ECLIPSEBIO
THE RNA GENOMICS COMPANY

m6A-eCLIP™ 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 m6A-eCLIP

The m6A-eCLIP (enhanced CrossLinking ImmunoPrecipitation) kit transforms RNA analysis by streamlining mRNA isolation, eCLIP sample prep, and library prep, using a robust and reproducible framework to identify and map methylation sites on target RNA. The methylated RNA is immunoprecipitated using a highly specific Eclipse Bioinnovations m6A antibody. RNA is chemically fragmented into 100 nucleotides or smaller fragments to generate high quality libraries.

The m6A-eCLIP kit utilizes the eCLIP technology based on the Van Nostrand et al. (Nature Methods, 2016) method which produces high quality libraries that can enable the user to achieve up to single nucleotide resolution.

The m6A-eCLIP kit offers:

High throughput and robust workflow

High reproducibility with accurate data

Unbiased and high specificity

Important Note

Before initiating this procedure thoroughly review the required equipment and materials list.

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. High-Salt Buffer contains SDS and other detergents. 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.
- Do not allow antibody-coupled beads to dry between wash steps.
- 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 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 100 ng to 2 µg of starting mRNA.

IMPORTANT: *Materials listed below are for m6A-eCLIP experimental set up ONLY. Additional equipment, materials and reagents are required for protein gel electrophoresis, membrane transfer, western blotting and imaging, and quantification of samples. See Appendix for supplemental information*.

Included with Kit

Item	Storage
Nitrocellulose membranes	Room temperature
Oligo(dT) beads	4 °C
2× Hybridization Buffer (2× HyB)	-20 °C
mRNA Elution Buffer	-20 °C
m6A Coupling Buffer	-20 °C
DNase Buffer	-20 °C
eCLIP Beads	4 °C
Bead Binding Buffer	4 °C
RNase Inhibitor Enzyme	-20 °C
DNase Enzyme	-20 °C
PSP Enzyme	-20 °C
RT Enzyme	-20 °C
Nuclease Enzyme	-20 °C
PNK Enzyme	-20 °C
Ligase Enzyme	-20 °C
Proteinase Enzyme	-20 °C
PCR mix	-20 °C
ssDNA Enzyme	-20 °C
High-Salt Buffer (HSB)	-20 °C
25× NoS (No-Salt) Buffer Concentrate	-20 °C
Proteinase Buffer	-20 °C
PSP Buffer	-80 °C
IP PNK Buffer	-80 °C
Bead Elution Buffer	-80 °C
Library Elution Buffer	-80 °C
RNA Ligation Buffer	-80 °C
ssDNA Ligation Buffer	-80 °C
2× Western Buffer	-80 °C
Input PNK Buffer	-80 °C
RT Buffer	-80 °C
IP RNA Adapter	-80 °C
Input RNA Adapter	-80 °C
ssDNA Adapter	-80 °C
RT Primer	-80 °C
qPCR Primers	-80 °C
Index primers	-80 °C

m6A IP antibody	4 °C
Protein G beads	4 °C

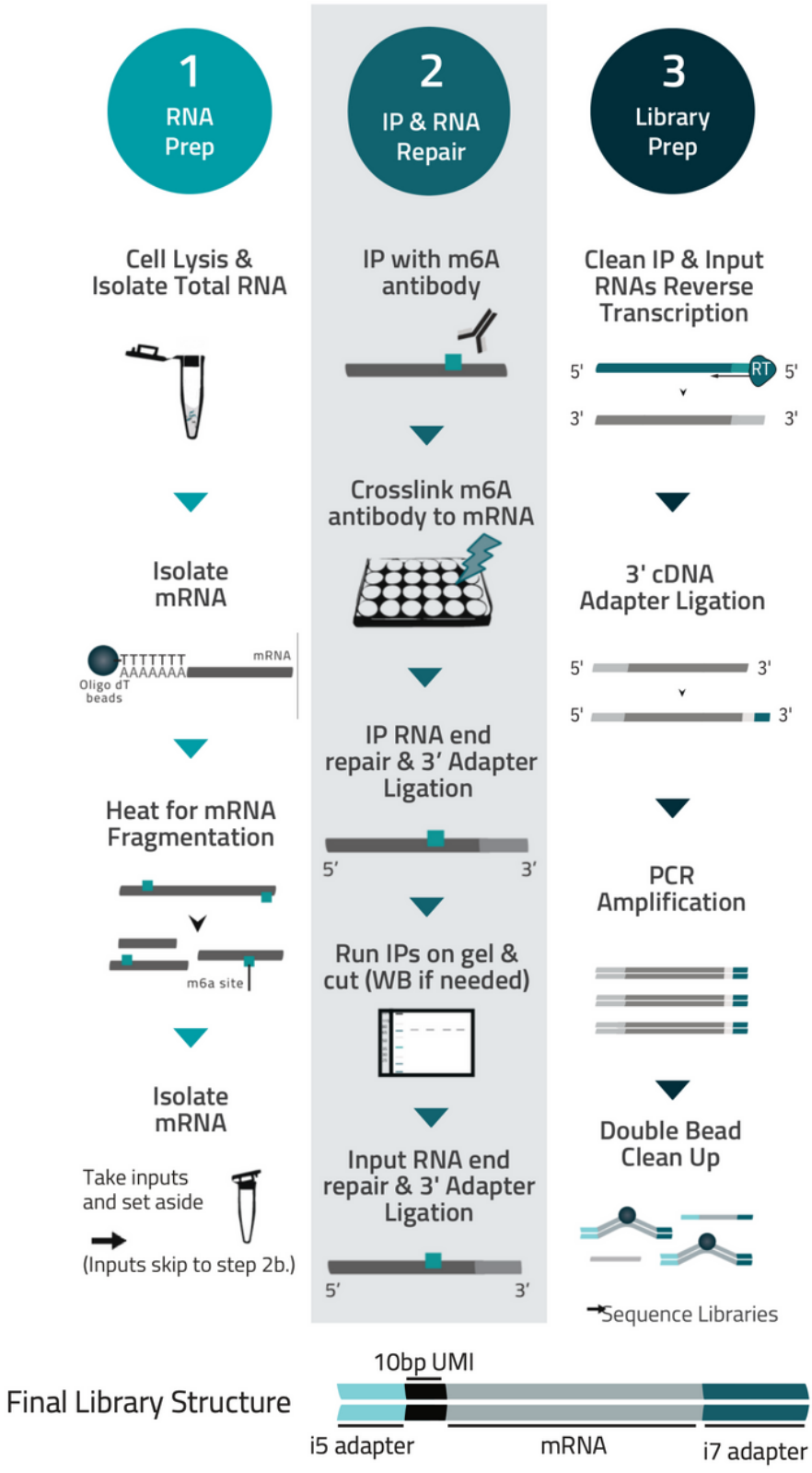
Equipment Not Included with Kit

Item	Source
Micro-centrifuge 5424R or equivalent	Eppendorf
Mini-centrifuge or equivalent	Corning LSE
254 nm UV-C Crosslinker	Fisher Scientific cat. #UVP95017401
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
Razor Blades, Surgical Carbon	Genesee Scientific cat. #38-100
Reagent Reservoirs	ThermoFisher cat. #95128093
RNA Clean & Concentrator -5	Zymo Research cat. #R1015/R1016
Direct-zol RNA MiniPrep or equivalent	Zymo Research cat # R2050
MinElute Gel Extraction Kit	Qiagen cat. #28604
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
Pyrex dish	Corning cat. #3175-10
24-well cell culture plates	ThermoFisher cat. #174899
Mini-Protean Tetra cell and supplies	BioRad cat. #165-8001
PowerPac HC Power Supply	BioRad cat. #1645052
Western Blotting Filter Paper, 7 cm × 8.4 cm	ThermoFisher cat. #84783
Polypropylene, Nalgene Tray	TaylorScientific cat. #14-6389-01

Reagents Not Included with Kit

Reagent	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
Spectra Multicolor Broad Range Protein Ladder	ThermoFisher Scientific cat. #26634
Pierce 20× TBS Tween 20 Buffer	ThermoFisher Scientific cat. #28360
20× NuPAGE Transfer Buffer	ThermoFisher Scientific cat. #NP00061
Methanol	Sigma-Aldrich cat. #494437

m6A-eCLIP Assay Workflow



Chapter 2 Poly(A) Selection

Overview

This section describes poly(A) selection from total RNA (see Appendix D: Total RNA Isolation for protocol). Here, mRNA is enriched by double Oligo(dT) capture to select for polyadenylated transcripts. 500 ng of final mRNA is then heat fragmented to 100-200 nucleotide pieces – 10 ng of the fragmented sample is saved for total RNA-seq (Input), and the remaining 490 ng is coupled to the m6A IP antibody (IP sample).

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)
- DNase Buffer (Thaw at room temperature then store on ice)
- RNase Inhibitor enzyme
- DNase enzyme
- Molecular Biology Grade Water
- 100% Ethanol

Preparation

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

Procedure

Poly(A)-RNA Isolation

1. Transfer **50 µg of total RNA** to a new 1.5 mL LoBind DNA tube.
2. If volume of RNA is less than 200 µL; bring volume up to 200 µL using **Molecular Biology Grade water**. 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 200 µL of **Oligo(dT) beads** into new 1.5 mL LoBind DNA tubes for each sample.
6. Add **100 µL** of **2× HyB** to each tube containing 200 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** to each 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 200 µL volume 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 according to **Table 1**.
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**).

