illumina®

# Illumina miRNA Prep

## Product Documentation

ILLUMINA PROPRIETARY
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# Overview

This resource explains how to prepare small RNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems using the Illumina miRNA Prep kit and PCR-based unique dual indexes (UDIs). The Illumina miRNA Prep kit allows for library preparation and multiplexing of up to 384 samples.

The Illumina miRNA Prep Solution facilitates both enhanced differential expression analysis using integrated unique molecular identifiers (UMIs) and discovery of novel miRNAs from cells, tissues, and biofluids. The required amount of template for a single Illumina miRNA Prep sequencing reaction can range from 1 ng to 500 ng purified total RNA.

The Illumina miRNA Prep workflow provides the following features:

- An optimized assay that does not require gel purification, excision, or elution, resulting in a reduction in hands-on time and workflow length.
- Methodology that uses modified oligonucleotides to prevent adapter dimerization in the sequencing library.
- Designed to minimize the presence of hY4 Y RNA, which is often observed in high levels in serum and plasma samples.
- Highly optimized reaction chemistry that reduces biases and background contaminants.
- Integration of unique molecular identifiers (UMIs) into the reverse transcription process enables unbiased and accurate miRNome-wide quantification of mature miRNAs by NGS systems.
- Primary and secondary data analysis solutions using the DRAGEN miRNA analysis software, developed to facilitate UMI counting, miRNA mapping, and differential expression analysis. Perform secondary analysis in the cloud via BaseSpace Sequence Hub (BSSH) and Illumina Connected Analytics (ICA).
  For details, refer to the following resources:
  - BaseSpace Sequence Hub support resources
  - Illumina Connected Analytics support resources

## Input Recommendations

The Illumina miRNA Prep kit is optimized to prepare miRNA and other similarly sized RNAs with a 3' hydroxyl group and a 5' phosphate group (such as piRNA) sequencing libraries for use with Illumina sequencing systems. RNA molecules that are 50 bp or smaller and have a 3' hydroxyl and 5' phosphate are included in the library.

Total RNA containing miRNA is the required starting material for the Illumina miRNA Prep kit. It is not necessary to enrich for small RNA.

- Quantify input RNA per the following recommendations:

  - Successful library preparation depends on accurate quantification of input RNA. Use multiple methods to verify results.
  - Use fluorometric-based methods, such as Qubit, for accurate quantification of input RNA. UV spectrophotometric-based methods measure any nucleotides present in the sample, including gDNA, dsDNA, ssDNA, and free nucleotides, which can give an inaccurate measurement of RNA.
  - Quantification methods depend on accurate pipetting methods. Make sure that the pipettes are calibrated. Avoid using pipettes at the extremes of volume specifications.
- Assess RNA quality per the following recommendations:
  - The 260/280 nm absorbance ratio is used as an indication of sample purity. Values from 1.9 through 2.1 indicate relatively pure RNA. The presence of DNA or small nucleic acid fragments, such as nucleotides, can compromise both absorbance measurements.
  - The spectral properties of nucleic acids depend on pH values. Illumina recommends preparing dilutions and measuring absorbance in 10 mM Tris-HCl, pH 7.5.
  - It is not useful to assess the concentration and purity of total RNA derived from fluids and/or exosomes.
  - Make sure that samples are free of contaminants.
- Assess RNA integrity per the following recommendations:
  - Confirm the integrity and size distribution of total RNA from cells and fresh or frozen tissue by using an automated analysis system. Use an RNA integrity score (RIS) or RNA integrity number (RIN) to assess RNA integrity.
  - For best results, aim for an RIN value of ≥ 8. However, successful miRNA library prep is possible with samples whose RIN values are ≤ 8. For samples with low RIN values, increase the sequencing reads allocated per sample to allow for RNA degradation products. This allowance also applies to 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.
- Cell and tissue samples: The recommended starting amount of total RNA is 100 ng. You can use the protocol with 1–500 ng total RNA.
- Serum and plasma samples: The recommended starting amount of total RNA is 5 µl RNA eluate when 200 µl serum/plasma is processed using either of the following kits:
  - QIAGEN miRNeasy Serum/Plasma Kit
  - QIAGEN miRNeasy Serum/Plasma Advanced Kit
- Exosome samples prepared from serum and plasma: The recommended starting amount of total RNA is 5 µl RNA eluate when 1 ml of serum or plasma is processed using the QIAGEN exoRNeasy kits.
- Samples with heparin are not compatible with Illumina miRNA Prep kit. Heparin is a potent reverse-transcription inhibitor. After total RNA isolation from heparinized samples, you must remove heparin.

⚠ Handle all plasma and serum samples as if they are potentially infectious agents.

# Consumables and Equipment

The Illumina miRNA Prep protocol requires the following Illumina-supplied and user-supplied consumables and equipment.

The protocol has been optimized using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Make sure that you have the required consumables and equipment before starting the protocol.

## Product Contents

The Illumina miRNA Prep protocol requires library prep reagents and index adapters. Index adapter kits are sold separately.

| Component | Kit Options | Catalog # |
|---|---|---|
| Library prep reagents | Illumina miRNA Prep (96 Samples) | 20145030 |
| Index adapters | Illumina miRNA UD Indexes (96 Indexes, 96 Samples)<br>One of the following sets: | |
| | • Set A<br>• Set B<br>• Set C<br>• Set D | • 20145031<br>• 20145032<br>• 20145033<br>• 20145034 |

### Reagent Kit Contents

Each Illumina miRNA Prep kit includes the components listed in the following tables.

Box 1 is shipped on dry ice or blue ice. Box 2 is shipped on blue ice. Store all reagent kit boxes in a constant-temperature freezer at the temperature specified. When stored correctly, you can use the Illumina miRNA Prep kit until the expiration date printed on the kit box.

