July 2020

QIAseq® miRNA Library Kit Handbook

Precision small RNA library prep for Illumina® NGS systems

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq miRNA Library Kit	**(12)**	**(96)**
Catalog no.	**331502**	**331505**
Number of reactions	**12**	**96**
Box 1 of 2		
QIAseq miRNA NGS 3' Adapter	12 µl	96 µl
QIAseq miRNA NGS 3' Buffer	24 µl	192 µl
QIAseq miRNA NGS 3' Ligase	12 µl	96 µl
QIAseq miRNA NGS RI	36 µl	288 µl
Nuclease-free Water	1 x 1.5 ml	2 x 1.5 ml
QIAseq miRNA NGS 5' Adapter	12 µl	96 µl
QIAseq miRNA NGS 5' Buffer	24 µl	192 µl
QIAseq miRNA NGS 5' Ligase	12 µl	96 µl
QIAseq miRNA NGS RT Initiator	24 µl	192 µl
QIAseq miRNA NGS RT Primer	24 µl	192 µl
QIAseq miRNA NGS RT Buffer	144 µl	1152 µl
QIAseq miRNA NGS RT Enzyme	12 µl	96 µl
QIAseq miRNA NGS Library Buffer	192 µl	1536 µl
HotStarTaq® DNA Polymerase	36 µl	288 µl
QIAseq miRNA NGS 3C Primer Assay	240 µl	240 µl
QIAseq miRNA NGS 5C Primer Assay	240 µl	240 µl
QIAseq miRNA NGS RTC Primer Assay	240 µl	240 µl
Box 2 of 2		
2x miRNA Ligation Activator	120 µl	2 x 600 µl
QIAseq Beads	10 ml	38.4 ml
QIAseq miRNA NGS Bead Binding Buffer	7 ml	54 ml

QIAseq miRNA NGS 12 Index IL		(12)
Catalog no.		331592
Number of reactions		12
Tube	**Index sequence**	
QIAseq miRNA NGS ILM Library Forward Primer		24 µl
QIAseq miRNA NGS ILM IDP1	ATCACG	4 µl
QIAseq miRNA NGS ILM IDP2	CGATGT	4 µl
QIAseq miRNA NGS ILM IDP3	TTAGGC	4 µl
QIAseq miRNA NGS ILM IDP4	TGACCA	4 µl
QIAseq miRNA NGS ILM IDP5	ACAGTG	4 µl
QIAseq miRNA NGS ILM IDP6	GCCAAT	4 µl
QIAseq miRNA NGS ILM IDP7	CAGATC	4 µl
QIAseq miRNA NGS ILM IDP8	ACTTGA	4 µl
QIAseq miRNA NGS ILM IDP9	GATCAG	4 µl
QIAseq miRNA NGS ILM IDP10	TAGCTT	4 µl
QIAseq miRNA NGS ILM IDP11	GGCTAC	4 µl
QIAseq miRNA NGS ILM IDP12	CTTGTA	4 µl

QIAseq miRNA NGS 48 Index IL		(96)
Catalog no.		331595
Number of reactions		(96)
Box 1 of 3		
QIAseq miRNA NGS ILM Library Forward Primer		192 µl
Box 2 of 3	**Index Sequence**	
QIAseq miRNA NGS ILM IDP1	ATCACG	4 µl
QIAseq miRNA NGS ILM IDP2	CGATGT	4 µl
QIAseq miRNA NGS ILM IDP3	TTAGGC	4 µl
QIAseq miRNA NGS ILM IDP4	TGACCA	4 µl
QIAseq miRNA NGS ILM IDP5	ACAGTG	4 µl
QIAseq miRNA NGS ILM IDP6	GCCAAT	4 µl
QIAseq miRNA NGS ILM IDP7	CAGATC	4 µl

QIAseq miRNA NGS 48 Index IL		(96)
Catalog no.		331595
Number of reactions		(96)
Box 2 of 3 (cont'd)	**Index Sequence**	
QIAseq miRNA NGS ILM IDP8	ACTTGA	4 µl
QIAseq miRNA NGS ILM IDP9	GATCAG	4 µl
QIAseq miRNA NGS ILM IDP10	TAGCTT	4 µl
QIAseq miRNA NGS ILM IDP11	GGCTAC	4 µl
QIAseq miRNA NGS ILM IDP12	CTTGTA	4 µl
QIAseq miRNA NGS ILM IDP13	AGTCAA	4 µl
QIAseq miRNA NGS ILM IDP14	AGTTCC	4 µl
QIAseq miRNA NGS ILM IDP15	ATGTCA	4 µl
QIAseq miRNA NGS ILM IDP16	CCGTCC	4 µl
QIAseq miRNA NGS ILM IDP17	GTAGAG	4 µl
QIAseq miRNA NGS ILM IDP18	GTCCGC	4 µl
QIAseq miRNA NGS ILM IDP19	GTGAAA	4 µl
QIAseq miRNA NGS ILM IDP20	GTGGCC	4 µl
QIAseq miRNA NGS ILM IDP21	GTTTCG	4 µl
QIAseq miRNA NGS ILM IDP22	CGTACG	4 µl
QIAseq miRNA NGS ILM IDP23	GAGTGG	4 µl
QIAseq miRNA NGS ILM IDP24	GGTAGC	4 µl
Box 3 of 3	**Index Sequence**	
QIAseq miRNA NGS ILM IDP25	ACTGAT	4 µl
QIAseq miRNA NGS ILM IDP26	ATGAGC	4 µl
QIAseq miRNA NGS ILM IDP27	ATTCCT	4 µl
QIAseq miRNA NGS ILM IDP28	CAAAAG	4 µl
QIAseq miRNA NGS ILM IDP29	CAACTA	4 µl
QIAseq miRNA NGS ILM IDP30	CACCGG	4 µl
QIAseq miRNA NGS ILM IDP31	CACGAT	4 µl
QIAseq miRNA NGS ILM IDP32	CACTCA	4 µl
QIAseq miRNA NGS ILM IDP33	CAGGCG	4 µl

QIAseq miRNA NGS 48 Index IL		**(96)**
Catalog no.		**331595**
Number of reactions		**(96)**
Box 3 of 3 (cont'd)	**Index Sequence**	
QIAseq miRNA NGS ILM IDP34	CATGGC	4 µl
QIAseq miRNA NGS ILM IDP35	CATTTT	4 µl
QIAseq miRNA NGS ILM IDP36	CCAACA	4 µl
QIAseq miRNA NGS ILM IDP37	CGGAAT	4 µl
QIAseq miRNA NGS ILM IDP38	CTAGCT	4 µl
QIAseq miRNA NGS ILM IDP39	CTATAC	4 µl
QIAseq miRNA NGS ILM IDP40	CTCAGA	4 µl
QIAseq miRNA NGS ILM IDP41	GACGAC	4 µl
QIAseq miRNA NGS ILM IDP42	TAATCG	4 µl
QIAseq miRNA NGS ILM IDP43	TACAGC	4 µl
QIAseq miRNA NGS ILM IDP44	TATAAT	4 µl
QIAseq miRNA NGS ILM IDP45	TCATTC	4 µl
QIAseq miRNA NGS ILM IDP46	TCCCGA	4 µl
QIAseq miRNA NGS ILM IDP47	TCGAAG	4 µl
QIAseq miRNA NGS ILM IDP48	TCGGCA	4 µl

QIAseq miRNA NGS 96 Index IL	(96)
Catalog no.	**331565**
Number of reactions	**96**
Box contains one MIIL-001 plate and 8-cap strips (12). MIIL-001 is a cuttable plate that contains a dried universal primer in every well combined with a different custom 8 base indexing primer (MIHTIL1 through MIHTIL96). MIHIL primers support indexing on Illumina NGS systems.	1

Table 1. QIAseq miRNA NGS 96 Index IL (cat. no. 331565) layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	MIHT1	MIHT9	MIHT17	MIHT25	MIHT33	MIHT41	MIHT49	MIHT57	MIHT65	MIHT73	MIHT81	MIHT89
B	MIHT2	MIHT10	MIHT18	MIHT26	MIHT34	MIHT42	MIHT50	MIHT58	MIHT66	MIHT74	MIHT82	MIHT90
C	MIHT3	MIHT11	MIHT19	MIHT27	MIHT35	MIHT43	MIHT51	MIHT59	MIHT67	MIHT75	MIHT83	MIHT91
D	MIHT4	MIHT12	MIHT20	MIHT28	MIHT36	MIHT44	MIHT52	MIHT60	MIHT68	MIHT76	MIHT84	MIHT92
E	MIHT5	MIHT13	MIHT21	MIHT29	MIHT37	MIHT45	MIHT53	MIHT61	MIHT69	MIHT77	MIHT85	MIHT93
F	MIHT6	MIHT14	MIHT22	MIHT30	MIHT38	MIHT46	MIHT54	MIHT62	MIHT70	MIHT78	MIHT86	MIHT94
G	MIHT7	MIHT15	MIHT23	MIHT31	MIHT39	MIHT47	MIHT55	MIHT63	MIHT71	MIHT79	MIHT87	MIHT95
H	MIHT8	MIHT16	MIHT24	MIHT32	MIHT40	MIHT48	MIHT56	MIHT64	MIHT72	MIHT80	MIHT88	MIHT96

Table 2. QIAseq miRNA NGS 96 Index IL (cat. no. 331565) index sequences

	1	2	3	4	5	6	7	8	9	10	11	12
A	ACAA GACG	TTGG ACCT	GACT TACG	TTGA GCAC	CGTT AGAG	AATA GGCC	GCTA GATC	AGGT AGGT	AGAG CCTT	CGTT CCTA	CGCC AATT	TATA CCGG
B	TGCC TTAC	GTTC GTTG	TCGG TAGA	CTGG TTCA	CAAC TTGG	AGAG TACC	CACA TGCA	GCAT ATGG	GATG GCTT	TCCG TCTT	TGAC GAAG	CTAG GAAC
C	CTCG AGAA	TTAC GGCA	GTGT GAAG	GCGT AACA	ACAC TGAG	GGTC TCAA	CCAT GTTC	CTCT TCTG	CACT ACGA	ACGA TCCT	GCCG ATTA	AGCG ATAG
D	CGAT GACA	AGAA CGCA	TCAA GCGT	AAGA CGAG	CCGA ATGT	AGAA TCGG	ACAC CAGT	CCGA CATA	ACTG CGAT	CAAG CAGA	CGGA ACAA	TCAT CGGA
E	AAGG AGCA	GGAC CATA	GAGA CACT	GTAA CCGA	GTCA CGAT	CGAT CTGT	GACA TCAC	CACT CTAC	CGTA CAAC	TTAG CTGC	GAAT CCTC	TACG TGAG
F	CACA CCTT	ATTG GCCA	ATGG TGAC	TAGC CAAC	CCGC TAAT	TGTA AGGC	GACT GGAA	GAAC GTAC	CCTT GCAT	AAGC AAGG	TGGT GTTG	AGGT CATC
G	AGCA GCAT	CTCA ATCC	GGAA GGTT	GGTT GTGA	GTGT TCGT	AATC CGGA	GCCT CATT	CTCC GTAT	CTGG AATG	ACCA CTTG	ATCG TAGG	ACGT ACAG
H	GGTA ACCT	CACA GAAG	AATC GCAG	CAAC CGAT	TTCC GATC	ACAG GTGA	CGAT TCAC	TGCG CAAT	CAAC GCTA	CTTC CACT	ACTT GGTG	TCTC CATG

Shipping and Storage

The QIAseq miRNA Library Kit is shipped in 2 boxes. Box 1 is shipped on dry ice or blue ice and Box 2 is shipped at room temperature. Upon receipt, all components in Box 1 should be stored immediately at –30 to –15°C in a constant-temperature freezer. All components in Box 2, except for the 2x miRNA Ligation Activator, should be stored immediately at 2–8°C. The 2x miRNA Ligation Activator should be stored at –30 to –15°C in a constant-temperature freezer. It can also be stored temporarily at 2–8°C for less than one month.