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

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

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 40 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. Magnetize immediately and transfer all supernatant to a new 1.5 mL LoBind DNA tube without disturbing beads and place on ice.
    - Note: Volume pulled from beads should be ~40 µL
56. Re-elute sample a second time by adding 41 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 (starting from when temperature is increased) on thermomixer.
61. Magnetize immediately and pool all supernatant with 1.5 mL LoBind DNA tube containing RNA (step 36) without disturbing beads.
    - Note: Total volume of mRNA will be around 80 µL.
62. Take ~80 µL sample into the following section (Measure mRNA Concentration).

Measure mRNA Concentration

mRNA 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 is based on the ratio of 28S rRNA to 18S rRNA. Oligo dT beads select out mRNA, so RIN is expected to be low due to depletion of 28/18S rRNA, but concentration of mRNA is still applicable. Expected mRNA yield is 1-3% of total RNA. For 50 µg starting RNA, expect 500 ng to 1.5 µg of final mRNA. Take sample into following section (mRNA Fragmentation)

Optional Stopping Point: *RNA samples to be stored at 80℃*
Next stopping point: 1 hour

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mRNA Fragmentation

1. Aliquot 420 ng of eluted mRNA to new signed 0.2 mL PCR tube strip and prepare **mRNA fragmentation mix** for each sample according to **Table 2**.

Table 2. mRNA fragmentation Mix (per sample)

Component	Volume (µL)
RNA + Molecular Biology Grade Water	67
DNase Buffer	8
RNase Inhibitor	2
DNase	3
Total:	*80*

2. Mix sample well.
3. Incubate samples in PCR machine: 37 °C for 10 minutes, 95 °C for 16 minutes and 5 °C for 10 sec, with lid at 98 °C.
4. Place samples on ice after incubation.

Chapter 3: Crosslink mRNA to m6A Antibody

Overview

In this section, Input material is saved and Immunoprecipitation samples are prepared for crosslinking. Fragmented RNA samples are first coupled with an m6A IP antibody and then crosslinked to the bound RNA fragments by UV irradiation. Lastly, samples are coupled overnight with protein G beads.

Consumables

- m6A IP antibody
- High-Salt Buffer (HSB) (Thaw at room temperature then store on ice)
- m6A Coupling Buffer (Thaw at room temperature then store on ice)
- RNase Inhibitor enzyme
- Molecular Biology Grade Water
- Protein G beads

Preparation

1. Thaw **m6A Coupling Buffer and High-Salt Buffer (HSB),** at room temperature then store on ice.
2. 254 nm UV-C mercury (Hg) bulbs **must** be used during crosslinking.

Procedure

- **Preparation Note**: Save Input material. Calculate concentration of fragmented mRNA (e.g., If 420 ng starting mRNA was used, the concentration should be (420 ng / 80 µL) = 5.25 ng/µL). Take 20 ng fragmented mRNA and freeze at -80 °C as Input sample.

Antibody-Coupling

1. Dilute 400 ng of fragmented mRNA with **m6A Coupling Buffer** to 393 µL.
2. Add 4 µL **RNase inhibitor enzyme** to each sample.
3. Add 3 µL (3 µg) **m6A IP antibody** to each sample. Total sample volume should be 400 µL.
4. Rotate for at least 2 hours up to 12 hours at **4 °C**.

Preparation of Protein G Beads for Coupling

1. Mix **Protein G beads** until homogeneous.
2. Transfer 10 µL of **Protein G beads** per sample into a 1.5 mL LoBind tube (e.g., for 8 samples use 80 µL of Protein G beads).
3. Dilute **HSB** according to **Table 3.** Invert to mix then store on ice.

Table 3. High-Salt Buffer (HSB) Dilution

Component	Volume (mL)
Molecular Biology Grade water	9
High-Salt Buffer	1
Total:	*10**

*this volume is sufficient for 9 samples

4. Add 5× volume (50 µL per sample) of chilled **diluted HSB** to the tube containing **Protein G beads.**
5. Place tube on DynaMag-2 magnet.
6. After separation is complete, discard supernatant without disturbing beads.
7. Remove tube from magnet.
8. Add 500 µL chilled **diluted HSB** to the tube.
9. Invert mix until homogeneous.
10. Place the tube on DynaMag-2 magnet.
11. After separation is complete, discard supernatant without disturbing beads.
12. Repeat steps 8-11 for a total of two washes.
    - **Note**: Do not discard undiluted **HSB**, store at 4 °C after use.
13. Remove tube from magnet and add 51 µL chilled **m6A Coupling Buffer** per sample to the tube (e.g., for 6 samples, add 306 µL chilled **m6A Coupling Buffer**).
14. Resuspend by pipetting until homogeneous.
15. Store on ice until samples are ready for bead-coupling.
    - Beads will be used in **Coupling Crosslinked Antibody-mRNA Complex to Protein G Beads** section.

Crosslink IP Samples and Couple Antibody-mRNA Complexes to Protein G Beads

1. Label 24-well plate according to samples being crosslinked.
2. Prepare a thin, flat layer of ice in a glass Pyrex dish and place a 24-well plate on top to chill. If not using flaked ice, add ~0.5 cm of water to ensure entire bottom of plate is in contact with either ice or ice water.
3. Transfer all antibody-coupled mRNA samples to each corresponding well on 24-well plate (from step **Antibody-Coupling section** step 4).
4. Place Pyrex dish containing 24-well plate (without lid) and samples into 254 nm UV-C Ultraviolet Crosslinker
5. Crosslink **twice** at Energy = 1500 (150 mJ/cm^2), removing dish containing samples from crosslinker between the rounds of crosslinking, allowing plate to cool on ice for 15-30 seconds.
6. Remove dish containing samples from Crosslinker allowing plate to cool on ice for 15-30 seconds.
7. Carefully transfer crosslinked samples to new 1.5 mL LoBind tubes and place on ice.

Coupling Crosslinked Antibody-mRNA Complexes to Protein G Beads

1. Resuspend washed Protein G beads (prepared in **Preparation of Protein G Beads for Coupling** section step 13) by pipetting.
2. Add 50 µL of resuspended Protein G bead solution to each crosslinked sample (**Crosslink IP Samples and Couple Antibody-mRNA Complexes to Protein G Beads section** step 5).
3. Rotate overnight at 4 °C.
   - **Preparation Note**: It is recommended to prepare **1× NoS Buffer (Table 4)** in advance for Western blot running and store at 4 °C overnight.

Table 4. 1× NoS Buffer Preparation

Component	Volume (mL)
Molecular Biology Grade Water	48
25× NoS (No-Salt) Buffer Concentrate	2
Total:	*50*

Chapter 4: Immunoprecipitation (IP) of Samples

Overview

This section describes immunoprecipitation and end repair of RNA samples, followed by ligation an adapter to the 3' end of bound transcripts.

Consumables

- PSP Buffer (Thaw at room temperature then store on ice)
- IP PNK Buffer (Thaw at room temperature then store on ice)
- RNA Ligation Buffer
- IP RNA Adapter
- PSP enzyme
- PNK enzyme
- RNase Inhibitor enzyme
- DNase enzyme
- Ligase enzyme
- 1× NoS (No-Salt) Buffer (Thaw at room temperature then store on ice)
  Undiluted High-Salt Buffer (HSB) (Thaw at room temperature then store on ice)
- Molecular Biology Grade Water

Preparation

1. Pre-warm Thermomixer to 37 °C.
2. Dilute 25× NoS (No-Salt) Buffer Concentrate to 1× **(Table 4)** if not previously prepared.

Procedure

First Immunoprecipitation Wash

1. Obtain immunoprecipitation (IP) tubes from (**Coupling Crosslinked Antibody-mRNA Complexes to Protein G Beads** step 3)
2. Put on DynaMag-2 magnet to separate beads,
3. Allow at least 1 minute for bead separation.
4. When separation is complete and liquid is transparent, carefully aspirate and discard supernatant without disturbing beads.
5. Remove IP tubes from magnet
6. Add 500 µL cold **HSB**.
7. Invert mix until homogeneous
8. Place on DynaMag-2 magnet.
9. While on magnet, slowly invert closed tubes as beads start to separate to capture any beads from cap.

10. When separation is complete and liquid is transparent, gently open tubes and discard supernatant without disturbing beads.
11. Remove IP tubes from magnet and add 500 µL cold **HSB**.
12. Close tubes well and vortex for 15 seconds.
13. Incubate on tube rotator for 3 min at room temperature, then place on magnet.
14. While on magnet, slowly invert closed tubes as beads start to separate to capture any beads from cap.
15. When separation is complete, and liquid is transparent, gently open tubes and discard supernatant without disturbing beads.
16. **Repeat steps 11-15 for additional round of wash.**
17. Remove IP tubes from magnet.
18. Add 500 µL cold **1× NoS Buffer**.
19. Gently invert mix until homogeneous.
20. Separate beads on magnet and remove supernatant without disturbing beads.
21. Remove IP tubes from magnet.
22. Add 500 µL cold **1× NoS Buffer**.
23. Gently invert mix until homogeneous.
24. Place samples back on magnet and allow 1 minute to separate.
25. When separation is complete, and liquid is transparent, gently open tubes and discard supernatant without disturbing beads.
26. **Spin all IP samples in mini-centrifuge for 3 seconds.**
27. Pipette and discard any excess liquid without disturbing beads.
28. Remove IP tubes from magnet.
29. Add 500 µL cold **1× NoS Buffer.**
30. Invert to mix until homogeneous.
31. Place samples on ice and proceed immediately to the next step.

