September 2023

QIAseq® miRNA Library Kit Handbook

Precision small RNA library prep for Thermo Fisher Scientific® NGS systems

Sample to Insight

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	4.8 mL	38.4 mL
Bead Binding Buffer	7 mL	54 mL

QIAseq miRNA NGS 12 Index TF		(12)
Catalog no.		331582
Number of reactions		12
Tube	Index sequence	
QMI TF Lib Rev Primer	n/a	24 µL
QMI TF IP1	CTAAGGTAA	10 µL
QMI TF IP2	TAAGGAGAA	10 µL
QMI TF IP3	AAGAGGATT	10 µL
QMI TF IP4	TACCAAGAT	10 µL
QMI TF IP5	CAGAAGGAA	10 µL
QMI TF IP6	CTGCAAGTT	10 µL
QMI TF IP7	TTCGTGATT	10 µL
QMI TF IP8	TTCCGATAA	10 µL
QMI TF IP9	TGAGCGGAA	10 µL
QMI TF IP10	CTGACCGAA	10 µL
QMI TF IP11	TCCTCGAAT	10 µL
QMI TF IP12	TAGGTGGTT	10 µL

QIAseq miRNA NGS 48 Index TF	(96)
Catalog no.	331585
Number of reactions	96
Box contains two MITF-001 plate and 8-cap strips (24). MITF-001 is a cuttable plate that contains a different indexing primer (QMI TF IP1 through QMI TF IP48) in 48 wells combined with a dried universal primer. QMI TF primers support indexing on Thermo Fisher Scientific NGS systems.	1

Table 1. QIAseq miRNA NGS 48 Index TF (cat. no. 331585) layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	QMI TF IP1	QMI TF IP9	QMI TF IP17	QMI TF IP25	QMI TF IP33	QMI TF IP41	Empty	Empty	Empty	Empty	Empty	Empty
B	QMI TF IP2	QMI TF IP10	QMI TF IP18	QMI TF IP26	QMI TF IP34	QMI TF IP42	Empty	Empty	Empty	Empty	Empty	Empty
C	QMI TF IP3	QMI TF IP11	QMITF IP19	QMI TF IP27	QMITF IP35	QMI TF IP43	Empty	Empty	Empty	Empty	Empty	Empty
D	QMI TF IP4	QMI TF IP12	QMI TF IP20	QMI TF IP28	QMI TF IP36	QMI TF IP44	Empty	Empty	Empty	Empty	Empty	Empty
E	QMI TF IP5	QMI TF IP13	QMI TF IP21	QMI TF IP29	QMI TF IP37	QMI TF IP45	Empty	Empty	Empty	Empty	Empty	Empty
F	QMI TF IP6	QMI TF IP14	QMI TF IP22	QMI TF IP30	QMI TF IP38	QMI TF IP46	Empty	Empty	Empty	Empty	Empty	Empty
G	QMI TF IP7	QMI TF IP15	QMI TF IP23	QMI TF IP31	QMI TF IP39	QMI TF IP47	Empty	Empty	Empty	Empty	Empty	Empty
H	QMI TF IP8	QMI TF IP16	QMI TF IP24	QMI TF IP32	QMI TF IP40	QMI TF IP48	Empty	Empty	Empty	Empty	Empty	Empty

Table 2. QIAseq miRNA NGS 48 Index TF (cat. no. 331585) index sequences

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTAAG GTAA	TGAGC GGAA	TCTAT TCGT	CCTGA GATA	TTCTCA TTGAA	TTCCA CTTCG	Empty	Empty	Empty	Empty	Empty	Empty
B	TAAGG AGAA	CTGAC CGAA	AGGCA ATTG	TTACA ACCT	TCGCA TCGTT	AGCAC GAAT	Empty	Empty	Empty	Empty	Empty	Empty
C	AAGAG GATT	TCCTC GAAT	TTAGT CGGA	AACCA TCCG	TAAGC CATTGT	CTTGAC ACCG	Empty	Empty	Empty	Empty	Empty	Empty
D	TACCA AGAT	TAGGT GGTT	CAGAT CCAT	ATCCG GAAT	AAGGA ATCGT	TTGGAG GCCAG	Empty	Empty	Empty	Empty	Empty	Empty
E	CAGAA GGAA	TCTAA CGGA	TCGCA ATTA	TCGAC CACT	CTTGAG AATGT	TGGAGC TTCCT	Empty	Empty	Empty	Empty	Empty	Empty
F	CTGCA AGTT	TTGGA GTGT	TTCGA GACG	CGAGG TTAT	TGGAGG ACGGA	TCAGT CCGAA	Empty	Empty	Empty	Empty	Empty	Empty
G	TTCGT GATT	TCTAG AGGT	TGCCA CGAA	TCCAA GCTG	TAACA ATCGG	TAAGGCA ACCA	Empty	Empty	Empty	Empty	Empty	Empty
H	TTCCG ATAA	TCTGG ATGA	AACCT CATT	TCTTA CACA	CTGAC ATAAT	TTCTA AGAGA	Empty	Empty	Empty	Empty	Empty	Empty

