

# 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 3' 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 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 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 ends. This method produces high quality libraries that enables the user to obtain detailed annotation of poly(A) sites.

The 3' 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 3' End-Seq libraries requires RIN above 3.

The 3' 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	
lce	0 – 4 °C	
Freeze	-80 °C	
Volume units		
μL is microliter	One millionth (10 <sup>-6</sup> ) of a liter	
<b>mL</b> is milliliter	One thousandth (10 <sup>-3</sup> ) 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.

<u>IMPORTANT:</u> Materials listed below are for 3' 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
Nuclease Enzyme	EZ-005	-20 °C
Ligase Enzyme	EZ-007	-20 °C
ssDNA Enzyme	EZ-010	-20 °C
RT4 Enzyme	EZ-011	-20 °C
3' End-zyme	EZ-013	-20 °C
PCR Mix	EZ-015	-20 °C
3' Buffer	BU-006	-80 °C
RT4 Buffer	BU-008	-80 °C
10X PNK Buffer	BU-012	-80 °C
0.1 M DTT	RM-020	-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
3' RNA Adapter	EB-AD-015	-80 °C
3' ssDNA Adapter	EB-AD-016	-80 °C
3' RT Primer	EB-PR-017	-80 °C
Oligo(dT) Primer	EB-PR-016	-80 °C
qPCR Primers	EB-PR-014	-80 °C
Index primers	(EB-PR-028 to EB-PR-031)	-80 °C
index primers	(EB-PR-035 to EB-PR-038)	-00 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	EdgeBio, cat. #57624
DynaMag-96 Side Magnet	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	ThermoFisher cat. #4346906
or equivalent	ווופווווטרואוופו למנ. #4540500
Falcon 15 mL Conical Centrifuge tubes	Fisher Scientific cat. #14-959-53A
Falcon 50 mL Conical Centrifuge tubes	Fisher Scientific cat. #14-432-22



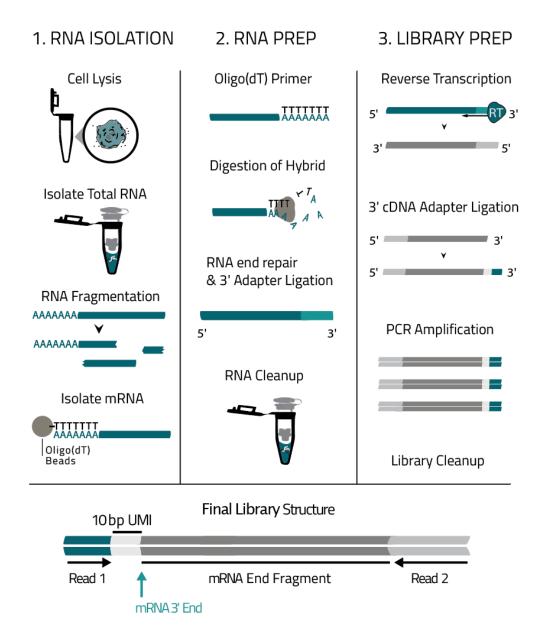
## **Reagents Not Included with Kit**

Item	Source
Ethanol, Pure, 200 proof, for Molecular	Sigma-Aldrich cat. #E7023-1L
Biology	Signa-Alunch cat. #E7023-1E
Nuclease-free Molecular Biology Grade	Corning/VWR cat. #95000-094
Water or UltraPure™ DEPC-Treated Water	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 (HCI)	Any
Agencourt AMPure XP	Beckman Coulter cat. #A63881
NEB LUNA Universal qPCR 2× Master Mix	New England BioLabs cat. #M3003S



# 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 3ug of total RNA to 100-200 nucleotide fragments followed by double oligo(dT) poly(A) selection (see <u>Appendix 1: Total RNA Isolation</u> for protocol). mRNA is then subjected to the 3' 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)
- > 10X PNK Buffer

#### **Preparation**

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

#### **Procedure**

#### Total RNA Fragmentation of 3' RNA Samples

- 1. For each sample transfer 3 µg of total RNA to a new 1.5 mL LoBind DNA tube.
- 1. Bring the sample volume up to 40 μL using **Molecular Biology Grade** water.
- 2. Add 5 μL of **10X PNK Buffer** into each tube of diluted RNA.
- 3. Incubate the RNA sample in thermomixer for 8 minutes at 95 °C, then immediately place on ice.
- 4. Immediately proceed to the next step.



#### Poly(A)-RNA Isolation

- 1. Transfer 3 μg of fragmented total RNA per sample (from step 3 in previous section), to a new 1.5 mL LoBind DNA tube.
- 2. Bring 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 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
- 6. Add 300 µL of **2× HyB** to each 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 μL **2× HyB** per sample.
  - Note: For 2 x 3' End-Seq samples transfer 2 x 100 μL of **2× 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 μL of **2× HyB** per sample.
  - Note: For 2 x 3' End-Seq samples transfer 2 x 100 μL of **2× HyB** to the tube with beads.
- 18. Pipette mix to combine until homogeneous.
- 19. Add 100 μL of beads in **2× HyB** to each tube containing 100 μL of denatured RNA (from step 4). Total volume per 3' End-Seq sample will be 200 μL.
- 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× HyB 5-fold according to **Table 1.**



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

Component	Volume (µL)
2× 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 µL 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 tube 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. Place the tube on magnet.
- 32. Discard supernatant.
- 33. Resuspend beads in 100 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 100 μL of **2× HyB** into eluted mRNA samples containing original **Oligo(dT) beads** for a total volume of 200 μL.
- 38. Incubate 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 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 tube on magnet as beads start to separate to capture any 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 µL **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 the tube 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 μL
- 56. Proceed to next step.



