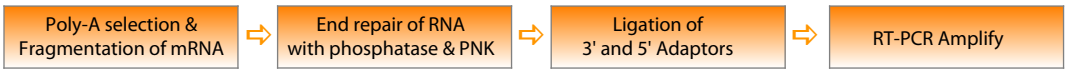
Directional mRNA-Seq Library Prep.
Pre-Release Protocol

Introduction

This protocol explains how to prepare directional, or strand specific, libraries from mRNA. Tech Support may be unable to provide detailed support for this pre-release protocol. The final released product may differ from pre-release protocols.

Libraries prepared by this method are compatible only with single read flowcells. The Small RNA sequencing primer (part # 1001375) and Single Read Sequencing Protocol should be used for the sequencing reactions.

Workflow



Pre-Release Protocol Required Components

Illumina Supplied Components

This protocol requires the Small RNA Sample Prep Kit with the v1.5 sRNA 3' Adaptor:

- (8 samples) FC-102-1009 or (40 samples) FC-102-1010

For mRNA selection and fragmentation, Illumina recommends using components from the mRNA-Seq library prep kit (RS-100-0801). Alternative poly-A selection methods may be used but have not been tested or verified. The output mRNA must be of high quality and free of inhibitors.

User Supplied Supplemental Components

See Small RNA Sample Prep Protocol for standard user supplied components plus the additional components listed below:

- 10 mM ATP (Epicenter Cat. # R109AT or any molecular grade substitute)
- T4 RNA Ligase 2, truncated with 10X T4 RNL2 truncated Reaction buffer (NEB, part # M0242S)
- 100 mM $MgCl_2$ (A 100 mM solution can be prepared from 1M $MgCl_2$ (USB Cat. #78641) or any molecular biology grade substitute)
- RNeasy MinElute Cleanup Kit including Buffers RLT and RPE, (Qiagen Cat. #74204)
  - Centrifugation can be used as an alternative to a vacuum manifold
- Qiagen Buffer EB, (or substitute 10 mM Tris-Cl, pH 8.5, per www.qiagen.com)
- Agencourt AMPure Kit, (BD p/n A29152, Agencourt p/n 000130)
- T4 Polynucleotide Kinase (PNK) with 10X PNK buffer [10 U/µl], (NEB Cat. # M0201S)
- Antarctic Phosphatase with 10X Phosphatase buffer [2 U/µl], (NEB Cat. # M0289S)

Dilution of Oligos & $MgCl_2$

NOTE It is recommended to prepare a fresh dilution of the adapters with each use.

Prepare 1:10 dilution of 10X v1.5 sRNA 3' Adaptor (p/n 15000263)

1. Dilute the 10X v1.5 sRNA 3' Adaptor by mixing 1µl with 9µl nuclease free H_2O.

Prepare 1:5 dilution of the SRA RT primer (p/n 15000597)

1. Dilute the SRA RT primer by mixing 1µl with 4µl nuclease free H_2O.

Prepare 100 mM $MgCl_2$ solution

1. Dilute the 1 M $MgCl_2$ solution by mixing 100 µl with 900 µl nuclease free H_2O

RNA Input Recommendations

NOTE This protocol is designed to start from eukaryotic total RNA and recommends using the poly A selection components from the mRNA-Seq library prep kit. Other mRNA selection methods may be used, provided the final volume is 16 µl.

Standard RNA Input: 1-2 µg Total RNA
--or--
50 ng purified mRNA

Best Practice Recommendations for Magnetic Bead Seperation

NOTE Do not allow the beads to dry during the entire process. During all wash steps, add buffers to the tube containing the beads while the tube is on the magnetic stand.

1. Place the tube containing the beads on the magnetic stand for 1–2 minutes to separate the beads and the buffer.
2. Exchange the buffer using a pipette while the tube is on the magnetic stand.