Shipping and Storage

The QIAseq miRNA Library Kit is shipped in 2 boxes:

- Box 1 is shipped on dry ice or blue 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 2x miRNA Ligation Activator included in Box 2 should be stored at 2–8°C or –30°C to –15°C in a constant-temperature freezer.

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

QIAseq index kits are sold separately and 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

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 TF, and QIAseq miRNA NGS 48 Index TF 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 Thermo Fisher Scientific NGS instruments. This highly optimized solution facilitates both enhanced differential expression analysis using integrated Unique Molecular Indexes (UMIs) and discovery of novel miRNAs 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 pre-sequencing 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 next-generation sequencing (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 12 samples using the QIAseq miRNA NGS 12 Index TF, or up to 48 samples in combination with the QIAseq miRNA NGS 48 Index TF.

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 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):

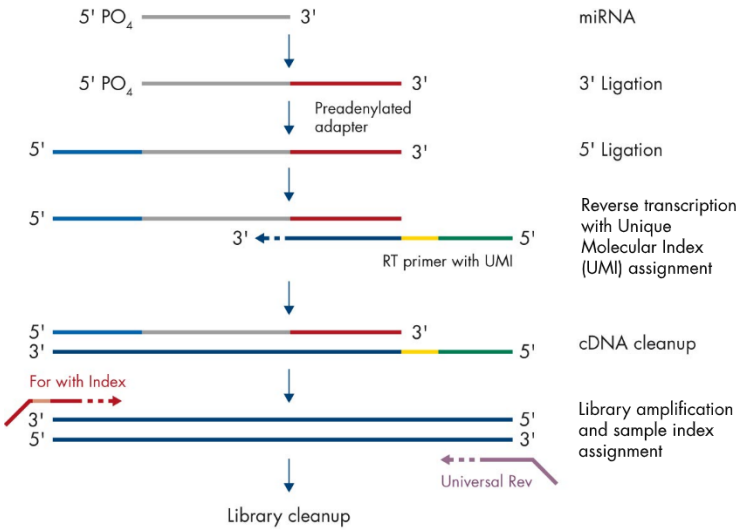

Figure 2. miRNA Sequencing Library preparation using the QIAseq miRNA Library Kit. Specially designed 3' and 5' adapters are ligated to mature miRNAs. The ligated miRNAs are then reverse-transcribed to cDNA using a reverse transcription (RT) primer with a UMI. No libraries are prepared from adapter dimers.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

- **3' Ligation**: A pre-adenylated 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 1 of 2 formats. In format 1, one of 12 wet forward primers is paired with a wet universal reverse primer from a tube (cat. no. 331582) to assign each sample a unique index. In format 2, one of 48 dried forward primers from a plate is paired with a dried universal reverse primer in the same plate (cat. no. 331585) to assign each sample a unique index. In format 2, library amplification reactions occur directly in the index plate, providing a convenient high-throughput (HT) indexing solution.
- **Library cleanup**: After library amplification, a cleanup of the miRNA library is performed using a magnetic bead–based method.

Next-generation sequencing on Ion Torrent NGS systems

Libraries prepared with the QIAseq miRNA Library Kit can be sequenced using a Thermo Fisher Scientific Ion Torrent® NGS system. QIAseq miRNA libraries require 100 bp single reads or 250 flows. It is recommended to allocate 5–10 million reads per sample.

