ECLIPSEBIO
THE RNA GENOMICS COMPANY

RBP-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.

Table of Contents

Reagents Included in Kit

Item	Storage
Nitrocellulose membranes	Room temperature
PVDF membranes	Room temperature
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
eCLIP Lysis Buffer	-20 °C
Proteinase Buffer	-20 °C
PSP Buffer	-80 °C
IP PNK Buffer	-80 °C
Input 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
RT Buffer	-80 °C
IP-E 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

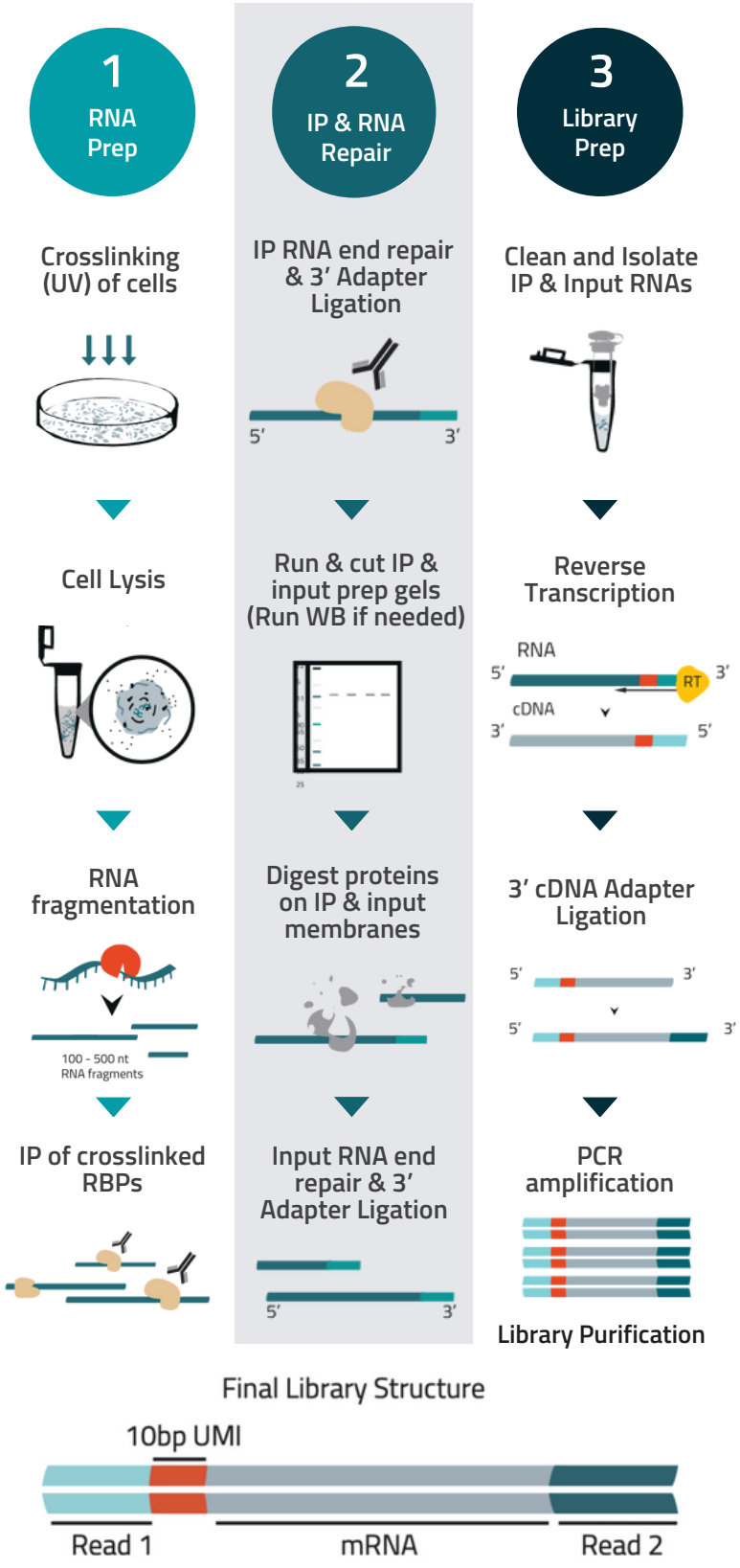
Reagents Not Included in Kit

Reagent	Source
Proteinase Inhibitor Cocktail	ThermoFisher Scientific cat. #87786
Ambion RNase-I, cloned, 100U/µL	ThermoFisher Scientific cat. #AM2294
Ethanol, Pure, 200 proof, for Molecular Biology	Sigma-Aldrich cat. #E7023-1L
Dynabeads	ThermoFisher Scientific cat. #11203D (Anti-Rabbit), #11202D (Anti-Mouse)
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

Required Equipment

Equipment	Source
Micro-centrifuge 5424R or equivalent	Eppendorf
Mini-centrifuge or equivalent	Corning LSE
Q800R2 Sonicator or equivalent	QSonica cat. #Q800R2-110
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. #R1013/R1014
MinElute Gel Extration Kit	Qiagen cat. #28606
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
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

RBP-eCLIP Workflow



Chapter 1: Overview

Introduction to RBP-eCLIP

The eCLIP (enhanced CrossLinking ImmunoPrecipitation) kit provides the ability to identify and map RBP binding sites on target RNA by streamlining eCLIP sample prep and library prep using a robust and reproducible framework.

The eCLIP kit utilizes the eCLIP technology based on the Van Nostrand et al. (Nature Methods, 2016) method.

The eCLIP kit offers:
High throughput and robust workflow
High reproducibility with accurate data
Unbiased target identification with 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 "Included with Kit" 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.

IMPORTANT: *Materials listed below are for 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*.

Chapter 2: Cell Lysis and RNA Assessment

Overview

This section describes cell lysis and RNA quality and quantity check using Agilent4200 TapeStation. Cell pellets stored at -80 °C are lysed in eCLIP Lysis Mix and homogenized via sonication. 1% of the homogenized lysate is aliquoted from each sample, and RNA isolation is performed to determine the volume of lysate required for immunoprecipitation and the amount of RNase required for optimal fragmentation.

Consumables

- eCLIP Lysis Buffer (thaw at room temperature then store on ice until use)
- Protease Inhibitor Cocktail **(not provided)**
- RNase Inhibitor (store on ice)
- Proteinase Buffer (thaw at room temperature then store on ice until use)
- Proteinase Enzyme (store on ice)
- Zymo RNA Clean and Concentrator-5 kit **(not provided)**
- Molecular Biology Grade Water **(not provided)**

Preparation

1. Set chiller on sonicator to 4 °C
2. Set chiller on centrifuge to 4 °C

Procedure

When starting an eCLIP experiment with a new cell type, always determine RNA quantity and RNase fragmentation conditions. We have determined that 1 U of RNase-I is sufficient for fragmentation of 5 µg of RNA. RNase-I unit definitions and concentrations vary by supplier. The above specifications are for Ambion RNase-I, cloned, 100 U/µL (AM2294).

Cell/Tissue Lysis

1. Prepare **eCLIP Lysis mix** for each crosslinked cell pellet according to **Table 1**.
   - **Note:** If lysing 2 or more samples, make a master mix by combining all components in a 15 mL or 50 mL conical tube pre-chilled on ice. Invert to mix then store on ice until use.

Table 1. eCLIP Lysis Mix (per one pellet of 20 million cells)

Reagent	Volume (µL)
eCLIP Lysis Buffer	1000
Protease Inhibitor Cocktail	5
RNase Inhibitor	10
Total:	1015

2. Retrieve tubes containing pellet(s) from -80 °C and quickly add 1 mL of cold **eCLIP lysis mix** (do not thaw pellets on ice first).
3. Gently pipette mix until sample is fully resuspended
4. Place cell tubes on ice for 5 minutes. During lysis, periodically pipette mix tubes slowly.
   - **Note:** Vortexing, shaking, and harsh pipetting should be avoided as this will cause foaming.
5. Transport samples to sonicator. If necessary, transfer to appropriate pre-chilled tubes for sonication equipment.
6. Sonicate at 4 °C to disrupt chromatin and fragment DNA (see **Table 2** below for settings).

Table 2. Sonicator Reference Settings

Sonicator	Energy setting	Set time	Cycles
QSonica Q800R	75% amplitude	5 minutes (total time is 10 min)	30 seconds ON / 30 seconds OFF

Digest Proteins

1. Following sonication, aliquot 10 µL of sonicated lysate for each sample into fresh, labeled 1.5 mL LoBind tubes.
2. Store remaining sample in aluminum cooling block on ice.
3. Prepare **Proteinase Master Mix** according to **Table 3** below in a fresh 1.5 mL LoBind tube. Pipette mix to combine then store on ice until use.
   - **Note:** Include 3% excess volume to correct for pipetting losses.

Table 3. Proteinase Master Mix (per sample)

Reagent	Volume (µL)
Proteinase Buffer	65
Proteinase Enzyme	15
Total:	80

4. Add 80 µL **Proteinase Master Mix** to 10 µL of aliquoted cell lysate. Pipette to mix.
5. Incubate in thermomixer at 37 °C for 10 minutes with interval mixing at 1,200 rpm.
6. After completion of first incubation, increase temperature to 50 °C without removing tubes and incubate samples for an additional 20 minutes with interval mixing at 1,200 rpm.
7. Place samples on ice.

Clean Samples with Zymo RNA Clean & Concentrator Kit

- **Preparative Note:** Ensure 100% Ethanol is added to the **RNA Wash Buffer** concentrate upon first usage.
- **Preparative Note:** Centrifugation steps are done at room temperature.

1. Add 30 µL of **Molecular Biology Grade Water** to each digested sample to bring the volume up to 120 µL.
2. Add 240 µL of **RNA Binding Buffer** to each sample. Pipette to mix.
3. Add 360 µL of **100% Ethanol** to each sample.
4. Pipette mix thoroughly.
5. Transfer all liquid (720 µL) to corresponding labeled filter-columns in collection tubes.
6. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
7. Add 400 µL of **RNA Wash Buffer** to each column.
8. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
9. Prepare **Zymo DNase mix** according to **Table 4** in a fresh 1.5 mL LoBind tube. Pipette mix to combine then store on ice until use.
   - **Note:** Include 3% excess volume to correct for pipetting losses.

