ECLIPSEBIO
THE RNA GENOMICS COMPANY

eSHAPE *Single RNA* ™ USER GUIDE

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.

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Chapter 1: Overview

Introduction to SHAPE *Single RNA*

The SHAPE *Single 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 imidazolide*), a structure probing agent, to create chemical adducts at the 2'OH on 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 *Single 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	
µL (or ul) is microliter	One millionth (10^{-6}) of a liter
mL (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°C.
- This protocol has been tested with 50ng to 200ng of starting IVT mRNA.

IMPORTANT: *Materials listed below are for SHAPE Single 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
SHAPE RT 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
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

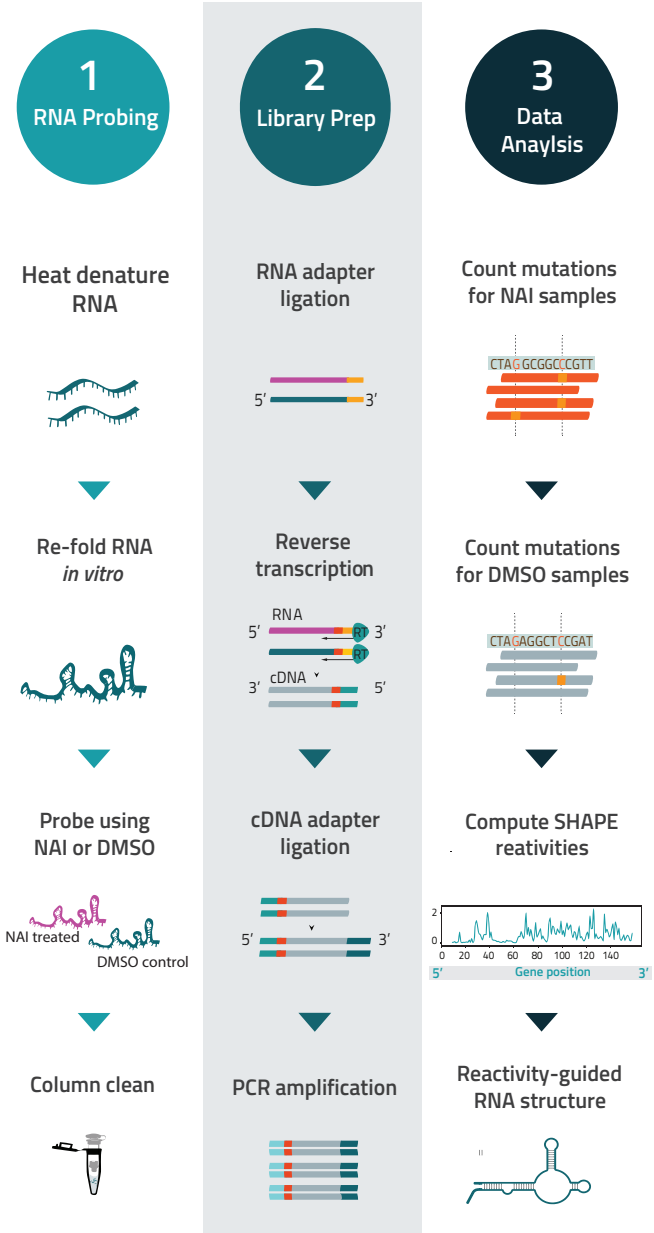
Eppendorf Thermomixer C	Eppendorf cat. #5382000015
DynaMag-2 Magnet	ThermoFisher Scientific cat. #12321D
MagWell™ Magnetic Separator 96 or DynaMag-96 Side Magnet	EdgeBio, cat. #57624 ThermoFisher Scientific cat. #12331D
Aluminum Cool Block	Diversified Biotech cat. #CHAM1000
Reagent Reservoirs	ThermoFisher 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 *Single RNA* Workflow

Overview

The SHAPE *Single 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 *in vitro* transcribed RNA molecules resulting from the T7 polymerase reaction is treated with DNase and cleaned. RNA molecules are linearized by heat, then incubated in SHAPE folding buffer to facilitate proper re-folding. RNA molecules are then treated with either NAI or DMSO control. The NAI reagent form 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

