



5' End-Seq™ 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 5' End-Seq

The End-Seq Kit transforms RNA analysis by streamlining mRNA isolation, mRNA transcript end preparation and library preparation. Using a robust and reproducible framework the 5' End-Seq kit allows for detection, amplification, and sequencing of the 5' ends of mRNA molecules.

The 5' End-Seq Kit is based on poly(A) selection of mRNA molecules followed by controlled enzymatic digestion of coding regions, reverse transcription of mRNA into cDNA and polymerase chain reaction amplification of 5' ends. This method produces high quality libraries that enables the user to obtain detailed annotation of transcription start sites.

The 5' End-Seq kit contains reagents for manual library preparation of up to 8 samples. The recommended input amount is 3 µg of total, non-crosslinked RNA per sample. Preparing 5' End-Seq libraries requires high integrity input material – RIN should exceed 7.

The 5' End-Seq Kit offers:

- High throughput and robust workflow
- High reproducibility with accurate data
- Unbiased target identification 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
Ice	0 – 4 °C
Freeze	-80 °C
Volume units	
µL is microliter	One millionth (10 ⁻⁶) of a liter
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) during all steps.
- During beads washing, ensure DNA LoBind 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 30 ng to 200 ng of starting mRNA.

IMPORTANT: *Materials listed below are for 5' End-Seq experimental set up ONLY. See Appendix for supplemental information.*



Reagents Included with Kit

Item	Part Number	Storage
eCLIP Beads	BD-001	4 °C
Oligo(dT) beads	BD-002	4 °C
Bead Binding Buffer	EB-BU-005	4 °C
2× Hybridization Buffer (2× HyB)	EB-BU-006	-20 °C
RNase Inhibitor	EZ-001	-20 °C
PSP Enzyme	EZ-003	-20 °C
Nuclease Enzyme	EZ-005	-20 °C
PNK Enzyme	EZ-006	-20 °C
Ligase Enzyme	EZ-007	-20 °C
ssDNA enzyme	EZ-010	-20 °C
RT4 Enzyme	EZ-011	-20 °C
5' End-zyne	EZ-014	-20 °C
PCR Mix	EZ-015	-20 °C
5' Buffer	BU-007	-80 °C
RT4 Buffer	BU-008	-80 °C
10X PNK Buffer	BU-012	-80 °C
100 mM ATP	RM-012	-80 °C
0.1 M DTT	RM-020	-80 °C
PSP Buffer	EB-BU-008	-80 °C
Bead Elution Buffer	EB-BU-011	-80 °C
RNA Ligation Buffer	EB-BU-013	-80 °C
ssDNA Ligation Buffer	EB-BU-014	-80 °C
mRNA Elution Buffer	EB-BU-017	-80 °C
5' RNA Adapter	EB-AD-011.2	-80 °C
5' ssDNA Adapter	EB-AD-013	-80 °C
5' RT Primer	EB-PR-015.2	-80 °C
qPCR Primers	EB-PR-014	-80 °C
Index Primers	(EB-PR-028 to EB-PR-031) (EB-PR-035 to EB-PR-038)	-80°C



Equipment and Materials 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
RNA Clean & Concentrator -5	Zymo Research cat. #R1015/R1016
Direct-zol RNA MiniPrep or equivalent	Zymo Research cat # R2050
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