#### Illumina miRNA Prep Box 1, Store at -25°C to -15°C

| Quantity | Reagent | Description |
|---|---|---|
| 1 | DP | DNA Polymerase |
| 1 | FPL | miRNA 5' Ligase |
| 2 | LB | miRNA Library Buffer |
| 1 | MFPB | miRNA 5' Buffer |

| Quantity | Reagent | Description |
|---|---|---|
| 1 | MRNH | miRNA RNase Inhibitor |
| 1 | MRTE | miRNA RT Enzyme |
| 1 | MTPA | miRNA 3' Adapter |
| 1 | MTPB | miRNA 3′ Buffer |
| 2 | NFW | Nuclease-Free Water |
| 1 | RTB | miRNA RT Buffer |
| 1 | RTP2 | miRNA RT Primer 2 |
| 1 | TPL | miRNA 3' Ligase |
| 1 | UA | UDI 5' Adapter |
| 1 | URTI | UDI RT Initiator |

### Illumina miRNA Prep Box 2, Store at 2°C to 8°C

| Quantity | Reagent | Description |
|---|---|---|
| 1 | LA | Ligation Activator* |
| 2 | MBB | miRNA Bead Binding Buffer |
| 2 | miPB | Illumina miRNA Purification Beads |

* You can store the Ligation Activator at 2–8°C or at -25°C to -15°C.

## Index Adapter Kits

Illumina miRNA UD Indexes kits are required for library construction with UDIs. The kits use 10 bp unique dual sample indexes. The plates are sealed with a pierceable foil seal. Each well is intended for single use.

Index kits are shipped on dry ice or blue ice. Store all components in a constant-temperature freezer.

### Illumina miRNA UD Indexes, Store at -25°C to -15°C

| Quantity | Reagent | Description |
|---|---|---|
| 1 | miRNA UD Indexes | 96 well plate containing 96 unique dual indexes |

To multiplex more than 96 libraries in a single sequencing run, combine kits with different UDI plates. Combine libraries prepared with the Illumina miRNA UD Indexes kit sets (A, B, C, D) to generate up to 384 libraries with different sample indexes for multiplex sequencing.

# User-Supplied Consumables and Equipment

## Consumables

| Consumable | Supplier |
|---|---|
| Ethanol 200 proof (absolute) for molecular biology (500 ml) | General lab supplier |
| Microcentrifuge tubes, nuclease free, 1.5–2 ml | General lab supplier |
| Microseal 'B' PCR Plate Sealing Film, adhesive, optical | Bio-Rad, catalog # MSB1001 |
| Nuclease-free water | Thermo Fisher, catalog # AM9937 or general lab supplier |
| PCR tubes or plates, select from:<br>• PCR Tubes and Caps<br>• PCR Strip Tubes and Caps 8-Strip<br>• Real-Time PCR Plates, 96-Well, with 8-Strip Flat Caps | The following suppliers or equivalent:<br>• VWR, catalog # 20170-012<br>• VWR, catalog # 93001-118<br>• VWR, catalog # 82006-664 (plates), # 83009-684 (caps) |
| Pipette tips, nuclease free, 10 µl | General lab supplier |
| Pipette tips, nuclease free, 20 µl | General lab supplier |
| Pipette tips, nuclease free, 200 µl | General lab supplier |
| Pipette tips, nuclease free, 1000 µl | General lab supplier |
| RNA extraction kit for extraction of pure, quantitated RNA. Recommendations for serum/plasma and exosome samples are as follows.<br>**[Serum and plasma samples]**<br>• miRNeasy Serum/Plasma Advanced Kit<br>• miRNeasy Serum/Plasma Kit<br>**[Exosome samples prepared from serum and plasma]**<br>• exoRNeasy Midi Kit (50)<br>• exoRNeasy Maxi Kit (50) | General lab supplier, or one of the following suppliers, depending on sample type:<br>**[Serum and plasma samples]**<br>• QIAGEN, catalog # 217204<br>• QIAGEN, catalog # 217184<br>**[Exosome samples]**<br>• QIAGEN, catalog # 77144<br>• QIAGEN, catalog # 77164 |
| Qubit Assay Tubes | Thermo Fisher Scientific, catalog # Q32856 |
| Qubit dsDNA HS Assay Kit | Thermo Fisher Scientific, catalog # Q32854 |

| Consumable | Supplier |
|---|---|
| The following consumables, depending on your analysis system:<br>• **[Bioanalyzer]** Agilent DNA 12000 Kit<br>• **[Fragment Analyzer]** Agilent DNF-464 HS Large Fragment Kit | One of the following suppliers, depending on the instrument:<br>• Agilent, catalog # 5067-1508<br>• Agilent, catalog # DNF-464-0500 |

## Equipment

| Equipment | Supplier |
|---|---|
| Benchtop microcentrifuge | General lab supplier |
| Freezer, constant-temperature, -20°C | General lab supplier |
| Magnet for bead cleanups, one of the following:<br>• **[Plates]** DynaMag-96 Side Skirted Magnet<br>• **[Tubes]** MagneSphere Technology Magnetic Separation Stand | One of the following suppliers:<br>• Thermo Fisher Scientific, catalog # 12331D<br>• Promega, catalog # Z5342 |
| Pipettes, multichannel or single channel, 10 µl | General lab supplier |
| Pipettes, multichannel or single channel, 20 µl | General lab supplier |
| Pipettes, multichannel or single channel, 200 µl | General lab supplier |
| Pipettes, multichannel or single channel, 1000 µl | General lab supplier |
| Qubit 4 Fluorometer | Thermo Fisher Scientific, catalog # Q33238 |
| Refrigerator, 4°C | General lab supplier |
| Thermal cycler | General lab supplier |
| One of the following analysis systems:<br>• 2100 Bioanalyzer Desktop System*<br>• 5300 Fragment Analyzer System | One of the following suppliers:<br>• Agilent, catalog # G2940CA<br>• Agilent, catalog # M5311AA |

* End of life announced. Refer to vendor site for more information.

