March 2024

QIAseq® miRNA Library Automation Kit Handbook

Precision small RNA library kit for Illumina® NGS systems using automated library preparation

Sample to Insight

Contents

Kit Contents

QIAseq miRNA Library Auto Kit	(384)
Catalog no.	331509
No. of reactions	384
Box 1 of 2	
QIAseq miRNA 3' Adapter	4 x 96 µL
QIAseq miRNA 3' Buffer	7 x 192 µL
QIAseq miRNA 3' Ligase	7 x 96 µL
QIAseq miRNA RI	5 x 288 µL
Nuclease-free Water	8 x 1.5 mL
QIAseq miRNA 5' Adapter	4 x 96 µL
QIAseq miRNA 5' Buffer	5 x 192 µL
QIAseq miRNA 5' Ligase	5 x 96 µL
QIAseq miRNA RT Initiator	8 x 192 µL
QIAseq miRNA RT Primer	4 x 192 µL
QIAseq miRNA RT Buffer	5 x 1152 µL
QIAseq miRNA RT Enzyme	5 x 96 µL
QIAseq miRNA Library Buffer	5 x 1536 µL
HotStarTaq® DNA Polymerase	5 x 288 µL
QIAseq miRNA 3C Primer Assay	4 x 240 µL
QIAseq miRNA 5C Primer Assay	4 x 240 µL
QIAseq miRNA RTC Primer Assay	4 x 240 µL
Box 2 of 2	
Ligation Activator	10 x 600 µL
QIAseq Beads	6 x 38.4 mL
QIAseq Bead Binding Buffer	6 x 54 mL

QIAseq miRNA 96 Index IL Auto A	(384)
Catalog no.	331569
Number of reactions	384
Each MIIL-001YX microRNA sample index plate is a hard shell plastic, 96 well plate, with 8 µL of pre-mixed indexing primers. The plate is covered with a pierceable foil seal supplied in a foil bag. Each well is intended for single use.	4 plates

Table 1. QIAseq miRNA 96 Index IL Auto A (cat. no. 331569) layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	MIHTIL1	MIHTIL9	MIHTIL17	MIHTIL25	MIHTIL33	MIHTIL41	MIHTIL49	MIHTIL57	MIHTIL65	MIHTIL73	MIHTIL81	MIHTIL89
B	MIHTIL2	MIHTIL10	MIHTIL18	MIHTIL26	MIHTIL34	MIHTIL42	MIHTIL50	MIHTIL58	MIHTIL66	MIHTIL74	MIHTIL82	MIHTIL90
C	MIHTIL3	MIHTIL11	MIHTIL19	MIHTIL27	MIHTIL35	MIHTIL43	MIHTIL51	MIHTIL59	MIHTIL67	MIHTIL75	MIHTIL83	MIHTIL91
D	MIHTIL4	MIHTIL12	MIHTIL20	MIHTIL28	MIHTIL36	MIHTIL44	MIHTIL52	MIHTIL60	MIHTIL68	MIHTIL76	MIHTIL84	MIHTIL92
E	MIHTIL5	MIHTIL13	MIHTIL21	MIHTIL29	MIHTIL37	MIHTIL45	MIHTIL53	MIHTIL61	MIHTIL69	MIHTIL77	MIHTIL85	MIHTIL93
F	MIHTIL6	MIHTIL14	MIHTIL22	MIHTIL30	MIHTIL38	MIHTIL46	MIHTIL54	MIHTIL62	MIHTIL70	MIHTIL78	MIHTIL86	MIHTIL94
G	MIHTIL7	MIHTIL15	MIHTIL23	MIHTIL31	MIHTIL39	MIHTIL47	MIHTIL55	MIHTIL63	MIHTIL71	MIHTIL79	MIHTIL87	MIHTIL95
H	MIHTIL8	MIHTIL16	MIHTIL24	MIHTIL32	MIHTIL40	MIHTIL48	MIHTIL56	MIHTIL64	MIHTIL72	MIHTIL80	MIHTIL88	MIHTIL96

Table 2. QIAseq miRNA 96 Index IL Auto A (cat. no. 331569) layout

	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 Automation Kit is shipped in 2 boxes.