Table 4. Zymo DNase mix (per sample)

Component	Volume (µL)
Zymo DNA Digestion Buffer	17.5
Zymo DNase 1	2.5
Total:	**20**

10. Add 20 µL of **Zymo DNase mix** directly to filter of each column.
11. Incubate at room temperature for 15 minutes to digest DNA.
12. Add 400 µL of **RNA Prep Buffer** to each column.
13. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
14. Add 480 µL of **RNA Wash Buffer** to each column.
15. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
16. Repeat steps 14-15 once for a total of 2 washes.
17. Place each spin column in a new collection tube. Discard used collection tubes.
18. 'Dry' spin at 10,000 x g for 90 seconds to remove any residual ethanol.
19. Transfer each filter-column to a fresh, labeled 1.5 mL LoBind tube. Discard used collection tubes.
20. Open columns' caps and allow to air dry for 3 minutes.
21. Elute all samples by adding 11 µL of **Molecular Biology Grade Water** directly to each filter.
22. Incubate at room temperature for 1 minute.
23. Centrifuge at 7,000 x g for 30 seconds. Keep flow-through.
24. Add additional 11 µL of **Molecular Biology Grade Water** directly to each filter.
25. Incubate at room temperature for 1 minute.

26. Centrifuge at 12,000 x g for 90 seconds. Keep flow-through. Discard filter-columns.
    - **Note:** Elution volume will be ~20 µL
27. If proceeding to next step, store all samples on ice.

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

Total RNA Quantification and RNase Determination

- **Note:** Protocol has been optimized using Agilent4200 TapeStation which quantifies both RNA concentration and RNA integrity. See Agilent4200 TapeStation manual for operational instructions.
- **Note:** RNA quantity is highly variable depending on cell type.

1. Measure RNA quantity using Agilent4200 TapeStation or equivalent.
2. Calculate the amount of RNA in the original lysate.
    a. Measured RNA concentration from TapeStation e.g. 74 ng/µL. Given 20 µL elution volume, the total mass of RNA is 74 ng/µL * 20 µL = 1480 ng
    b. Being that we removed 10 µL (1%) of cell lysate to conduct RNA isolation; calculate the total amount of RNA in 1 mL = 1000 / 10 = 100 * 1480 ng = 148000 ng/mL. **If cell pellets were resuspended in a different volume, adjust number accordingly.**
    c. Convert concentration from ng/µL to µg/mL = 148000 / 1000 = 148 µg/mL
3. The amount of RNA for each IP sample is **100 µg.**
    a. 100 / 148 = 0.675 mL x 1000 = 675 µL
    b. Therefore, 675 µL of cell lysate is aliquoted for the experiment.
4. Calculate the amount of RNase required for optimal fragmentation.
    a. Given that 1U of RNase-I is sufficient to fragment 5 µg of RNA, we calculate the number of RNase-I units that is sufficient to fragment the amount of RNA in our sample e.g. 100 µg / 5 µg = 20
    b. RNase-I stock solution has a concentration of 100U / µL, e.g. there are 1000U in 10 µL. To obtain 20U in 10 µL, we need to dilute the stock solution 1000 / 20 = 50 times.
    c. RNase-I stock solution is diluted 1:50 and 10 µL is used to digest 100 µg of RNA in cell lysate.

Chapter 3: RBP ImmunoPrecipitation (IP)

Overview

This section describes immunoprecipitation of protein-RNA complexes. Dynabeads beads (magnetic beads pre-coupled to a secondary antibody) are coupled to the primary antibody for one hour at room temperature. Dynabead selection is based on host species of primary antibody. RNase is added to the lysate and RNA is fragmented into ~200 nucleotide fragments. 100 µg of fragmented RNA is then incubated with the coupled beads overnight.

Consumables

- 1× DPBS **(not provided)**
- DNase enzyme (store on ice)
- RNase-I **(not provided)**
- Primary antibody (thaw at room temperature then store on ice until use) **(not provided)**
- Dynabeads **(not provided)**
- eCLIP Lysis Buffer (thaw at room temperature then store on ice until use)

Preparation

1. Prewarm Thermomixer to 37 °C
2. Invert **eCLIP Lysis Buffer** to mix before use.
3. Place **High-Salt Buffer (HSB)** and **25× NoS (No-Salt) Buffer Concentrate** at 4 °C overnight to thaw.

Procedure

Coupling Primary Antibody to Magnetic Beads Pre-coupled with Secondary Antibody

1. Mix **Dynabeads** until homogeneous.
2. Transfer 50 µL **Dynabeads** per sample into a fresh 1.5 mL LoBind tube (e.g., for 3 samples use 150 µL of secondary beads).
3. Add 200 µL of **eCLIP Lysis Buffer** (chilled) to the tube with secondary Dynabeads.
4. Place the tube on DynaMag-2 magnet.
5. After separation is complete and supernatant is transparent (~ 1 minute), carefully aspirate and discard supernatant without disturbing beads.
6. Remove the tube from the magnet.

7. Add 500 µL **eCLIP Lysis Buffer** (chilled) to the tube, close tube, and invert mix until homogeneous.
8. Place the tube on DynaMag-2 magnet.
9. After separation is complete and supernatant is transparent (~ 1 minute), carefully aspirate and discard supernatant without disturbing beads.
10. Remove the tube from the magnet.
11. Repeat steps 7-10 once for a total of two washes.
12. Add 200 µL **eCLIP Lysis Buffer** (chilled) per sample to the tube (e.g., for 3 samples add 600 µL of eCLIP Lysis Buffer). If coupling volume exceeds 600 µL, split into multiple tubes.
13. Add 5 µg of **primary antibody** per sample to the tube containing washed beads.
14. Place tube on tube rotator and allow beads and antibody to couple for 1 hour at room temperature.

RNA Fragmentation

- **Preparative Note:** Chill centrifuge to 4 °C for pelleting of cellular debris if not done previously
- **Preparative Note:** Prewarm Thermomixer to 37 °C if not done previously.

1. For each sample, determine the volume of lysate required for 100 µg of RNA (refer to calculation performed in previous section, **Chapter 2: Cell Lysis and RNA Assessment**).
2. For each sample, aliquot the determined volume for 100 µg of RNA into a fresh labeled 1.5 mL LoBind tube and place on ice.
3. Bring the total volume up to 1 mL with **eCLIP Lysis mix** (see **Table 1**).
4. Dilute **RNase-I** in **1× DPBS** according to calculation (e.g., for 100 µg of RNA, dilute RNase-I 50-fold with **1× DPBS**).
5. Add 5 µL of **DNase** to each sample on ice.
6. Add 10 µL of **diluted RNase-I** to each sample on ice. Proceed immediately to next step.
7. Incubate in thermomixer at 37 °C for 5 minutes with interval mixing at 1,200 rpm to fragment RNA.
8. Immediately following incubation, move all samples to ice for 3 minutes.
9. Centrifuge samples at 12,000 x g for 3 minutes at 4 °C to pellet cellular debris.
10. Transfer supernatant (clarified lysate) to fresh labeled 1.5 mL LoBind tubes without disturbing cell pellet.
11. Leave clarified supernatant on ice until **Immunoprecipitation** in the following step.
12. Discard cell pellets.

Immunoprecipitation

1. After 1 hour, remove antibody-coupled magnetic bead tubes from rotator.
2. To each antibody-coupled magnetic bead tube, add 500 µL **eCLIP Lysis Buffer** (chilled).
3. Invert mix until homogenous.
4. Put tubes on DynaMag-2 magnet to separate beads and allow at least 1 minute for bead separation.

5. When separation is complete and liquid is transparent, carefully aspirate and discard supernatant without disturbing beads.
6. Remove tube from magnet.
7. Add 1 mL of clarified lysate containing fragmented RNA (RNA Fragmentation, step 11) to each antibody-coupled magnetic bead tube and slowly pipette to mix until homogeneous.
8. Rotate immunoprecipitation tubes containing fragmented RNA and antibody-coupled magnetic beads overnight at 4 °C.

Stopping Point: *Samples rotate overnight at 4 °C for up to 16 hours*

Chapter 4: IP RNA 3’ Adapter Ligation

Overview

This section describes the 5’ and 3’ end repair of RNA, followed by ligation of an adapter to the 3’ end of bound transcripts.

Consumables

- PSP Buffer (thaw at room temperature then store on ice until use)
- IP PNK Buffer (thaw at room temperature then store on ice until use)
- RNA Ligation Buffer (thaw at room temperature then keep at room temperature)
- IP-E RNA Adapter (thaw at room temperature then store on ice until use)
- PSP enzyme (store on ice)
- PNK enzyme (store on ice)
- RNase Inhibitor enzyme (store on ice)
- DNase enzyme (store on ice)
- Ligase enzyme (store on ice)
- 1× NoS (No-Salt) Buffer (Gently invert 5 times to mix then store on ice until use)
- High-Salt Buffer (HSB) (Gently invert 5 times to mix then store on ice until use)
- Molecular Biology Grade Water **(not provided)**

Preparation

1. Pre-warm Thermomixer to 37 °C
2. Dilute 25× NoS (No-Salt) Buffer Concentrate
3. Centrifugation steps are done at room temperature

Procedure

Prepare Wash Buffer

1. Dilute 25× NoS Buffer (stored at 4 °C from Chapter 2) according to **Table 5** in a 50 mL conical tube. Invert to mix 1× NoS Buffer and store on ice.

Table 5. 1× NoS Buffer preparation

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

Take Input Samples

1. Obtain immunoprecipitation (IP) tubes (**Immunoprecipitation,** Chapter 3, step 8).
2. Pipette mix until homogenous.
3. Transfer 30 µL of mixed lysate (including beads) to a new 1.5 mL DNA LoBind tube for each IP sample.
   - **Note:** This will be the Size-matched Input sample. Label accordingly.
4. Store Input samples on ice until Chapter 6: SDS-PAGE and Membrane Transfer.
5. Aliquot an additional 30 µL of mixed lysate in new tubes as backup Input samples and store at -80 °C.

