November 2023

QIAseq® miRNA Library Kit Handbook for G4® Sequencing Platform

Precision small RNA library prep for Singular Genomics G4 Sequencing Platform using unique dual indexes (UDIs)

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

* The QIAseq miRNA NGS 5' Adapter and QIAseq miRNA NGS RT Initiator are not usable when libraries are prepared using Singular Genomic UDIs. Instead, the UDI 5' Adapter and UDI RT Initiator from RT Initiator & Adapter UDI Kit (384) should be used.

† The QIAseq miRNA NGS 3C Primer Assay, QIAseq miRNA NGS 5C Primer Assay, and QIAseq miRNA NGS RTC Primer Assay are not usable when QIAseq miRNA libraries are prepared using Singular Genomics UDIs.

QIAseq miRNA Library Kit	(12)	(96)
Catalog no.	331502	331505
Number of reactions	12	96
QIAseq Beads	10 mL	38.4 mL
Bead Binding Buffer	7 mL	54 mL

RT Initiator & Adapter UDI Kit (384)	(384)
Catalog no.	331507
Number of reactions	384
UDI 5′ Adapter (96)	4 x 96 µL
UDI RT Initiator (96)	4 x 192 µL

Important: To successfully build a miRNA library for Singular Genomics G4 sequencing, the following must be ordered:

- From QIAGEN (Table 1)
  - QIAseq miRNA library Kit (choose 331502 or 331505)
  - RT initiator & Adapter UDI Kit

and

- From Singular Genomics (Table 2)
  - SG UDI Primer kit (choose 700,134 or 700,135 or 700,136)

Table 1. QIAGEN kits

Catalog number	Product name	Number of reactions
331502	QIAseq miRNA Library Kit (12)	12
331505	QIAseq miRNA Library Kit (96)	96
331507	RT Initiator & Adapter UDI Kit (384)	384

The SG UDI Primer kit provides primers designed to support the preparation of indexed libraries or make existing libraries containing SP1 and SP2 sequences compatible for G4 Sequencing Platform.

See *Adapters and Indices for the G4 Sequencing Platform Reference Guide* for more information about Singular Genomics primers and adapters.

Table 2. Singular Genomics kits

Material number	Product name	Number of reactions
700,134	SG UDI Primers (1-96)	96
700,135	SG UDI Primers Set A (1-24)	24
700,136	SG UDI Primers Set B (25-48)	24

One SG UDI Primer kit contains:

Components	Container	Description
UDI Primer Plate	96-well Plate	Plate with Indexed Singular Genomics S1/SP1 PCR primers and S2/SP2 PCR primers, each at 5 µM concentration in at least 10 µL per well.

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 stored at –30°C to –15°C in a constant-temperature freezer.
    - The RT Initiator & Adapter UDI Kit (384) is shipped on dry ice or blue ice. Upon receipt, store immediately at –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.

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.

Body fluids such as serum or plasma of all human and animal subjects are considered potentially infectious. Take all necessary precautions recommended by the appropriate authorities in the country of use when working with body fluids.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each QIAseq miRNA kit 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 the Singular Genomics G4 Sequencing Platform. 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. 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).

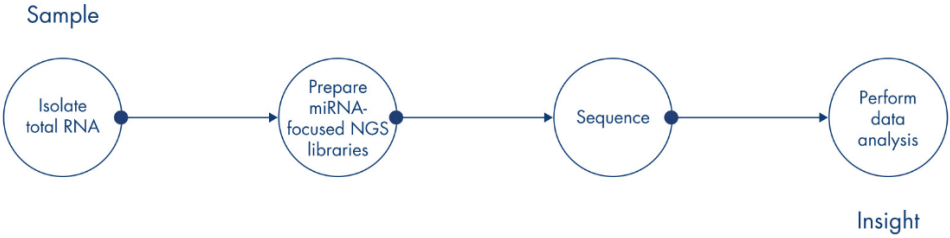

Figure 1. QIAGEN's Sample to Insight QIAseq miRNA workflow.

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 3' hydroxyl and 5' phosphate groups. 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 96 samples with PCR-based unique dual indexes (UDIs, ordered directly from Singular Genomics). Usage of UDIs effectively mitigates the risk of read misassignment. This is enabled by filtering misassigned reads during the demultiplexing of individual samples, thus generating highly accurate output data.