- Box 1 is shipped on dry ice. Upon receipt, all components in Box 1 should be stored immediately at –30°C to –15°C in a constant-temperature freezer.
- Box 2 is shipped on blue ice. This should be stored immediately at 2–8°C. The Ligation Activator included in Box 2 can be stored at 2–8°C or stored at –30°C to –15°C.

When stored correctly, the QIAseq miRNA Library Automation Kit is good until the expiration date printed on the kit box.

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

Intended Use

The 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 Automation Kit and the QIAseq miRNA 96 Index IL Auto A 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).

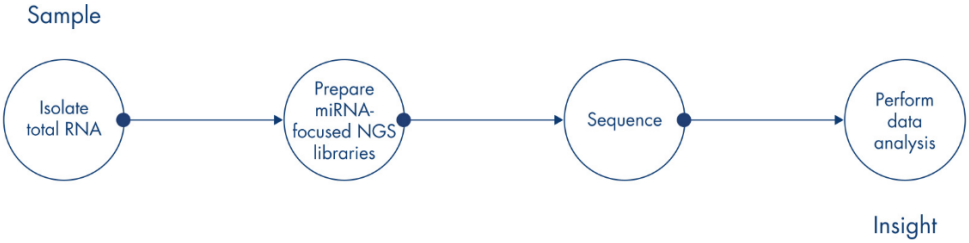


Figure 1. QIAGEN's Sample to Insight QIAseq miRNA workflow.

Principle and procedure

Mature miRNAs are naturally occurring, 22-nucleotide, noncoding RNAs that mediate posttranscriptional 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. The QIAseq miRNA Library Auto Kit (384) with the QIAseq miRNA 96 Index IL Auto A (384) provides enough reagents for automated library preparation of up to 384 samples and multiplexing of up to 96 samples per flow cell lane on Illumina NGS instruments.

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):

