TruSeq® Stranded mRNA Library Prep for NeoPrep™ Reference Guide

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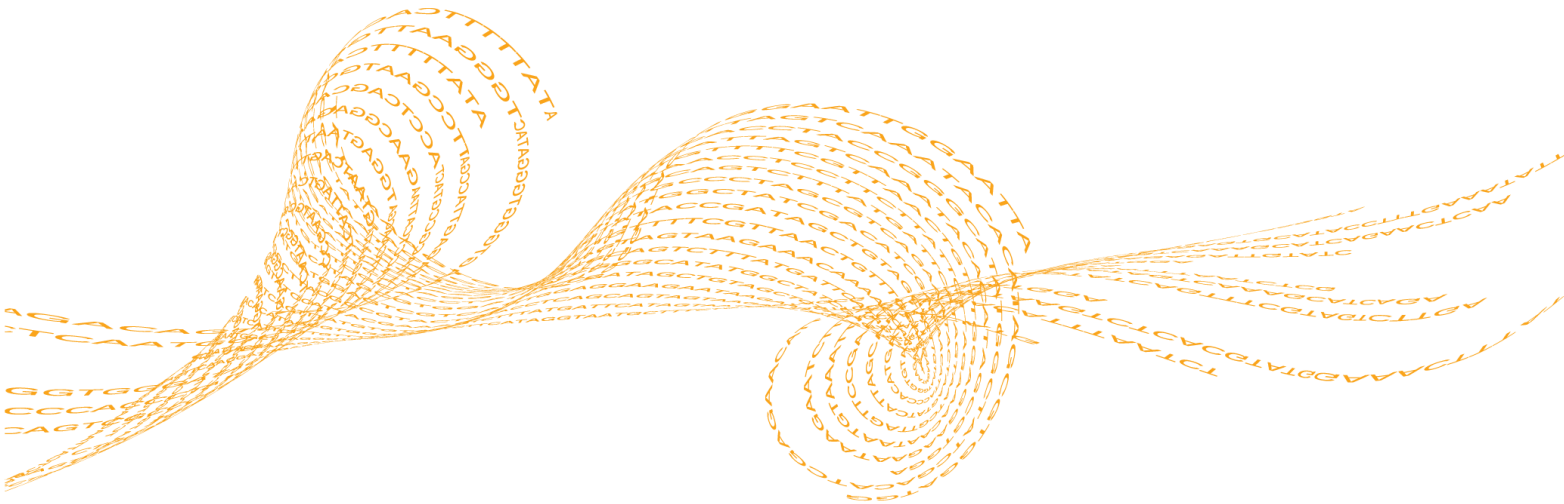

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Introduction

This protocol explains how to convert the mRNA in total RNA into a library of template molecules of known strand origin using the reagents provided in the Illumina® TruSeq® Stranded mRNA Library Prep Kit for NeoPrep™. The resulting libraries are ready for subsequent cluster generation and sequencing.

The protocol offers:

- Streamlined workflow
- All reagents required for library prep, quantification, and normalization are included
- 30 minutes hands-on time
- Strand information on RNA transcript
- Library capture of both coding RNA and multiple forms of noncoding RNA that are polyadenylated
- A disposable library card allowing for simultaneous preparation of up to 16 mRNA samples
- Use of 16 default index adapters, plus 8 alternate index adapters, allowing up to 24-plex pooling with additional NeoPrep runs.
- Compatibility with no indexing or a lower indexing pooling level

Additional Resources

Visit the TruSeq Stranded mRNA Library Prep Kit for NeoPrep kit support page on the Illumina website for documentation, software downloads, training resources, and information about compatible Illumina products.

The following documentation is available for download from the Illumina website.

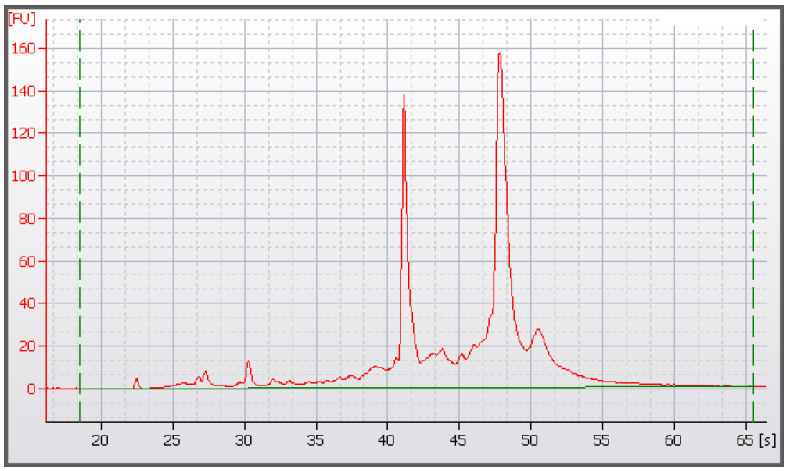
Resource	Description
Custom Protocol Selector	support.illumina.com/custom-protocol-selector.html A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
TruSeq Stranded mRNA Library Prep for NeoPrep Protocol Guide (document # 15059581)	Provides only protocol instructions. The protocol guide is intended for experienced users.
TruSeq Stranded mRNA Library Prep for NeoPrep Checklist (document # 15068682)	Provides a checklist of the protocol steps. The checklist is intended for experienced users.
NeoPrep Library Prep System Guide (document # 15049720)	Provides an overview of instrument components and software, instructions for performing library prep runs, and procedures for proper instrument maintenance and troubleshooting.
Illumina Experiment Manager Guide (document # 15031335) and *IEM NeoPrep Quick Reference Card (document # 15061111)*	Provide information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
BaseSpace help (help.basespace.illumina.com)	Provides information about the BaseSpace® sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment.
TruSeq Library Prep Pooling Guide (document # 15042173)	Provides TruSeq pooling guidelines for preparing libraries for Illumina sequencing systems that require balanced index combinations. Review this guide before beginning library preparation.

