eCLIP: Antibody Immunoprecipitation Validation Protocol

Required materials:

1. Cell pellet (non-crosslinked is fine) of 5 x 10^6 cells
2. Antibody (5 ug per sample plus appropriate amount for western)
3. Magnetic separator (ThermoFisher DynaMag-2 or equivalent)
4. Sonicator (BioRuptor, Qsonica Q800R2 or equivalent)
5. Western blotting equipment
6. Thermomixer (or equivalent; for 65°C sample denaturation)
7. Dark room or ECL Western Imager

Buffer	Provided by	Long-term Storage
eCLIP Lysis Buffer	Eclipse BioInnovations	-20°C
High-Salt Buffer (HSB)	Eclipse BioInnovations	-20°C
25× No-Salt Buffer (NoS Buffer)	Eclipse BioInnovations	-20°C
2× Western Buffer	Eclipse BioInnovations	-80°C

Reagents

Dynabeads M-280 sheep anti-rabbit (for rabbit primary antibodies)*			
*for western blot with TruBlot secondary	10 mg/ml	LifeTech	11204D
(or Dynabeads Protein G)	30 mg/ml	LifeTech	37002D

Standard Western blotting reagents (or equivalent):
PVDF membrane

Day 1

Prepare eCLIP Lysis mix

- Pre-chill **eCLIP Lysis Buffer**
- Per sample (5 million cells): add **2.75 µl 200× Protease Inhibitor Cocktail III** to **500 uL** eCLIP Lysis Buffer, mix

Couple antibody to magnetic beads

- **Beads and antibodies:**
  - Use **50 µl beads** per sample
    - rabbit antibodies: use sheep anti-rabbit beads
    - mouse antibodies: use sheep anti-mouse beads
    - others – use Protein G or Protein A beads as recommended
  - Use **5 µg antibody** per sample

- **Prepare beads:**
  - Magnetically separate beads, remove supernatant
  - Wash beads 2x in 500 µl cold **eCLIP Lysis Buffer**
  - Resuspend beads in 100 µl cold **eCLIP Lysis Buffer**

- **Bind antibody:**
    - Add antibody (5 µg) to 50 µl washed beads
    - Rotate, room temp, 45 min

Lyse cells

- Retrieve cell pellets from -80°C freezer
- Immediately add 500uL cold **eCLIP Lysis mix** to each pellet of 5×10^6 cells, pipette to resuspend
- Lyse 5 mins on ice

Sonicate & clarify lysate (while ab+bead binding):

- Sonicate in Bioruptor at 'low' setting, 4°C, 5 min, 30sec on / 30 sec off
- Centrifuge 15,000g, 4°C, 3min
- Transfer supernatant to a new tube

Capture RBP-RNA complexes on beads

- Wash antibody beads 2x in 500 µl cold **eCLIP Lysis Buffer**
- Remove 20 µL (4%) of Sample as backup inputs for western; store at -20°C
    - ✓ This is a backup input sample in case the **input sample** is not properly taken in Day 2
- Add remainder to washed antibody beads
- Rotate 4 degC, 2 h or overnight (in cold room)

Prepare buffers for Day 2

- Dilute 25× **NoS Buffer** according to **Table 1** in a 50mL conical tube. **Mix 1× NoS Buffer and store at 4°C overnight**.

Table 1. 1× NoS Buffer preparation

Component	Volume (mL)
Molecular Biology Grade Water	**48**
25× NoS Buffer	**2**
Total volume	***50***

- **Thaw High-Salt Buffer (HSB) at 4°C overnight.**

Day 2

Step: SAVE INPUT SAMPLES:

Remove Input Samples (Note: these are the true input samples)

- Mix samples well
- To new tube, take 20 µL (4%) of each sample, store at 4°C – this is the **Input Sample**

Prepare buffers and equipment

- Pre-warm Thermomixer to 65°C.
- Remove **High-Salt Buffer (HSB) from 4°C**. Gently invert 5 times to mix and store on ice.
- Remove **1× NoS Buffer from 4°C.** Gently invert 5 times to mix and store on ice.

Wash beads

- Wash 3× with 500µL cold **High-Salt Buffer (HSB)**
- Wash 2x with 500µL cold **1× NoS Buffer**

Prepare samples for gel loading

- **IP-Bead samples**
  - **Remove s/n,** add **40** µl cold **1× NoS Buffer**, resuspend beads well
  - Move 20 µl to new tube #1 = **IP-WB samples**
  - Remaining 20 µL = **backup sample**, store at -20°C or -80°C

- For **IP-WB samples:**
  - **IP-WB samples** 20 µl

  Add:
  - **2× Western Buffer** 20 µl

- For **input samples**, mix:

Input sample:	1x	0.1x
Input sample	20 µl /	2 µl (Saved in Step: SAVE INPUT SAMPLES)
1× NoS Buffer	0 µl /	18 µl
2× Western Buffer	20 µl /	20 µl

- Denature all samples in Thermomixer, 1150 rpm, 65°C, 10 min
- Cool on ice 1 min, spin briefly in minifuge
- For **all samples**, magnetically separate prior to loading (IP AND Inputs have beads)
- Load COLD gel (4-12% Bis-Tris, N-well, 1.5 mm), e.g.

1	2	3
Input (1x)	Input (0.1x)	IP

(**Note**: 10 well gels can load entire 30 µl sample; for other gels, load 15 µl and save remainder as backup -20°C. If load only 20 µl, backup another 20 µl of denatured samples)

- Run at 150V in 1× MOPS running buffer, 75 min or until dye front is at the bottom

Transfer to membranes

- **Prepare transfer:**

  Note: the below is for standard (wet) transfer. We also commonly use rapid transfer methods (such as ThermoFisher iBlot2 system) here as well; if using these systems, follow protocols provided by manufacturer.

  - (Have pre-prepared COLD (4 deg) transfer buffer with methanol: 1× NuPAGE transfer buffer, 10% methanol
  - Prepare PVDF membrane(s): pre-flash 10 s in methanol, move to transfer buffer with methanol
  - Wet sponges and Whatman papers in transfer buffer with methanol
  - Assemble transfer stacks, from bottom to top (black side of stack holder on bottom):

    1× sponge – 2× Whatman paper – gel – membrane – 2× Whatman paper – 1× sponge

  Transfer:

  - overnight 30V (preferred) OR
  - 2 hr 200 mA (if doing this, only hook up one transfer box per power supply)

Day 3

Develop COLD membrane

- Block in 5% milk in TBST, room temp, 30 min
- Probe with primary antibody: 0.2 µg/ml (1:5000 for a 1 mg/ml Bethyl antibody stock; can be different per antibody) in 5% milk in TBST, room temp, 1 hr.
- Wash 3x with TBST, 5 min
- Probe with secondary antibody: 1:4000 Rabbit TrueBlot HRP in in 5% milk in TBST, room temp, 1–3 hours
  - (Note: if western fails or signal is low, 1:1000 gives higher signal)
- Wash 3× with TBST, 5 min
- Mix equal volumes of ECL Buffer A + Buffer B (or 40:1 of ECL Plus Buffer 1 to Buffer 2), add to membrane and incubate (mix/rotate) for 1-5 min. (1ml final volume per membrane)
- Develop 30 sec & 5 min, then judge signal (15 min maximum; if 15 sec is still too bright, expose two films