CLAP Protocol

BioT transfection:

1. Grow cells (i.e., HEK293T) to 70-80% confluency for day of transfection.
2. The following is for transfection using BioT reagent (Bioland) on a 10 cm plate of adherent cells:
    a. In a 1.5 mL tube, mix 500 µL of 1X PBS and 5 µg of desired plasmid.
    b. Add 7.5 µL of BioT (µL of BioT scales 1.5x with µg of plasmid) *directly* to mixture, making sure to avoid the sides of the tube.
    c. Mix thoroughly by pipetting up and down.
    d. Incubate mixture for 15 minutes at room temperature, then sprinkle the mixture drop-wise over cells.
3. Incubate cells for 24-48 hours prior to crosslinking and collection.

UV-crosslinking and cell collection:

1. Chill 1X PBS prior to crosslinking.
2. Remove media carefully from cells and wash once with room temperature 1X PBS.
3. Aspirate PBS before crosslinking.
4. Remove lid from cells and UV-crosslink using "Energy" setting, set at 2500 x 100 µJ/cm^2.
5. Immediately add 1 mL of ice-cold PBS to plate.
6. Scrape cells down, pipette up and down to resuspend and aliquot into 1.5 mL tubes.
7. Spin down cells for 5 minutes at 1000 x g.
8. Remove PBS from cells and either proceed to **Lysis** or flash freeze and store at -80°C for future use.

Prepare HaloLink Resin:

1. Aliquot 50 µL of HaloLink Resin™ per capture condition (this is 200 µL of the 25% slurry from the bottle).
2. Spin at 2000 x g for 1 minute and discard supernatant.
3. Add 5X the volume of resin of 1X PBS + 0.1% Triton-X, vortex for 5 seconds, then spin down at 2000 x g for 1 min and discard supernatant.
4. Wash the resin twice more in this way.
5. Block resin: add 200 µL of 1% w/v BSA in PBS (RNase-free) to each resin and incubate at room temperature with rotation (i.e., HulaMixer) for 20 minutes.
6. Spin down at 2000 x g for 1 min and discard supernatant.
7. Wash resin three times using 1X PBS + 0.1% Triton-X.
8. Add 2X the volume of resin of 1X PBS + 0.1% Triton-X and keep on ice until lysate is ready.
9. When ready, spin down resin at 2000 x g for 2 minutes and discard supernatant prior to adding lysate.

Lyse cells:

1. Prepare Lysis Buffer (1X):

Stock component	**Volume**	
RIPA Buffer	1000 µL	
50X **Promega* protease inhibitor cocktail**	20 µL	***some protease inhibitors block HaloTag**
TURBO DNase	10 µL	
100X Mn^{2+}/Ca^{2+} mix	10 µL	
RNase inhibitor (i.e., RiboLock)	5 µL	
Total	1045 µL	

2. Add 1 mL of Lysis Buffer to each cell pellet and break up the pellet by pipetting up and down several times.
3. Incubate for 10min on ice.
4. Sonicate cells (optional for HEK293T cells):
    a. For mouse ES cells, we use a Branson sonicator and sonicate at 20% amplitude for 30 seconds, 0.7 s on, 2.3 s off) at 3-4 W.
5. Heat at 37°C for 10 minutes with shaking (1200 rpm on ThermoMixer) to allow for increased DNAse activity.
6. Spin down cells at 4°C at 15000 x g for 2 minutes to clarify lysate.
7. Move supernatant to a new tube and keep on ice for the remainder of protocol unless otherwise specified.
    a. Save 50 µL of lysate (keep as a separate aliquot) to use for input library processing.
    b. Save an additional 20 µL of lysate (keep as a separate aliquot) to use for expression testing by Halo-ligand AlexaFluor.
8. Add lysate to 50 µL of prepared HaloLink resin and incubate for either 1-2 hours at room temperature or 4°C overnight, with rotation (i.e., HulaMixer).