Integrated reaction controls

The QIAseq miRNA Library Kit contains integrated reaction controls to monitor 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 ("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 **ngsdataanalysis2.qiagen.com/QIAseqmiRNA/analysisfile**. Here, UMIs are counted and miRNA sequences are mapped. Secondary data analysis for traditional gene expression calculations is also available at **dataanalysis.qiagen.com/QIASeqmiRNA/arrayanalysis.php**. 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:
  - QIAxcel Connect (QIAGEN)
  - 2100 Bioanalyzer (Agilent®)
  - Fragment Analyzer (Agilent)
- 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 Thermo Fisher Scientific sequencers. Generally speaking, an RNA molecule that is 50 bp or smaller, and has a 3′ hydroxyl and 5′ phosphate, will be robustly included in the library.
- 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 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.
  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 measuring 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 assess RNA integrity using a RNA integrity score or (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 have 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 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 Indexes (331582)” or “Protocol: Library Amplification using HT Plate Indexes (331585)”.
- During setup of the sequencing run, select **.UBAM** and choose **Ion Xpress Adapter Sample Index System**. To make use of the UMIs, the recommended protocol is 100 bp single read or 250 flows.

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 has 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.
- The 3' ligation reactions are very viscous. To mix, pipette slowly and thoroughly (pipette up and down 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 and 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 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°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 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: Pipette slowly when mixing the reaction. The 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: Pipette 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.
- 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 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 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°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 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. Briefly centrifuge, mix by pipetting up and down 10 to 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
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 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°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 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 (°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 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 (next page). 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 (previous page).

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 Bead Binding Buffer to create QMN Beads.
- QIAseq Beads and the subsequently prepared QMN 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.
- After preparation, the QMN Beads need to be placed on ice.

Procedure

1. Thoroughly vortex QIAseq Beads and 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 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.

4. Remove the tube from the magnet stand, and carefully pipette (buffer is viscous) 150 µL of Bead Binding Buffer onto the beads. Thoroughly vortex to completely resuspend the bead pellet. Briefly centrifuge and immediately separate 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 pipette 400 µL of 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 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 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 beads have fully migrated).
   Important: Ensure that the beads have fully migrated before proceeding.

6. Discard the supernatant and keep the beads.

   Important: Ensure as much supernatant as possible has been removed.

   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: It is important to completely remove all traces of ethanol after the second wash. Briefly centrifuge (centrifuge plates at 2000 rpm) and return the tubes/plates to the magnet 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 magnet 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 magnet 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 magnet stand.

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

    Return the tubes/plates to the magnet stand for ~2 min (or until beads have fully migrated).

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

12. Transfer 15 µL of eluted DNA to new tubes/plates.

13. Proceed to "Protocol: Library Amplification using Tube Indexes (331582)" or "Protocol: Library Amplification using HT Plate Indexes (331585)". Alternatively, the completed cDNA cleanup product can be stored at –30°C to –15°C in a constant-temperature freezer.

Protocol: Library Amplification using Tube Indexes (331582)

Important points before starting

- This library amplification protocol uses tubes indexes from QIAseq miRNA NGS 12 Index TF (331582). If using QIAseq miRNA NGS 48 Index TF (331585), proceed to "Protocol: Library Amplification using HT Plate Indexes (331585)".
- 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.
- 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, required index primer(s)* from QIAseq miRNA 12 Index TF 331582, and QMI TF Lib Rev Primer. Mix by flicking the tube. Centrifuge briefly to collect residual liquid from the sides of the tubes.

   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.

* **Note**: QMI TF IP1 through IP12 are options, and the respective index sequences are listed in the tables found under "Kit Contents".

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 indexes

Component	Volume/reaction
Product from "Protocol: cDNA Cleanup"	15 µL
QIAseq miRNA NGS Library Buffer	16 µL
HotStarTaq DNA Polymerase	3 µL
QMI TF IP1 through IP12 (Index Primer)*	2 µL
QMI TF Lib Rev Primer	2 µL
Nuclease-Free Water	42 µL
Total volume	**80 µL**

* Up to 12 different QMI TF IP 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 (next page).

Table 13. Library amplification protocol

Step	Time	Temperature
Hold	15 min	95°C
3-step cycling (see Table 14 for no. 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 fresh 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 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 fresh tubes. Discard the tubes containing the beads.

   Important: Do not discard the supernatant at this step.

10. Add 130 µL of QMN Beads to the 145 µL supernatant. 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 ethanol after the second wash. Briefly centrifuge and return the tubes to the magnet 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 magnet 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 magnet stand, elute the DNA by adding 17 µL of Nuclease-Free Water to the tubes. Subsequently close and remove the tubes from the magnet stand.
18. Carefully pipette up and down until all beads are thoroughly resuspended, briefly centrifuge, and incubate at room temperature for 2 min.

