

# mRNA Sequencing Sample Preparation Guide

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# Introduction

This protocol explains how to convert total RNA into a library of template molecules suitable for high throughput DNA sequencing for subsequent cluster generation.

The first step in the workflow involves purifying the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, the mRNA is fragmented into small pieces using divalent cations under elevated temperature. Then the cleaved RNA fragments are copied into first strand cDNA using reverse transcriptase and random primers. This is followed by second strand cDNA synthesis using DNA Polymerase I and RNaseH. These cDNA fragments then go through an end repair process, the addition of a single 'A' base, and then ligation of the adapters. These products are then purified and enriched with PCR to create the final cDNA library.

## Sample Prep Workflow

You need two days to complete this protocol.

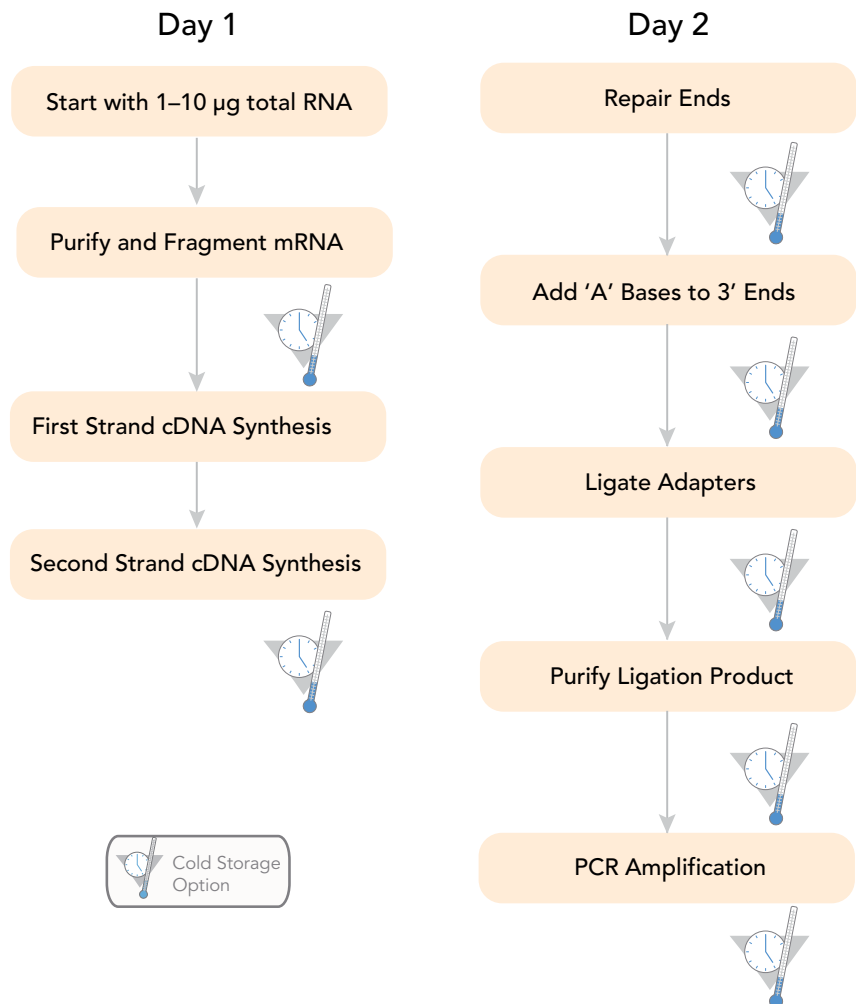


Figure 1 mRNA-Seq Sample Preparation Workflow

# RNA Input Recommendations

## Best Practices

RNA is highly susceptible to degradation by RNase enzymes. RNase enzymes are present in cells and tissues and can be carried on hands, labware, or even dust. They are very stable and difficult to inactivate. For these reasons, it is important to follow best laboratory practices while preparing and handling RNA samples.