QIAseq Index Kits are shipped on dry ice or blue ice. Upon receipt, all components in each box should be stored immediately at –30 to –15°C in a constant-temperature freezer.

Intended Use

All QIAseq miRNA products 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 miRNA Library Kit, QIAseq miRNA NGS 12 Index IL , QIAseq miRNA NGS 48 Index IL, and QIAseq miRNA NGS 96 Index IL is tested against predetermined specifications to ensure consistent product quality.

Introduction

QIAseq miRNA enables Sample to Insight, precision next-generation sequencing (NGS) of mature miRNAs on Illumina NGS instruments. This highly optimized solution facilitates both enhanced differential expression analysis using integrated unique molecular indices (UMIs) and novel discovery of miRNA from cells, tissues, and biofluids. The required amount of template for a single QIAseq miRNA sequencing reaction can range from 500 ng to as little as 1 ng of purified total RNA.

In recent years, NGS has emerged as a highly advanced research tool for both high-throughput miRNA expression analysis and novel miRNA discovery. Among commercially available solutions, QIAseq miRNA defines a new generation of small RNA sequencing products and includes several distinct features not found in other sequencing kits. The standard QIAseq miRNA procedure does not require gel purification, excision, and elution, which substantially reduces the required hands-on time and noticeably shortens the length of the whole workflow. Proprietary methodology utilizing modified oligonucleotides efficiently prevents adapter–dimerization in the sequencing library and the highly optimized reaction chemistry virtually eliminates biases and background contaminants, facilitating the preparation of robust, miRNA-specific libraries. The kit also integrates UMIs into the reverse transcription process, enabling unbiased and accurate miRNome-wide quantification of mature miRNAs by NGS. Should a library fail presequencing quality control (QC), in-line controls are included in the library generation procedure to allow the use of real-time PCR for fast and efficient troubleshooting. Both primary and secondary data analysis solutions have been developed to facilitate rapid and robust UMI counting, miRNA mapping and differential expression analysis. Overall, QIAseq miRNA offers an unrivaled Sample to Insight solution for differential expression analysis and discovery of novel miRNAs using NGS (Figure 1).

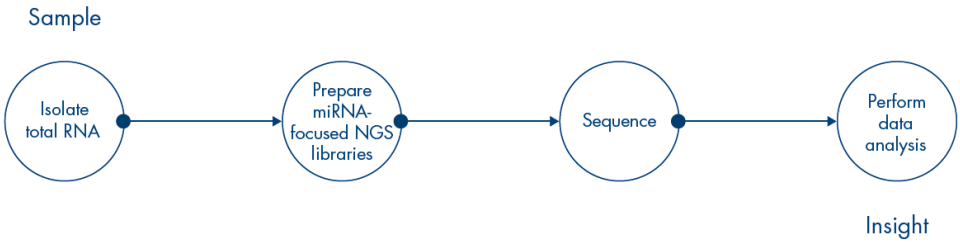


Figure 1. QIAGEN's Sample to Insight QIAseq miRNA workflow.

Principle and procedure

Mature miRNAs are naturally occurring, 22-nucleotide, noncoding RNAs that mediate post-transcriptional gene regulation. Unlike most cellular RNAs, mature miRNAs possess both a 3' hydroxyl group and a 5' phosphate group. This allows adapters to be specifically ligated to both the 3' end and 5' end of miRNAs enabling universal reverse transcription and library preparation of mature miRNAs, while minimizing the background from other RNA species. In addition, the QIAseq miRNA Library Kit enables library preparation and multiplexing of up to twelve samples using QIAseq miRNA NGS 12 Index IL, up to 48 samples in combination with QIAseq miRNA NGS 48 Index IL or up to 96 samples with QIAseq miRNA NGS 96 Index IL.

Universal cDNA synthesis and library preparation of miRNA

In an unbiased reaction, adapters are ligated sequentially to the 3' and 5' ends of miRNAs. Subsequently, universal cDNA synthesis with UMI assignment, cDNA cleanup, library amplification and library cleanup are performed. Proprietary methodology using modified oligonucleotides virtually eliminates the presence of adapter–dimers in the sequencing library, effectively removing a major contaminant often observed during sequencing. Additionally, the kit is designed to also minimize the presence of hY4 Y RNA, which is often observed in high levels in serum and plasma samples.

The following reactions are part of the workflow (Figure 2):

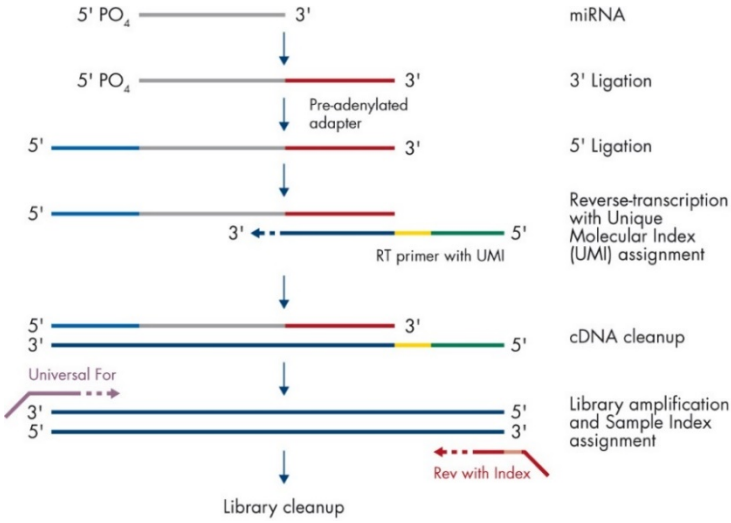


Figure 2. miRNA sequencing library preparation using the QIAseq miRNA Library Kit. Specially designed 3' and 5' adapters are ligated to mature miRNAs. The ligated miRNAs are then reverse transcribed to cDNA using a reverse transcription (RT) primer with a UMI. No libraries are prepared from adapter–dimers. Following cDNA cleanup, library amplification occurs with a universal forward primer and indexing reverse primers. Following a final library cleanup, the miRNA library is then ready for QC and subsequent NGS.

- **3' ligation**: A preadenylated DNA adapter is ligated to the 3' ends of all miRNAs. The QIAseq miRNA NGS 3' Ligase is highly optimized for efficient ligation as well as prevention of undesired side products.
- **5' ligation**: An RNA adapter is ligated to the 5' end of mature miRNAs.
- **cDNA synthesis**: The reverse transcription (RT) primer contains an integrated UMI. The RT primer binds to a region of the 3' adapter and facilitates conversion of the 3'/5' ligated miRNAs into cDNA while assigning a UMI to every miRNA molecule. During reverse transcription, a universal sequence is also added that is recognized by the sample indexing primers during library amplification.
- **cDNA cleanup**: After reverse transcription, a cleanup of the cDNA is performed using a streamlined magnetic bead-based method.

- **Library amplification**: Library amplification is accomplished using one of two formats. In format 1, a wet universal forward primer from a tube is paired with 1 of 48 wet reverse primers from tubes (cat. no. 331592 and 331595) to assign each sample a unique index. In format 2, a dried universal forward primer from a plate is paired with 1 of 96 dried reverse primers in the same plate (cat. no. 331565) to assign each sample a unique custom index. In format 2, library amplification reactions occur directly in the index plate, providing a convenient HT indexing solution. The unbiased amplification of all miRNAs in a single reaction ensures that sufficient target is present for NGS.
- **Library cleanup**: After library amplification, a cleanup of the miRNA library is performed using a streamlined magnetic bead-based method.

NGS on Illumina NGS systems

miRNA sequencing libraries prepared with the QIAseq miRNA Library Kit can be sequenced using an Illumina NGS system (MiSeq® Personal Sequencer, NextSeq 500/550, HiSeq® 1000, HiSeq 1500, HiSeq 2000, HiSeq 2500, HiSeq 4000, NovaSeq™ 6000, and GAIIx). QIAseq miRNA Library Kit derived libraries require 75 bp single reads. It is recommended to allocate 5–10 million reads per sample. A 50 bp single read protocol can be used if there is no desire to include the UMIs. If a 50 bp single read protocol is used, primary data analysis cannot be performed using the QIAseq miRNA Primary Data Analysis pipeline. To use the QIAseq miRNA Primary Data Analysis pipeline, UMIs must be sequenced.

Integrated reaction controls

The QIAseq miRNA Library Kit contains integrated reaction controls to monitor the 3' ligation, 5' ligation, and reverse transcription (Table 3). Together, the controls monitor critical steps of the workflow. If library QC (Protocol: miRNA Library Presequencing QC) is unsuccessful (if, for instance, no peak is observed during Bioanalyzer® analysis), these controls can be assessed using real-time PCR. This helps to determine if the absence of a library is due to a technical or

sample issue (see Appendix B: Real-time PCR Troubleshooting) and at which step the library preparation failed.

Table 3. QIAseq miRNA Library Kit reaction controls

Control	Purpose
QIAseq miRNA NGS 3' Ligation Control (miC3')	Assessment of 3' ligation performance
QIAseq miRNA NGS 5' Ligation Control (miC5')	Assessment of 5' ligation performance
QIAseq miRNA NGS RT Control (miCRT)	Assessment of reverse transcription performance

Data analysis

Primary analysis is available at **geneglobe.qiagen.com**. Here, UMIs are counted and miRNA sequences are mapped. Secondary data analysis for traditional gene expression calculations is also available through the same portal. Using the UMI counts for each miRNA, the software performs differential expression analysis and presents the results in a variety of visual formats.

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.