In Vitro NAI Treatment of RNA

1. Aliquot 100 ng IVT mRNA 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 µL 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 µL 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 µL 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 µL of **2M NAI** to each sample (or 9 µL 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 µL total volume) in a thermocycler at 37°C for 15 minutes. Place and keep samples on ice.
10. Add 23 µL **molecular biology grade water** to each sample for a total volume of 50 µL.
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 100µL of **RNA Binding Buffer** to the 50µL of each RNA sample. Pipette mix.
2. Add 150µL 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 × g for 30 seconds. Discard flow-through.
5. Add 400µL **RNA Prep Buffer** to each column.
6. Centrifuge at 7,000 × g for 30 seconds. Discard flow-through.
7. Add 480µL **RNA Wash Buffer** to each column.
8. Centrifuge at 7,000 × 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 × 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 11µL of **molecular biology grade water** directly to filter.
15. Incubate at room temperature for 1 minute.
16. Centrifuge at 12,000 × 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 from the previous chapter (~9 µL, ~100 ng RNA) in to a new 0.2 mL strip tube. Place and keep samples on ice.
2. Add 8 µL 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 (µL)
RNase Inhibitor	1
PSP Enzyme	2
Total:	**3**

2. Add 3 µL 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
Total:	**75**

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.

- **Note:** If your IVT RNA molecule length is <400 nucleotides (before fragmentation), refer to **Appendix 1** for steps 1-23**.** If your RNA is >400 nucleotides, proceed with the steps below.

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 µL 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 Adapter Ligation

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
- 10X FS SHAPE 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 µL 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
Total:	**13.5**

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. Ad 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 µL 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 µL 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 µL) 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 µL 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 (µL)
10X FS SHAPE Buffer	2
MTL	0.24
100 mM DTT	1
RNase Inhibitor	0.4
Molecular Biology Grade Water	5.76
SHAPE RT Enzyme	0.6
Total:	**10**

6. Pipette up and down 10 times to mix. Liquid should turn light brown. Store on ice.
7. Add 10 µL 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 µL 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 µL **0.5M EDTA** (pH 8) to each sample.
6. Pipette to mix.
7. Add 3 µL **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 µL 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 µL **eCLIP Beads** to a new 1.5 mL DNA LoBind tube (e.g., for 2 samples transfer 14 µL of eCLIP beads to tube).
3. Ad 5x volume of **Bead Binding Buffer** to beads (e.g., for 2 samples, transfer add 70 µL buffer to 14 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 90 µL **Bead Binding Buffer** per sample.
6. Add 87 µL of washed **eCLIP Beads** to each tube of ~30 µL ligated cDNA sample. Pipette mix until sample is homogeneous.
7. Add 68 µL **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 µL 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 µL 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

Component	Volume (µL)
ssDNA Ligation Buffer	6.5
Ligase	1
ssDNA Enzyme	0.3
Total:	**7.8**

2. Slowly add 7.8 µL 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 µL of **Bead Elution Buffer** to each 10 µL adapter-ligated cDNA sample. Pipette mix until homogeneous.
2. Add 45 µL of **Bead Binding Buffer** to each sample. Pipette mix.
3. Add 31 µL 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 µL 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 µL 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 µL) 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**.

Table 1. qPCR Master Mix

Component	Volume (µL)
qPCR Mix (NEB Luna) 2x	5
qPCR Primers	4
Total:	**9**

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
Total volume	**40**

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 = 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	**6**
70°C	30 seconds	
72°C	40 seconds	
Extra N cycles (N = Ct value – 9)		
98°C	15 seconds	**N***
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 µL of **AMPure XP beads** to each 40 µL 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 µL 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 20µL 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 µL 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 °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: Zymo RNA Column Cleanup Protocol for Smaller RNA Molecules (<400 nts)

Consumables

- RNA Clean & Concentrator -5 Kit (Zymo Research cat. #R1015/R1016)
- 100% Ethanol
- 80% Ethanol
- Bead Elution Buffer

Procedure

1. Sample volume is ~95 µL. Add 2 sample volumes (190 µL) of **RNA Binding Buffer** to each repaired RNA sample. Mix by pipetting.
2. Add 3 sample volumes of **100% EtOH** (285 µL) to each sample. Mix by pipetting.
3. Transfer entire sample volume (~570 µL) to a new filter column placed in a collection tube.
4. Centrifuge at 7,000 x g for 30 seconds.
5. Take eluent and put through filter column again (double-bind).
6. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
7. Add 400 µL **RNA Prep Buffer** to each column.
8. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
9. Add 480 µL **RNA Wash Buffer** to each column
10. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
11. Repeat steps 9-10 for a total of two washes.
12. Centrifuge column at 12,000 x g for 1 minute with emptied collection tube.
13. Carefully transfer filter column to a new 1.5 mL LoBind tube (avoid liquid in collection tube).
14. Discard flow-through and collection tube.
15. Open column caps and allow to air dry for 2 minutes or until column is completely dry.
16. Elute all samples by adding 8 µL of **Bead Elution Buffer** directly to filter.
17. Incubate at room temperature for 1 minute.
18. Centrifuge at 12,000 x g for 90 seconds.
19. Place RNA samples on ice if continuing to next step or freeze at -80C until you are ready to proceed with the protocol (on page 23).