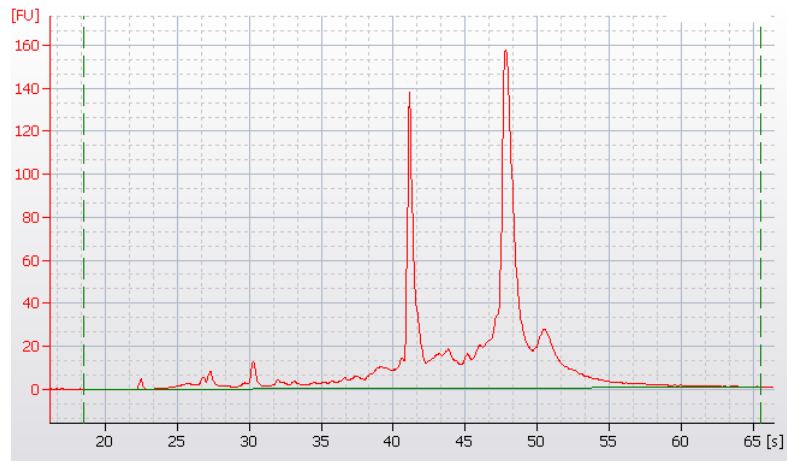
- ▶ When harvesting total RNA, use a method that quickly disrupts tissue and isolates and stabilizes RNA
- ▶ Wear gloves and use sterile technique at all times
- ▶ Reserve a set of pipettes for RNA work. Use sterile RNase-free filter pipette tips to prevent cross-contamination
- ▶ Use disposable plasticware that has been certified to be RNase-free. Illumina recommends the use of non-sticky sterile RNase-free microfuge tubes. These should not be used for other lab work.
- ▶ All reagents should be prepared from RNase-free components, including ultrapure water
- ▶ Store RNA samples by freezing. Keep samples on ice at all times while working with them. Avoid extended pauses in the protocol until the RNA has been reverse transcribed into DNA
- ▶ Use RNase/DNase decontamination solution to decontaminate work surfaces and equipment

## Starting Material

### Total RNA Input

This protocol is suitable for 1–10  $\mu\text{g}$  of total RNA. Lower amounts may result in inefficient ligation and low yield. The protocol has been optimized using 1  $\mu\text{g}$  of high-quality universal human reference total RNA as input. Use of RNA from other species, tissues, or qualities may require further optimization with regard to the initial input amount and selection of the desired bands during the final gel excision.

It is very important to use high-quality RNA as the starting material. Use of degraded RNA can result in low yield, overrepresentation of the 5' ends of the RNA molecules, or failure of the protocol. Illumina recommends that you check total RNA integrity following isolation using an Agilent Technologies 2100 Bioanalyzer with an RNA Integrity Number (RIN) value greater than 8. Alternatively, a formaldehyde 1% agarose gel can be run and the integrity of RNA judged upon staining with ethidium bromide. High quality RNA shows a 28S rRNA band at 4.5 kb that should be twice the intensity of the 18S rRNA band at 1.9 kb. Both kb determinations are relative to a RNA 6000 ladder. The mRNA will appear as a smear from 0.5–12 kb. Figure 2 is a Universal Human Reference (UHR) starting RNA Bioanalyzer trace.



**Figure 2** Starting RNA Bioanalyzer Trace

### Purified mRNA Input

Alternately, previously isolated mRNA can be used as starting material. Use the entire fraction of mRNA purified from 1–10  $\mu\text{g}$  of total RNA. Begin this protocol at the mRNA fragmentation step, omitting mRNA purification.

### Positive Control

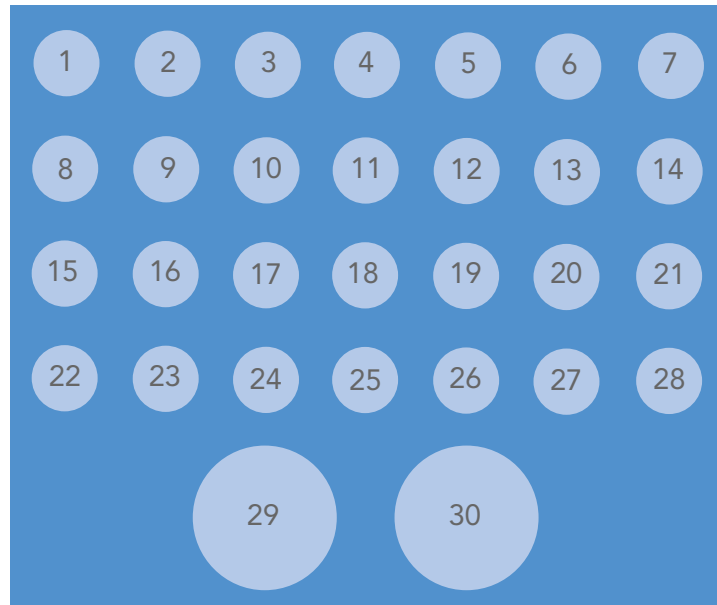
Illumina recommends using Stratagene Human UHR total RNA (catalog # 740000) as a positive control sample for this protocol.