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):

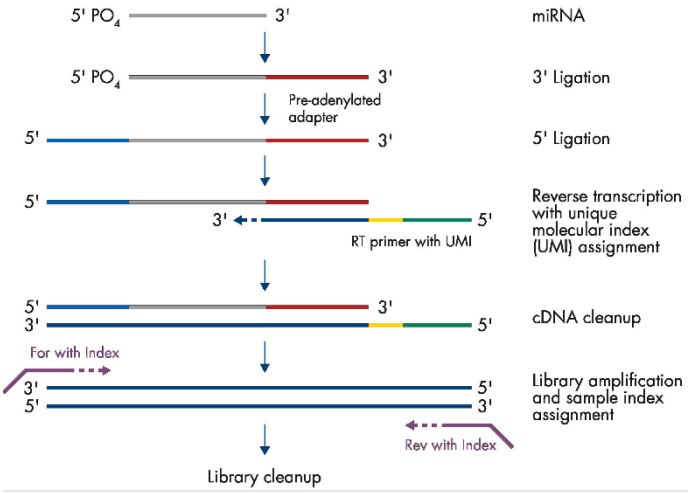

Figure 2. miRNA sequencing library preparation using the QIAseq miRNA Library Kit. Specially designed 3' and 5' adapters are ligated to mature miRNAs. The ligated miRNAs are then reverse transcribed to cDNA using a reverse transcription (RT) primer with a UMI. No libraries are prepared from adapter dimers. Following the cDNA cleanup, library amplification is performed using forward and reverse primers each containing a unique index, giving each sample a UDI pair. 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. 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. 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.

- **Library amplification and Sample indexing**: Library amplification is accomplished by using an aliquot from a single well of the plate from the SG UDI Primer kit. In each well, a unique Index 1 is premixed with a unique Index 2; the premixed oligos will be used in a library amplification reaction to assign each sample a UDI. The specific index sequences used in the QI UDI Primers plates are listed in the **SG UDI Primers Overview** available at **www.singulargenomics.com/g4/support/**
- **Library cleanup**: After library amplification, a cleanup of the miRNA library is performed using a magnetic bead-based method.

NGS on Singular Genomics G4 Platform

Libraries prepared with the QIAseq miRNA Library Kit and SG UDI Primers can be sequenced using the G4 Sequencing Platform. For this platform, QIAseq miRNA Library Kit libraries require 72 bp single reads with 12 bp dual indexing. 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, data analysis cannot be performed using the RNA-seq Analysis Portal (RAP); to use RAP, UMIs must be sequenced.

Data Analysis using RNA-seq Analysis Portal

Both primary and secondary data analysis and even some interpretation analysis is performed using the RNA-seq Analysis Portal at **www.rnaportal.qiagen.com/rnaportalui/.** Here, UMIs are counted and miRNA sequences are mapped. The same application also performs secondary differential miRNA expression analysis based on traditional calculations. Finally, the software presents the results in a table and two different visual formats while also providing insights into upstream regulators and overlapping biological pathways and diseases.

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:
    - 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. 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 the purification of total RNA including miRNA (Table 3).

Table 3. Recommended kits for purification of total RNA containing miRNA

Kits	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:
  - **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 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 that the 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 SG UDI Primer Kits“.
- To make use of the UMIs, the recommended protocol is 72 bp single read (Read 1) with 12 bp dual indexing. A 50 bp single read protocol can be used if there is no desire to include the UMIs, but the RNA-seq Analysis Portal (RAP) 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 input amounts or serum/plasma samples, the QIAseq miRNA NGS 3' Adapter must be diluted according to Table 4.
- 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 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 the 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 the 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 the QIAseq miRNA NGS RI and QIAseq miRNA NGS 3′ RNA Ligase from the –30°C to –15°C freezer just before use, and place both 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 NGS miRNA 3' Adapter using nuclease-free water according to Table 4. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

Table 4. 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 1:20
Serum/Plasma	Dilute 1:5

4. On ice, prepare the 3' ligation reaction according to Table 5. 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 5. 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

† 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 has 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.

5. Add the 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 UDI 5' Adapter is provided with the RT Initiator & Adapter UDI Kit (384).