First Immunoprecipitation Wash

1. Put IP tubes on DynaMag-2 magnet to separate beads.
2. Allow at least 1 minute for bead separation.
3. When separation is complete and liquid is transparent, carefully aspirate and discard supernatant without disturbing beads.
4. Remove IP tubes from magnet.
5. Add 500 µL cold **High-Salt Buffer (HSB)**.
6. Invert mix until homogeneous.
7. Place on DynaMag-2 magnet.
8. While on magnet, slowly invert closed tubes as beads start to separate to capture any beads from cap.
9. When separation is complete, and liquid is transparent, gently open tubes and discard supernatant without disturbing beads.
10. Repeat steps 4-9 twice for a total of three HSB washes.
11. Remove IP tubes from magnet.
12. Add 500 µL cold **1× NoS Buffer**.
13. Invert mix until homogeneous.
14. Place on DynaMag-2 magnet.
15. While on magnet, slowly invert closed tubes as beads start to separate to capture any beads from cap.
16. When separation is complete, and liquid is transparent, gently open tubes and discard supernatant without disturbing beads.
17. Repeat steps 11-15 once for a total of two **1× NoS washes.**

18. Remove IP tubes from magnet.
19. Spin all IP samples in mini-centrifuge for 3 seconds.
20. Place samples back on magnet and allow 30 seconds to separate.
21. Pipette to discard any excess liquid without disturbing beads.
22. Add 500 µL cold **1× NoS Buffer**.
23. Gently invert mix until homogeneous.
24. 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 6** in a fresh 1.5mL DNA 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 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 30 seconds to separate.
6. Pipette and discard any excess liquid, careful not to let the beads dry.
7. Add 50 µL **IP PSP Master Mix** to each tube.
8. Pipette mix until homogenous.
9. Incubate in thermomixer at 37 °C for 10 minutes with interval mixing at 1,250rpm.
10. Continue to the next step during the incubation.

IP RNA 3'-End Repair

1. Prepare **IP PNK Master Mix** according to **Table 7** 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 7. 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 (**IP RNA 5'-end repair**, step 9) is complete, add 150 µL **IP PNK Master Mix** to each IP tube at room temperature.
3. Pipette mix until homogenous.
4. Incubate in thermomixer at 37 °C for 20 minutes with interval mixing at 1,250 rpm.

Second Immunoprecipitation Wash

- **Preparation 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**.
2. Invert mix until homogeneous.
3. Place on DynaMag-2 magnet.
4. While on magnet, slowly invert tubes as beads start to separate to capture any beads from cap.
5. When separation is complete, and liquid is transparent, gently open tubes and discard supernatant without disturbing beads.
6. Remove IP tubes from magnet.
7. Repeat steps 1-6 once for a total of two HSB washes.
8. Add 500 µL cold **1× NoS Buffer**.
9. Gently invert mix until homogenous.
10. Place on DynaMag-2 magnet.
11. While on magnet, slowly invert tubes as beads start to separate to capture any beads from cap.
12. When separation is complete, and liquid is transparent, gently open tubes and discard supernatant without disturbing beads.
13. Remove IP tubes from magnet.
14. Repeat steps 8-13 once for a total of two **1× NoS washes**
15. Spin all samples in mini-centrifuge for 3 seconds
16. Place samples back on magnet and allow 30 seconds to separate.
17. Pipette to discard any excess liquid without disturbing beads.
18. Remove IP tubes from magnet.
19. Add 500 µL cold **1× NoS Buffer**.
25. Invert to mix until homogeneous.
26. Store on ice and proceed to the next step.

RNA Adapter Ligation to Immunoprecipitation Samples

1. Prepare **IP Ligation Master Mix** according to **Table 8** 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 8. 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 and allow at least 1 minute for bead separation.
4. Remove and discard supernatant.
5. Spin all samples in mini-centrifuge for 3 seconds.
6. Place samples back on magnet and allow 30 seconds to separate.
7. Pipette and discard any excess liquid, taking care to not disturb beads.
8. Add 3 µL of **IP-E 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.

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. Invert mix until homogeneous.
16. Place samples on ice.

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 and Input samples and Chemiluminescent Biotinylated RNA Detection for IP samples only. It is recommended to perform these steps during IP RNA 3'-adapter ligation.

Consumables

- NuPAGE 4-12% Bis-Tris gels **(not provided)**
- 20× NuPAGE MOPS SDS Running Buffer (store at 4°C until use) **(not provided)**
- 20× NuPAGE Transfer Buffer (store at 4°C until use) **(not provided)**
- Spectra Multicolor Broad Range Protein Ladder (thaw at room temperature then store on ice until use) **(not provided)**
- 2× Western Buffer (thaw at room temperature then store on ice until use)
- 100% Methanol
- Molecular Biology Grade Water (not provided)

Preparation

1. Remove pre-cast gels from plastic and wash with rinse 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) and analytical gels (for western blotting) required for IP and Input samples; as well as the numner of Biotin gels (for RNA detection) required for IP samples only (see **Example: Preparative Gel Loading Scheme**, **Example: Analytical Gel Loading Scheme** and **Example: Biotin Gel Loading Scheme** below).

2. Label the appropriate number of **pre-cast NuPAGE 4-12% Bis-Tris gels** with sample/ladder information. For the preparative gels, reserve wells between samples for ladders. This will simplify size-selection later on (see **Example: Preparative Gel Loading Scheme** below).

Example: Preparative Gel Loading Scheme (IP and 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	Input 1	Low Conc. Ladder	Input 2	Low Conc. Ladder	1× WB

Example: Analytical Gel Loading Scheme (IP and Input samples)

Well	1	2	3	4	5	6	7	8	9	10
Sample/ Ladder	High Conc. Ladder	IP 1	IP 2	IP 3	IP4	Input 1	Input 2	Input 3	Input 4	Low Conc. Ladder

Example: Biotin Gel Loading Scheme (IP samples ONLY)

Well	1	2	3	4	5	6	7	8	9	10
Sample/ Ladder	High Conc. Ladder	IP 1	IP 2	IP 3	IP4	IgG Ctrl	1× WB	1× WB	1× WB	1× WB

3. Dilute 20× NuPAGE MOPS SDS Running Buffer to create **1× Running Buffer** according to **Table 9**.
4. Shake manually to mix, then store on ice.

Table 9. 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 10.**
6. Shake manually to mix, then store on ice.

Table 10. 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 11** and store on ice.

Table 11. 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.*

Proceed immediately to next chapter

Next Stopping point: ~30 minutes

Chapter 6: SDS-PAGE and Membrane Transfer

Overview

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

Consumables

- 2× Western Buffer (thawed in previous section)
- 1× Running Buffer (diluted in previous section)
- High-concentration ladder (diluted in previous section)
- Low-concentration ladder (diluted in previous section)

Preparation

1. Pre-heat thermomixer to 65 °C.

Procedure

Elution of IP and Input RBP-RNA Complexes for Electrophoresis

1. Prepare 1× Western Buffer according to **Table 13** below and store on ice.

Table 13. 1× Western Buffer Preparation (per sample)

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

2. Move all IP tubes from ice (**Third Immunoprecipitation Wash** in Chapter 4, step 16) 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 30 seconds for separation.

6. Pipette and discard any excess liquid without disturbing beads.
7. Add 30 µL of **1× Western Buffer** to IP samples.
8. Pipette mix until homogenous and then store on ice.
9. Add 30 µL of **2× Western Buffer** to Input samples (**Take Input Samples** in Chapter 4, step 5).
10. Pipette mix until homogenous and then store on ice.
11. Incubate all samples (IP and Input) on thermomixer at 65 °C for 10 minutes with interval mixing at 1,200 rpm.
12. Move all samples to ice for 2 minutes.
13. Spin all samples in mini-centrifuge for 3 seconds.
14. Move all tubes from ice to DynaMag-2 magnet and allow at least 1 minute for bead separation.
15. Remove supernatants from beads and place in fresh, labeled tubes.
16. Discard tubes containing beads.

Optional Stopping Point: *If stopping here, store denatured samples at -80 °C*
Next stopping point: ~4 hours

Load SDS-PAGE Preparative, Analytical, and Biotin Gels

1. Assemble electrophoresis tank (see Appendix B1: SDS-PAGE for assembly instructions).
2. Remove sticker from all labeled gels.
3. Place labeled **preparative**, **analytical** and biotin gels inside of 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 gels.
8. Load 20 µL of IP samples and 20 µL of Input samples into appropriate wells of **preparative gels**.
   - **Note:** Ensure all wells in the middle of the gels are filled, as empty wells can cause uneven sample electrophoresis. Empty wells are filled with 1× WB.
9. Add 55 µL of **1× Western Buffer** to 10 µL of remaining IP samples. Pipette to mix.
10. Add 20 µL of diluted IP samples and 20 µL of Input samples into appropriate wells of **analytical gels.**
11. Add 20 µL of diluted IP samples into appropriate wells of **biotin gels.**
12. Run gels at constant 160 V until the lower dye front reaches the bottom of the gel (typically 75-90 minutes).

13. Remaining ~20 µL of IP and Input samples can be frozen at -80 °C as backup.
    - **Note:** 20 µL of backup Input samples are sufficient for preparative gels or western blot. 20 µL of diluted backup IP samples are sufficient for analytical gels (western blot or RNA visualization).

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.
5. Gel is now ready to be transferred.
6. Prepare membranes for transfer (see **Appendix B2: Membrane Transfer** for detailed instructions).
    - **Note:** For **preparative gels** and **RNA visualization gels**, 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:** The **analytical gel** can be transferred using a variety of 'wet', 'dry', and 'semi-dry' transfer methods. For Western Blot, we recommend the same 'wet' transfer to PVDF membrane for optimal imaging results. If using PVDF membranes for western blot, be sure to activate membrane in methanol prior to use (see Appendix). If using other methods, follow manufacturer's recommended protocol.
7. Assemble transfer chamber and transfer stacks (see **Appendix B2: Membrane Transfer** if using Mini Protean Tetra Apparatus).
8. 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: Size-selection and Proteinase Digestion

Overview

This section describes the size-selection of protein-bound RNA fragments and subsequent digestion by proteinase. Following transfer, the section of membrane corresponding to the protein size plus 75 kDa is cut out and placed in a tube. Proteinase is then added to digest proteins, leaving only free RNA. Lastly, the RNA is cleaned and concentrated with using columns.

Consumables

- Proteinase Buffer (thaw at room temperature then store on ice)
- Proteinase Enzyme (store on ice)
- Razor blades **(not provided)**
- Microscope slides **(not provided)**
- RNA Clean and Concentrator-5 (Zymo Kit) **(not provided)**
- Molecular Biology Grade Water (not provided)

Preparation

1. If performing western blot, prepare 5% milk in TBST or other blocking agent prior to disassembly of transfer chamber.
2. Prewarm Thermomixer to 37 °C.