## mRNA-Seq Sample Preparation Kit Contents

Check to ensure that you have all of the reagents identified in this section before proceeding to sample preparation.

### Kit Contents, Box 1 **Store at -15° to -25°C**

This box is shipped on dry ice. As soon as you receive your kit, store the following components at -15° to -25°C.



**Figure 3** mRNA-Seq Sample Prep Kit, Box 1, part # 1004814

1. Ultra Pure Water, part # 1001913
2. 10 mM Tris Buffer, part # 1002115
3. 5X Fragmentation Buffer, part # 1005084
4. Fragmentation Stop Solution, part # 1004826
5. Glycogen, part # 1001664
6. Random Primers, part # 1004784
7. 25 mM dNTPs Mix, part # 11318102
8. RNase Inhibitor, part # 15003548
9. GEX Second Strand Buffer, part # 1000562
10. RNaseH, part # 1000576
11. DNA Polymerase I, part # 1000577
12. 10X End Repair Buffer, part # 1004819
13. T4 DNA Polymerase, part # 1000514
14. Klenow DNA Polymerase, part # 1000515
15. T4 PNK, part # 1005082

16. 10X A-Tailing Buffer, part # 1002105
17. 1 mM dATP, part # 11318081
18. Klenow Exo-, part # 11318090
19. 2X Rapid T4 DNA Ligase Buffer, part # 1004792
20. PE Adapter Oligo Mix, part # 1001782
21. T4 DNA Ligase, part # 1004790
22. 5X Phusion™ Buffer (Finnzymes Oy), part # 1000585
23. PCR Primer PE 2.0, part # 1001784
24. PCR Primer PE 1.0, part # 1001783
25. Phusion DNA Polymerase (Finnzymes Oy), part # 1000584
26. Empty
27. Empty
28. Empty
29. Bead Binding Buffer, part # 1002118
30. Bead Washing Buffer, part # 1004800



Briefly centrifuge the tubes before use, as the contents may have settled on the sides.

## Kit Contents, Bag 1

### Store at 2° to 8°C

This bag (part # 1004815) is shipped at 4°C. As soon as you receive your kit, store the contents at 2° to 8°C.

- ▶ Sera-Mag Magnetic Oligo(dT) Beads, part # 1004815

## User-Supplied Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation.

**Table 1** *User-Supplied Consumables*

| Consumable  | Supplier                     |
|---|------------------------------|
| 1.5 ml RNase-free non-sticky tube   | Ambion, part # AM12450       |
| 3 M NaOAc, pH 5.2   | General lab supplier         |
| 50x TAE Buffer  | General lab supplier         |
| 70% EtOH  | General lab supplier         |
| 100% EtOH   | General lab supplier         |
| 100 bp DNA Ladder   | Invitrogen, part # 10488-058 |
| 6X DNA loading dye  | General lab supplier         |
| Certified Low-Range Ultra Agarose   | BIO-RAD, part # 161-3106     |
| GeneCatcher Disposable Gel Excision Kit   | Gel Company, part # PKB6.5   |
| MinElute PCR Purification Kit   | QIAGEN, part # 28004         |
| QIAquick Gel Extraction Kit   | QIAGEN, part # 28704         |
| QIAquick PCR Purification Kit   | QIAGEN, part # 28104         |
| SuperScript II Reverse Transcriptase with 100 mM DTT and 5X First Strand Buffer | Invitrogen, part # 18064-014 |

**Table 2** *Equipment Checklist*

| Consumable                    | Supplier             |
|-------------------------------|----------------------|
| 4°C microcentrifuge           | General lab supplier |
| Heat block (2)                | General lab supplier |
| Magnetic stand                | General lab supplier |
| Room temperature tube rotator | General lab supplier |
| Thermal cycler                | General lab supplier |



# Purify the mRNA

This process purifies the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads.

