

eSHAPE *Total RNA*™  
USER GUIDE *In vitro*

# RNA GENOMICS SOLUTIONS

Robust next-gen technologies to simplify  
the complexity of RNA discovery and  
therapeutic development

*This product is for research use only and is not intended for diagnostic or therapeutic uses.*



## Table of Contents

<b>Chapter 1: Overview</b> .....	<b>4</b>
Introduction to SHAPE .....	4
Important Note .....	4
Precautions .....	5
Included with Kit .....	5
Equipment and Reagents Not Included with Kit .....	6
<b>Chapter 2: SHAPE Total RNA in vitro Workflow</b> .....	<b>9</b>
Workflow overview .....	9
<b>Chapter 3: In Vitro NAI Probing of RNA</b> .....	<b>10</b>
Overview .....	10
Consumables .....	10
Procedure .....	10
In Vitro NAI Probing of RNA .....	10
RNA Cleanup of NAI Treated Samples .....	11
<b>Chapter 4: End Repair Protocol</b> .....	<b>12</b>
Overview .....	12
Consumables .....	12
Procedure .....	12
RNA Fragmentation .....	12
RNA End Repair .....	13
Column Cleanup of Repaired RNA .....	13
<b>Chapter 5: 3' End Adapter Ligation</b> .....	<b>15</b>
Overview .....	15
Consumables .....	15
Procedure .....	15
3' RNA Adapter Ligation .....	15
RNA Silane Bead Cleanup .....	16
Reverse Transcription with SHAPE Modifications .....	18
cDNA End Repair .....	19
cDNA Bead Cleanup .....	19
cDNA 3' Adapter Ligation on Beads .....	21



<b>Chapter 6: Library Amplification and Preparation for Sequencing</b> .....	<b>22</b>
Overview.....	22
Consumables.....	22
Procedure.....	22
cDNA Bead Cleanup.....	22
Sample Quantification by qPCR.....	24
PCR Amplification with Index Primer Addition.....	25
AMPure Bead Library Cleanup.....	27
Library Quantification.....	28
Pool and Sequence Final Library.....	28
<b>Appendix 1: Zymo Cleanup For Small RNAs (&lt;200 nt)</b> .....	<b>29</b>
<b>Adapter and primer sequences</b> .....	<b>39</b>



# Chapter 1: Overview

## Introduction to SHAPE *Total RNA*

---

The SHAPE *Total RNA* Kit provides an efficient workflow to obtain RNA nucleotide flexibility at *in vitro* conditions. Folded RNA molecules are probed with NAI (*2-methylnicotinic acid imidazolid*), a structure probing agent, to create chemical adducts at the 2'OH in the backbones of unpaired nucleotides. These adducts induce mutations in the cDNA library during reverse transcription. After sequencing, the positions of these mutations are analyzed using bioinformatics tools to deduce nucleotide flexibility, which is used to guide the folding of RNA molecules.

The SHAPE *Total RNA* Kit offers:

- **High throughput and robust workflow**
- **High reproducibility with accurate data**
- **Unbiased with high specificity**

### Important Note

Before using the procedures in this guide, review the required equipment and materials list of contents of the kit and list of materials and equipment not provided with the kit.

This procedure is to be followed by trained lab personnel.

Term	Temperature
Room Temperature	20 – 25 °C (68 – 77 °F)
Ice	0 – 4 °C
Freeze	-80 °C
Volume units	
<b>μL</b> (or ul) is microliter	One millionth ( $10^{-6}$ ) of a liter
<b>mL</b> (or ml) is milliliter	One thousandth ( $10^{-3}$ ) of a liter



## Precautions

This kit contains chemicals which can be hazardous. Enzyme buffers contain reducing agents and nucleotide solutions. Personal protection equipment (PPE) should be worn during the entirety of this procedure.

- Use 1.5 mL DNA LoBind tubes (Eppendorf) or 0.2 mL DNA/RNase free strip tubes during all steps.
- During beads washing, ensure tubes are completely closed.
- Beads used in nucleic acid cleanup steps should be completely dried before elution.
- Store all reagents on ice between steps unless otherwise indicated.
- When not in use, store all reagents at temperature indicated in “Day x Reagents” section.
- Use only calibrated pipettes. An additional 3% volume is recommended and listed for all master mix calculations to account for volume inaccuracy.
- TipOne® RPT Ultra Low Retention Filter Tips from USA Scientific are strongly recommended for entire experiment.
- Always completely resuspend beads before taking aliquot or adding beads to sample.
- All Thermomixer incubations are done with interval mixing (15 seconds ON/15 seconds OFF).
- Store all enzymes at  $-20^{\circ}\text{C}$ .
- This protocol has been tested with 50ng to 500ng of starting RNA.

*IMPORTANT: Materials listed below are for SHAPE Total RNA experimental set up ONLY. See Appendix for supplemental information.*

## Included with Kit

Item	Storage
Bead Binding Buffer	4°C
eCLIP Beads	4°C
RNase Inhibitor Enzyme	-20°C
DNase Enzyme	-20°C
PSP Enzyme	-20°C
SHAPE RT Enzyme	-20°C



Nuclease Enzyme	-20°C
PNK Enzyme	-20°C
Ligase Enzyme	-20°C
PCR Mix	-20°C
ssDNA Enzyme	-20°C
NAI, 2M	-20°C
DMSO	-20°C
100 mM DTT	-20°C
Index Primers (8)	-80°C
PSP Buffer	-80°C
Bead Elution Buffer	-80°C
RNA Ligation Buffer	-80°C
ssDNA Ligation Buffer	-80°C
SHAPE PNK Buffer	-80°C
SHAPE Folding Buffer	-80°C
10X FS SHAPE Buffer	-80°C
SHAPE RNA Adapter	-80°C
ssDNA Adapter	-80°C
RT Primer	-80°C
qPCR Primers	-80°C