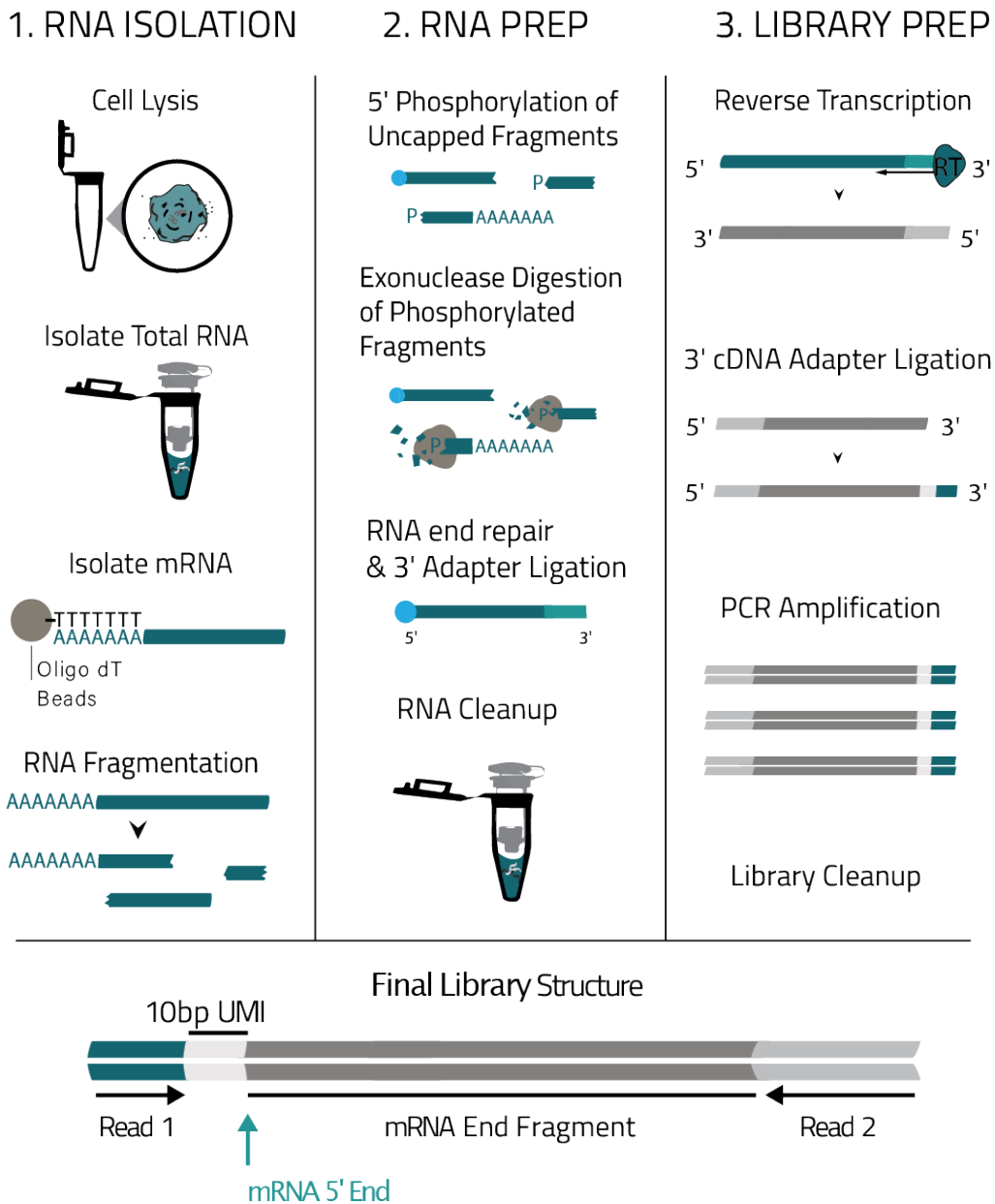
Reagents Not Included with Kit

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
DPBS, Corning	VWR cat. #21-031-CV
EDTA (0.5 M), pH 8.0, RNase-free	ThermoFisher Scientific cat. #AM9261
1 M Sodium Hydroxide solution (NaOH)	Sigma-Aldrich cat. #79724-100ML
1 M Hydrogen Chloride (HCl)	Any
Agencourt AMPure XP	Beckman Coulter cat. #A63881
NEB LUNA Universal qPCR 2× Master Mix	New England BioLabs cat. #M3003S



5' End-Seq Workflow

The following protocol is a method to enrich specifically for transcript 5' ends and prepare libraries for sequencing. Sequencing of 5' End-Seq libraries will map all alternative transcription start sites. The 5' End-Seq protocol takes approximately three days from RNA isolation to finished library.



Chapter 2: Poly(A)-RNA Isolation

Overview

This section describes poly(A) selection from total RNA (see [Appendix 1: Total RNA Isolation](#) for protocol). Here, mRNA is enriched by double Oligo(dT) capture to select for polyadenylated transcripts. mRNA is then subjected to the 5' End-Seq protocol.

Consumables

- Molecular Biology Grade Water
- Oligo(dT) beads
- 2× 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 °C.

Procedure

Poly(A)-RNA Isolation

1. For each sample transfer 3 µg of total RNA to a new 1.5 mL LoBind DNA tube.
2. Bring the sample volume up to 100 µL using **Molecular Biology Grade** water.
 - **Note:** If RNA volume exceeds 100 µL, continue with volume and increase volume of **2× HyB** when resuspending washed Oligo(dT) beads so final concentration of **HyB** is 1× during binding.
3. Incubate the RNA sample in thermomixer for 2 minutes at 60 °C with interval mixing.
4. After incubation immediately place RNA sample on ice. Keep on ice while washing beads.
5. Transfer 25 µL of **Oligo(dT) beads** per sample into a new 1.5 mL LoBind DNA tube. Keep RNA and beads separate.
 - **Note:** For 2 x 5' End-Seq samples transfer 2 x 25 µL of **Oligo(dT) beads** to a new 1.5 mL LoBind DNA tube.
6. Add 300 µL of **2× HyB** to the tube containing Oligo(dT) beads, invert tube to mix.
7. Place the tube on DynaMag-2 magnet and allow 1 minute for beads to separate.