IP RNA 5'-end repair

1. Prepare **IP PSP Master Mix** according to **Table 5** in a fresh 1.5 mL DNA LoBind tube. Pipette mix to combine and store on ice until use.

- **Note:** Include 3% excess volume to correct for pipetting losses.

Table 5. IP PSP Master Mix (per sample)

Component	Volume/ IP (µL)
Molecular Biology Grade Water	23
PSP Buffer	20
RNase Inhibitor	2
DNase	2
PSP enzyme	3
Total:	***50***

2. Move all IP tubes from ice to DynaMag-2 magnet and allow at least 1 minute for bead separation.

3. Remove and discard supernatant.
4. Spin all samples in mini-centrifuge for 3 seconds.
5. Place samples back on magnet and allow 1 minute to separate.
6. Pipette and discard away any excess liquid, careful not to let the beads dry.
7. Add 50 µL **IP PSP Master Mix** to each tube
8. Spin all samples in mini-centrifuge for 3 seconds.
9. Incubate in thermomixer at 37 °C for 10 minutes with interval mixing at 1,250 rpm.
10. Continue to the next step during the incubation.

IP RNA 3'-end repair

1. Prepare **IP PNK master** mix according to **Table 6** in a fresh 1.5 mL LoBind tube. Pipette mix to combine and store on ice until use.
   - **Note:** Include 3% excess volume to correct for pipetting losses.

Table 6. IP PNK master mix (per sample)

Component	Volume/ IP (µL)
IP PNK Buffer	146
PNK enzyme	4
Total:	*150*

2. When IP RNA 5'-end repair is complete, add 150 µL **IP PNK master mix** to each IP tube.
3. Incubate in thermomixer at 37 °C for 20 minutes with interval mixing at 1,250 rpm.

Second Immunoprecipitation Wash

- **Note**: Fully thaw **RNA Ligation Buffer** and **IP RNA Adapter** at room temperature, then store **IP RNA Adapter** on ice and **RNA Ligation Buffer** at room temperature.

1. To each IP sample, add 500 µL cold **HSB**, close tubes.
2. Invert mix until homogeneous.
3. Place on DynaMag-2 magnet.
4. Slowly invert closed tubes as beads start to separate to capture any beads from cap.
5. When separation is complete, discard supernatant without disturbing beads.
6. Remove IP tubes from magnet and add 500 µL cold **HSB.**
7. Invert mix until homogeneous.
8. Separate beads on magnet and remove supernatant without disturbing beads.
9. Remove IP tubes from magnet and add 500 µL cold **1× NoS Buffer.**
10. Gently invert mix until homogeneous.
11. Separate beads on magnet and remove supernatant without disturbing beads.
12. Remove IP tubes from magnet
13. Add 500 µL cold **1× NoS Buffer**.
14. Gently invert mix until homogeneous.
15. Place samples back on magnet and allow 1 minute to separate.

16. When separation is complete, and liquid is transparent, gently open tubes and discard supernatant without disturbing beads.
17. Spin all IP samples in mini-centrifuge for 3 seconds.
18. Pipette and discard any excess liquid without disturbing beads.
19. Remove IP tubes from magnet.
20. Add 500 µL cold **1× NoS Buffer**.
21. Invert to mix until homogeneous.
22. Store on ice and proceed to the next step.

RNA Adapter Ligation to Immunoprecipitation Samples

1. Prepare **IP Ligation Master Mix** according to **Table 7** in a fresh 1.5 mL LoBind tube.
   - **Note:** Keep Ligation mix at room temperature, do not place on ice.
   - **Note:** Include 3% excess volume to correct for pipetting losses.

Table 7. IP Ligation Master Mix (per sample)

Component	Volume/IP (µL)
Molecular Biology Grade Water	6.2
RNA Ligation Buffer	18
RNase Inhibitor	0.4
Ligase	2.4
Total:	*27*

2. Ligation mix is highly viscous, pipette slowly to mix and spin down.
3. Move all IP samples from ice to DynaMag-2 magnet.
4. Incubate for at least 1 minute until beads fully separate then discard supernatant.
5. Spin all samples in mini-centrifuge for 3 seconds.
6. Place samples back on magnet and allow 1 minute to separate.
7. Pipette and discard away any excess liquid, taking care to not disturb beads.
8. Add 3 µL of **IP RNA Adapter** to each IP sample.
9. Add 27 µL of **IP ligation master mix** to each IP sample.
10. Pipette mix all components until homogeneous.
11. Incubate IP tubes at room temperature for 1 hour on tube rotator.
    - **Note:** It is recommended to proceed to the next chapter, **Preparation for SDS-PAGE**, while IP samples are ligating.

Third Immunoprecipitation Wash

1. Add 500 µL cold **1× NoS Buffer** to beads, invert mix until homogeneous,
2. Place on DynaMag-2 magnet.
3. Slowly invert closed tubes as beads start to separate to capture any beads from cap.
4. After separation is complete, discard supernatant without disturbing beads.
5. Remove IP tubes from magnet
6. Add 500 µL cold **HSB**.

7. Invert mix until homogeneous.
8. Place IP tube on DynaMag-2 magnet.
9. Remove supernatant as done in previous steps.
10. Add 500 µL cold 1× NoS Buffer.
11. Invert mix until homogeneous.
12. Separate beads on magnet and remove supernatant as done in previous steps.
13. Remove IP tubes from magnet.
14. Add 500 µL cold 1× NoS Buffer.
15. Gently invert mix until homogeneous.
16. Store on ice and proceed to the next step.

Proceed immediately to next chapter

Next Stopping point: ~45 minutes

Chapter 5: Preparation for SDS-PAGE and Membrane Transfer

Overview

This section describes the preparative steps required to perform SDS-PAGE on IP samples. It is recommended to perform these steps during IP RNA 3'-adapter ligation.

Consumables

- NuPAGE 4-12% Bis-Tris gels
- 20× NuPAGE MOPS SDS Running Buffer
- 20× NuPAGE Transfer Buffer
- Spectra Multicolor Broad Range Protein Ladder (thaw at room temperature then store on ice)
- 2× Western Buffer (thaw at room temperature then store on ice)
- 100% Methanol
- Molecular Biology Grade Water

Preparation

1. Remove pre-cast gels from plastic and rinse with clean water prior to use.

Procedure

The following protocol is for pre-cast NuPage 4-12% Bis-Tris gels, which have neutral pH and are ideal for RNA stability. NuPAGE Tris-Acetate gels have been tested and are recommended for large molecular weight proteins (>200 kDa) but require different running buffer (see manufacturer recommendations). We have not validated other pre-cast or manually cast polyacrylamide gel formulations.

Prepare Reagents for SDS-PAGE

1. Determine the number of preparative gels (for size selection) required for all IP and Input samples (see **Example: Preparative Gel Loading Scheme** below).
2. Label the appropriate number of **pre-cast NuPAGE 4-12% Bis-Tris gels** with sample/ladder information, reserve wells between samples for ladders.

Example: Preparative Gel Loading Scheme (2 IP samples + 2 Input samples)

Well	1	2	3	4	5	6	7	8	9	10
Sample/Ladder	High Conc. Ladder	IP 1	Low Conc. Ladder	IP 2	Low Conc. Ladder	IP 3	Low Conc. Ladder	IP 4	Low Conc. Ladder	

3. Dilute 20× NuPAGE MOPS SDS Running Buffer to create **1× Running Buffer** according to **Table 8.**
4. Shake manually to mix, then store on ice.

Table 8. 1× Running Buffer Preparation

Component	Volume (mL)
Molecular Biology Grade Water	1000
20× NuPAGE MOPS SDS Running Buffer	53
Total:	***1053****

** This volume is sufficient volume for 2 gel chambers (for a total of 4 gels).*

5. Dilute 20× NuPAGE Transfer Buffer to create **1× Transfer Buffer** according to **Table 9**.
6. Shake manually to mix, then store on ice.

Table 9. 1× Transfer Buffer Preparation

Component	Volume (mL)
Molecular Biology Grade Water	1000
20× NuPAGE Transfer Buffer	59
100% Methanol	118
Total:	***1177****

**This volume is sufficient for 1 standard transfer chamber, which holds 2 transfer stacks (2 gels).*

7. Dilute protein ladders according to **Table 10** and store on ice.

Table 10. Protein Ladder Dilutions

	Component (µL)			
Ladder Concentration	Molecular Biology Grade Water	Spectra Multicolor Broad Range Protein Ladder	2× Western Buffer	*Total volume*
High*	60	43	60	163
Low*	150	23	150	323

**High concentration ladder dilution volume is sufficient to run 8 wells; adjust according to sample number.*

**Low concentration ladder dilution volume is sufficient to run 16 wells; adjust according to sample number.*

Chapter 6: SDS-PAGE and Membrane Transfer

Overview

This section describes the running of IP samples on SDS-PAGE and subsequent transfer to membranes. IP samples are denatured in western buffer and loaded onto gels. SDS-PAGE is performed for ~90 minutes. Lastly, samples are horizontally transferred from gels to membranes for size-selection and western blotting.

Consumables

- 2× Western Buffer
- 1× Running Buffer (diluted in previous chapter)
- High-concentration ladder (diluted in previous chapter)
- Low-concentration ladder (diluted in previous chapter)
- Bead Elution Buffer

Preparation

1. Running and transfer buffer should be made previously and pre-chilled to 4 °C. Buffers can be stored at 4 °C for up to 1 month.
2. Place 2× Western Buffer and Spectra Multicolor Broad Range Protein Ladder at room temperature until fully thawed.
3. NuPAGE MOPS SDS Running Buffer according to **Table 8** (Page 24).