### Equipment and Reagents Not Included with Kit

Item	Source
Micro-centrifuge 5424R or equivalent	Eppendorf
PolyA Selection Module	Eclipsebio
rRNA Depletion Module	
Mini-centrifuge or equivalent	Corning LSE
Tube Rotator	VWR cat. #10136-084
T100 Thermal Cycler or equivalent	BioRad cat. #1861096



StepOne qPCR or equivalent	ThermoFisher Scientific cat. #4376357
Eppendorf Thermomixer C	Eppendorf cat. #5382000015
DynaMag-2 Magnet	ThermoFisher Scientific cat. #12321D
MagWell™ Magnetic Separator 96 or DynaMag-96 Side Magnet	EdgeBio, cat. #57624 ThermoFisher Scientific cat. #12331D
Aluminum Cool Block	Diversified Biotech cat. #CHAM1000
Reagent Reservoirs	ThermoFisher cat. #95128093
0.2 mL PCR 8-tube strip with 8-cap strips	VWR cat. #20170-004
1.5 mL DNA LoBind Micro- centrifuge tubes	Eppendorf cat. #022431021
MicroAmp Fast Optical 96-well reaction plate or equivalent	ThermoFisher cat. #4346906
Falcon 15 mL Conical Centrifuge tubes	Fisher Scientific cat. #14-959-53A
Falcon 50 mL Conical Centrifuge tubes	Fisher Scientific cat. #14-432-22
HiScribe T7 High Yield RNA Synthesis Kit	New England BioLabs cat. #E2040S
Monarch RNA Cleanup Kit (500 µg)	New England BioLabs cat. #T2050S
RNA Clean & Concentrator -5	Zymo Research cat. #R1015/R1016



Item	Source
Ethanol, Pure, 200 proof, for Molecular Biology	Sigma-Aldrich cat. #E7023-1L
Nuclease-free Molecular Biology Grade Water or UltraPure™ DEPC-Treated Water	Corning/VWR cat. #95000-094 ThermoFisher Scientific cat. #750023
EDTA (0.5 M), pH 8.0, RNase-free	ThermoFisher Scientific cat. #AM9261
1M Sodium Hydroxide solution (NaOH)	Sigma-Aldrich cat. #79724-100ML
1M Hydrogen Chloride (HCl)	Any
Agencourt AMPure XP	Beckman Coulter cat. #A63881
NEB LUNA Universal qPCR 2× Master Mix	New England BioLabs cat. #M3003S

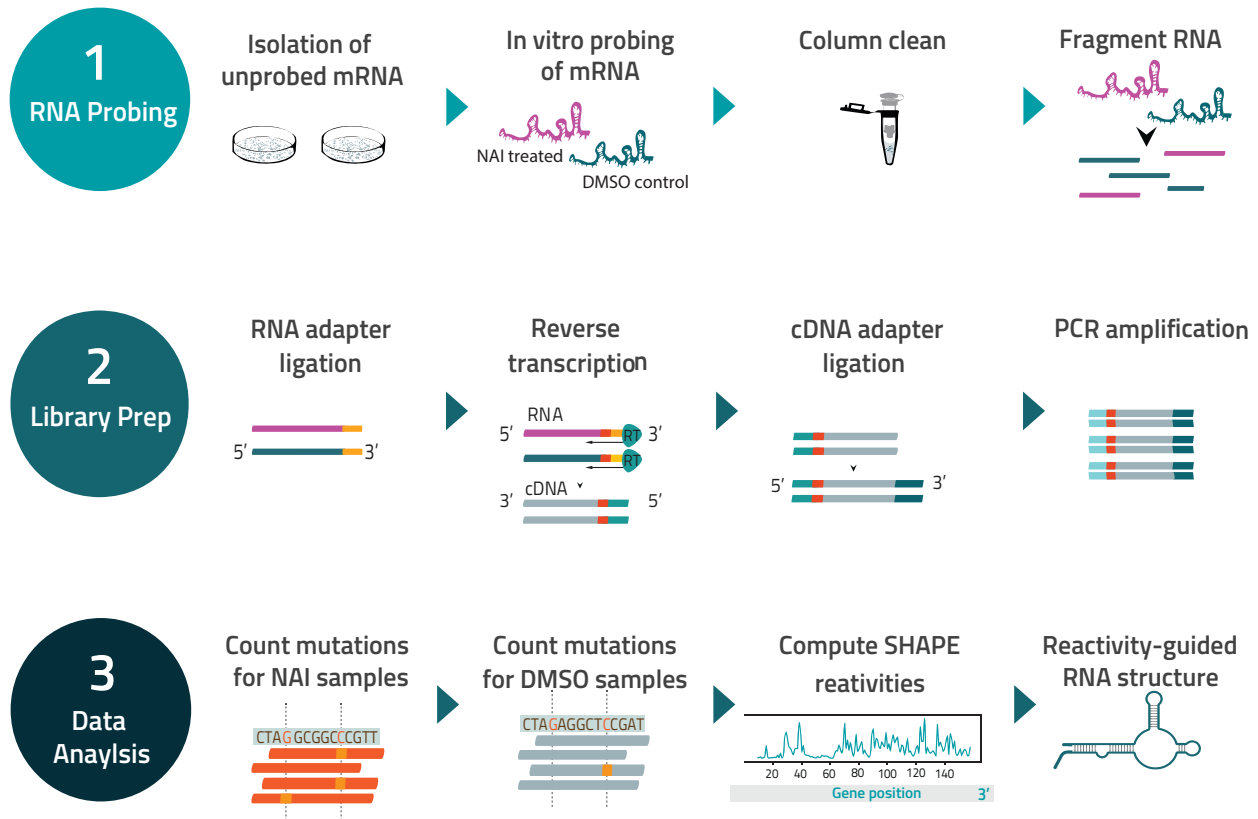




# Chapter 2: SHAPE *Total RNA* Workflow

## Overview

The SHAPE *Total RNA* protocol is outlined below, including steps for RNA probing, library preparation and the data analysis portion, which is handled separately from this protocol. The wet lab portions of this protocol take about 3 days to complete, with about 2.5 hours spent on RNA probing.