- Nuclease-free pipette tips and tubes
- Microfuge tubes (1.5–2 ml)
- PCR tubes (0.2 ml individual tubes or tubes strips) (VWR cat. no. 20170-012 or 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 cat. no. 12331D)
- **Library QC Option 1**:
  - 2100 Bioanalyzer® (Agilent)
  - Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)
- **Library QC Option 2**:
  - PAGE gel-related equipment and consumables to prepare and run a 6% PAGE TBE gel
- **Library Concentration Readings**:
  - Qubit™ Fluorometer (Thermo Fisher Scientific)
  - Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific cat. no. Q32854)
  - Qubit Assay Tubes (Thermo Fisher Scientific cat. no. Q32856)

Important Notes

- The QIAseq miRNA Library Kit has been optimized to prepare miRNA (and other similarly sized RNAs with a 3' hydroxyl group and a 5' phosphate group such as piRNA) sequencing libraries for use with Illumina sequencers.

  Total RNA containing miRNA is the required starting material for the QIAseq miRNA Library Kit. It is not necessary to enrich for small RNA. QIAGEN provides a range of solutions for the purification of total RNA including miRNA (Table 4).

Table 4. Recommended kits for purification of total RNA containing miRNA

Kit	Cat. no.	Starting material
miRNeasy Micro Kit	217084	Small amounts of cells and tissue
miRNeasy Mini Kit	217004	Animal/human tissues and cells
miRNeasy 96 Kit	217061	Animal/human tissues and cells
miRNeasy FFPE Kit	217504	FFPE tissue samples
miRNeasy Serum/Plasma Kit	217184	Animal and human plasma and serum
miRNeasy Serum/Plasma Advanced Kit	217204	Animal and human plasma and serum
QIAamp ccfDNA/RNA Kit	55184	Animal and human plasma and serum
exoRNeasy Midi Kit	77144	Animal and human plasma and serum
exoRNeasy Maxi Kit	77164	Animal and human plasma and serum

- Ensure that total RNA samples are of high quality relative to their sample type. For additional information, please see "Appendix C: General Remarks on Handling RNA". **RNA quantification**: Determine the concentration and purity of total RNA isolated from cells and fresh/frozen tissues by measuring the absorbance in a spectrophotometer. As the spectral properties of nucleic acids are highly dependent on pH, we recommend preparing dilutions and measure absorbance in 10 mM Tris·Cl, pH 7.5, instead of RNase-free water. Pure RNA has an A_{260}:A_{280} ratio of 1.9–2.1 in 10 mM Tris·Cl, pH 7.5. It is not useful to assess the concentration and purity of total RNA derived from fluids and/or exosomes.

  RNA integrity: The integrity and size distribution of total RNA from cells and fresh/frozen tissue can be confirmed using an automated analysis system (such as the QIAxcel® Advanced System or the Agilent 2100 Bioanalyzer) that assesses RNA integrity using an RNA integrity score (RIS) or RNA integrity number (RIN). Although the RIN should ideally

be ≥8, successful miRNA library prep is still possible with samples whose RIN values are ≤8. However, for samples with low RIN values, the sequencing reads allocated per sample should be increased to allow for RNA degradation products. This is also the case with FFPE-derived RNA samples, which typically have low RIN values. It is not useful to assess the RNA integrity of total RNA derived from fluids and/or exosomes.

- When working with cell and tissue samples, the recommended starting amount of total RNA is 100 ng. The protocol can be used with 1–500 ng of total RNA.
- When working with serum and plasma samples, the recommended starting amount of total RNA is 5 µl of the RNA eluate when 200 µl of serum/plasma has been processed using the miRNeasy Serum/Plasma Kit or miRNeasy Serum/Plasma Advanced Kit.
- When working with exosome samples prepared from serum and plasma samples, the recommended starting amount of total RNA is 5 µl of the RNA eluate when 1 ml of serum/plasma has been processed using the exoRNeasy kits.
- Ensure reaction components are added in the order listed.
- Ensure reactions are thoroughly mixed, as well as prepared and incubated at the recommended temperatures. Due to the viscosity of the ligation reactions, correct preparation is crucial for a successful experiment.
- If the workflow is not expected to be completed in one day, convenient stopping points are indicated at the end of particular sections, including "Protocol: cDNA Cleanup" and "Protocol: Library Amplification Using Tube Indices (331592/331595)" or "Protocol: Library Amplification Using HT Plate Indices (331565)".
- If the miRNA library (approximately 180 bp on a Bioanalyzer or 173 bp on a PAGE gel) is not detectable during "Protocol: miRNA Library Presequencing QC", it is highly recommended to perform real-time PCR quality control (see "Appendix B: Real-time PCR Troubleshooting"). During the real-time PCR quality control, 3 controls are targeted to assess 3' ligation, 5' ligation, and reverse transcription efficiency. Performing this QC assesses whether or not the library preparation procedure (3' Ligation, 5' Ligation, Reverse Transcription, QIAseq miRNA NGS Bead Preparation, cDNA Cleanup, Library Amplification, and Library Cleanup) has been performed correctly and can provide important insight for troubleshooting.
- During setup of the sequencing run, select **FASTQ Only** and choose **TruSeq Small RNA** from the Sample Prep Kit dropdown menu. To make use of the UMIs, the recommended protocol is 75 bp single read. A 50 bp single read protocol can be used if there is no desire to include the UMIs, but the QIAseq miRNA primary data analysis pipeline cannot be used.

Protocol: 3' Ligation

Important points before starting

- When working with cell and tissues samples, the recommended starting amount of total RNA is 100 ng.
- When working with serum and plasma samples, the recommended starting amount of total RNA is 5 µl of the RNA eluate when 200 µl of serum/plasma have been processed using either the miRNeasy Serum/Plasma Advanced Kit or miRNeasy Serum/Plasma Kit. The recommended starting amount of total RNA is 5 µl of the RNA eluate when 1 ml of serum/plasma has been processed using the exoRNeasy kits.
- When working with low total RNA inputs amounts or serum/plasma samples, the QIAseq miRNA NGS 3' Adapter must be diluted according to Table 5.
- Set up the 3' ligation reactions on ice, adding the components in the order listed.
- The 3' ligation reactions are very viscous. To mix, pipet slowly and thoroughly (pipet up and down at least 15–20 times).
- Do not vortex QIAseq miRNA NGS RI, QIAseq miRNA NGS 3' Ligase, template RNA, or the 3' ligation reactions.
- Upon completion of the 3' ligation reactions, proceed immediately to "Protocol: 5' Ligation".

Procedure

1. Thaw template RNA on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and return to ice.
2. Prepare reagents required for the 3' ligation reactions. Thaw QIAseq miRNA NGS 3' Adapter, QIAseq miRNA NGS 3' Buffer, 2x miRNA Ligation Activator, and Nuclease-free Water at room temperature (15–25°C). Mix each solution by flicking the tubes. Centrifuge the tubes briefly to collect any residual liquid from the sides of the tubes and keep at room temperature.

   Remove QIAseq miRNA NGS RI and QIAseq miRNA NGS 3' RNA Ligase from the –30 to –15°C freezer just before use, and place on ice. Return both enzymes to the freezer immediately after use.

3. If working with low RNA inputs or serum/plasma samples, dilute the QIAseq miRNA NGS 3' Adapter using nuclease-free water according to Table 5. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

Table 5. Dilution of the QIAseq miRNA NGS 3' Adapter

Template RNA input (total RNA)	Adapter dilution
500 ng	Use undiluted
100 ng	Use undiluted
10 ng	Dilute 1:5
1 ng	Dilute 1:10
Serum/Plasma	Dilute 1:5

4. On ice, prepare the 3' ligation reaction according to Table 6. Briefly centrifuge, mix by pipetting up and down 15–20 times, and centrifuge briefly again.

   Important: Pipet slowly when mixing the reaction. 2x miRNA Ligation Activator is very viscous.

   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 6. Setup of 3' ligation reactions

Component	Volume/reaction
Nuclease-free Water	Variable
QIAseq miRNA NGS 3' Adapter*	1 µl
QIAseq miRNA NGS RI	1 µl
QIAseq miRNA NGS 3' Ligase	1 µl
QIAseq miRNA NGS 3' Buffer	2 µl
2x miRNA Ligation Activator	10 µl
Template RNA (added in step 5)	Variable†‡
Total volume	**20 µl**

* For low input and serum/plasma RNA, the QIAseq miRNA NGS 3' Adapter must be diluted according to Table 5.

† For cell and tissue samples, the recommended starting amount of total RNA is 100 ng.

‡ For serum/plasma samples, the recommended starting amount of total RNA is 5 µl of the RNA eluate when 200 µl of serum/plasma have been processed using either the miRNeasy Serum/Plasma Advanced Kit or the miRNeasy Serum/Plasma Kit. The recommended starting amount of total RNA is 5 µl of the RNA eluate when 1 ml of serum/plasma have been processed using the exoRNeasy kits.

5. Add template RNA to each tube containing the 3' ligation Master Mix. Briefly centrifuge, mix by pipetting up and down 15–20 times, and centrifuge briefly again.

   Important: Pipet slowly to mix. The reaction mix is very viscous.

6. Incubate for 1 h at 28°C.
7. Incubate for 20 min at 65°C.
8. Hold at 4°C.

   Important: Hold at 4°C for at least 5 min.

9. Proceed immediately to "Protocol: 5' Ligation".

Protocol: 5' Ligation

Important points before starting

- The entire 20 µl 3' ligation reaction completed in "Protocol: 3' Ligation" is the starting material for the 5' ligation reaction.
- The 5' ligation components are added directly to the tube containing the completed 3' ligation reaction.
- When working with low RNA inputs or serum/plasma samples, the QIAseq miRNA NGS 5' Adapter must be diluted according to Table 7.
- Set up the 5' ligation reactions on ice, adding the components in the order listed.
- The 5' ligation reactions are very viscous. Pipet slowly and thoroughly (pipet up and down 15–20 times) to mix the reaction.
- Do not vortex the QIAseq miRNA NGS RI, QIAseq miRNA NGS 5' Ligase, or 5' ligation reactions.
- Upon completion of the 5' ligations reactions, proceed immediately to "Protocol: Reverse Transcription".

Procedure

1. Prepare reagents required for the 5' ligation reactions. Thaw QIAseq miRNA NGS 5' Adapter and QIAseq miRNA NGS 5' Buffer at room temperature. Mix by flicking the tube. Centrifuge the tube briefly to collect residual liquid from the sides of the tube and keep at room temperature.

   Remove QIAseq miRNA NGS RI and QIAseq miRNA NGS 5' Ligase from the –30 to –15°C freezer just before preparation of the Master Mix, and place on ice. Return both enzymes to the freezer immediately after use.
2. If working with low RNA inputs or serum/plasma samples, dilute the QIAseq miRNA NGS 5' Adapter using nuclease-free water according to Table 7. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

Table 7. Dilution of the QIAseq miRNA NGS 5' Adapter

Template RNA Input (total RNA)	Adapter dilution
500 ng	Use undiluted
100 ng	Use undiluted
10 ng	Dilute 1:2.5
1 ng	Dilute 1:5
Serum/Plasma	Dilute 1:2.5

3. On ice, prepare the 5' ligation reaction according to Table 8, adding the components in the order listed. Briefly centrifuge, mix by pipetting up and down 10–15 times, and centrifuge briefly again.

   Important: Pipet slowly when mixing the reaction. The reaction mix is very viscous.