Procedure

Size-selection of Protein Bound RNA

1. After transfer is complete, disassemble transfer chamber and transfer stacks (see **Appendix B2: Membrane Transfer** if using Mini Protean Tetra Apparatus).
2. Briefly rinse preparative membranes, analytical membranes, and biotin membranes in a container with **1× DPBS**.
3. Place **preparative membranes** in plastic cover sheet.
   - **Note:** If desired, preparative membranes can be frozen in plastic cover sheet at -20 °C until western blot is finished.

4. For **analytical membranes**, proceed immediately to western blot protocol (see **Appendix C: Western Blot Image Preparation**) and RNA visualization protocol (see **Appendix D: Chemiluminescent Biotinylated-RNA Detection**).
5. With **preparative membrane** still in cover sheet, place on top of flat cutting surface.
6. Using a razor blade, carefully cut a rectangular section out of the membrane corresponding to the region from protein size plus 75 kDa above (reference loading scheme; IP and Input samples should be in lanes between ladders).
7. Separate membrane cut-out from plastic cover sheet.
8. Place rectangular cut-out on top of clean cutting surface such as a microscope slid and cut into 1-2 mm slices.
9. Carefully place membrane slices into a fresh, labeled 1.5 mL LoBind tube.
10. Discard razor.
11. Repeat steps 6-10 for each IP and Input sample.
12. Discard cover sheet.

Proteinase Digestion of IP and Input Samples

1. Prepare **Proteinase Master Mix** according to **Table 14** below in a fresh 1.5 mL LoBind tube.
   - **Note:** Include 3% excess volume to correct for pipetting losses.

Table 14. Proteinase Master Mix (per sample)

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

1. Add 127 µL of **Proteinase Master Mix** to each sample tube containing membrane slices and ensure all membrane slices are submerged.
2. Incubate in thermomixer at 37 °C for 20 minutes with interval mixing at 1,200 rpm.
3. After completion of first incubation, increase temperature to 50 °C and continue incubation in thermomixer at 50 °C for 20 minutes with interval mixing at 1,200 rpm.

Clean all IP and Input Samples with Zymo RNA Clean & Concentrator Kit

- **Preparative Note:** Ensure 100% Ethanol is added to the **RNA Wash Buffer** concentrate upon first usage.
- **Preparative Note:** Centrifugation steps are done at room temperature.

1. For each sample, transfer all liquid (~125 µL) from proteinase digestion into fresh, labeled DNA LoBind tubes. This contains the eluted RNA sample.
2. Discard membrane slices.
3. Add 250 µL of **RNA Binding Buffer** to the 125 µL of eluted RNA sample. Pipette to mix.
4. Add 375 µL of **100% Ethanol** to the tubes.

5. Pipette mix thoroughly.
6. Transfer all liquid (750 µL) to corresponding labeled filter-columns in collection tubes.
7. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
8. Add 400 µL of RNA Prep Buffer to each column.
9. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
10. Add 480 µL of RNA Wash Buffer to each column.
11. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
12. Repeat steps 10-11 once for a total of 2 washes.
13. Place each spin column in a new collection tube. Discard used collection tubes.
14. 'Dry' spin at 10,000 x g for 1 minute to remove any residual ethanol.
15. Transfer each filter-column to a new labeled 1.5 mL LoBind tube. Discard used collection tubes.
16. Open columns' caps and allow to air dry for 3 minutes.
17. Elute all samples by adding 11 µL of Molecular Biology Grade Water directly to each filter.
18. Incubate at room temperature for 1 minute.
19. Centrifuge at 12,000 x g for 90 seconds. Discard filter-columns.
    - Note: Elution volume will be ~10 µL.
20. If proceeding to next step, store all samples on ice. IP samples can remain on ice or be frozen until Chapter 9: Reverse Transcription and cDNA Adapter Ligation.

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

Chapter 8: Input RNA 3’ Adapter Ligation

Overview

This section describes the 5’ and 3’ end repair of Input RNA, followed by ligation of an adapter to the 3’ end of Input RNA transcripts.

Consumables

- PSP buffer (thaw at room temperature then store on ice until use)
- RNase Inhibitor (store on ice)
- PSP Enzyme (store on ice)
- Input PNK Buffer (thaw at room temperature then store on ice until use)
- DNase Enzyme (store on ice)
- PNK Enzyme (store on ice)
- RNA Ligation Buffer (thaw at room temperature then store at room temperature until use)
- Input RNA Adapter (thaw at room temperature then store on ice until use)
- Ligase Enzyme (store on ice)
- eCLIP beads (resuspend until homogeneous then store at room temp)
- Bead Binding Buffer (store on ice until use)
- 100% Ethanol **(not provided)**
- Bead Elution Buffer (thaw at room temperature then store on ice until use)

Preparation

1. Prewarm Thermomixer to 37 °C.
2. 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 15** 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 15. Input PSP Master Mix (per sample)

Reagent	Volume (µL)
PSP Buffer	8
RNase Inhibitor	1
PSP Enzyme	2
Total:	11

3. Add 11 µL of **Input PSP Master Mix** to each 10 µL Input sample. Pipette to mix.
4. Incubate in Thermomixer at 37 °C for 10 minutes with interval mixing at 1,200 rpm.

3'-End Repair of Input RNA

1. Prepare **Input PNK Master Mix** according to **Table 16** 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 16. Input PNK Master Mix (per sample)

Reagent	Volume (µL)
Input PNK Buffer	70
DNase	1
PNK Enzyme	4
Total:	75

3. Add 75 µL of **Input PNK Master Mix** to each Input sample. Pipette to mix.
4. Incubate in thermomixer for 20 minutes at 37°C with interval mixing at 1,200 rpm.

Clean Input Samples with Zymo RNA Clean & Concentrator Kit

- **Preparative Note:** Ensure 100% Ethanol is added to the **RNA Wash Buffer** concentrate upon first usage.
- **Preparative Note:** Centrifugation steps are done at room temperature.

1. Add 200 µL of **RNA Binding Buffer** to the 96 µL of eluted RNA sample.
2. Add 300 µL of **100% Ethanol** to the tubes.
3. Pipette mix thoroughly.
4. Transfer all liquid (~600 µL) to corresponding labeled filter-columns in collection tubes.
5. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
6. Add 400 µL of **RNA Prep Buffer** to each column.
7. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
8. Add 480 µL of **RNA Wash Buffer** to each column.
9. Centrifuge at 7,000 x g for 30 seconds. Discard flow-through.
10. Repeat steps 8-9 once for a total of 2 washes.

11. Place each spin column in a new collection tube. Discard used collection tubes.
12. 'Dry' spin at 10,000 x g for 1 minute to remove any residual ethanol.
13. Transfer each filter-column to a new labeled 1.5 mL LoBind tube. Discard used collection tubes.
14. Open columns' caps and allow to air dry for 3 minutes.
15. Elute all samples by adding 11 µL of **Molecular Biology Grade Water** directly to each filter.
16. Incubate at room temperature for 1 minute.
17. Centrifuge at 12,000 x g for 90 seconds. Discard filter-columns.
    - **Note:** Elution volume will be ~10 µL.
18. If proceeding to next step, store all samples on ice.

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

Input Sample Adapter Ligation

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

1. Prepare **Input Ligation Master Mix** according to **Table 17** 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.
- **Note**: RNA Ligation Buffer is very viscous, and the master mix will require thorough mixing.

Table 17. Input Ligation Master Mix (per sample)

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

3. Add 5 µL of eluted Input RNA into pre-labeled 0.2 mL strip tubes. Store remaining repaired Input RNA at -80 °C as backup.
4. Add 2 µL of **Input RNA Adapter** to each Input RNA sample. Pipette to mix.
5. Spin samples in mini-centrifuge for 3 seconds to draw all liquid to the bottom of the tube.
6. Incubate tubes at 65 °C for 2 minutes in thermal cycler with the lid preheated to 70 °C.
7. Immediately place samples on ice for 1 minute.
8. Add 13.5 µL of **Input Ligation Master Mix** to the adapter-added Input RNA sample.
9. Slowly pipette mix until homogeneous.
10. Incubate the samples for 1 hour at room temperature on tube rotator.

Input RNA Bead Cleanup

1. For each Input sample, add 10 µL of **eCLIP Beads** to a fresh 1.5 mL LoBind tube (e.g., for 5 samples use 50 µL of eCLIP Beads).
2. Wash eCLIP beads prior to addition to samples:
    a. Add 5× volume of **Bead Binding Buffer** (e.g., for 5 samples, add 250 µL of Bead Binding Buffer).
    b. Pipette mix until sample is homogeneous.
    c. Place tube on DynaMag-2 magnet.
    d. When separation is complete and supernatant is transparent, carefully aspirate and discard supernatant without disturbing beads.
    e. Remove tube from magnet.
3. 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 5 samples add 315 µL of Bead Binding Buffer).
4. Transfer 60 µL of washed **eCLIP Beads** to each 20 µL tube of Input RNA sample. Pipette to mix.
5. Add 60 µL of **100% Ethanol** to each sample.
6. Pipette mix until homogeneous.
7. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
8. Place on magnet for 30 seconds until separation is complete and supernatant is transparent.
9. Carefully aspirate and discard supernatant without disturbing beads.
10. Add 150 µL of **80% Ethanol** without disturbing beads.
11. Move samples to different positions on magnet to wash thoroughly.
12. Carefully add an additional 150 µL of **80% Ethanol.**
13. Place on magnet for 30 seconds until separation is complete and supernatant is transparent.
14. Carefully aspirate and discard supernatant.
15. Repeat steps 10-14 for a total of two washes.
16. Spin capped samples in mini-centrifuge for 3 seconds to draw all liquid to the bottom of the tube.
17. Place tube back on 96-well magnet.
18. Incubate on magnet for 10 seconds until separation is complete and supernatant is transparent.
19. While on magnet aspirate and discard all residual liquid without disturbing beads.
20. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
21. Once completely dry, carefully remove tubes from magnet.
22. Add 10 µL **Bead Elution Buffer** to each sample.
23. Pipette mix until sample is homogeneous.
24. Incubate for 5 minutes at room temperature.
25. After incubation, move tubes to 96-well magnet.
26. Incubate on magnet for 30 seconds until separation is complete and supernatant is transparent.
27. Transfer supernatant (containing eluted RNA) to new 0.2 mL strip tubes.

