

Collibri™ Stranded RNA Library Prep Kit for Illumina® Systems

USER GUIDE

SMALL RNA SEQUENCING WORKFLOW

for use with Illumina® next generation sequencing (NGS) platforms

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Product information

Product description

The Invitrogen™ Collibri™ Stranded RNA Library Prep Kit is designed for robust construction of cDNA libraries for strand-specific RNA sequencing on the Illumina® NGS (next generation sequencing) platforms.

This user guide describes the library preparation procedure of small RNA species from 100 ng to 3 µg of total RNA sample. The protocol is suitable for any total RNA, including cfDNA and FFPE samples. Multiplexing of libraries can be carried out using up to 96 single-indexed and dual-indexed primers, enabling single read or paired-end sequencing. For convenience, the kit provides color-coded components for visual tracking of the library preparation progress. Inert dyes in the reagents do not interfere with enzymatic reactions and do not compromise library prep and sequencing results.

Note: For an overview of the technology used in the Collibri™ Stranded RNA Library Prep Kit, see “Technology overview” on page 9.

IMPORTANT! Due to the differences between standard library cDNA preparation and small RNA library preparation workflows, the amount of reagents in the Collibri™ Stranded RNA Library Prep Kit is sufficient for:

- 12 small RNA library preparations with the 24-prep size kit (Cat. No. [A38994024](#) or [A38996024](#))
 - 50 small RNA library preparations with the 96-prep size kit (Cat. No. [A38994096](#) or [A38996096](#))
-

Note: For more information on how to co-sequence single-indexed libraries with dual-indexed libraries see “Sequence the prepared library” on page 28.

Kit contents and storage

The Colibri™ Stranded RNA Library Prep Kit is available in 24- and 96-prep sizes. However, due to the differences between standard library cDNA preparation and small RNA library preparation workflows, the 24-prep and 96-prep kits contain sufficient reagents for 12 or 50 small RNA preparations.

Component	Cap color		Amount		Storage
			24 preps ^[1]	96 preps ^[2]	
Library Prep Kit					
10X Fragmentation Buffer	Blue		24 µL	96 µL	-20°C
RNase III	Yellow		24 µL	96 µL	
RNA End Repair Enzyme	Green		24 µL	96 µL	
Water, nuclease-free	White		1.25 mL	4 × 1.25 mL	
2X Adaptor Mix	Blue		240 µL	4 × 240 µL	
2X Ligation Buffer	Yellow		600 µL	4 × 600 µL	
10X Ligation Enzyme Mix	White		120 µL	4 × 120 µL	
2.5X RT Buffer	Red		960 µL	4 × 960 µL	
10X SuperScript™ IV Enzyme Mix	White		240 µL	4 × 240 µL	
2X Library Amplification Master Mix	Blue		600 µL	2 × 600 µL	
10X Index Primer Mix ^[3]	—		5 µL/well (24 wells)	5 µL/well (96 wells)	
Library Cleanup Kit					
Dynabeads™ Cleanup Beads	Orange		10 mL	40 mL	2°C to 8°C
Wash Buffer (Concentrated)	Blue		4.5 mL	18 mL	IMPORTANT! Do not freeze.
Elution Buffer	White		5 mL	20 mL	

^[1] Contains sufficient reagents for 12 small RNA preparations.

^[2] Contains sufficient reagents for 50 small RNA preparations.

^[3] i7 indices pre-mixed with universal or indexed i5 PCR primer, which allow up to 24 or 96 samples to be multiplexed, are included in the kit (supplied in 10X Index Primer Mix Plate format). Each well in the 10X Index Primer Mix contains 5 µL of primer mix, sufficient for amplification/barcoding of one library. See “i7 index sequences and locations in the primer mix plate” on page 30 for single indexing and “Unique dual index sequences and locations in the primer mix plate” on page 33 for dual indexing.

Required materials not supplied

For the Safety Data Sheet (SDS) of any chemical not distributed by Thermo Fisher Scientific, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Unless otherwise indicated, all materials are available through [thermofisher.com](https://www.thermofisher.com).

MLS: Fisher Scientific™ ([fisherscientific.com](https://www.fisherscientific.com)) or other major laboratory supplier.

Item	Source
Thermal cycler with heated lid, such as:	
ProFlex™ 96-well PCR System	4484075
VeritiPro™ 96-well Thermal Cycler	A48141
Magnetic rack – one of the following:	
Invitrogen™ DynaMag™ -2 Magnet (for 1.5-mL tubes)	12321D
Invitrogen™ DynaMag™ -96 Side Magnet (for 96-well 0.2-mL plates)	12331D
Invitrogen™ Magnetic Stand-96 (for 96-well, U-bottom microplates)	AM10027
Other equipment and reagents:	
Agilent™ 2100 Bioanalyzer™ Instrument ^[1]	Agilent™, G2938A
Agilent™ High Sensitivity DNA Kit ^[1]	Agilent™, 5067-4626
Agilent™ RNA 6000 Pico Kit	Agilent™, 5067-1513
Benchtop microcentrifuge	MLS
(Optional) Refrigerated microcentrifuge	MLS
Vortex mixer	MLS
Heating block and/or thermomixer	MLS
0.2-mL nuclease-free PCR tubes and/or 96-well 0.2-mL PCR plates	MLS
Nuclease-free 1.5-mL tubes, such as: <ul style="list-style-type: none"> Thermo Scientific™ ABgene™ 96-Well 1.2-mL Polypropylene Deepwell Storage Plate Eppendorf™ DNA LoBind™ Tubes, 1.5-mL 	<ul style="list-style-type: none"> Thermo Scientific™, AB1127 Eppendorf™, 022431021
Cooling rack for 0.2-mL PCR tubes/plates	MLS
Calibrated single-channel or multi-channel pipettes (1 µL– 1,000 µL)	MLS
Nuclease-free pipette tips	MLS
Ethanol 96–100%, molecular biology grade	MLS

(continued)

Item	Source
Water, nuclease free	R0581 R0582
PureLink™ Quick Gel Extraction Kit	K210012 K210025
E-Gel™ EX Agarose Gels, 4%	G401004
Gel Knife	EI9010
E-Gel™ 50 bp DNA Ladder	10488099
E-Gel™ Power Snap Electrophoresis System	G8300
(Optional) Safe Imager™ 2.0 Blue-Light Transilluminator	G6600
(Optional) Novex™ TBE Gels, 6%, 10 well	EC6265BOX
(Optional) TBE Buffer (Tris-borate-EDTA) (10X)	MLS or B52
(Optional) DNA Loading Dye, such as Invitrogen™ BlueJuice™ Gel Loading Buffer (10X)	10816015
(Optional) Low range DNA ladder, such as Thermo Scientific™ GeneRuler™ 50 bp DNA Ladder, ready-to-use	SM0373
(Optional) Mini Gel Tank	A25977
(Optional) SYBR™ Safe DNA Gel Stain	S33102
(Optional) Ammonium Acetate (5 M)	MLS or AM9070G
(Optional) Glycogen (5 mg/mL)	MLS or AM9510
(Optional) Invitrogen™ mirVana™ miRNA Isolation Kit, with phenol	AM1560
(Optional) Agencourt™ AMPure™ XP	Beckman Coulter™, A63881
(Optional) Qubit™ 4 Fluorometer, with WiFi	Q33238
(Optional) Qubit™ RNA BR Assay Kit	Q10210
(Optional) Invitrogen™ Collibri™ Library Quantification Kit	A38524100 A38524500
(Optional) Human Brain Total RNA	AM7962
(Optional) Collibri™ Library Amplification Master Mix with Primer Mix	A38540050 A38540250

^[1] You can also use comparable method to assess the quality of prepared library.