8. Slowly invert closed tube on magnet as beads start to separate to capture beads from cap.
9. When supernatant is transparent, discard supernatant without disturbing beads.
10. Remove the tube from magnet and add 100 μ L **2 \times HyB** per sample.
 - **Note:** For 2 x 5' End-Seq samples transfer 2 x 100 μ L of **2 \times HyB** to the tube with beads.
11. Invert the tube to mix until homogeneous.
12. Place the tube on DynaMag-2 magnet.
13. Allow 1 minute for beads to separate.
14. Slowly invert closed tube 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 the tube from magnet and add 100 μ L of **2 \times HyB** per sample.
 - **Note:** For 2 x 5' End-Seq samples transfer 2 x 100 μ L of **2 \times HyB** to the tube with beads.
18. Pipette mix to combine until homogeneous.
19. Add 100 μ L of beads suspended in **2 \times HyB** to each tube containing 100 μ L of denatured RNA (from step 4). Total volume per 5' End-Seq sample will be 200 μ L.
20. Place the 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 (μ L)
2 \times Hybridization Buffer (HyB)	300
Molecular Biology Grade Water	1200
Total:	1500

22. After incubation, place the tube containing beads and RNA on DynaMag-2 magnet.
23. Allow 1 minute for beads to separate.
24. Slowly invert closed tube on magnet as beads start to separate to capture any beads from cap.
25. When separation is complete, discard supernatant without disturbing beads.
26. Remove the tube from magnet and add 745 μ L of **diluted HyB (Table 1)**.
27. Invert to mix until homogeneous.
28. Place the 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 the tube in mini-centrifuge for 2 seconds. Place the tubes on magnet.
32. Discard supernatant.
33. Resuspend beads in 100 μ 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 the tube on ice for 2 minutes.
37. Add 100 μ L of **2 \times HyB** into eluted mRNA samples containing original **Oligo(dT) beads** for a total volume of 200 μ L.
38. Incubate sample on tube rotator for 20 minutes at room temperature.
39. Once rotation is complete place the tube containing beads and RNA on DynaMag-2 magnet.
40. Allow 1 minute for beads to separate.
41. Slowly invert closed tube on magnet as beads start to separate to capture beads from cap.
42. When separation is complete, discard supernatant without disturbing beads.
43. Remove the tube from magnet.
44. Add 745 μ L of **diluted HyB (Table 1)**.
45. Invert to mix until homogeneous.
46. Place the tube on DynaMag-2 magnet.
47. Allow 1 minute for beads to separate.
48. Slowly invert closed tube on magnet as beads start to separate to capture beads from cap.
49. When separation is complete and supernatant is transparent, aspirate and discard supernatant without disturbing beads.
50. Spin the tube in mini-centrifuge for 15 seconds. Place the tube on magnet.
51. Aspirate all residual liquid.
52. Add 11 μ 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:** Volume will be ~9 μ L
56. Proceed to next step.



Chapter 3: 5' End Sample Protocol

Overview

The 5' End-Seq protocol uses intact mRNA as starting material. Poly(A)+ mRNA is first heat fragmented and subsequently 5' dephosphorylated using PSP enzyme, before it is 5' phosphorylated using PNK. The 5'-P mRNA fragments are then digested by 5' End-zye from 5'→ 3', leaving behind only mRNA fragments with 5'-caps.

Consumables

- 100 mM ATP
- RNase Inhibitor
- PNK Enzyme
- 5' Buffer
- 5' End-zye
- PSP Buffer
- PSP Enzyme
- 10X PNK buffer
- Bead Elution Buffer
- 100% EtOH
- Zymo RNA Clean and Concentrator-5 Kit
- Molecular Biology Grade Water

Procedure

RNA Fragmentation of 5' Samples

1. Aliquot all mRNA isolated from 3 µg total RNA (~30 ng) to a new 0.2 mL tube. Sample volume will be ~9 µL.
2. Add 8 µL of **PSP buffer** to each tube of mRNA.
3. Place samples in the thermocycler at 95 °C for 5 minutes, then place immediately on ice.