   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 8. Setup of 5' ligation reactions

Component	Volume/reaction
3' ligation reaction (already in tube)	20 µl
Nuclease-free Water	15 µl
QIAseq miRNA NGS 5' Buffer	2 µl
QIAseq miRNA NGS RI	1 µl
QIAseq miRNA NGS 5' Ligase	1 µl
QIAseq miRNA NGS 5' Adapter*	1 µl
Total volume	**40 µl**

* For low input and serum/plasma RNA, the QIAseq miRNA NGS 5' Adapter must be diluted according to Table 7.

4. Incubate for 30 min at 28°C.
5. Incubate for 20 min at 65°C.
6. Hold at 4°C.
7. Proceed immediately to "Protocol: Reverse Transcription".

Protocol: Reverse Transcription

Important points before starting

- The entire 40 µl 5' ligation reaction completed in "Protocol: 5' Ligation" is the starting material for the reverse transcription reaction.
- The reverse transcription components are added directly to the tube containing the completed 5' ligation reaction.
- When working with low RNA inputs or serum/plasma samples, the QIAseq miRNA NGS RT Primer must be diluted according to Table 10.
- Set up reverse transcription reactions on ice.
- Do not vortex the QIAseq miRNA NGS RI, QIAseq miRNA NGS RT Enzyme, or reverse transcription reactions.
- Upon completion of the reverse transcription reactions, proceed immediately to "Protocol: Preparation of QIAseq miRNA NGS Beads (QMN Beads)".

  Note: This protocol can be performed while the reverse transcription reactions are incubating.

Procedure

1. Prepare reagents required for the reverse transcription reactions. Thaw QIAseq miRNA NGS RT Initiator, QIAseq miRNA NGS RT Buffer, and QIAseq miRNA NGS RT Primer at room temperature. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes, and keep at room temperature.

   Remove QIAseq miRNA NGS RI and QIAseq miRNA NGS RT Enzyme from the –30 to –15°C freezer just before preparation of the Master Mix, and place on ice. Return both enzymes to the freezer immediately after use.
2. Add 2 µl QIAseq miRNA NGS RT Initiator to each tube. Briefly centrifuge, mix by pipetting up and down 15–20 times, and centrifuge briefly again.
3. Incubate the tubes as described in Table 9.

Table 9. Incubation of tubes with QIAseq miRNA NGS RT Initiator

Time	Temperature
2 min	75°C
2 min	70°C
2 min	65°C
2 min	60°C
2 min	55°C
5 min	37°C
5 min	25°C
∞*	4°C

* Hold until setup of the RT reaction.

4. If working with low RNA inputs or serum/plasma samples, dilute the QIAseq miRNA NGS RT Primer using nuclease-free water according to Table 10.

Table 10. Dilution of the QIAseq miRNA NGS RT Primer

Template RNA input (total RNA)	RT Primer dilution
500 ng	Use undiluted
100 ng	Use undiluted
10 ng	Dilute 1:5
1 ng	Dilute 1:10
Serum/Plasma	Dilute 1:5

5. On ice, prepare the reverse transcription reaction according to Table 11. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly 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. Setup of reverse transcription reactions

Component	Volume/reaction
5' ligation reaction + QIAseq miRNA NGS RT Initiator (already in tube)	42 µl
QIAseq miRNA NGS RT Primer*	2 µl
Nuclease-free Water	2 µl
QIAseq miRNA NGS RT Buffer	12 µl
QIAseq miRNA NGS RI	1 µl
QIAseq miRNA NGS RT Enzyme	1 µl
Total volume	**60 µl**

* For low input and serum/plasma RNA, the QIAseq miRNA NGS RT Primer must be diluted according to Table 10.

6. Incubate for 1 h at 50°C.
7. Incubate for 15 min at 70°C.
8. Hold at 4°C.

   Important: Hold at 4°C for at least 5 min.
9. Proceed to "Protocol: Preparation of QIAseq miRNA NGS Beads (QMN Beads)".

Protocol: Preparation of QIAseq miRNA NGS Beads (QMN Beads)

Important points before starting

- This protocol prepares the QIAseq miRNA NGS Beads, hereafter referred to as QMN Beads. QIAseq Beads are rebuffered with QIAseq miRNA NGS Bead Binding Buffer to create QMN Beads.
- **Important**: QIAseq Beads and the subsequently prepared QMN Beads need to be homogenous. This necessitates working quickly and resuspending the beads thoroughly immediately before use. If a delay in the protocol occurs, simply vortex the beads again.
- **Important**: After preparation, the QMN Beads need to be placed on ice.

Procedure

1. Thoroughly vortex QIAseq Beads and QIAseq miRNA NGS Bead Binding Buffer to ensure that the beads are in suspension and homogenously distributed. Do not centrifuge the reagents.

   Important: QIAseq Beads need to be homogenous. This necessitates working quickly and thoroughly resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads again.

2. Carefully add 400 µl of QIAseq Beads (bead storage buffer is viscous) to a 2 ml microfuge tube. This amount of beads is sufficient to perform "Protocol: cDNA Cleanup" and the cleanup associated with library amplification for one sample. Briefly centrifuge and immediately separate beads on a magnet stand.

   Note: Beads for up to 4 samples (1.6 ml) can be prepared at one time in a single 2 ml tube. If beads for multiple samples are processed together, simply scale up the amounts of QIAseq Beads and QIAseq miRNA NGS Binding Buffer added below.

3. When beads have fully migrated, carefully remove and discard the supernatant.

   Note: At this step, it is acceptable to leave a small amount of supernatant in the tube.

4. Remove the tube from the magnet stand, and carefully pipet (buffer is viscous) 150 µl of QIAseq miRNA NGS Bead Binding Buffer onto the beads. Thoroughly vortex to completely resuspend the bead pellet. Briefly centrifuge and immediately separate the beads on a magnet stand.

5. When beads have fully migrated, carefully remove and discard the supernatant.

   Note: Without disturbing the beads, ensure that as much supernatant as possible has been removed.

6. Remove the tube from the magnet stand and carefully pipet 400 µl of QIAseq miRNA NGS Bead Binding Buffer onto the beads (buffer is viscous). Thoroughly vortex to completely resuspend the bead pellet.

   Preparation of the QMN Beads is now complete. If the beads will not be used immediately, store beads on ice or at 2–8°C.

   Note: QMN Beads can be stored at 2–8°C for up to one week.

7. Proceed to “Protocol: cDNA Cleanup“.

Protocol: cDNA Cleanup

Important points before starting

- The entire 60 µl cDNA synthesis completed in "Protocol: Reverse Transcription" is the starting material for the cleanup procedure.
- The QMN Beads prepared in "Protocol: Preparation of QIAseq miRNA NGS Beads (QMN Beads)" are required for the cleanup procedure.
- Beads cleanups can be performed in tubes or plates. When working with plates, perform brief centrifugations at 2000 rpm for 2 min.
- Prepare fresh 80% ethanol using nuclease-free water.
- **Important**: Following ethanol washes, beads must be completely dried. Specific recommendations are given to remove excess ethanol.

Procedure

1. Ensure that the QMN 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 again.
2. Centrifuge the tubes/plates containing the cDNA reactions.
3. Add 143 µl of QMN Beads to tubes/plates containing the cDNA reactions. Vortex for 3 s and centrifuge briefly.
   Note: When working with plates, centrifuge at 2000 rpm for 2 min.
   Note: If plates are warped, transfer mixtures to new plates.
4. Incubate for 5 min at room temperature.
5. Place the tubes/plates on a magnet stand for ~4 min or until the beads have fully migrated.

   Note: Ensure that the beads have fully migrated before proceeding.

6. Discard the supernatant and keep the beads.

   Note: Do not remove the tubes/plates from the magnet stand.

7. With the beads still on the magnet stand, add 200 µl of 80% ethanol. Immediately remove and discard the ethanol wash.

8. Repeat the wash by adding 200 µl of 80% ethanol. Immediately remove and discard the second ethanol wash.

   Important: Completely remove all traces of ethanol after the second wash. Briefly centrifuge (centrifuge plates at 2000 rpm) and return the tubes/plates to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol.

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

   Note: Visually inspect that the pellets are completely dry and that all residual ethanol has evaporated. Residual ethanol can hinder amplification efficiency in the subsequent library amplification reactions. Depending on humidity, extended drying time may be required.

10. With the beads still on the magnetic stand, elute the DNA by adding 17 µl of nuclease-free water to the tubes/plates. Subsequently close/cover and remove the tubes/plates from the magnetic stand.

11. Carefully pipet up and down until all the beads are thoroughly resuspended, briefly centrifuge, and incubate at room temperature for 2 min.

12. Return the tubes/plates to the magnetic stand for ~2 min or until the beads have fully migrated.

    Note: Ensure that the beads have fully migrated before proceeding.

13. Transfer 15 µl of eluted DNA to new tubes/plates.

14. Proceed to "Protocol: Library Amplification Using Tube Indices (331592/331595)" or "Protocol: Library Amplification Using HT Plate Indices (331565)". Alternatively, the completed cDNA cleanup product can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: Library Amplification Using Tube Indices (331592/331595)

Important points before starting

- This library amplification protocol uses tube indices from QIAseq miRNA NGS 12 Index IL (331592) or QIAseq miRNA NGS 48 Index IL (331595). If using QIAseq miRNA NGS 96 Index IL (331565), proceed to "Protocol: Library Amplification Using HT Plate Indices (331565)".
- 15 µl of the product from "Protocol: cDNA Cleanup" is the starting material for the library amplification procedure.
- Set up library amplification reactions on ice.
- Do not vortex the HotStarTaq DNA Polymerase or library amplification reactions.
- **Important**: During bead cleanups, beads must be completely dried following the ethanol washing step. Specific recommendations are given to remove excess ethanol.

Procedure

1. Prepare reagents required for the library amplification reactions. Thaw QIAseq miRNA NGS Library Buffer, QIAseq miRNA NGS ILM Library Forward Primer, and required index primer(s) – QIAseq miRNA NGS ILM IPD1 through IPD48 are options, and the respective index sequences are listed in Table 1 (page 7) and Table 2 (page 8) – from QIAseq miRNA index kits 331592/331595. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes.

   Remove HotStarTaq DNA Polymerase from the –30 to –15°C freezer just before preparation of the Master Mix, and place on ice. Return HotStarTaq DNA Polymerase to the freezer immediately after use.

2. On ice, prepare the library amplification reaction according to Table 12. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly 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 12. Setup of library amplification reactions when using tube indices

Component	Volume/reaction
Product from "Protocol: cDNA Cleanup"	15 µl
QIAseq miRNA NGS Library Buffer	16 µl
HotStarTaq DNA Polymerase	3 µl
QIAseq miRNA NGS ILM Library Forward Primer	2 µl
QIAseq miRNA NGS ILM IPD1 through IPD48 (Index Primer)*	2 µl
Nuclease-free water	42 µl
Total volume	**80 µl**

* Up to 48 different QIAseq miRNA NGS ILM IPD primers (Index Primers) are available for use.