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

Chapter 9: Reverse Transcription, cDNA Adapter Ligation

Overview

This section describes the reverse transcription of IP and Input RNA into cDNA. Reverse transcription is performed, followed by several steps to remove dNTPs and template RNA. ssDNA samples are then cleaned using beads and overnight ligation is performed to attach an adapter to the 5' ends of cDNA.

Consumables

- eCLIP beads (resuspend until homogeneous)
- Bead Binding Buffer (store on ice until use)
- RT Enzyme (store on ice)
- RNase Inhibitor (store on ice)
- Nuclease Enzyme (store on ice)
- ssDNA Enzyme (store on ice)
- Ligase Enzyme (store on ice)
- RT Buffer (thaw at room temperature then store on ice until use)
- RT Primer (thaw at room temperature then store on ice until use)
- ssDNA Adapter (thaw at room temperature then store on ice until use)
- ssDNA Ligation Buffer (thaw at room temperature then store on ice until use)
- 0.5 M EDTA **(not provided)**
- 1 M NaOH **(not provided)**
- 1 M HCl **(not provided)**
- 80 & 100% Ethanol

Preparation

1. Preheat PCR thermal cycler block to 65 °C (with lid set at 70 °C).

Procedure

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 (**IP Sample Cleanup** section step 27).
2. For each IP RNA sample, transfer 9 µL into a new, labeled 0.2 mL 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.
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 1.5 mL 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 – 15 minutes (with lid set to 45 °C).

cDNA End Repair of IP and Input Samples

1. Add **2.5 µL** of **Nuclease** to each sample.
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 – 10 minutes (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. Wash **eCLIP beads** prior to addition to samples.
3. For each IP and Input cDNA 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).
4. Add 5× volume of **Bead Binding Buffer** (e.g., for 4 samples add 100 µL buffer to 20 µL of eCLIP beads). Pipette mix until sample is homogeneous.
5. Place tube on DynaMag-2 magnet. When separation is complete and supernatant is clear, carefully aspirate and discard supernatant without disturbing beads.
6. Remove tube from magnet.
7. Resuspend eCLIP beads in **93 µL** of **Bead Binding Buffer** per sample.
8. Pipette up and down until beads are fully resuspended.
9. Add 90 µL of washed **eCLIP Beads** to each IP and Input cDNA sample.
10. Pipette mix until sample is homogeneous.
11. Add 90 µL of **100% Ethanol** to each IP and Input cDNA sample.
12. Pipette mix until homogeneous.
13. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
14. **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.**
15. Allow to incubate for 1 minute or until separation is complete and liquid is transparent.
16. Carefully discard supernatant without disturbing beads.
17. Add 150 µL of **80% Ethanol**.
18. Move samples to different positions on magnet to wash thoroughly.
19. Add an additional 150 µL of **80% Ethanol**.
20. Incubate on magnet for 30 seconds until separation is complete and supernatant is transparent.
21. Carefully aspirate and discard all supernatant while on magnet.
22. Repeat steps 17-21 once for a total of two washes.
23. Spin capped samples in mini-centrifuge for 5 seconds to draw all liquid to the bottom of the tube.
24. Place tube back on 96-well magnet.
25. Incubate on magnet for 10 seconds until separation is complete and supernatant is transparent.
26. Using fine tips, aspirate and discard all residual liquid without disturbing beads while on magnet.
27. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
    - **Note**: Do not over dry samples.
28. Once completely dry, carefully remove tubes from magnet.

29. Resuspend beads in 2.5 µL of **ssDNA Adapter**.
30. Pipette to mix until homogeneous.
31. Incubate in thermal cycler at 70 °C for 2 minutes with the lid at 75 °C.
32. Following incubation, immediately place on ice for 1 minute.

IP and Input cDNA Ligation

1. Prepare **cDNA Ligation Master Mix** according to **Table 17** in a fresh 1.5 mL 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 10: 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

- Library Elution Buffer (store on ice until use)
- Bead Binding Buffer (store on ice until use)
- Bead Elution Buffer (thaw at room temperature then store on ice until use)
- qPCR Primers (thaw at room temperature then store on ice until use)
- Index Primers (thaw at room temperature then store on ice until use)
- PCR Mix (thaw at room temperature then store on ice until use)
- NEB LUNA Universal qPCR Master Mix (**not provided;** thaw at room temperature then store on ice until use)
- AMPure XP Beads (**not provided**; equilibrate to room temperature and mix until homogenous before use)
- Molecular Biology Grade Water **(not provided)**

Procedure

Ligated cDNA IP and Input Sample Cleanup

1. Obtain ligated-cDNA samples from tube rotator.
2. To each IP and Input cDNA sample, add 5 µL of **Bead Elution Buffer.**
3. Add 45 µL of **Bead Binding Buffer**. Pipette to mix.
4. Add 45 µL of **100% Ethanol** to each sample and pipette mix until homogeneous.
5. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
6. Place strip-tube on 96-well magnet and allow to incubate for 1 minute or until separation is complete and liquid is transparent.
7. Carefully aspirate and discard supernatant without disturbing beads.

8. Add 150 µL of **80% Ethanol** without disturbing beads.
9. Move samples to different positions on magnet to wash thoroughly.
10. Carefully add an additional 150 µL of **80% Ethanol.**
11. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
12. Carefully aspirate and discard supernatant.
13. Repeat steps 7-11 for a total of two washes.
14. Spin capped samples in mini-centrifuge for 3 seconds to draw all liquid to the bottom of the tube.
15. Place tube back on 96-well magnet.
16. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
17. While on magnet, aspirate and discard all residual liquid without disturbing beads.
18. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
19. Once completely dry, carefully remove tubes from magnet.
20. Add 25 µL **Bead Elution Buffer** to each sample.
21. Pipette mix until sample is homogeneous.
22. Incubate for 5 minutes at room temperature.
23. After incubation, move tubes to 96-well magnet.
24. Incubate on magnet for 30 seconds until separation is complete and supernatant is transparent.
25. Transfer supernatant (containing eluted cDNA) to new 0.2 mL strip tubes.

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

---

cDNA IP and Input Sample Quantification by qPCR

1. Prepare **qPCR Master Mix** for the appropriate number of reactions according to **Table 20** in a fresh 1.5 mL LoBind tube.

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

Table 20. 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 21** for suggested 96-well layout).

Table 21. 96-well qPCR plate layout for 16 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 on ice until PCR in the next section.
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

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

1. Thaw Index primers at room temperature until fully melted. Shake to mix and spin in mini-centrifuge for 3 seconds. Store on ice until use.
2. Prepare PCR amplification reaction mix according to **Table 22** 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 22. PCR Amplification Reaction 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

   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 26.**

Table 26. PCR Amplification Program

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

*N should be **≥ 1** and **< 14**.

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

AMPure Library PCR Product Cleanup

- **Preparative Note**: Allow AMPure XP beads (not provided) to equilibrate at room temperature for 5 minutes.

1. Manually shake or vortex AMPure XP beads to resuspend until homogeneous.
2. Add 60 µL of **AMPure XP beads** into each 40 µL PCR sample.
3. Pipette to mix until sample is homogeneous.
4. Incubate at room temperature for 10 minutes, with pipette mixing every 5 minutes.
5. Place strip-tube on 96-well magnet and allow to incubate for 1 minute or until separation is complete and liquid is transparent.
6. Carefully aspirate and discard supernatant without disturbing beads.
7. Add 150 µL of **80% Ethanol** without disturbing beads.
8. Move samples to different positions on magnet to wash thoroughly.
9. Carefully add an additional 150 µL of **80% Ethanol.**
10. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
11. Carefully aspirate and discard supernatant.
12. Repeat steps 7-11 for a total of two washes.
13. Spin capped samples in mini-centrifuge for 3 seconds to draw all liquid to the bottom of the tube.
14. Place tube back on 96-well magnet.
15. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
16. While on magnet, aspirate and discard all residual liquid without disturbing beads.
17. Allow beads to air dry for 5 minutes or until beads no longer appear wet and shiny.
18. Once completely dry, carefully remove tubes from magnet.
19. Add 20 µL **Library Elution Buffer** to each sample.
20. Pipette mix until sample is homogeneous.
21. Incubate for 5 minutes at room temperature.
    - **Note:** Do not transfer the 20 µL of eluted sample to new strip-tube.
22. Proceed immediately to next step.

Gel Purification of IP and Input Samples

- **Note**: The following protocol is for standard gel electrophoresis equipment. Refer to APPENDIX E: E-GEL PURIFICATION for protocol using the E-Gel system, which can improve recovery.

Prepare 3% Low Melting Temperature Agarose Gel

1. Mix 3 grams of low melting temperature agarose per 100 mL of 1× TBE in a microwave-safe flask. Choose final volume based on available gel tray.

2. Microwave for 30 seconds on 'HIGH' setting, followed by swirling to mix. Repeat until agarose is fully melted.
   - **Note**: low melting temperature agarose tends to boil over rapidly.
3. Cool for 10 minutes at room temperature.
4. Add 1:10000 volume of SybrSafe (or equivalent). Swirl to mix.
5. Pour into gel tray. Ensure combs used can support loading 25 µL of sample per well.

Prepare Samples and Run Gel

1. Add 6 µl 6× OrangeG buffer (2× final concentration) to each 18 µl of sample and pipette up and down until homogeneous.
2. Prepare two 50 bp ladder samples in Orange G buffer (Per well: 0.5 µl ladder + 2 µL Orange G + 7.5 µL H_2O)
3. Load gel. It is recommended to load samples in every other well, with intervening wells filled with Orange G buffer diluted in water. Load ladder on both sides of the gel. See Example 4 for a model for a 2 replicate (4 library) experiment.

Example 4. PCR gel loading schematic

Well #	1	2	3	4	5	6	7	8	9	10
Sample/ Ladder	50 bp Ladder	IP Rep. 1	1× Orange G	IP Rep. 2	1× Orange G	Input Rep. 1	1× Orange G	Input Rep. 2	50 bp Ladder	1× Orange G

4. Run gel at 95 V for 50 mins.
   - **Note**: Longer running gives better resolution but larger cut sizes.

Excise Library from Gel

1. For each sample, take and label a new 15 mL conical tube.
2. Weigh empty tube on analytical balance and record weight.
3. Under blue light illumination, cut a gel slice from 175-350 bp and place into the 15 mL conical tube. Use a fresh razor blade for each sample.
   - **Note**: Adapters alone (including RNA adapter) are 142 bp. Fragments smaller than 165 bp will yield reads too short to properly map.
   - **Note**: Blue light illumination is preferred to UV illumination, which can cause DNA damage. If using UV illumination, minimize illumination time (use razor blades to mark ladder lanes and perform gel isolation with UV off if possible).
5. Discard used razor into sharps container.
6. Repeat steps 1 – 5 for all samples.