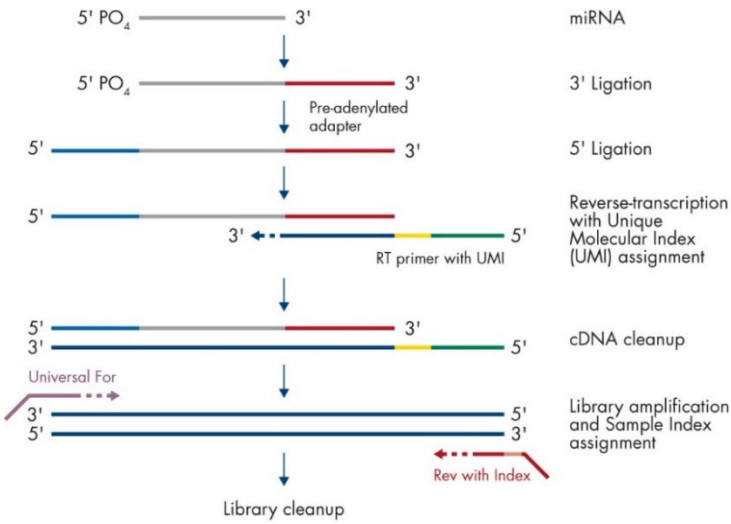


Figure 2. miRNA sequencing library preparation using the QIAseq miRNA Library Kit. Specially designed 3' and 5' adapters are ligated to mature miRNAs. The ligated miRNAs are then reverse transcribed to cDNA using a reverse transcription (RT) primer with a UMI. No libraries are prepared from adapter–dimers. 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 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 a premixed universal forward primer paired with 1 of 96 reverse primers (cat. no. 331569) to assign each sample a unique index. 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 Automation 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 Auto Kit (cat. no. 331509) 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 3' Ligation Control (miC3')	Assessment of 3' ligation performance
QIAseq miRNA 5' Ligation Control (miC5')	Assessment of 5' ligation performance
QIAseq miRNA 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 93001118) 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 the 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 A260:A280 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 QIAseq miRNA 96 Index IL Auto A (cat. no. 331569)“.
- 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 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**. 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 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, pipette slowly and thoroughly (pipette up and down at least 15–20 times).
- Do not vortex QIAseq miRNA RI, QIAseq miRNA 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 3' Adapter, QIAseq miRNA 3' Buffer, 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 RI and QIAseq miRNA 3' RNA Ligase from the –3015°C 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 3' Adapter using Nuclease-free Water according to Table 5. Briefly centrifuge, mix by pipetteting up and down 12 times, and centrifuge briefly again.

Table 5. Dilution of the QIAseq miRNA 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: Pipette slowly when mixing the reaction. The 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.

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: Pipette slowly to mix. The reaction mix is very viscous.

6. Incubate for 1 h at 28°C.

Table 6. Setup of 3' ligation reactions

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

* For low input and serum/plasma RNA, the QIAseq miRNA 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 has been processed using the exoRNeasy kits.

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 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. Pipette slowly and thoroughly (pipette up and down 15–20 times) to mix the reaction.
- Do not vortex the QIAseq miRNA RI, QIAseq miRNA 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 5' Adapter and QIAseq miRNA 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 RI and QIAseq miRNA 5' Ligase from the –30 °C 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 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 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: Pipette 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 (µL)
3' ligation reaction (already in tube)	20
Nuclease-free Water	15
QIAseq miRNA 5' Buffer	2
QIAseq miRNA RI	1
QIAseq miRNA 5' Ligase	1
QIAseq miRNA 5' Adapter*	1
Total volume	**40**

* For low input and serum/plasma RNA, the QIAseq miRNA 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 RT Primer must be diluted according to Table 10.
- Set up reverse transcription reactions on ice.
- Do not vortex the QIAseq miRNA RI, QIAseq miRNA RT Enzyme, or reverse transcription reactions.
- Upon completion of the reverse transcription reactions, proceed immediately to "Protocol: Preparation of QIAseq miRNA 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 RT Initiator, QIAseq miRNA RT Buffer, and QIAseq miRNA 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 RI and QIAseq miRNA RT Enzyme from the –30°C 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 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 RT Initiator

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

* Hold until setup of the RT reaction.

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

Table 10. Dilution of the QIAseq miRNA 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

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. Dilution of the QIAseq miRNA RT Primer

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

* For low input and serum/plasma RNA, the QIAseq miRNA 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 Beads (QMN Beads)".

Protocol: Preparation of QIAseq miRNA Beads (QMN Beads)

Important points before starting

- This protocol prepares the QIAseq miRNA Beads, hereafter referred to as QMN Beads. QIAseq Beads are rebuffered with QIAseq 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 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 Bead 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.

Remove the tube from the magnet stand, and carefully pipette (buffer is viscous) 150 µL of QIAseq 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.

4. 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.

5. Remove the tube from the magnet stand and carefully pipette 400 µL of QIAseq 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.

6. 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 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 pipette 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 QIAseq miRNA 96 Index IL Auto A (cat. no. 331569)". Alternatively, the completed cDNA cleanup product can be stored at –30°C to –15°C in a constant-temperature freezer.

Protocol: Library Amplification Using QIAseq miRNA 96 Index IL Auto A (cat. no. 331569)

Important points before starting

- This library amplification protocol uses plate indices from QIAseq miRNA 96 Index IL Auto A (cat. no. 331569).
- 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 Library Buffer, and required index plates. Mix by flicking the tube or plate, and centrifuge the tube or plate briefly to collect residual liquid from the sides of the tube and plate.
   Remove HotStarTaq DNA Polymerase from the –30 °C 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. From the QIAseq miRNA 96 Index IL Auto A (cat. no. 331569) plate, select the desired wells that will be used to assign each sample a unique index.
   Note: This is a pierceable plate that contains both a universal primer and a custom 8 base pair indexing primer (MIHTIL1 through MIHTIL96) in every well. The layout is described in Table 12.