RNA Input Recommendations

Total RNA Input

- The protocol is optimized for 25–100 ng of total RNA.
  - Do not use more than 100 ng of total RNA.
  - A lower than specified input amount can result in low yield and increased duplicates.
- The protocol has been tested using 25–100 ng of high-quality universal human reference total RNA as input.
- Determine the quality of the RNA starting material.
  - Use an Agilent RNA 6000 Nano Kit or Advanced Analytical Standard Sensitivity RNA Analysis Kit to determine the quality of your starting material.
  - Do not use low quality or degraded RNA with this protocol. Use of degraded RNA can result in low yield, overrepresentation of the 3' ends of the RNA molecules, or failure of the protocol.
  - Check total RNA integrity following isolation:
    - For samples with an RNA Integrity Number (RIN) value ≥ 8, use an Agilent Technologies 2100 Bioanalyzer.
    - For samples with an RNA Quality Number (RQN) value > 8, use an Advanced Analytical Fragment Analyzer.
  - Using RNA with DNA contamination results in an underestimation of the amount of RNA used.
- Include a DNase step with the RNA isolation method to ensure purity and accurate quantification of the sample.
- The following figure shows a Universal Human Reference (UHR) starting RNA Bioanalyzer trace.

Figure 1 Starting RNA Bioanalyzer Trace



  - Alternatively, run a formaldehyde 1% agarose gel and determine the integrity of RNA upon staining with ethidium bromide.
    - High-quality RNA shows a 28S ribosomal RNA (rRNA) band at 4.5 kb with 2X the intensity of the 18S rRNA band at 1.9 kb.
    - Both kb determinations are relative to an RNA 6000 ladder.
    - The mRNA appears as a smear from 0.5–12 kb.

Positive Control

Use Agilent Technologies Human UHR total RNA (catalog # 740000) as a positive control sample for this protocol.

Pipette and Tip Requirements

Use the following required pipettes and tips. Other pipettes and tips are not supported and can result in reagents not dispensing properly and run failure.

Table 1 Required User-Supplied Pipettes and Tips

Volume	Use	Product Name	Supplier
20 µl	≤ 20 µl	Pipet-Lite XLS+ 8-channel LTS, 2 µl to 20 µl	Rainin, catalog # L8-20XLS+
		One of the following: • LTS tips 20 µl. Presterilized. Filter • ART Barrier Pipette Tips 20 µl; 20 µl SoftFit-L	• Rainin, catalog # RT-L10F • Fisher Scientific, catalog # 2749RI
200 µl	21–200 µl, 20 µl in Unload Library step	Pipet-Lite XLS+ 8-channel LTS, 20 µl to 200 µl	Rainin, catalog # L8-200XLS+
		One of the following: • LTS tips 200 µl. Presterilized. Filter • ART Barrier Pipette Tips 200 µl; 200 µl SoftFit-L	• Rainin, catalog # RT-L200F • Fisher Scientific, catalog # 2769RI

Sealing a Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Centrifuge
  - Thermal cycling
- Apply the adhesive seal to cover the plate and seal with a rubber roller.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates.

Handling the Library Card

- To avoid instrument damage, do not place the library card guide on the library card during library card verification or a run.
- Use the library card latch release to load and remove the library card from the library card stage.
  - Do not snap the library card into place.
  - Oil and reagents in a used library card can splash out of the card and onto the instrument.
- Hold the used library card level when removing it from the instrument to avoid spilling its contents.

Library Card Loading Guidelines

- Load the library card while it is on the library card stage to avoid spilling or disturbing the loaded contents.
- The NeoPrep Control Software guides you through the steps to set up a run and load the library card. Use the loading procedures in this guide as a reference.
- Perform *Set Up Run and Load Library Card* preparation and initial software setup while the sample plate is on the thermal cycler.
- Do not open the compartment door during library card verification or a run.
- Change your gloves after loading the oil.
- Transfer contents from the reagent plate to the corresponding wells on the library card.
- Reference the corresponding colors and well labels on the reagent plate and library card guides.
- Make sure that pipettes are calibrated before beginning. Uncalibrated pipettes can lead to reagents not dispensing properly, resulting in run failure.
- Use the pipettes and tips specified in *Pipette and Tip Requirements* on page 7 and *Consumables and Equipment* on page 29. Other pipettes and tips are not supported and can result in reagents not dispensing properly and run failure.
- Use a multichannel pipette to load reagents, samples, and adapters.
- To avoid instrument damage, make sure that the library card guide is removed from the library card before starting the run.

Library Card Loading Techniques

- Use proper library card loading techniques and specified loading angles.
- Pipette to the first stop to avoid creating bubbles.
- Insert pipette tips perpendicular to the well.
- Insert the pipette tips to the bottom of the well while dispensing. Do not lift the tips until the reagents are dispensed completely.
- Dispense at an angle by pointing pipette tips under the well label and dotted well outline on the library card guide.
- The pipette loading angle depends on the item being dispensed. The angle is specified in each step of the control software loading guide and is depicted in the protocol.
- An icon represents the loading angle and the volume is specified on the control software loading guide. For example, ( 5 µl)

Icon	Description
	Point the pipette tip toward the well label and dotted well outline on the left.
	Point the pipette tip perpendicular in the well.
	Point the pipette tip toward the well label and dotted well outline on the right.

- Increase the pipette angle if liquid is not dispensing from the pipette tips.

Handling Samples

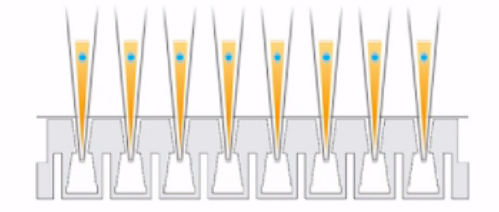
- Always track the location of each sample.
- Change tips between each sample to avoid cross-contamination.
- Do not centrifuge samples before loading.
- Touch and keep the pipette tips at bottom of the well while mixing before loading onto the library card. Use the same tips to load the sample onto the library card.
- Some space might be present in the pipette tips during transfer from the sample plate to the library card.

Collecting Libraries

- Unload the library card while it is on the library card stage.
- The NeoPrep Control Software guides you through the steps to unload the library card. Use the procedures in this guide as a reference.
- Do not use a 20 µl pipette. It does not fit properly into the library card well.
- Insert pipette tips perpendicularly and touch the tips to the bottom of the collection wells.
- Hold down the library card with one hand while removing the tips from the collection ports to prevent any movement of the card.
- An icon represents the required pipette angle, and the volume is specified on the control software unloading guide. For example, ( 20 µl)

- Inspect each pipette tip to make sure that a blue library droplet is present in the tips indicated by the control software.

  Figure 2 Library Droplet in Pipette Tips

- If a blue library droplet is not visible in each expected pipette tip, do the following:
  - Transfer the extracted liquid to the corresponding plate well containing RSB.
  - Do not dispense the liquid back into the library card, which can introduce air gaps and interfere with library extraction.
  - Use a single-channel pipette to repeat the transfer 1 time for the wells that did not contain the blue droplet. Do not attempt the transfer more than 2 times.
- Vigorously pipette up and down in RSB to dislodge the blue library droplet from the pipette tip.