Technology overview

The Collibri™ Stranded RNA Library Prep Kit combines the SuperScript™ IV Reverse Transcriptase, Dynabeads™ magnetic particles, and Platinum™ SuperFi™ DNA Polymerase to enable the generation of high quality sequencing-ready libraries.

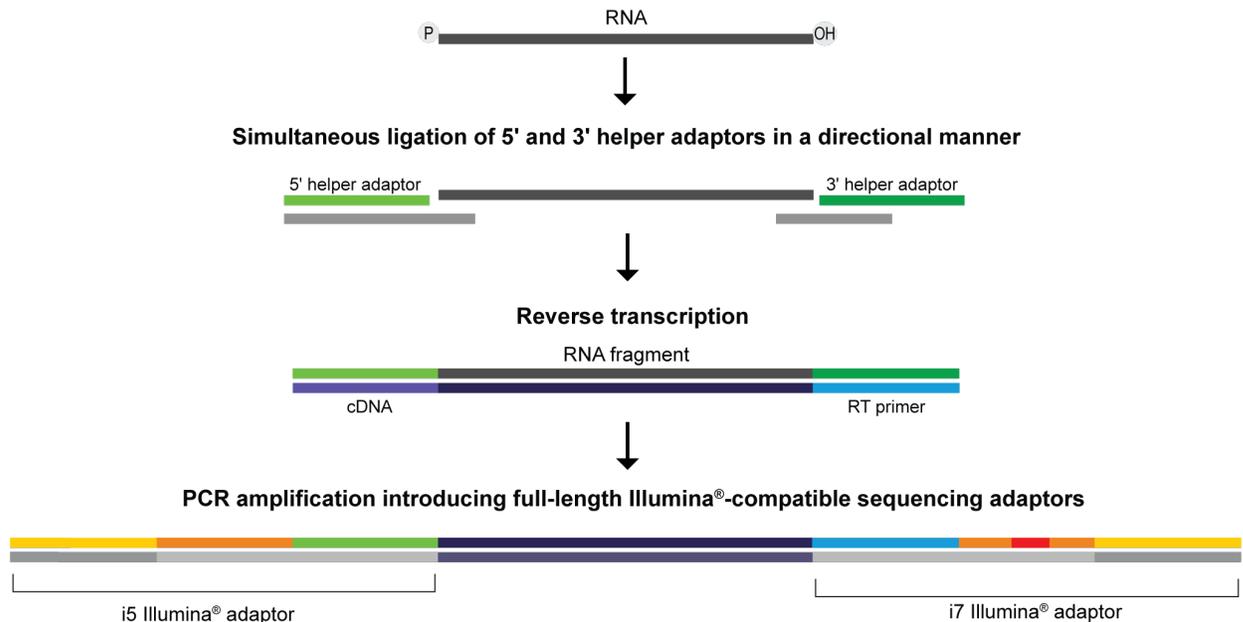


Figure 1 Simplified schematic of the technology used in the Collibri™ Stranded RNA Library Prep Kit

Hybridization and ligation to the Adaptor Mix

Following magnetic bead-based enrichment, small RNA sample is hybridized with helper Adaptor Mix, which is a set of RNA/DNA oligonucleotides with single-stranded degenerate sequence at one end and a defined sequence at the other end. Ligation enzyme mix is then added to the mixture to ligate the hybridized adaptors.

Reverse transcription

Next, RNA population with ligated adaptors is reverse transcribed using the SuperScript™ IV Enzyme Mix to generate cDNA. cDNA is then PCR amplified, which introduces full-length Illumina®-compatible sequencing adaptors and generates single-indexed ready-to-sequence libraries compatible with single-read or paired-end sequencing.

Indexing PCR

The Index Primer Mix plate contains 24 or 96 single-indexed barcoded PCR primers (i7 indices) that are pre-mixed with the universal i5 primer. i7 indices are introduced during the library amplification step using the Collibri™ Library Amplification Master Mix.



Cleanup and quantification

Gel-based size selection is recommended for complete removal of side products and extraction of libraries with insert sizes of interest. For best results, we recommend qPCR-based quantification of libraries using the Invitrogen™ Collibri™ Library Quantification Kit before proceeding to sequencing.



Enrich for small RNA (if starting material is total RNA)

You can enrich the small RNA species from any total RNA sample from 100 ng to 3 µg.

Note: This protocol does not provide a range of required starting concentration (from total RNA) because of the different possible amounts of miRNA present in samples. You can perform additional quantification, if needed, using the Qubit™ microRNA Assay Kit (Cat. No. [Q32880](#)).

Note: If your sample is already enriched for small RNA, omit this step and proceed directly to “Hybridize and ligate the adaptors”.

Required materials

Use the components from the Library Cleanup Kit, equilibrated to room temperature:

- Dynabeads™ Cleanup Beads

Note: Agencourt™ AMPure™ XP beads can be used as an alternative without any modifications to the protocol)

- Wash Buffer (diluted with 96–100% ethanol)

Other materials and equipment:

- 96–100% ethanol, molecular biology grade
- Magnetic rack (see “Technology overview” on page 9)
- Water, nuclease-free
- Heating block at 65°C

Before you begin

- Ensure that your samples are in 1.5-mL LoBind™ tubes or 1.2-mL 96-well plates. The protocol is **not** suitable for 0.2-mL PCR tubes or plates.
- Ensure that 96–100% Ethanol was added to the Wash Buffer before first use.
- Ensure that the Dynabeads™ Cleanup Beads and the Wash Buffer are at room temperature.
- Pre-heat the heating block to 65°C.
- Gently vortex the Dynabeads™ Cleanup Beads to completely resuspend the magnetic beads in the solution.

Purify small RNA species

1. Bring up the sample volume to 50 μL with nuclease-free water.
2. Mix the total RNA (50 μL) with 90 μL of Dynabeads™ Cleanup Beads by pipetting or vortexing until you have obtained a homogenous suspension.
3. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 5 minutes at room temperature.
4. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 5 minutes or until the beads have formed a tight pellet.
5. Keeping the reaction tube or plate on the magnetic rack, carefully transfer the supernatant (~140 μL) into a new tube or well using a pipette. Avoid aspirating the beads, because they will carry over the ribosomal RNA to the next step.

Note: If the pellet of magnetic particles was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

6. Add 70 μL of Dynabeads™ Cleanup Beads and 90 μL of 96–100% ethanol to the supernatant. Mix by pipetting or vortexing until you have obtained a homogenous suspension.
7. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 5 minutes at room temperature.
8. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 10 minutes or until the beads have formed a tight pellet.
9. Keeping the reaction tube or plate on the magnetic rack, carefully transfer the supernatant (~300 μL) into a new tube or well using a pipette. Avoid aspirating the beads.