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

    Note: Ensure that the beads have fully migrated before proceeding.
19. Transfer 15 µL of eluted DNA to new tubes. This is the miRNA Sequencing Library. 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: Library Amplification using HT Plate Indexes (331585)

Important points before starting

- This library amplification protocol uses plate indexes from QIAseq miRNA NGS 48 Index TF (331585). If using QIAseq miRNA NGS 12 Index TF (331582), proceed to "Protocol: Library Amplification using Tube Indexes (331582)".
- 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.
- 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°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. Open the QIAseq miRNA NGS 48 Index TF index plate, and choose the wells required for amplification.
   Note: This is a cuttable plate that contains a dried indexing primer (QMI TF IP1 through QMI TF IP48) in wells 1–48 and a dried universal primer in wells 1–48. The layout is described in Table 15 (next page).

Note: During reaction setup in step 2, components are added directly to the plate. It is recommended to either perform reactions in sets of 8 or 12.

Table 15. QIAseq miRNA NGS 48 Index TF index plate (MITF-001)

	1	2	3	4	5	6	7	8	9	10	11	12
A	QMI TF IP1	QMI TF IP9	QMI TF IP17	QMI TF IP25	QMI TF IP33	QMI TF IP41	Empty	Empty	Empty	Empty	Empty	Empty
B	QMI TF IP2	QMI TF IP10	QMI TF IP18	QMI TF IP26	QMI TF IP34	QMI TF IP42	Empty	Empty	Empty	Empty	Empty	Empty
C	QMI TF IP3	QMI TF IP11	QMITF IP19	QMI TF IP27	QMITF IP35	QMI TF IP43	Empty	Empty	Empty	Empty	Empty	Empty
D	QMI TF IP4	QMI TF IP12	QMI TF IP20	QMI TF IP28	QMI TF IP36	QMI TF IP44	Empty	Empty	Empty	Empty	Empty	Empty
E	QMI TF IP5	QMI TF IP13	QMI TF IP21	QMI TF IP29	QMI TF IP37	QMI TF IP45	Empty	Empty	Empty	Empty	Empty	Empty
F	QMI TF IP6	QMI TF IP14	QMI TF IP22	QMI TF IP30	QMI TF IP38	QMI TF IP46	Empty	Empty	Empty	Empty	Empty	Empty
G	QMI TF IP7	QMI TF IP15	QMI TF IP23	QMI TF IP31	QMI TF IP39	QMI TF IP47	Empty	Empty	Empty	Empty	Empty	Empty
H	QMI TF IP8	QMI TF IP16	QMI TF IP24	QMI TF IP32	QMI TF IP40	QMI TF IP48	Empty	Empty	Empty	Empty	Empty	Empty

Note: Indexing primers and a universal primer are pre-dried as single-use plates. During reaction setup, components are added directly to the plate. There is no need to reconstitute and transfer indexes 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: Reaction components are added directly to plate MITF-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 MITF-001

Component	Volume/reaction (µL)
Product from "Protocol: cDNA Cleanup"	15
QIAseq miRNA NGS Library Buffer	8
HotStarTaq DNA Polymerase	1.5
Nuclease-Free Water	15.5
Total volume	**40**

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 (°C)
Hold	15 min	95
3-step cycling (see Table 18 for no. of cycles)		
Denaturation	15 s	95
Annealing	30 s	60
Extension	15 s	72
Hold	2 min	72
Hold	∞*	4

* 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 beads have fully migrated.

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

10. Keep the supernatant, and transfer 72.5 µL of the supernatant to new plates. Discard the plates containing the beads.

    Important: Do not discard the supernatant at this step.

11. To the 72.5 µL 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 magnet 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 magnet 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 magnet stand, elute the DNA by adding 17 µL of Nuclease-Free Water to the plates. Subsequently cover and remove the plates from the magnet 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 magnet 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 point before starting

- A portion of the 15 µL miRNA Sequencing Library from "Protocol: Library Amplification using Tube Indexes (331582)" or "Protocol: Library Amplification using HT Plate Indexes (331585)" is the starting material for the library QC. When not in use, store the miRNA Sequencing Library on ice.

