3' End-Seq User Guide

RNA Genomics Solutions

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

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 3’ End-Seq

The End-Seq Kit transforms RNA analysis by streamlining mRNA isolation, mRNA end sample and library preparation. Using a robust and reproducible framework the 3’ End-Seq kit allows for detection, amplification, and sequencing of the 3' ends of mRNA molecules.

The 3’ End-Seq Kit is based on polyA selection of mRNA molecules followed by controlled enzymatic digestion of coding regions, reverse transcription of mRNA into cDNA and polymerase chain reaction amplification of ends. This method produces high quality libraries that enables the user to obtain detailed annotation of polyA sites.

The 3’ End-Seq 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
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 End-Seq experimental set up ONLY. See Appendix for supplemental information.*

Included with Kit

Item	Storage
Oligo(dT) Beads	4 °C
eCLIP Beads	4 °C
Bead Binding Buffer	4 °C
10X PNK Buffer	-80 °C
2× Hybridization Buffer (2× HyB)	-20 °C
mRNA Elution Buffer	-20 °C
Oligo(dT) Primer	-20 °C
3′ End-zyme	-20 °C
RNase Inhibitor Enzyme	-20 °C
DNase Enzyme	-20 °C
RT4 Enzyme	-20 °C
Nuclease Enzyme	-20 °C
Ligase Enzyme	-20 °C
PCR mix	-20 °C
ssDNA Enzyme	-20 °C
3′ Buffer	-80 °C
Bead Elution Buffer	-80 °C
RNA Ligation Buffer	-80 °C
ssDNA Ligation Buffer	-80 °C
0.1 M DTT	-80 °C
RT4 Buffer	-80 °C
Oligo(dT) Primer	-80 °C
3' RNA Adapter	-80 °C
3' ssDNA Adapter	-80 °C
3' RT Primer	-80 °C
qPCR Primers	-80 °C
Index primers	-80 °C

Equipment Not Included with Kit

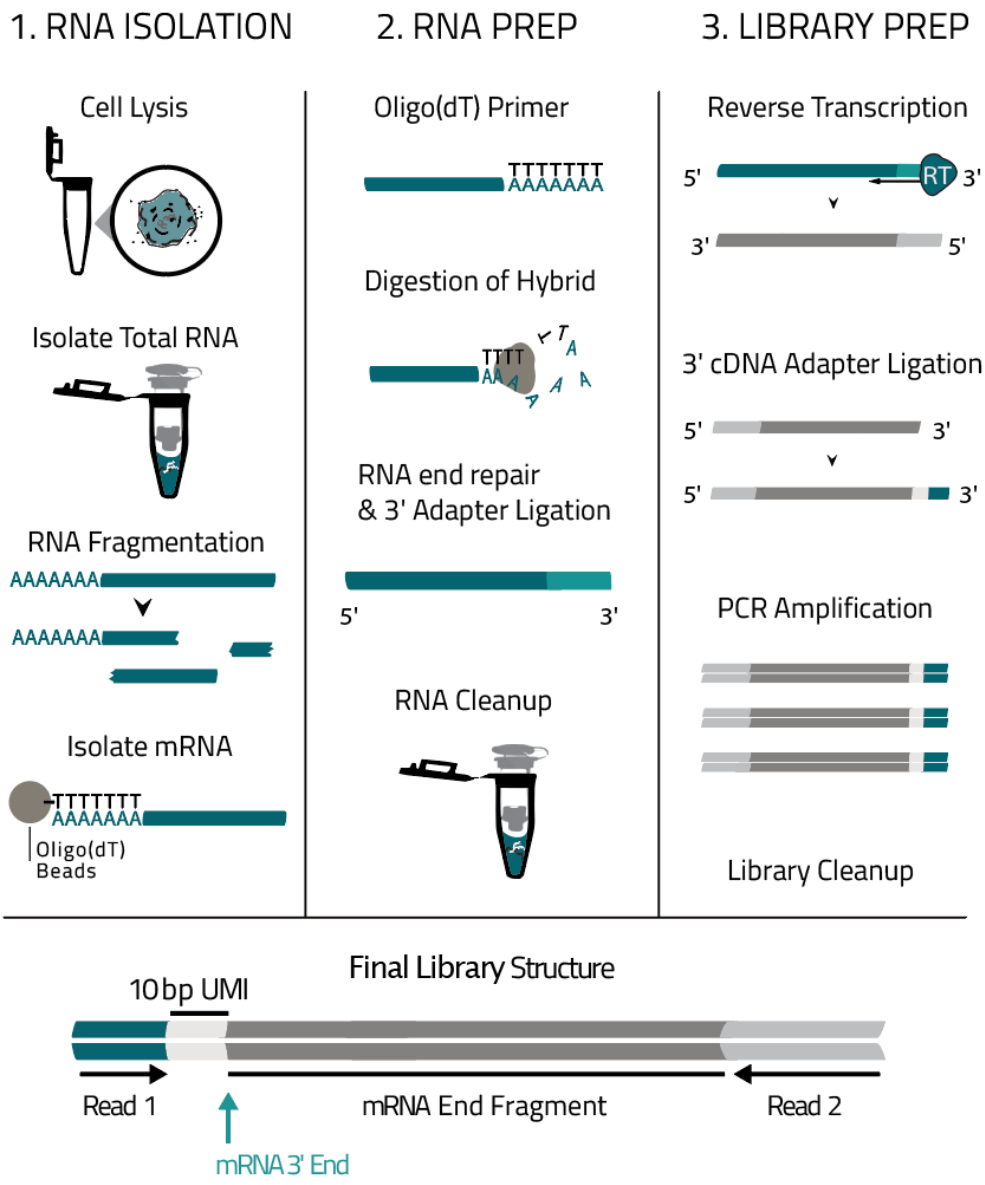
Item	Source
Micro-centrifuge 5424R or equivalent	Eppendorf
Mini-centrifuge or equivalent	Corning LSE
Tube Rotator	VWR cat. #10136-084
T100 Thermal Cycler or equivalent	BioRad cat. #1861096
StepOne qPCR or equivalent	ThermoFisher Scientific cat. #4376357
Eppendorf Thermomixer C	Eppendorf cat. #5382000015
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
AMPure XP beads	Agencourt