Expression testing (i.e., by HaloTag AlexaFluor):

1. Take 20 µL of soluble lysate and add 1.5 µL of a 1:60 dilution of HaloTag AlexaFluor 660 ligand (Promega). Mix thoroughly by pipetting up and down.
2. Incubate mixture for 20 minutes at room temperature in the dark.
3. Make mastermix of Gel Loading Buffer:

    a. For each sample, this is 4 µL of 10X Bolt™ Sample Reducing Agent, 10 µL of 4X LDS, and 4 µL of H_2O per sample.
4. To stop the reaction, add 1X Gel Loading Buffer to each sample.
5. Denature samples at 90°C for 6 minutes, then bring to room temperature.
6. Run 40 µL of sample on Bis-Tris 4-12% protein gel at 200 V for 45 minutes, or until the dye front has passed out of the gel.
7. After gel is finished running, image gel directly on LI-COR Odyssey.

CLAP washes:

Buffers should be made fresh and warmed at 70°C prior to usage. Buffer recipes are collated at the end of this document.

1. Spin down captures at 2000 x g for 2 minutes and remove flowthroughs carefully.
    a. The resin may have a different consistency depending on degree of protein capture. With strong expression, we noticed the post-capture sepharose often resembled gels.
2. Wash resin twice using 400 µL of RIPA Buffer by vortexing resin briefly, spinning down at 2000 x g for 1 minute, and removing the buffer carefully.
    a. Be sure to not disturb the resin! It is okay to leave a bit of leftover buffer after each wash.
3. For each subsequent wash, use 400 of each µL buffer, incubate with mixing (1200 rpm on ThermoMixer) at 90°C for 3 minutes, spin down resin at 2000 x g for 30 seconds, and carefully remove buffer:
    a. CLAP Wash 1 (NLS CLAP Wash Buffer) ▢ ▢ ▢
    b. CLAP Wash 2 (1M NaCl CLAP Wash Buffer) ▢ ▢ ▢
    c. CLAP Wash 3 (8M Urea CLAP Wash Buffer) ▢ ▢ ▢
    d. CLAP Wash 4 (Tween-20 CLAP Wash Buffer) ▢ ▢ ▢
    e. CLAP Wash 5 (TEV CLAP Wash Buffer) ▢ ▢ ▢
4. After final wash, if planning to check capture by western blot, split the resin 90:10 (90% for **Proteinase K elution** and 10% for **TEV cleavage and elution**).
5. Spin down resin at 7500 x g for 1 minute and discard supernatant.

Proteinase K elution:

1. Add 100 µL of NLS CLAP Wash Buffer and 10 µL of proteinase K to each sample.
2. Incubate with shaking (1200 rpm on ThermoMixer) at 50°C for 20 minutes.
    a. Digest the inputs at this time in the same way (add 100 µL of NLS CLAP Wash Buffer and 10 µL proteinase K to 50 µL input).
3. Transfer slurry to a Pierce™ Micro-Spin column and spin down at 2000 x g for 30 seconds.
4. Purify RNA using Zymo RNA Clean & Concentrator-5 (>17 nt protocol).
5. Elute in 15 µL of H_2O.
6. Proceed to **Library preparation** or freeze samples at -20°C for storage.

TEV cleavage and elution:

This step is to check for successful protein capture of each target.

1. Make 1X TEV reaction mastermix:
    a. For 100 µL of buffer, add the following: 5 µL of 20X ProTEV Buffer, 1 µL of 100 mM DTT, and 3 µL of ProTEV in 91 µL of H_2O.
2. Add 20 µL of TEV reaction mastermix to each resin and incubate at 30°C for 120 minutes with shaking (1200 rpm on a ThermoMixer).
3. Stop reaction by adding Gel Loading Buffer to 1X concentration.
4. Boil resin at 80°C for 6 minutes.
5. Transfer slurry to a Pierce™ Micro-Spin column and spin down at 2000 x g for 30 seconds.
6. Load all of each sample on a protein gel and proceed with western blot.