3. Program the thermal cycler according to Table 13. The correct number of cycles depends on the original RNA input and is shown in Table 14.

Table 13. Library amplification protocol

Step	Time	Temperature
Hold	15 min	95°C
3-step cycling (see Table 14 for number of cycles)		
Denaturation	15 s	95°C
Annealing	30 s	60°C
Extension	15 s	72°C
Hold	2 min	72°C
Hold	∞*	4°C

* Hold at 4°C for at least 5 min.

Table 14. Cycles of library amplification

Original RNA input (total RNA)	Cycle number
500 ng	13
100 ng	16
10 ng	19
1 ng	22
Serum/Plasma	22

4. Place the library amplification reaction in the thermal cycler and start the run.

   Important: Upon completion of the protocol, hold at 4°C for at least 5 min.

5. Add 75 µl of QMN Beads to tubes.

   Note: Ensure the QMN 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.

6. Briefly centrifuge the 80 µl library amplification reactions, and transfer 75 µl to the tubes containing the QMN Beads. Vortex for 3 s and briefly centrifuge.

7. Incubate for 5 min at room temperature.

8. Place tubes on a magnet stand for approximately 4 min or until the beads have fully migrated.

   Note: Ensure that the beads have fully migrated before proceeding.

9. Keep the supernatant, and transfer 145 µl of the supernatant to new tubes. Discard the tubes containing the beads.

   Important: Do not discard the supernatant at this step.

10. To the 145 µl supernatant, add 130 µl of QMN Beads. Vortex for 3 s and briefly centrifuge.

11. Incubate at room temperature for 5 min.

12. Place the tubes on a magnet stand until beads have fully migrated.

    Note: Ensure that the beads have fully migrated before proceeding.

13. Discard the supernatant and keep the beads.

    Note: Do not remove the tubes from the magnet stand.

14. With the beads still on the magnet stand, add 200 µl of 80% ethanol. Immediately remove and discard the ethanol wash.

15. Repeat the wash by adding 200 µl of 80% ethanol. Immediately remove and discard the second ethanol wash.

    Note: It is important to completely remove all traces of the ethanol wash after the second wash. Briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol.

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

    Note: Visually inspect that the pellet is completely dry and that all residual ethanol has evaporated. Depending on humidity, extended drying time may be required.

17. With the beads still on the magnetic stand, elute the DNA by adding 17 µl of nuclease-free water to the tubes. Subsequently close and remove the tubes from the magnetic stand.

18. Carefully pipet up and down until all beads are thoroughly resuspended; briefly centrifuge and incubate at room temperature for 2 min.

19. Place the tubes on the magnetic stand for ~2 min (or until beads have cleared).

    Note: Ensure that the beads have fully migrated before proceeding.

20. Transfer 15 µl of eluted DNA to new tubes. This is the miRNA Sequencing Library.

21. Proceed to "Protocol: miRNA Library Presequencing QC". Alternatively, the completed miRNA Sequencing Library can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: Library Amplification Using HT Plate Indices (331565)

Important points before starting

- This library amplification protocol uses plate indices from QIAseq miRNA NGS 96 Index IL (331565). If using QIAseq miRNA NGS 12 Index IL (331592) or QIAseq miRNA NGS 48 Index IL (331595), proceed to "Protocol: Library Amplification Using Tube Indices (331592/331595)".
- 15 µl of the product from "Protocol: cDNA Cleanup" is the starting material for the library amplification procedure.
- Set up library amplification reactions on ice.
- Do not vortex the HotStarTaq DNA Polymerase or library amplification reactions.
- **Important**: During bead cleanups, beads must be completely dried following the ethanol washing step. Specific recommendations are given to remove excess ethanol.

Procedure

1. Prepare reagents required for the library amplification reactions. Thaw QIAseq miRNA NGS Library Buffer, mix by flicking the tube, and centrifuge the tube briefly to collect residual liquid from the sides of the tubes.
   Remove HotStarTaq DNA Polymerase from the –30 to –15°C freezer just before preparation of the Master Mix and place on ice. Return HotStarTaq DNA Polymerase to the freezer immediately after use.
2. Open the QIAseq miRNA NGS 96 Index IL index plate, and choose the wells required for amplification.
   Note: This is a cuttable plate that contains a dried universal primer in every well and a custom 8-base indexing primer (MIHTIL1 through MIHTIL96) in every well. The layout is described in Table 15.
   Note: During reaction setup in step 3, components are added directly to the plate. It is recommended to perform reactions in sets of either 8 or 12.

Table 15. QIAseq miRNA NGS 96 Index IL index plate (MIIL-001)

	1	2	3	4	5	6	7	8	9	10	11	12
A	ACAA GACG	TTGG ACCT	GACT TACG	TTGA GCAC	CGTT AGAG	AATA GGCC	GCTA GATC	AGGT AGGT	AGAG CCTT	CGTT CCTA	CGCC AATT	TATA CCGG
B	TGCC TTAC	GTTC GTTG	TCGG TAGA	CTGG TTCA	CAAC TTGG	AGAG TACC	CACA TGCA	GCAT ATGG	GATG GCTT	TCCG TCTT	TGAC GAAG	CTAG GAAC
C	CTCG AGAA	TTAC GGCA	GTGT GAAG	GCGT AACA	ACAC TGAG	GGTC TCAA	CCAT GTTC	CTCT TCTG	CACT ACGA	ACGA TCCT	GCCG ATTA	AGCG ATAG
D	CGAT GACA	AGAA CGCA	TCAA GCGT	AAGA CGAG	CCGA ATGT	AGAA TCGG	ACAC CAGT	CCGA CATA	ACTG CGAT	CAAG CAGA	CGGA ACAA	TCAT CGGA
E	AAGG AGCA	GGAC CATA	GAGA CACT	GTAA CCGA	GTCA CGAT	CGAT CTGT	GACA TCAC	CACT CTAC	CGTA CAAC	TTAG CTGC	GAAT CCTC	TACG TGAG
F	CACA CCTT	ATTG GCCA	ATGG TGAC	TAGC CAAC	CCGC TAAT	TGTA AGGC	GACT GGAA	GAAC GTAC	CCTT GCAT	AAGC AAGG	TGGT GTTG	AGGT CATC
G	AGCA GCAT	CTCA ATCC	GGAA GGTT	GGTT GTGA	GTGT TCGT	AATC CGGA	GCCT CATT	CTCC GTAT	CTGG AATG	ACCA CTTG	ATCG TAGG	ACGT ACAG
H	GGTA ACCT	CACA GAAG	AATC GCAG	CAAC CGAT	TTCC GATC	ACAG GTGA	CGAT TCAC	TGCG CAAT	CAAC GCTA	CTTC CACT	ACTT GGTG	TCTC CATG

Universal primer and indexing primers are predried as single-use plates. During reaction setup, components are added directly to the plate. There is no need to reconstitute and transfer indices to a separate plate.

3. On ice, prepare the library amplification reaction according to Table 16. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

   Note: Reactions components are added directly to plate MIIL-001.

   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 16. Setup of library amplification reactions when using HT index plate MIIL-001

Component	Volume/reaction
Product from "Protocol: cDNA Cleanup"	15 µl
QIAseq miRNA NGS Library Buffer	8 µl
HotStarTaq DNA Polymerase	1.5 µl
Nuclease-free water	15.5 µl
Total volume	**40 µl**

4. Program the thermal cycler according to Table 17. The correct number of cycles depends on the original RNA input and is shown in Table 18.

Table 17. Library amplification protocol

Step	Time	Temperature
Hold	15 min	95°C
3-step cycling (see Table 18 for number of cycles)		
Denaturation	15 s	95°C
Annealing	30 s	60°C
Extension	15 s	72°C
Hold	2 min	72°C
Hold	∞*	4°C

* Hold at 4°C for at least 5 min.

Table 18. Cycles of library amplification

Original RNA input (total RNA)	Cycle number
500 ng	13
100 ng	16
10 ng	19
1 ng	22
Serum/Plasma	22

5. Place the library amplification reaction in the thermal cycler and start the run.

   Important: Upon completion of the protocol, hold at 4°C for at least 5 min.

6. Briefly centrifuge the 40 µl library amplification reactions.

7. Add 37.5 µl of QMN Beads to plates containing the cDNA reactions. Vortex for 3 s and centrifuge briefly.

   Note: Ensure the QMN 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 again.

   Note: When working with plates, centrifuge at 2000 rpm.

   Note: If plates are warped, transfer mixtures to new plates.

8. Incubate for 5 min at room temperature.
9. Place plates on a magnet stand for approximately 4 min or until the beads have fully migrated.

   Note: Ensure that the beads have fully migrated before proceeding.

10. Keep and transfer the supernatant to new plates. Discard the plates containing the beads.

    Important: Do not discard the supernatant at this step.

11. To the supernatant, add 65 µl of QMN Beads. Vortex for 3 s, and briefly centrifuge.
12. Incubate at room temperature for 5 min.
13. Place the plates on a magnet stand until beads have fully migrated.

    Note: Ensure that the beads have fully migrated before proceeding.

14. Discard the supernatant and keep the beads.

    Note: Do not remove the tubes from the magnet stand.

15. With the beads still on the magnet stand, add 200 µl of 80% ethanol. Immediately remove and discard the ethanol wash.
16. Repeat the wash by adding 200 µl of 80% ethanol. Immediately remove and discard the second ethanol wash.

    Note: It is important to completely remove all traces of ethanol after the second wash. Briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol.

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

    Note: Visually inspect that the pellet is completely dry and that all residual ethanol has evaporated. Depending on humidity, extended drying time may be required.

18. With the beads still on the magnetic stand, elute the DNA by adding 17 µl of nuclease-free water to the plates. Subsequently cover and remove the plates from the magnetic stand.

19. Carefully pipet up and down until all beads are thoroughly resuspended. Briefly centrifuge, and incubate at room temperature for 2 min.

20. Place the plates on the magnetic stand for ~2 min (or until beads have cleared).

    Note: Ensure that the beads have fully migrated before proceeding.

21. Transfer 15 µl of eluted DNA to new plates. This is the miRNA Sequencing Library.

22. Proceed to "Protocol: miRNA Library Presequencing QC". Alternatively, the completed miRNA Sequencing Library can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: miRNA Library Presequencing QC

Important points before starting

- A portion of the 15 µl miRNA Sequencing Library from "Protocol: Library Amplification Using Tube Indices (331592/331595)" or "Protocol: Library Amplification Using HT Plate Indices (331565)" is the starting material for the library QC. When not in use, store the miRNA Sequencing Library on ice.
- Performing 1 of 2 options is recommended for library QC. "Procedure: Option 1" involves the use of an Agilent Bioanalyzer 2100. "Procedure: Option 2" involves use of PAGE gel electrophoresis.