3' End-Seq Workflow

The following protocol is a method to enrich specifically for transcript 3′ ends and prepare libraries for sequencing. Sequencing of 3′ End-Seq libraries will map all alternative poly(A) sites. The 3′ End-Seq protocol takes approximately three days from RNA isolation to finished library.



Chapter 2: Poly(A)-RNA Isolation

Overview

This section describes heat fragmentation of total RNA followed by poly(A) selection (see **Appendix 1: Total RNA Isolation** for protocol). mRNA is then subjected to the 3' protocol.

Consumables

- 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)
- 10X PNK Buffer
- Molecular Biology Grade Water

Preparation

1. Centrifugation steps are done at room temperature.
2. Prewarm thermomixer to 60 °C.

Procedure

Total RNA Fragmentation of 3’ RNA Samples

The 3' End-Seq Protocol uses fragmented total RNA as starting material. 3 µg of total RNA is heat fragmented to 100-200 nucleotide fragments before mRNA is enriched by double oligo(dT) capture.

1. Aliquot 3 µg of total RNA per 3' Sample into new tubes.
2. Add **Molecular Biology Grade** water up to 40 µL.
3. Add 5 µL of **10X PNK Buffer** into each tube of diluted RNA.
4. Place samples at 95 °C for 8 min, then immediately place on ice.

Poly(A)-RNA Isolation

1. Transfer 3 µg of total RNA per sample to a new 1.5 mL LoBind DNA tube.
2. If volume of RNA is < 200 µL; bring volume up to 200 µL using **Molecular Biology Grade** water.
   - **Note**: If RNA volume exceeds 200 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 RNA in thermomixer for 2 minutes at 60 °C with interval mixing.
4. After incubation immediately place RNA samples on ice.
5. Transfer 100 µL of **Oligo(dT) beads** per sample into a new 1.5 mL LoBind DNA tubes. Keep RNA and beads separate.
6. Add 100 µL of **2× HyB** to each tube containing 100 µL 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 300 µL **2× 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 200 µL of **2× HyB**.
18. Pipette mix to combine until homogeneous.
19. Add entire volume (200 µL) of beads in **2× HyB** to 200 µL 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× HyB 5-fold according to **Table 1**.