  Do not use the QIAseq miRNA NGS 5' Adapter provided with the QIAseq miRNA Library Kit.
- 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 UDI 5' Adapter must be diluted according to Table 6.
- 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 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 the reagents required for the 5' ligation reactions. Thaw UDI 5' Adapter [supplied in the RT Initiator & Adapter UDI Kit (384)] and QIAseq miRNA NGS 5' Buffer at room temperature. Mix by flicking the tubes, centrifuge briefly to collect residual liquid from the sides of the tube, and keep at room temperature.
2. Remove QIAseq miRNA NGS RI and QIAseq NGS 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.
3. If working with low RNA inputs or serum/plasma samples, dilute the UDI 5' Adapter using nuclease-free water according to Table 6. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

Table 6. Dilution of the UDI 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 1:10
Serum/Plasma	Dilute 1:2.5

4. On ice, prepare the 5' ligation reaction according to Table 7, 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 7. Setup of 5' ligation reactions

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

* For low input and serum/plasma RNA, the UDI 5' Adapter must be diluted according to Table 6.

5. Incubate for 30 min at 28°C.
6. Incubate for 20 min at 65°C.
7. Hold at 4°C.
8. 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 9.
- Set up reverse transcription reactions on ice.
- Do not vortex the QIAseq miRNA NGS RI, QIAseq miRNA NGS RT Enzyme, or reverse transcription reactions.
- Be certain to use the UDI RT Initiator (provided with the RT Initiator & Adapter UDI Kit (384).

  Do not use the QIAseq miRNA NGS RT Initiator provided with the QIAseq miRNA Library Kit.
- 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 UDI RT Initiator [supplied in the RT Initiator & Adapter UDI Kit (384)], QIAseq miRNA NGS RT Buffer, and QIAseq miRNA NGS RT Primer at room temperature. Mix by flicking the tubes, 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 UDI 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 8.

Table 8. Incubation of tubes with UDI RT Initiator

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

* Hold until setup of the RT reaction.

4. If working with low RNA inputs or serum/plasma samples, dilute the QIAseq miRNA RT Primer using nuclease-free water according to Table 9.

Table 9. 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 1:20
Serum/Plasma	Dilute 1:5

5. On ice, prepare the reverse transcription reaction according to Table 10. Briefly centrifuge, mix by pipetting up and down 15–20 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 10. Setup of reverse transcription reactions

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

* For low input and serum/plasma RNA, the QIAseq miRNA NGS RT Primer must be diluted according to Table 9.

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 Bead Binding Buffer to create QMN Beads.
- 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.
- 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 magnetic stand.

   Note: Beads for up to 4 samples (1.6 mL) can be prepared at the same 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 magnetic 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 magnetic 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 magnetic 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 1 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 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.
- 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 magnetic stand for ~4 min or until the 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 magnetic stand.

7. With the beads still on the magnetic 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 efficiency of 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 SG UDI Primer Kits". Alternatively, the completed cDNA cleanup product can be stored at –30°C to –15°C in a constant-temperature freezer.

Protocol: Library Amplification Using SG UDI Primer Kits

Important points before starting

- This library amplification protocol uses plate UDIs from SG UDI Primer kits, purchased directly from Singular Genomics.
- 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, the beads must be completely dried following the ethanol washing step. Specific recommendations are given to remove excess ethanol.

Procedure

1. Prepare the reagents required for the library amplification reactions. Thaw the QIAseq miRNA NGS Library Buffer and required SG UDI Primer plate. Mix by flicking the tube or plate, and centrifuge the tubes/plate briefly to collect residual liquid.

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

2. Open the SG UDI Primer plate and pierce the wells required for amplification to assign each sample a unique index.

   Note: This is a pierceable plate that contains both an index 1 and index 2 UDI pair in every well.

3. On ice, prepare the library amplification reaction according to Table 11. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

   Note: Reaction components are added first with the addition of the SG UDI Primers added last to assign each sample a unique dual 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, adding both the cDNA and index pair separately.

Table 11. Setup of library amplification reactions when using plate indexes

Component	Volume/reaction (µL)
Product from "Protocol: cDNA Cleanup"	15
QIAseq miRNA NGS Library Buffer	10
HotStarTaq DNA Polymerase	1.5
Sample index pair from a well of SG UDI Primers plate*	2
Nuclease-free water	21.5
Total volume	**50**

* Up to 96 SG UDI indexes are available for use.

4. Program the thermal cycler according to Table 12. The correct number of cycles depends on the original RNA input and is shown in Table 13.

Table 12. Library amplification protocol

Step	Time	Temperature
Hold	15 min	95°C
3-step cycling (see Table 13 for number of cycles)		
Denaturation	15 s	95°C
Annealing	30 s	60°C
Extension	15 s	72°C
Hold	2 min	72°C
Hold	∞*	4°C

* Hold at 4°C for at least 5 min.