## Chapter 3: *In Vitro* NAI Probing of RNA

---

### Overview

The SHAPE Total RNA protocol is compatible with total RNA, mRNA or rRNA depleted RNA. We recommend performing either polyA selection or rRNA depletion to avoid rRNA contamination in your sequencing libraries. Appendix 1 describes the process of isolating mRNA from total RNA using the Eclipsebio polyA selection module (sold separately). If you plan to conduct rRNA depletion in place of polyA selection, we recommend selecting a rRNA depletion kit with a large total RNA capacity like the riboPOOL kit from siTOOLS BioTech. For both polyA selection and rRNA depletion you need to start with about 5-6 ug total RNA. This chapter describes the linearization and re-folding of RNA molecules. RNA molecules are then treated with either NAI or DMSO control. The NAI reagent forms adducts with the 2'OH in the RNA backbone of unpaired bases found in structurally flexible regions. These adducts result in mutations which can be used to infer the structure of an RNA molecule.

### Consumables

- SHAPE Folding Buffer
- NAI, 2M
- DMSO
- Molecular Biology Grade Water

### Isolation of mRNA from total RNA

See Appendix 1 for mRNA isolation using oligodT beads.

### *In Vitro* NAI Treatment of RNA

1. Aliquot 100 ng RNA to new 0.2 mL strip tubes. Place and keep samples on ice.
2. Add **molecular biology grade water** up to a total volume of 12  $\mu$ L for each sample.
3. Place samples in a thermocycler at 95°C for 2 minutes. Place and keep samples on ice.
  - **Note!** If you prefer to not denature and re-fold your RNA, add 6  $\mu$ L of **SHAPE Folding Buffer** to each sample and skip straight to step 6 without incubating the RNA at 95°C.
4. Immediately proceed to next step.
5. Add 6  $\mu$ L of **SHAPE Folding Buffer** to each sample. Pipette to mix.
6. Incubate samples in thermocycler at 37°C for 20 minutes. Place and keep samples on ice.



7. Immediately proceed to next step.
8. Add 9  $\mu\text{L}$  of **2M NAI** to each sample (or 9  $\mu\text{L}$  of **DMSO** for control samples). Pipette to mix.
  - **Note!** NAI is dissolved in DMSO and takes a while to thaw completely. Before using NAI, we recommend pipetting the entire amount into a pipette tip to visually confirm that there are no undissolved crystals in the solution.
9. Incubate samples (~27  $\mu\text{L}$  total volume) in a thermocycler at 37°C for 15 minutes. Place and keep samples on ice.
10. Add 23  $\mu\text{L}$  **molecular biology grade water** to each sample for a total volume of 50  $\mu\text{L}$ .
11. **Immediately** proceed to the next step.

### RNA Cleanup of NAI Treated Samples

This chapter uses the Zymo RNA Clean and Concentrator-5 kit to clean RNA and the reagents for this can be found in the Zymo RNA Clean and Concentrator-5 kit.

1. Add 100 $\mu\text{L}$  of **RNA Binding Buffer** to the 50 $\mu\text{L}$  of each RNA sample. Pipette mix.
2. Add 150 $\mu\text{L}$  of **100% EtOH**, pipette 10 times to mix.
3. Transfer the entire sample to a new filter column placed in a collection tube.
4. Centrifuge at 7,000  $\times$  g for 30 seconds. Discard flow-through.
5. Add 400 $\mu\text{L}$  **RNA Prep Buffer** to each column.
6. Centrifuge at 7,000  $\times$  g for 30 seconds. Discard flow-through.
7. Add 480 $\mu\text{L}$  **RNA Wash Buffer** to each column.
8. Centrifuge at 7,000  $\times$  g for 30 seconds. Discard flow-through.
9. Repeat step 7-8 for a total of two washes.
10. Centrifuge the column at 10,000  $\times$  g for 3 minutes with emptied collection tube.
11. Carefully transfer filter column to a new 1.5mL LoBind tube (avoid liquid in collection tube).
12. Discard flow-through and collection tube.
13. Open column caps and allow to air dry for 2 minutes or until column is completely dry.
14. Elute all samples by adding 11 $\mu\text{L}$  of **molecular biology grade water** directly to filter.
15. Incubate at room temperature for 1 minute.
16. Centrifuge at 12,000  $\times$  g for 90 seconds.
17. Place RNA samples on ice if continuing to the next step.



---

**Optional Stopping Point:** *If stopping here, RNA samples should be stored at -80 °C*

*Next stopping point: 2 hours*

---



# Chapter 4: End Repair Protocol

---

## Overview

RNA is fragmented using heat to generate the appropriate lengths for library preparation. RNA is dephosphorylated in preparation for adapter ligation. An RNA adapter is then ligated to the 3' end of the RNA.

## Consumables

- PSP Buffer
- PSP Enzyme
- RNase Inhibitor
- SHAPE PNK Buffer
- DNase enzyme
- PNK Enzyme
- Bead Elution Buffer

## RNA Fragmentation

1. Aliquot all eluted RNA (~9  $\mu$ L) to a new 0.2 mL strip tube. Place and keep samples on ice.
2. Add 8  $\mu$ L of **PSP Buffer** to each tube of RNA.
3. Incubate samples in thermocycler (starting from room temperature, not pre-heated) at 95°C for 2 minutes and 30 seconds, with lid set to 98°C. Place and keep samples on ice.