Table 1. Dilution of 2× Hybridization Buffer (per sample)

Component	Volume (µL)
2x Hybridization Buffer (HyB)	300
Molecular Biology Grade water	1200
Total:	**1500**

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 µL 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 200 µL **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 200 µL of **2× HyB** into eluted mRNA samples containing original **Oligo(dT) beads** to have total volume of 400 µL.
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 µL of **diluted HyB** (**Table 1**).
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 20 µL **Molecular Biology Grade** water to bead pellet.

53. Pipette mix until homogeneous.
54. Incubate sample in thermomixer for 2 minutes at 60 ° 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 should be ~20 µL
56. Re-elute sample a second time by adding 21 µL **Molecular Biology Grade** water to the beads.
57. Pipette mix until homogeneous.
58. Place sample in thermomixer set at 60 °C with interval mixing.
59. Increase temperature to 70 °C, allow sample to transition temperatures
60. Incubate for a total of 3 minutes from the time the samples were placed on the thermomixer.
61. Immediately place tubes on magnet and pool all supernatant with sample from step 56 containing RNA without disturbing beads.
    - **Note**: Total volume of mRNA will be around 40 µL.

Chapter 3: 3’ End Sample Protocol

Overview

In this section of the 3’ End-Seq Protocol, Oligo(dT) primers are added to poly(A)+ mRNA, leaving poly(A) tails as double stranded molecules. The 3’ End-zyme is then used to trim away double stranded poly(A) tails.

Consumables

- Oligo(dT) Primer
- RNase Inhibitor
- 3’ Buffer
- 3’ End-zyme
- Molecular Biology Grade Water
- Zymo RNA Clean and Concentrator-5 Kit
- 100% EtOH

Procedure

Oligo(dT) Primer Annealing and 3’ End-zyme Digestion

1. Aliquot all eluted mRNA to a new 0.2 mL strip tube.
2. Prepare **Oligo(dT) Primer Master Mix** for each sample according to **Table 2.**

Table 2. Oligo(dT) Primer Master Mix (per sample)

Component	Volume (µL)
RNA Sample	40 (all)
Oligo(dT) Primer	2.5
3’ Buffer	5
Molecular Biology Grade Water	2.5
Total:	**50 µL**

3. Add 50 µL of **Oligo(dT) Primer Master Mix** to each sample. Mix thoroughly.
4. Incubate samples in thermocycler: 75 °C for 1 minute, 42 °C for 5 minutes, then slowly cooling to 32 °C (0.1 rate of cooling).
5. While the sample is incubating, prepare **3’ End-zyme Master Mix** according to **Table 3**.

Table 3. 3' End-zyme Master Mix (per sample)

Component	Volume (µL)
RNase Inhibitor	1
3' End-zyme	2
Total:	**3 µL**

6. When sample reaches 32 °C, add 3 µL **3' End-zyme Master Mix** to each sample**.** Mix thoroughly.
7. Incubate sample for 1 hour at 37 °C.
8. Place sample on ice. Sample volume should be 53 µL.

RNA Cleanup of 3' Samples

- **Note:** This section uses the Zymo RNA Clean and Concentrator-5 kit to clean RNA.

1. Add 100 µL of **RNA Binding Buffer** to the 53 µL of 3' mRNA 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 × g for 30 seconds. Discard flow-through.
5. Add 400 µL **RNA Prep Buffer** to each column.
6. Centrifuge at 7,000 × g for 30 seconds. Discard flow-through.
7. Add 480 µL **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 1 minute with emptied 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 µL 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. Transfer entire sample (~6 µL) to new 0.2 mL strip tube.
18. Store on ice if proceeding to the next step.

Optional Stopping Point: *If stopping here, 3' RNA samples should be stored at -80 °C.*

Next stopping point: ~2 hours

Chapter 4: 3’ End Sample Adapter Ligation

Overview

The previous chapters have described the selection of 3’ 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

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

Procedure

RNA Adapter Ligation

1. Aliquot entire sample (~6 µL) into new 0.2 mL strip tubes.
2. Place on ice.
3. Add 2 µL of **3' RNA Adapter** to each 3' sample.
4. Pipette to mix. Close caps and spin in mini centrifuge for 5 seconds.
5. Incubate tubes at 65 °C for 2 minutes in thermocycler.
6. Place on ice for 1 minute.
7. Prepare **RNA Ligation Master Mix** according to **Table 4**.