Procedure

Elution of IP m6A-Ab-RNA complexes for electrophoresis

1. Prepare 1× Western Buffer according to **Table 11** below and store on ice.

Table 11. 1× Western Buffer Preparation

Reagent	Volume (µL)
Molecular Biology Grade Water	30
2× Western Buffer	30
Total:	60

2. Move all IP tubes from ice to DynaMag-2 magnet and allow at least 1 minute for bead separation.
3. Remove and discard supernatant.
4. Spin all samples in mini-centrifuge for 3 seconds.
5. Place samples back on magnet and allow 1 minute for separation.
6. Pipette and discard any excess liquid without disturbing beads.
7. Add 21 µL of **1× Western Buffer** to IP samples.

8. Pipette mix until homogenous and then store on ice.
9. Incubate IP samples on thermomixer at 65 °C for 10 minutes with interval mixing at 1,200 rpm.
10. Move all samples to ice for 2 minutes.
11. Spin all samples in mini-centrifuge for 3 seconds.
12. Move all tubes from ice to DynaMag-2 magnet and allow at least 1 minute for bead separation.
13. Remove supernatants (containing m6A-Ab-RNA complexes) from beads and place in fresh, labeled tubes.
14. Discard tubes containing beads.

Optional Stopping Point: *If stopping here, eluted cDNA samples should be stored at -80 °C*
Next stopping point: ~4 hours

Load SDS-PAGE preparative gel

1. Assemble electrophoresis tank (see Appendix A: SDS-PAGE for assembly instructions).
2. Remove sticker from all labeled gels.
3. Place labeled **preparative** gel inside electrophoresis tank and clamp shut.
4. Add **1× Running Buffer** to central chamber of electrophoresis tank until outer chambers are halfway filled (~500 mL).
5. Remove combs from gels.
6. Wash wells of gels by gently pipetting 1 mL of **1× Running Buffer** from central chamber into wells.
7. Load 20 µL of **High Concentration Ladder** and **Low Concentration Ladder** into appropriate wells of preparative gel.
8. Load 20 µL of IP samples into appropriate wells of **preparative gel.**
   - **Note:** Ensure all wells in the middle of the gels are filled, as empty wells can cause uneven sample electrophoresis.
9. Run gels at constant 160 V until the lower dye front reaches the bottom of the gel (typically 75-90 minutes).
   - Optional: During polyacrylamide gel-electrophoresis continue to **Chapter 7: Processing of Input RNA samples.**
10. Continue to WB Gel Transfer of IP samples when gel run is completed.

Prepare gels and transfer to membrane

1. After SDS-PAGE is complete, remove gel cassette(s) and place face down on work-space surface.
2. Disassemble the gel cassette by carefully inserting the gel knife into the gap between the two plates of the cassette. Gently push up and down on the handle to 'crack' the cassette but not fully separate the top and bottom plates. Continue until all 3 sealed edges have been released.

3. Carefully separate the top and bottom cassette plates, with the gel attached to one of the two plates.
   - **Note:** If the gel remains attached to both plates, briefly submerging the cassette apparatus in **1× NuPAGE Transfer Buffer** will loosen the gel sufficiently to separate the plates without tearing the gel.
   - **Note:** If using the gel knife to separate the gel from the plate, ensure that the knife is wetted with transfer buffer to avoid tearing the gel.
4. Trim off wells on top of gel and 2-3 mm on bottom of gel using gel knife. Gel is now ready to be transferred.
5. Prepare membranes for transfer (see **Appendix B: Membrane Transfer** for detailed instructions).
   - **Note:** We recommend using the provided nitrocellulose membranes, which were identified to have decreased background RNA contamination (see Van Nostrand, *et al.* Methods Mol Biol. 2017 (PMID 28766298)).
   - **Note:** We recommend using a 'wet' transfer method as modeled in this protocol.
6. Assemble transfer chamber and transfer stacks (see **Appendix B: Membrane Transfer** if using Mini Protean Tetra Apparatus).
7. Run transfer for 2 hours at constant 200 mA or at constant 30 V overnight at 4 °C.
   - **Note:** If transferring for 2 hours at constant 200 mA, surround as much of the outside of the apparatus with ice as possible to avoid overheating. If using more than one Mini-Protean Tetra apparatus, connect only one per power supply.

Chapter 7: Chapter Processing of Input RNA samples

Overview

This chapter describes end repair of Input RNA, followed by RNA adapter ligation.

Consumables

- PSP buffer (Thaw at room temperature then store on ice)
- Input PNK (Thaw at room temperature then store on ice)
- RNA Ligation Buffer (Thaw at room temperature then store at room temperature)
- Input RNA Adapter (Thaw at room temperature then store on ice)
- Bead Elution Buffer (Thaw at room temperature then store on ice)
- eCLIP Beads (Take out of 4 °C and resuspend until homogeneous)
- Bead Binding Buffer

Preparation

1. Prewarm thermomixer to 37 °C 10 minutes with interval mixing.
2. Ensure **100% EtOH** is added to **RNA Wash Buffer** concentrate upon first usage.
3. Centrifugation steps are done at room temperature.
4. Prepare fresh 80% ethanol in Molecular Biology Grade water in a fresh 50 mL tube. Store at room temperature for up to 1 week.

Procedure

5'-End repair of Input RNA

1. Prepare **Input PSP Master Mix** according to **Table 12** below in a fresh 1.5 mL LoBind tube.
2. Mix then store on ice until use.
   - **Note:** Include 3% excess volume to correct for pipetting losses

Table 12. Input PSP Master Mix (per sample)

Component	Volume/Input (µL)
PSP Buffer	8
RNase Inhibitor	1
PSP enzyme	2
Total:	*11*

3. Dilute each 20 ng of saved fragmented Input mRNA (from **Antibody-Coupling** section step 4) to 10 µL with **Molecular Biology Grade** water.
4. Add 11 µL of **Input PSP Master Mix** to each 10 µL Input sample, mix by flicking.
5. Spin in mini-centrifuge for 3 seconds.
6. Incubate in Thermomixer for 10 minutes at 37 °C with interval mixing at 1,250rpm.

3'-End repair of Input RNA

1. Prepare **Input PNK Master Mix** according to **Table 13** in a fresh 1.5 mL LoBind tube.
2. Mix then store on ice until use.
   - **Note:** Include 3% excess volume to correct for pipetting losses

Table 13. Input PNK master mix (per sample)

Component	Volume/Input (µL)
Input PNK Buffer	70
DNase	1
PNK enzyme	4
Total:	*75*

3. Add 75 µL of **Input PNK master mix** to each tube.
4. Mix by flicking, spin in mini-centrifuge for 3 seconds.
5. Incubate in Thermomixer for 20 minutes at 37 °C with interval mixing at 1,250 rpm.

Clean Repaired Input Samples

1. Add 200 µL of **RNA Binding Buffer** to the 95 µL of each end-repaired Input RNA. Pipette mix.
2. Add 300 µL of **100% EtOH**, pipette 10 times to mix.
3. Transfer the entire sample to a new filter column placed in a collection tube.
4. Centrifuge at 7,000 × g for 30 seconds. Discard flow-through.
5. Add 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 11 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. Place RNA samples on ice if continuing to the next step.

Optional Stopping Point: *If stopping here, Input RNA samples should be stored at -80 °C*
Next stopping point: ~2 hours

Input Sample Adapter Ligation

- **Preparation Note:** Preheat PCR thermal cycler block to 65 °C (with lid set at 70 °C).

1. Add 5 µL of **repaired Input RNA** (from **Clean Repaired Input Samples section step 17**) into pre-labeled 0.2 mL strip tubes, temporarily place on ice. Store remaining repaired Input RNA at -80 °C as backup.
2. Add 2 µL of **Input RNA Adapter** to each Input RNA tube, pipette mix.
3. Spin samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
4. Incubate tubes at 65 °C for 2 minutes in thermal cycler with the lid preheated to 70°C.
5. Immediately place samples on ice for 1 minute.
6. Add 13.5 µL of **Input Ligation Master Mix (Table 14)** adapter-added Input RNA sample.

Table 14. Input Ligation Master Mix (per sample)

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

7. Pipette mix until homogeneous.
8. Spin samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
9. Incubate the samples for 1 hour at room temperature on tube rotator.

Input RNA bead cleanup

1. Take **eCLIP Beads** out of 4 °C and resuspend until homogeneous.
2. For each Input sample, add 10 µL of eCLIP Beads to a new 1.5 mL LoBind tube (e.g., for 2 Input samples transfer 20 µL of eCLIP Beads into new 1.5 mL LoBind tube).
3. Wash **eCLIP Beads** prior to addition to samples.
    a. Add 5× volume of **Bead Binding Buffer** (e.g., for 2 Input samples add 100 µL buffer to 20 µL of eCLIP Beads).
    b. Pipette up and down to mix until sample is homogeneous.
    c. Place tube on DynaMag-2 magnet.
    d. When separation is complete and supernatant is clear, carefully aspirate and discard supernatant without disturbing beads.
    e. Remove tube from magnet.
4. Resuspend **eCLIP Beads** in 63 µL of Bead Binding Buffer per sample and pipette up and down until beads are fully resuspended. (e.g., for 3 Input samples resuspend beads in the same tube with 189 µL of **Bead Binding Buffer**.)
5. Transfer 60 µL of washed **eCLIP Beads** to each 20 µL tube of ligated Input RNA sample.
6. Pipette up and down to mix until sample is homogeneous.
7. Add 60 µL of **100% EtOH** to each sample.
8. Pipette mix until homogeneous.

9. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
10. Incubate on magnet for 30 seconds until separation is complete and supernatant is transparent.
11. Carefully aspirate and discard supernatant without disturbing beads.
12. Add 320 µL of **80% EtOH** to each sample.
13. Carefully aspirate and discard supernatant without disturbing beads.
14. Add 160 µL of **80% EtOH** without disturbing beads.
15. Move samples to different positions on magnet to wash thoroughly.
16. Carefully add an additional 160 µL of **80% EtOH.**
17. Incubate on magnet for 30 seconds until separation is complete and supernatant is transparent.
18. Carefully aspirate and discard all supernatant while on magnet.
19. Repeat steps 14-18 for a total of two 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. While on magnet aspirate and discard all residual liquid without disturbing beads.
24. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
25. Once completely dry, carefully remove tubes from magnet.
26. Add 10 µL **Bead Elution Buffer** to each sample.
27. Pipette up and down to mix until sample is homogeneous.
28. Incubate for 5 minutes at room temperature.
29. After incubation, move tubes to 96-well magnet.
30. Incubate on magnet for 30 seconds until separation is complete and supernatant is transparent.
31. Transfer whole sample to new 0.2 mL strip tubes.
32. Store Input RNA samples at -80 °C or continue to Reverse Transcription.
    - **Note**: If stopping here, store **IP nitrocellulose membrane** flat in sheet protector at -20 °C.

Optional Stopping Point: *If stopping here, Input RNA samples should be stored at -80 °C*
Next stopping point: ~4 hours

Chapter 8: mRNA Recovery, Reverse Transcription, cDNA Adapter Ligation

Overview

The following chapter describes mRNA Recovery, Reverse Transcription, and cDNA Adapter Ligation. IP RNA is recovered from the nitrocellulose membrane by proteinase digestion. Next, reverse transcription is performed for both IP and Input samples, followed by removal of dNTPs and template RNA. ssDNA samples are then cleaned, and overnight adapter ligation is performed.

Consumables

- 0.5 M EDTA
- 1 M NaOH
- 1 M HCl
- 100% Ethanol
- 80% Ethanol
- eCLIP Beads (Take out of 4 °C and resuspend until homogeneous)
- Bead Binding Buffer
- Proteinase Buffer (Thaw at room temperature)
- Proteinase Enzyme
- RT Enzyme
- RNase Inhibitor
- Nuclease Enzyme
- ssDNA Enzyme
- Ligase Enzyme
- RT Buffer (Thaw at room temperature then store on ice)
- RT Primer (Thaw at room temperature then store on ice)
- ssDNA Adapter
- ssDNA Ligation Buffer

Preparation

1. Ensure **100% EtOH** is added to **RNA Wash Buffer** concentrate upon first usage.
2. Centrifugation steps are done at room temperature.
3. Preheat PCR thermal cycler block to 65 °C (with lid set at 70 °C).

Procedure

IP Membrane Cutting and mRNA Recovery

1. Place membrane with plastic sheet protector on a flat, hard surface, such as a glass coverslip.
2. Using a clean razor blade for each sample, carefully cut the region from 30 kDa to 150 kDa from the IP nitrocellulose membrane (see **Figure 1**).

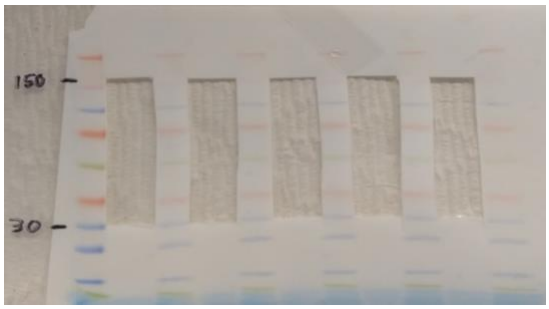

Figure 1. IP nitrocellulose membrane region

3. Remove excised membrane piece from sheet protector and place on glass coverslip.
   - **Note**: To prevent membrane from drying out, 10 µL of Molecular Biology Grade water can be added to each strip.
4. Further cut the membrane piece into 1-2 mm square slices.
5. Carefully transfer all slices to a new 1.5 mL LoBind tube.
6. Spin samples in mini-centrifuge for 5 seconds to collect membrane slices at the bottom.
7. Store tubes on ice until all samples are isolated then proceed immediately to the next step.

Digest RBP-RNA Complexes

1. Prepare **Proteinase Master Mix** according to **Table 15** in a fresh 1.5 mL LoBind tube.
   - **Note:** Include 3% excess volume to correct for pipetting losses.

Table 15. Proteinase Master Mix (per sample)

Component	Volume (µL)
Proteinase Buffer	110
Proteinase	17
Total:	***127***

2. Add 127 µL **Proteinase Master Mix** to each sample tube containing membrane slices.
3. Incubate in Thermomixer at 37 °C for 20 minutes.
4. Incubate in Thermomixer at 50 °C for 20 minutes with interval mixing at 1,250 rpm.
   - **Note**: It is important to ensure all membrane slices are submerged during incubation; move slices with a clean pipette tip if necessary.

IP Sample Cleanup

1. Spin in mini-centrifuge for 5 seconds to collect membrane slices at the bottom.
2. Transfer all liquid (~125 µL) for each sample from proteinase digestion into new DNA LoBind tube. This contains the eluted RNA sample.
3. Discard tubes containing membrane slices.
4. Add 250 µL of **RNA Binding Buffer** to the 125 µL of eluted RNA sample.
5. Pipette mix up and down.
6. Add 375 µL of **100% EtOH** and mix thoroughly.
7. Transfer all liquid (750 µL) to corresponding filter columns
8. Centrifuge at 7,000 × g for 30 seconds, then discard flow-through.
9. Add 400 µL **RNA Prep Buffer** to each column.
10. Centrifuge at 7,000 × g for 30 seconds, then discard flow-through.
11. Using new pipette tip for each column, add 480 µL **RNA Wash Buffer** to each column.
    - **Note**: Making sure to wash the rim of the filter column.
12. Centrifuge at 7,000 × g for 30 seconds, then discard flow-through.
13. Repeat steps 11-12 for a total of two washes.
14. Centrifuge the column at 10,000 × g for 1 minute with emptied collection tube.
15. Carefully transfer filter column to a new labeled 1.5 mL LoBind tube.
16. Discard flow-through and used collection tube.
18. Open column caps and allow to air dry for 2 minutes or until column is completely dry.
17. Elute all samples by adding 11 µL of **Molecular Biology Grade Water** directly to filter.
18. Incubate at room temperature for 1 minute.
19. Centrifuge at 12,000 × g for 90 seconds.
20. If proceeding immediately to next step, store all RNA samples on ice.

Optional Stopping Point: *If stopping here, RNA samples should be stored at -80°C*
Next stopping point: ~3 hours

Reverse Transcription of IP and Input Sample Reagent Preparation

1. Add 1.5 µL of **RT Primer** into the 0.2 mL strip tube containing ~9 µL of Input samples ("**Input RNA bead Cleanup"** section step 36).
2. For each IP RNA sample, transfer entire sample (~9 µL) into a new, labeled 0.2mL strip tube.
3. Add 1.5 µL of **RT Primer** into IP RNA.
4. Mix, and spin all samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
5. Incubate at 65 °C for 2 minutes in thermal cycler with the lid preheated to 70 °C.
6. After incubation, immediately transfer to ice for 1 minute.
7. Adjust the thermal cycler block temperature to 54 °C – 20 minutes (with lid set to 65 °C).

Reverse transcription of IP and Input RNA

1. Prepare **Reverse Transcription Master Mix** according to **Table 16** in a fresh 1.5mL LoBind tube.
2. Pipette to mix.
3. Store samples on ice until use.
   - **Note:** Include 3% excess volume to correct for pipetting losses.

Table 16. Reverse Transcription Master-Mix (per sample)

Component	Volume (µL)
RT Buffer	9.2
RNase Inhibitor	0.2
RT enzyme	0.6
Total:	10

4. Add 10 µL of the **Reverse Transcription Master Mix** to each sample leaving samples on ice. Pipette to mix.
5. Spin samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
6. Immediately incubate samples at 54 °C for 20 minutes in thermal cycler with the lid at 65 °C.
7. After incubation, immediately place samples on ice.
8. Adjust thermal cycler block temperature to 37 °C (with lid set to 45 °C).

cDNA End Repair of IP and Input Samples

1. Add **2.5 µL** of **Nuclease** to each sample. Pipette to mix.
2. Spin samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
3. Incubate in thermal cycler at 37 °C for 15 minutes with the lid at 45 °C.
4. Remove the strip-tube and place samples on ice.
5. Adjust thermal cycler block to 70 °C (with lid set to 75 °C).
6. Add 1 µL of **0.5 M EDTA (pH 8)** to each sample.
7. Pipette samples up and down gently 5 times to mix.
8. Add 3 µL of **1 M NaOH** to each sample.
9. Pipette samples up and down gently 5 times to mix.