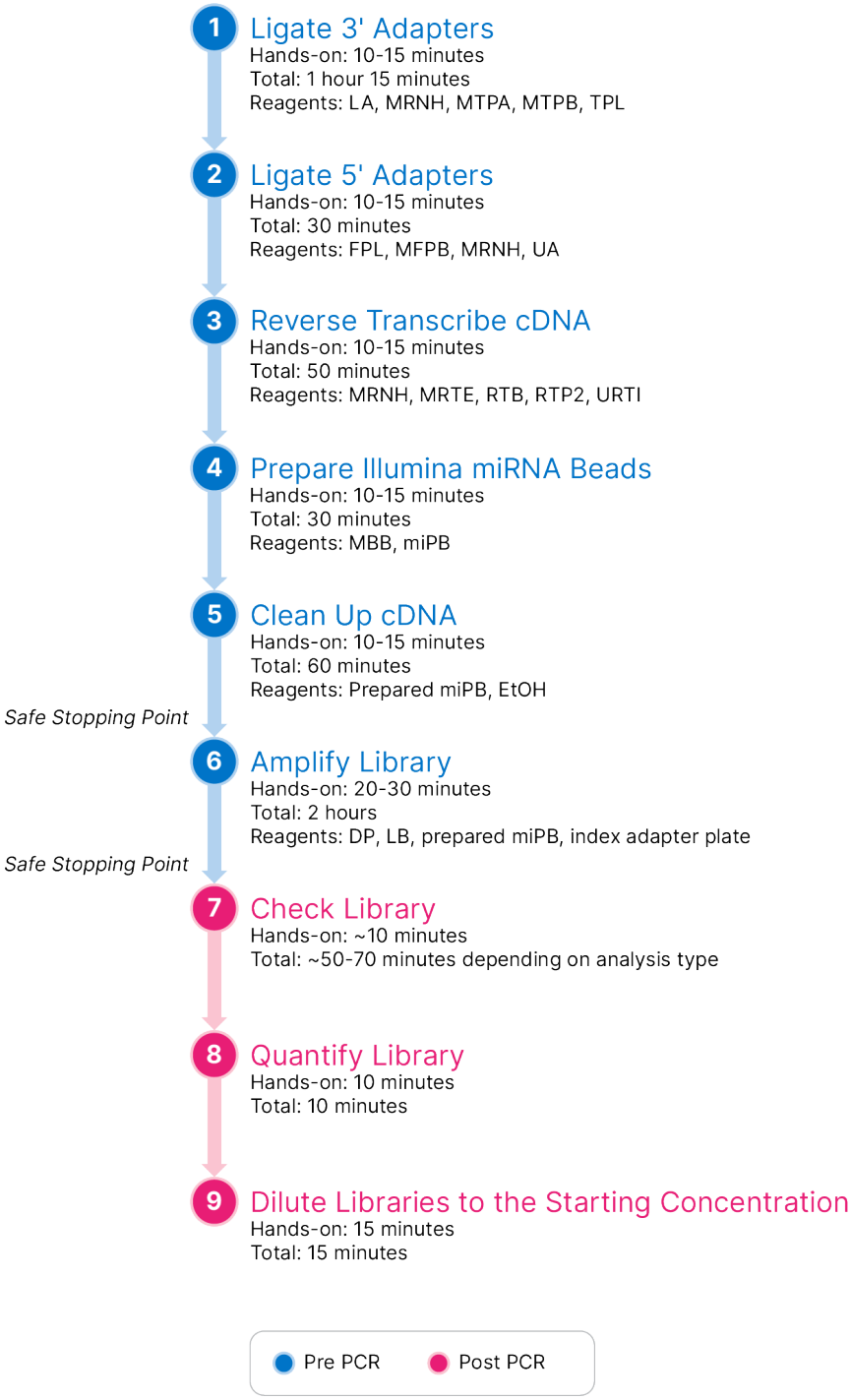
# Protocol

This section describes the Illumina miRNA Prep protocol.

- Review the planned complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Before proceeding, confirm the kit contents and make sure that you have the required components, equipment, and consumables. This protocol requires library prep reagents and index adapters. Index adapter plates are ordered separately. Refer to *Product Contents* on page 6 for details.
- To prevent adapter dimerization, use ≥ 1 ng total RNA and make sure to add reaction components in the order listed in the protocol.
- Follow the protocol steps in the order shown, using the specified volumes and incubation parameters. Changes in volumes, timing, or steps can result in failure of assay quality control metrics.
- The Illumina miRNA Prep protocol is time sensitive. Unless a safe stopping point is specified, proceed immediately to the next step.
- Make sure that reactions are thoroughly mixed, and are prepared and incubated at the recommended temperatures. Due to the viscosity of the ligation reactions, correct preparation is crucial for a successful experiment.

# Illumina miRNA Prep Workflow

The following diagram illustrates the Illumina miRNA Prep workflow. Time estimates are based on preparing 96 samples.



# Warnings and Precautions

- Wear protective equipment, including eye protection, gloves, and laboratory coat.
- Do not use any assay components beyond their stated expiration date on the assay box label. Assay lots are identified on the assay box label. Store the assay components at the specified temperature.
- Failure to follow the procedures as outlined can result in erroneous results or significant reduction in sample quality.
- For additional environmental, health, and safety information, refer to the safety data sheets (SDS) at support.illumina.com/sds.html.

# Tips and Techniques

**Protocol Continuity**

- Follow the protocol in the order described using the specified parameters.
- Avoid extended pauses until RNA is converted into double-stranded cDNA.
- Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

**Avoiding Cross-Contamination**

- When adding or transferring samples or reagent master mixes, change tips between ***each sample***.
- When adding index adapters or primers with a multichannel pipette, change tips between ***each row*** or ***each column***. If using a single channel pipette, change tips between ***each sample***.
- Remove unused index adapter plates from the working area.

**Handling Reagents and RNA**

- Avoid multiple freeze-thaw cycles of input RNA.
  - You can store RNA in RNase-free water or TE buffer at -85°C to -65°C for up to one year.
  - If you must reuse the sample, aliquot into separate tubes for single-use.
- Keep thawed reagents on ice until needed. Promptly return all reagents to storage after use.
- When not in use, seal plates and close lids to limit contamination.

**Handling Beads**

- Use beads at room temperature.
- Do not use beads that have been stored at < 2°C.
- Vortex beads before each use and frequently throughout the protocol to resuspend. Resuspended beads are evenly distributed and homogenous in color.
- Dispense liquid directly onto bead pellets so that beads on the side of the wells are wetted.
- Use the applicable magnetic stand as follows.
  - DynaMag-96 Side Skirted Magnet—Use for plates.

  - MagneSphere Technology Magnetic Separation Stand—Use for tubes.
- When the plate is on the magnetic stand, do not agitate the plate or disturb the bead pellet.
- If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, then wait until the liquid is clear (~2 minutes).
- If beads adhere to well walls, centrifuge at 280 × g for 3 seconds, and then pipette to resuspend.

**Sealing Plates and Tubes**

- Before starting the following steps in the protocol, seal the plate or cap the tube firmly. Improper sealing leads to cross-contamination.
  - Shaking steps
  - Centrifuge steps
  - Thermal cycling steps
  - Vortexing steps
- If a plate does not have its own seal, use Microseal 'B' adhesive seal. Microseal 'B' adhesive seal is effective at -40°C to 110°C.
  - Cover the plate with the seal, and then seal firmly with a rubber roller or wedge.
  - After each use, discard seals.