Removal of 5' Phosphates of 5' End-Seq Samples

1. Prepare **PSP Master Mix** according to **Table 2**.



Table 2. PSP Master Mix (per sample)

Component	Volume (μL)
RNase Inhibitor	1
PSP Enzyme	2
Total:	3

2. Add 3 μL of **PSP Master Mix** to each 17 μL fragmented mRNA sample. Pipette mix to combine.
3. Incubate in thermocycler at 37 °C for 10 minutes, followed by 75 °C for 5 minutes.
4. Place sample on ice.

5' Phosphorylation of 5' End-Seq Samples

1. Prepare **PNK Master Mix** according to **Table 3**.

Table 3. PNK Master Mix (per sample)

Component	Volume (μL)
RNase Inhibitor	1
10X PNK Buffer	5
100 mM ATP	0.5
PNK Enzyme	3.5
Molecular Biology Grade water	20
Total:	30

2. Add 30 μL of **PNK Master Mix** to each 20 μL fragmented mRNA sample.
3. Incubate in thermocycler at 37 °C for 30 minutes.
4. Place sample on ice.

RNA Cleanup of 5' End-Seq Samples

- **Note:** This section uses the Zymo RNA Clean and Concentrator-5 kit to clean RNA. Before the first usage, prepare the kit based on manufacturer's protocol.

1. Add 100 μL of **RNA Binding Buffer** to the 50 μL of each phosphorylated 5' End-Seq sample. Pipette mix.
2. Add 150 μL of **100% EtOH** and mix thoroughly.
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 μL **RNA Prep Buffer** to each column.
6. Centrifuge at $7,000 \times g$ for 30 seconds. Discard flow-through.
7. Add 480 μL **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 1 minute with empty 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 12 μL 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. Re-elute all samples by adding 12 μL of **Molecular Biology Grade** water directly to filter.
18. Incubate at room temperature for 1 minute.
19. Centrifuge at $12,000 \times g$ for 90 seconds.
20. Transfer entire sample ($\sim 23 \mu\text{L}$) to new 0.2 mL strip tube.
21. Store on ice if proceeding to the next step.

Optional Stopping Point: *If stopping here, 5' RNA samples should be stored at -80°C*

Next stopping point: ~ 2 hours

Digestion of 5'-P mRNA Fragments in 5' End-Seq Samples

1. Prepare **5' End-zyyme Master Mix** in a new 1.5 mL LoBind tube according to **Table 4**.

Table 4. 5' End-zyyme Master Mix (per sample)

Component	Volume (μL)
5' Buffer	3
RNase Inhibitor	1
5' End-zyyme	3
Total:	7



2. Add 7 μ L **5' End-zyme Master Mix** to each 23 μ L sample. Pipette mix to combine.
3. Incubate at 30 °C for 75 minutes.
4. Add 20 μ L **Molecular Biology Grade** water to each sample, for a total volume of 50 μ L.
5. Proceed directly to the next step.

RNA Cleanup of 5' End-Seq Samples

➤ **Note:** This section uses the Zymo RNA Clean and Concentrator-5 kit to clean RNA. Before the first usage, prepare the kit based on manufacturer's protocol.

1. Add 100 μ L of **RNA Binding Buffer** to the 50 μ L of each sample. Pipette mix.
2. Add 150 μ L of **100% EtOH** and mix thoroughly.
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 μ L **RNA Prep Buffer** to each column.
6. Centrifuge at 7,000 \times g for 30 seconds. Discard flow-through.
7. Add 480 μ 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 1 minute with empty collection tube.
11. Carefully transfer filter column to a new 1.5 mL 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 8 μ 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, 5' RNA samples should be stored at -80 °C*

Next stopping point: ~2 hours



Chapter 4: 5' End-Seq Sample Adapter Ligation

Overview

The previous chapters have described the selection of 5' ends in the samples. This section describes adapter ligation and reverse transcription, followed by removal of dNTPs and template RNA. ssDNA samples are then cleaned, and overnight adapter ligation is performed.