10. Incubate tubes at 70 °C for 10 minutes in thermal cycler with the lid at 75 °C.
11. Place strip-tube on ice for 10 seconds.
12. Add 3 µL of **1 M HCl** to each sample.
13. Proceed immediately to the next step.

cDNA IP and Input Sample Bead Cleanup

- **Preparation Note**: Thaw **ssDNA Adapter** and **ssDNA Ligation Buffer** at room temperature until completely melted then store ssDNA Adapter on ice and ssDNA Ligation Buffer at room temperature.
- **Preparation Note**: Prepare fresh **80% Ethanol** in Molecular Biology Grade water in a fresh 50 mL tube if was 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 IP and Input sample, add 5 µL of eCLIP Beads to a new 1.5 mL LoBind tube (e.g., for 4 samples transfer 20 µL of eCLIP Beads).
3. Wash **eCLIP Beads** prior to addition to samples.
    a. Add 5× volume of **Bead Binding Buffer** (e.g., for 4 samples add 100 µL buffer to 20 µL of eCLIP Beads
    b. Pipette mix until sample is homogeneous.
    c. Place tube on DynaMag-2 magnet.
    d. When separation is complete and supernatant is clear, carefully aspirate and discard supernatant without disturbing beads.
    e. Remove tube from magnet.
4. Resuspend eCLIP Beads in **93 µL** of **Bead Binding Buffer** per sample.
5. Pipette mix until beads are fully resuspended.
6. Add 90 µL of washed **eCLIP Beads** to each IP and Input cDNA sample.
7. Pipette up and down to mix until sample is homogeneous.
8. Add 100 µL of **100% EtOH** to each IP and Input cDNA sample.
9. Pipette mix until homogeneous.
10. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
11. **Move samples to fresh strip tube: place a new, labeled 0.2 mL strip tube on 96-well magnet and transfer sample from old to new strip tube.**
12. Allow to incubate for 1 minute or until separation is complete and liquid is transparent.
13. Carefully discard supernatant without disturbing beads.
14. Add 150 µL of **80% EtOH**.
15. Move samples to different positions on magnet to wash thoroughly.
16. Add an additional 150 µL of **80% EtOH**.
17. Incubate on magnet for 30 seconds until separation is complete and supernatant is transparent.
18. Carefully aspirate and discard all supernatant while on magnet.
19. Repeat steps 14-18 once for a total of two 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. Using fine tips, 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 2.5 µL of **ssDNA Adapter**.
27. Pipette to mix until homogeneous.
28. Incubate in thermal cycler at 70 °C for 2 minutes with the lid at 75 °C.
29. Following incubation, immediately place on ice for 1 minute.

IP and Input cDNA Ligation on Beads

1. Prepare cDNA Ligation master mix according to **Table 17** in a fresh 1.5mL LoBind tube. Pipette mix to combine (do not vortex). Use immediately.
    - **Note:** Include 3% excess volume to correct for pipetting losses

Table 17. 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 **cDNA Ligation master mix** to each sample from previous section **cDNA IP and Input Sample Bead Clean Up**) and pipette mix until homogeneous.
3. Incubate at room temperature overnight on a tube rotator.

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

Chapter 9: Library Amplification and Preparation for Sequencing

Overview

This section describes the 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
- qPCR Primers
- NEB LUNA Universal qPCR 2× Master Mix (Thaw at room temperature then keep on ice)
- PCR mix
- Bead Binding Buffer
- Agencourt AMPure XP beads
- Molecular Biology Grade Water
- 80% and 100% Ethanol

Procedure

Ligated cDNA IP and Input Sample Bead Cleanup

1. Add 5 µL of **Bead Elution Buffer to** 10 µL adapter-ligated IP and Input cDNA sample from **IP and Input cDNA Ligation on Beads** (p.37, step 3).
2. Mix until sample is homogeneous.
3. Add 45 µL of **Bead Binding Buffer** to each IP and Input sample.
4. Pipette mix until homogeneous.
5. Add 45 µL of **100% EtOH** to each sample.
6. Pipette mix until homogeneous.
7. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
8. Move samples to 96-well magnet.
9. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
10. Carefully discard supernatant without disturbing beads.
11. Add 150 µL of **80% EtOH**.
12. Move samples to different positions on magnet to wash thoroughly.
13. Add an additional 150 µL of **80% EtOH**.
14. Incubate on magnet for 30 seconds until separation is complete and supernatant is transparent.
15. Carefully discard supernatant while on magnet.
16. Repeat steps 11-15 once for a total of two washes.

17. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
18. Place tube back on 96-well magnet.
19. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
20. Carefully aspirate all remaining supernatant while on magnet.
21. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
    - **Note**: Do not over dry samples.
22. Carefully remove tubes from magnet.
23. For each sample, resuspend beads in 25 µL **Bead Elution Buffer**.
24. Pipette up and down to mix until sample is homogeneous.
25. Incubate for 5 minutes at room temperature.
26. Move tubes to 96-well magnet.
27. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
28. Transfer 25 µL to new 0.2 mL strip tubes.
29. Store samples on ice if proceeding to the next step.

Optional Stopping Point: *If stopping here, eluted cDNA samples should be stored at -80 °C.*
Next stopping point: ~2 hours

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cDNA IP and Input sample quantification by qPCR

1. Prepare **qPCR Master Mix** for the appropriate number of reactions according to **Table 18** in a fresh 1.5 mL LoBind tube.
    - **Note:** Include 3% excess volume to correct for pipetting losses

Table 18. qPCR Master Mix (per sample)

Component	Volume (µL)
NEB LUNA Universal qPCR 2× Master Mix	5
qPCR Primers	4
Total:	*9*

2. Obtain and label a 96- or 384-well qPCR reaction plate (**See Table 19** for suggested 96-well layout).

Table 19. 96-well qPCR plate layout for 21 samples

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

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

3. Add 1 µL of eluted cDNA samples to 9 µL of **Molecular Biology Grade Water** for a 1:10 dilution.
4. Add 9 µL of **qPCR Master Mix** into all assay wells on ice.
5. Add 1 µL of each diluted cDNA (or water 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 × 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.
   - **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

10. Record qPCR Ct values for all samples.
11. Set threshold to 0.5 – this recommendation is for StepOnePlus System.
    - **Note:** Typical acceptable Ct values range from 10 to 23 (with Input samples typically <10). 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 of IP and Input cDNA and Dual Index Addition

- **Preparation Note**: For library pooling strategies, see Illumina documentation. We recommend multiplexing at least 8 libraries with diverse indexes.
- **Preparation Note**: See APPENDIX: SEQUENCING SPECIFICATIONS for read structure.

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 20** in fresh 0.2 mL PCR strip-tubes. Keep tubes on ice.

- **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 20. 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. Pipette mix to combine.
4. Spin samples in mini-centrifuge for 3 seconds to draw all liquid to the bottom of the tube.
5. Keep samples on ice.
6. Refer to Ct values recorded to calculate the appropriate number of cycles for each sample. 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 N = 4 and Total number of PCR cycles equal 10 (6+4).

7. Run PCR for the specific number of cycles calculated for each sample according to the PCR program shown in **Table 21.**

Table 21. 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 ≥ 2 and ≤ 13.

8. Immediately put samples on ice to cool following PCR amplification.

First AMPure library PCR product cleanup

1. Allow AMPure XP beads (not provided) to equilibrate at room temperature for 5 minutes.
2. Manually shake AMPure XP beads to resuspend until homogeneous.
3. Add 70 µL of **AMPure XP beads** into each 40 µL PCR reaction.
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**.
10. Move samples to different positions on magnet to wash thoroughly.
11. Add an additional 150 µL of **80% EtOH**.
12. Incubate on magnet for at least 30 seconds until supernatant is transparent.
13. Aspirate and discard all supernatant.
14. Repeat steps 9 - 13 for a total of two washes.
15. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
16. Place tube back on 96-well magnet.
17. Incubate on magnet for at least 30 seconds until supernatant is transparent.
18. Aspirate and discard all residual liquid without disturbing beads.
19. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
20. Once completely dry, carefully remove tubes from magnet.
21. For each sample, resuspend beads in 20µL of **Molecular Biology Grade water**.
22. Pipette up and down to mix until sample is homogeneous.

Second AMPure library PCR product cleanup

1. Add 35 µL **AMPure XP beads** into each 20 µL sample with beads.
2. Pipette up and down until homogeneous.
3. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
4. Move samples to 96-well magnet.
5. Incubate until separation is complete and supernatant is transparent.
6. Carefully aspirate and discard supernatant without disturbing beads.
7. Add 150 µL of **80% EtOH**.
8. Move samples to different positions on magnet to wash thoroughly.
9. Add an additional 150 µL of **80% EtOH**.
10. Incubate on magnet for at least 30 seconds until supernatant is transparent.
11. Aspirate and discard all supernatant.
12. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
13. Place tube back on 96-well magnet.
14. Incubate on magnet for 30 seconds until supernatant is transparent.
15. Aspirate and discard all residual liquid without disturbing beads.

16. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny. Do not over dry.
17. Once completely dry, carefully remove tubes from magnet.
18. Resuspend beads in 20 µL of Molecular Biology Grade water.
19. Pipette up and down to mix until sample is homogeneous.
    - Note: Do not use other buffers for final library.
20. Incubate 5 minutes at room temperature.
21. After incubation, move tubes to 96-well magnet.
22. Incubate on magnet for 1 minute until supernatant is transparent.
23. Transfer 20 µL to a new 0.2 mL strip-tube.
24. 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. 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 A: SDS-PAGE Gel Electrophoresis

Required Equipment and Materials

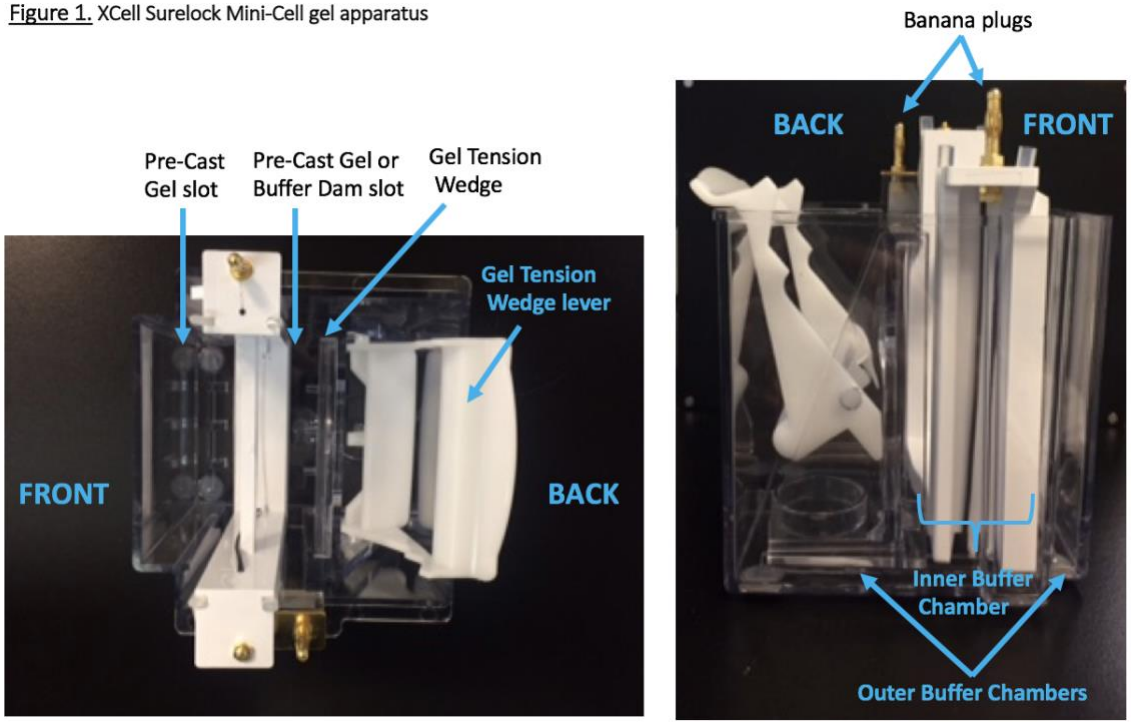
Item	Source
NuPAGE 4-12% Bis-Tris Gels, 1.0mm, 12-well	ThermoFisher Scientific cat. #NP0322BOX
NuPAGE 4-12% Bis-Tris Gels, 1.0mm, 10-well	ThermoFisher Scientific cat. #NP0321BOX
XCell Surelock Mini-Cell	ThermoFisher Scientific cat. #EI0001
PowerEase 300W Power Supply (230VAC)	ThermoFisher Scientific cat. #PS0301
Polypropylene, Nalgene Tray	TaylorScientific cat. #14-6389-01

Reagents

Not Included in kit	
Item	**Source**
20× NuPAGE MOPS SDS Running Buffer	ThermoFisher Scientific cat. #NP001
Molecular Biology Grade Water, Corning	VWR cat. #95000-094
Spectra Multicolor Broad Range Protein Ladder	ThermoFisher Scientific cat. #26634

Included in kit		
Item	**Source**	**Storage**
2× Western Buffer	Eclipse Bioinnovations	-80 °C

Figure 1. XCell Surelock Mini-Cell gel apparatus



Procedure

Prepare NuPAGE 4-12% Bis-Tris gels

1 All samples will be run on two gels (PREPARATIVE), select 10-well or 12-well gel(s) according to sample number and loading scheme.
2 Cut open gel cassette pouch with scissors, remove cassettes from pouch and remove white sealing tape from bottom of gel cassette – rinse with clean water (diH_2O). Dry with kimwipes.

Prepare XCell Surelock Mini-Cell gel apparatus for loading gel cassettes

1. Rinse XCell Surelock Mini-Cell gel apparatus thoroughly with dH_2O then dry with kimwipes.
2. Place XCell Surelock Mini-Cell gel apparatus into Polypropylene Nalgene ice tray.
3. Insert first labeled NuPAGE 4-12% Bis-Tris gel cassette in either the FRONT 'Pre-Cast Gel slot' or in the BACK 'Pre-Cast Gel slot'.
4. If only running one gel insert 'Buffer Dam' in BACK portion of apparatus.
5. Insert 'Gel Tension Wedge'.
6. Add 200 mL of cold Running Buffer into 'Inner Buffer Chamber'.
7. Pull forward on the 'Gel Tension Wedge level' until lever stops, and the gels or gel/Buffer Dam appears snug.
8. Add remaining cold Running Buffer into 'Inner Buffer Chamber'. Continue to fill until Outer Buffer Chambers are approximately half full (roughly 500-550 mL per whole chamber).
9. Carefully remove gel comb(s).
10. Rinse gel wells with cold Running Buffer from the Outer Buffer Chamber using a p1000 pipettor. Be careful not to disrupt well walls of gel, this will create difficulties when loading samples. Check carefully for broken wells.
11. Carefully load all ladders (see m6A-eCLIP Protocol for volume) in predesignated wells by lowering the tip to the bottom of the well and slowly pipetting ladder without carry over.
12. Next carefully load all samples (see m6A-eCLIP Protocol for volume) in predesignated wells by lowering the tip to the bottom of the well and slowly pipetting sample without carry over.
13. Load any undesignated wells with diluted Western Buffer.
14. Once all wells have been filled, place cell safety lid with cables on XCell Surelock gel apparatus. The lid can only be firmly closed if the (-) electrode is aligned over the banana plug. If the lid is not properly seated, error message can appear on power supply.
15. With the Power Supply off, connect the electrode cords. Check labels: Red to (+) and black to (-).
16. Turn on the power.
17. Adjust settings to 150 V and run for 75 minutes or until dye front is at the bottom of gel.
18. Cover hole in cell safety lid with a paper towel.
19. Pour a sufficient amount of ice into Nalgene ice tray so XCell Surelock Mini-Cell gel apparatus is ¾ covered then remove the paper towel from lid.

Disassembling XCell Surelock Mini-Cell gel apparatus

1. At the end of run turn off the power and disconnect the cables from power supply. Remove the lid and unlock the lever.
2. Remove gel cassette(s) and place on workspace surface.
3. Discard Running Buffer accordingly.
4. Store all reagents under appropriate storage conditions.
5. Rinse XCell Surelock Mini-Cell gel apparatus and all associated parts with clean water.
6. Dry thoroughly.

APPENDIX B: Membrane Transfer

Required Equipment and Materials

Item	Source
Mini-Protean Tetra cell and supplies	BioRad cat. #165-8001
PowerPac HC Power Supply	BioRad cat. #1645052
Western Blotting Filter Paper, 7cm × 8.4cm	ThermoFisher cat. #84783
Polypropylene, Nalgene Tray	TaylorScientific cat. #14-6389-01

Reagents

Not Included in kit	
Item	**Source**
Pierce 20× TBS Tween 20 Buffer	ThermoFisher Scientific cat. #28360
20× NuPAGE Transfer Buffer	ThermoFisher Scientific cat. #NP00061
Methanol	Sigma-Aldrich cat. #494437
DPBS	VWR cat. #21-031-CV
Molecular Biology Grade Water	VWR catalog #95000-094

Included in kit	
Item	**Source**
Nitrocellulose membrane	Eclipse Bioinnovations
PVDF membrane	Any

Procedure

Prepare Mini-Protean Tetra System

1. Rinse Mini-Protean Tetra cell thoroughly with dH_2O and dry with kimwipes.
2. Place cell into transfer tank.

Prepare transfer stack for PREPARATIVE gel transfer

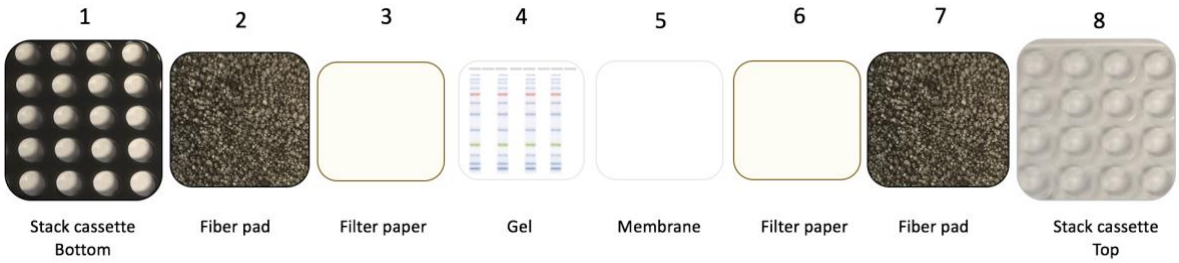


Figure 2. Transfer stack layer composition

1. Place stack cassette into Pyrex drying dish with the black side facing down (Refer to **Figure 2**).
2. Pour chilled 1× NuPAGE Transfer Buffer into Pyrex drying dish so that the black side of stack cassette (1) is submerged.
3. Wet 'Fiber pad' in 1× NuPAGE Transfer Buffer and place on top of the black side of stack cassette. Roll with a Blotting Roller to remove air bubbles.
4. Fully saturate a piece of Western blotting 'Filter paper' in 1× NuPAGE Transfer Buffer. Place wetted filter paper on top of Fiber pad. Use Blotting Roller to remove any bubbles between layers.
5. Carefully move gel from cassette to top of wetted filter paper using gel knife (see Figure 3). Ensure gel knife is wetted with transfer buffer so not to tear the gel.