Procedure: Option 1 (Agilent Bioanalyzer 2100)

1. Analyze 1 µl of the miRNA Sequencing Library on an Agilent Bioanalyzer using a High Sensitivity DNA chip according to the manufacturer's instructions. A miRNA-sized library is approximately 180 bp, and a piRNA-sized library is approximately 188 bp. Typical miRNA-sized library results are shown in Figure 3.

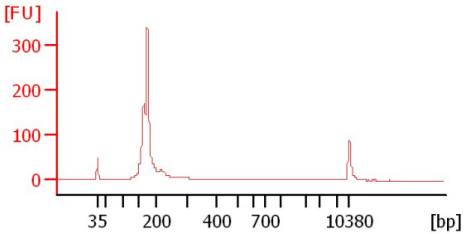


Figure 3. Bioanalyzer trace of miRNA-sized library prepared with the QIAseq miRNA Library Kit.

2. If a large peak (greater than 25% of the height of the miRNA peak) is observed at approximately 157 bp (adapter–dimer), or if other undesired bands are noted, gel excision on the remainder of the miRNA Sequencing Library is recommended to select the specific library of interest (see "Appendix A: Gel Size Selection of Library").

Note: If no library is observed, assess the integrated reaction controls using real-time PCR (see "Appendix B: Real-time PCR Troubleshooting") to determine if the absence of a library is due to a technical issue.

Note: To prevent adapter–dimerization, use 1 ng or more of total RNA and ensure that all reaction components have been added in the order listed.

3. Proceed to "Protocol: Determining Library Concentration and Read Allocation per Sample".

Procedure: Option 2 (PAGE gel electrophoresis)

1. Prepare a 6% PAGE TBE gel.
2. Load 3 µl of the library cleanup product on the gel; use a 25 bp DNA ladder for size reference.
3. Run the gel at 120V for approximately 1 h or until the dye front has reached the bottom of the cassette.
4. Take an image of the gel. A miRNA-sized library is approximately 173 bp, and a piRNA-sized library is approximately 181 bp. Typical results are shown in Figure 4.

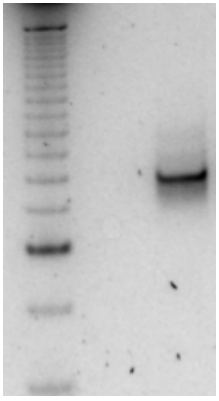


Figure 4. PAGE gel of miRNA-sized library prepared with the QIAseq miRNA Library Kit.

5. If a prominent band is observed at approximately 150 bp (adapter–dimer), or if other undesired bands are observed, perform gel excision on the remainder of the miRNA Sequencing Library to select the specific library of interest (see "Appendix A: Gel Size Selection of Library").

   Note: If no library is observed, assess the integrated reaction controls using real-time PCR (see "Appendix B: Real-time PCR Troubleshooting") to determine if the absence of a library is due to a technical issue.

   Note: To prevent adapter–dimerization, use 1 ng or more of total RNA and ensure that all reaction components have been added in the order listed.

6. Proceed to "Protocol: Determining Library Concentration and Read Allocation per Sample".

Protocol: Determining Library Concentration and Read Allocation per Sample

Important points before starting

- A portion of the 15 µl miRNA Sequencing Library from "Protocol: Library Amplification Using Tube Indices (331592/331595)" or "Protocol: Library Amplification Using HT Plate Indices (331565)" is the starting material for the library QC. When not in use, store the library on ice.
- A Qubit Fluorimeter is recommended to determine the library concentration.

Procedure

1. Determine the concentration of 2 µl of the miRNA Sequencing Library on a Qubit Fluorimeter according to the manufacturer's instructions.
2. Determine the molarity of each sample (in nM) using the following equation. The equation is for a miRNA-sized library.

$$(X\ ng/\mu l)(10^6)/(112450) = Y\ nM$$

3. Dilute individual libraries to 4 nM using nuclease-free water.
4. If multiplexing, combine libraries in equimolar amounts and mix well.
   Important: It is recommended to allocate 5–10 million reads per sample.

Protocol: Preparation for Sequencing

Important points before starting

- The diluted individual or multiplexed 4 nM library from "Protocol: Determining Library Concentration and Read Allocation per Sample" is the starting material for sequencing.
- For complete instructions on how to denature sequencing libraries and set up a sequencing run, please refer to the system-specific Illumina documents.
- Tube indices (331592/331595) use the TruSeq Small RNA 6 bp sample indices.
- HT plate indices (331565) use custom 8 bp sample indices

Sequencing preparations when using Tube Indices (331592/331595)

1. **Sample dilution and pooling preparation**: Prepare the diluted individual or multiplexed 4 nM libraries using the "Standard Normalization Method" protocol in the system-specific Illumina document (i.e., *MiSeq System Denature and Dilute Libraries Guide*, *NextSeq System Denature and Dilute Libraries Guide*, etc.).
2. **Load libraries**: Load libraries and set up the sequencing run using the system-specific Illumina document (i.e., *MiSeq System User Guide*, *NextSeq System User Guide*, etc.). The recommended final library concentration is 10 pM for the MiSeq and 1.2 pM for the NextSeq.
3. **Sequencing run setup**: Select **FASTQ Only** and choose **TruSeq Small RNA** from the Sample Prep Kit dropdown menu.

   The recommended protocol is 75 bp single read. A 50 bp single read protocol can be used if there is no desire to include the UMIs.

   The index sequences for QIAseq miRNA NGS ILM IPD1 through IPD48 are listed in the tables found under "Kit Contents", and the IPD indices are the TruSeq Small RNA 6 bp index sequences.

Sequencing preparations when using HT Plate Indices (331565)

MiSeq

1. Go to **www.qiagen.com/shop/sequencing/qiaseq-mirna-ngs** and select **Product Resources** > **Instrument Technical Documents** to find and download the *MiSeq Custom miRNA* template.
2. The sample sheet already contains all relevant information to use with the instrument.
3. Open the CSV file, delete any MIHTIL indices that will not be used in the experiment, and save the file with a new name.
4. Copy the file into the "Sample Sheet" folder on the MiSeq instrument.
5. When ready to perform run, select the file.
6. **Sample dilution and pooling**: Dilute libraries to 4 nM for the MiSeq. Then, combine libraries with different sample indexes in equimolar amounts if similar sequencing depth is needed for each library.
7. **Library preparation and loading**: Prepare and load library to load on a MiSeq according to the *MiSeq System Denature and Dilute Libraries Guide*. Dilute the denatured library pool a second time, mixing 300 µl denatured library with 300 µl HT1 buffer. The final library concentration is 10 pM on a MiSeq (V3 chemistry).
8. Upon completion of the sequencing run, proceed with "Protocol: Primary and Secondary Data Analysis".

NextSeq: Setup custom library prep kit in BaseSpace Sequence Hub

The steps outlined here are intended for users generating sequencing data on a NextSeq and using the BaseSpace Sequence Hub or a BaseSpace Onsite Sequence Hub system for data analysis, which requires the use of the **Prep** tab for setup. To ensure proper sample index demultiplexing of the custom 8 bp HT plate indices (331565), a custom library prep kit must be created and uploaded through the **Prep** tab. To add a custom library prep kit for the QIAseq miRNA Library Kit RNA Library, perform the following steps:

1. Go to **www.qiagen.com/shop/sequencing/qiaseq-mirna-ngs** and select **Product Resources** > **Instrument Technical Documents** to find and download the *NextSeq Custom miRNA* template.
2. Log in to BaseSpace or BaseSpace Onsite and go to the **Prep** tab screen.
3. From the Prep tab start page, select **Biological Samples**.
4. Choose the samples and click **Prep Libraries**.
5. From the **Library Prep Kit** drop-down menu, select **+Custom Library Prep Kit**. On the screen that appears (Figure 5), name the custom kit **Custom8bpmiRNA** and specify any other options, such as read types (**Single Read**), indexing strategies (**Single Index**) and default read cycles (Read 1 Cycles **75**). Then, click **Choose .csv File** and select **libraryprep_template_Custom8bp_miRNA.csv**.
6. Click **Create New Kit** to generate library prep kit "Custom8bpmiRNA." This new kit now appears in the drop-down menu and is ready for any future runs.

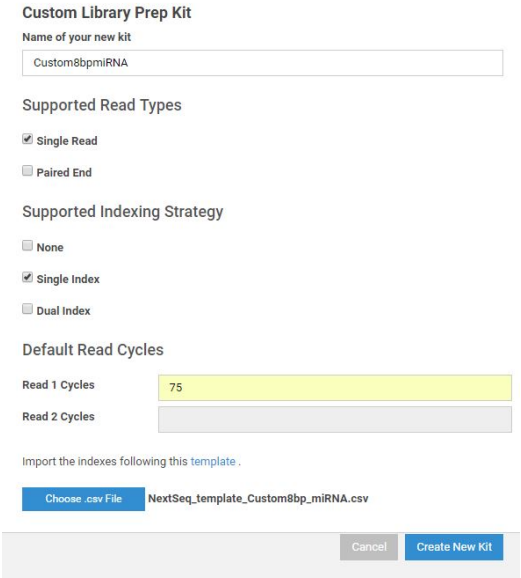


Figure 5. Custom library prep kit setup for the QIAseq miRNA 8 bp Library Kit.

NextSeq: Run planning and sequencing preparations

1. From the drop-down menu on the **Libraries** tab (Figure 6), select library prep kit **Custom8bpmiRNA**, check individual sample and drag it into corresponding well to assign INDEX 1.

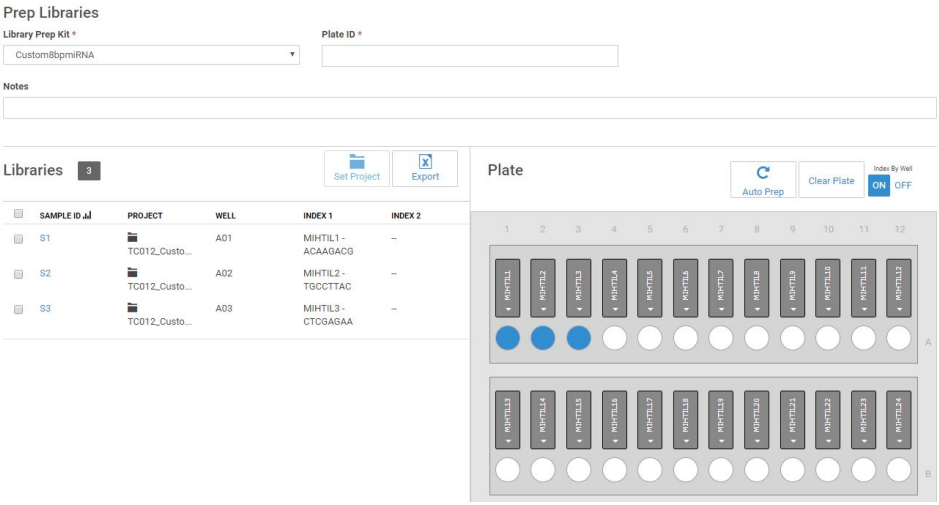


Figure 6. Assigning sample indices in the Libraries tab.