Extract Libraries from Gel Slices

- **Note:** Centrifugation steps are done at room temperature.

1. Label new filter columns from MinElute Gel Extraction Kit (columns are stored at 4 °C).

2. Weigh each 15 mL tube containing library gel slices and record weight. Subtract weight of tubes containing gel slices from weight of empty tube previously measured to identify the weight of gel slices. Record weight of gel slice.
3. Add 5× volume of Buffer QG to each tube (e.g., for **80 mg** gel weight add **400 µL** Buffer QG).
4. Rotate tubes at room temperature until completely melted. Do not heat samples.
5. Once gel has completely melted, add 1× volume of isopropanol (e.g. for **80 mg** gel, add **80 µL** isopropanol). Pipette to mix. Transfer total volume to corresponding MinElute filter column (if volume exceeds **700 µL,** spin in multiple steps).
6. Spin columns in mini-centrifuge 5,000 × g for 1 minute. Discard flow-through.
7. If volume exceeded 700 µL, transfer remaining sample onto the same MinElute filter column and centrifuge. Discard flow-through. Repeat until all sample has been used.
8. **Prepare Buffer QG + 20% isopropanol** (See **Table 24**; volumes are for 1 sample, adjust accordingly).

Table 24. Buffer QG + 20% Isopropanol

Component	Volume (µL)
Buffer QG	400
Isopropanol	100
Total:	***500***

9. **Add 500 µL of Buffer QG + 20% isopropanol** to each MinElute column and spin column in mini-centrifuge 5,000 × g for 1 minute. Discard flow-through.
10. **Add 500 µL of Buffer PE** (ensure ethanol has been added per vendor recommendation). Spin columns in mini-centrifuge 5,000 × g for 1 minute. Discard flow-through.
11. **Repeat step 15 once** more for two total Buffer PE washes.
12. Open caps and remove any liquid left above internal column ring. Allow columns to air dry for at least 3 minutes. Once dry, carefully move columns to fresh 1.5. mL tubes that have been appropriately labeled.
13. **Add 20 µL of Molecular Biology Grade Water** directly to center of column. Incubate at room temperature for 1 minute.
14. Spin samples at 12,000 × g for 30 seconds.
15. To improve yield, re-elute samples by taking 15 µL of flow-through and adding it directly back to the same MinElute column. Spin again at 12,000 × g for 90 seconds.
16. Store samples on ice if proceeding to library quantitation.

Optional Stopping Point: If stopping here, freeze libraries 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 A1: Preparation and UV Crosslinking of Adherent Cells

Required Equipment and Materials

Item	Source
UV Crosslinker with **254-nm wavelength UV bulbs**	UVP CL-1000 Ultraviolet Crosslinker or equivalent
Liquid Nitrogen	Any
1×DPBS	Corning cat. #21-031-CV or equivalent
Trypan blue stain	Thermo Fisher Scientific, cat. #15250061 or equivalent live cell counting assay
Standard cell counting system	Hemocytometer or automated cell counter

Procedure

Cell viability validation (prior to crosslinking)

1. Use Trypan blue stain (Thermo Fisher Scientific, cat. #15250061) or equivalent live cell counting assay to valuate cell viability.
2. Cell viability should be > 95% to ensure intact RNA.
3. Cells should be grown to a proper confluence, in most cases grow cells to 75% confluence.

Wash cells

1. Aspirate spent media.
2. Wash the plate gently with 1× DPBS at room temperature (15 mL for a 15 cm plate).
3. Carefully aspirate 1× DPBS.
4. Add enough 1× DPBS to just cover the plate (5 mL for a 15 cm plate)
   - **Note:** If all plates are at equal cell density, one plate can be sacrificed for counting – this plate would be dissociated (with trypsin, accutase, or equivalent) and cell number (per plate) counted at this stage. **This is recommended for cell types that require chemical dissociation** enzymes to dissociate and be properly counted, as this is not recommended post-crosslinking.

UV crosslinking

1. Place the tissue culture plate on leveled ice or a cooling block pre-chilled to 4 °C
2. Place the above (plate plus ice or cooling block) into the UV cross-linker.
   - **Notes:**
     - **Ensure the plate is leveled**.
     - **Remove tissue culture plate lid** for crosslinking.
3. Crosslink at 254-nm UV with an energy setting of 400 mJoules/cm^2.

- Note: this is a setting of 4000 on many cross-linkers which display values in 0.1 mJoules/cm^2

4. While keeping the cells on ice, use a cell scraper (Corning, cat. #CLS3010-10EA) to scrape the plate.
5. Transfer the cells to a 50 mL conical tube.
6. Wash plate once with 10 mL of 1× DPBS and add to the same 50 mL tube.
7. Gently resuspend until the sample is homogeneous.
8. Count cell concentration (either with automated cell counter or hemocytometer).
   - Note: ensure cells are re-suspended well before counting.
   - Note: for cells that do not easily dissociate into single cells, a separate plate of cells can be counted instead (see note above).
9. Centrifuge the 50 mL conical tube at 200 x g for 5 minutes at room temperature.
10. Aspirate and discard supernatant.
11. Resuspend in the desired amount of 1× DPBS for flash freezing (typically 20×10^6 cells per mL).
12. Transfer desired amount into 1.5 mL Eppendorf Safe-Lock Tubes (typically 1 mL of 20×10^6 cells per mL).
13. Spin down at 200 × g for 5 minutes at room temperature.
14. Aspirate the supernatant and freeze cell pellets quickly by submerging the 1.5 mL Eppendorf tubes completely in liquid nitrogen.
15. After frozen (at least thirty seconds), remove from the liquid nitrogen and store at -80 °C.

APPENDIX A2: Preparation and UV Crosslinking of Suspension Cells

Required materials:

1. UV crosslinker with **254-nm** wavelength UV bulbs (UVP CL-1000 Ultraviolet Crosslinker or equivalent). Please, check bulbs carefully before start experiment. Bulbs must be **254-nm**, **UV-C**, mercury bulbs.
2. Liquid nitrogen (sufficient to submerge tubes in appropriate container).
3. 1× DPBS (Corning cat. #21-031-CV or equivalent).
4. Trypan blue stain (Thermo Fisher Scientific, cat. #15250061 or equivalent live cell counting assay).
5. Standard cell counting system (hemocytometer or automated cell counter).

Procedure

Cell viability validation (prior to crosslinking):

1. Use Trypan blue stain (Thermo Fisher Scientific, cat. #15250061) or equivalent live cell counting assay to evaluate cell viability.
2. Cell viability should be > 95% to ensure intact RNA.

Preparation of suspension cells:

1. Pool all cells per biosample (if multiple plates):
2. Transfer cells with media to 50mL conical tube(s).
3. Centrifuge at 200 × g for 5 minutes at room temperature.
4. Aspirate spent media.

Wash cells:

1. Resuspend the pellet(s) in 25 mL of 1× DPBS at room temperature.
2. Count cell concentration (either with automated cell counter or hemocytometer).
3. Spin down remaining sample in 50 mL conical tube(s) at 200 g for 5 minutes at room temperature.
4. Aspirate supernatant.
5. Resuspend cells to no more than 20 × 10^6 cells per mL.

UV crosslinking:

1. Aliquot at most 60 × 10^6 cells (re-suspended in 1× DPBS) in at least 3 mL total volume to a standard 10cm tissue culture grade plate.
   - **Note**: Ensure the cells are evenly dispersed and the plate is fully covered (3 mL should be sufficient volume for 10 cm plate).
2. Place the tissue culture plate on leveled ice or a cooling block pre-chilled to 4 °C.
3. Place the above (plate plus ice or cooling block) into the UV crosslinker.

- Notes:
  - Ensure the plate is leveled.
  - Remove tissue culture plate lid for crosslinking.

4. Cross-ink at 254-nm UV with an energy setting of 400 mJoules/cm^2.
   - Note: this is a setting of 4000 on many cross-linkers which display values in 0.1 mJoules/cm^2).
5. After crosslinking is completed, transfer cells to a 50 mL conical tube.
6. Wash plate once with 7 mL of 1× DPBS and add to the same 50 mL tube.
7. Count cell concentration (either with automated cell counter or hemocytometer).
   - Note: ensure cells are re-suspended well before counting.
8. Centrifuge the 50 mL conical tube at 200 × g for five minutes at room temperature.
9. Aspirate and discard supernatant.
10. Resuspend in the desired amount for flash freezing.
    - Typically, 20 × 10^6 cells per mL.
11. Transfer desired amount into 1.5 mL Eppendorf Safe-Lock Tubes (or equivalent).
    - Typically, 1 mL of 20 × 10^6 cells per mL.
12. Spin down at 200 × g for five minutes at room temperature.
13. Aspirate the supernatant and freeze by submerging the epi-tubes completely in liquid nitrogen.
14. After frozen (at least thirty seconds), remove from the liquid nitrogen and store at -80 °C.