Handling Library Separation Tube Strips

- Label the tubes to support tracking sample location.
- Use the wells of a plate or another device to hold the library separation tube strips upright.
- Do not centrifuge library separation tube strips.

Library Prep Workflow

Figure 3 Workflow Diagram

Samples are first manually prepared with RNA Purification Beads 2 (RPB2). Then, oil, samples, reagents, and adapters are loaded into the library card for a run on the NeoPrep System. A run includes library prep and optional quantification and normalization. The NeoPrep Control Software guides you through the run setup and library card loading steps. After the run is complete, the control software guides you through the process of collecting your libraries from the library card and separating them from the oil. If NeoPrep System performs quantification and normalization, the libraries are ready for pooling, denaturing, and clustering.

Before proceeding:

- Review Best Practices, available on the Illumina website. See *Additional Resources* on page 4.
- Review *Supporting Information* on page 27. Confirm kit contents and make sure that you have the requisite equipment and consumables for this protocol.

Select Samples and Indexes

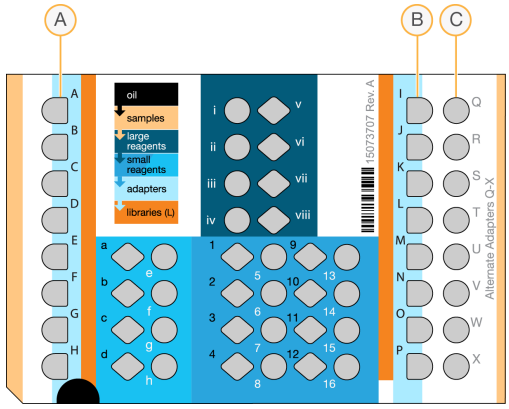
Before beginning library preparation, plan for your NeoPrep System run.

1 Select the samples to use for library prep. Each kit is single-use for 1 NeoPrep System run and each NeoPrep System run prepares up to 16 samples.

2 Plan the sample locations on the sample plate and the library card.
- Place samples 1–8 in column 1, A–H
- Place samples 9–16 in column 2, A–H

3 Use the default index adapters in the order that they are arranged in the reagent plate and arrange the samples used with those index adapters accordingly.

Figure 4 TruSeq Stranded mRNA Library Prep for NeoPrep Index Adapters



A Adapters A–H (default for samples 1–8)
B Adapters I–P (default for samples 9–16)
C Adapters Q–X (alternate)

- Indexes are single-use.
- Each sample requires a unique index in a library prep run.
- Each kit includes 24 single-index adapters, allowing for pooling up to 24 samples with multiple NeoPrep System runs.
- For the index adapter layout, see *Reagent Plate Contents* on page 27.
- For the index adapter sequences, see *Index Adapter Sequences* on page 31. The TruSeq Stranded mRNA Library Prep for NeoPrep adapters are TruSeq LT single-index adapters.
- Review the planning steps in the *TruSeq Library Prep Pooling Guide (document # 15042173)* for Illumina sequencing systems that require balanced index combinations.

4 [Optional] Use IEM or BaseSpace Prep tab to record information about your samples and indexes. The information is used during the run setup.

Prepare Samples for Loading

This process binds the polyA containing mRNA molecules using oligo-dT attached magnetic beads in preparation for loading into the library card.

Consumables

- RPB2 (RNA Purification Beads 2)
- 96-well 0.3 ml PCR plate
- Microseal 'B' adhesive seal
- Nuclease-free ultrapure water
- Total RNA (25–100 ng)

Preparation

1. Remove RPB2 from 2°C to 8°C storage and let stand for at least 15 minutes to bring to room temperature.
2. Save the following mRNA Denaturation program on a thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 65°C for 5 minutes
   - 25°C for 5 minutes
   - 25°C hold
3. Make sure that pipettes are calibrated before beginning. Uncalibrated pipettes can lead to reagents not dispensing properly, resulting in run failure.

Procedure

1. Dilute 25–100 ng total RNA with nuclease-free ultrapure water to a final volume of 12.5 µl in each well of a new PCR plate. Pipette to mix. Do not vortex.
2. Vortex RPB2 to resuspend.
3. Add 12.5 µl RPB2 to each well. Pipette to mix.
4. Place on the thermal cycler and run the mRNA Denaturation program.

Set Up Run and Load Library Card

This process describes how to set up a NeoPrep System run, which includes loading oil, samples, reagents, and adapters into the library card. Load the library card and start the run within 90 minutes.

The NeoPrep Control Software guides you through the steps to set up a run and load the library card. Use the procedures in this section as a reference. For more information on the NeoPrep Control Software, see the *NeoPrep Library Prep System Guide (document # 15049720)*.

Consumables

- Library card
- Library card guide
- Oil vial
- Oil funnel
- DMB (Digital Microfluidics Beads)
- TruSeq Stranded mRNA - NeoPrep reagent plate
- RNase/DNase-free 8-tube strips (2)
- [Optional] RSB (Resuspension Buffer)

WARNING

The reagent plate contains hazardous materials. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and a laboratory coat. Handle the used reagent plate as chemical waste. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region. For more information, see the SDS for this kit at support.illumina.com/sds.html.

About Reagents

Small reagents 9–16 must remain free of RNase contamination. The foil on the reagent plate is not guaranteed to be RNase-free. Therefore, to prevent the pipette tips from touching the foil and contaminating these reagents, use a clean 8-tube strip to pierce the foil on these reagent wells before transferring the reagents.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
Reagent plate	-25°C to -15°C	Let stand for 15 minutes to bring to room temperature.
DMB	2°C to 8°C	Let stand for 10 minutes to bring to room temperature.
RSB	2°C to 8°C	Let stand for 10 minutes to bring to room temperature.

Set Up the Run

1 Vortex the reagent plate for 3 seconds.

2 Centrifuge at 600 × g for 5 seconds.
If you are not using the reagent plate immediately, set aside on ice.

3 Select **Prepare Libraries** on the NeoPrep System Welcome screen.

4 Do the following and then select **Next**.
- If running in BaseSpace mode, select a run.
- If running in standalone mode, use the following options to select a protocol:
  - Click **Select by barcode**, and then scan the reagent plate barcode or enter the reagent plate serial number.
  - Click **Select by name**, and then select **TruSeq Stranded mRNA**.