Consumables

- Molecular Biology Grade Water
- 5' RNA Adapter
- RNA Ligation Buffer
- RNase Inhibitor
- eCLIP Beads
- 80% and 100% EtOH
- 5' RT Primer
- RT4 Buffer
- RT4 Enzyme
- 0.1 M DTT
- Nuclease
- 0.5 M EDTA
- 1 M NaOH
- 1 M HCl
- 5' ssDNA Adapter
- ssDNA Ligation Buffer
- Ligase
- ssDNA Enzyme

Preparation

1. Thaw RNA ligation buffer and 5' RNA adapter on ice.
2. Thaw samples on ice if stored at -80 °C.

Procedure

RNA Adapter Ligation

1. Aliquot entire sample (~6 µL) into new 0.2 mL strip tube.
2. Place the tube on ice.



3. Add 2 μ L of **5' RNA Adapter** to each sample. Pipette mix to combine.
4. Close caps and spin in mini centrifuge for 5 seconds.
5. Incubate tube at 65 °C for 2 minutes in thermocycler.
6. Place on ice for 1 minute.
7. Prepare **RNA Ligation Master Mix** according to **Table 5**.

Table 5. RNA Ligation Master Mix (per sample)

Component	Volume (μ L)
RNA Ligation Buffer	12
RNase Inhibitor	0.3
Ligase	1.2
Total:	13.5

8. Add 13.5 μ L **RNA Ligation Master Mix** to each sample. Pipette mix to combine..
9. Spin down samples briefly in mini centrifuge.
10. Incubate samples at room temperature for 1 hour on a tube rotator.

Ligated RNA Cleanup

- **Preparation Note:** Prepare fresh **80% Ethanol** in **Molecular Biology Grade** water in a fresh 50 mL tube if not done previously. Store at room temperature for up to 1 week. Keep tube closed tightly.

1. Take **eCLIP Beads** out of 4 °C and resuspend until homogeneous.
2. For each RNA sample, transfer 5 μ L of **eCLIP Beads** to a new 1.5 mL DNA LoBind tube (e.g., for 4 samples transfer 20 μ L of eCLIP Beads).
3. Add 5 \times volume of **Bead Binding Buffer** (e.g., for 4 samples add 100 μ L buffer to 20 μ L of eCLIP Beads). Pipette mix until the solution is homogeneous.
4. Place the tube on DynaMag-2 magnet. When separation is complete and supernatant is clear, carefully aspirate and discard supernatant without disturbing beads.
5. Remove the tube from magnet.
6. Resuspend eCLIP Beads in **62 μ L** of **Bead Binding Buffer** per sample. (e.g., for 4 samples add 248 μ L of buffer).
7. Pipette mix until beads are fully resuspended.
8. Add 60 μ L of washed **eCLIP Beads** to each sample.
9. Pipette mix until sample is homogeneous.



10. Add 45 μ L of **100% EtOH** to each sample.
11. Pipette mix until homogeneous. Transfer sample to new strip tube after mixing.
12. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
13. Place tubes on magnet. Allow to incubate for 1-2 minutes or until separation is complete and liquid is transparent.
14. Carefully discard supernatant without disturbing beads.
15. Wash beads by adding 200 μ L of **80% EtOH** without disturbing beads. Move sample to different positions on the magnet for about 45 seconds to wash.
16. Carefully discard supernatant without disturbing beads.
17. Repeat step 15 - 16 for a total of 3 washes.
18. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
19. Place the tube back on 96-well magnet.
20. Incubate on magnet for 10 seconds until separation is complete and supernatant is transparent.
21. Aspirate and discard all residual liquid without disturbing beads while on magnet.
22. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
 - **Note:** Do not over dry samples.
23. Once completely dry, carefully remove tubes from magnet.
24. Resuspend beads in 10 μ L of **Molecular Biology Grade** water.
25. Pipette mix until homogeneous.
26. Incubate for 5 minutes at room temperature.
27. Place strip tubes with samples on magnet.
28. Transfer entire sample (~9 μ L) from beads to new 0.2 mL strip tube. Discard beads.
29. Place the tube on ice.

Preparation

1. Thaw 5' RT primer, RT4 buffer, 0.1M DTT, and RT4 Enzyme on ice.

Reverse Transcription

1. Add 1.5 μ L **5' RT Primer** to each 5' End-Seq sample.
2. Briefly spin samples down on the mini-centrifuge.
3. Incubate in the thermocycler at 65 °C for 2 minutes.
4. Transfer to ice immediately.
5. Prepare the **Reverse Transcription Master Mix** according to **Table 6**.