Note: If the pellet of magnetic particles was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

10. Add 110 μL of Dynabeads™ Cleanup Beads and 330 μL of 96–100% ethanol to the supernatant. Mix by pipetting or vortexing until you have obtained a homogenous suspension.
11. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 10 minutes at room temperature.
12. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 10–15 minutes or until the beads have formed a tight pellet.
13. Carefully remove and discard the supernatant using a pipette.
14. Keeping the reaction tube or plate on the magnet, add 500 μL of Wash Buffer (pre-mixed with ethanol), then incubate for 30 seconds at room temperature.

IMPORTANT! Do **not** resuspend the magnetic particles in Wash Buffer.

15. Carefully remove and discard the supernatant using a pipette.
16. Repeat steps 14–15 once.
17. To remove the residual ethanol, briefly centrifuge the reaction tube or plate, place it back on the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
18. Keeping the reaction tube or plate on the magnet, air dry the magnetic particles for 5–10 minutes at room temperature or until there are no droplets of ethanol left on the walls of the tube or plate.

IMPORTANT! Do not over-dry by prolonged incubation for more than 10 minutes. Over-drying significantly decreases the elution efficiency.

19. Add 17 μL of nuclease-free water to each sample, remove the tube or plate from the magnetic rack, then mix well by pipetting or vortexing until all of the beads are fully resuspended.
20. Place the tube or plate in the heating block at 65°C and incubate for 5 minutes.
21. Place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
22. For each sample, collect 15 μL of the supernatant into a new 0.2-mL PCR tube or plate.

STOPPING POINT Store the enriched small RNA samples at -70°C , or immediately proceed to the hybridization and ligation step.

Hybridize and ligate the adaptors

Required materials

Use the components from the Library Prep Kit:

- 2X Adaptor Mix
- 2X Ligation Buffer
- 10X Ligation Enzyme Mix

Other materials and equipment:

- Enriched small RNA (from step 22 on page 13)
- Thermal cycler with heated lid (see “Required materials not supplied” on page 7)

Hybridize and ligate the adaptors

1. Prepare the RNA-adaptor hybridization reaction mixture in the same 0.2-mL PCR tube or plate containing the purified small RNA sample (from step 22 on page 13):

Component	Volume
Enriched small RNA sample (clear)	15 µL
2X Adaptor Mix (blue) ^[1]	5 µL
Total volume (blue mixture):	20 µL

^[1] To avoid excessive formation of side-products, use lower than standard amount of adaptors.

2. Pipet or vortex the hybridization reaction mixture to mix it thoroughly.
3. Run the hybridization reaction in a thermal cycler using a cooling ramp rate of $-0.5^{\circ}\text{C}/\text{second}$:

Temperature	Time
65°C	10 minutes
20°C	5 minutes

4. Prepare a ligation master mix with 5% of excess volume to compensate for pipetting error:

Component	Volume (+5%)				
	1 library	6 libraries	24 libraries	96 libraries	N libraries
2X Ligation Buffer (yellow)	26.25 µL	157.5 µL	630 µL	2520 µL	$N \times 26.25 \mu\text{L}$
10X Ligation Enzyme Mix (clear)	5.25 µL	31.5 µL	126 µL	504 µL	$N \times 5.25 \mu\text{L}$
Total volume (yellow mixture):	31.5 µL	189 µL	756 µL	3024 µL	$N \times 31.5 \mu\text{L}$

IMPORTANT! If the 2X Ligation Buffer contains a precipitate, warm the tube at 37°C for 2-5 minutes or until the precipitate is dissolved. 2X Ligation Buffer is very viscous; pipet slowly to dispense it accurately.

5. Add the RNA ligation reagents (ligation master mix from step 4) to the same reaction tube or plate containing the hybridization reaction mixture (from step 3):

Component	Volume
RNA-adaptor hybridization mixture (blue)	20 μ L
Ligation master mix (yellow)	30 μ L
Total volume (green mixture):	50 μL

6. Vortex the ligation reaction mixture to mix it thoroughly, then centrifuge it briefly to collect all the droplets at the bottom.
7. Incubate the ligation reaction in a thermal cycler for 15 minutes at 20°C.

IMPORTANT! Set the temperature of the thermal cycler lid to match the block temperature, turn OFF the heated lid, or leave the thermal cycler open during the incubation.

Perform reverse transcription (RT)

Required materials

Use the components from the Library Prep Kit:

- 2.5X RT Buffer
- 10X SuperScript™ IV Enzyme Mix

Other materials and equipment:

- Enriched small RNA (from step 7 on page 15)
- Thermal cycler with heated lid (see “Required materials not supplied” on page 7)

Perform reverse transcription

1. Prepare a reverse transcription master mix with 5% of excess volume to compensate for pipetting error:

Component	Volume (+5%)				
	1 library	6 libraries	24 libraries	96 libraries	N libraries
2.5X RT Buffer (red)	42 µL	252 µL	1008 µL	4032 µL	N × 42 µL
10X SuperScript™ IV Enzyme Mix (clear)	10.5 µL	63 µL	252 µL	1008 µL	N × 10.5 µL
Total volume (red mixture):	52.5 µL	315 µL	1260 µL	5040 µL	N × 52.5 µL

2. Add the reverse transcription reagents to the same reaction tube or plate containing the ligation reaction mixture:

Component	Volume
Adaptor-ligated RNA mixture (green)	50 µL
Reverse transcription master mix (red)	50 µL
Total volume (purple mixture):	100 µL

3. Vortex the reverse transcription reaction mixture to mix it thoroughly, then centrifuge it briefly to collect all the droplets at the bottom.
4. Incubate the reverse transcription reaction in a thermal cycler with the lid temperature set to 85-95°C.

Temperature	Time
50°C	10 minutes
85°C	5 minutes

Purify small cDNA fragments

Required materials

Use components from the Library Cleanup Kit, equilibrated to room temperature:

- Dynabeads™ Cleanup Beads

Note: Agencourt™ AMPure™ XP beads can be used as an alternative without any modifications to the protocol.

- Wash Buffer (diluted with 96–100% ethanol)
- Elution Buffer

Other materials and equipment:

- 96–100% ethanol, molecular biology grade
- Magnetic rack (see “Required materials not supplied” on page 7)
- Water, nuclease-free

Before you begin

- Ensure that your samples are in 1.5-mL LoBind™ tubes or 1.2-mL 96-well plates.

Note: The protocol is **not** suitable for 0.2-mL PCR tubes or plates.

- Ensure that 96–100% Ethanol was added to Wash Buffer before first use.
- Ensure that the Dynabeads™ Cleanup Beads and Wash Buffer are at room temperature.
- Gently vortex the Dynabeads™ Cleanup Beads to completely resuspend the magnetic beads in solution.

Purify small cDNA fragments

1. Transfer the reverse transcription reaction mixture (100 µL) to a 1.5-mL LoBind™ tube or a 1.2-mL DeepWell™ plate.
2. Mix the reverse transcription reaction mixture with 180 µL of Dynabeads™ Cleanup Beads by pipetting or vortexing until you have obtained a homogenous suspension.
3. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 5 minutes at room temperature.
4. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 5 minutes or until the beads have formed a tight pellet.
5. Keeping the reaction tube or plate on the magnetic rack, carefully transfer the supernatant (~280 µL) into a new tube or well using a pipette. Avoid aspirating beads.