5 Configure the run. Select **Next**.
For more information, see *Configure the Run* in the *NeoPrep Library Prep System Guide (document # 15049720)*.
- The following processes are available:
  - **Prep Library**—Prepares libraries and must be selected.
  - [Optional] **Quantify**—Quantifies samples during the run, after library prep is complete.
  - [Optional] **Normalize**—Normalizes the final libraries to 10 nM, after quantification is complete. This option can only be selected if Quantify is also selected.
- Configuration options for TruSeq Stranded mRNA are as follows:

Table 2 Configuration Options

Parameter	Default	Options
Sample Count	16	1–16
PCR Cycles	15	12–20

NOTE
Illumina recommends default PCR cycles.

6 Review the run and sample information. Select **Next**.
For more information, see *Confirm Run* in the *NeoPrep Library Prep System Guide (document # 15049720)*.

7 Enter the consumable tracking information. Select **Next**.
For more information, see *Track Consumables* in the *NeoPrep Library Prep System Guide (document # 15049720)*.

8 Open the library card compartment door, slide the latch release to the right, and then place the library card on the library card stage.

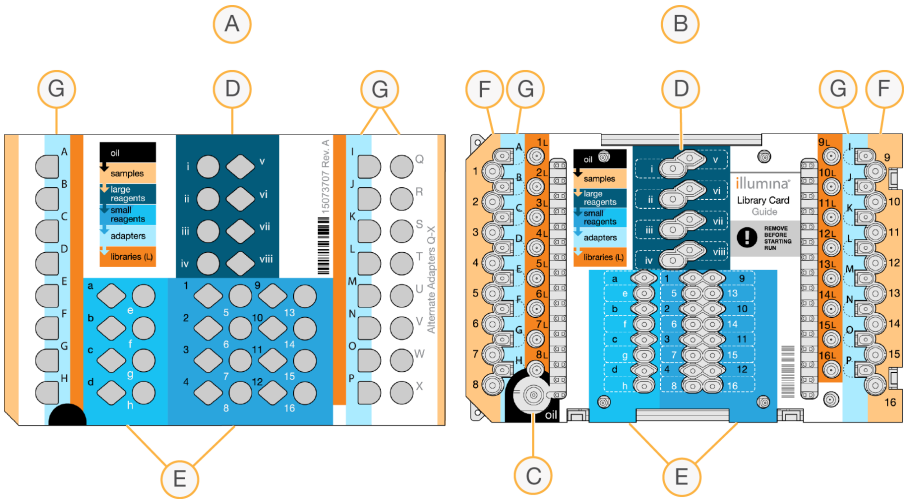
WARNING
To avoid instrument damage, make sure that the library card guide is not on the library card.

9 Close the library card compartment door. Select **Verify Library Card**. Do not open the compartment door during library card verification.

Load the Library Card

1 When library card verification is complete, open the library card compartment door and place the library card guide on the library card.

Figure 5 Reagent Plate to Library Card Transfer Layout



A Reagent plate
B Library card
C Oil
D Large reagents
E Small reagents
F Samples
G Adapters

2 Load the entire contents of the oil vial into the library card using the oil funnel. Wait 3 minutes for the oil to drain. Change your gloves after loading the oil.

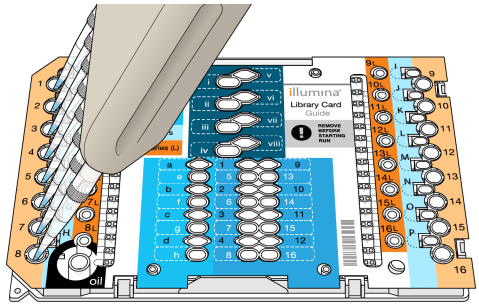
WARNING
Use the pipette tips specified in *Pipette and Tip Requirements* on page 7 and *Consumables and Equipment* on page 29. Other tips are not supported and can result in reagents not dispensing properly and run failure.

The loading angle of the pipette depends on the item being dispensed. The angle is specified in each step of the control software loading guide and is depicted in these procedures.

3 Insert pipette tips to the bottom of the wells of the prepared sample plate. Pipette up and down 1 time to mix.

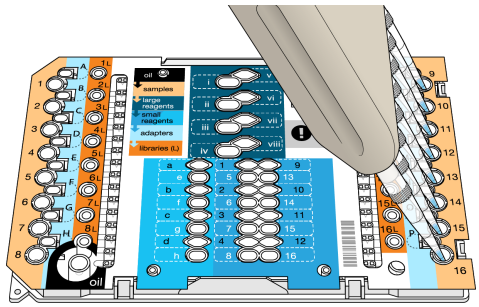
4 Transfer 25 µl of prepared samples 1–8.

Figure 6 Loading Samples 1–8



5 Transfer 25 µl of prepared samples 9–16.

Figure 7 Loading Samples 9–16

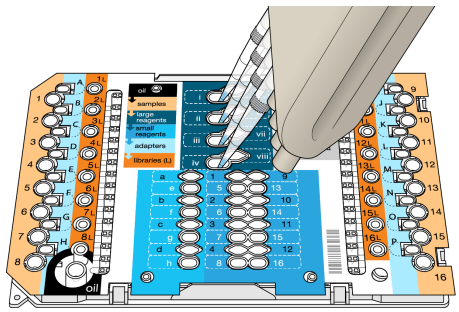


6 If you are preparing < 16 samples, add 25 µl RSB to empty sample wells.

NOTE
If you are preparing < 9 samples, the NeoPrep Control Software does not provide the RSB loading instructions for wells 9–16. Add 25 µl RSB to each empty sample well 9–16.

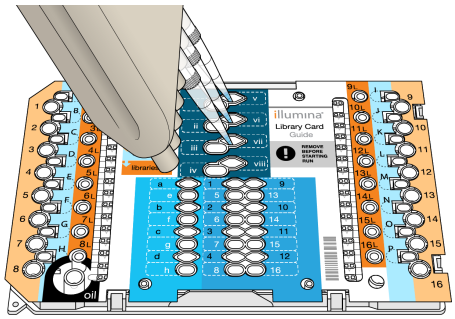
7 Transfer 125 µl of the large reagents i–iv.