## Consumables Illumina-Supplied

- ▶ Sera-Mag Magnetic Oligo(dT) Beads
- ▶ Bead Binding Buffer
- ▶ Bead Washing Buffer
- ▶ Ultra Pure Water
- ▶ 10 mM Tris Buffer

## Best Practice Using the Magnetic Stand

Follow these guidelines throughout the sample preparation protocol to prevent the beads from drying out.



Allow sufficient time for all of the beads to be captured by the magnetic stand, not just until a pellet is visible.

1. Place the tube containing the beads on the magnetic stand for at least 1–2 minutes to separate the beads and the buffer.



Allow all of the beads long enough to be captured by the magnetic stand, not just until you see a pellet.

2. Exchange the buffer using a pipette while the tube is on the magnetic stand.
3. Resuspend the beads thoroughly by vortexing with 0.5-1 second pulses.



It is critical that the beads are thoroughly resuspended in the solution.

4. Centrifuge the samples in a benchtop microcentrifuge for 1-2 seconds to remove any beads or liquid from the walls of the tube.
5. Repeat steps 1 through 4 as required.

## Procedure

It is important to follow this procedure exactly to ensure reproducibility. Allow the beads to fully pellet against the magnetic stand, for at least 1–2 minutes. Remove the supernatant from the beads immediately while the beads are still pelleted against the magnetic stand. Do not allow the pellets to dry.



Illumina recommends you use 1–10 µg of total RNA.



You may also start this protocol with 100 ng of mRNA. If so, proceed to the next section, *Fragment the mRNA* on page 12.

1. Preheat one heat block to 65°C and the other heat block to 80°C.
2. Dilute the total RNA with nuclease-free water to 50 µl in a 1.5 ml RNase-free non-sticky tube.
3. Heat the sample in a preheated heat block at 65°C for 5 minutes to disrupt the secondary structures and then place the tube on ice.
4. Aliquot 15 µl of Sera-Mag oligo(dT) beads into a 1.5 ml RNase-free non-sticky tube.
5. Wash the beads two times with 100 µl of Bead Binding Buffer and remove the supernatant.
6. Resuspend the beads in 50 µl of Bead Binding Buffer and add the 50 µl of total RNA sample from step 3.
7. Rotate the tube from step 6 at room temperature for 5 minutes and remove the supernatant.
8. While the tube is incubating, aliquot 50 µl of Binding Buffer to a fresh 1.5 ml RNase-free non-sticky tube.
9. After the 5 minutes incubation, wash the beads from step 7 twice with 200 µl of Washing Buffer and remove the supernatant.
10. Add 50 µl of 10 mM Tris-HCl to the beads and then heat in the preheated heat block at 80°C for 2 minutes to elute the mRNA from the beads.
11. Immediately put the tube on the magnet stand, transfer the supernatant (mRNA) to the tube from step 8. Do not discard the used beads.
12. Place the samples aside and wash the beads twice with 200 µl of Washing Buffer.
13. Heat the samples in the preheated heat block at 65°C for 5 minutes to disrupt the secondary structures and then place the samples on ice.
14. Add the iced 100 µl of the mRNA sample from step 13 to the washed beads and rotate it at room temperature for 5 minutes, then remove the supernatant.

15. Wash the beads twice with 200  $\mu$ l of Washing Buffer and remove the supernatant.
16. Add 17  $\mu$ l of 10 mM Tris-HCl to the beads and heat in the preheated heat block at 80°C for 2 minutes to elute the mRNA from the beads.
17. Immediately put the tube on the magnet stand and then transfer the supernatant (mRNA) to a fresh 200  $\mu$ l thin-wall PCR tube.  
The resulting amount of mRNA should be approximately 16  $\mu$ l.