Table 6. Reverse Transcription Master Mix (per sample)

Component	Volume (µL)
Molecular Biology Grade Water	4.3
RT4 Buffer	4
0.1 M DTT	1
RNase Inhibitor	0.2
RT4 Enzyme	0.5
Total:	10.0

6. Add 10.0 µL **Reverse Transcription Master Mix** to each sample. Keep on ice.
7. Flick samples to mix, briefly spin in mini-centrifuge.
8. Incubate in thermocycler at 54 °C for 20 minutes. Keep the lid at 65 °C.
9. Place samples on ice.

cDNA End Repair

1. Add 2.5 µL **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. Keep the lid at 45 °C.
4. Add 1 µL **0.5 M EDTA** to each sample.
5. Pipette to mix.
6. Add 3 µL **1 M NaOH** to each sample.
7. Pipette to mix.
8. Incubate in thermocycler at 70 °C for 10 minutes.
9. Place on ice for 2 minutes.
10. Add 3 µL of **1 M HCl** to each sample.
11. Proceed directly to the next step.

cDNA Bead Cleanup

- **Preparation Note:** Prepare fresh **80% Ethanol** in **Molecular Biology Grade** water in a fresh 50 mL tube if not done previously. Store at room temperature for up to 1 week. Keep tube closed tightly.
 - **Preparation Note:** Thaw **5' ssDNA Adapter** and **ssDNA Ligation Buffer**.
1. Take **eCLIP Beads** out of 4 °C and resuspend until homogeneous.



2. For each cDNA sample, transfer 5 μ L of **eCLIP Beads** to a new 1.5 mL DNA LoBind tube (e.g. for 4 samples transfer 20 μ L of eCLIP Beads).
3. Add 5 \times volume of **Bead Binding Buffer** (e.g., for 4 samples add 100 μ L buffer to 20 μ L of eCLIP Beads). Pipette to mix until the solution is homogeneous.
4. Place the tube on DynaMag-2 magnet. When separation is complete and supernatant is clear, carefully aspirate and discard supernatant without disturbing beads.
5. Remove the tube from magnet.
6. Resuspend eCLIP Beads in **92 μ L** of **Bead Binding Buffer** per sample. (e.g., for 4 samples add 368 μ L buffer).
7. Pipette up and down until beads are fully resuspended.
8. Add 90 μ L of washed **eCLIP Beads** to each cDNA sample.
9. Pipette mix until sample is homogeneous.
10. Add 67 μ L of **100% EtOH** to each cDNA sample.
11. Pipette mix until homogeneous. Transfer sample to new strip tube, this will yield a more defined bead pellet at the end.
12. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
13. Place the tube on magnet. Allow to incubate for 1-2 minutes or until separation is complete and liquid is transparent.
14. Carefully discard supernatant without disturbing beads.
15. Wash beads by adding 200 μ L of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet for about 45 seconds to wash.
16. While on the magnet, carefully discard supernatant without disturbing beads.
17. Repeat step 15-16 for a total of 3 washes.
18. Spin capped sample in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
19. Place the tube back on magnet.
20. Incubate on magnet for 10 seconds until separation is complete and supernatant is transparent.
21. Aspirate and discard all residual liquid without disturbing beads while on magnet.
22. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
 - **Note:** Do not over dry samples. Once completely dry, carefully remove tubes from magnet.
23. Resuspend beads in 2.5 μ L **5' ssDNA Adapter**. Pipette the ssDNA Adapter directly to beads.
24. Pipette to mix.
25. Incubate in thermocycler at 70 $^{\circ}$ C for 2 minutes. Keep lid at 75 $^{\circ}$ C.
26. Place sample on ice.



cDNA Ligation on Beads

1. Prepare the **cDNA Ligation Master Mix** according to **Table 7**.

Table 7. cDNA Ligation Master Mix (per sample)

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

2. Slowly add 7.8 μL of the **cDNA Ligation Master Mix** to each sample. The master mix is viscous.
3. Incubate all samples at room temperature overnight on a rotator.

Stopping Point: *Samples rotate overnight at room temperature for up to 16 hours.*



Chapter 5: 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 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 (Thaw and keep at room temperature)
- 50(5,6,7,8) Index Primer
- 70(5,6,7,8) Index Primer
- Bead Binding Buffer
- NEB LUNA Universal qPCR 2× Master Mix (Thaw at room temperature then keep on ice)
- qPCR Primers
- PCR mix
- AMPure XP beads
- 80% and 100% EtOH
- Molecular Biology Grade Water

Procedure

cDNA Bead Cleanup

- **Preparation Note:** Prepare fresh **80% Ethanol** in **Molecular Biology Grade** water in a fresh 50mL tube if not done previously. Store at room temperature for up to 1 week. Keep tube closed tightly.
1. Transfer 30 µL of **Bead Binding Buffer** to each sample containing beads and cDNA. Pipette mix until homogeneous.
 2. Add 23 µL of **100% EtOH** to each sample. Pipette mix.
 3. Transfer sample to new strip tubes.
 4. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.