## RNA End Repair

1. Prepare **PSP Master Mix** for each sample according to **Table 1**, on ice.

**Table 1. PSP Master Mix**

Component	Volume ( $\mu$ L)
RNase Inhibitor	1
PSP Enzyme	2
<b>Total:</b>	<b>3</b>

2. Add 3  $\mu$ L of **PSP Master Mix** to each fragmented RNA sample.



3. Mix by flicking then spin in mini-centrifuge for 3 seconds.
4. Incubate in thermocycler at 37°C for 10 minutes. Place and keep samples on ice.
5. Prepare **PNK Master Mix** for each sample according to **Table 2**, on ice.

**Table 2. PNK Master Mix**

Component	Volume (µL)
SHAPE PNK Buffer	70
DNase enzyme	1
PNK Enzyme	4
<b>Total:</b>	<b>75</b>

6. Add 75 µL of **PNK Master Mix** to each tube containing 20 µL of sample.
7. Mix by flicking, then spin in mini-centrifuge for 3 seconds.
8. Incubate in thermocycler at 37°C for 20 minutes.

### Column Cleanup of Repaired RNA: 200 nt Cutoff Protocol

This chapter uses the Zymo RNA Clean and Concentrator-5 kit to clean RNA and the reagents for this can be found in the Zymo RNA Clean and Concentrator-5 kit.

1. Add 1 sample volume (100 µL) of **RNA Binding Buffer, per sample**, to a new 15 mL conical tube.
2. Add 1 sample volume (100 µL) of **100% EtOH, per sample**, to the same 15 mL conical tube. Mix by pipetting.
3. Add 190 µL of the **RNA Binding Buffer + 100% EtOH** mixture to each sample. Mix by pipetting.
4. Transfer entire sample to a new filter column placed in a collection tube.
5. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
6. Add 400 µL **RNA Prep Buffer** to each column.
7. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
8. Add 480 µL **RNA Wash Buffer** to each column
9. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
10. Repeat steps 8-9 for a total of two washes.
11. Centrifuge column at 12,000 x g for 3 minutes with emptied collection tube.



12. Carefully transfer filter column to a new 1.5 mL LoBind tube (avoid liquid in collection tube).
13. Discard flow-through and collection tube.
14. Open column caps and allow to air dry for 2 minutes or until column is completely dry.
15. Elute all samples by adding 8  $\mu$ L of **Bead Elution Buffer** directly to filter.
16. Incubate at room temperature for 1 minute.
17. Centrifuge at 12,000 x g for 90 seconds.
18. Place and keep RNA samples on ice if continuing to next step.

---

**Optional Stopping Point:** *If stopping here, RNA samples should be stored at -80*  
*Next stopping point: ~1.5 hours*

---



# Chapter 5: 3' End Ligation and Reverse Transcription

---

## Overview

This section describes 3' RNA adapter ligation and reverse transcription, followed by the removal of dNTPs and template RNA. ssDNA samples are then cleaned and overnight adapter ligation is performed.

## Consumables

- SHAPE RNA Adapter
- RNA Ligation Buffer
- RNase Inhibitor
- Ligase
- RT Primer
- SHAPE RT Buffer
- MTL
- 100mM DTT
- SHAPE RT Enzyme
- Nuclease
- 0.5M EDTA (pH 8.0)
- 1M NaOH
- 1M HCl
- eCLIP Beads
- Bead Binding Buffer
- Bead Elution Buffer
- 80% and 100% Ethanol
- Molecular Biology Grade Water

## 3' RNA Adapter Ligation

1. Add the **repaired RNA** from the previous elution step (~5-6 uL) to new 0.2 mL strip tubes and place on ice.
2. Add 2  $\mu$ L of **SHAPE RNA Adapter** to each RNA tube. Pipette mix.
3. Spin samples in mini-centrifuge for 5 seconds to draw all liquid to bottom of the tube.
4. Incubate tubes at 65°C for 2 minutes in thermocycler with lid preheated to 70°C.





5. Immediately place tubes on ice, keep samples on ice.
6. Prepare **Ligation Master Mix** for each sample according to **Table 1**:

**Table 1. Ligation Master Mix**

Component	Volume (µL)
RNA Ligation Buffer	12
RNase Inhibitor	0.3
Ligase	1.2
<b>Total:</b>	<b>13.5</b>

7. Add 13.5 µL of **Ligation Master Mix** to each sample RNA sample with adapter. Pipette mix until homogeneous.
8. Spin samples in mini-centrifuge for 5 seconds to draw all liquid to bottom of the tube.
9. Incubate samples for 1 hour at room temperature on tube rotator.

### RNA Silane Bead Cleanup

1. Take **eCLIP Beads** out of 4 °C and resuspend until homogeneous.
2. For each sample, transfer 10 µL **eCLIP Beads** to a new 1.5 mL DNA LoBind tube (e.g., for 2 samples transfer 20 µL of eCLIP beads to tube).
3. Add 5x volume of **Bead Binding Buffer** to beads (e.g., for 2 samples, transfer add 100 µL buffer to 20 µL of eCLIP beads). Pipette mix until sample is homogeneous.
4. Place tube on DynaMag-2 magnet. When separation is complete and supernatant is clear, carefully aspirate and discard supernatant without disturbing beads.
5. Remove tube from magnet and resuspend **eCLIP Beads** in 63 µL **Bead Binding Buffer** per sample.
6. Add 60 µL of washed **eCLIP Beads** to each tube of 20 µL ligated RNA sample. Pipette mix until sample is homogeneous.
7. Add 45 µL **100% EtOH** to each sample. Pipette mix until homogeneous.
8. Incubate at room temperature for 10 minutes with pipette mixing every 5 minutes.
9. Place tubes on magnet for 30 seconds or until separation is complete and supernatant is transparent.