# Fragment the mRNA

This process fragments the mRNA into small pieces using divalent cations under elevated temperature.

## Consumables

### Illumina-Supplied

- ▶ 5X Fragmentation Buffer
- ▶ Fragmentation Stop Solution
- ▶ Glycogen
- ▶ Ultra Pure Water

### User-Supplied

- ▶ 3 M NaOAC, pH 5.2
- ▶ 70% EtOH
- ▶ 100% EtOH

## Procedure

1. Preheat a PCR thermal cycler to 94°C.
2. Prepare the following reaction mix in a 200 µl thin wall PCR tube:
  - 5X Fragmentation Buffer (4 µl)
  - mRNA (16 µl)
 The total volume should be 20 µl.
3. Incubate the tube in a preheated PCR thermal cycler at 94°C for exactly 5 minutes.
4. Add 2 µl of Fragmentation Stop Solution.
5. Place the tube on ice.
6. Transfer the solution to a 1.5 ml RNase-free non-sticky tube.
7. Add the following to the tube and incubate at -80°C for 30 minutes or overnight as desired:
  - 3 M NaOAC, pH 5.2 (2 µl)
  - Glycogen (2 µl)
  - 100% EtOH (60 µl)



The protocol can be safely stopped here. Illumina does not recommend stopping the protocol at any other point while the sample is RNA. If you are stopping here, store the samples at -15° to -25°C.

8. Centrifuge the tube at 14,000 rpm (20,200 relative centrifugal force) for 25 minutes at 4°C in a microcentrifuge.
9. Carefully pipette off the EtOH without dislodging the RNA pellet.  
The RNA pellet will be small and almost colorless. To avoid dislodging it, remove the EtOH in several steps, removing 90% at each step and switching to smaller pipette tips for the next step.

- 10.** Without disturbing the pellet, wash the pellet with 300  $\mu$ l of 70% EtOH.
- 11.** Centrifuge the pellet and carefully pipette out the 70% EtOH.
- 12.** Air dry the pellet for 10 minutes at room temperature.
- 13.** Resuspend the RNA in 11.1  $\mu$ l of RNase-free water.

## Synthesize the First Strand cDNA

This process reverse transcribes the cleaved RNA fragments into first strand cDNA using reverse transcriptase and random primers.

### Consumables **Illumina-Supplied**

- ▶ 25 mM dNTP Mix
- ▶ Random Primers
- ▶ RNase Inhibitor

### User-Supplied

- ▶ SuperScript II
- ▶ 100 mM DTT (included with SuperScript II)
- ▶ 5X First Strand Buffer (included with SuperScript II)

### Procedure

1. Assemble the following reaction in a 200  $\mu$ l thin wall PCR tube:
  - Random Primers (1  $\mu$ l)
  - mRNA (11.1  $\mu$ l)The total volume should be 12.1  $\mu$ l.
2. Incubate the sample in a PCR thermal cycler at 65°C for 5 minutes, and then place the tube on ice.
3. Set the PCR thermal cycler to 25°C.
4. Mix the following reagents in the order listed in a separate tube.  
Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples.
  - 5X First Strand Buffer (4  $\mu$ l)
  - 100 mM DTT (2  $\mu$ l)
  - 25 mM dNTP Mix (0.4  $\mu$ l)
  - RNase Inhibitor (0.5  $\mu$ l)The total volume should be 6.9  $\mu$ l.
5. Add 6.9  $\mu$ l of mixture to the PCR tube and mix well.
6. Heat the sample in the preheated PCR thermal cycler at 25°C for 2 minutes.
7. Add 1  $\mu$ l SuperScript II to the sample and incubate the sample in a thermal cycler with following program:
  - a. 25°C for 10 minutes
  - b. 42°C for 50 minutes
  - c. 70°C for 15 minutes
  - d. Hold at 4°C
8. Place the tube on ice.