Library preparation

DNase and end repair:

1. Add RNA to FastAP mix according to the table below:

10X PNK Buffer	3 µL
PNK enzyme	3 µL
Turbo DNase	1 µL
ddH_2O	3 µL
Total	**10 µL**

2. For inputs only (which need thorough DNase treatment and RNA fragmentation):
    a. Bring inputs up to 44 µL in H_2O.
    b. Add 5 µL of 10X TURBO DNase buffer and 1 µL of TURBO DNase to each sample.
    c. Incubate at 37°C for 15 minutes with shaking (1200 rpm on a ThermoMixer).
    d. Purify RNA using Zymo RNA Clean & Concentrator-5 (>17 nt protocol).
    e. Elute in 16 µL H_2O.
    f. Add 2 µL of 10X FastAP buffer to cleaned RNA. Move samples to strip tubes.
    g. Fragment RNA for 90 seconds at 91°C, then hold at 4°C.
    h. Proceed to FastAP reaction by adding 1 µL of FastAP and 1 µL of RiboLock on top of the reaction.
3. Incubate for 10 minutes at 37°C with shaking (1200 rpm on a ThermoMixer).
4. Add PNK mix on top of reaction:

10X PNK Buffer	3 µL
PNK enzyme	3 µL
Turbo DNase	1 µL
ddH_2O	3 µL L
Total	**10 µL**

5. Incubate for an additional 10 minutes at 37°C with shaking (1200 rpm on a ThermoMixer).
6. Purify RNA using Zymo RNA Clean & Concentrator-5 (>17 nt protocol).

RNA ligation:

1. To each sample, add 1.5 µL of DMSO and 1 µL of **20µM RIL19 adapter**.
    a. 5’ – /Phosphate/rArGrArUrCrGrGrArArGrArGrCrGrUrCrGrUrG/3ddC/ – 3’
2. Heat at 65°C for 2 minutes and hold at 4°C.
3. Prepare Ligation mastermix (make more than needed since PEG 8000 is viscous):

10X T4 RNA Ligase Buffer	2 µL
ATP (100 mM)	0.2 µL
50% PEG 8000	6 µL
RNase inhibitor (i.e., RiboLock)	0.3 µL
T4 RNA Ligase, High concentration	0.3 µL
Total	**8.8 µL**

4. Add 8.8 µL of Ligation mastermix to each sample.
5. Add an additional 1 µL of T4 RNA Ligase to each sample and pipette up and down.
6. Incubate for 1 hour and 15 minutes with shaking (1600 rpm at room temperature, 1 minute on, 5 minutes off).
7. Clean samples using SILANE beads (Dynabeads):
    a. Per sample, wash 12 µL SILANE beads twice with 50 µL of RLT (QIAGEN).
    b. Resuspend beads in 60 µL of RLT and add to sample. Mix thoroughly and let sit for 1 minute.
    c. Add 50 µL of 100% ethanol. Mix thoroughly and let sit for 2 minutes.
    d. Magnetize beads and remove supernatant.
    e. Wash beads three times with 80% ethanol.
    f. Elute in 13 µL of H_2O.

Reverse transcription:

1. Add 1 µL of **5µM AR17** to each sample.
    a. 5' – ACACGACGCTCTTCCGA – 3'
2. Heat at 65°C for 2 minutes and hold at 4°C.
3. Make reverse transcription mastermix:

5X RT Buffer	4 µL
10 mM dNTP	2 µL
RNase inhibitor (i.e., RiboLock)	0.5 µL
Maxima RT	1 µL
Total	**7.5 µL**

4. Add 7.5 µL of reverse transcription mastermix to each sample.
5. Incubate at 50°C for 20 minutes, then drop to 4°C and hold.
    a. Potential stopping point: do not heat inactivate, store samples directly in 4°C.
6. Add 4 µL of ExoI and incubate samples at 37°C for 15 minutes.
7. Quench reaction by adding 1 µL of 0.5 M EDTA and placing samples on ice for 3 minutes.

8. To fragment RNA, add 2.5 µL of 1M NaOH and heat samples at 80°C for 6 minutes (hold at 4°C).
9. Neutralize samples by adding 2.5 µL of 1M HCl.
10. Clean samples using SILANE beads (Dynabeads):
    a. Per sample, wash 12 µL SILANE beads twice with 50 µL of RLT (QIAGEN).
    b. Resuspend beads in 90 µL of RLT and add to sample. Mix thoroughly and let sit for 1 minute.
    c. Add 75 µL of 100% ethanol. Mix thoroughly and let sit for 2 minutes.
    d. Magnetize beads and remove supernatant.
    e. Wash beads three times with 80% ethanol.
    f. Elute in 16 µL of H_2O.

cDNA splint ligation:

1. We use an annealed "splint" 3TR3 adapter for cDNA ligation:
    a. SplintTop-3TR3-Spcr: CAGACGTGTGCTCTTCCGATCTNNNNNN/3SpC3/
    b. SplintBot-3TR3-5Phos: /5Phos/AGATCGGAAGAGCACACGTCTG/3SpC3/
    c. Splint structure is depicted below:

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5'       AGATCGGAAGAGCACACGTCTG
         ||||||||||||||||||||||
3' NNNNNNTCTAGCCTTCTCGTGTGCAGAC
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2. Make splint ligation mastermix:

2X Instant Sticky Mastermix	15 µL
45 µM 3TR3 Splint	1 µL
cDNA	14 µL
Total	**30 µL**

3. Add 15 µL of splint ligation mastermix to cDNA samples.
4. Incubate for 1 hour (or overnight) at room temperature with shaking (1600 rpm on a ThermoMixer, 1 minute on, 5 minutes off).
5. Clean samples using SILANE beads (Dynabeads):
    a. Per sample, wash 12 µL SILANE beads twice with 50 µL of RLT (QIAGEN).
    b. Resuspend beads in 90 µL of RLT and add to sample. Mix thoroughly and let sit for 1 minute.
    c. Add 75 µL of 100% ethanol. Mix thoroughly and let sit for 2 minutes.
    d. Magnetize beads and remove supernatant.
    e. Wash beads three times with 80% ethanol.
    f. Elute in 35 µL of H_2O.
6. Proceed to **PCR** using 21 µL of elution (remainder can be saved for later re-amplification).

PCR:

2PUNI + 2PBC (12.5 µM each)	4 µL
2X Q5 mastermix	25 µL
cDNA sample	21 µL
Total	**50 µL**

Temp (°C)	Time (s)	Cycles
98	60	1 cycle
98	15	4 cycles
69	15	
72	90	
98	15	10 cycles (CLAP elutions) OR 4 cycles (CLAP inputs)
72	90	
72	120	

1. Add 1.2x the volume of SPRI beads (i.e., 60 µL for a 50 µL PCR) to each sample.
2. Mix thoroughly and let sit for 5-10 minutes.
3. Magnetize SPRI beads for 3 minutes and remove supernatant.
4. Wash twice using 150 µL of 80% ethanol (made fresh).
5. Elute in 13 µL of H_2O.

Buffers:

1X PBS + 0.1% Triton-X:

Stock solution	Volume
10X PBS	5 mL
10% Triton X-100	500 µL
Ultrapure dH_2O	44.5 mL
Total	50 mL

RIPA Buffer:

Stock solution	Volume
1M HEPES, pH 7.4	2 mL
5M NaCl	800 µL
10% DOC	2 mL
20% SDS	200 µL
10% NP-40	4 mL
Ultrapure dH_2O	31 mL
Total	40 mL

NLS CLAP Wash (CLAP Wash 1):

Stock solution	Volume
20% NLS	4 mL
500mM EDTA	800 µL
10X PBS	4 mL
Ultrapure dH_2O	31.2 mL
Total	40 mL

1M NaCl CLAP Wash (CLAP Wash 2):

Stock solution	Volume
1M HEPES, pH 7.4	2 mL
5M NaCl	8 mL
10% NP40	400 µL
Ultrapure dH_2O	29.6 mL
Total	40 mL

8M Urea CLAP Wash (CLAP Wash 3):

Stock solution	Volume
1M HEPES, pH 7.4	2 mL
10% NP-40	400 µL
8M Urea	37.2 mL
Total	40 mL

Tween-20 CLAP Wash (CLAP Wash 4):

Stock solution	**Volume**
1M HEPES, pH 7.4	2 mL
10% NP-40	400 µL
10% Tween-20	400 µL
Ultrapure dH_2O	37.2 mL
Total	40 mL

TEV CLAP Wash (CLAP Wash 5):

Stock solution	**Volume**
1M HEPES, pH 7.4	2 mL
10% NP-40	400 µL
500mM EDTA	80 µL
Ultrapure dH_2O	37.52 mL
Total	40 mL

100X Mn^{2+}/Ca^{2+} mix:

Stock solution	**Volume**
1M $MnCl_2$	250 µL
1M $CaCl_2$	50 µL
Total	1000 µL