# **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% FtOH

#### **Procedure**

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

- 1. Aliquot all eluted, fragmented mRNA samples to new 0.2 mL strip tubes. Sample volume will be ~9 µL.
- 2. Add **Molecular Biology Grade** water up to 40 μL.
- 3. Prepare Oligo(dT) Primer Master Mix according to Table 2.

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

Component	Volume (µL)
Oligo(dT) Primer	2.5
3' Buffer	5
Molecular Biology Grade Water	2.5
Total:	10

- 4. Add 10 μL of **Oligo(dT) Primer Master Mix** to each sample. Mix thoroughly.
- 5. Incubate sample in thermocycler at 75 °C for 1 minute, 42 °C for 5 minutes, then slowly cooling to 32 °C (0.1 rate of cooling).
- 6. 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

- 7. When sample reaches 32 °C, add 3  $\mu$ L **3' End-zyme Master Mix** to each sample. Pipette mix to combine.
- 8. Incubate sample for 1 hour at 37 °C.
- 9. Place sample on ice.

#### RNA Cleanup of 3' 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 53 μL of each 3' 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.
- 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  $12,000 \times g$  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-Seq 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

- Molecular Biology Grade Water
- > 3' RNA Adapter
- > RNA Ligation Buffer
- RNase Inhibitor
- eCLIP Beads
- > 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

#### **Preparation**

- 1. Thaw RNA ligation buffer and 3' 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 tubes.
- 2. Place the tube on ice.



- 3. Add 2 µL of **3' RNA Adapter** to each sample. Pipette mix to combine.
- 4. 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

- 8. Add 13.5 µL RNA Ligation Master Mix to each sample. Pipette mix to combine...
- 9. Spin down sample briefly in mini centrifuge.
- 10. Incubate sample 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 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 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 μL** of **Bead Binding Buffer** per sample (e.g., for 4 samples add 248 μL of buffer).
- 7. Pipette mix until beads are fully resuspended.
- 8. Add 60 µL of washed **eCLIP Beads** to each sample.
- 9. Pipette mix until sample is homogeneous.



- 10. Add 45  $\mu$ L of **100% EtOH** to each 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 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 µL of **Molecular Biology Grade** water.
- 25. Pipette to mix until homogeneous.
- 26. Incubate for 5 minutes at room temperature.
- 27. Place strip tubes with samples on magnet.
- 28. Transfer entire sample (~9 µL) from beads to new 0.2 mL strip tubes. Discard beads.
- 29. Place the tube on ice.

#### **Preparation**

1. Thaw 3' RT primer, RT4 buffer, 0.1M DTT, and RT4 Enzyme on ice.

#### **Reverse Transcription**

- 1. Add 1.5 µL **3' RT Primer** to each 3' End-Seq 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)

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 HCI** 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** out of 4°C and resuspend until homogeneous.



- 2. For each cDNA sample, transfer 5  $\mu$ L of **eCLIP Beads** to a new 1.5 mL DNA LoBind tube (e.g., for 4 samples transfer 20  $\mu$ L 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 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 μL** of **Bead Binding Buffer** per sample (e.g., for 4 samples add 368 μL buffer).
- 7. Pipette up and down until beads are fully resuspended.
- 8. Add 90 µL of washed **eCLIP Beads** to each cDNA sample.
- 9. Pipette mix until sample is homogeneous.
- 10. Add 67 µL 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 with 200 µL 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 µL **3' ssDNA Adapter**. Pipette the ssDNA Adapter directly to beads.
- 24. Pipette to mix.
- 25. Incubate in thermocycler at 70 °C for 2 minutes. Keep lid at 75 °C.
- **26.** Place sample 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

- 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 samples 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 samples 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 samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
- 11. Place the tube back on 96-well 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 tubes 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 7.** 

Table 7. qPCR Master Mix (per sample)

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 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
Α												
В	water	water	water	water	water	water	water	water	water	water	water	
С	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	
н												

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

- 3. **Dilute cDNA for qPCR** 10-fold: in a fresh 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 sample (or Bead Elution Buffer for negative controls) into the designated well.
  - Note: Store remaining diluted cDNA samples at -20 °C.
- 6. Cover the plate with a MicroAmp adhesive film and seal with MicroAmp adhesive film applicator.
- 7. Spin plate at  $3,000 \times g$  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:

 $(95 \, ^{\circ}\text{C} - 10 \, \text{sec}, 65 \, ^{\circ}\text{C} - 30 \, \text{sec}) \times 32 \, \text{cycles};$  no melting curve

- 11. Record qPCR Ct values for all samples.
- 12. 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 minicentrifuge 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, 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**

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.

Total cycles = 
$$Ct - 3$$
  
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 Ct = 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			
70°C	30 seconds	6		
72°C	40 seconds			
Extra N cycles (N = Ct value – 9)				
98°C	15 seconds	N*		
72°C	45 seconds	IV		
72°C	1 minute			
4°C	∞			
Total number of PCR cycles 6+N				

\*N should be  $\geq$  1 and < 14.

- 2. Run PCR for the specific number of cycles calculated for each sample.
- 3. Immediately put samples on ice.

#### AMPure Library PCR Cleanup of 3' End-Seq Samples

- 1. Allow AMPure XP beads 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 12 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.
  - Note: Do not use other buffers for final library.
- 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. 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**

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  $12,000 \times g$  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 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.
  - ightharpoonup **Note:** If post-elution volume is < 25  $\mu$ L add an additional 30  $\mu$ 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 **R**NA Integrity **N**umber **(RIN)**.