APPENDIX B1: SDS-PAGE Gel Electrophoresis

Required Equipment and Materials

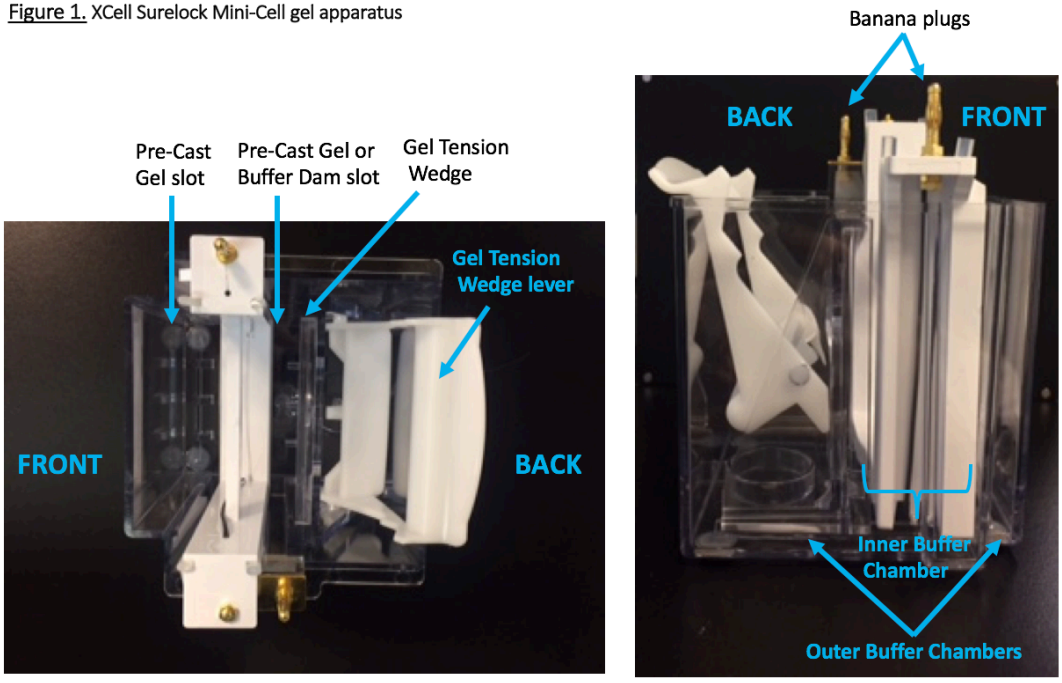
Item	Source
NuPAGE 4-12% Bis-Tris Gels, 1.0mm, 12-well	ThermoFisher Scientific cat. #NP0322BOX
NuPAGE 4-12% Bis-Tris Gels, 1.0mm, 10-well	ThermoFisher Scientific cat. #NP0321BOX
XCell Surelock Mini-Cell	ThermoFisher Scientific cat. #EI0001
PowerEase 300W Power Supply (230VAC)	ThermoFisher Scientific 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**
2× Western Buffer	Eclipse Bioinnovations

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 and ANALYTICAL), 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 (dH_2O). Dry with kimwipes.

Prepare XCell Surelock Mini-Cell gel apparatus for loading gel cassettes (Refer to Figure 1 for specific locations).

1. Load and start running PREPARATIVE gel before ANALYTICAL gel.
2. Rinse XCell Surelock Mini-Cell gel apparatus thoroughly with dH_2O.
3. Place XCell Surelock Mini-Cell gel apparatus into Polypropylene Nalgene ice tray.
4. 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'.
5. If only running one gel insert 'Buffer Dam' in BACK portion of apparatus.
6. Insert 'Gel Tension Wedge'.
7. Add 200 mL of cold Running Buffer into 'Inner Buffer Chamber'.
8. Pull forward on the 'Gel Tension Wedge level' until lever stops, and the gels or gel/Buffer Dam appears snug.
9. 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).
10. Carefully remove gel comb(s).
11. Rinse gel wells with cold Running Buffer from the Outer Buffer Chamber. Be careful not to disrupt well walls of gel, this will create difficulties when loading samples. Check carefully for broken wells.
12. Carefully load all ladders (see **Chapter 6: SDS-PAGE and Membrane Tranfer** for volume) in pre-designated wells by lowering the tip to the bottom of the well and slowly pipetting ladder without carry over.
13. Next carefully load all samples (see eCLIP Protocol for volume) in pre-designated wells by lowering the tip to the bottom of the well and slowly pipetting sample without carry over.
14. Load any undesignated wells with diluted Western Buffer.
15. 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.
16. With the Power Supply off, connect the electrodes. **Check labels: Red to (+) and black to (-)**.
17. Turn on the power.
18. Adjust settings to **150 V** and run for **75 minutes** or until dye front is at the bottom of gel.
19. Cover hole in cell safety lid with a paper towel.

20. 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 B2: 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, 7 cm × 8.4 cm	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

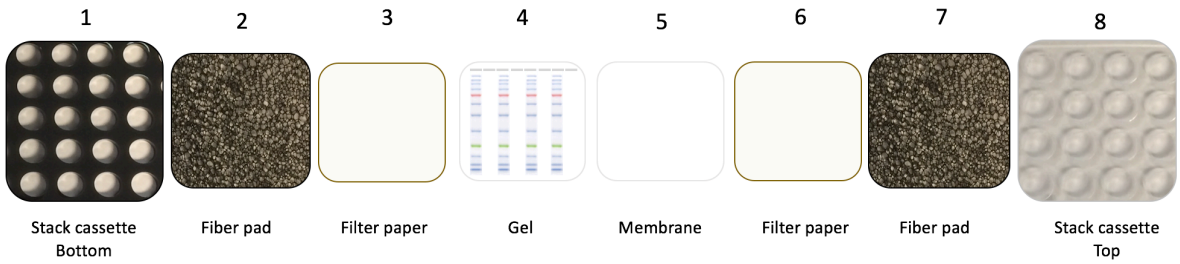


Figure 2. Transfer stack layer composition

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.

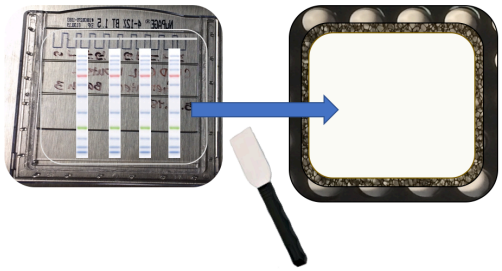


Figure 3. Gel transfer schematic

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).

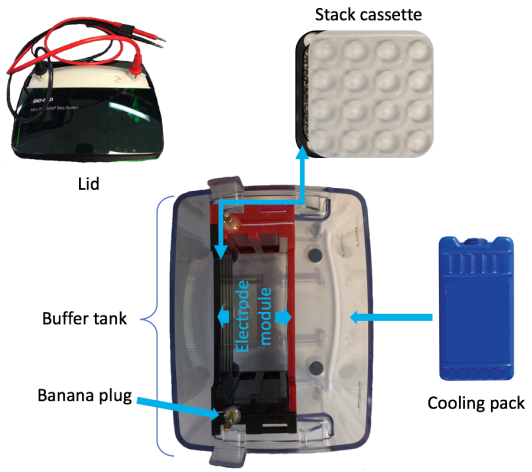


Figure 5. Mini-Protean Tetra components

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 900 mL 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 1mm 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: Western Blot Image Preparation

Required Equipment and Materials

Item	Source
Analytical precision balance, Fisher Scientific Mettler Toledo New Classic ME or equivalent	Fisher Scientific cat. #01-912-401
Tube Rotator	VMR cat. #10136-084
Bench Rocker 2D Rocker	Genesee Scientific cat. #31-201
GeneMate Western Blot Boxes, Blotting Containers	VWR cat. #490016-640).
Forceps 7×7 Teeth, Straight, standard, 4.75 in	ThermoFisher cat. #3120024
50mL Conical Sterile Polypropylene Centrifuge Tubes	Fisher Scientific cat. #12-565-268

Reagents

Not Included in kit	
Item	**Source**
TBST (Pierce 20× TBS Tween-20 Buffer)	ThermoFisher Scientific cat. #28360
Pierce ECL Western Blotting Substrate	ThermoFisher cat. #32109
Primary Antibody	Variable
Secondary Antibody	Variable
Nonfat Dry Milk, 500 g/U	Genesee Scientific cat. #20-241
Molecular Biology Grade Water, Corning	VWR cat. #95000-094

Procedure

Prepare antibody blocking reagents

1. Dilute 20× TBS Tween 20 Buffer according to **Table 1**. Mix then store at room temperature.

Table 1. 1× TBS Tween Buffer

Component	Volume (mL)
Molecular Biology Grade Water	950
20× TBS Tween 20 Buffer	50
Total:	**1000**

2. Place an empty 50 mL conical tube on an Analytical precision balance then tare the balance so that it reads '0'. Remove 50 mL tube from balance and carefully add 2.5 grams of Nonfat Dry Milk.
3. Fill 50 mL conical tube containing dry milk with 50 mL of 1× TBS Tween Buffer.
4. Shake 50 mL conical tube manually to partially suspend Nonfat Dry Milk in solution then place on tube rotator to mix until homogenous.
5. Once solution is homogeneous, add 15 mL of 1× TBST + 5% milk into a Blotting container. Store remaining 1× TBS Tween + 5% milk at room temperature.

Blocking non-specific antibody binding

1. If transfer membrane is larger than Blotting container carefully trim away any unwanted edges.
2. If transfer membrane contains more than one antibody carefully cut out each ladder and sample lane according to antibody and place in separate Blotting containers containing 1× TBST + 5% milk. Label each Blotting container with antibody name.
3. Move Blotting container to Bench Rocker and allow to mix for 30 minutes at room temperature.

Probe membrane(s) with primary antibody

1. After 30-minute blocking incubation, carefully discard 1× TBST + 5% milk solution, forceps can be used to ensure membrane(s) do not fall out. Add 10mL of fresh 1× TBS Tween Buffer + 5% milk to Blotting container(s).
2. Add 0.2 μg/mL of primary antibody to appropriate container(s). Final volume is 10 mL; adjust volume according to concentration on primary antibody label. (For a 1 mg/mL stock; add 2.0 μL primary antibody to 10 mL of 1× TBS Tween Buffer + 5% milk). Check antibody recommendations.
3. Gently swirl Blotting container to mix antibody. Incubate on Bench Rocker at room temperature for a minimum of 1 hour or at 4 °C overnight.

Wash primary antibody probed membrane(s)

1. Carefully discard 1× TBS Tween + 5% milk solution, forceps can be used to ensure membrane(s) do not fall out.
2. Add 15 mL of fresh 1× TBS Tween Buffer to Blotting container(s). Place Blotting container(s) with membrane(s) to Bench Rocker and wash for 5 minutes.
3. Discard solution as done in previous step.
4. Repeat steps 2- 3 for a total of three washes.

Probe membrane(s) with secondary antibody

1. Add 10 mL of fresh 1× TBS Tween Buffer + 5% milk to Blotting container(s).

2. Add 0.1 µg/mL of secondary antibody to appropriate container(s). Final volume is 10 mL; adjust volume according to concentration on secondary antibody label. (For a 1 mg/mL stock; add 1 µL secondary antibody to 10 mL of 1× TBS Tween Buffer + 5% milk).
3. Gently swirl Blotting container to mix antibody. Incubate on Bench Rocker at room temperature for 1 hour.