Note: If the pellet of magnetic particles was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

6. Add 220 μL of Dynabeads™ Cleanup Beads and 330 μL of 96–100% ethanol to the supernatant. Mix by pipetting or vortexing until you have obtained a homogenous suspension.
7. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 10 minutes at room temperature.
8. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 10–15 minutes or until the beads have formed a tight pellet.
9. Carefully remove and discard the supernatant using a pipette.
10. Keeping the reaction tube or plate on the magnet, add 500 μL of Wash Buffer (pre-mixed with ethanol), then incubate for 30 seconds at room temperature.

IMPORTANT! Do **not** resuspend the magnetic particles in Wash Buffer.

11. Carefully remove and discard the supernatant using a pipette.
12. Repeat steps 10–11 once.
13. To remove the residual ethanol, briefly centrifuge the reaction tube or plate, place it back on the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
14. Keeping the reaction tube or plate on the magnet, air dry the magnetic particles for 5–10 minutes at room temperature or until there are no droplets of ethanol left on the walls of the tube or plate.

IMPORTANT! Do **not** over-dry by prolonged incubation for more than 10 minutes. Over-drying significantly decreases the elution efficiency.

15. Add 22 μL of Elution Buffer to each sample, remove the tube or plate from the magnetic rack, then mix well by pipetting or vortexing until all of the beads are fully resuspended.
16. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 2 minutes at room temperature.
17. Place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
18. For each sample, collect 20 μL of the supernatant into a new 0.2-mL PCR tube or plate.

STOPPING POINT Store the cDNA samples at -20°C , or immediately proceed to the indexing PCR.

Amplify the cDNA (Indexing PCR)

Required materials

Use components from the Library Prep Kit:

- 2X Library Amplification Master Mix
- 10X Index Primer Mix

Other materials and equipment:

- Purified cDNA (from step 18 on page 18)
- Thermal cycler with heated lid (see “Required materials not supplied” on page 7)

Amplify the cDNA (Indexing PCR)

1. For each cDNA sample, set up the PCR reaction mixture in the same PCR tube or plate containing the purified cDNA sample (from step 18 on page 18):

Component	Volume
cDNA	20 µL
2X Library Amplification Master Mix (blue)	25 µL
10X Index Primer mix (yellow)	5 µL
Total volume (green mixture):	50 µL

IMPORTANT! Before use, briefly centrifuge the 10X Index Primer Mix plate to avoid cross-contamination of indices.

2. Run the reactions in a thermal cycler with the lid temperature set to 105°C:

Stage	Number of cycles ^[1]	Temperature	Time
Activate the enzyme	1 cycle	98°C	30 seconds
Denature	12–15 cycles	98°C	10 seconds
Anneal		60°C	30 seconds
Extend		72°C	30 seconds
Final extension	1 cycle	72°C	1 minute
Hold	1 cycle	4°C	hold

^[1] The number of PCR cycles depends on the starting amount of RNA (i.e., input RNA), tissue, and species. This protocol was optimized using 500 ng–1 µg of Human Brain Total RNA and 13 PCR cycles.

Purify the amplified cDNA

Required materials

Use components from the Library Cleanup Kit, equilibrated to room temperature:

- Dynabeads™ Cleanup Beads

Note: Agencourt™ AMPure™ XP beads can be used as an alternative without any modifications to the protocol.

- Wash Buffer (diluted with 96–100% ethanol)
- Elution Buffer

Other materials and equipment:

- 96–100% ethanol, molecular biology grade
- Magnetic rack (see “Required materials not supplied” on page 7)
- Water, nuclease-free

Before you begin

- Ensure that 96–100% Ethanol was added to Wash Buffer before first use.
- Ensure that the Dynabeads™ Cleanup Beads, Wash Buffer, and Elution Buffer are at room temperature.
- Gently vortex the Dynabeads™ Cleanup Beads to completely resuspend the magnetic beads in solution.

Purify the amplified cDNA

1. Mix the PCR reaction mixture (50 μ L) (from step 2 on page 19) with 100 μ L of Dynabeads™ Cleanup Beads by pipetting or vortexing until you have obtained a homogenous suspension.
2. Briefly centrifuge the tube or plate containing the amplified cDNA and bead mixture to collect all the droplets at the bottom, then incubate for 5 minutes at room temperature.
3. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet.
4. Keeping the tube or plate on the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all of the supernatant is removed.

Note: If the pellet of magnetic particles was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

5. Keeping the tube or plate on the magnet, add 200 μ L of Wash Buffer (pre-mixed with ethanol), then incubate for 30 seconds at room temperature.

Note: Do **not** resuspend the magnetic particles in Wash Buffer.

6. Carefully remove and discard the supernatant using a pipette.
7. Repeat steps 5–6.
8. To remove the residual ethanol, briefly centrifuge the tube or plate, place it back on the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
9. Keeping the reaction tube or plate on the magnet, air dry the magnetic particles for 1 minute at room temperature or until there are no droplets of ethanol left on the walls of the tube or plate.

Note: Do **not** over-dry by prolonged incubation for more than 5 minutes. Over-drying significantly decreases the elution efficiency.

10. Remove the tube or plate from the magnetic rack, add 42 μ L of Elution Buffer, then mix well by vortexing.
11. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 1 minute at room temperature.
12. Place the tube or plate in the magnetic rack for 2–3 minutes or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
13. Without removing the tube or plate from the magnetic rack, transfer 40 μ L of the supernatant to a new tube or plate for storage.

Note: If the pellet of magnetic particles was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

STOPPING POINT Store the amplified cDNA at -20°C , or immediately proceed to the size selection step.

Perform size selection using 4% E-Gel™ EX cassette

Required materials

- Purified amplified cDNA libraries (from step 13 on page 21)
- E-Gel™ Power Snap Plus Electrophoresis System (see “Required materials not supplied” on page 7)
- E-Gel™ EX Agarose Gels (see “Required materials not supplied” on page 7)
- Gel Knife (see “Required materials not supplied” on page 7)
- E-Gel™ 50 bp DNA Ladder (see “Required materials not supplied” on page 7)
- PureLink™ Quick Gel Extraction Kit (see “Required materials not supplied” on page 7)

Run the cDNA libraries on a 4% E-Gel™ EX cassette

1. Prepare the E-Gel™ Power Snap Electrophoresis System, then load the E-Gel™ 50 bp DNA Ladder and the samples into E-Gel™ cassette wells according to the manufacturer’s instructions. For best results, we recommend that you split each sample between two wells (2 × 20 µL).
2. Select the program **E-Gel EX 4%** on the E-Gel™ Power Snap Device and set the run time to **20 minutes**.
3. Run the electrophoresis.

Open the E-Gel™ EX cassette and excise the target band

1. Place the E-Gel™ EX cassette on a bench with the wells facing up.
2. Insert the sharp edge of the gel knife in the groove between the cassette halves, then lever the knife up and down. Repeat for every edge of the cassette.
3. Open the cassette and excise the target band using a clean scalpel. The ~140–160 bp bands correspond to the small RNA library with ~10–30 bp inserts (miRNA, piRNA, etc.). For other small RNA species the target band size may be different. Note that adaptors add ~130 bp to the insert size length.
4. Transfer the excised gel slices, corresponding to the each target library, to a new 1.5-mL tube.