Table 12. QIAseq miRNA 96 Index IL Auto A (cat. no. 331569) index sequences

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

The combined universal primer and indexing primers are in single-use plates. During reaction setup, indices are transferred directly to the reaction to assign each sample a unique index.

3. On ice, prepare the library amplification reaction according to Table 13. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

   Note: Reactions components are added first with the addition of the indexes from plate MIIL-001 YX added last to assign each sample a unique index.

   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. Setup of library amplification reactions when using QIAseq miRNA 96 Index IL Auto A (cat. no. 331569)

Component	Volume/reaction (µL)
Product from "Protocol: cDNA Cleanup"	15
QIAseq miRNA Library Buffer	8
HotStarTaq DNA Polymerase	1.5
QIAseq miRNA 96 Indexes IL Auto (from plate)	1
Nuclease-free Water	14.5
Total volume	**40**

4. Program the thermal cycler according to Table 14. The correct number of cycles depends on the original RNA input and is shown in Table 15.

Table 14. Library amplification protocol

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

* Hold at 4°C for at least 5 min.

Table 15. 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 pipette 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°C 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 QIAseq miRNA 96 Index IL Auto A (cat. no. 331569)" 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.

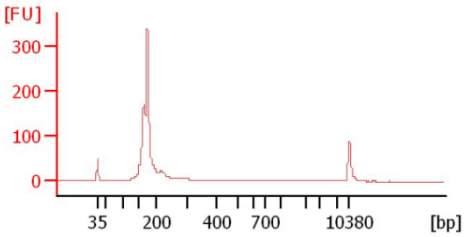


Figure 3. Bioanalyzer trace of miRNA-sized library prepared with the QIAseq miRNA Library Kit.

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.

2. 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.

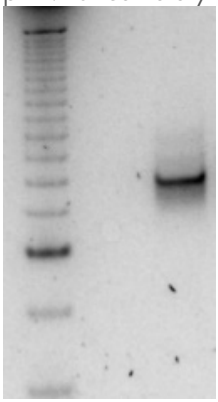


Figure 4. PAGE gel of miRNA-sized library prepared with the QIAseq miRNA Library Kit.

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 QIAseq miRNA 96 Index IL Auto A (cat. no. 331569)" 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: Preparation for Sequencing" 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.
- QIAseq miRNA 96 Index IL Auto A (cat. no. 331569) uses custom 8 bp sample indices.

Generation of sample sheets for Illumina Instruments

Index sequences for QIAseq miRNA 96 Index IL Auto A (cat. no. 331569) are available for download at www.qiagen.com. To make sequencing preparation more convenient, ready-to-use templates that include sample sheets containing all QIAseq miRNA 96 Index IL Auto A (cat. no. 331569) index sequences are available for the different sequencing instruments, MiSeq, MiniSeq, NextSeq, HiSeq, and NovaSeq at **www.qiagen.com** These can be imported and edited using the Illumina Local Run Manager or any text editor. Make sure to download the appropriate sample sheet for the Illumina systems depending on whether you are using Local Run Manager or manually configuring the sequencing run.

All Illumina Instruments

1. Go to **www.qiagen.com/products/discovery-and-translational-research/next-generation-sequencing/metagenomics/qiaseq-mirna-ngs** and select **Product Resources** > **Instrument Technical Documents** to find and download the appropriate QIAseq miRNA Automation templates.
2. The sample sheet already contains all relevant information to use with the instrument.
3. Open the CSV file, delete any 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 or upload the "Sample Sheet" into Local Run Manager for Illumina instruments: MiSeq, MiniSeq, and NextSeq.
5. When ready to perform run, select the file.
6. **Sample dilution and pooling**: Dilute individual libraries to 4 nM except for the NovaSeq dilute individual libraries to 10 nM. Then, combine libraries with different sample indexes in equimolar amounts if similar sequencing depth is needed for each library.