2. Once indices are assigned, select pool on the **Pools** tab and then click **Plan Run**.
   Under "Plan Run" (Figure 7):
   From the **Instrument** drop-down menu, select **NextSeq**.
   Check **Single Read**, and verify **75** for **Read 1 Cycles**.
   Check **Single Index**, and verify **8** for **Index 1 Cycles**.
3. **Sample dilution and pooling**: Dilute libraries to 1 nM for NextSeq. Then, combine libraries with different sample indexes in equimolar amounts, if similar sequencing depth is needed for each library.

4. **Library preparation and loading**: Prepare and load library to load on a NextSeq according to the *NextSeq System Denature and Dilute Libraries Guide*. The final library concentration is 1.2 pM on a NextSeq.
   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).
5. Upon completion of the sequencing run, proceed with "NextSeq run parameters".

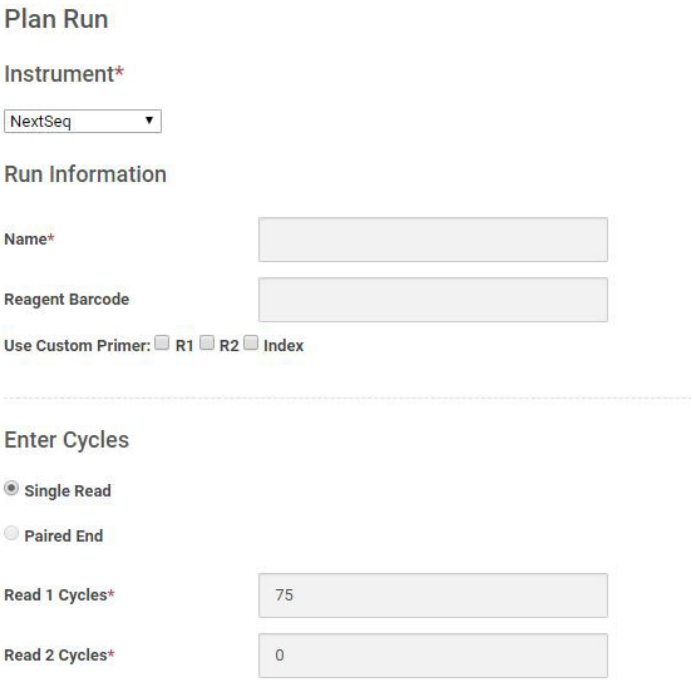


Figure 7. NextSeq run parameters.

Protocol: Primary and Secondary Data Analysis

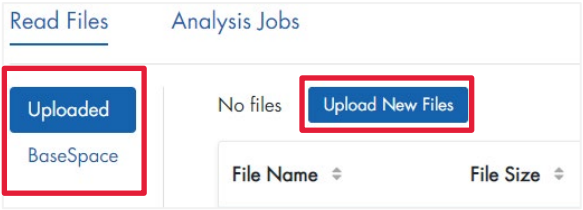
Important points before starting

- Primary analysis is available at **geneglobe.qiagen.com**.
- Through this portal, UMIs are counted and miRNA sequences are mapped.
- **Important**: To ensure a proper secondary data analysis, all samples must be processed in the same miRNA Quantification Job during primary analysis. If FASTQ or FASTQ.GZ files have been derived from different sequencing runs, combine them into one miRNA Quantification Job.
- Secondary data analysis for traditional gene expression calculations is available at **geneglobe.qiagen.com**. Using the UMI counts for each miRNA, the software performs differential expression analysis and presents the results in a variety of visual formats.

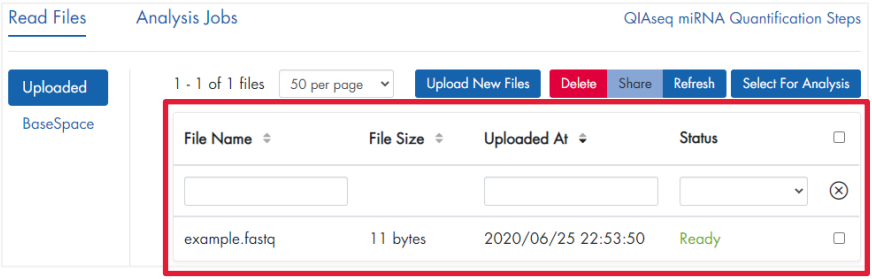
Primary data analysis

1. Go to **geneglobe.qiagen.com/analyze**.

   If prompted, log in to the portal.
2. Select **NGS** and **QIAseq miRNA Library Kit – Primary Quantification**.
3. Click **Start Analysis**.
4. Under the **Read Files** tab, select **Uploaded** > **Upload New Files** to upload files from your computer, or select **BaseSpace** to work with files from BaseSpace.



5. Add FASTQ or FASTQ.GZ files. These will appear in the **Read Files** work area.



6. Check the box beside each file you would like to map, and then click **Select for Analysis**.
7. Under the **Analysis Jobs** tab:
   7a. In **Read Files**, confirm that the correct files are listed.
   7b. Fill out **Job Title**.
   7c. In **QIAseq Spike-ins Added**, select **Yes** or **No**.
   7d. In **Species**, select the correct species from the drop-down list.
   7e. In **File Lanes**, select the applicable option from the drop-down list: **1-lane** (MiSeq/HiSeq/NextSeq concatenated) or **4-lane** (NextSeq [individual lane files])
8. Click Analyze.
9. Periodically, click **Refresh**. Job status will change from **Queued** to **In Progress** and, ultimately, to **Done Successfully**.
10. Click **Report File** to receive the primary analysis output file, or click **Secondary Analysis** to immediately proceed to secondary analysis.

Secondary data analysis – directly from primary analysis

1. Under **Analysis setup**, go to **Sample Manager** to define sample group.
2. Under **Analysis setup**, go to **Select Normalization Method** to choose the normalization option for the data.
3. Under **Analysis**, observe the Fold-Regulation and Fold-Change results.
4. Under **Plots & charts**, observe visual representations of the data.
5. Under **Export data**, choose what data you want to export, and then click **Export**.

Secondary data analysis – directly from GeneGlobe Data Analysis Center

1. Go to **geneglobe.qiagen.com/analyze**.

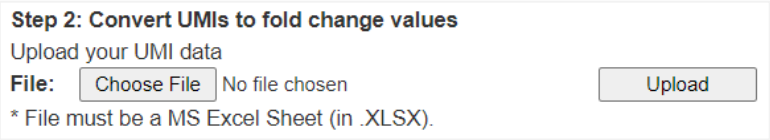
   If prompted, log in to the portal.

2. Select **NGS** and **QIAseq miRNA Library Kit – Secondary Analysis**.
3. Click **Start Analysis**.
4. Upload the miRNA primary analysis report file:

   4a. Select **Choose File**.

   4b. A browser window opens. Browse for the file you want to upload, select the file, and click **Open** in the browser window.

   4c. Select **Upload**.



5. Under **Analysis setup**, go to **Sample Manager** to define sample group.
6. Under **Analysis setup**, go to **Select Normalization Method** to choose the normalization option for the data.
7. Under **Analysis**, observe the Fold-Regulation and Fold-Change results.
8. Under **Plots & charts**, observe visual representations of the data.
9. Under **Export data**, choose what data you want to export, and then click **Export**.

Troubleshooting Guide

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

	Comments and suggestions
During cDNA cleanup or library cleanup, not enough sample can be pipetted	
a) Excess evaporation may have occurred during the previous reaction, or sample may not have been centrifuged prior to cleanup	Check that caps on tubes have a secure fit and that samples are centrifuged prior to cDNA cleanup.
During cDNA cleanup or library cleanup, supernatant does not completely clear after 4–6 min	
This is not a significant issue	Sometimes, samples do not completely clarify. This is possibly due to the cold temperature of the buffer. Simply proceed with the cleanup.
During library prep QC, no library is observed	
a) 3' ligation reaction has not been properly mixed	Once all components have been added to the reaction, briefly centrifuge, mix by pipetting up and down 15–20 times, and centrifuge briefly again.
b) Excess ethanol from the cDNA cleanup has been carried over to the amplification reaction	After the second wash, briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol.
c) Reaction inhibitors are present in the RNA sample, or the reactions were not set up correctly	Perform "Appendix B: Real-time PCR Troubleshooting". During this, 3' ligation, 5' ligation, and RT controls built into the kit are assessed using qPCR. The controls are then interpreted to separate technical issues from sample issues. If the controls exhibit C_T values <28, it suggests that the RNA sample may be compromised. If the controls exhibit C_T values >28, the RNA samples may be compromised, or the experiments could be set up incorrectly. Please review all protocols and ensure that "Appendix B: Real-time PCR Troubleshooting" has been performed correctly.

		Comments and suggestions
During library prep QC, prominent adapter–dimer band is observed at 150 bp (greater than 25%)		
a)	Ensure that the QIAseq miRNA NGS RT Initiator has been added as indicated, between the 5' ligation and RT reactions, and the correct temperature profile has been set up for the initiation	Double check the RT reaction setup.
b)	Ensure that 3' ligation and 5' ligation components were added to their respective reactions in the order listed	Double check 3' ligation and 5' ligation reaction setup.
During library prep QC, a prominent product of approximately 225 bp is observed		
a)	QIAseq Beads were not rebuffered with QIAseq miRNA NGS Binding Buffer to produce QMN Beads	Rebuffer QIAseq Beads with QIAseq miRNA NGS Binding Buffer to produce QMN Beads.
b)	Each reaction was not held at 4°C for 5 min.	At the end of each reaction (3' ligation, reverse transcription, and library amplification) hold at 4°C for at least 5 min.
miRNA Sequencing Library concentrations are too low to obtain a 4 nM library		
Not necessarily a problem		If Library QC suggests the library is of good quality and simply low in concentration, use 2 nM library instead, or sequence the maximum amount possible of that library (either individually or in multiplex with other samples). At the same time, keep all libraries being multiplexed at comparable concentrations.
During primary data analysis, Unique Molecular Indices (UMIs) are not present		
a)	A read length shorter than 75 bp may have been performed	Resequence and ensure that 75 bp single reads are performed.
What are the sequences of the 3' and 5' adapters?		
a)	3' adapter	AACTGTAGGCACCATCAAT
b)	5' adapter	GTTCAGAGTTCTACAGTCCGACGATC

Appendix A: Gel Size Selection of Library

This protocol describes excision of a library from a 6% TBE PAGE gel.

Important points before starting

- The miRNA Sequencing Library from "Protocol: Library Amplification Using Tube Indices (331592/331595)" or "Protocol: Library Amplification Using HT Plate Indices (331565)" is the starting material for gel excision.
- PAGE-gel-related equipment and consumables to prepare and run a 6% PAGE TBE gel are required.
- 5x GelPilot® DNA Loading Dye (cat. no. 239901) or equivalent is required.
- 25 bp DNA Ladder (Thermo Fisher Scientific cat. no. 10597-011) or equivalent is required.
- SYBR® Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific cat. no. S11494) is required.
- Gel Breaker Tubes (Fisher Scientific cat. no. NC0462125) are required.
- Corning® Costar® Spin-X® Centrifuge Tube Filters (Fisher Scientific cat. no. 07-200-387) are required.
- 3 M NaOAc, pH 5.2 is required.
- Linear Acrylamide is required.