10. Carefully aspirate and discard supernatant without disturbing beads.
11. Add 200  $\mu$ L of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet to wash (~30 seconds).
12. Carefully discard supernatant without disturbing beads.
13. Repeat steps 11-12 for a total of 3 washes.
14. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
15. Place tube back on 96-well magnet.
16. Incubate on magnet until for 10 seconds or until separation is complete and supernatant is transparent.
17. Aspirate and discard all residual liquid without disturbing beads while on magnet
18. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
  - **Note:** Do not over-dry samples.
19. Once completely dry, carefully remove tube from magnet.
20. Add 11  $\mu$ L of **Bead Elution Buffer** to each sample.
21. Pipette mix up and down until sample is homogeneous.
22. Incubate for 5 minutes at room temperature.
23. After incubation, move tubes to 96-well magnet. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
24. While on magnet, carefully transfer entire sample (~9  $\mu$ L) from beads to new 0.2 mL strip tubes. Discard beads.
25. Place samples on ice if continuing to next step.

---

**Optional Stopping Point:** *If stopping here, RNA samples should be stored at -80 °C*

*Next stopping point: ~5 hours*

---



## Reverse Transcription with SHAPE Modifications

1. Add 1.5  $\mu\text{L}$  of **RT Primer** to each ligated RNA sample.
2. Flick to mix and spin samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
3. Incubate in thermocycler at 65°C for 2 minutes with lid preheated to 70°C.
4. After incubation, immediately transfer to ice for 1 minute.
5. Prepare **Reverse Transcription Master Mix** for each sample according to **Table 2** in a new 1.5 mL LoBind DNA tube.

**Table 2. Reverse Transcription Master Mix**

Component	Volume ( $\mu\text{L}$ )
SHAPE RT Buffer	2
MTL	0.24
100 mM DTT	1
RNase Inhibitor	0.4
Molecular Biology Grade Water	5.76
SHAPE RT Enzyme	0.6
<b>Total:</b>	<b>10</b>

6. Pipette up and down 10 times to mix. Liquid should turn light brown. Store on ice.
7. Add 10  $\mu\text{L}$  of the **Reverse Transcription Master Mix** to each sample while on ice.
8. Close strip tubes well and flick to mix.
9. Spin samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
10. Immediately incubate samples in thermocycler at **45°C for 3 hours** with lid preheated to 60°C.
11. After incubation, place samples on ice and proceed immediately to the next step.

## cDNA End Repair

1. Add 2.5  $\mu\text{L}$  of **Nuclease** to each sample.
2. Flick tubes to mix, spin down briefly on mini-centrifuge.
3. Incubate in thermocycler at 37°C for 15 minutes with lid at 45°C.



4. Place samples on ice.
5. Add 1  $\mu\text{L}$  **0.5M EDTA** (pH 8) to each sample.
6. Pipette to mix.
7. Add 3  $\mu\text{L}$  **1M NaOH** to each sample.
8. Pipette to mix.
9. Incubate in thermocycler at 70°C for 10 minutes with lid at 75°C.
10. Place on ice for 2 minutes.
11. Add 3  $\mu\text{L}$  of **1M HCl** to each sample.
12. Proceed directly to next step.

### cDNA Bead Cleanup

- **Preparation Note:** Thaw ssDNA Adapter and ssDNA Ligation Buffer at room temperature until completely melted then store ssDNA Adapter on ice and ssDNA Ligation Buffer at room temperature.
1. Take **eCLIP Beads** out of 4°C and resuspend until homogeneous.
  2. For each sample, transfer 7  $\mu\text{L}$  **eCLIP Beads** to a new 1.5 mL DNA LoBind tube (e.g., for 2 samples transfer 14  $\mu\text{L}$  of eCLIP beads to tube).
  3. Add 5x volume of **Bead Binding Buffer** to beads (e.g., for 2 samples, transfer add 70  $\mu\text{L}$  buffer to 14  $\mu\text{L}$  of eCLIP beads). Pipette mix until sample is homogeneous.
  4. Place tube on DynaMag-2 magnet. When separation is complete and supernatant is clear, carefully aspirate and discard supernatant without disturbing beads.
  5. Remove tube from magnet and resuspend **eCLIP Beads** in 90  $\mu\text{L}$  **Bead Binding Buffer** per sample.
  6. Add 87  $\mu\text{L}$  of washed **eCLIP Beads** to each tube of ~30  $\mu\text{L}$  ligated cDNA sample. Pipette mix until sample is homogeneous.
  7. Add 68  $\mu\text{L}$  **100% EtOH** to each sample. Pipette mix until homogeneous.
  8. Transfer samples to new 0.2 mL strip tubes and incubate at room temperature for 10 minutes with pipette mixing every 5 minutes. It is important to transfer to new strip tubes at this point to avoid beads sticking to the side of the tube.
  9. Place tubes on magnet for 30 seconds or until separation is complete and supernatant is transparent.



10. Carefully aspirate and discard supernatant without disturbing beads.
11. Add 200  $\mu\text{L}$  of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet to wash (~30 seconds).
12. Carefully discard supernatant without disturbing beads.
13. Repeat steps 11-12 for a total of 3 washes.
14. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
15. Place tube back on 96-well magnet.
16. Incubate on magnet until for 10 seconds or until separation is complete and supernatant is transparent.
17. Aspirate and discard all residual liquid without disturbing beads while on magnet
18. Allow beads to air dry for ~5 minutes or until beads no longer appear wet and shiny.
  - **Note:** Do not over-dry samples.
19. Once completely dry, carefully remove tube from magnet.
20. Resuspend beads in 2.5  $\mu\text{L}$  of **ssDNA Adapter**.
21. Pipette mix up and down until sample is homogeneous.
22. Incubate in thermocycler at 70°C for 2 minutes with lid at 75°C.
23. After incubation, immediately place on ice for 1 minute.