Purify the target library

Purify the target library using the PureLink™ Quick Gel Extraction Kit protocol for **≤2% agarose gels**.

STOPPING POINT Store the final library at –20°C, or proceed to quality control and sequencing.

(Optional) Excise the target band from polyacrylamide gel

Required materials

Use components from the Library Cleanup Kit, equilibrated to room temperature:

- Dynabeads™ Cleanup Beads (Agencourt™ AMPure™ XP beads can be used as an alternative without any modifications to the protocol)
- Wash Buffer (diluted with 96–100% ethanol)
- Elution Buffer

Other materials and equipment:

- Purified amplified cDNA libraries (from step 13)
- Thermomixer (see “Required materials not supplied” on page 7)
- 96–100% ethanol, molecular biology grade
- Magnetic rack (see “Required materials not supplied” on page 7)
- Novex™ TBE Gel, 6%, 10-well (see “Required materials not supplied” on page 7)
- 10X TBE Buffer (see “Required materials not supplied” on page 7)
- Mini Gel Tank (see “Required materials not supplied” on page 7)
- DNA Loading Dye (see “Required materials not supplied” on page 7)
- Low range DNA ladder (see “Required materials not supplied” on page 7)
- SYBR™ Safe DNA Gel Stain (see “Required materials not supplied” on page 7)
- (Optional) Ammonium Acetate (5 M) (see “Required materials not supplied” on page 7)
- (Optional) Glycogen (5 mg/mL) (see “Required materials not supplied” on page 7)
- (Optional) Ice cold 96–100% ethanol, molecular biology grade
- (Optional) Ice cold 70% ethanol, molecular biology grade
- (Optional) Refrigerated microcentrifuge

Before you begin

- Prepare 2 L of 1X TBE Buffer.
- Insert Novex™ TBE Gel into the Gel Tank, then fill the Gel Tank with 1X TBE Buffer up to the marked fill line.
- Preheat the thermomixer to 37°C.
- Ensure that 96–100% Ethanol was added to Wash Buffer before first use.
- Ensure that the Dynabeads™ Cleanup Beads, Wash Buffer, and Elution Buffer are at room temperature.
- Gently vortex the Dynabeads™ Cleanup Beads to completely resuspend the magnetic beads in the solution before use.

Run the cDNA libraries on a polyacrylamide gel

1. Mix each purified amplified cDNA library (40 μ L) with 4 μ L of 10X Gel Loading Dye.
2. Load 5 μ L of low range DNA ladder into one well on the 6% 10-well Novex™ TBE Gel.
3. Load each amplified cDNA library mixed with 10X Gel Loading Dye into a separate well on the 6% 10-well Novex™ TBE Gel. For best results, we recommend that you split each sample into two wells (2 \times 20 μ L).
4. Run the gel for 1 hour at 120 V or until the blue dye reaches the bottom of the gel. Do **not** let the blue dye exit the gel.
5. Remove the gel from the Gel Tank, stain it with SYBR™ Safe DNA Gel Stain in a clean container for 2–3 minutes, then view the gel on a UV transilluminator or the Safe Imager™ 2.0 Blue-Light Transilluminator.
6. Excise the target band with a clean scalpel. The ~140–160 bp bands correspond to the small RNA library with ~10–30 bp inserts (miRNA, piRNA, *etc.*). For other small RNA species, the target band size may be different. Note that the adaptors add ~130 bp to the insert size length.
7. Place the gel slice in a 1.5-mL LoBind™ tube and soak in 100 μ L of Elution Buffer.
8. Incubate the gel slices in Elution Buffer at 37°C for at least 2 hours. For best results, incubate the gel slices in Elution Buffer at 37°C overnight.
9. Transfer the eluate into a new 1.5-mL LoBind™ tube.

Concentrate the final library (Option 1)

1. Add RNase-free water to the eluate (from step 9 on page 24) to bring the sample volume to 180 μL .
2. Add 11 μL of 5 M ammonium acetate and 4 μL of 5 mg/mL glycogen, then vortex to mix.
3. Add 600 μL of ice cold 96–100% ethanol, then vortex to mix.
4. Incubate the samples at -20°C for at least 1 hour.
5. Centrifuge the samples at $10,000 \times g$ for 30 minutes at 4°C , then remove and discard all supernatant.
6. Add 200 μL of ice cold 70% ethanol.
7. Centrifuge at $10,000 \times g$ for 5 minutes at 4°C , then remove and discard all supernatant.
8. Repeat steps 6–7 once for a total of two washes.
9. Centrifuge briefly to collect any residual supernatant, then remove and discard all supernatant.
10. Air dry the pellet at room temperature for 5 minutes.
11. Dissolve the pellet in 20 μL of Elution Buffer.

STOPPING POINT Store the final library at -20°C , or proceed to quality control and sequencing.

Concentrate the final library (Option 2)

1. Add 200 μL of Dynabeads™ Cleanup Beads and 100 μL of 96–100% ethanol to the eluate (~100 μL , from step 9 on page 24). Mix by pipetting or vortexing until you have obtained a homogeneous suspension.
2. Briefly centrifuge the tube containing the final cDNA and bead mixture to collect all the droplets at the bottom, then incubate for 10 minutes at room temperature.
3. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet.
4. Keeping the tube or plate on the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all of the supernatant is removed.

Note: If the pellet of magnetic particles was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

5. Keeping the tube or plate on the magnet, add 200 μL of Wash Buffer (pre-mixed with ethanol), then incubate for 30 seconds at room temperature.

IMPORTANT! Do **not** resuspend the magnetic particles in Wash Buffer.

6. Carefully remove and discard the supernatant using a pipette.

7. Repeat steps 5–6 once.
8. To remove the residual ethanol, briefly centrifuge the tube or plate, place it back on the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
9. Keeping the reaction tube or plate on the magnet, air dry the magnetic particles for 1 minute at room temperature or until there are no droplets of ethanol left on the walls of the tube or plate.

IMPORTANT! Do **not** over-dry by prolonged incubation for more than 5 minutes. Over-drying significantly decreases the elution efficiency.

10. Remove the tube or plate from the magnetic rack, add 22 μ L of Elution Buffer, then mix well by vortexing.
11. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 1 minute at room temperature.
12. Place the tube or plate in the magnetic rack for 2–3 minutes or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
13. Without removing the tube or plate from the magnetic rack, transfer 20 μ L of the supernatant to a new tube or plate for storage.

Note: If the pellet of magnetic particles was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

STOPPING POINT Store the final library at -20°C , or proceed to quality control and sequencing.

Assess the size distribution of the amplified cDNA

Required materials

- Agilent™ 2100 Bioanalyzer™ Instrument (Agilent™, Cat. No. G2938A)
- Agilent™ High Sensitivity DNA Kit (Agilent™, Cat. No. 5067-4626)
- Nuclease-free water

Note: You can also use a comparable method to assess the yield and size distribution of the prepared libraries.

Analyze the size distribution of the amplified cDNA library

1. Remove 1 μ L from each prepared small cDNA library.
2. Analyze 1 μ L of the diluted cDNA library using the appropriate chip on the Agilent™ 2100 Bioanalyzer™ Instrument with the Agilent™ High Sensitivity DNA Kit.