Figure 8 Loading Large Reagents i–iv



8 Transfer 125 µl of the large reagents v–vii.

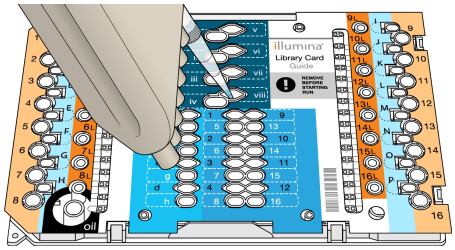
Figure 9 Loading Large Reagents v–vii



9 Vortex DMB until well-dispersed. Do not centrifuge DMB.

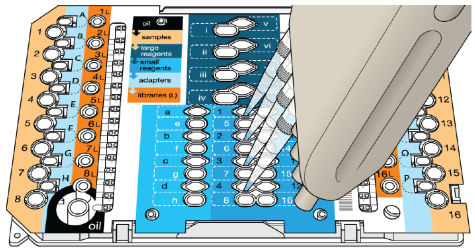
10 Add 80 µl DMB to the large reagent well viii.

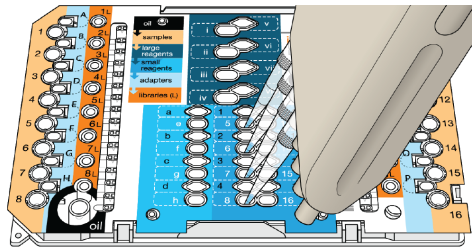
Figure 10 Loading Large Reagent viii



11 Transfer 15 µl of small reagents 1–4, and then 5–8.

Figure 11 Loading Small Reagents 1–4, 5–8



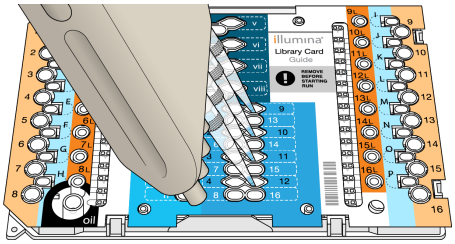


12 For small reagents 9–12:

a Use a clean 8-tube strip to pierce the foil on the reagent wells. Discard the 8-tube strip.

b Transfer 15 µl of each reagent.

Figure 12 Loading Small Reagents 9–12

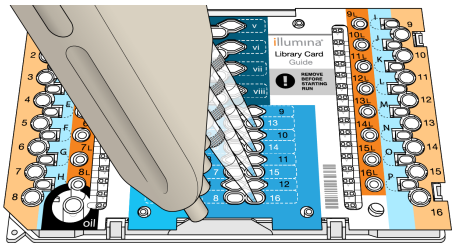


13 For small reagents 13–16:

a Use a clean 8-tube strip to pierce the foil on the reagent wells. Discard the 8-tube strip.

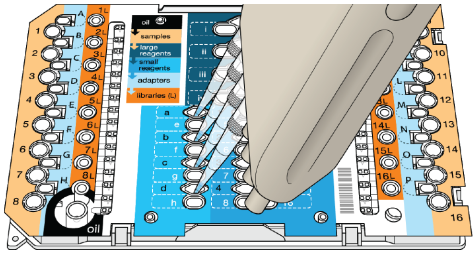
b Transfer 15 µl of each reagent.

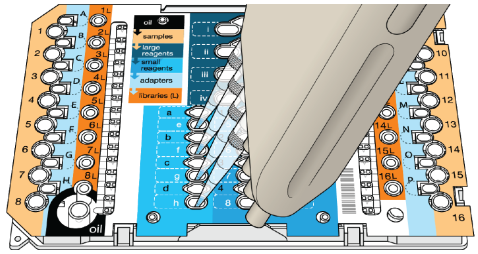
Figure 13 Loading Small Reagents 13–16



14 Transfer 5 µl of small reagents a–d, and then e–h.

Figure 14 Loading Small Reagents a–d, e–h



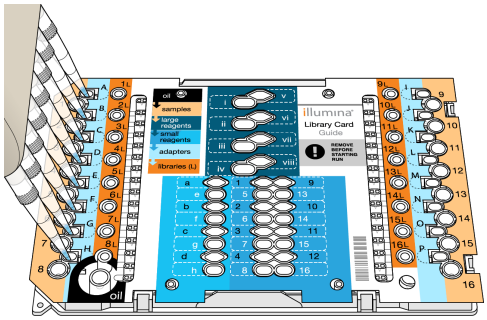


NOTE

For steps 15 and 16, if you are not using the default index adapter layout, the control software specifies which adapter to transfer to each library card well.

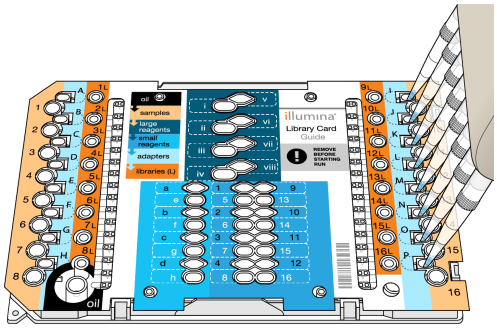
15 Transfer 3 µl of adapters A–H.

Figure 15 Loading Adapters A–H



16 Transfer 3 µl of adapters I–P.

Figure 16 Loading Adapters I–P



NOTE
If you are preparing < 9 samples, the NeoPrep Control Software does not provide the adapter loading instructions for adapters I–P. Transfer 3 µl of adapters I–P.

17 Remove the library card guide. Keep it for later use during the unloading process.

WARNING
To avoid instrument damage, make sure that the library card guide is removed from the library card.

18 Close the library card compartment door. Select **Start Run**. Do not open the compartment door until the run is complete.

19 When the run is complete, select **Next**. Libraries can remain at room temperature on a library card for up to 3 days after a run is complete.

Unload Libraries

This process describes how to collect libraries from the library card, separate libraries from the oil, and unload the library card from the instrument.

The NeoPrep Control Software guides you through the steps to unload the library card. Use the procedures in this section as a reference. For more information on the NeoPrep Control Software, see the *NeoPrep Library Prep System Guide (document # 15049720)*.

Consumables

- RSB (Resuspension Buffer)
- Library card guide
- Library separation tube strips (2)
- 96-well 0.3 ml PCR plates (2)
- Microseal 'B' adhesive seals

WARNING

The used library card contains hazardous materials. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and a laboratory coat. Handle the used library card as chemical waste. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region. For more information, see the SDS for this kit at support.illumina.com/sds.html.