# Synthesize the Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand generating double-stranded cDNA.

## Consumables

### Illumina-Supplied

- ▶ Ultra Pure Water
- ▶ GEX Second Strand Buffer
- ▶ 25 mM dNTP Mix
- ▶ RNaseH
- ▶ DNA Pol I

### User-Supplied

- ▶ QIAquick PCR Purification Kit (QIAGEN, part # 28104)

## Procedure

1. Preheat a PCR thermal cycler to 16°C.
2. Add 62.8 µl of ultra pure water to the first strand cDNA synthesis mix.
3. Add the following reagents to the mix:
  - GEX Second Strand Buffer (10 µl)
  - 25 mM dNTP Mix (1.2 µl)
4. Mix well and incubate on ice for 5 minutes or until well-chilled.
5. Add the following reagents:
  - RNaseH (1 µl)
  - DNA Pol I (5 µl)
6. Mix well and incubate at 16°C in a thermal cycler for 2.5 hours.
7. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample and elute in 50 µl of QIAGEN EB buffer.
8. At this point, the sample is in the form of double-stranded DNA.



The protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C.

## Perform End Repair

This process converts the overhangs into blunt ends using T4 DNA polymerase and Klenow DNA polymerase. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs.

### Consumables

#### Illumina-Supplied

- ▶ 10X End Repair Buffer
- ▶ 25 mM dNTP Mix
- ▶ T4 DNA Polymerase
- ▶ Klenow DNA Polymerase
- ▶ T4 PNK
- ▶ Ultra Pure Water

#### User-Supplied

- ▶ QIAquick PCR Purification Kit (QIAGEN, part # 28104)

### Procedure

1. Preheat one heat block to 20°C and the other heat block to 37°C.
2. Prepare the following reaction mix in a 1.5 ml RNase-free non-sticky tube:
  - Eluted DNA (50 µl)
  - Water (27.4 µl)
  - 10X End Repair Buffer (10 µl)
  - 25 mM dNTP Mix (1.6 µl)
  - T4 DNA Polymerase (5 µl)
  - Klenow DNA Polymerase (1 µl)
  - T4 PNK (5 µl)The total volume should be 100 µl.
3. Incubate the sample in a heat block at 20°C for 30 minutes.
4. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample and elute in 32 µl of QIAGEN EB buffer.



The protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C.



## Adenylate 3' Ends

This process adds an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, using the polymerase activity of Klenow fragment (3' to 5' exo minus). This prepares the DNA fragments for ligation to the adapters, which have a single 'T' base overhang at their 3' end.

### Consumables Illumina-Supplied

- ▶ A-Tailing Buffer
- ▶ 1 mM dATP
- ▶ Klenow exo (3' to 5' exo minus) (3  $\mu$ l)

### User-Supplied

- ▶ MinElute PCR Purification Kit (QIAGEN, part # 28004)



#### NOTE

This process requires a MinElute column rather than a normal QIAquick column.

### Procedure

1. Prepare the following reaction mix in a 1.5 ml RNase-free non-sticky tube:
  - Eluted DNA (32  $\mu$ l)
  - A-Tailing Buffer (5  $\mu$ l)
  - 1 mM dATP (10  $\mu$ l)
  - Klenow exo (3' to 5' exo minus) (3  $\mu$ l)The total volume should be 50  $\mu$ l.
2. Incubate the sample in a heat block at 37°C for 30 minutes.
3. Follow the instructions in the MinElute PCR Purification Kit to purify the sample and elute in 23  $\mu$ l of QIAGEN EB buffer.



The protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C.

## Ligate the Adapters

This process ligates adapters to the ends of the DNA fragments, preparing them to be hybridized to a single read flow cell.



### NOTE

Libraries constructed with this kit can be sequenced on a PE flow cell and give PE reads, however Illumina's post-sequencing analysis does not support PE mRNA reads.

### Consumables **Illumina-Supplied**

- ▶ 2X Rapid T4 DNA Ligase Buffer
- ▶ PE Adapter Oligo Mix
- ▶ T4 DNA Ligase

### User-Supplied

- ▶ MinElute PCR Purification Kit (QIAGEN, part # 28004)

### Procedure

1. Prepare the following reaction mix in a 1.5 ml RNase-free non-sticky tube:
  - Eluted DNA (23  $\mu$ l)
  - 2X Rapid T4 DNA Ligase Buffer (25  $\mu$ l)
  - PE Adapter Oligo Mix (1  $\mu$ l)
  - T4 DNA Ligase (1  $\mu$ l)

The total volume should be 50  $\mu$ l.
2. Incubate the sample at room temperature for 15 minutes.
3. Follow the instructions in the MinElute PCR Purification Kit to purify the sample and elute in 10  $\mu$ l of QIAGEN EB buffer.