5. Place tubes on magnet. Allow to incubate for 1-2 minutes or until separation is complete and liquid is transparent.
6. Carefully discard supernatant without disturbing beads.
7. Wash beads by adding 200 µL of **80% EtOH** without disturbing beads. Move sample to different positions on the magnet to wash.
8. Allow beads to settle and carefully discard supernatant without disturbing beads.
9. Repeat step 7-8 for a total of 3 washes.
10. Spin capped sample in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
11. Place the tube back on magnet.
12. Incubate on magnet for 10 seconds until separation is complete and supernatant is transparent.
13. Aspirate and discard all residual liquid without disturbing beads while on magnet.
14. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
 - **Note:** Do not over dry samples.
15. Once completely dry, carefully remove tube from magnet. Resuspend beads in 25 µL **Bead Elution Buffer**.
16. Incubate in room temperature for 5 minutes.
17. Place beads on magnet, allow 1 minute for beads to fully clear the elution buffer.
18. Transfer eluted cDNA to new 0.2 mL strip tubes.

Sample Quantification by qPCR

1. Prepare the **qPCR Master Mix** according to **Table 8**.

Table 8. qPCR Master Mix (per sample)

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



- Label a 96- or 384-well qPCR reaction plate (See Table 9. for suggested 96-well layout).

Table 9. 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 duplicate. Negative controls use water in place of cDNA.

- Dilute cDNA for qPCR** 10-fold: in a fresh strip-tube, **mix 9 μ L of Molecular Biology Grade water and 1 μ L of sample.**
- Add 9 μ L of **qPCR Master Mix** into all assay wells on ice.
- Add 1 μ L of each diluted cDNA sample (or Bead Elution Buffer for negative controls) into the designated well.
 - **Note:** Store remaining diluted cDNA samples at -20°C .
- Cover the plate with a MicroAmp adhesive film and seal with MicroAmp adhesive film applicator.
- Spin plate at $3,000 \times g$ for 1 minute.
- Run qPCR assay according to the user manual for the specific instrument in your laboratory.
- Run parameters appropriate for SYBR.
- Note: For example, for the StepOnePlus qPCR system the appropriate program is:
 - $95^{\circ}\text{C} - 30 \text{ sec}$
 - $(95^{\circ}\text{C} - 10 \text{ sec}, 65^{\circ}\text{C} - 30 \text{ sec}) \times 32 \text{ cycles}$; no melting curve
- Record qPCR Ct values for all samples.
- Set threshold to 0.5 – this recommendation is for StepOnePlus System.
 - **Note:** 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, Dual Index Addition and Bead Cleanup

- **Preparation Note:** For library pooling strategies, see Illumina documentation. Per lane, we recommend multiplexing at least 8 libraries with diverse indexes.
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 10** in fresh 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 10. 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:	40

3. Close strip tube, spin sample for 5 seconds to draw all liquid to the bottom of the tube. Keep on ice and quickly proceed to next step.

PCR Cycle Calculation

1. Refer to Ct values recorded to calculate the appropriate number of cycles for each sample according to **Table 11**. Use formula to calculate $N = Ct - 9$, where N is the number of cycles performed using the second (two-step) cycling conditions.

$$\text{Total cycles} = Ct - 3$$

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

$$\text{TOTAL number of PCR cycles for final library amplification} = 6 + N.$$

- **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 11. 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.

AMPure Library PCR Cleanup of 5' End-Seq Samples

- Allow AMPure XP beads to equilibrate at room temperature for 5 minutes.
- Manually shake AMPure XP beads to resuspend until homogeneous.
- Add 64 µL of **AMPure XP beads** into each 40 µL PCR sample.
- Pipette up and down until sample is homogeneous.
- Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
- Move samples to 96-well magnet.
- Incubate until separation is complete and supernatant is transparent.
- Carefully aspirate and discard supernatant without disturbing beads.
- Add 150 µL of **80% EtOH** to wash beads.
- Incubate on magnet for at least 30 seconds until supernatant is transparent.
- Move samples a few times to different positions on magnet to wash thoroughly.
- Aspirate and discard all supernatant.
- Repeat steps 9 - 12 for a total of two washes.
- Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
- Place tube back on 96-well magnet.



