reaction formats allowing the indexing of 48 DNA and 48 RNA libraries using one plate of Set A or Set B. In each indicated well of the cuttable plate, there are dried N7 index primers for either DNA or RNA. The plates can be cut in columns to enable indexing of the desired number of samples.

Two plates of Set A or Set B are included for each index kit for making a total of 96 DNA and 96 RNA libraries. By combining Set A and Set B, up to 96 DNA and 96 RNA libraries can be multiplexed. Each well in the plate is single use.

Procedure

1. Prepare the reagents required for target enrichment.
   1a. Thaw TEPCR Buffer, 5x; Multimodal DHS Panel (DNA); and Multimodal VHS Panel (RNA); and bring QIAseq Multimodal N7 SW Plate to room temperature.
   1b. Mix by flicking the tube, and then centrifuge briefly.

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

2. Prepare the target enrichment mix according to Table 32. Briefly centrifuge, mix by pipetting up and down 10–12 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 32. Reaction mix for target enrichment

Component	Separate DNA	Separate RNA
Sample (from "Protocol: Reverse Transcription", page 40)	10.2 µl	10.2 µl
TEPCR Buffer, 5x	4 µl	4 µl
Multimodal DHS Panel (DNA)	5 µl	0 µl
Multimodal VHS Panel (RNA)	0 µl	4 µl
HotStarTaq Polymerase (6 U/µl)	0.8 µl	0.8 µl
Nuclease-free Water	0 µl	1 µl
Total	**20 µl**	**20 µl**

3. Add the 20 µl target enrichment reaction mix into a well of a QIAseq Multimodal N7 Plate SW (Table 33 and Table 34), which are cuttable plates that contain predispensed, dried N7 index primers for either DNA (columns 1–6) or RNA (columns 7–12).

   Note: The plates can be cut in columns to enable indexing of the desired number of samples.

   Important: Put the unused plate in the foil back and keep in –20°C for long-term storage.

   Important: Index primers for DNA and RNA should be used in pairs. For example: use well A1 for DNA and well A7 for RNA for sample 1 (S1); use well B1 for DNA and well B7 for RNA for sample 2 (S2), and so on.

Table 33. Layout of QIAseq Multimodal N7 Plates SW, 48 reactions Set A

	1		2		3		4		5		6		7		8		9		10		11		12	
A	S1	DNAp-M031	S9	DNAp-M009	S17	DNAp-M017	S25	DNAp-M025	S33	DNAp-M033	S41	DNAp-M041	S1	RNAp-M049	S9	RNAp-M057	S17	RNAp-M065	S25	RNAp-M073	S33	RNAp-M081	S41	RNAp-M089
B	S2	DNAp-M032	S10	DNAp-M010	S18	DNAp-M018	S26	DNAp-M026	S34	DNAp-M034	S42	DNAp-M042	S2	RNAp-M050	S10	RNAp-M058	S18	RNAp-M066	S26	RNAp-M074	S34	RNAp-M082	S42	RNAp-M090
C	S3	DNAp-M033	S11	DNAp-M011	S19	DNAp-M019	S27	DNAp-M027	S35	DNAp-M035	S43	DNAp-M043	S3	RNAp-M051	S11	RNAp-M059	S19	RNAp-M067	S27	RNAp-M075	S35	RNAp-M083	S43	RNAp-M091
D	S4	DNAp-M034	S12	DNAp-M012	S20	DNAp-M020	S28	DNAp-M028	S36	DNAp-M036	S44	DNAp-M044	S4	RNAp-M052	S12	RNAp-M060	S20	RNAp-M068	S28	RNAp-M076	S36	RNAp-M084	S44	RNAp-M092
E	S5	DNAp-M035	S13	DNAp-M013	S21	DNAp-M021	S29	DNAp-M029	S37	DNAp-M037	S45	DNAp-M045	S5	RNAp-M053	S13	RNAp-M061	S21	RNAp-M069	S29	RNAp-M077	S37	RNAp-M085	S45	RNAp-M093
F	S6	DNAp-M036	S14	DNAp-M014	S22	DNAp-M022	S30	DNAp-M030	S38	DNAp-M038	S46	DNAp-M046	S6	RNAp-M054	S14	RNAp-M062	S22	RNAp-M070	S30	RNAp-M078	S38	RNAp-M086	S46	RNAp-M094
G	S7	DNAp-M007	S15	DNAp-M015	S23	DNAp-M023	S31	DNAp-M031	S39	DNAp-M039	S47	DNAp-M047	S7	RNAp-M055	S15	RNAp-M063	S23	RNAp-M071	S31	RNAp-M079	S39	RNAp-M087	S47	RNAp-M095
H	S8	DNAp-M008	S16	DNAp-M016	S24	DNAp-M024	S32	DNAp-M032	S40	DNAp-M040	S48	DNAp-M048	S8	RNAp-M056	S16	RNAp-M064	S24	RNAp-M072	S32	RNAp-M080	S40	RNAp-M088	S48	RNAp-M096