Table 4. RNA Ligation Master Mix (per sample)

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

8. Add 13.5 µL **RNA Ligation Master Mix** to each sample. Pipette to mix.
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** (provided) out of 4 °C and resuspend until homogeneous.
2. For each RNA sample, transfer 5 µL of eCLIP Beads to a new 1.5 mL DNA LoBind tube (e.g., for 4 samples transfer 20 µL of eCLIP Beads).
3. Add 5× volume of **Bead Binding Buffer** (e.g., for 4 samples 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.

6. Resuspend eCLIP Beads in **62 µL** of **Bead Binding Buffer** per sample.
7. Pipette mix until beads are fully resuspended.
8. Add 60 µL of washed **eCLIP Beads** to each RNA sample.
9. Pipette mix until sample is homogeneous.
10. Add 45 µL of **100% EtOH** to each RNA sample.
11. Pipette mix until homogeneous. Transfer samples to new strip tubes 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 with 200 µL of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet to wash.
16. Carefully discard supernatant without disturbing beads.
17. Add 200 µL of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet to wash.
18. Carefully discard supernatant without disturbing beads.
19. Repeat step 17-18 for a total of 3 washes.
20. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
21. Place tube back on 96-well magnet.
22. Incubate on magnet for 10 seconds until separation is complete and supernatant is transparent.
23. Aspirate and discard all residual liquid without disturbing beads while on magnet.
24. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
    - **Note**: Do not over dry samples.
25. Once completely dry, carefully remove tubes from magnet.
26. Resuspend beads in 10 µL of **Molecular Biology Grade** water.
27. Pipette to mix until homogeneous.
28. Incubate for 5 minutes at room temperature.
29. Place strip tubes with samples on magnet.
30. Transfer entire sample (~9 µL) from beads to new 0.2 mL strip tubes. Discard beads.
31. Place on ice.

Reverse Transcription

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

Table 5. Reverse Transcription Master Mix (per sample)

Volume (µL)	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 µL**

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 Thermo Cycler 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 Thermo Cycler 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 50mL tube if not done previously. Store at room temperature for up to 1 week. Keep tube closed tightly.
- **Preparation Note**: Thaw **3' ssDNA Adapter** and **ssDNA Ligation Buffer**.

1. Take **eCLIP Beads** (provided) out of 4°C and resuspend until homogeneous.
2. For each RNA sample, transfer 5 µL of **eCLIP Beads** to a new 1.5 mL DNA LoBind tube (e.g., for 4 samples transfer 20 µL of eCLIP Beads).
3. Add 5× volume of **Bead Binding Buffer** (e.g., for 4 samples 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.
6. Resuspend eCLIP Beads in **92 µL** of **Bead Binding Buffer** per sample.
7. Pipette up and down until beads are fully resuspended.
8. Add 90 µL of washed **eCLIP Beads** to each RNA sample.
9. Pipette up and down to mix until sample is homogeneous.
10. Add 67 µL of **100% EtOH** to each RNA sample.
11. Pipette mix until homogeneous. Transfer samples to new strip tube.
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 with 200 µL of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet to wash.
16. Carefully discard supernatant without disturbing beads.
17. Add 200 µL of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet to wash.
18. Carefully discard supernatant without disturbing beads.
19. Repeat step 17-18 for a total of 3 washes.
20. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
21. Place tube back on 96-well magnet.

22. Incubate on magnet for 10 seconds until separation is complete and supernatant is transparent.
23. Aspirate and discard all residual liquid without disturbing beads while on magnet.
24. 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.
25. Resuspend beads in 2.5 µL **3' ssDNA Adapter**.
26. Pipette to mix.
27. Incubate in thermocycler at 70 °C for 2 minutes. Keep lid at 75 °C.
28. Place samples on ice.

cDNA Ligation on Beads

1. Prepare the **cDNA Ligation Mix** according to **Table 6**.

Table 6. cDNA Ligation Master Mix (per sample)

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

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

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 Beads
- 80% and 100% EtOH
- Molecular Biology Grade Water

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 samples to new strip tube.
4. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
5. Allow to incubate for 1-2 minutes or until separation is complete and liquid is transparent.
6. Carefully discard supernatant without disturbing beads.