16. Incubate on magnet for 10 seconds until supernatant is transparent.
17. Aspirate and discard all residual liquid without disturbing beads.
18. Allow beads to air dry for 3 minutes or until beads no longer appear wet and shiny.
19. Once completely dry, carefully remove tubes from magnet.
20. For each sample, resuspend beads in 20 μ L of **Molecular Biology Grade** water.
 - **Note:** Do not use other buffers for final library.
21. Pipette up and down to mix until sample is homogeneous.
22. Incubate 5 minutes at room temperature.
23. After incubation, move tubes to 96-well magnet.
24. Incubate on magnet for 1 minute until supernatant is transparent.
25. Transfer 20 μ L to a new 0.2 mL strip tube. Discard beads.
26. 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 hours if pooling immediately*

Library Quantification

Libraries can be quantified using a variety of methods. This protocol has been optimized using Agilent4200 TapeStation, 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. See provider information or Illumina website for additional details, especially for sequencing color-balancing.

Protocol End



Appendix 1: Total RNA Isolation

Required Reagents

<i>Not Included in kit</i>	
Item	Source
Direct-zol RNA Isolation Kit or equivalent	Zymo cat. # R2071

Procedure

Total RNA Isolation

1. Obtain cells for RNA isolation either fresh or from frozen pellets.
2. Resuspend cells in 500 μ L of **TRI Reagent**.
3. Mix until sample is homogeneous.
 - **Note:** If sample is very viscous, add additional **TRI Reagent** in 100 μ L increments.
4. Centrifuge sample at 12,000 \times g at room temperature for 3 minutes to pellet cellular debris.
5. Transfer supernatant to a fresh 1.5 mL LoBind DNA tube. Supernatant may be viscous.
6. Add an equal volume of **100% Ethanol** to supernatant and pipette mix thoroughly.
7. Use one Zymo-Spin IIC column per 5 million cells used.
8. Split volume in increments of 600 μ L into each Zymo-Spin IIC column in a collection tube.
9. Centrifuge at 5,000 \times g for 1 minute or until all liquid has passed through filter.
10. Rebind flow-through a second time by transferring it from the collection tube back to the filter.
11. Centrifuge at 5,000 \times g for 1 minute or until all liquid has passed through filter.
12. Discard the flow-through.
13. Repeat with remaining lysate-ethanol mixture until entire volume has been transferred.
14. Add 400 μ L **RNA Wash Buffer** to each column. Centrifuge and discard flow-through.
15. In a new 1.5 mL DNA LoBind tube, prepare **DNase Master Mix** according to **Table 1**.

Table 1. DNase Master Mix (per sample)

Component	Volume (μ L)
Zymo DNA Digestion Buffer	75
Zymo DNase I	5
Total:	80



16. Add 80 μ L **DNase Master Mix** directly to each column and incubate at room temperature for 15 minutes.
17. Add 400 μ L **Direct-zol RNA PreWash** to each column.
18. Centrifuge at $5,000 \times g$ for 1 minute or until all liquid has passed through filter.
19. Discard the flow-through.
20. Repeat steps 14-16 for a total of two washes.
21. Add 700 μ L **RNA Wash Buffer** to column.
22. Centrifuge at $5,000 \times g$ for 1 minute or until all liquid has passed through filter.
23. Discard flow-through.
24. Add 350 μ L **RNA Wash Buffer** to column.
25. Centrifuge at $5,000 \times g$ for 1 minute or until all liquid has passed through filter.
26. Discard flow-through.
27. Repeat step 21-23 for a total of two washes.
28. Transfer column filter to a new collection tube.
29. Spin at $12,000 \times g$ for 2 min to dry column filter.
30. Transfer filter to a new 1.5 mL LoBind DNA tube.
31. Open cap and allow filter to air dry for 2 minutes.
32. Add 50 μ L of **Molecular Biology Grade** water to filter in each column to elute RNA.
33. Allow to incubate at room temperature for 1 minute.
34. Centrifuge at $12,000 \times g$ for 90 seconds.
 - **Note:** For high concentrations of RNA, elute in $<50 \mu$ L and $>25 \mu$ L
35. Re-elute RNA by transferring flow-through back to filter and centrifuge at $15,000 \times g$ for 3 min.
 - **Note:** If post-elution volume is $< 25 \mu$ L add an additional 30 μ L of Molecular Biology Grade water to filter and spin at $15,000 \times g$ for 3 minutes
36. Put samples on ice.

Optional Stopping Point: RNA samples to be stored at -80°C

Next stopping point: 2-3 hours

RNA and RIN Measurement Overview

Total RNA can be measured using a variety of methods. This protocol has been optimized using Agilent 4200 TapeStation with Agilent's High Sensitivity RNA ScreenTape, which measures both total RNA concentration and **RNA Integrity Number (RIN)**.