### **cDNA 3' Adapter Ligation on Beads**

1. Prepare **cDNA Ligation Master Mix** according to Table 3 in a new 1.5 mL LoBind DNA tube. Flick tube to mix (do not vortex). Use immediately.

**Table 3. cDNA Ligation Master Mix**

<b>Component</b>	<b>Volume (<math>\mu\text{L}</math>)</b>
ssDNA Ligation Buffer	6.5
Ligase	1
ssDNA Enzyme	0.3
<b>Total:</b>	<b>7.8</b>



2. Slowly add 7.8  $\mu$ L of **cDNA Ligation Master Mix** to each sample (beads and ssDNA Adapter). Pipette mix until homogeneous.
3. Incubate at room temperature **overnight** on a tube rotator.

---

**Stopping Point:** Ligation **overnight**

---



# Chapter 6: Library Amplification and Preparation for Sequencing

---

## Overview

This section describes PCR amplification of cDNA. Samples that ligated overnight are first cleaned using eCLIP beads, then qPCR is run to determine the number of cycles needed for amplification, followed by PCR amplification of all samples. Lastly, amplified libraries are cleaned using AMPure beads and eluted in water for subsequent pooling and sequencing.

## Consumables

- Bead Elution Buffer
- Bead Binding Buffer
- NEB Luna Universal qPCR 2x Master Mix
- qPCR Primers
- 50 (5,6,7,8) Index Primer
- 70 (5,6,7,8) Index Primer
- PCR Mix
- AMPure Beads
- 100% Ethanol
- 80% Ethanol
- Molecular Biology Grade Water

## cDNA Bead Cleanup

1. Add 5  $\mu\text{L}$  of **Bead Elution Buffer** to each 10  $\mu\text{L}$  adapter-ligated cDNA sample. Pipette mix until homogeneous.
2. Add 45  $\mu\text{L}$  of **Bead Binding Buffer** to each sample. Pipette mix.
3. Add 31  $\mu\text{L}$  of **100% EtOH** to each sample. Pipette mix until homogeneous.
4. Transfer samples to new 0.2 mL strip tubes and incubate at room temperature for 10 minutes with pipette mixing every 5 minutes.
5. Move samples to 96-well magnet.
6. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.



7. Carefully discard supernatant without disturbing beads.
8. Add 200  $\mu\text{L}$  of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet to wash.
9. Carefully discard supernatant without disturbing beads.
10. Repeat steps 8-9 for a total of 3 washes.
11. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
12. Place tube back on 96-well magnet.
13. Incubate on magnet until for 10 seconds or until separation is complete and supernatant is transparent.
14. Aspirate and discard all residual liquid without disturbing beads while on magnet
15. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
  - **Note:** Do not over-dry samples
16. Once completely dry, carefully remove tube from magnet.
17. For each sample, resuspend in 21  $\mu\text{L}$  of **Bead Elution Buffer**.
18. Pipette mix up and down until sample is homogeneous.
19. Incubate for 5 minutes at room temperature.
20. After incubation, move tubes to 96-well magnet. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
21. While on magnet, carefully transfer entire sample (~20  $\mu\text{L}$ ) from beads to new 0.2 mL strip tubes. Discard beads.
22. Place samples on ice if continuing to next step.

---

**Safe Stopping Point:** *samples can be frozen and stored until next use.*

---





## Sample Quantification by qPCR

1. Prepare the **qPCR Master Mix** for the appropriate number of reactions according to **Table 1**.  
x
2. Label a 96- or 384-well qPCR reaction plate (see **Table 2** for suggested 96-well layout).

**Table 2. 96-well qPCR plate layout for 21 samples**

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	water	water	water	water	water	water	water	water	water	water	water	
C	water	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Neg Control	water	
D	water	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Neg Control	water	
E	water	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Neg Control	water	
F	water	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Neg Control	water	
G	water	water	water	water	water	water	water	water	water	water	water	
H												

*Note: We recommend running each sample in biological duplicate. Negative controls use water in place of cDNA.*

3. **Dilute cDNA for qPCR** 10-fold: in a new 0.2 mL strip tube, mix **9 µL of molecular biology grade water** and **1 µL of sample**.
4. Add 9 µL of **qPCR Master Mix** into all assay wells on ice.
5. Add 1 µL of each diluted cDNA (or Bead Elution Buffer for negative controls) into the designated well.
  - **Note:** Store remaining diluted cDNA samples at -20°C.
6. Cover plate with MicroAmp adhesive film and seal with film applicator.
7. Mix, then spin plate at 3,000 x g for 1 minute.
8. Run qPCR assay according to the user manual for the specific instrument in your laboratory.
9. Run parameters appropriate for SYBR.
10. Note: For example, for the StepOnePlus qPCR system the appropriate program is:



95°C – 30 sec

(95°C – 10 sec, 65°C – 30 sec) × 32 cycles; No melting curve

11. Record qPCR Ct values for all sample.

- **Set threshold to 0.5** – this recommendation is for StepOnePlus System. Typical acceptable Ct values range from 10 to 23. For robust estimation, Ct values for samples should be ≥ 10. If values are below 9, dilute the 1:10 diluted cDNA an additional 10-fold, and re-perform qPCR using the 1:100 diluted cDNA.