Procedure

1. Analyze 1 µL of the miRNA Sequencing Library on a QIAxcel Connect, Agilent Bioanalyzer, or Fragment Analyzer according to the manufacturer's instructions. A miRNA-sized library is approximately 166 bp, and a piRNA-sized library is approximately 174 bp. If there is a peak at approximately 151–158 bp, this comprises RNA fragments or small RNAs other than miRNAs; these are common to see in total RNA samples, being particularly strong in biofluid total RNA samples. Even if a peak is observed at 151–158 bp, there are still likely miRNAs present in the sample. Any RNA that has a 3′ OH and 5′ PO_4, and is approximately 50 bp and smaller, should be robustly captured by the QIAseq miRNA Library Kit. A typical miRNA-sized library is shown in Figure 3 (next page).

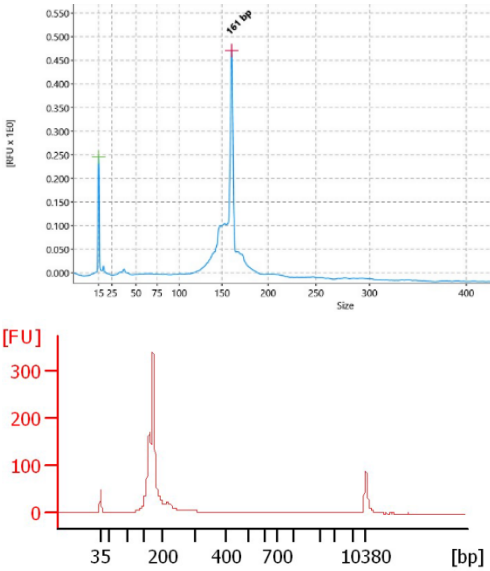

Figure 3. QIAxcel Connect (top) and Bioanalyzer (bottom) traces of miRNA-sized libraries prepared with the QIAseq miRNA Library Kit.

2. If a large peak (greater than 25% of the height of the miRNA peak) is observed at approximately 144 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 (Appendix A: Gel Size Selection of Library).

   Note: If no library is observed, assess the integrated reaction controls using real-time PCR (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.

3. Proceed to "Protocol: Determining Library Concentration".

Protocol: Determining Library Concentration

Important points before starting

- A portion of the 15 µL miRNA Sequencing Library from "Protocol: Library Amplification using Tube Indexes (331582)" or "Protocol: Library Amplification using HT Plate Indexes (331585)" is the starting material for the library QC. When not in use, store the library on ice.
- A Qubit Fluorimeter (Thermo Fisher Scientific) 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 166 bp library, using 650 g/mol as the average weight for a single DNA bp.

$$(X\,\text{ng/µL})(10^6)/(107900) = Y\,\text{nM}$$

Protocol: Preparation for Sequencing

Important points before starting

- For complete instructions on how to set up a sequencing run, please refer to system-specific Thermo Fisher Scientific documents.
- Following recommendations for library dilution concentrations and library loading concentrations are based on a Qubit™ Fluorimeter:
  - Ion Chef: 50 pM
  - Ion OneTouch™: 4 pM
- **Note:** The sample indexes of the QIAseq miRNA Library Kits are compatible with the Ion Xpress Adapter Sample Index System
- Important: Perform 100 bp read length or 250 flows for optimal results. Our data analysis tool requires complete read length from UMIs to miRNAs.
- Set up the sequencing run without a reference genome to get unaligned **.bam** files for our data analysis. In addition, due to the special read structure of QIAseq miRNA libraries, reads start with 12 bp UMI.
- Do not run the plugin or Ion reporter. Due to the special structure of the library, it poses a problem for correct read processing.

Procedure

1. Pooling and Templating:
   1a. Dilute individual libraries to 4 nM (or similar) using Nuclease-Free Water.
   1b. If multiplexing, combine libraries in equimolar amounts and mix well.
   1c. For the Ion Chef, further dilute the multiplexed library to 50 pM, with a final volume of 25 µL.
2. Sign in to the Torrent Suite™ Software, click the **Plan** tab, and select **DNA** for research application and **Other** for Target Technique. Plan an S5 run with the following settings:

2a. Using the **Planned Run** wizard, in the **Create Plan** tab, make the appropriate selections as below:

- Template Name: **Ion RNA - miRNA**
- Run Plan Name (required): [Enter a planned run name]
- Analysis Parameters: **Default (Recommended)**
- Number of barcodes: [Enter the number of samples]

Important: Do not select a reference genome.

Click **Next**.