Table 13. Cycles of library amplification

Original RNA input (total RNA)	Cycle number
500 ng	13
100 ng	16
10 ng	19
1 ng	24
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 50 µL library amplification reactions, tubes/plates, upon thermal cycler completion and add 47 µL of QMN Beads to each library amplification reaction.

   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.

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

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

7. Vortex for 3 s and briefly centrifuge.
8. Incubate for 5 min at room temperature.
9. Place tubes/plates on a magnetic stand for approximately 4 min or until the beads have fully migrated.

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

10. Keep the supernatant, and transfer 92 µL of the supernatant to new tubes/plates. Discard the tubes containing the beads.

    Important: Do not discard the supernatant at this step.

11. To the 92 µL supernatant, add 83 µL of QMN Beads. Vortex for 3 s and briefly centrifuge.
12. Incubate at room temperature for 5 min.

13. Place the tubes/plates on a magnetic 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/plates from the magnetic stand.

15. With the beads still on the magnetic 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 tubes. Subsequently close and remove the tubes 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 tubes/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 tubes. This is the miRNA Sequencing Library.

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

Protocol: miRNA Library QC

Important points before starting

- A portion of the 15 µL miRNA Sequencing Library from "Protocol: Library Amplification Using SG UDI Primer Kits" 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, etc., according to the manufacturer's instructions. 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 miRNA-sized library is approximately 200 bp, and a piRNA-sized library is approximately 208 bp. If there is a peak at approximately 185–192 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 185–192 bp, there are still likely miRNAs present in the sample. A peak at 150 bp is inconsequential and will not affect sequencing. A typical miRNA-sized library is shown in Figure 3.

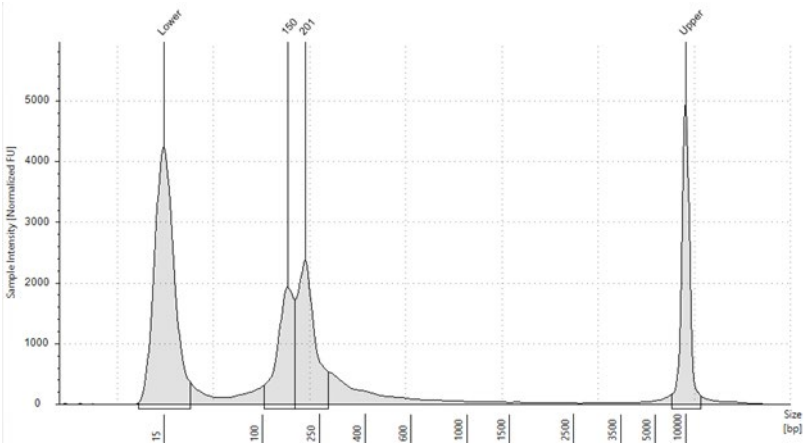

Figure 3. TapeStation trace of a library prepared from 100 ng XpressRef Universal RNA.

2. If a large peak (greater than 25% of the height of the miRNA peak) is observed at approximately 178 bp (adapter dimer), or if other undesired bands are noted, gel excision on the remainder of the miRNA Sequencing Library is recommended to select the specific library of interest (see "Appendix A: Gel Size Selection of Library"). As noted above, s peak at 150 bp is inconsequential and will not affect sequencing.

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

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

Protocol: Determining Library Concentration

Important points before starting

- A portion of the 15 µL miRNA Sequencing Library from "Protocol: Library Amplification Using SG UDI Primer Kits" 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 200 bp library, using 650 g/mol as the average weight for a single DNA bp.

$$(X\ \text{ng/µL})(10^6)/(130{,}000) = Y\ \text{nM}$$

Protocol: Preparation for Sequencing

Important points before starting

- For complete instructions on how to denature sequencing libraries and set up a sequencing run, please refer to the G4 Sequencing Platform User Guide or **G4 Sequencing Platform QRC**, available from **www.singulargenomics.com/g4/support/**
- SG UDIs are 12 bp unique dual sample indexes.
- See **www.singulargenomics.com/** to download SG UDI index sequences. To make sequencing preparation more convenient, the online Singular Genomics Sample Sheet Generator is available from **www.singulargenomics.com/g4/support/**
  - For more instructions about the sample sheet setup, refer to the G4 Sequencing Platform User Guide or G4 Sequencing Platform QRC available from **www.singulargenomics.com/g4/support/**

  Note: Although not required, the Singular Genomics PhiX Control Library (Singular Genomics, cat. No. 700120) can be added to the sequencing run. For more information, please refer to the PhiX DNA Control Overview available at **www.techwriting.singulargenomics.com/G4-Documentation/PhiX-Control-Overview-600010.pdf**
- It is recommended to allocate 5–10 million reads per sample.
- Sequencing run setup.