**Plate Transfers**

- When transferring volumes between plates, transfer the specified volume from each well of the first plate to the corresponding well of the second plate.

**Centrifugation**

- At any step, centrifuge at 280 × g for 3 seconds to consolidate liquid or beads in the bottom of the well to prevent sample loss.

# Ligate 3' Adapters

This step ligates a pre-adenylated DNA adapter to the 3' end of the miRNAs.

When working with cell and tissue samples, the recommended starting amount of total RNA is 100 ng. For details, refer to *Input Recommendations* on page 4.

If you are working with low total RNA input amounts or with serum or plasma samples, you must dilute the miRNA 3' Adapter. Instructions are provided in the following *Preparation* on page 14 section.

## Consumables

- LA (Ligation Activator)
- MRNH (miRNA Inhibitor)
- MTPA (miRNA 3' Adapter)

- MTPB (miRNA 3′ Buffer)
- TPL (miRNA 3′ Ligase)
- NFW (Nuclease-free water)
- 96-well PCR plate or PCR tubes
- Microcentrifuge tubes, 1.5–2 ml

## About Reagents

- TPL and MRNH
  - Remove from the -25°C to -15°C freezer immediately before preparing the Master Mix. Place on ice.
  - Aliquot into smaller volumes upon first thaw to avoid repeated freeze-thaw cycles.
  - Avoid vortexing.
  - Return to the freezer immediately after use.

## Preparation

1. Thaw the template RNA on ice. Mix gently, centrifuge briefly to collect residual liquid from the sides of the tubes, and then return to ice.

   Avoid vortexing the template RNA.

2. Prepare the following consumables:

| Reagent | Storage | Instructions |
|---|---|---|
| LA | 2°C to 8°C | Thaw at room temperature. |
| MTPA | -25°C to -15°C | Thaw at room temperature. |
| MTPB | -25°C to -15°C | Thaw at room temperature. |
| Nuclease-free water | -25°C to -15°C | Thaw at room temperature. |

3. Flick each tube to mix, and then centrifuge briefly to collect residual liquid from the sides of the tube. Keep at room temperature.
4. If you are working with low RNA inputs or with serum or plasma samples, dilute the MTPA as follows.
   a. Add nuclease-free water to the MTPA, as specified in the following table.

| Template RNA Input (Total RNA) | MTPA Dilution |
|---|---|
| 500 ng | Use undiluted |
| 100 ng | Use undiluted |
| 10 ng | Dilute 1:5 |

| Template RNA Input (Total RNA) | MTPA Dilution |
|---|---|
| 1 ng | Dilute 1:20 |
| Serum or plasma | Dilute 1:5 |

b. Centrifuge the dilution briefly.

c. Pipette up and down 12 times to mix, and then centrifuge briefly a second time.

5. Save the following Lig3 program on the thermal cycler:
   - Choose the preheated lid option and set to 100°C
   - Reaction volume is 20 µl
   - 28°C for 1 hour
   - 65ºC for 20 minutes
   - 4°C for 5 minutes
   - Hold at 4°C
6. Immediately before preparing the 3' ligation Master Mix, prepare the following consumables:

| Reagent | Storage | Instructions |
|---|---|---|
| MRNH | -25°C to -15°C | Place on ice. Return to the freezer immediately after use. |
| TPL | -25°C to -15°C | Place on ice. Return to the freezer immediately after use. |

## Procedure

1. Working on ice, prepare the 3′ ligation Master Mix as follows.

a. Add the following volumes to the tube ***in the order listed.*** For multiple reactions, multiply each volume by the number of samples plus 10% extra.

| Component | Volume per Reaction (µl) |
|---|---|
| Nuclease-free water | Variable |
| MTPA (diluted as needed)[1] | 1 |
| MRNH | 1 |
| TPL | 1 |
| MTPB | 2 |
| LA | 10 |
| Template RNA (added in step 3) | Variable[2] |
| Total volume per reaction | 20 µl |

[1] For low input, serum, or plasma RNA, the MTPA must be diluted (refer to *Preparation* on page 14 section).
[2] For cell and tissue samples, the recommended starting amount of total RNA is 100 ng. For serum and plasma samples and exosome samples prepared from serum/plasma, the recommended starting amount is 5 µl RNA eluate. Processing details differ depending on the sample type and extraction kit used. For details, refer to *Input Recommendations* on page 4.

b. Centrifuge briefly.

Avoid vortexing the 3' ligation Master Mix reactions.

c. Pipette up and down slowly 15–20 times to mix, and then centrifuge briefly a second time.

Pipette slowly when mixing. The LA reagent is highly viscous.

2. Distribute the 3′ ligation Master Mix into one PCR plate well or PCR tube per sample.
3. Add template RNA to each well or tube containing the 3′ ligation Master Mix.
4. Place on the thermal cycler and run the Lig3 program.
5. Proceed immediately to *Ligate 5′ Adapters* on page 16.

# Ligate 5' Adapters

This step ligates an RNA adapter to the 5' end of mature miRNAs.

If you are working with low total RNA input amounts or with serum or plasma samples, you must dilute the UDI 5' Adapter. Instructions are provided in the following *Preparation* on page 17 section.

## Consumables

- FPL (miRNA 5' Ligase)
- MFPB (miRNA 5' Buffer)

- MRNH (miRNA Inhibitor)
- UA (UDI 5' Adapter)
- NFW (Nuclease-free water)
- Microcentrifuge tubes, 1.5–2 ml

## About Reagents

- FPL and MRNH
  - Remove from the -25°C to -15ºC freezer immediately before preparing the Master Mix. Place on ice.
  - Aliquot into smaller volumes upon first thaw to avoid repeated freeze-thaw cycles.
  - Avoid vortexing.
  - Return to the freezer immediately after use.