### NOTE

You can spin the column twice as long after QIAGEN PE removal to ensure complete ethanol removal.



The protocol can be safely stopped here. If you are stopping, store the samples at  $-15^{\circ}$  to  $-25^{\circ}\text{C}$ .

## Purify the cDNA Templates

This process purifies the products of the ligation reaction on a gel to select a size range of templates for downstream enrichment.

### Consumables

#### User-Supplied

- ▶ Certified Low-Range Ultra Agarose
- ▶ 50X TAE Buffer
- ▶ Distilled Water
- ▶ 100 bp DNA Ladder
- ▶ 6X DNA Loading Dye
- ▶ GeneCatcher Disposable Gel Excision Kit
- ▶ QIAquick Gel Extraction Kit (QIAGEN, part # 28704)

### Procedure

1. Prepare a 50 ml, 2% agarose gel with distilled water and TAE. Final concentration of TAE should be 1X at 50 ml.
2. Load the samples as follows:
  - 2  $\mu$ l 100 bp DNA Ladder in the first well
  - 10  $\mu$ l DNA elute from the ligation step mixed with 2  $\mu$ l of 6X DNA Loading Dye in the second well
  - 2  $\mu$ l 100 bp DNA Ladder in the third well

Using ladders on both sides of a sample help locate the gel area to be excised as the band is not visible.



#### NOTE

For handling multiple samples, leave one empty lane between samples and ladders to prevent cross-contamination. Do not run more than two samples on the same gel to avoid contamination.

3. Run the gel at 120 V for 60 minutes.
4. Excise a region of gel with a clean gel excision tip and remove the gel slice by centrifuging it into a microcentrifuge tube. The gel slice should contain the material in the 200 bp ( $\pm$ 25 bp) range. See Figure 4.



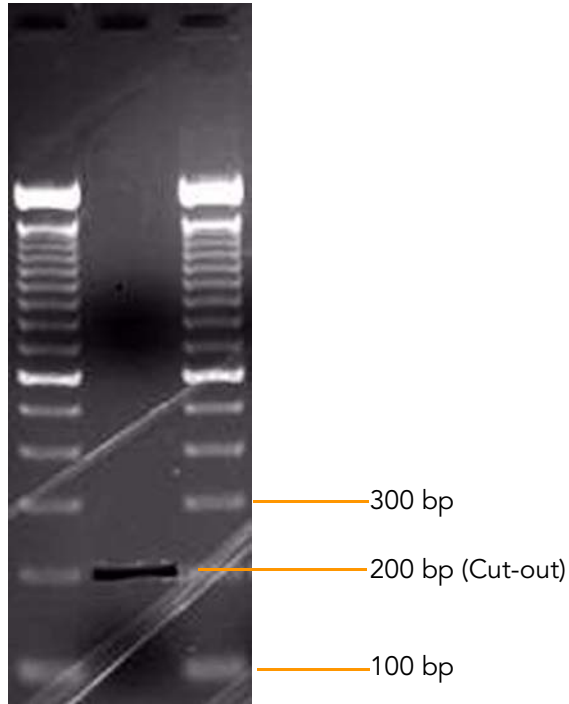
#### NOTE

Depending on the application, the excised band can be up to 500 bp if desired. However, it should be a thin slice  $\pm$  25 bp.



#### NOTE

It is normal to not see any visible DNA on the gel.



*Figure 4* 2% Agarose Gel

5. Follow instructions in the QIAquick Gel Extraction Kit to purify the sample and elute in 30  $\mu$ l of QIAGEN EB buffer. (Be sure to add isopropanol per the manufacturer's instructions.)



The protocol can be safely stopped here. If you are stopping, store the samples at  $-15^{\circ}$  to  $-25^{\circ}\text{C}$ .

## Enrich the Purified cDNA Templates

This process uses PCR to amplify the cDNA in the library. The PCR is performed with two primers that anneal to the ends of the adapters.