Appendix 2: G-block Amplification and RNA synthesis

Overview

This section describes the amplification of a G-block or other DNA template using two rounds of PCR. The first round is performed using G-block-adapter specific primers to amplify the G-block DNA. The second round amplifies the product from the first round using gene specific primers. If you do not have any adapter primers, use the gene specific primers for both rounds of PCR. The resulting PCR product is then transcribed into in RNA using an overnight T7 RNA polymerase reaction.

Consumables

- G-block oligo or DNA template oligo
- G-block Adapter Forward Primer (if applicable)
- G-block Adapter Reverse Primer (if applicable)
- Gene Specific Forward Primer
- Gene Specific Reverse Primer
- PCR Mix (NEB Q5 Master Mix)
- eCLIP Beads
- Bead Binding Buffer
- Bead Elution Buffer
- 80% and 100% Ethanol
- DNase Buffer
- DNase enzyme
- RNase Inhibitor
- Molecular Biology Grade Water
- HiScribe T7 High Yield RNA Synthesis Kit
- NEB Monarch RNA Cleanup Kit (500 µg)

Preparation

- Reconstitute lyophilized G-block Adapter Primers (Forward & Reverse) by adding **molecular biology grade water** to make a 100 µM primer stock.

- Reconstitute lyophilized Gene Specific Primers (Forward & Reverse) by adding **molecular biology grade water** to make a 100 µM primer stock.

  Reconstitute lyophilized G-block by adding **molecular biology grade water** to make a 10 ng/µL G-block stock.

Procedure

PCR1 Amplification

1. Prepare **PCR1 Reaction Mix** according to **Table 1** for each G-block in a 1.5 mL LoBind DNA Tube. Mix thoroughly.

Table 1. PCR1 Reaction Mix (prepare for EACH G-block)

Component	Volume (µL)
Molecular Biology Grade water	90
G-block (10 ng/µL)	6
Forward Primer (100 µM)	2
Reverse Primer (100 µM)	2
PCR Mix	100
Total:	**200**

2. Divide each 200 µL reaction into 4 x 50 µL volumes in 0.2 mL strip tubes. Spin down briefly in centrifuge and keep on ice.
3. Run samples in thermocycler using **PCR1 amplification program** in **Table 2**.

Table 2. PCR1 amplification program

Temperature	Time	Cycles
98°C	30 seconds	
98°C	15 seconds	**8**
65-70°C (primer specific)	30 seconds	
72°C	1 minute	
72°C	2 minutes	
4°C	∞	

4. Pool all PCR1 reaction products from the same G-block/template back into a 1.5 mL LoBind DNA tube (200 µL total).

PCR1 Cleanup

1. Take **eCLIP Beads** out of 4°C and resuspend until homogeneous.
2. For each sample, transfer 100 µL **eCLIP Beads** to a new 1.5 mL DNA LoBind tube.
3. Ad 5x volume of **Bead Binding Buffer** to 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 600 µL **Bead Binding Buffer** per sample.
6. Add 600 µL of washed **eCLIP Beads** to each tube of pooled PCR1 product. Pipette mix until sample is homogeneous.
7. Add 300 µL **100% EtOH** to beads and PCR1 product. Pipette mix until homogeneous.
8. Place tube on tube rotator for 15 minutes at room temperature.
9. Place tube on DynaMag-2 magnet and allow 1 minute for beads to separate.
10. Slowly invert closed tubes on magnet as beads start to separate to capture beads from cap.
11. When supernatant is transparent, discard supernatant without disturbing beads.
12. Remove tube from magnet and add 400 µL of 80% EtOH.
13. Invert tube to mix until homogeneous.
14. Place tube on DynaMag-2 magnet and allow 1 minute for beads to separate.
15. Slowly invert closed tubes on magnet as beads start to separate to capture beads from cap.
16. Aspirate and discard all residual liquid without disturbing beads while on magnet.
17. Repeat steps 12-16 for a total of two washes.
18. Aspirate and discard all residual liquid without disturbing beads while on magnet.
19. Allow beads to air dry for 5-10 minutes or until beads no longer appear wet and shiny
20. Once completely dry, carefully remove tube from magnet and add 50 µL of **molecular biology grade water**.
21. Pipette mix to resuspend beads and incubate for 5 minutes at room temperature.
22. Place tube on magnet and transfer all supernatant to new 1.5 mL DNA LoBind tube.
23. Re-elute sample by adding another 50 µL of **molecular biology grade water** to beads.
24. Pipette mix to resuspend beads and incubate for 5 minutes at room temperature.