Expected results

Typical small RNA library size is in the range of 140–160 bp.

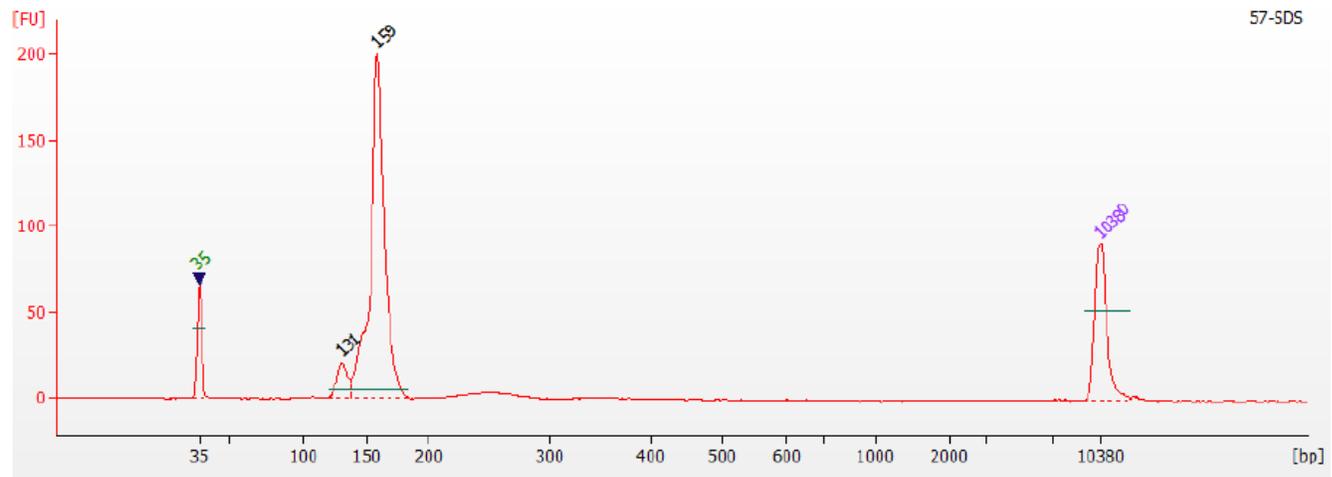


Figure 2 Typical Agilent™ 2100 Bioanalyzer™ Instrument trace of a small RNA library prepared from Human Brain Total RNA using the Collibri™ Stranded RNA Library Prep Kit workflow for small RNA sequencing.

Next steps

Quantify the prepared library by qPCR

We strongly recommend that you perform qPCR quantification of prepared libraries using the Invitrogen™ Collibri™ Library Quantification Kit (available separately from Thermo Fisher Scientific, Cat. Nos. [A38524100](#), [A38524500](#)) before proceeding to sequencing.

Sequence the prepared library

Denature, dilute, and load the libraries according to the standard guidelines appropriate for the Illumina® NGS platform you are using.

To pool single indexed libraries prepared with the Collibri™ Stranded RNA Library Prep Kit with dual indexed libraries, you will need to indicate i5 index sequence in the sample sheet. For samples prepared with the Collibri™ Stranded RNA Library Prep Kit, use the following i5 index sequence:

- TCTTTCCC for MiSeq™, Hi-Seq™ 2000, Hi-Seq™ 2500, and NovaSeq™ 6000 instruments.
- AGATCTCG for MiniSeq™, NextSeq™, iSeq™ 100, Hi-Seq™ X, Hi-Seq™ 3000, and Hi-Seq™ 4000 instruments.

Do **not** perform Index 2 (i5) Read when sequencing libraries prepared with the Collibri™ Stranded RNA Library Prep Kit only.

Note: Do **not** pool together different sizes of Collibri™ kits containing the same type of indexed adaptors. For the highest throughput, use [A38996096](#) and [A38996096](#) together. This allows you to sequence a total of 100 small RNA samples.



Troubleshooting

Observation	Possible cause	Recommended action
Low yield and/or poor size distribution obtained in the amplified library	Low recovery rates after cleanup.	Ensure that the Dynabeads™ Cleanup Beads suspension is at room temperature and the magnetic particles are thoroughly resuspended before use.
		Ensure that the appropriate volume of ethanol is added to the bottle of concentrated Wash Buffer before first use.
	Suboptimal number of PCR cycles.	Increase the number of PCR cycles.
Extremely low yield and/or no PCR products	Enzymatic reaction or the purification step failed.	Minimize the time spent above –20°C for the 10X Ligation Enzyme Mix and the 10X SuperScript™ IV Enzyme Mix.
Residual adaptors (~60 bp) or adaptor dimers (~130 bp) are visible on the Agilent™ Bioanalyzer™ trace	Inaccurate excision of the target band.	If the overall amount of adaptor dimers exceeds 10%, repeat library preparation and take care not to excise bands that are <140 bp.
The color of the reaction mixture after the setup of the reverse transcription reaction is not purple.	Some of the colored reaction components were not added or were added in wrong quantities.	Make sure to pipet accurate volumes of reagents.
		Make sure to pipet the viscous Ligation Buffer carefully to aspirate the correct volume.
The color of the reaction mixture after the setup of PCR is not green.	Some of the colored reaction components were not added or were added in wrong quantities.	The color of reaction mixture after the setup of ligation reaction should be green.
		Make sure to pipet accurate volumes of reagents.
		Make sure to pipet the viscous Ligation Buffer carefully to aspirate the correct volume.
		The color of reaction mixture after the setup of ligation reaction should be green.



Index sequences and plate layouts

i7 index sequences and locations in the primer mix plate

The following tables list the i7 index sequences (Table 1) and the location of the indices in the 10X Index Primer Mix Plate for 96 preps (Table 2) and 24 preps (Table 3).

i7 index sequences

Table 1 i7 index sequences

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
IX001	ATGCCTAA	IX025	AACTCACC	IX049	ACGTATCA	IX073	AATGTTGC
IX002	GAATCTGA	IX026	GCTAACGA	IX050	GTCTGTCA	IX074	TGAAGAGA
IX003	AACGTGAT	IX027	CAGATCTG	IX051	CTAAGGTC	IX075	AGATCGCA
IX004	CACTTCGA	IX028	ATCCTGTA	IX052	CGACACAC	IX076	AAGAGATC
IX005	GCCAAGAC	IX029	CTGTAGCC	IX053	CCGTGAGA	IX077	CAACCACA
IX006	GACTAGTA	IX030	GCTCGGTA	IX054	GTGTTCTA	IX078	TGGAACAA
IX007	ATTGGCTC	IX031	ACACGACC	IX055	CAATGGAA	IX079	CCTCTATC
IX008	GATGAATC	IX032	AGTCACTA	IX056	AGCACCTC	IX080	ACAGATTC
IX009	AGCAGGAA	IX033	AACGCTTA	IX057	CAGCGTTA	IX081	CCAGTTCA
IX010	GAGCTGAA	IX034	GGAGAACA	IX058	TAGGATGA	IX082	TGGCTTCA
IX011	AAACATCG	IX035	CATCAAGT	IX059	AGTGGTCA	IX083	CGACTGGA
IX012	GAGTTAGC	IX036	AAGGTACA	IX060	ACAGCAGA	IX084	CAAGACTA
IX013	CGAACTTA	IX037	CGCTGATC	IX061	CATACCAA	IX085	CCTCCTGA
IX014	GATAGACA	IX038	GGTGCGAA	IX062	TATCAGCA	IX086	TGGTGGTA
IX015	AAGGACAC	IX039	CCTAATCC	IX063	ATAGCGAC	IX087	AACAACCA
IX016	GACAGTGC	IX040	CTGAGCCA	IX064	ACGCTCGA	IX088	AATCCGTC
IX017	ATCATTCC	IX041	AGCCATGC	IX065	CTCAATGA	IX089	CAAGGAGC
IX018	GCCACATA	IX042	GTACGCAA	IX066	TCCGTCTA	IX090	TTCACGCA
IX019	ACCACTGT	IX043	AGTACAAG	IX067	AGGCTAAC	IX091	CACCTTAC