Procedure

1. Prepare a 6% PAGE TBE gel.
2. Adjust the volume of the miScript® Sequencing Library to 24 µl using nuclease-free water. Add 6 µl of 5x GelPilot DNA Loading Dye and mix thoroughly.
3. Distribute the mixture across 3 lanes of the 6% PAGE TBE gel.
4. Run the gel at 120V for 1 h or until the dye front has reached the bottom of the cassette.

5. Remove the gel from the cassette and stain with 1x SYBR® Gold for 10 min.
6. Excise the library of choice.

   Note: A miRNA-sized library is approximately 173 bp, and a piRNA-sized library is approximately 181 bp.
7. Place each excised band in a 0.5 ml Gel Breaker tube in a 2 ml tube and centrifuge at max speed for 2 min.
8. Soak the debris in 250 µl 0.3 M sodium acetate.
9. Rotate at room temperature for at least 2 h.
10. Transfer eluate and gel debris to a Corning Costar Spin-X Centrifuge Tube Filter column and centrifuge for 2 min at max speed.
11. Recover eluate and add 1 µl of Linear Acrylamide and 750 µl of 100% ethanol.
12. Vortex and incubate at –80°C for at least 1 h.
13. Centrifuge at 14000 x *g* for 30 min at 4°C.
14. Remove supernatant without disturbing the pellet.
15. Wash the pellet with 500 µl of 80% ethanol.
16. Centrifuge at 14,000 x *g* for 30 min at 4°C.
17. Remove alcohol and air-dry the pellet at 37°C for 10 min.
18. Resuspend pellet in 15 µl water.

Appendix B: Real-time PCR Troubleshooting

Three control primers are provided to assess reaction performance using real-time PCR:

- QIAseq miRNA NGS 3C Primer Assay
- QIAseq miRNA NGS 5C Primer Assay
- QIAseq miRNA NGS RTC Primer Assay

These primers target the miC3', miC5', and miCRT controls, respectively, whose purpose is detailed in Table 3. If library QC (Protocol: miRNA Library Presequencing QC) is unsuccessful (if, for example, no peak is observed during Bioanalyzer analysis), these controls can be used to determine if the absence of a library is due to a technical or sample issue (Appendix B: Real-time PCR Troubleshooting).

Important points before starting

- A portion of the 15 µl miRNA Sequencing Library from "Protocol: Library Amplification Using Tube Indices (331592/331595)" or "Protocol: Library Amplification Using HT Plate Indices (331565)" is the starting material for the library QC.
- The miScript SYBR® Green PCR Kit (cat. no. 218073, 218075, or 218076) is required for this quality control procedure.
- **Important**: The 10x miScript Universal Primer is not used during real-time PCR. The individual primer assays contain both a forward and a reverse primer instead.
- The PCR must start with an initial incubation step of 15 min at 95°C to activate HotStarTaq DNA Polymerase (included in the 2x QuantiTect® SYBR® Green PCR Master Mix).
- **Important**: The recommended number of real-time PCR cycles is 35.
- For the real-time PCR quality control cycling program, there is no need to perform dissociation curve analysis of the PCR product(s).
- Do not vortex the miRNA Sequencing Library or the components of the miScript SYBR® Green PCR Kit.

- If using the iCycler iQ™, iQ5, or MyiQ™, well factors must be collected at the beginning of each experiment. Well factors are used to compensate for any system or pipetting nonuniformity. For details, refer to the user manual supplied with the instrument or Technical Information: Using QuantiTect SYBR® Green Kits on Bio-Rad® cyclers available at **www.qiagen.com**

Procedure

1. Prepare reagents required for the real-time PCR troubleshooting. Thaw control primers and components of the miScript SYBR® Green PCR Kit (2x QuantiTect SYBR® Green PCR Master Mix and nuclease-free water) at room temperature. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes, and keep at room temperature.

   Important: The 10x miScript Universal Primer is not used during real-time PCR.

2. Dilute 1 µl of the miRNA Sequencing Library as described in Table 19. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes, and keep at room temperature.

Table 19. Dilution of miRNA Sequencing Library for real-time PCR troubleshooting

Number of library amplification cycles	Dilution of sequencing library
13	1 µl + 4 µl water
16	1 µl + 49 µl water
19	1 µl + 499 µl water
22	Step 1: 1 µl +49 µl water Step 2: Dilute 1 µl of Step 1 + 99 µl water Use step 2 for qPCR

3. For each sample, prepare a Master Mix for either a 10 µl per well reaction volume (used in 384-well plates), a 25 µl per well reaction volume (used in 96-well plates), or a 20 µl per well reaction volume (used in the Rotor-Disc® 100), according to Table 20. Mix gently and thoroughly.

   Important: Reaction mix contains everything except the control primers. These are added in step 5.

Table 20. Setup of real-time PCR troubleshooting

Component	Master Mix (for 384-well)	Master Mix (for 96-well)	Master Mix (for Rotor-Disc 100)
2x QuantiTect SYBR® Green PCR Master Mix	20 µl	50 µl	40 µl
Control Primer Assay (added in step 5)	–	–	–
Nuclease-free Water	12 µl	36 µl	28 µl
Diluted library amplification product	4 µl	4 µl	4 µl
Total volume	**36 µl**	**90 µl**	**72 µl**

4. For each sample, dispense Master Mix into 3 individual wells of an empty plate/Rotor-Disc (9 µl for 384-well plates, 22.5 µl for 96-well plates, 18 µl for Rotor-Disc 100).
5. Into each sample's 3 wells containing Master Mix, dispense one of the respective 3 control primers (1 µl for 384-well plates, 2.5 µl for 96-well plates, 2 µl for Rotor-Disc 100).
6. Carefully seal the plate or disc tightly with caps, film, or Rotor-Disc Heat-Sealing Film.
7. Centrifuge for 1 min at 1000 x *g* at room temperature (15–25°C) to remove bubbles.

   Note: This step is not necessary for reactions set up in Rotor-Discs.
8. Program the real-time cycler according to Table 21.

   Note: For the real-time PCR quality control cycling program, there is no need to perform dissociation curve analysis of the PCR product(s).

Table 21. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR Initial activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling (35 cycles)*†‡§			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension¶	30 s	70°C	Perform fluorescence data collection.

* For Bio-Rad models CFX96™ and CFX384™: adjust the ramp rate to 1°C/s.

† For Eppendorf® Mastercycler® ep *realplex* models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%.

‡ If using a Roche® LightCycler® 480, adjust the ramp rate to 1°C/s.

§ If using a Roche LightCycler 480, use 45 cycles.

¶ Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM® 7000 or 34 s with the Applied Biosystems 7300 and 7500.

9. Place the plate/Rotor-Disc in the real-time cycler and start the cycling program.
10. When the run is finished, analyze the data. First, define the baseline:

    Use the "Linear View" of the amplification plot to determine the earliest visible amplification. Set the baseline from cycle 2 to two cycles before the earliest visible amplification. The number of cycles used to calculate the baseline can be changed and should be reduced if high template amounts are used.

    Note: Ensure that baseline settings are the same across all PCR runs associated with the same experiment to allow comparison of results.

    Define the threshold. Use a logarithmic amplification plot to set the threshold so that the log-linear range of the curve can be easily identified. Using the "Log View" of the amplification plot, place the threshold above the background signal but within the lower half of the log-linear range of the amplification plot. The threshold should never be set in the plateau phase. The absolute position of the threshold is less critical than its consistent position across PCR runs.

    Note: Ensure that threshold settings are the same across all PCR runs in the same analysis to allow comparison of results.
11. Export the C_T values according to the manual supplied with the real-time PCR cycler.
12. Interpret the C_T values for the miC3', miC5', and miCRT as follows:

    If all the C_T values are less than 28, the individual reaction steps have been performed correctly. If the library preparation had failed QC, this might indicate that the sample was compromised.

    If the C_T values for some or all of the controls are greater than 28, either the respective step of library preparation has not been performed correctly or the sample has been compromised. Ensure that the Real-time PCR troubleshooting protocol has been performed correctly. For comments and suggestions, see the "Troubleshooting Guide."

Appendix C: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. To create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice.

To remove RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment (e.g., pipettes and electrophoresis tanks), general laboratory reagents can be used. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water, or rinse with chloroform* if the plasticware is chloroform resistant. To decontaminate electrophoresis tanks, clean with detergent* (e.g., 0.5% SDS), rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol resistant), and allow to dry.

* 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.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed and oven baked at 240°C for 4 hours or more (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate), as described in "Solutions" below.

Solutions

Note: QIAGEN solutions, such as the components found in the miScript Single Cell qPCR Kit, are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong but not absolute inhibitor of RNases. DEPC is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated, and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers.* DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA–RNA or RNA–RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Ordering Information

Product	Contents	Cat. no.
QIAseq miRNA Library Kit (12)	For 12 sequencing prep reactions: 3' ligation, 5' ligation, reverse transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331502
QIAseq miRNA Library Kit (96)	For 96 sequencing prep reactions: 3' ligation, 5' ligation, reverse transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331505
QIAseq miRNA NGS 12 Index IL (12)	Sequencing adapters, primers and indexes compatible with Illumina platforms. 12 indexes for 12 samples	331592
QIAseq miRNA NGS 48 Index IL (96)	Sequencing adapters, primers and indexes compatible with Illumina platforms. Two 48 indexes for 96 samples	331595
QIAseq miRNA NGS 96 Index IL (96)	Sequencing adapters, primers and custom indexes compatible with Illumina platforms. Cuttable HT format (dried primers) with 96 indexes for 96 samples.	331565
miScript SYBR® Green PCR Kit (200)	For 200 reactions: QuantiTect SYBR® Green PCR Master Mix, miScript Universal Primer	218073
miScript SYBR® Green PCR Kit (1000)	For 1000 reactions: QuantiTect SYBR® Green PCR Master Mix, miScript Universal Primer	218075
miScript SYBR® Green PCR Kit (2000)	For 2000 reactions: QuantiTect SYBR® Green PCR Master Mix, miScript Universal Primer	218076

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
07/2019	In the QIAseq miRNA Library Kit, the location of the 2x miRNA Ligation Activator was changed from Box 1 to Box 2.
03/2020	Change in the volume of QIAseq Beads in Box 2 from 4.8 ml to 10 ml.
07/2020	Changed description of MIIL-001 plates to “cuttable”, from the original “breakable”. Corrected erroneous cross-references (table or step numbers). Updated exoRNeasy Kit recommendations in Table 4. Updated data analysis URLs to geneglobe.qiagen.com. Corrected content of first bullet in “Protocol: cDNA Cleanup”. Updated the procedures in “Protocol: Primary and Secondary Data Analysis”.

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