### PCR Amplification with Index Primer Addition

1. Thaw Index primers at room temperature until fully melted. Shake to mix and spin in mini-centrifuge for 5 seconds. Store on ice until use.
2. Prepare **PCR Amplification Reaction Mix** according to **Table 3** in new 0.2 mL strip-tubes.
  - **Note:** If samples are going to be multiplexed during high-throughput sequencing, ensure that all samples to be pooled together have a unique combination of indexing primers.

**Table 3. PCR amplification reaction mix contents (prepare individually for each sample)**

Component	Volume (µL)
Ligated cDNA	16
50(5,6,7,8) Index Primer	2
70(5,6,7,8) Index Primer	2
PCR mix	20
<b>Total volume</b>	<b>40</b>

3. Close strip tubes, pipette mix with multi-channel. Spin samples for 5 seconds to draw all liquid to the bottom of the tube. Keep on ice and quickly proceed to next step.
4. Refer to Ct values recorded to calculate the appropriate number of cycles for each sample according to **Table 13**. Use formula to calculate  $N = Ct - 9$ , where N is the number of cycles performed using the second (two-step) cycling conditions.

- **PCR Cycle Calculation:**

Total cycles = Ct – 3 (adjusting for 1:10 dilution of cDNA)



$$N = \text{Total cycles} - 6 = Ct - 9$$

TOTAL number of PCR cycles for final library amplification = 6+N.

- **Note:** e.g. If Ct = 13.1, then N = 4 and Total number of PCR cycles equal 10 (6+4).
- **Note:** Arrange PCR tubes such that reactions with equal *Total Cycles* are together. Remove tubes after Total Cycle numbers have been reached. If using this method, be sure to remove tubes before transition from 72°C to 95°C to avoid denaturation of amplified library.

**Table 13. PCR amplification program**

Temperature	Time	Cycles
98°C	30 seconds	
98°C	15 seconds	<b>6</b>
70°C	30 seconds	
72°C	40 seconds	
<b>Extra N cycles (N = Ct value - 9)</b>		
98°C	15 seconds	<b>N*</b>
72°C	45 seconds	
72°C	1 minute	
4°C	∞	
Total number of PCR cycles		6+N

\*N should be ≥ 1 and < 14.

- Run PCR for the specific number of cycles calculated for each sample.
- Immediately put samples on ice to cool following PCR amplification.

### AMPure Bead Library Cleanup

1. Allow **AMPure XP beads** (not provided) to equilibrate at room temperature for 5 minutes.
2. Manually shake **AMPure XP beads** to resuspend until homogeneous.



3. Add 64  $\mu\text{L}$  of **AMPure XP beads** to each 40  $\mu\text{L}$  PCR reaction.
4. Pipette up and down until sample is homogeneous.
5. Transfer samples to new 0.2 mL strip tubes and incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
6. Move samples to 96-well magnet.
7. Incubate until separation is complete and supernatant is transparent.
8. Carefully aspirate and discard supernatant without disturbing beads.
9. Add 200  $\mu\text{L}$  of **80% EtOH** to wash beads.
10. Incubate on magnet for at least 30 seconds until supernatant is transparent.
11. Aspirate and discard all supernatant.
12. Repeat steps 9 - 11 for a total of two washes.
13. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
14. Place tube back on 96-well magnet.
15. Incubate on magnet for 10 seconds until supernatant is transparent.
16. Aspirate and discard all residual liquid without disturbing beads.
17. Allow beads to air dry for 3 minutes or until beads no longer appear wet and shiny.
18. Once completely dry, carefully remove tubes from magnet.
19. For each sample, resuspend beads in 20 $\mu\text{L}$  of **Molecular Biology Grade water**.
20. Pipette up and down to mix until sample is homogeneous.
21. Incubate 5 minutes at room temperature.
22. After incubation, move tubes to 96-well magnet.
23. Incubate on magnet for 1 minute until supernatant is transparent.
24. Transfer 20  $\mu\text{L}$  of sample to a new 0.2 mL strip-tube.
25. Store samples on ice if proceeding to the next step.

---

**Optional Stopping Point:** *If stopping here, libraries should be quickly frozen at  $-80^{\circ}\text{C}$*

*Next stopping point: ~20 minutes or ~2 hrs if pooling immediately*

---

## Library Quantification



Libraries can be quantified using a variety of methods. Protocol has been optimized using Agilent4200 TapeStation and a D1000 tape kit, which quantifies both library concentration, molarity and size distribution. See Agilent4200 TapeStation manual for operation instructions.

### **Pool and Sequence Final Library**

Final library pooling concentration and conditions are typically set by high-throughput sequencing provider. At Eclipsebio we typically pool these samples at equimolar rates and clean the pool using 1X AMPure XP beads. See provider information or Illumina website for additional details, especially for sequencing color-balancing.

---

***Protocol End***

---



# Appendix 1: mRNA (polyA RNA) selection from total RNA

---

## Overview

This section describes poly(A) selection from total RNA. Here, mRNA is enriched by double Oligo(dT) capture to select for polyadenylated transcripts. The expected yield of polyA RNA is ~1-2% of the total RNA. Keep in mind that the maximum volume for RNA fragmentation (the first step after mRNA selection is 8  $\mu$ L per 100 ng mRNA. Adjust the elution volume accordingly.