25. Place tube on magnet and pool supernatant in same 1.5 mL tube.
    - **Note**: Total volume of DNA will be around 100 µL.
26. Measure PCR1 product on Tapestation using **D5000** tape with product at **1:5 dilution**. Mix **4 µL of molecular biology grade water** and **1 µL of sample**.

    - **Note:** You will need **400 ng** of product for **PCR2** step.

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

Next stopping point ~2 hours

PCR2 Amplification

1. Prepare **PCR2 Reaction Mix** according to **Table 3** for each sample in a 1.5mL LoBind DNA Tube. Mix thoroughly.

Table 3. PCR2 Reaction Mix (prepare for each PCR1 product)

Component	Volume (µL)
PCR1 Product	*Vol. for 400 ng*
Gene Specific Forward Primer T7 (100 µM)	4
Gene Specific Reverse Primer T7 (100 µM)	4
PCR Mix	200
Molecular Biology Grade Water	*Add up to 400 µL total*
Total:	**400**

2. Divide each 400 µL reaction into 8 x 50 µL volumes in 0.2 mL strip tubes. Spin down briefly in centrifuge and keep on ice.
3. Run samples in thermocycler using **PCR2 program** in **Table 4**.

Table 4. PCR2 amplification program

Temperature	Time	Cycles
98°C	30 seconds	
98°C	15 seconds	**8**
70°C (primer specific)	30 seconds	
72°C	2 minutes	
72°C	2 minutes	
4°C	∞	

4. Pool each **PCR2** reaction product back into a 1.5 mL LoBind DNA tube (400 µL total).

PCR2 Cleanup

1. Take **eCLIP Beads** out of 4°C and resuspend until homogeneous.
2. For each sample, transfer 60 µL **eCLIP Beads** to a new 1.5 mL DNA LoBind tube.
3. Add 5x volume of **Bead Binding Buffer** to 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 **1.2 mL Bead Binding Buffer** per sample.
    - **Note!** Total sample volume with eCLIP Beads, PCR2 product and 100% EtOH will be ~2.1 mL, use a bigger tube or split across multiple 1.5 mL DNA LoBind tubes.
6. Add 400 µL of pooled **PCR2** product to the washed beads. Pipette mix until sample is homogeneous.
7. Add 500 µL **100% EtOH** to beads and PCR2 product. Pipette mix until homogeneous.
8. Place tubes on tube rotator for 15 minutes at room temperature.
9. Place tube on DynaMag-2 magnet and allow 1 minute for beads to separate.
10. Slowly invert closed tubes on magnet as beads start to separate to capture beads from cap.
11. When supernatant is transparent, discard supernatant without disturbing beads.
12. Remove tube from magnet and add 500 µL of **80% EtOH**.
13. Invert tube to mix until homogeneous.
14. Place tube on DynaMag-2 magnet and allow 1 minute for beads to separate.

15. Slowly invert closed tubes on magnet as beads start to separate to capture beads from cap.
16. Aspirate and discard all residual liquid without disturbing beads while on magnet.
17. Repeat steps 12-16 for a total of two washes.
    - **Note!** On final wash, pool all beads from the same sample together into one tube before placing on magnet.
18. Aspirate and discard all residual liquid without disturbing beads while on magnet.
19. Allow beads to air dry for 5-10 minutes or until beads no longer appear wet and shiny
20. Once completely dry, carefully remove tube from magnet and add 21 µL of **Bead Elution Buffer**.
21. Pipette mix to resuspend beads and incubate for 5 minutes at room temperature.
22. Place tube on magnet and transfer all supernatant to new 1.5 mL DNA LoBind tube.
23. Re-elute sample by adding another 21 µL of **Bead Elution Buffer** to beads.
24. Pipette mix to resuspend beads and incubate for 5 minutes at room temperature.
25. Place tube on magnet and pool supernatant in same 1.5 mL tube.
    - **Note**: Total volume of DNA will be around 40 µL.
26. Measure PCR2 product on Tapestation using **D5000** tape with product at **1:100 dilution**. Mix **99 µL of molecular biology grade water** and **1 µL of sample**.
    - **Note:** You will need at least 1 µg of product for the **T7 RNA synthesis** step.