2b. In the **Ion Reporter** tab, under **Ion Reporter Account**, select **None**. Click **Next.**

2c. In the **Research Application** tab: Under **Research Application**, select **DNA**. Under **Target Technique**, select **Other**. Click **Next**.

2d. In the **Kits** tab, select the following:

- Instrument: Ion GeneStudio™ S5 System
- Leave Sample Preparation Kit blank
- Leave Library Kit Type blank
- Chip Type: [Select the appropriate chip type from the drop-down list]
- Leave Control Sequence blank
- Barcode Set: IonXpress
- Template Kit: IonChef
- Flows: 250
- Sequencing Kit: Ion S5 Sequencing Kit
- Advanced Settings: Use Recommended Defaults

Click **Next**.

2e. In the **Plugins** tab, select **FileExporter**. Click **Next**.

2f. In the **Projects** tab, select or create the appropriate project. Click **Next**.

2g. Under the **Plan** tab:

- Run Plan Name (required): [Enter a planned run name]
- Analysis Parameters: Default (Recommended)
- Sample Tube Label: [Enter or scan the barcodes of the Ion Chef Library Sample Tubes]

- Chip Barcode: [Enter or scan the barcodes of the chip]
- Monitoring thresholds:
  Bead loading (%) <30
  Key signal (1-100) <30
  Usable sequence (%) <30

3. When you have completed your selections, click **Plan Run** to save the run. The run is listed on the **Planned Runs** page under the name that you specified and is automatically used by the Ion Chef System when the associated sample is loaded.
4. Run the Ion Chef system according to manufacturer's instructions.
   Note: If the template is prepared by Ion OneTouch instead of Ion Chef: In the **Kits** tab, for **Template Kit**, select **OneTouch**. Other sequencing parameters set up on Ion S5 should be the same as above described with the Ion Chef.
5. When the run is complete, unload the Ion Chef Instrument and sequence the chips immediately on Ion S5 according to manufacturer's instructions.
6. When the run is complete, navigate to the report page on the Ion Torrent Server. Locate the **Output Files** section near the end of the report (Figure 5).

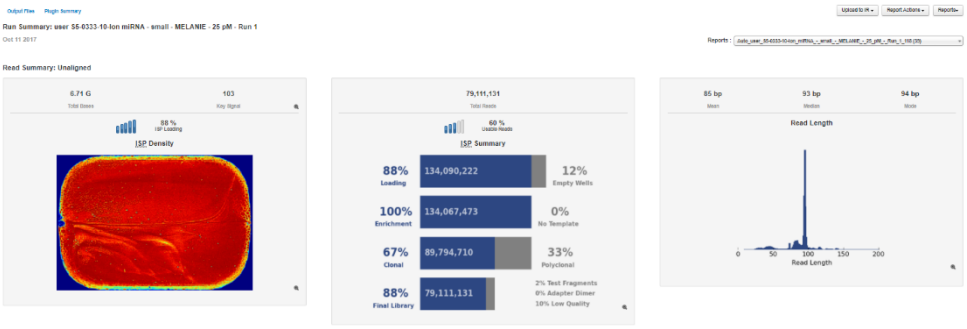

Figure 4. Ion Torrent Server Report Page

7. Click the **UBAM** button in the row corresponding to the individually indexed samples and column labeled **Files** in the table (Figure 6, next page). These are the unaligned reads in BAM format, with the index separated for each sample. Save the UBAM file to your local

disk. The file is usually several hundreds of megabytes to several gigabytes, depending on the size of the sequencing chip being used.

8. Proceed to “Protocol: Data Analysis“.

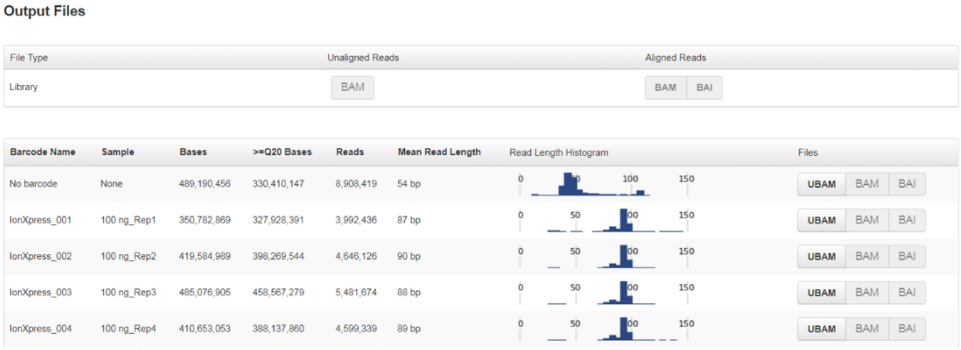

Figure 5. Output files, unaligned reads in BAM format.