7. Pipette mix until homogeneous. Transfer samples to new strip tube.
8. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
9. Place tubes on magnet. Allow to incubate for 1-2 minutes or until separation is complete and liquid is transparent.
10. Carefully discard supernatant without disturbing beads.
11. Wash beads with 200 µL of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet to wash.
12. Carefully discard supernatant without disturbing beads.
13. Add 200 µL of **80% EtOH** without disturbing beads. Move samples to different positions on the magnet to wash.
14. Carefully discard supernatant without disturbing beads.
15. Repeat step 13-14 for a total of 3 washes.
16. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
17. Place tube back on 96-well magnet.
18. Incubate on magnet for 10 seconds until separation is complete and supernatant is transparent.
19. Aspirate and discard all residual liquid without disturbing beads while on magnet.
20. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
    - **Note**: Do not over dry samples.
21. Once completely dry, carefully remove tubes from magnet. Resuspend beads in 25 µL **Bead Elution Buffer**.
22. Incubate in room temperature for 5 minutes.
23. Place beads on magnet, allow 1 minute for beads to fully clear the elution buffer.
24. Transfer eluted cDNA to new 0.2 mL strip tubes.

Sample Quantification by qPCR

1. Prepare the **qPCR Master Mix** according to **Table 7**.

Table 7. qPCR Master Mix (per sample)

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

2. Label a 96- or 384-well qPCR reaction plate (**See Table 8** for suggested 96-well layout).

Table 8. 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.

1. **Dilute cDNA for qPCR** 10-fold: in a fresh strip-tube, **mix 9 µL of Molecular Biology Grade** water and **1 µL of sample.**
2. Add 9 µL of **qPCR Master Mix** into all assay wells on ice.
3. 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.
4. Cover the plate with a MicroAmp adhesive film and seal with MicroAmp adhesive film applicator.
5. Mix, then spin plate at 3,000 × g for 1 minute.
6. Run qPCR assay according to the user manual for the specific instrument in your laboratory.
7. Run parameters appropriate for SYBR.
8. 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
9. Record qPCR Ct values for all samples.
10. 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 9** 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 9. 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 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.

PCR Cycle Calculation

1. Refer to Ct values recorded to calculate the appropriate number of cycles for each sample according to **Table 10.** Use formula to calculate N = Ct – 9, where N is the number of cycles performed using the second (two-step) cycling conditions.

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

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

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 10. 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**.

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

AMPure Library PCR Cleanup of 3' Samples

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** into each 40 µL PCR sample.
4. Pipette up and down until sample is homogeneous.
5. 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 150 µL of **80% EtOH** to wash beads.
10. Incubate on magnet for at least 30 seconds until supernatant is transparent.
11. Move samples a few times to different positions on magnet to wash thoroughly.
12. Aspirate and discard all supernatant.
13. Repeat steps 9 - 11 for a total of two 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 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 µL of **Molecular Biology Grade** water.
21. Pipette to mix until sample is homogeneous.
    - **Note**: Do not use other buffers for final library.
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 µL to a new 0.2 mL strip tube.
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

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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

Not Included in kit	
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 µL of **TRI Reagent**.
3. Mix until sample is homogeneous.
   - **Note**: If sample is very viscous, add additional **TRI Reagent** in 100 µL increments.
4. Centrifuge sample at 12,000 × 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 µL into each Zymo-Spin IIC column in a collection tube.
9. Centrifuge at 5,000 × 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 × 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 µL **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**.
16. Add 80 µL **DNase Master Mix** directly to each column and incubate at room temperature for 15 minutes.

Table 1. DNase Master Mix (per sample)

Component	Volume (µL)
Zymo DNA Digestion Buffer	75
Zymo DNase I	5
Total:	**80 µL**

17. Add 400 µL **Direct-zol RNA PreWash** to each column.
18. Centrifuge at 5,000 × 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 µL **RNA Wash Buffer** to column
22. Centrifuge at 5,000 × g for 1 minute or until all liquid has passed through filter.
23. Discard flow-through.
24. Add 350 µL **RNA Wash Buffer** to column
25. Centrifuge at 5,000 × 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 × 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 µL 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 × g for 90 seconds.
    - **Note**: For high concentrations of RNA, elute in <50 µL and >25 µL
35. Re-elute RNA by transferring flow-through back to filter and centrifuge at 15,000 × g for 3 min.
    - **Note:** If post-elution volume is < 25 µL add an additional 30 µL of Molecular Biology Grade water to filter and spin at 15,000 × 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 **R**NA **I**ntegrity **N**umber (**RIN**).