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

3. Resuspend the beads thoroughly by vortexing.
4. Repeat steps 1 through 3 as required.

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Poly-A Selection of mRNA from Total RNA

Do not allow the beads to dry during the entire process. During all wash steps, add buffers to the tube containing the beads while the tube is on the magnetic stand.

mRNA Purification using Sera-mag oligo(dT) beads

1. Dilute the total RNA with nuclease-free water to 50 µl in a 1.5 ml RNase-free, non-sticky tube.
2. Heat the sample at 65°C for 5 minutes then place the tube on ice.
3. Aliquot 15 µl of Sera-mag oligo(dT) beads into a 1.5 ml RNase-free non-sticky tube.
4. Wash the beads two times with 100 µl of Bead Binding Buffer and remove the supernatant.
5. Resuspend the beads in 50 µl of Bead Binding Buffer and add the 50 µl of total RNA sample from step 2.
6. Aliquot 50 µl of Binding Buffer to a fresh 1.5 ml RNase-free non-sticky Eppendorf tube.
7. Rotate the tube from step 5 at room temperature for 5 minutes and remove the supernatant.
8. Wash the beads twice with 200 µl of Washing Buffer and remove the supernatant.
9. Add 50 µl of 10 mM Tris-HCl to the beads and then heat at80°C for 2 minutes to elute the mRNA from the beads.
10. Immediately put the tube on the magnet stand, transfer the supernatant (mRNA) to the tube from step 6, and add 200 µl of Washing Buffer to the beads.
11. Heat the samples at 65°C for 5 minutes to disrupt the secondary structures and then place the samples on ice.
12. Wash the beads from step 9 twice with 200 µl of Washing Buffer and remove the supernatant.
13. Add 100 µl of the mRNA sample from step 11 rotate it at room temperature for 5 minutes, and remove the supernatant.
14. Wash the beads twice with 200 µl of Washing Buffer and remove the supernatant.
15. Add 17 µl of 10 mM Tris-HCl to the beads and heat at 80°C for 2 minutes to elute the mRNA from the beads.
16. Immediately put the tube on the magnet stand and then transfer the supernatant (mRNA) to a fresh 200 µl thin-wall PCR tube. The resulting amount of mRNA should be approximately 16 µl.

Fragmentation of purified mRNA

mRNA Fragmentation Reaction

1. Mix the following components in the 200 µl PCR tube:
   - 5X fragmentation buffer (4 µl)
   - purified mRNA (16 µl)
2. Incubate the tube in PCR thermal cycler at 94°C for exactly 8 minutes then immediatly transfer to ice and add 2 µl of fragmentation stop solution.

RNeasy Purification of Fragmented mRNA

1. Transfer the sample to a new 1.5 ml eppendorf tube and adjust the volume to 100 µl using nuclease free H_2O. Add 350 µl of RLT buffer and mix well.
2. Add 675 µl of 100% EtOH and mix well by inversion. Immediately proceed to step 3.
3. Load a RNeasy MinElute spin column onto a vacuum manifold. Transfer the entire sample to the spin column.
4. Wash the column with 500 µl of RPE buffer.
5. Wash the column with 750 µl 80% EtOH.
6. Place column in a 2 ml collection tube with the lid open and spin at >10K RPM for 5 min.
7. Transfer the column to a new eppendorf tube. Elute using 17 µl nuclease free H_2O and spin at >10K RPM for 1 minute to elute. The recovered volume should be ~16 µl.

Phosphatase Treatment of Fragmented mRNA

Phosphatase Reaction

1. Mix the following in 200 µl PCR tube containing the fragmented mRNA:
   - Fragmented mRNA (16 µl)
   - 10X phospatase buffer (2 µl)
   - Antarctic phosphatase (1 µl)
   - RNaseOUT (1 µl)
2. Incubate in a thermal cycler with the following protocol:
   - 37°C for 30 minutes
   - 65°C for 5 minutes
   - 4°C indefinite hold

PNK Treatment of Fragmented mRNA

PNK Reaction

1. Add the following components to the 200 µl PCR tube from the previous step:
   - nuclease-free H_2O (17 µl)
   - 10X phosphatase buffer (5 µl)
   - ATP (10 mM) (5 µl)
   - RNaseOUT (1 µl)
   - PNK (2 µl)
2. Incubate in a thermal cycler at 37°C for 60 minutes then 4°C hold.