Preparation

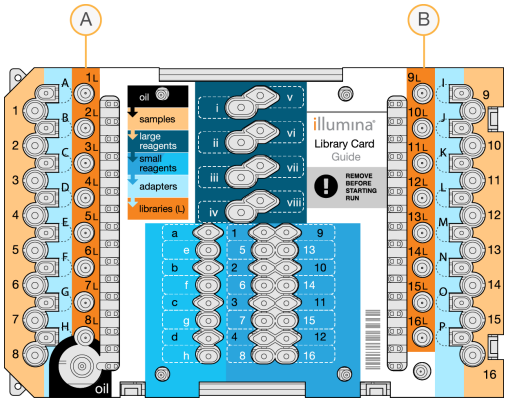
1. Remove RSB from 2°C to 8°C storage and bring to room temperature.
2. Label wells of 2 new PCR plates 1–16.
3. Label tubes of a library separation tube strip 1–8 and another library separation tube strip 9–16.

Procedure

1. Add 10 µl RSB to each well of a new PCR plate labeled 1–16.
2. Open the library card compartment door and place the library card guide on the library card.

3 Use a 200 µl pipette to transfer 20 µl from library card collection wells 1L–8L, and then 9L–16L to corresponding wells 1–16 of the plate. Pipette to mix.

Figure 17 Library Card Collection Wells



A Collection wells 1L–8L
B Collection wells 9L–16L

4 Centrifuge briefly.

5 Transfer the entire volume from plate wells 1–8, and then 9–16 to the center indent in the membrane of the corresponding library separation tubes 1–16.

6 Let stand for 10 seconds while the oil is absorbed in the tubes.

7 Transfer the entire volume from library separation tubes 1–8, and then 9–16 to the corresponding wells 1–16 of a new PCR plate.

8 Remove the library card and library card guide from the library card stage.

9 Discard the library card in accordance with applicable standards.

10 Close the library card compartment door, and then select **Home**.

11 Select from the following options:

Table 3 Post Run Options

NeoPrep System Quantification	NeoPrep System Normalization	Pooling Required	Then...
Yes	Yes	No	The protocol stops here. The final library is normalized to 10 nM. Proceed to cluster generation. For more information, see the system guide for your Illumina sequencing platform.
Yes	Yes	Yes	Proceed to *Pool Libraries*.
Yes	No	Yes or No	Proceed to *[Optional] Normalize Libraries Manually*.
No	No	Yes or No	Proceed to *[Optional] Check Libraries Manually*.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 2 months.

[Optional] Check Libraries Manually

If quantification was not performed on the NeoPrep System, perform quantification of your DNA libraries and quality control analysis.

Consumables

- One of the following for quantification:
  - KAPA Library Quantification Kit
  - Fluorometric quantification with dsDNA binding dye reagents
- One of the following for quality check:
  - Agilent DNA 7500 Kit
  - Agilent High Sensitivity DNA Kit

Quantify Libraries

If quantification was performed on the NeoPrep System, you do not need to perform this process.

To achieve the highest-quality data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of the flow cell. Optimizing cluster densities requires accurate quantification of DNA libraries.

1 Quantify the libraries using qPCR according to the Illumina *Sequencing Library qPCR Quantification Guide (document # 11322363)*.

Check Library Quality

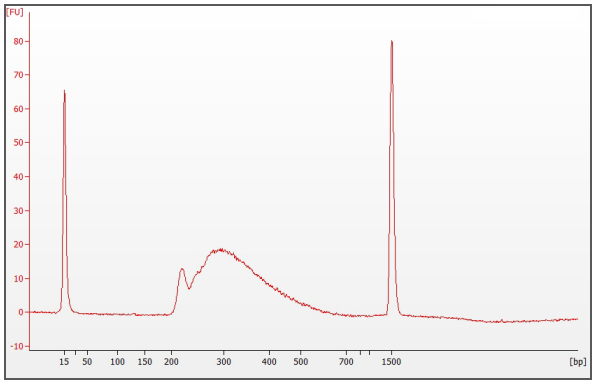
1 If using a Standard Sensitivity NGS Fragment Analysis Kit on an Advanced Analytical Fragment Analyzer:

   a Dilute the DNA library 1:1 with RSB.
   b Run 1 µl diluted DNA library.

2 If using a DNA 1000 chip on an Agilent Technologies 2100 Bioanalyzer, run 1 µl undiluted DNA library.

3 Check the size and purity of the sample. Expect the final product to be a band at ~300 bp.

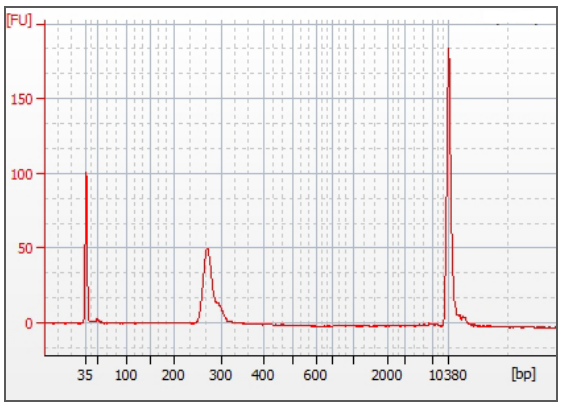
Figure 18 Example Library Size Distribution with Peak at ~300 bp (Before Normalization)



NOTE

A blue dye is used in the TruSeq Stranded mRNA Library Prep for NeoPrep reagents to aid in loading and collection. The dye appears as a characteristic peak at 200–250 bp and is not indicative of issues with the final library. Figure 19 shows the distribution of only the blue dye.

Figure 19 Reagent Blue Dye Distribution



[Optional] Normalize Libraries Manually

This process describes how to prepare DNA libraries for cluster generation when normalization was not performed on the NeoPrep System. DNA libraries are normalized to 10 nM.

Do not perform this process if normalization was performed on the NeoPrep System.

Consumables

- 96-well midi plate
- Microseal 'B' adhesive seal
- Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20

Preparation

1. If the DNA library plate was stored, thaw it at room temperature, and then centrifuge at 280 × g for 1 minute.
2. Label wells of a new 96-well midi plate 1–16.

Procedure

1. Transfer 5 µl from each well of the library plate to the corresponding wells of a midi plate.
2. Normalize each library to 10 nM with Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20. Pipette to mix.
   Depending on the quantification yield data of each sample library, the final volume in the plate can vary from 5–250 µl.
3. Select from the following options:
   - For libraries that do not require pooling, the protocol stops here. Proceed to cluster generation.
   - For libraries that require pooling, proceed to *Pool Libraries*.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 2 months.

Pool Libraries

This process describes how to pool normalized DNA libraries in equal volumes. Do not perform this process if you are not pooling libraries.