Protocol: Data Analysis

Important point before starting

- To ensure a proper secondary data analysis, all samples must be processed in the same miRNA Quantification Job during primary analysis. If UBAM files have been derived from different sequencing runs, combine them into one miRNA Quantification Job.

Procedure

1. For primary data analysis, please visit **ngsdataanalysis2.qiagen.com/QIAseqmiRNA/analysisfile**.
2. For secondary data analysis (indirect access, not linked from primary analysis), please visit **dataanalysis.qiagen.com/QIASeqmiRNA/arrayanalysis.php**.

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/support/faqs/**). 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.	
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.
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 magnet stand. Remove the ethanol with a 200 µL pipette first, and then use a 10 µL pipette to remove any residual ethanol.

		Comments and suggestions
c)	Reaction inhibitors are present in the RNA sample, or the reactions were not set up correctly	See "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.

During library prep QC, prominent adapter dimer band is observed at 144 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.

During library prep QC, a prominent product of approximately 225 bp is observed.

a)	QIAseq Beads were not rebuffered with Bead Binding Buffer to produce QMN Beads.	Rebuffer QIAseq Beads with 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.

During primary data analysis, Unique Molecular Indexes (UMIs) are not present.

b)	A read length shorter than required may have been performed.	When using Thermo Fisher Scientific systems, 100 bp single reads or 250 flows are recommended.

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 Indexes (331582)" or "Protocol: Library Amplification using HT Plate Indexes (331585)" 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 (Thermo Fisher Scientific, cat. no. 07-200-387) are required. Requires 3 M NaOAc, pH 5.2.
- Linear Acrylamide is required.

Procedure

1. Prepare a 6% PAGE TBE gel.
2. Adjust the volume of the miRNA 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 three lanes of the 6% PAGE TBE gel.
4. Run the gel at 120 V 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: On a PAGE gel, an miRNA-sized library is approximately 159 bp and a piRNA-sized library is approximately 167 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 NGS 3C Primer Assay, QIAseq miRNA NGS 5C Primer Assay and 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), these controls can be used to determine if the absence of a library is due to a technical or sample issue.

Important points before starting

- A portion of the 15 µL miRNA Sequencing Library from "Protocol: Library Amplification using Tube Indexes (331582)" or "Protocol: Library Amplification using HT Plate Indexes (331585)" 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, 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.
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 of the 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 (°C)	Additional comments
PCR Initial activation step	15 min	95	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling (35 cycles)*†‡§			
Denaturation	15 s	94	Perform fluorescence data collection.
Annealing	30 s	55	
Extension¶	30 s	70	

* 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. The threshold should be set using a logarithmic amplification plot 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 “Appendix B: Real-time PCR Troubleshooting“ has been performed correctly. For comments and suggestions, see the “Troubleshooting Guide.”

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 12 Index TF (12)	Sequencing adapters, primers and indexes compatible with Thermo Fisher platforms; 12 indexes for 12 samples	331582
QIAseq miRNA 48 Index TF (96)	Sequencing adapters, primers and indexes compatible with Thermo Fisher platforms; two 48 indexes for 96 samples	331585
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
07/2020	Changed description of MITF-001 plates to “cuttable”, from the original “breakable”. Updated data analysis URLs to **geneglobe.qiagen.com**. Updated exoRNeasy Kit recommendations in Table 4. Corrected typos. Moved 2x miRNA Ligation Activator to Box 2 instead of Box 1 in QIAseq miRNA Library Kit contents. Changed precise storage temperatures to temperature ranges. Updated the procedures in “Protocol: Primary and Secondary Data Analysis”.
09/2023	Updated component names. Updated “Shipping and Storage” section. Deleted “Library QC Option 2” under ”Equipment and Reagents to Be Supplied by User”. Updated “Important Notes” and the protocols. Updated all Data Analysis sections with new URL addresses for the primary and secondary analysis data analysis applications. Corrected the cat. no. of QIAseq miRNA 12 Index TF on page 28 from 331592 to 331582. Removed “Appendix C: General Remarks on Handling RNA.”

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