Column Purification of PNK Treated mRNA

Column Purification Instructions

1. Transfer the sample to a new 1.5 ml eppendorf tube and adjust the volume to 100 µl using nuclease free H_2O. Add 350 µl of RLT buffer and mix well.
2. Add 675 µl of 100% EtOH and mix well by inversion. Immediately proceed to step 3.
3. Load a RNeasy MinElute spin column onto a vacuum manifold. Transfer the entire sample to the spin column.
4. Wash the column with 500 µl of RPE buffer.
5. Wash the column with 750 µl 80% EtOH.
6. Place column in a 2 ml collection tube with the lid open and spin at >10K RPM for 5 min.
7. Transfer the column to a new eppendorf tube. Elute using 18 µl nuclease free H_2O and spin at >10K RPM for 1 minute to elute. The recovered volume should be ~17 µl.
8. Dry down the sample using a speedvac at room temperature. Resuspend in 6 µl H_2O .

Ligate 3' and 5' RNA Adapters

This protocol does not use the Illumina-supplied SRA 3' Adaptor nor the 10X T4 Ligase buffer. Instead, these components are replaced with the diluted v1.5 sRNA 3' Adaptor and the NEB supplied 10X T4 T4 RNL2 truncated buffer.

3' and 5' Adaptor Ligation Reactions

1. Set up the ligation reactions in a sterile, RNase-free 200 µl PCR tube:
   - RNA sample (6 µl)
   - Diluted v1.5 sRNA 3' Adaptor (1 µl)
2. Incubate at 70°C for 2 minutes and store on ice.
3. Add the following reagents:
   - 10X T4 RNL2 truncated Reaction buffer (NEB-supplied) (1 µl)
   - 100 mM $MgCl_2$ (0.8 µl)
   - RNaseOUT (0.5 µl)
4. Mix well and spin down prior to adding enzyme:
   - T4 RNA Ligase 2, truncated (1.5 µl)
5. Incubate at 22°C for 1 hour in a thermal cycler.
6. With 5 minutes remaining in the above incubation, determine the required amount of the 5' adapter from the stock and heat an aliquot at 70°C for 2 minutes then transfer tube to ice.
7. Add the following reagents to the ligation mixture from Step 4 and mix well:
   - 10 mM ATP (1 µl)
   - SRA 5' adaptor (1 µl)
8. Mix well and spin down prior to adding enzyme:
   - T4 RNA ligase (1 µl)
9. Incubate at 20°C for 1 hours in a thermal cycler.

Reverse Transcribe and Amplify the Adapter-ligated RNA

User-supplied Components

- Use 4 µl from the 5' and 3' adapter-ligated RNA reaction mix for the RT reaction
- SuperScript II Reverse Transcriptase with 100 mM DTT and 5X first strand buffer (Invitrogen, part # 18064-014)

Template Preparation

1. Combine the following in a sterile, RNase-free 200 µl PCR tube:
   - 5' and 3' ligated RNA (4.0 µl)
   - Diluted SRA RT primer (1.0 µl)

   The total volume should be 5 µl.
2. Heat the mixture at 70°C in a thermal cycler for 2 minutes.
3. Spin tube to collect and place the tube on ice.

Dilute the 25 mM dNTP Mix

1. Premix the following reagents in a separate, sterile, RNase-free, 200 µl PCR tube. Multiply each volume by the number of samples being prepared. Make 10% extra master mix if you are preparing multiple samples.
   - nuclease-free H_2O (0.5 µl)
   - 25 mM dNTP mix (0.5 µl)

   The total volume should be 1 µl per sample.
2. Label the tube "12.5 mM dNTP Mix."

Prepare the RT Reaction Master Mix

1. Premix the following reagents in the listed order in a separate tube. Multiply each volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.
   - 5X first strand buffer (2 µl)
   - 12.5 mM dNTP (0.5 µl)
   - 100 mM DTT (2.0 µl)
   - RNaseOUT (0.5 µl)
2. Heat to 48°C for 3 minutes then add:
   - Superscript II RT (1 µl)
3. Incubate at 44°C for 60 minutes

Reverse Transcribe and Amplify the Adapter-ligated RNA (cont'd)

Prepare the PCR Reaction Master Mix

1. Premix the following reagents in the listed order in a separate tube. Multiply each volume by the number of samples being prepared. Make 10% extra reagent mix if you are preparing multiple samples.

   - 5X Phusion HF buffer (10 µl)
   - Primer GX1 (1.0 µl)
   - Primer GX2 (1.0 µl)
   - 25 mM dNTP mix (0.5 µl)
   - Phusion DNA Polymerase (0.5 µl)
   - nuclease-free H_2O (27 µl)

   The total volume should be 40 µl per sample.