## Preparation

1. Prepare the following consumables:

| Reagent | Storage | Instructions |
|---|---|---|
| MFPB | -25°C to -15°C | Thaw at room temperature. |
| UA | -25°C to -15°C | Thaw at room temperature. |

2. Flick each tube to mix, and then centrifuge briefly to collect residual liquid from the sides of the tube. Keep at room temperature.
3. If you are working with low RNA inputs or with serum or plasma samples, dilute the UA as follows.
   a. Add nuclease-free water to the UA, as specified in the following table.

| Template RNA Input (Total RNA) | UA Dilution |
|---|---|
| 500 ng | Use undiluted |
| 100 ng | Use undiluted |
| 10 ng | Dilute 1:2.5 |
| 1 ng | Dilute 1:10 |
| Serum or plasma | Dilute 1:2.5 |

   b. Centrifuge the dilution briefly.
   c. Pipette up and down 12 times to mix, and then centrifuge briefly a second time.
4. Save the following Lig5 program on the thermal cycler:
   - Choose the preheated lid option and set to 100°C
   - Reaction volume is 40 µl

- 28°C for 30 minutes
- 65°C for 20 minutes
- Hold at 4°C

5. Immediately before preparing the 5′ ligation reaction, prepare the following consumables:

| Reagent | Storage | Instructions |
|---|---|---|
| FPL | -25°C to -15°C | Place on ice. Return to the freezer immediately after use. |
| MRNH | -25°C to -15°C | Place on ice. Return to the freezer immediately after use. |

## Procedure

1. Working on ice, prepare the 5′ ligation reaction as follows.
   a. Add the following volumes to the tube containing the 3' ligation reaction ***in the order listed***. For multiple reactions, multiply each volume by the number of samples plus 10% extra.

| Component | Volume per Reaction (µl) |
|---|---|
| 3' ligation reaction (already in tube) | 20 |
| Nuclease-free water | 15 |
| MFPB | 2 |
| MRNH | 1 |
| FPL | 1 |
| UA (dilute as needed)* | 1 |
| Total volume per reaction | 40 |

* For low input, serum, or plasma RNA, the UA must be diluted (refer to *Preparation* on page 17 section).

   b. Centrifuge briefly.

   Avoid vortexing the 5' ligation reactions.

   c. Pipette up and down slowly 10–15 times to mix, and then centrifuge briefly a second time.

   Pipette slowly when mixing. The reaction mix is highly viscous.

2. Place on the thermal cycler and run the Lig5 program.
3. Proceed immediately to *Reverse Transcribe cDNA* on page 19.

# Reverse Transcribe cDNA

In this step, the ligated miRNAs are reverse transcribed to cDNA using a reverse transcription primer with a unique molecular identifier (UMI).

The RT primer binds to a region of the 3' adapter and facilitates conversion of the 3'/5' ligated miRNAs into cDNA. The primer also assigns a UMI to every miRNA molecule. During reverse transcription, a universal sequence is added.

If you are working with low total RNA input amounts or with serum or plasma samples, you must dilute the miRNA RT Primer 2. Instructions are provided in the following *Preparation* on page 19 section.

## Consumables

- MRNH (miRNA RNase Inhibitor)
- MRTE (miRNA RT Enzyme)
- RTB (miRNA RT Buffer)
- RTP2 (miRNA RT Primer 2)
- URTI (UDI RT Initiator)
- **[Low input, serum, or plasma RNA]** NFW (Nuclease-free water)
- Microcentrifuge tubes, 1.5–2 ml

## About Reagents

- MRNH and MRTE
  - Remove from the -25°C to -15°C freezer immediately before preparing the Master Mix. Place on ice.
  - Aliquot into smaller volumes upon first thaw to avoid repeated freeze-thaw cycles.
  - Avoid vortexing.
  - Return to the freezer immediately after use.

## Preparation

1. Prepare the following consumables:

| Reagent | Storage | Instructions |
|---|---|---|
| RTB | -25°C to -15°C | Thaw at room temperature. |
| RTP2 | -25°C to -15°C | Thaw at room temperature. |
| URTI | -25°C to -15°C | Thaw at room temperature. |

2. Flick each tube to mix, and then centrifuge briefly to collect residual liquid from the sides of the tube. Keep at room temperature.
3. If you are working with low RNA inputs or with serum or plasma samples, dilute the RTP2 as follows. Add nuclease-free water to the RTP2, as specified in the following table.

| 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 or plasma | Dilute 1:5 |

4. Save the following RT1 program on the thermal cycler:
   - Choose the preheated lid option and set to 100°C
   - Reaction volume is 42 µl
   - 75°C for 2 minutes
   - 70°C for 2 minutes
   - 65°C for 2 minutes
   - 60°C for 2 minutes
   - 55°C for 2 minutes
   - 37°C for 5 minutes
   - 25°C for 5 minutes
   - Hold at 4°C
5. Save the following RT2 program on the thermal cycler:
   - Choose the preheated lid option and set to 100°C
   - Reaction volume is 60 µl
   - Incubate at 50°C for 1 hour
   - Incubate at 70ºC for 15 minutes
   - 4°C for 5 minutes
   - Hold at 4°C
6. Immediately before preparing the reverse transcription reaction, prepare the following consumables:

| Reagent | Storage | Instructions |
|---|---|---|
| MRNH | -25°C to -15°C | Place on ice. Return to the freezer immediately after use. |
| MRTE | -25°C to -15°C | Place on ice. Return to the freezer immediately after use. |

### Procedure

1. Add 2 µl URTI to each tube containing the 5' ligation reaction.
2. Centrifuge briefly.
3. Pipette up and down 15–20 times to mix, and then centrifuge briefly a second time.
4. Place on the thermal cycler and run the RT1 program. Hold at 4°C until ready to prepare the reverse transcription reaction.
5. Working on ice, prepare the reverse transcription reaction as follows.
   Add the following volumes to the tube containing the 5' ligation reaction ***in the order listed.*** For multiple reactions, multiply each volume by the number of samples plus 10% extra.

| Component | Volume per Reaction (µl) |
|---|---|
| 5' ligation reaction + URTI (already in tube) | 42 |
| RTP2 (dilute as needed)* | 2 |
| Nuclease-free water | 2 |
| RTB | 12 |
| MRNH | 1 |
| MRTE | 1 |
| Total volume per reaction | 60 |

   * For low input, serum, and plasma RNA, the RTP2 must be diluted (refer to *Preparation* on page 19 section).

   Avoid vortexing the reverse transcription reactions.
6. Place on the thermal cycler and run the RT2 program.
7. Proceed immediately to *Prepare Illumina miRNA Beads* on page 21. You can perform this step while the reverse transcription reactions are incubating.

# Prepare Illumina miRNA Beads

This step prepares the Illumina miRNA Beads by rebuffering them with miRNA Bead Binding Buffer.