Wash secondary antibody probed membrane(s)

1. Carefully discard 1× TBS Tween + 5% milk solution, forceps can be used to ensure membrane(s) do not fall out.
2. Add 15 mL of fresh 1× TBS Tween Buffer to Blotting container(s). Place Blotting container(s) with membrane(s) to Bench Rocker and wash for 5 minutes.
3. Discard solution as done in previous step.
4. Repeat steps 2- 3 for a total of three washes.

Stain membrane(s) with Pierce ECL Western Blotting Substrates

1. Mix 1.5 mL of ECL Reagent A and 1.5 mL of ECL Reagent B to clean Blotting container. Gently swirl to combine.
2. Place Blotting container(s) with membrane(s) on Bench Rocker and allow to mix at room temperature for 2 minutes (incubation time will depend on ECL reagents).
3. Carefully discard ECL solution, forceps can be used to ensure membrane(s) do not fall out.
4. Scan membrane starting from 2 minutes normal sensitivity settings (for Azure Gel Imaging system).

APPENDIX D: Chemiluminescent Biotinylated - RNA Detection

Required Equipment and Materials:

Item	Source
Bench Rocker 2D Rocker	Genesee Scientific cat. #31-201
GeneMate Western Blot Boxes, Blotting Containers	VWR cat. #490016-640).
Forceps 7×7 Teeth, Straight, standard, 4.75 in	ThermoFisher cat. #3120024
50 mL Conical Sterile Polypropylene Centrifuge Tubes	Fisher Scientific cat. #12-565-268

Reagents:

Not Included in kit	
Item	**Source**
Molecular Biology Grade Water	VWR cat. #95000-094

Included in kit	
Item	**Source**
ECL A	Eclipse Bioinnovations
ECL B	Eclipse Bioinnovations
Biotin Blocking Buffer	Eclipse Bioinnovations
Biotin 4× Wash Buffer	Eclipse Bioinnovations
Biotin EQ Buffer	Eclipse Bioinnovations
Biotin HRP	Eclipse Bioinnovations

Procedure

Block membrane(s)

1. Warm the **Biotin Blocking Buffer** and **Biotin 4× Wash Buffer** until all particulates are dissolved (~30 minutes).
2. **Add 4 mL of Biotin Blocking Buffer** to an empty Blotting container.
3. If transfer membrane is larger than Blotting container, carefully trim away any unwanted edges. Place membrane in Blotting Container with Biotin Blocking Buffer and **incubate on Bench Rocker for 15 minutes at room temperature.**

Probe membrane(s) with Biotin HRP

1. Add **12.5 µL of Biotin HRP** directly to blotting container with membrane and blocking buffer.
2. Gently mix. **Incubate on Bench Rocker for 15 minutes at room temperature**.

Wash membrane(s)

1. Prepare 1× Wash Buffer according to Table 1. below.

Table 1. 1× Wash Buffer

Component	Volume (mL)
Molecular Biology Grade Water	24
Biotin 4×Wash Buffer	8
Total:	**32**

* *This volume is appropriate for approximately 1 membrane.*

2. Transfer membrane(s) to new container(s) and add **8 mL 1× Wash Buffer**. Incubate on bench rocker for 5 minutes.
3. Discard 1× Wash Buffer.
4. **Repeat steps 2 - 3 three times** (without changing containers) for a **total of 4 washes.**
5. Transfer membrane(s) to new container(s) and **add 4 mL of Biotin EQ Buffer**. Incubate on bench rocker for 5 minutes.

Stain membrane(s) with Chemiluminescent Substrate Solution

1. Prepare Chemiluminescent Substrate Solution by adding **1.5 mL ECL A to 1.5 mL ECL B.**
   - **Note:** Exposure to intense light can harm the solution. For best results keep the solution in an amber bottle and avoid prolonged exposure to any intense light.
2. Remove membrane(s) from Biotin EQ Buffer and carefully blot an edge of the membrane on a paper towel to remove excess buffer. Place membrane in a clean Blotting container.
6. Add enough Chemiluminescent Substrate Solution to completely cover **the surface of the membrane(s)** (~3 mL). Incubate on bench rocker for 5 minutes.
3. Carefully discard Substrate Solution. Forceps(s) can be used to ensure membrane(s) do not fall out.
4. Scan membrane(s) starting from 1-minute normal sensitivity settings.
   - **Note:** Significantly less exposure time may be required depending on amount of starting material.

APPENDIX E: E-Gel Purification

Required Equipment and Materials

Item	Source
E-gel Safe Imager	ThermoFisher Scientific cat. #G6500
E-gel iBase Power System	ThermoFisher Scientific cat. #G6400
E-gel EX Agarose Gel 2%	ThermoFisher Scientific cat. #G402002
Mettler Toledo NewClassic ME Analytical Balances or equivalent	Fisher Scientific cat. #01-912-401
Tube Rotator	VWR cat. #10136-084
Razor Blades, Surgical Carbon	Genesee Scientific cat. #38-100

Reagents

Not Included in kit	
Item	**Source**
Ultra-low range DNA Ladder	ThermoFisher Scientific cat. #10597012
Isopropanol	Sigma Aldrich cat. #190764-500ML
Molecular Biology Grade Water	VWR cat. #95000-094
MinElute Gel Extration Kit	Qiagen cat. #28606

Included in kit	
Item	**Source**
Library Elution Buffer	Eclipse Bioinnovations

Procedure

Prepare E-Gel

1. Attach the power cord of the iBase device to the power inlet and outlet.
2. Open E-gel package and carefully remove protective comb from the gel.
3. Slide the gel cassette into the two electrode connections on the iBase device. The base illuminates a red light to indicate that the cassette is inserted correctly.

Dilute and load ladder on 2× agarose gel

1. Dilute ladder according to Table 1 and carefully load 20 µL in the first and last well on the gel.

Table 1. Ultra-low range DNA ladder dilution

Component	Volume (µL)
Ultra-low range DNA ladder	10
Molecular Biology Grade Water	240
Total:	*250*

Load and run samples on 2% agarose gel

2. Load 20 µL of each IP and Input libraries into separate wells, skipping the first and last wells that contain the ladder. Load 20 µL of Library Elution Buffer into any empty wells.
3. Run gel for 5 minutes using size-select E-gel system program #9.
4. Check sample separation after 5 minutes using built-in or standard blue-light transilluminator. Standard UV-light trans-illuminator can be also used. If samples are not adequately separated, remove gel cassette from iBase and put on ice for 3-5 minutes to cool-down, then put gel cassette back into iBase device and run for an additional 5 minutes using same program #9.
5. Remove gel cassette from iBase and inspect again using blue light transilluminator. If samples have adequately separated proceed with step 4. If samples have not adequately separated, cool gel again on ice for 3-5 minutes and run for additional 3 minutes.

Cut libraries from E-gel

1. Label new 1.5 mL LoBind tubes with specific sample names then weigh each empty tube on analytical balance and record weight of empty tube.
2. Using gel cassette opener, remove top E-gel cover. Be careful – gel cassette can be tough. While gel is under transilluminator, extract libraries using a fresh razor for each sample (typically from 175-300 bps). Carefully cut gel slice containing library cross wise to produce smaller gel squares. Move gel slices into appropriately labeled tube.
3. Discard razor into sharps container.
4. Repeat steps 2 – 3 for all samples.

Extract libraries from gel slices

1. Label new filter columns from QIAquick Gel Extraction Kit (columns are stored at 4 °C).
2. Weigh each 1.5 mL tube containing library gel slices and record. Subtract weight of tubes containing gel slices from weight of empty tube previously measured (equals the weight of gel slices). Record weight of library.
3. Add 5× volume of Buffer QG to each tube (i.e., for 80 mg gel weight, add 400 µL Buffer QG).
4. Ensure tubes are fully close and move to tube rotator. Allow tubes to rotate at room temperature until completely melted. Do not heat – use room temperature (23 °C)!

5. Once gel has completely melted, add 2× volume of isopropanol (i.e., for 80 mg gel, add 160 µL isopropanol). Pipette mix thoroughly then transfer total volume to corresponding filter column (if volume exceeds 700 µL spin in multiple steps).
6. Spin columns in micro mini-centrifuge 5,000 × g for 1 minute.
7. Add remaining sample volume if necessary, from step 5 and transfer flow-through back into original tubes or bind product again by transferring flow-through onto column filter if volume did not exceed 700 µL.
8. Spin columns in micro mini-centrifuge 5,000 × g for 1 minute.
9. If volume did exceed 700 µL, transfer sample from 1.5 mL tubes back onto column and the flow-through from step 7 back into original tubes. Spin again as done previously. Discard flow-through. Finally transfer remaining volume from tubes onto filter and spin as done previously. Discard flow-through.
10. Prepare Buffer QG + 20% isopropanol (See **Table 2**; volumes are for 1 sample, adjust accordingly)

Table 2. Buffer QG + 20% isopropanol

Component	Volume (µL)
Buffer QG	400
Isopropanol	100
Total:	**500**

11. Add 500 µL of Buffer QG + 20% isopropanol to each column.
    Spin column in mini-centrifuge 5,000 × g for 1 minute then discard flow-through.
12. Wash column sides, including column rim with 500µL Buffer PE using fresh tip for each column.
    Spin columns in mini-centrifuge 5,000 × g for 1 minute then discard flow-through.
13. Wash column sides, including rim with fresh 500 µL Buffer PE using fresh tip for each sample, centrifuge at 12,000 × g for 2 minutes then discard flow-through.
14. Carefully move columns to fresh 1.5 mL tubes that have been appropriately labeled with sample name.
15. Open caps and remove any liquid left above internal column ring. Allow columns to air dry for at least 3 minutes. Once dry, add 15 µL of Molecular Biology Grade Water directly to center of column, incubate at room temperature for 1 minute then spin all samples at 12,000 × g for 30 seconds.
16. Re-elute samples by taking 15 µL of flow-through and adding it directly on filter of same column. Spin again at 12,000 × g for 90 seconds.
17. Immediately proceed to quantifying libraries using Agilent 4200 Tapestation or immediately freeze at –80 °C for long term storage.

Appendix F: 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:

[[link 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 eCLIP-seq method are typically sequenced using standard SE50 or SE75 conditions on the Illumina HiSeq, NovaSeq, or NextSeq platforms. eCLIP-seq 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 25 million reads per eCLIP-seq dataset.

How deeply to sequence an eCLIP-seq dataset is a challenging balance between cost and sufficient read depth to detect true binding events. 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 athttps://www.biorxiv.org/content/early/2018/10/05/179648)*. However, we have found that targeting 25 million reads 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