PCR Amplification

1. Add 40 µl of PCR master mix into a sterile, nuclease free 200 µl PCR tube.
2. Add 10 µl of product from reverse transcription reaction.
3. Amplify the PCR in the thermal cycler using the following PCR protocol:

   a. 30 seconds at 98°C

   b. 12 cycles of:
   10 seconds at 98°C
   30 seconds at 60°C
   15 seconds at 72°C

   c. 10 minutes at 72°C

   d. Hold at 4°C


NOTE Amplification conditions may vary based on RNA input amount, tissue type and species. This protocol is optimized for 1 µg of total RNA from Human Brain; other samples may require further optimization.

Purification of Library Using AMPure Beads

Purification of PCR product : Round 1

1. Add the following to a new non-stick, nuclease-free 1.5 ml eppendorf tube:
   - AMPure bead solution (65 µl)
   - PCR Reaction Mix (50 µl)
2. Vigorously vortex the tube to ensure thorough mixing. Incubate at room temperature for 5 minutes.
3. Place the tube in the magnetic stand for 5-10 minutes to allow complete capture of the beads. After the suspension is completely clear, carefully remove and discard the supernatant. Do not disturb the bead pellet.
4. With the tube on the magnetic stand, carefully rinse the bead pellet with 500 µl of 70% EtOH and discard the supernatant. Do not disturb the beads.
5. With the tube on the magnetic stand, perform a second wash by carefully rinsing the bead pellet with 500 µl of 70% EtOH. Discard the supernatant. Do not disturb the beads.
6. Carefully spin down any remaining EtOH using a mini-centrifuge. Replace the tube in the magnetic stand to capture the beads. Remove any remaining EtOH, taking care to retain the bead pellet.
7. Open the tube lid and transfer to a 37°C heat block for 3 minutes or until the beads are completely dry. Small cracks can be observed in the dried bead pellet surface.
8. Add 30 µl of Qiagen Buffer EB directly to the pellet and soak the beads for 2 minutes. Mix thoroughly using a P20 pipettor to pipette a 20 µl volume up and down 15-20 times. Ensure beads are completely rehydrated and re-suspended.
9. Place the tube in the magnetic stand for at least 2 minute to allow complete capture of the beads. When suspension is clear, transfer the library-containing supernatant to a new tube.

Purification of PCR product : Round 2

1. Add the following to a new non-stick, nuclease-free 1.5 ml eppendorf tube:
   - AMPure bead solution (39 µl)
   - Purified library from Round 1 (30 µl)
2. Vigorously vortex the tube to ensure thorough mixing. Incubate at room temperature for 5 minutes.
3. Place the tube in the magnetic stand for 5-10 minutes to allow complete capture of the beads. After the suspension is completely clear, carefully remove and discard the supernatant. Do not disturb the bead pellet.
4. With the tube on the magnetic stand, carefully rinse the bead pellet with 500 µl of 70% EtOH and discard the supernatant. Do not disturb the beads.
5. With the tube on the magnetic stand, perform a second wash by carefully rinsing the bead pellet with 500 µl of 70% EtOH. Discard the supernatant. Do not disturb the beads.

Purification of Library Using AMPure Beads (cont'd)

Purification of PCR product: Round 2 (cont'd)

6. Carefully spin down any remaining EtOH using a mini-centrifuge. Replace the tube in the magnetic stand to capture the beads. Remove any remaining EtOH, taking care to retain the bead pellet.
7. Open the tube lid and transfer to a 37°C heat block for 3 minutes or until the beads are completely dry. Small cracks can be observed in the dried bead pellet surface.
8. Add 10 µl of Qiagen Buffer EB directly to the pelletand soak the beads for 2 minutes. Mix thoroughly using a P20 pipettor to pipette a 10 µl volume up and down 15-20 times. Ensure beads are completely rehydrated and re-suspended.
9. Place the tube in the magnetic stand for at least 2 minute to allow complete capture of the beads. When suspension is clear, transfer the library-containing supernatant to a new tube.

Characterizing the purified library

- The final purified product can be visualized using an Agilent Bioanalyzer. The product peak should be between 200-250 nucleotides in length.
- The peak size and range is indicative of the purity and quantity of the library.