You can prepare beads for up to four samples (1.6 ml) at one time in a single 2 ml tube. If processing beads for multiple samples together, scale up the amounts of miPB and MBB specified in the procedure.

Before and after preparation, the Illumina miRNA Beads must be homogenous. This requirement means that you must work quickly and resuspend the beads thoroughly immediately before use. If a delay in the protocol occurs, vortex the beads again.

After preparation, place the Illumina miRNA Beads on ice.

## Consumables

- MBB (miRNA Bead Binding Buffer)
- miPB (Illumina miRNA Beads)
- Microcentrifuge tubes, 2 ml

## About Reagents

- Avoid centrifugation of both MBB and miPB reagents before combining them (step 6 of the following procedure).
- miPB
  - The beads must be homogenous. Work quickly and resuspend the beads thoroughly immediately before use. If a delay in the protocol occurs, vortex the beads again.

## Procedure

1. Vortex miPB and MBB thoroughly to make sure that the beads are in suspension and homogenously distributed. Avoid centrifugation.
2. Add 400 µl miPB carefully (bead storage buffer is viscous) to a 2 ml microcentrifuge tube.
   This volume of beads is sufficient to perform the subsequent cDNA cleanup and the cleanup associated with library amplification for one sample.
3. Centrifuge briefly and immediately place on a magnetic stand. Wait until the liquid is clear.
4. When the liquid is clear, carefully remove and discard the supernatant.
   It is acceptable to leave a small amount of supernatant in the tube after this step.
5. Remove the tube from the magnetic stand.
6. Pipette 150 µl MBB carefully (buffer is viscous) onto the beads.
7. Vortex thoroughly to resuspend the bead pellet completely.
8. Centrifuge briefly and immediately place on a magnetic stand. Wait until the liquid is clear.
9. When the liquid is clear, carefully remove and discard the supernatant.
   Without disturbing the beads, make sure that as much supernatant as possible has been removed.
10. Remove the tube from the magnetic stand and pipette 400 µl MBB carefully (buffer is viscous) onto the beads.
11. Vortex thoroughly to resuspend the bead pellet completely.

12. Place the prepared miPB on ice.

**SAFE STOPPING POINT**

If you are not using the prepared miPB immediately, cap the tube and store the beads on ice or at 2°C to 8°C.

You can store prepared miPB at 2°C to 8°C for up to 7 days.

# Clean Up cDNA

In this step, a cleanup of the miRNA library is performed using a magnetic bead-based method.

The entire 60 µl cDNA synthesis prepared in *Reverse Transcribe cDNA* on page 19 is the starting material for this step. The Illumina miRNA Beads (miPB) prepared in the *Prepare Illumina miRNA Beads* on page 21 procedure are required for the cleanup procedure.

- You can perform the cleanup procedure in tubes or plates. When working with plates, perform brief centrifugations at 2000 rpm for 2 minutes.
- Following ethanol washes, beads must be completely dry. Recommended procedures for removing excess ethanol are provided.

## Consumables

- Prepared miPB
- Freshly prepared 80% ethanol (EtOH)
- 96-well PCR plates or microcentrifuge tubes, 1.5–2 ml
- Microseal 'B' adhesive film

## About Reagents

- Prepared miPB
  - The beads must be thoroughly mixed at all times. This requirement means that you must work quickly and resuspend the beads thoroughly immediately before use. If a delay in the protocol occurs, vortex the beads again.

## Procedure

1. Centrifuge the tubes or plates containing the cDNA reactions.
2. Add 143 µl prepared miPB to each reaction.
3. Mix as follows.
   - **[Tubes]** Vortex for 3 seconds and centrifuge briefly.

- **[Plates]** Vortex for 3 seconds and centrifuge at 2000 rpm for 2 minutes. If the plates are warped, transfer the solution to new plates.

4. Incubate at room temperature for 5 minutes.
5. Place on the magnetic stand and wait until the liquid is clear (~4 minutes).

   Make sure that the liquid is clear before proceeding.

6. Keep on the magnetic stand and do as follows.
   a. Discard the supernatant.
   b. With a 20 µl pipette, remove any residual supernatant.
7. With the beads still on the magnetic stand, wash the beads as follows.
   a. Add 200 µl fresh 80% EtOH to each tube or plate well.
   b. Remove and discard the EtOH wash immediately.
8. Wash beads a **second** time.
9. With the beads still on the magnetic stand, remove residual EtOH as follows.
   a. With a 200 µl pipette, remove as much EtOH as possible.
   b. With a 10 µl pipette, remove any residual EtOH.

   It is important to completely remove all traces of EtOH after the second wash.

10. Air-dry on the magnetic stand for 10 minutes at room temperature.
    Visually inspect the bead pellets to confirm that they are completely dry and that all residual EtOH has evaporated.

    Residual EtOH can hinder efficiency of the subsequent library amplification reactions. Depending on humidity, you might need to extend the drying time.

11. Keep on the magnetic stand and elute the DNA by adding 17 µl nuclease-free water to each tube or plate well.
12. Remove from the magnetic stand.
13. Pipette up and down carefully until the beads are resuspended, and then seal.
14. Centrifuge briefly. If working with plates, centrifuge at 2000 rpm for 2 minutes.
15. Incubate at room temperature for 2 minutes.
16. Return to the magnetic stand and wait until the liquid is clear (~2 minutes).

    Make sure that the liquid is clear before proceeding.

17. Remove the seal and transfer 15 µl eluted DNA to a new tube or to the corresponding well of a new PCR plate.

**SAFE STOPPING POINT**

If you are stopping, cap the tube or seal the plate and store at -25°C to -15°C in a constant-temperature freezer for up to 7 days.

# Amplify Library

This step amplifies the ligated DNA fragments and assigns each sample a unique dual index. After library amplification, a cleanup of the miRNA library is performed using a magnetic bead-based method. To confirm the indexes of libraries selected for low plexity pooling have the appropriate color balance, refer to the *Index Adapters Pooling Guide (document # 1000000041074)*.

Following ethanol washes, beads must be completely dry. Recommended procedures for removing excess ethanol are provided.