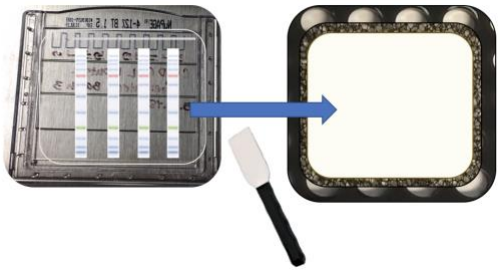


Figure 3. Gel transfer schematic

6. Analytical gel: PVDF membrane transfer
    a. Let PVDF membrane sit in 100% Methanol for 1 minute.
    b. Using tweezers, remove PVDF membrane from methanol and transfer to 1× NuPAGE Transfer Buffer. Let sit for 1 minute.

7. Preparative gel: Nitrocellulose membrane transfer
    a. Let nitrocellulose membrane equilibrate in transfer buffer for 1 minute

8. Analytical and Preparative gels:
    a. Using tweezers, carefully place the membrane on top of the polyacrylamide gel. Use Blotting Roller to remove any bubbles between layers.

b. Fully saturate a second piece of filter paper in 1× NuPAGE Transfer Buffer and place on top of 'Membrane'. Use Blotting Roller to remove any bubbles.
c. Wet second Fiber pad in 1× NuPAGE Transfer Buffer and roll out any bubbles. Place gray Fiber pad on top of the Filter paper. Ensure that no parts are sticking outside of the stack cassette that would prevent insertion into apparatus. Close the stack cassette firmly and lock with the white latch.

Assembling and running the Mini-Protean Tetra System

1. Assemble the Mini-Protean Tetra System apparatus (Refer to **Figure 5** for component identification).

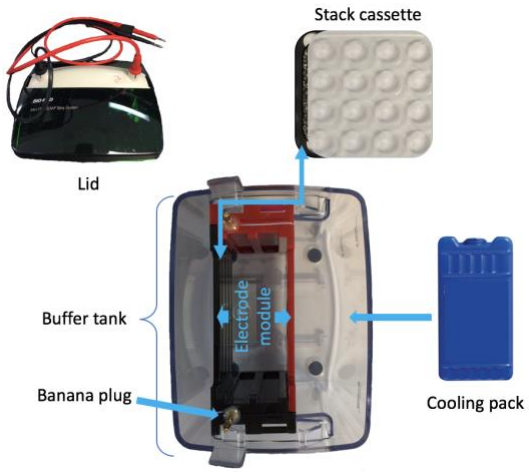


Figure 5. Mini-Protean Tetra components

2. When placing the stack cassette into the 'Electrode module' it is **essential** that the **black** (cathode -) side of the cassette is **facing the black side** of the module.
3. After all stack cassettes have been loaded, place a blue cooling ice pack (stored at -20°C) in Mini-Protean Tetra apparatus. Pour 1× NuPAGE Transfer Buffer from Pyrex tray into the apparatus.
4. Add remaining 900mL of 1× NuPAGE Transfer Buffer inside the electrode module between the two gel stack cassettes. Continue adding any remaining Transfer Buffer to the chamber until chamber is full (see indicator line on transfer tank). Securely close the lid by placing the electrodes over the 'Buffer tank banana plugs'.
5. Connect cables to corresponding colors on power supply and turn on power supply.
6. Run gel transfer overnight (> 10 hours) in a cold (4 °C) room at **constant 30 Volts.**

- **Note**: Transfer can alternatively be performed for **2 hours** at constant **200 mA.** If doing so, surround as much of the outside of the apparatus with ice as possible to avoid overheating. If using more than one Mini-Protean Tetra apparatus, connect only one per power supply or increase current to 210 mA. For 1 mm gels, 90 min at 210 mA can be enough to complete transfer (connect only one chamber with 1-2 gels per power supply).

Appendix C: Sequencing Specifications

IMPORTANT: *If user has purchased an eCLIP kit with data analysis package (*Eclipse BioInnovations *cat. #ECEKD-0001) single-end sequencing must be performed in order to be compatible with Eclipse Bio's software pipeline.*

Sequencing parameters: Eclipse Bio's eCLIP kit is based on the single-end eCLIP variant described in:

https://www.ncbi.nlm.nih.gov/pubmed/28766298
Van Nostrand EL, Nguyen TB, et al.
Robust, Cost-Effective Profiling of RNA Binding Protein Targets with Single-end Enhanced Crosslinking and Immunoprecipitation (seCLIP). Methods Mol Biol. 2017;1648:177-200. PMID: 28766298.

Libraries generated using the m6A-eCLIP method are typically sequenced using standard SE100 conditions on the Illumina HiSeq, NovaSeq, or NextSeq platforms. m6A-eCLIP libraries are compatible with paired-end sequencing if desired by the user, however due to the small size of typical eCLIP RNA fragments (~200bp), most fragments are fully sequenced in standard single-end formats.

Sequencing depth: Eclipse Bio's target is 40-50 million reads per m6A-eCLIP dataset.

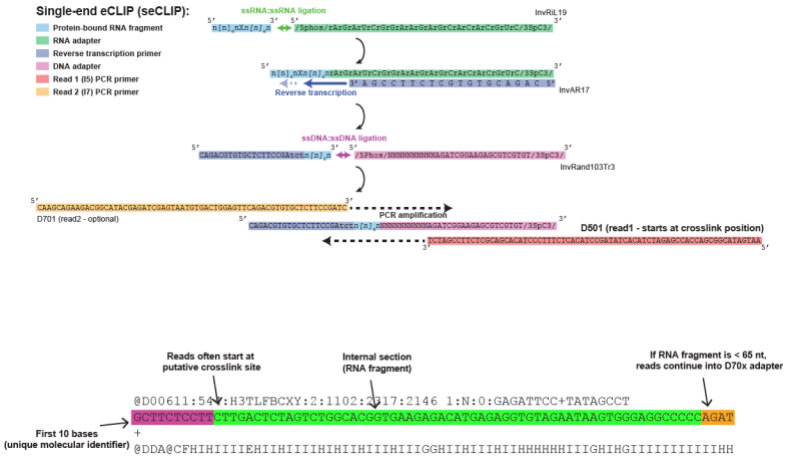
How deeply to sequence an m6A-eCLIP dataset is a challenging balance between cost and sufficient read depth to detect true binding events. In an effort to experimentally address this question, an analysis of eCLIP-seq datasets for 150 RNA binding proteins suggested that for 90% of datasets, saturation of peak information occurred at or below 8.5 million reads *(See Supplementary Fig. 11 of Van Nostrand EL, et al. A Large-Scale Binding and Functional Map of Human RNA Binding Proteins. Nature (Accepted, in press) (preprint available at https://www.biorxiv.org/content/early/2018/10/05/179648)*. However, we have found that targeting 25 million reads (for eCLIP) provides better coverage for abundant, broadly binding RNA binding proteins (such as HNRNPs) while still allowing pooling of ~14 eCLIP libraries per standard Illumina HiSeq 4000 lane.

- **Adapter sequences:** Illumina TruSeq HT adapters (provided)
  RNA adapter: 5Phos/rArGrArUrCrGrGrArArGrArGrCrArCrArCrGrUrCrUrG/3SpC3/
  ssDNA adapter: 5Phos/NNNNNNNNNNAGATCGGAAGAGCGTCGTGT/3SpC3/

Index primer sequences: Illumina dual index primers (provided)

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

Read structure model



Appendix D: Total RNA Isolation

Reagents

Not Included in kit	
Item	Source
Direct-zol RNA isolation kit or equivalent	Zymo cat. # R2071
Spectra Multicolor Broad Range Protein Ladder	ThermoFisher Scientific cat. #26634

Procedure

Total RNA Isolation

1. Obtain 10 million cells for RNA isolation either fresh or from frozen pellets.
2. Resuspend cells in 500 µL of **TRIzol**.
3. Mix until sample is homogeneous.
   - **Note**: If sample cannot easily be pipetted due to viscosity, add additional **TRIzol** 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 600µL 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 **Direct-zol RNA PreWash** to each column.
15. Centrifuge at 5,000 × g for 1 minute or until all liquid has passed through filter.
16. Discard the flow-through.
17. Repeat steps 14-16 for a total of two washes.
18. Add 700 µL **RNA Wash Buffer** to column
19. Centrifuge at 5,000 × g for 1 minute or until all liquid has passed through filter.
20. Discard flow-through.
21. Add 350 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. Repeat step 21-23 for a total of two washes.
25. Transfer column filter to a new collection tube.
26. Spin at 12,000 × g for 2 min to dry column filter.
27. Transfer filter to a new 1.5 mL LoBind DNA tube.

28. Open cap and allow filter to air dry for 2 minutes.
29. Add 50 µL of **Molecular Biology Grade Water** to filter in each column to elute RNA.
30. Allow to incubate at room temperature for 1 minute.
31. Centrifuge at 12,000 × g for 90 seconds.
    - **Note**: For high concentrations of RNA, elute in <50 µL and >25 µL
32. 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
33. Put samples on ice.

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**). RNA must have a RIN score of 7 or higher to continue.