Table 1 *i7* index sequences (continued)

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
IX020	CTGGCATA	IX044	ACATTGGC	IX068	CCATCCTC	IX092	AAGACGGA
IX021	ACCTCCAA	IX045	ATTGAGGA	IX069	AGATGTAC	IX093	ACACAGAA
IX022	GCGAGTAA	IX046	GTCGTAGA	IX070	TCTTCACA	IX094	GAACAGGC
IX023	ACTATGCA	IX047	AGAGTCAA	IX071	CCGAAGTA	IX095	AACCGAGA
IX024	CGGATGTC	IX048	CCGACAAC	IX072	CGCATACA	IX096	ACAAGCTA

Location of i7 indices in the primer mix plate

Table 2 Location of i7 indices in 10X Index Primer Mix Plate - i7 indices for 96 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	IX001	IX009	IX017	IX025	IX033	IX041	IX049	IX057	IX065	IX073	IX081	IX089
B	IX002	IX010	IX018	IX026	IX034	IX042	IX050	IX058	IX066	IX074	IX082	IX090
C	IX003	IX011	IX019	IX027	IX035	IX043	IX051	IX059	IX067	IX075	IX083	IX091
D	IX004	IX012	IX020	IX028	IX036	IX044	IX052	IX060	IX068	IX076	IX084	IX092
E	IX005	IX013	IX021	IX029	IX037	IX045	IX053	IX061	IX069	IX077	IX085	IX093
F	IX006	IX014	IX022	IX030	IX038	IX046	IX054	IX062	IX070	IX078	IX086	IX094
G	IX007	IX015	IX023	IX031	IX039	IX047	IX055	IX063	IX071	IX079	IX087	IX095
H	IX008	IX016	IX024	IX032	IX040	IX048	IX056	IX064	IX072	IX080	IX088	IX096

Table 3 Location of i7 indices in 10X Index Primer Mix Plate - i7 indices for 24 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	IX001	IX009	IX017	—	—	—	—	—	—	—	—	—
B	IX002	IX010	IX018	—	—	—	—	—	—	—	—	—
C	IX003	IX011	IX019	—	—	—	—	—	—	—	—	—
D	IX004	IX012	IX020	—	—	—	—	—	—	—	—	—
E	IX005	IX013	IX021	—	—	—	—	—	—	—	—	—
F	IX006	IX014	IX022	—	—	—	—	—	—	—	—	—
G	IX007	IX015	IX023	—	—	—	—	—	—	—	—	—
H	IX008	IX016	IX024	—	—	—	—	—	—	—	—	—

Unique dual index sequences and locations in the primer mix plate

The following tables list the unique dual (UD) index sequences (Table 4) and the location of the indices in the 10X Index Primer Mix Plate for 96 preps (Table 5) and 24 preps (Table 6).

Unique dual (i7 and i5) index sequences

Table 4 Unique dual (i7 and i5) index sequences

UDI index primer mix name	i7 index	i5 index for entry on sample sheet (NovaSeq™, MiSeq™, Hi-Seq™ 2000/2500)	i5 index for entry on sample sheet (MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ X) ^[1]
UDI001	CCTTCTAC	GGCGAATA	TATTCGCC
UDI002	GGTCGTAT	GTTGCATG	CATGCAAC
UDI003	CGTAGACA	TCGTAGAC	GTCTACGA
UDI004	ATGTCACG	AGACAGCT	AGCTGTCT
UDI005	CAAGAAGC	AGGTCTCA	TGAGACCT
UDI006	CACGGATA	CTGAACAG	CTGTTCAG
UDI007	ACAGGATG	ACGTTGTC	GACAACGT
UDI008	ACACAACC	GGCTCAAT	ATTGAGCC
UDI009	TGCTGACT	GACACAGA	TCTGTGTC
UDI010	ATCGGAGA	AGACACAG	CTGTGTCT
UDI011	ATTAGCGG	TGGTTCAC	GTGAACCA
UDI012	TAGCCACT	CGATGGAT	ATCCATCG
UDI013	AGCACACA	CCACAGAA	TTCTGTGG
UDI014	GTTAAGCG	TGTGTCAG	CTGACACA
UDI015	GGTTGGTT	ATGGCGAT	ATCGCCAT
UDI016	AACGCATG	TCGACGAA	TTCGTCGA
UDI017	TAGTCAGC	TAGGCTAC	GTAGCCTA
UDI018	ACTGATGC	ACACCTCT	AGAGGTGT
UDI019	CTATGTGG	GAATAGGC	GCCTATTC
UDI020	ATAGTCGG	GAATCAGG	CCTGATTC
UDI021	AAGTACGC	CGCATTAC	GTAATGCG

Table 4 Unique dual (i7 and i5) index sequences (continued)

UDI index primer mix name	i7 index	i5 index for entry on sample sheet (NovaSeq™, MiSeq™, Hi-Seq™ 2000/2500)	i5 index for entry on sample sheet (MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ X) ^[1]
UDI022	CAACAGGT	CGCAACAT	ATGTTGCG
UDI023	GGATCACA	GATGGCAA	TTGCCATC
UDI024	ACTAAGCC	TACCGTGA	TCACGGTA
UDI025	AAGTGGCT	TCGTGCAT	ATGCACGA
UDI026	CCATCGTA	CGTGCTAA	TTAGCACG
UDI027	CGACAATC	AATGACGG	CCGTCATT
UDI028	GTTGGCTT	ACAAGAGC	GCTCTTGT
UDI029	AGTGAGGA	ACCGCTAT	ATAGCGGT
UDI030	TTCATGCG	AGTGCTGT	ACAGCACT
UDI031	TTATGGCC	GTACTAGC	GCTAGTAC
UDI032	TCGATGCT	CGGTCTAT	ATAGACCG
UDI033	CGTAACGA	ATCCACGA	TCGTGGAT
UDI034	CTGTATGC	GTCATCTG	CAGATGAC
UDI035	GTTACGGT	TGTCTAGC	GCTAGACA
UDI036	ACATGCCA	TCAACGGT	ACCGTTGA
UDI037	CTATACCG	CATCTCGA	TCGAGATG
UDI038	TATGGTCC	ATCGATGG	CCATCGAT
UDI039	TCAGGCTA	TGTAAGGC	GCCTTACA
UDI040	TAGTGGTG	CACATGGT	ACCATGTG
UDI041	CAGTGATC	CATGTTGG	CCAACATG
UDI042	ATCCGCTT	GCATCTGA	TCAGATGC
UDI043	GTTGTCGA	ATCCAACG	CGTTGGAT
UDI044	GTTCTTGG	ATTCTCGC	GCGAGAAT
UDI045	ATGCTGGT	GACGATGT	ACATCGTC
UDI046	GTGATCCA	TTGAGACG	CGTCTCAA
UDI047	GACAATCG	GTAGATGC	GCATCTAC