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

T7 RNA synthesis

1. Prepare **T7 Reaction Mix** according to **Table 5** for each eluted PCR2 product.

Table 5. T7 Reaction Mix (prepare for each PCR2 product)

Component	Volume (µL)
1 µg PCR2 Product + H2O	40
T7 Reaction Buffer (10X)	10
ATP (100 mM)	10
GTP (100 mM)	10
CTP (100 mM)	10
UTP (100 mM)	10
T7 RNA Polymerase Mix	10
Total:	**100**

2. Combine all components from the **T7 Reaction Mix** in a 1.5 mL DNA LoBind tube. Use at least 1 µg PCR2 product.
3. Incubate all samples in thermomixer at 37°C, overnight, with interval shaking.
   - **Note!** T7 Reaction incubation can also be done for 4-6 hours at 37°C with interval shaking.

Stopping Point: *Samples incubate overnight at 37°C on thermomixer for up to 16 hours*

T7 RNA Cleanup

1. Remove samples from thermomixer and measure each T7 reaction on RNA tape at **1:100 dilution**. Mix **99 µL of molecular biology grade water** and **1 µL of sample**.

   - Take note of the total RNA yield of each sample. Each column in the NEB RNA Cleanup kit holds a maximum of 500 µg RNA. For example; if the T7 Reaction yields 750 µg of RNA, use 2 NEB RNA Cleanup columns.

2. Prepare **DNase Master Mix** for each T7 Reaction tube according to **Table 1**.

Table 1. DNase Master Mix

Component	Volume (µL)
DNase Buffer	30
RNase Inhibitor	3
DNase enzyme	13
Molecular Biology Grade Water	154
Total:	**200**

4. Add 200 µL **DNAse Master Mix** to each T7 Reaction tube. Mix thoroughly.
5. Incubate samples on thermomixer at 37°C for 30 minutes without shaking.
6. Using the calculated RNA yield from step 1, determine the number of filter columns to use for each sample (500 µg max per column).
7. For each 300 µL of sample, add 600 µL of **RNA Cleanup Binding Buffer.** Pipette mix.
8. For each 300 µL of sample, add 900 µL of **100% EtOH**. Mix thoroughly.
9. Add up to 900 µL of sample to a filter column placed in a collection tube.
10. Centrifuge at 14,000 x g for 1 minute. Discard flow-through.
11. Add remainder of sample onto column and centrifuge at 14,000 x g for 1 minute. Discard flow-through.
12. Add 500 µL **RNA Cleanup Wash Buffer** to each column.
13. Centrifuge at 14,000 x g for 1 minute. Discard flow-through
14. Repeat steps 11-12 for a total of two washes.
15. Centrifuge dry filter column at 14,000 x g for 2 minutes with emptied collection tube.

16. Carefully transfer filter column to a new 1.5 mL LoBind DNA tube. Discard flow-through and collection tube.
17. Open column caps and allow to air dry for 2 minutes or until filter is completely dry.
18. Elute all samples by adding 50 µL of **molecular biology grade water** directly to filter.
19. Incubate at room temperature for 5 minutes.
20. Centrifuge at 14,000 x g for 1 minute.
21. Re-elute all samples by adding another 50 µL of **molecular biology grade water** directly to filter.
22. Incubate at room temperature for 5 minutes
23. Centrifuge at 14,000 x g for 1 minute.
24. Keep all samples on ice if proceeding to the next step.
    - **Note:** Total volume of RNA will be ~100 µL
25. Measure each sample on RNA tape at **1:100 dilution**. Mix **99 µL of molecular biology grade water** and **1 µL of sample**.
    - Final RNA probe concentration should be above 1 µg/µL.
26. Adjust final RNA probe concentration to ~1 µg/µL with **molecular biology grade water**.

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

Next stopping point: 2 hours

Adapter and index primer sequences

- Illumina TruSeq HT adapters (provided)

RNA adapter: 5Phos/rArGrArUrCrGrGrArArGrArGrCrArCrArCrGrUrCrUrG/3SpC3/

ssDNA adapter: 5Phos/NNNNNNNNNNAGATCGGAAGAGCGTCGTGT/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	GTACTGAC