## Consumables

- DP (DNA Polymerase)
- LB (miRNA Library Buffer)
- Illumina miRNA UD Indexes adapter plate
- Prepared miPB (Illumina miRNA Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well PCR plates
- Microcentrifuge tubes, 1.5–2 ml
- Microseal 'B' adhesive film

## About Reagents

- DP
  - Remove from the -25°C to -15°C freezer immediately before preparing the Master Mix. Place on ice.
  - Aliquot into smaller volumes upon first thaw to avoid repeated freeze-thaw cycles.
  - Avoid vortexing.
  - Return to the freezer immediately after use.
- Illumina miRNA UD Indexes
  - Each well is single-use and contains a premixed Index 1 (i7) and Index 2 (i5) UDI pair.
  - During the reaction setup, add components from the index adapter plate directly to the tube or plate.
- Prepared miPB

– The beads must be thoroughly mixed at all times. This requirement means that you must work quickly and resuspend the beads thoroughly immediately before use. If a delay in the protocol occurs, vortex the beads again.

## Preparation

1. Prepare the following consumables:

| Reagent | Storage | Instructions |
|---|---|---|
| LB | -25°C to -15°C | Thaw at room temperature. Flick to mix, and then centrifuge briefly. |
| Index plate | -25°C to -15°C | Thaw at room temperature. Flick to mix, and then centrifuge briefly. |

2. Save the following Amp program on a thermal cycler using the appropriate number of amplification cycles. The number of cycles (X) depends on the original RNA input and is shown in the subsequent table.
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 50 µl
   - 95°C for 15 minutes
   - X cycles of:
     - 95°C for 15 seconds (Denaturation)
     - 60°C for 30 seconds (Annealing)
     - 72°C for 15 seconds (Extension)
   - 72°C for 2 minutes
   - 4°C for 5 minutes
   - Hold at 4°C

   When amplifying multiple samples together on one plate, make sure that the input for each sample is the same.

| Original RNA input (Total RNA) | Number of Cycles (X) |
|---|---|
| 500 ng | 13 |
| 100 ng | 16 |
| 10 ng | 19 |
| 1 ng | 24 |
| Serum or plasma | 22 |

3. Immediately before starting the procedure, prepare the following consumable:

| Reagent | Storage | Instructions |
|---|---|---|
| DP | -25°C to -15°C | Place on ice. Return to the freezer immediately after use. |

## Procedure

1. If you are resuming the protocol after a safe stopping point, proceed as follows.
   - Centrifuge the sealed tube or PCR plate containing the cleaned up cDNA at 280 × g for 10 seconds.
2. Open the index adapter plate. Using a new pipette tip for each well, pierce the foil covering the wells that you intend to use.
3. Working on ice, prepare the library amplification reaction as follows.
   Add the following volumes to each tube or PCR plate well containing the cleaned up cDNA.
   - Add reaction components first. Add UD indexes last.
   - For multiple reactions, multiply each volume by the number of samples plus 10% extra.

| Component | Volume per Reaction (µl) |
|---|---|
| Cleaned up cDNA (already in tube/plate) | 15 |
| LB | 10 |
| DP | 1.5 |
| Sample index from index plate well* | 2 |
| Nuclease-free water | 21.5 |
| Total volume | 50 |

* Up to 384 unique Illumina miRNA UD Indexes are available for use. Use a different UDI for each sample.

Avoid vortexing the library amplification reactions.

4. Centrifuge briefly.
5. Pipette up and down 12 times to mix, seal, and then centrifuge briefly a second time.
6. Place on the preprogrammed thermal cycler and run the Amp program.

   At the end of the program, hold at 4°C for at least 5 minutes.
7. Remove the library amplification reactions from the thermal cycler.
8. Centrifuge as follows.
   - **[Tubes]** Centrifuge briefly.
   - **[Plates]** Centrifuge at 2000 rpm for 2 minutes. If the plates are warped, transfer the solution to new plates.
9. Add 47 µl prepared miPB to each reaction.

10. Vortex for 3 seconds and centrifuge briefly. If working with plates, centrifuge at 2000 rpm for 2 minutes.
11. Incubate at room temperature for 5 minutes.
12. Place on the magnetic stand and wait until the liquid is clear (~4 minutes).

    Make sure that the liquid is clear before proceeding.

13. Retain the supernatant and transfer 92 µl supernatant to new tubes or plate wells. Discard the tubes containing the beads.

    Make sure to retain the supernatant.

14. Add 83 µl prepared miPB to the 92 µl supernatant.
15. Vortex for 3 seconds and centrifuge briefly. If working with plates, centrifuge at 2000 rpm for 2 minutes.
16. Incubate at room temperature for 5 minutes.
17. Place on the magnetic stand and wait until the liquid is clear (~4 minutes).

    Make sure that the liquid is clear before proceeding.

18. Keep on the magnetic stand and do as follows.
    a. Discard the supernatant.
    b. With a 20 µl pipette, remove any residual supernatant.
19. With the beads still on the magnetic stand, wash the beads as follows.
    a. Add 200 µl fresh 80% EtOH to each tube or plate well.
    b. Remove and discard the EtOH wash immediately.
20. Wash beads a **second** time.
21. With the beads still on the magnetic stand, remove residual EtOH as follows.
    a. With a 200 µl pipette, remove as much EtOH as possible.
    b. With a 10 µl pipette, remove any residual EtOH.

    It is important to completely remove all traces of EtOH after the second wash.

22. Air-dry on the magnetic stand for 10 minutes at room temperature.
    Visually inspect the bead pellets to confirm that they are completely dry and that all residual EtOH has evaporated.

    Residual EtOH can hinder efficiency of the subsequent library amplification reactions. Depending on humidity, you might need to extend the drying time.

23. Keep on the magnetic stand and elute the DNA by adding 17 µl nuclease-free water to each tube or plate well.
24. Remove from the magnetic stand.

25. Pipette up and down carefully until the beads are resuspended, and then seal.
26. Centrifuge briefly. If working with plates, centrifuge at 2000 rpm for 2 minutes.
27. Incubate at room temperature for 2 minutes.
28. Return to the magnetic stand and wait until the liquid is clear (~2 minutes).

    Make sure that the liquid is clear before proceeding.

29. Remove the seal and transfer 15 µl eluted DNA to new tubes. This product is the miRNA sequencing library.

**SAFE STOPPING POINT**

If you are stopping, cap the tubes or seal the plates and store at -25°C to -15°C in a constant-temperature freezer for up to 30 days.

Avoid repeated freeze-thaw cycles to prevent degradation.