Table 4 Unique dual (i7 and i5) index sequences (continued)

UDI index primer mix name	i7 index	i5 index for entry on sample sheet (NovaSeq™, MiSeq™, Hi-Seq™ 2000/2500)	i5 index for entry on sample sheet (MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ X) ^[1]
UDI048	GGTATTCC	GTGTGTGT	ACACACAC
UDI049	AGGACCTA	GCTACACA	TGTGTAGC
UDI050	CATTCGTG	AATCGACG	CGTCGATT
UDI051	GCTTCATC	TCACATGC	GCATGTGA
UDI052	ACACGTGA	CAGTCACA	TGTGACTG
UDI053	AGTCTTCG	TTAGAGCG	CGCTCTAA
UDI054	CGATACGT	ATGAGTGC	GCACATCAT
UDI055	CCTCATCA	GATTGGCA	TGCCAATC
UDI056	GGTTCTTG	CATCAACC	GGTTGATG
UDI057	AGAGCTTC	GAGAGACT	AGTCTCTC
UDI058	CTAACCGT	ATTGGCCA	TGGCCAAT
UDI059	TCCACTCA	GTATTGCG	CGCAATAC
UDI060	GCATGTTG	CTAAGACC	GGTCTTAG
UDI061	GCAACTTC	CCAACACT	AGTGTGGG
UDI062	TACGTCGT	TTGTTCGG	CGGAACAA
UDI063	GATGTCTG	TTCTCACC	GGTGAGAA
UDI064	TTGCGTTC	GAAGAGCT	AGCTCTTC
UDI065	TACTAGCG	TCCTTGGT	ACCAAGGA
UDI066	GCAGTTCA	TTACGTCG	CGACGTAA
UDI067	CACTTCTC	TTCTTCC	GGAAGGAA
UDI068	AAGCCTGT	CTTCTGCT	AGCAGAAG
UDI069	TTGACCTG	CTCTCTCA	TGAGAGAG
UDI070	GATCCTCA	TGAAGGTG	CACCTTCA
UDI071	TCCAACCTG	CACACATC	GATGTGTG
UDI072	TAGCTGTC	GAGCATCT	AGATGCTC
UDI073	CTAGACTC	TGGCTTCA	TGAAGCCA

Table 4 Unique dual (i7 and i5) index sequences (continued)

UDI index primer mix name	i7 index	i5 index for entry on sample sheet (NovaSeq™, MiSeq™, Hi-Seq™ 2000/2500)	i5 index for entry on sample sheet (MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ X) ^[1]
UDI074	GCCAAGAA	ACTAGGTG	CACCTAGT
UDI075	TCACTCAC	ACCGTATC	GATACGGT
UDI076	TCTCAAGG	AACCGTCT	AGACGGTT
UDI077	ATGGTCAC	GACTTGTG	CACAAGTC
UDI078	GATCAGTG	AAGCTAGG	CCTAGCTT
UDI079	CGTTGAAG	TGGCGATA	TATCGCCA
UDI080	CTTCCAAC	GTGTACTG	CAGTACAC
UDI081	TCCGAGAT	CGTACGTT	AACGTACG
UDI082	GGTACGAA	CCGCTATA	TATAGCGG
UDI083	GAGGTAAC	CAGGTGTT	AACACCTG
UDI084	TACCGGAT	CGGTACTA	TAGTACCG
UDI085	CTCCTGAA	ACACTCTG	CAGAGTGT
UDI086	TAGGAGAG	TGCTTGTC	GACAAGCA
UDI087	TGGCTGAT	GCAAGCTT	AAGCTTGC
UDI088	GTGAGTAG	GGATGCTA	TAGCATCC
UDI089	AACGTGAC	AGTGGTTG	CAACCACT
UDI090	CCGTTGAT	CGATGTTC	GAACATCG
UDI091	AGCCGTAA	TGTCGCTT	AAGCGACA
UDI092	TCAGTAGG	GAAGCCTA	TAGGCTTC
UDI093	GGATACAC	ACCTGTTC	GAACAGGT
UDI094	GTCACCAT	GTCTCCTA	TAGGAGAC
UDI095	TGAACCAC	CFACTCTT	AAGAGTCG
UDI096	CCGCATAT	TGCACTTC	GAAGTGCA

^[1] Sequencing on the MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, and Hi-Seq™ X systems follow a different dual-indexing workflow than other Illumina® systems, which require the reverse complement of the i5 index adaptor sequence.

Location of unique dual indices in the primer mix plate

Table 5 Location of unique dual (UD) indices in 10X Index Primer Mix Plate - UD indices for 96 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI001	UDI009	UDI017	UDI025	UDI033	UDI041	UDI049	UDI057	UDI065	UDI073	UDI081	UDI089
B	UDI002	UDI010	UDI018	UDI026	UDI034	UDI042	UDI050	UDI058	UDI066	UDI074	UDI082	UDI090
C	UDI003	UDI011	UDI019	UDI027	UDI035	UDI043	UDI051	UDI059	UDI067	UDI075	UDI083	UDI091
D	UDI004	UDI012	UDI020	UDI028	UDI036	UDI044	UDI052	UDI060	UDI068	UDI076	UDI084	UDI092
E	UDI005	UDI013	UDI021	UDI029	UDI037	UDI045	UDI053	UDI061	UDI069	UDI077	UDI085	UDI093
F	UDI006	UDI014	UDI022	UDI030	UDI038	UDI046	UDI054	UDI062	UDI070	UDI078	UDI086	UDI094
G	UDI007	UDI015	UDI023	UDI031	UDI039	UDI047	UDI055	UDI063	UDI071	UDI079	UDI087	UDI095
H	UDI008	UDI016	UDI024	UDI032	UDI040	UDI048	UDI056	UDI064	UDI072	UDI080	UDI088	UDI096

Table 6 Location of unique dual (UD) indices in 10X Index Primer Mix Plate - UD indices for 24 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI001	UDI009	UDI017	—	—	—	—	—	—	—	—	—
B	UDI002	UDI010	UDI018	—	—	—	—	—	—	—	—	—
C	UDI003	UDI011	UDI019	—	—	—	—	—	—	—	—	—
D	UDI004	UDI012	UDI020	—	—	—	—	—	—	—	—	—
E	UDI005	UDI013	UDI021	—	—	—	—	—	—	—	—	—
F	UDI006	UDI014	UDI022	—	—	—	—	—	—	—	—	—
G	UDI007	UDI015	UDI023	—	—	—	—	—	—	—	—	—
H	UDI008	UDI016	UDI024	—	—	—	—	—	—	—	—	—



Safety



WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the “Documentation and Support” section in this document.



Chemical safety



WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.



WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.



WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.



Biological hazard safety



WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.



WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
<https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf>
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
www.who.int/publications/i/item/9789240011311



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 - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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