### Consumables

#### Illumina-Supplied

- ▶ 5X Phusion Buffer (Finnzymes Oy)
- ▶ Phusion DNA Polymerase (Finnzymes Oy)
- ▶ PCR Primer PE 1.0
- ▶ PCR Primer PE 2.0
- ▶ 25 mM dNTP Mix
- ▶ Ultra Pure Water

#### User-Supplied

- ▶ QIAquick PCR Purification Kit (QIAGEN, part # 28104)

### Procedure

1. Prepare the following PCR reaction mix in a 200  $\mu$ l thin wall PCR tube (Make 10% extra reagent for multiple samples):
  - 5X Phusion Buffer (10  $\mu$ l)
  - PCR Primer PE 1.0 (1  $\mu$ l)
  - PCR Primer PE 2.0 (1  $\mu$ l)
  - 25 mM dNTP Mix (0.5  $\mu$ l)
  - Phusion DNA Polymerase (0.5  $\mu$ l)
  - Water (7  $\mu$ l)

The total volume should be 20  $\mu$ l.
2. Add 30  $\mu$ l of purified ligation mix (from step 5 of the previous section) to the 200  $\mu$ l PCR tube.
3. Amplify using the following PCR process:
  - a. 30 seconds at 98°C
  - b. 15 cycles of:
    - 10 seconds at 98°C
    - 30 seconds at 65°C
    - 30 seconds at 72°C
  - c. 5 minutes at 72°C
  - d. Hold at 4°C
4. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample and elute in 30  $\mu$ l of QIAGEN EB buffer.



The protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C.

## Validate the Library

Illumina recommends performing the following quality control analysis on your sample library to quantify the DNA concentration.

1. Load 1  $\mu$ l of the resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA specific chip such the Agilent DNA-1000.

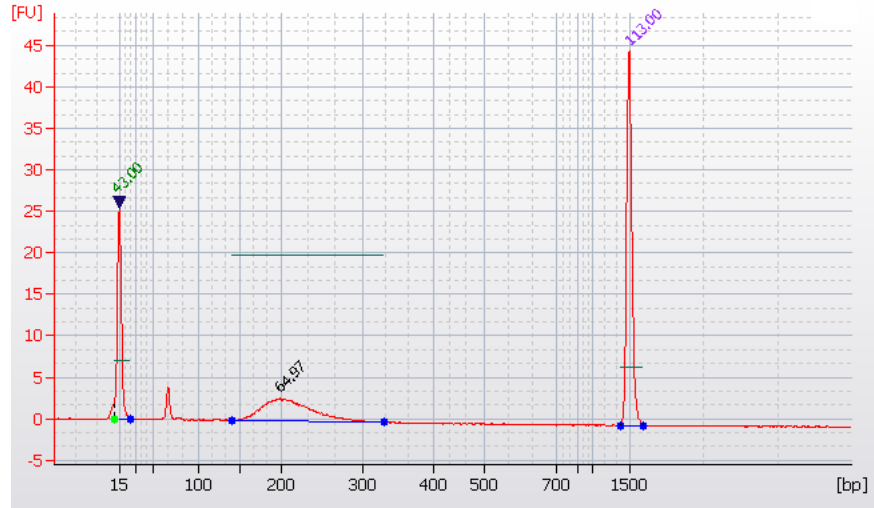


Figure 5 Final mRNA-Seq Library Bioanalyzer Trace

2. Check the size, purity, and concentration of the sample. The final product should be a distinct band at approximately 200 bp.

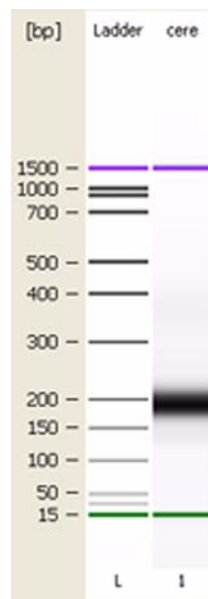


Figure 6 200 bp PCR Product

You can confirm the final product by cloning 1  $\mu$ l of the product into Invitrogen Zero Blunt TOPO vector, and sequence using conventional technology.



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