   Note: For the NovaSeq, the final pooled library concentration recommendation is between 1.0–1.5 nM yielding a final loading concentration of between 200–300 pM on the NovaSeq.
7. **Library preparation and loading**: Prepare and load the pooled library on an Illumina instrument according to the specific Illumina instrument guide. Dilute the denatured library pool a second time, to obtain a final library concentration as stated below in Table 16.

Table 16. Recommended final library loading concentrations for Illumina Instruments

Illumina sequencing instrument	Illumina-specific documentation	Final library concentration (pM)
iSeq	*iSeq 100 System Guide*	75
MiSeq	*MiSeq System Guide*	10
MiniSeq	*MiniSeq System Guide*	1.2
NextSeq	*NextSeq 500 System Guide or NextSeq 550 System Guide*	1.2
NextSeq 1000/2000	*NextSeq 1000 and 2000 Denature and Dilute Libraries Guide (1000000139235 v03)*	75
NovaSeq 6000	*NovaSeq 6000 Sequencing System Guide*	200–300

8. Sequencing run setup: Select **FASTQ Only**.

   The recommended protocol is 75 bp single read with 8 bp single indexing. A 50 bp single read protocol can be used if there is no desire to include the UMIs.

9. Upon completion of the sequencing run, proceed with either the “Protocol: Data Analysis Using GeneGlobe Web Based Analysis Tools” or the “Protocol: Legacy Primary and Secondary Data Analysis“.

Protocol: Data Analysis Using GeneGlobe Web Based Analysis Tools

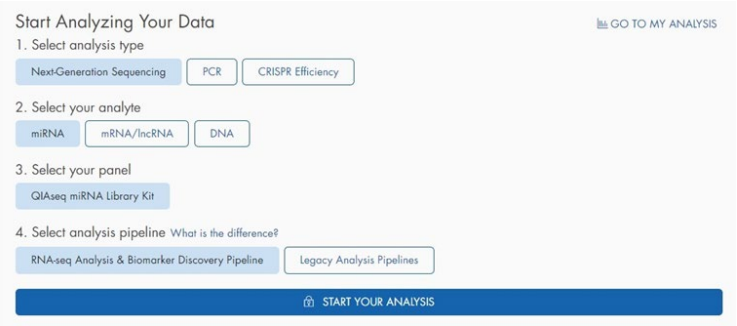
Important points before starting

- Primary and secondary analysis tools are available at **geneglobe.qiagen.com**
- The RNA-seq Analysis & Biomarker Discovery Pipeline uses QIAGEN CLC Biomedical Workbench for read alignment, UMI counting and differential expression, and Ingenuity Pathway Analysis to return the top hits from the QIAGEN knowledge base for canonical pathways, upstream regulators, and diseases.
- Using the RNA-seq Analysis & Biomarker Discovery Pipeline, FASTQ files can be uploaded, miRNA sequences are aligned, and UMIs are counted. Differential miRNA expression will be calculated and visualized using interactive volcano plots. Differentially expressed miRNAs will be queried against the QIAGEN knowledge base for canonical pathways, upstream regulators, and diseases and biological functions. Important microRNAs can then be identified, and digital PCR and qPCR assays are easily found for biological verification.
- For each alignment, a credit is deducted from your account. Credits for using the RNA-seq Analysis & Biomarker Discovery Pipeline are included with QIAseq library kits. Credits can also be purchased for using the RNA-seq Analysis portal with non-QIAGEN kits through **www.qiagen.com**
- The legacy analysis pipelines contain online tools for read alignment, UMI counting, and differential expression. These pipelines for data analysis must have all of the data uploaded simultaneously, and the read alignment and UMI counting must be completed in one session.