Table 34. Layout of QIAseq Multimodal N7 Plates SW, 48 reactions Set B

	1		2		3		4		5		6		7		8		9		10		11		12	
A	DNAp-M097	S57	DNAp-M105	S65	DNAp-M113	S73	DNAp-M121	S81	DNAp-M129	S89	DNAc-M137	S49	RNAp-M145	S57	RNAp-M153	S65	RNAp-M161	S73	RNAc-M169	S81	RNAp-M177	S89	RNAp-M185	
B	DNAp-M098	S58	DNAp-M106	S66	DNAp-M114	S74	DNAp-M122	S82	DNAp-M130	S90	DNAp-M138	S50	RNAp-M146	S58	RNAp-M154	S66	RNAp-M162	S74	RNAp-M170	S82	RNAp-M178	S90	RNAp-M186	
C	DNAp-M099	S59	DNAp-M107	S67	DNAp-M115	S75	DNAp-M123	S83	DNAp-M131	S91	DNAp-M139	S51	RNAp-M147	S59	RNAp-M155	S67	RNAp-M163	S75	RNAp-M171	S83	RNAp-M179	S91	RNAp-M187	
D	DNAp-M100	S60	DNAp-M108	S68	DNAp-M116	S76	DNAp-M124	S84	DNAp-M132	S92	DNAp-M140	S52	RNAp-M148	S60	RNAp-M156	S68	RNAp-M164	S76	RNAp-M172	S84	RNAp-M180	S92	RNAp-M188	
E	DNAc-M101	S61	DNAp-M109	S69	DNAp-M117	S77	DNAp-M125	S85	DNAc-M133	S93	DNAp-M141	S53	RNAp-M149	S61	RNAp-M157	S69	RNAc-M165	S77	RNAp-M173	S85	RNAp-M181	S93	RNAp-M189	
F	DNAp-M102	S62	DNAp-M110	S70	DNAp-M118	S78	DNAp-M126	S86	DNAp-M134	S94	DNAp-M142	S54	RNAp-M150	S62	RNAp-M158	S70	RNAp-M166	S78	RNAp-M174	S86	RNAp-M182	S94	RNAp-M190	
G	DNAp-M103	S63	DNAp-M111	S71	DNAp-M119	S79	DNAp-M127	S87	DNAp-M135	S95	DNAp-M143	S55	RNAp-M151	S63	RNAp-M159	S71	RNAp-M167	S79	RNAp-M175	S87	RNAp-M183	S95	RNAc-M191	
H	DNAp-M104	S64	DNAp-M112	S72	DNAc-M120	S80	DNAp-M128	S88	DNAp-M136	S96	DNAp-M144	S56	RNAp-M152	S64	RNAp-M160	S72	RNAp-M168	S80	RNAp-M176	S88	RNAp-M184	S96	RNAp-M192	

4. Briefly centrifuge, mix by pipetting up and down 8 times, and then briefly centrifuge again.

   Note: If only a column is used, cut that column from the cuttable plate and proceed to the next step.

5. Program a thermal cycler using the cycling conditions based on the number of DNA or RNA primers (not combined DNA+RNA).

Table 35. Cycling conditions for target enrichment if primers <1500

Step	Time	Temperature
Initial denaturation	13 min	95°C
	2 min	98°C
8 cycles	15 sec	98°C
	10 min	68°C
Hold	5 min	72°C
	∞	4°C

Table 36. Cycling conditions for target enrichment if number of primers ≥1500/tube

Step	Time (1500–12,000 primers/tube)	Time (>12,000 primers/tube)	Temperature
Initial denaturation	13 min	13 min	95°C
	2 min	2 min	98°C
6 cycles	15 s	15 s	98°C
	15 min	30 min	65°C
1 cycle	5 min	5 min	72°C
Hold	5 min	5 min	4°C
Hold	∞	∞	4°C

6. Place the target enrichment reaction in the thermal cycler and start the run.
7. Once the run has finished, add 80 µl of Nuclease-free Water to bring each sample to 100 µl.
8. Add 100 µl QIAseq Beads and mix by vortexing or by pipetting up and down several times.
9. Incubate for 5 min at room temperature.
10. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates).
11. After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Important: Do not discard the beads, because they contain the DNA of interest.
12. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
13. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µl pipette first, spin down briefly, and then use a 10 µl pipette to remove any residual ethanol.
14. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required. Ethanol carryover to the next universal PCR step will affect PCR efficiency.
15. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 14 µl Nuclease-free Water. Mix well by pipetting.
16. Return the tube/plate to the magnetic rack until the solution has cleared.
17. Transfer 12 µl of the supernatant to clean tubes/plate.
18. Proceed to “Protocol: qPCR Determination of Universal PCR Cycles”, page 51. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Ordering Information