# Check Library

This step checks the quality of the final library. A portion of the 15 µl miRNA sequencing library from the *Amplify Library* on page 25 procedure is the starting material for library QC.

When not in use, store the miRNA sequencing library on ice.

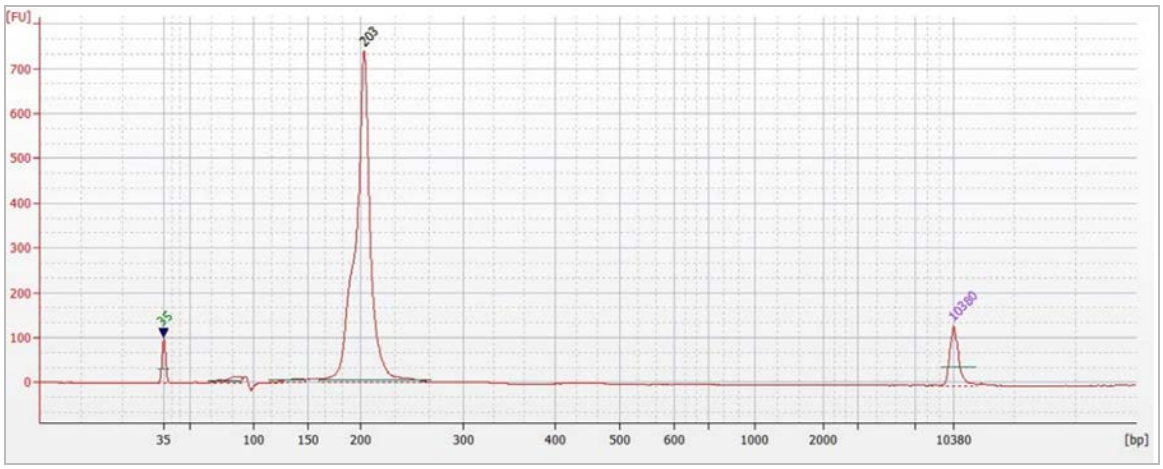
- A miRNA-sized library is approximately 200 bp. A piRNA-sized library is approximately 208 bp.
- If there is a peak at approximately 185–192 bp, the peak comprises RNA fragments or small RNAs other than miRNAs. RNA fragments or small RNAs are common in total RNA samples, being particularly strong in biofluid total RNA samples.
- Even if a peak is observed at 151–158 bp, miRNAs are likely present in the sample. Any RNA that has a 3' OH and 5' PO4, and is approximately 50 bp and smaller, should be captured by the Illumina miRNA Prep kit.

**Procedure**

1. Analyze 1 µl library using one of the following analysis systems, following the instructions provided by the manufacturer:
   - Agilent 2100 Bioanalyzer and DNA 1000 Kit.
   - Fragment Analyzer and D1000 Screen Tape.

   The following example shows a Bioanalyzer QC trace for a typical miRNA-sized library prepared from 1 ng XpressRef Universal RNA.

   Figure 1 Example Bioanalyzer Trace




# Quantify Library

A portion of the 15 µl miRNA sequencing library from the *Amplify Library* on page 25 step is the starting material for this step. When not in use, store the miRNA sequencing library on ice.

Illumina recommends using a Qubit fluorometer to determine the library concentration.

Perform the following to check the quantity and quality of the purified library prep product.

1. Quantify 2 µl miRNA sequencing library using a Qubit fluorometer and Qubit dsDNA HS Assay Kit. Follow the instructions provided by the manufacturer.

# Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration. For details on sequencing system compatibility, refer to the Illumina support site.

The final loading concentrations are a starting point and general guideline. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

1. Calculate the molarity value of the libraries. Use the following formula:
   Use 200 bp as the average library size. The formula uses 660 g/mol as the average weight for a single DNA bp.

$$\frac{ng/\mu l \times 10^6}{660\frac{g}{mol} \times average\ library\ size\ (bp)} = Molarity\ (nM)$$

2. Using the molarity value, dilute libraries to the starting concentration for your system.
   If pooling, combine libraries with different sample indexes in equimolar amounts if similar sequencing depth is needed for each library.

| Sequencing System | Starting Concentration (nM) | Final Loading Concentration (pM) |
|---|---|---|
| iSeq 100* | 4 | 75 |
| MiSeq* | 4 | 10 |
| MiniSeq* | 4 | 1.2 |
| NextSeq 500/550 | 4 | 1.2 |
| NextSeq 1000/2000 | 2 | **[Onboard Denature & Dilute]** 650<br>**[Manual Denature & Dilute]** 75 |
| NovaSeq 6000 | 10 | 200–300 |

* End of life announced. Refer to the Illumina website for more information.

3. Follow the denature and dilute instructions for your system to dilute libraries to the final loading concentration.
   - For iSeq 100, refer to the *iSeq 100 System Product Documentation*.
   - For the following sequencing systems, refer to the Denature and Dilute Protocol Generator and follow the Standard loading protocol:
     - MiSeq
     - MiniSeq
     - NextSeq 500/550
     - NovaSeq 6000
   - For NextSeq 1000/2000, refer to the Denature and Dilute Protocol Generator and follow the Onboard or Manual protocol.
4. Prepare and load the pooled library on the sequencing system according to the specific Illumina sequencing system product documentation.
   - Although not required, you can add PhiX to the sequencing run. For the optimal PhiX percentage, refer to the denature and dilute instructions for your system.
   - Illumina miRNA Prep libraries require 1 x 72 bp read length with 10 bp dual indexing. Illumina recommends allocating 5–10 million reads per sample.

# Resources & References

The Illumina miRNA Prep support pages on the Illumina support site provide additional resources. Always check the support pages for the latest versions.

## Revision History

| Document | Date | Description of Change |
|---|---|---|
| Document # 200071379 v01 | November 2025 | • Updated input sample recommendations to include information about samples with heparin.<br>• Updated overview information for consumables and equipment list and index adapter kits.<br>• Corrected ethanol supplier reference.<br>• Corrected miPB reagent reference in Amplify Library procedure. |
| Document # 200071379 v00 | September 2025 | Initial release. |

# Technical Assistance

For technical assistance, contact Illumina Technical Support.

| | |
|---|---|
| **Website:** | www.illumina.com |
| **Email:** | techsupport@illumina.com |

**Safety data sheets (SDSs)**—Available on the Illumina website at support.illumina.com/sds.html.

**Product documentation**—Available for download from support.illumina.com.

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