Consumables

- 96-well 0.3 ml PCR plate
- Microseal 'B' adhesive seal

Procedure

1. Determine the number of samples to combine for each pool. Do not pool samples with the same index.
2. Transfer 5 µl of each library to be pooled from the library plate to a single well of a new PCR plate. Pipette to mix.
   The total volume in each well is 5 times the number of combined sample libraries. For example, the volume for 2 samples is 10 µl, the volume for 12 samples is 60 µl, or the volume for 16 samples is 80 µl.
3. Proceed to cluster generation. For more information, see the system guide for your Illumina sequencing platform.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 2 months.

Supporting Information

The protocols described in this guide assume that you have reviewed the contents of this section, confirmed your kit contents, and obtained all the required consumables and equipment.

TruSeq Stranded mRNA Library Prep Kit for NeoPrep Kit

Make sure that you have the items identified in this section in your TruSeq Stranded mRNA Library Prep Kit for NeoPrep (catalog # NP-202-1001) before starting the protocol. The kit contains 3 boxes.

Box 1, Store at 15°C to 30°C

Quantity	Description
2	Library separation tube strips
1	NeoPrep Oil vial
1	Oil funnel
1	Library Card Guide
1	NeoPrep Library Card, 16-samples

Box 2, Store at -25°C to -15°C

This box contains a TruSeq Stranded mRNA Library Prep for NeoPrep reagent plate covered by a reagent plate guide.

Reagent Plate Contents

The TruSeq Stranded mRNA Library Prep for NeoPrep reagent plate is a single-use consumable. It consists of a 96-well foil-sealed plate prefilled with library prep adapters and reagents for a single TruSeq Stranded mRNA Library Prep for NeoPrep run.

Figure 20 Reagent Plate

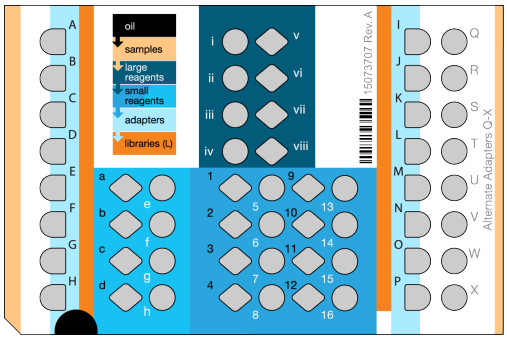


Table 4 Default Adapters

Well	Reagent	Description	Well	Reagent	Description
A	NR006	Adapter Index 6	I	NR001	Adapter Index 1
B	NR013	Adapter Index 13	J	NR010	Adapter Index 10
C	NR012	Adapter Index 12	K	NR020	Adapter Index 20
D	NR014	Adapter Index 14	L	NR008	Adapter Index 8
E	NR005	Adapter Index 5	M	NR025	Adapter Index 25
F	NR015	Adapter Index 15	N	NR011	Adapter Index 11

Well	Reagent	Description	Well	Reagent	Description
G	NR019	Adapter Index 19	O	NR018	Adapter Index 18
H	NR021	Adapter Index 21	P	NR023	Adapter Index 23

Table 5 Alternate Adapters

Well	Reagent	Description	Well	Reagent	Description
Q	NR002	Adapter Index 2	U	NR003	Adapter Index 3
R	NR004	Adapter Index 4	V	NR009	Adapter Index 9
S	NR007	Adapter Index 7	W	NR022	Adapter Index 22
T	NR016	Adapter Index 16	X	NR027	Adapter Index 27

NOTE

The large and small reagents and adapters are not the same as manual TruSeq library prep reagents and adapters. Do not use these reagents for manual TruSeq library prep and do not use the reagents and adapters in the manual TruSeq library prep kits for NeoPrep library prep.

Table 6 Large Reagents

Well	Reagent	Description
i	BWS3	Bead Wash Solution 3
ii	ESL	Elution Solution
iii	BBS	Bead Binding Solution
iv	QDR	Quant Dye Reagent
v	BWS3	Bead Wash Solution 3
vi	ESL	Elution Solution
vii	BBS	Bead Binding Solution
viii	–	Empty

Table 7 Small Reagents

Well	Reagent	Description	Well	Reagent	Description
a	FAM	FAM Dye	5	EPM2	Enhanced PCR Mix 2
b	DBW	Droplet Blue Water	6	EPM2	Enhanced PCR Mix 2
c	DBW	Droplet Blue Water	7	PPC2	PCR Primer Cocktail 2
d	DBW	Droplet Blue Water	8	ESL	Elution Solution
e	DBW	Droplet Blue Water	9	ELB2	Elution Buffer 2
f	DBW	Droplet Blue Water	10	BBB2	Bead Binding Buffer 2
g	DBW	Droplet Blue Water	11	FSA2	First Strand Synthesis Mix 2
h	FAM	FAM Dye	12	BWB2	Bead Washing Buffer 2
1	ESL	Elution Solution	13	EPH2	Elute, Prime, Fragment High Mix 2
2	LIG4	Ligation Mix	14	EPH2	Elute, Prime, Fragment High Mix 2
3	ATL3	A-Tailing Mix	15	SMM2	Second Strand Marking Master Mix 2
4	ERP4	End Repair Mix	16	BWB2	Bead Washing Buffer 2

Box 3, Store at 2°C to 8°C

This box is shipped at room temperature. When you receive your kit, store this box at 2°C to 8°C.

Quantity	Reagent	Description
1	RSB	Resuspension Buffer
1	DMB	Digital Microfluidics Beads
1	RPB2	RNA Purification Beads 2

Consumables and Equipment

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

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

Pipettes and Tips

Use the pipettes and tips specified. Other pipettes and tips are not supported and can result in reagents not dispensing properly and run failure.