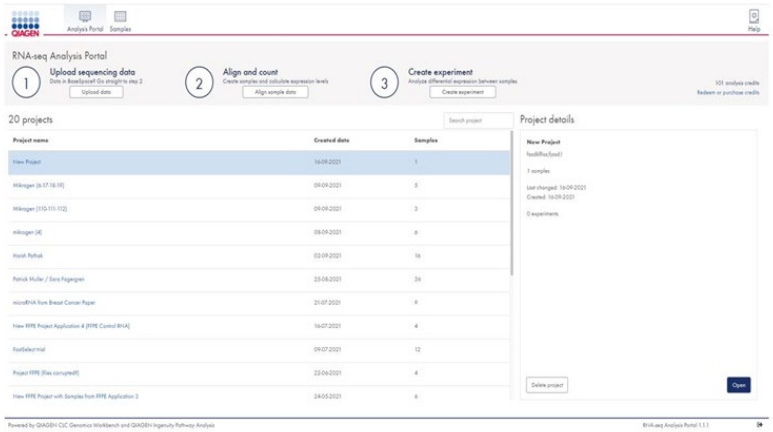
Data analysis steps

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

Log into GeneGlobe with your username and password.



2. Select **Next-Generation Sequencing > miRNA > QIAseq miRNA Library Kit > RNA-seq Analysis & Biomarker Discovery Pipeline**.
3. Click **Start Your Analysis**.
4. Follow the 3 steps: Upload sequencing data, Align and count, and Create an experiment. Directions on how to use the RNA-seq analysis portal can be accessed by clicking Help at the upper-right corner.



Protocol: Legacy Primary and Secondary Data Analysis

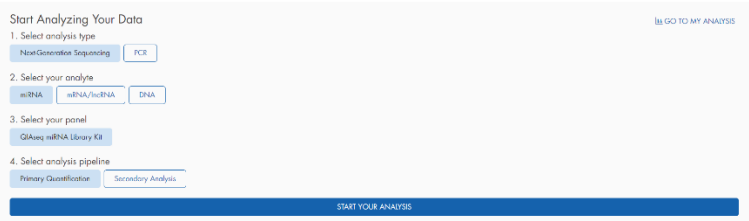
Important points before starting

- 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

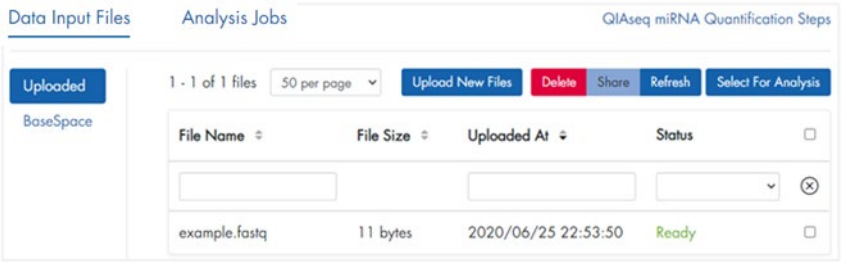
1. Go to **geneglobe.qiagen.com/analyze**.

   If prompted, log in to the portal.
2. Select **Next-Generation Sequencing** > **miRNA** > **QIAseq miRNA Library Kit** > **Primary Quantification**.
3. Click **Start Your Analysis**.



4. Under the **Data Input 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 **Data Input 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.

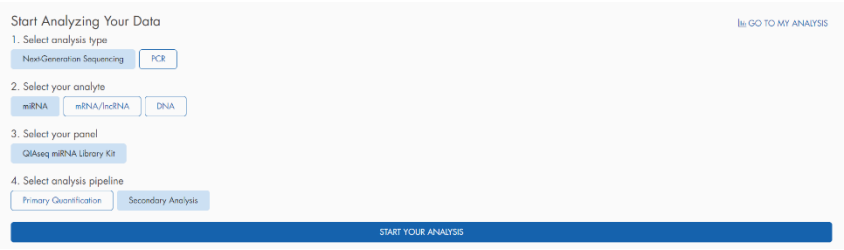
Secondary data analysis – directly from primary analysis

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 **Next-Generation Sequencing** > **miRNA** > **QIAseq miRNA Library Kit** > **Secondary Analysis**.
3. Click **Start Your Analysis**.