Product	Contents	Cat. no.
QIAseq Multimodal Panel (12)*	Kit containing **all** reagents (except indexes) for multimodal (DNA and RNA) sequencing; fixed small panel for a total of 12 samples: 12 DNA and 12 RNA libraries	333932
QIAseq Multimodal Panel (96)*	Kit containing **all** reagents (except indexes) for multimodal (DNA and RNA) sequencing; fixed small panel for a total of 96 samples: 96 DNA and 96 RNA libraries	333935
QIAseq Multimodal HC Panel (12)*	Kit containing **all** reagents (except indexes) for multimodal (DNA and RNA) sequencing; fixed high content (HC) panel for a total of 12 samples: 12 DNA and 12 RNA libraries	333942
QIAseq Multimodal HC Panel (96)*	Kit containing **all** reagents (except indexes) for multimodal (DNA and RNA) sequencing; fixed high content (HC) panel for a total of 96 samples: 96 DNA and 96 RNA libraries	333945
QIAseq Multimodal Custom Panel (96)*	Kit containing **all** reagents (except indexes) for multimodal (DNA and RNA) sequencing; custom panel for a total of 96 samples: 96 DNA and 96 RNA libraries	333955
QIAseq Multimodal Index I (12)*	Box containing oligos, enough to process a total of 12 samples, for indexing up to a total of 12 samples (12 for DNA and 12 for RNA libraries) for multimodal panel sequencing on Illumina platforms	333962

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Product	Contents	Cat. no.
QIAseq Multimodal Index I Set A (96)*	Box containing oligos, enough to process a total of 96 samples, for indexing up to a total of 96 libraries (48 for DNA and 48 for RNA) in one run for multimodal panel sequencing on Illumina platforms; Set A (of A and B) required for multiplexing 192 libraries (96 for DNA and 96 for RNA) in one run	333965
QIAseq Multimodal Index I Set B (96)*	Box containing oligos, enough to process a total of 96 samples, for indexing up to a total of 96 libraries (48 for DNA and 48 for RNA) in one run for multimodal panel sequencing on Illumina platforms; Set B (of A and B) required for multiplexing 192 libraries (96 for DNA and 96 for RNA) in one run	333975
QIAseq Multimodal Index I Set A SW (96)*	Box containing oligos, enough to process a total of 96 samples, for indexing up to a total of 96 libraries (48 for DNA and 48 for RNA) in one run for multimodal panel sequencing on Illumina platforms using the "separated targeted enrichment" workflow; Set A (of A and B) required for multiplexing 192 libraries (96 for DNA and 96 for RNA) in one run	333985
QIAseq Multimodal Index I Set B SW (96)*	Box containing oligos, enough to process a total of 96 samples, for indexing up to a total of 96 libraries (48 for DNA and 48 for RNA) in one run for multimodal panel sequencing on Illumina platforms using the "separated targeted enrichment" workflow; Set B (of A and B) required for multiplexing 192 libraries (96 for DNA and 96 for RNA) in one run	333995

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Product	Contents	Cat. no.
Related products		
QIAseq Library Quant Array Kit	Reagents for quantification of libraries prepared for Illumina or Ion Torrent® platforms; array format	333304
QIAseq Library Quant Assay Kit	Reagents for quantification of libraries prepared for Illumina or Ion Torrent platforms; assay format	333314
QIAseq DNA QuantiMIZE Array Kit	qPCR arrays for optimizing amount of input DNA and PCR cycling conditions for targeted enrichment of FFPE DNA	333404
QIAseq DNA QuantiMIZE Assay Kit	qPCR assays for optimizing amount of input DNA and PCR cycling conditions for targeted enrichment of FFPE DNA	333414
QIAamp® DNA Mini Kit (50)	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, collection tubes (2 ml), reagents and buffers	51304
QIAamp Circulating Nucleic Acid Kit (50)	For 50 DNA preps: QIAamp Mini Columns, Tube Extenders (20 ml), QIAGEN Proteinase K, carrier RNA, buffers, VacConnectors, and collection tubes (1.5 ml and 2 ml)	55114
AllPrep DNA/RNA Mini Kit (50)	For 50 minipreps: AllPrep DNA Spin Columns, RNeasy Mini Spin Columns, collection tubes, RNase-free water, and buffers	80204
AllPrep DNA/RNA FFPE Kit (50)	50 RNeasy MinElute Spin Columns, 50 QIAamp MinElute Spin Columns, collection tubes, RNase-free reagents, and buffers	80234
PAXgene Blood DNA Kit (25)	Processing tubes and buffers for 25 preparations	761133

Product	Contents	Cat. no.
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	Inquire
GeneRead™ DNA FFPE Kit (50)	QIAamp MinElute® columns, proteinase K, UNG, collection tubes (2 ml), buffers, deparaffinization solution, RNase A	180134

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Document Revision History

Date	Changes
10/2019	Initial release
6/2020	Removed Index sequences from HB. Index sequences can be found on **qiagen.com/QIAseqMultimodalPanels**. Removed references to the Ingenuity Variant Analysis (IVA) tool. Updated volumes of clean up steps after DNA ligation Updated the following sections: Kit Contents (Optical Thin-wall 8-cap Strips), Storage, Principle and procedure, Nucleic acid input amount and Sequencing depth section, Protocol: Combined Targeted DNA+RNA Enrichment in a Single Tube Protocol: qPCR Determination of Universal PCR Cycles, Protocol: Universal PCR, and Protocol: Sequencing Setup on Illumina MiSeq and NextSeq. Layout and formatting changes

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