Pipettes and Tips	Supplier
Pipet-Lite XLS+ 8-channel LTS, 2 µl to 200 µl	Rainin, catalog # L8-20XLS+
One of the following 20 µl pipette tips: • LTS tips 20 µl. Presterilized. Filter • ART Barrier Pipette Tips 20 µl; 20 µl SoftFit-L	• Rainin, catalog # RT-L10F • Fisher Scientific, catalog # 2749RI
Pipet-Lite XLS+ 8-channel LTS, 20 µl to 200 µl	Rainin, catalog # L8-200XLS+
One of the following 200 µl pipette tips: • LTS tips 200 µl. Presterilized. Filter • ART Barrier Pipette Tips 200 µl; 200 µl SoftFit-L	• Rainin, catalog # RT-L200F • Fisher Scientific, catalog # 2769RI

Consumables

Consumable	Supplier
1000 µl barrier pipette tips	General lab supplier
96-well 0.3 ml skirtless PCR plates, or Twin.tec 96-well PCR plates	E&K Scientific, part # 480096, or Eppendorf, part # 951020303
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658

Consumable	Supplier
[Optional - positive control] Human UHR total RNA	Agilent Technologies, part # 740000
[Optional - for starting material quality assessment] One of the following: • Standard Sensitivity RNA Analysis Kit (20nt Lower Marker) • Agilent RNA 6000 Nano Kit	• Advanced Analytical Technologies, part # DNF-489 • Agilent Technologies, part # 5067-1511
[Optional - for library quality control] One of the following: • Standard Sensitivity NGS Fragment Analysis Kit, 1–6000 bp (500 samples) • DNA 1000 Kit	• Advanced Analytical Technologies, part # DNF-473-0500 • Agilent Technologies, part # 5067-1504
[Optional - for library quality control] One of the following: • Standard Sensitivity NGS Fragment Analysis Kit, 1–6000 bp (500 samples) • DNA 1000 Kit	• Advanced Analytical Technologies, part # DNF-473-0500 • Agilent Technologies, part # 5067-1504
[Optional - for manual normalization] 96-well storage plates, round well, 0.8 ml (midi plate)	Fisher Scientific, part # AB-0859
[Optional - for manual normalization] Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20	General lab supplier

Equipment

Equipment	Supplier
96-well thermal cycler (with heated lid)	General lab supplier
NeoPrep Library Prep System	Illumina, catalog # SE-601-1001
Microplate centrifuge	General lab supplier
Vortexer or microplate shaker	General lab supplier
[Optional - for library quality control] One of the following: • Fragment Analyzer Automated CE System • 2100 Bioanalyzer Desktop System	• Advanced Analytical Technologies, part # FSv2-CE2 or FSv2-CE10 • Agilent Technologies, part # G2940CA

Index Adapter Sequences

The TruSeq Stranded mRNA Library Prep Kit for NeoPrep contains the following index adapter sequences.

- The indexes are TruSeq LT single-index adapters.
- The index numbering is not contiguous. There is no Index 17, 24, or 26.
- The sequence contains 7 bases. The seventh base, shown in parenthesis (), is not included in the Index Read. Record only the first 6 bases in a sample sheet. For indexes 13 and above, the seventh base (in parentheses) might not be A, which is seen in the cycle 7 of the Index Read.

  For more information on the number of cycles used to sequence the Index Read, see the system guide for your Illumina sequencing platform.

Table 8 Indexed Adapter Sequences

Adapter	Sequence		Adapter	Sequence
NR0001	ATCACG(A)		NR0013	AGTCAA(C)
NR0002	CGATGT(A)		NR0014	AGTTCC(G)
NR0003	TTAGGC(A)		NR0015	ATGTCA(G)
NR0004	TGACCA(A)		NR0016	CCGTCC(C)
NR0005	ACAGTG(A)		NR0018	GTCCGC(A)
NR0006	GCCAAT(A)		NR0019	GTGAAA(C)
NR0007	CAGATC(A)		NR0020	GTGGCC(T)
NR0008	ACTTGA(A)		NR0021	GTTTCG(G)
NR0009	GATCAG(A)		NR0022	CGTACG(T)
NR0010	TAGCTT(A)		NR0023	GAGTGG(A)
NR0011	GGCTAC(A)		NR0025	ACTGAT(A)
NR0012	CTTGTA(A)		NR0027	ATTCCT(T)

Acronyms

Acronym	Definition
ATL	A-Tailing Mix
BBB	Bead Binding Buffer
BBS	Bead Binding Solution
BWB	Bead Washing Buffer
BWS	Bead Wash Solution

Acronym	Definition
DMB	Digital Microfluidics Beads
ELB	Elution Buffer
EPH	Elute, Prime, Fragment High Mix
EPM	Enhanced PCR Mix
ERP	End Repair Mix
ESL	Elution Solution
FAM	FAM Dye
FSA	First Strand Synthesis Mix
LIG	Ligation Mix
PPC	PCR Primer Cocktail
QDR	Quant Dye Reagent
QSD	Quant Standard
RSB	Resuspension Buffer
SMM	Second Strand Marking Master Mix

Revision History

Document	Date	Description of Change
Document # 15049725 v03	June 2016	• In Pipette and Tip Requirements, clarified that the 200 µl pipette used to pipette 20 µl in Unload Library step. • Replaced quantification and normalization procedures that were removed in v01. • Changed name of Validate Libraries section to Check Libraries.
Document # 15049725 v02 Material # 20000945	March 2016	• Corrected order of steps in Load the Library Card section to pipette to mix samples in the sample plate before samples are loaded into the library card.

Document # 15049725 v01	January 2016	• Removed quantification and normalization procedures and information
		• Added Custom Protocol Selector to *Additional Resources*
		• Moved Revision History to back of booklet
		• Clarify to mix by pipette when diluting RNA in *Prepare Samples for Loading*
		• Changed loading order to load samples first
		• Changed loading workflow on reagent plate guide and library card guide
		• Added 3 minute wait to drain oil from vial into library card
		• Removed step to pipette samples before transferring to library card
		• Renamed button Home at the conclusion of unloading libraries
		• *Consumables* table:
		  • Removed 20 µl and 200 µl pipettes. They are specified in *Pipettes and Tips*.
		  • Removed 1000 µl pipettes as they are standard lab items
		  • Added kits for starting material quality assessment
		• Moved *Acronyms* to end of Supporting Information

Part # 15049725 Rev. C	June 2015	Added required pipettes to: • *Pipette Tip Requirements* • *Consumables and Equipment* Removed part numbers and package layouts from *Kit Contents* Updated reagent names in *Reagent Plate Contents*
Part # 15049725 Rev. B	April 2015	Added required pipette tip and calibration information to: • New *Pipette Tip Requirements* • *Consumables and Equipment* • *Tips and Techniques* • *Load the Library Card* Changed BaseSpace resource reference to helpcenter
Part # 15049725 Rev. A	March 2015	Initial release.

Notes

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 9 Illumina General Contact Information

Website	www.illumina.com
Email	techsupport@illumina.com

Table 10 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Japan	0800.111.5011
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
China	400.635.9898	Singapore	1.800.579.2745
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	Taiwan	00806651752
Hong Kong	800960230	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000
Italy	800.874909		

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

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

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