## Consumables

- Molecular Biology Grade Water
- Oligo(dT) beads
- 2 $\times$  Hybridization Buffer (HyB) (Thaw at room temperature then store on ice)
- mRNA Elution Buffer (Thaw at room temperature then store on ice)

## Preparation

1. Centrifugation steps are done at room temperature.
2. Prewarm thermomixer to 60  $^{\circ}$ C.

## Procedure

### Poly(A)-RNA Isolation

1. Per sample for eSHAPE Total RNA, transfer 6  $\mu$ g of total RNA (RIN>7 is recommended) to a new 1.5 mL LoBind DNA tube.
2. If volume of total RNA is <100  $\mu$ L; bring volume up to 100  $\mu$ L using **Molecular Biology Grade** water.
  - **Note:** If RNA volume exceeds 100  $\mu$ L, continue with volume and increase volume of **2 $\times$  HyB** when resuspending washed Oligo dT beads so final concentration of **HyB** is 1 $\times$  during binding.
3. Incubate RNA in thermomixer for 2 minutes at 60  $^{\circ}$ C with interval mixing.
4. After incubation immediately place RNA samples on ice.
5. Transfer 50  $\mu$ L of **Oligo(dT) beads** per sample into a new 1.5 mL LoBind DNA tubes. Keep RNA and beads separate.
6. Add 50  $\mu$ L of **2 $\times$  HyB** to each tube containing 50  $\mu$ L Oligo(dT) beads, invert tube to mix.



7. Place tube on DynaMag-2 magnet and allow 1 minute for beads to separate.
8. Slowly invert closed tubes on magnet as beads start to separate to capture beads from cap.
9. When supernatant is transparent, discard supernatant without disturbing beads.
10. Remove tube from magnet and add 100  $\mu\text{L}$  **2 $\times$  HyB** per sample.
11. Invert tube to mix until homogeneous.
12. Place tube on DynaMag-2 magnet.
13. Allow 1 minute for beads to separate.
14. Slowly invert closed tubes on magnet as beads start to separate to capture beads from cap.
15. When separation is complete, discard supernatant without disturbing beads.
16. Repeat steps 10-15 for a total of two washes.
17. Remove tube from magnet and add 100  $\mu\text{L}$  of **2 $\times$  HyB**.
18. Pipette mix to combine until homogeneous.
19. Add entire volume (100  $\mu\text{L}$ ) of beads in **2 $\times$  HyB** to 100  $\mu\text{L}$  of denatured RNA (from step 4).
20. Place tube containing RNA and beads on tube rotator for 20 minutes at room temperature.
21. While the sample is rotating, dilute 2 $\times$  HyB 5-fold according to **Table 1**.

**Table 1. Dilution of 2 $\times$  Hybridization Buffer (per sample)**

Component	Volume ( $\mu\text{L}$ )
2 $\times$ Hybridization Buffer (HyB)	300
Molecular Biology Grade Water	1200
<b>Total:</b>	<b>1500</b>

22. Place tube containing beads and RNA on DynaMag-2 magnet
23. Allow 1 minute for beads to separate.
24. Slowly invert closed tubes while on magnet as beads to separate to capture any beads from cap.
25. When separation is complete, discard supernatant without disturbing beads.
26. Remove tube from magnet and add 745  $\mu\text{L}$  of **diluted HyB (Table 1)**.
27. Invert to mix until homogeneous.
28. Place tube on DynaMag-2 magnet and allow 1 minute for beads to separate.
29. Slowly invert closed tubes while on magnet to capture any beads from cap.
30. When separation is complete, discard supernatant without disturbing beads.
31. Spin tube in mini-centrifuge for 2 seconds.



32. Discard supernatant.
33. Resuspend beads in 100  $\mu$ L **mRNA Elution Buffer**.
34. Pipette mix to combine until homogeneous.
35. Incubate sample in thermomixer for 2 minutes at 60 °C with interval mixing.
36. After incubation immediately place on ice for 2 minutes.
37. Add 100  $\mu$ L of **2 $\times$  HyB** into eluted mRNA samples containing original **Oligo(dT) beads** to have total volume of 200  $\mu$ L.
38. Incubate on tube rotator for 20 minutes at room temperature.
39. Once rotation is complete place tube containing beads and RNA on DynaMag-2 magnet.
40. Allow 1 minute for beads to separate.
41. Slowly invert closed tubes to separate and capture any beads from cap.
42. When separation is complete, discard supernatant without disturbing beads.
43. Remove tube from magnet.
44. Add 745  $\mu$ L of **diluted HyB (Table 2)**.
45. Invert to mix until homogeneous.
46. Place tube on DynaMag-2 magnet.
47. Allow 1 minute for beads to separate.
48. Slowly invert closed tubes to separate and capture any beads from cap.
49. When separation is complete and supernatant is transparent, aspirate and discard supernatant without disturbing beads.
50. Spin tube in mini-centrifuge for 15 seconds.
51. Aspirate all residual liquid.
52. Add 11  $\mu$ L **Molecular Biology Grade** water to bead pellet.
53. Pipette mix until homogeneous.
54. Incubate sample in thermomixer for 3 minutes at 70 °C with interval mixing.
55. Immediately place tubes on magnet and transfer all supernatant to a new 0.2 mL strip tube without disturbing beads and place on ice.
  - **Note:** Total volume of mRNA will be approximately ~8  $\mu$ L. Bring entire volume to step 1 on page 10 of this protocol.





## Adapter and index primer sequences

---

- Illumina TruSeq HT adapters (provided)

**RNA adapter:** 5Phos/rArGrArUrCrGrGrArArGrArGrCrArCrArCrGrUrCrUrG/3SpC3/

**ssDNA adapter:** 5Phos/NNNNNNNNNAGATCGGAAGAGCGTCGTGT/3SpC3/ (

**Index primer sequences:** Illumina dual index primers (provided)

i7 index name	i7 bases on Sample Sheet	i5 index name	i5 bases bases on Sample Sheet
705	ATTCAGAA	505	AGGCGAAG
706	GAATTCGT	506	TAATCTTA
707	CTGAAGCT	507	CAGGACGT
708	TAATGCGC	508	GTA CTGAC