  The recommended protocol is 72 bp single read with 12 bp dual indexing. A 50 bp single read protocol can be used if there is no desire to include the UMIs.
- Upon completion of the sequencing run, proceed with "Protocol: Data Analysis using GeneGlobe Web-Based Analysis Tools".

Protocol: Data Analysis using GeneGlobe Web-Based Analysis Tools

Important points before starting

- To analyze the data generated from the QIAseq miRNA Library Kit, you must first activate your analysis credits by entering the lot number from Box # into the following site: **www.geneglobe.qiagen.com/analyze/analysis-credits/redeem-credits**
- Both primary and secondary data analysis and even some interpretation analysis is performed using the RNA-seq Analysis Portal at **www.rnaportal.qiagen.com/rnaportalui/**

Procedure

1. Once all FASTQ files are ready and analysis credits are activated, navigate to the RNA-seq Analysis Portal using the web address above or by following the prompts on the GeneGlobe Analyze page at **www.geneglobe.qiagen.com/analyze**
2. Follow the instructions provided by the User manual available for download from the Help link in the top right corner of the website to upload sequencing data, align and count reads, and create experiments for differential miRNA expression analysis.

Troubleshooting Guide

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

	Comments and suggestions
During cDNA cleanup or library cleanup, not enough sample can be pipetted	
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, the supernatant does not completely clear after 4 – 6 minutes	
This is not a significant issue	Sometimes, samples do not completely clarify. This is possibly due to the cold temperature of the buffer. Simply proceed with the cleanup.
During library prep QC, no library is observed	
a) 3' ligation reaction has not been properly mixed	Once all components have been added to the reaction, briefly centrifuge, mix by pipetting up and down 15–20 times, and centrifuge briefly again.
b) Excess ethanol from the cDNA cleanup has been carried over to the amplification reaction	After the second wash, briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200 µL pipette first, and then use a 10 µL pipette to remove any residual ethanol.

	Comments and suggestions
During library prep QC, prominent adapter dimer band is observed at 178 bp (greater than 25%)	
a) Ensure that the UDI RT Initiator has been added as indicated, between the 5' ligation and RT reactions, and the correct temperature profile has been set up for the initiation	Double check the RT reaction setup.
b) Ensure that 3' ligation and 5' ligation components were added to their respective reactions in the order listed	Double check 3′ ligation and 5′ ligation reaction setup.
miRNA Sequencing Library concentrations are too low to obtain a 4 nM library	
Not necessarily a problem	If Library QC suggests the library is of good quality and simply low in concentration, use 2 nM library instead, or sequence the maximum amount possible of that library (either individually or in multiplex with other samples). At the same time, keep all libraries being multiplexed at comparable concentrations.
During primary data analysis, Unique Molecular Indexes (UMIs) are not present	
A read length shorter than 72 bp may have been performed	Resequence and ensure that 72 bp single reads with 12 bp dual indexes are performed.

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 SG UDI Primer Kits" 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 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 3 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: A miRNA-sized library is approximately 200 bp, and a piRNA-sized library is approximately 208 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.

Ordering Information

Product	Contents	Cat. no.
QIAGEN		
QIAseq miRNA Library Kit (12)	For 12 sequencing prep reactions: 3' ligation, 5' ligation, reverse transcription, cDNA cleanup, library amplification, and library cleanup reagents	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	331505
RT Initiator & Adapter UDI Kit (384)	For 384 sequencing prep reactions: UDI-specific 5' adapter and RT initiator	331507
Singular Genomics		
SG UDI Primers [1-96]	For 96 reactions, Plate with indexed Singular Genomics S1/SP1 PCR primers and S2/SP2 PCR primers, each at 5 µM concentration.	700,134
SG UDI Primers Set A [1-24]	For 24 reactions, Plate with indexed Singular Genomics S1/SP1 PCR primers and S2/SP2 PCR primers, each at 5 µM concentration.	700,135
SG UDI Primers Set B [25-48]	For 24 reactions, Plate with indexed Singular Genomics S1/SP1 PCR primers and S2/SP2 PCR primers, each at 5 µM concentration.	700,136

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

Document Revision History

Date	Changes
11/2023	Initial release.