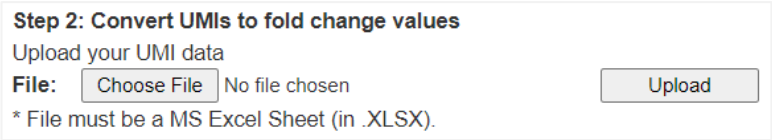


4. Upload the miRNA primary analysis report file:

   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 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		
a)	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.
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 CT values <28, it suggests that the RNA sample may be compromised. If the controls exhibit CT 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.
During library prep QC, prominent adapter–dimer band is observed at 150 bp (greater than 25%)		
a)	Ensure that the QIAseq miRNA 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.

		Comments and suggestions
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 Bead Binding Buffer to produce QMN Beads	Rebuffer QIAseq Beads with QIAseq Bead 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

a)	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

Contact Information

For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support**, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**).

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 QIAseq miRNA 96 Index IL Auto A (cat. no. 331569)" 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. 07200387) 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 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 14,000 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 3C Primer Assay
- QIAseq miRNA 5C Primer Assay
- QIAseq miRNA 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 QIAseq miRNA 96 Index IL Auto A (cat. no. 331569)" is the starting material for the library QC.
- The QuantiTect® SYBR® Green PCR Kit (cat. no. 204143 or 204145) is required for this quality control procedure.
- 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 QuantiTect 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 QuantiTect SYBR® Green PCR Kit 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.
2. Dilute 1 µL of the miRNA Sequencing Library as described in Table 17. 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 17. 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 18. Mix gently and thoroughly.

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

Table 18. Setup of real-time PCR troubleshooting

Component	Master Mix (µL)	Component (µL)	Master Mix (µL)
2x QuantiTect SYBR® Green PCR Master Mix	20	50	40
Control Primer Assay (added in step 5)	–	–	–
Nuclease-free Water	12	36	28
Diluted library amplification product	4	4	4
Total volume	36	90	72

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 19.

   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 19. 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)*†‡§			Perform fluorescence data collection.
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension¶	30 s	70°C	

* 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

* 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.

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.

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 QuantiTect SYBR Green PCR 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

* 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 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 Library Auto Kit (384)	For 384 sequencing prep reactions on automation platforms. Includes at least 50% more volume of reagents for: 3' ligation, 5' ligation, reverse transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331509
QIAseq miRNA 12 Index IL (12)	Sequencing adapters, primers and indexes compatible with Illumina platforms. 12 indexes for 12 samples	331592
QIAseq miRNA 48 Index IL (96)	Sequencing adapters, primers and indexes compatible with Illumina platforms. Two 48 indexes for 96 samples	331595
QIAseq miRNA 96 Index IL (96)	Sequencing adapters, primers and custom indexes compatible with Illumina platforms. Cuttable HT format	331565

Product	Contents	Cat. no.
	(dried primers) with 96 indexes for 96 samples	
QIAseq miRNA 96 Index IL Auto A (384)	Hard plastic plate with 8 ul per well (liquid primers) of pre-mixed sample index primers supplied with a pierceable foil seal in a foil bag. 4 plates suppled per order	331569
QuantiTect SYBR Green PCR Kit (200)	For 200 x 50 µL reactions: 3 x 1.7 mL 2x QuantiTect SYBR Green PCR Master Mix, 2 x 2 mL RNase-Free Water	204143
QuantiTect SYBR Green PCR Kit (1000)	For 1000 x 50 µL reactions: 25 mL 2x QuantiTect SYBR Green PCR Master Mix, 20 mL RNase-Free Water	204145

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

Revision	Description
06/2021	Initial revision
03/2024	Updated the URL for the Generation of Sample Sheets to Templates. Included NextSeq 1000/2000 loading concentration recommendation in Table 16. Included Data Analysis Using GeneGlobe to the new RNA-seq Analysis & Biomarker Discovery Pipeline.

Limited License Agreement for QIAseq miRNA Library Automation Kit, Illumina NGS Systems

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