Covalent linkage and Affinity Purification (CLAP) protocol

1. Transient Transfection (HEK293 adherent cells)

Plate cells at desired density and grow until cells are at 80% confluent

We have transfected and captured proteins successfully from both 6 well and 10cm plates

Follow the steps in the CLAP Fugene Transfection protocol below.

Cells are crosslinked 16-24hrs post transfection

2. UV Crosslinking

Remove media from cells and place in Spectrolinker XL-1500, irradiate using "Energy" set at 2500x100 uJ/cm^2

Immediately add 4mL of 1X ice cold PBS

Scrape cells down, pipette up and down to resuspend and aliquot into four 1.5 mL microcentrifuge tubes (1mL a piece)

Spin cells down for 5 minutes at 1500 x g

Remove PBS from cells and flash freeze (pellets can be kept at -80*C until ready for lysis)

3. Lysis

Make RIPA Buffer and keep on ice

50 mM HEPES pH 7.4	500	1M	
100 mM NaCl	200	5M	
1% NP-40	1000	10%	
0.5% Na-Deoxycholate	500	10%	
0.1% SDS	50	20%	
ddH20	7750		
Total	10000		

Make Lysis Mix and keep on ice

100× Mn/Ca Mix (50mM CaCl2, 250mM MnCl2)

RIPA buffer	1000	1M	

1X Promega PIC	20	50X	**SOME PROTEASE INHIBITORS BLOCK HALO ENZYME**
Turbo DNAse	10		
1x Mn/Ca mix	10	100x	
RNase Inhibitor	5		
Total	1045		

Begin lysis add 1 mL Lysis Mix to a 5M-15M cell pellet breaking up the cell pellet by pipetting up and down, place on ice for 10 minutes (sonication is optional for HEK cells, four mouse ES cells Sonicate at 15% power 30secs 0.7 on, 2.3 off)

Remove DNA from the lysate and solubilize chromatin by placing the lysate at 37C for 10 minutes

Pellet insoluble debris by spinning at 15000xg for 2 minutes ((Pellet = insoluble junk, Supernatant = Soluble Halo Protein used for expression testing/capture)

4. Expression Testing with AlexaFluor 660 Halo Ligand

Take 20 uL of the soluble lysate from step 13 and 1.5 uL of a 1:60 dilutation of Promega Alexfluor 660 ligand (Catalog Number G8471)

Incubate at room temperature for 20 minutes (keep in dark)

To stop the reaction add SDS loading buffer to 1X final concentration and run on SDS page gel

After gel is finished running image gel directly on LICOR (Use an Untransfected Lysate as negative control)

5. Resin Preparation

50 mM HEPES pH 7.4
10 ug/mL blocked 9mer (IDT - NNNNNNNNN-C3spacer)
100 ug/mL BSA

Place 25% slurry of HaloTag resin into a 1.5 ml tube, spin at 2000 x g for 1 min and discard supernatant. (Use 200uL (50 uL resin per sample))

Add 5X bead/resin volume of *1x PBS + 0.1% Triton X-100* to resin and vortex for 5 sec; spin at 2000 x g for 1 min and discard supernatant.

Repeat previous wash step two additional times. ☐ ☐

Add 2X bead/resin volume of 1X *SURF & TURF Buffer* to the tube, vortex for 5 sec and incubate the tube for 20 minutes at room temperature on end-over-end mixer.

Spin at 2000 x g for 1 min and discard supernatant.

Add 5X bead/resin volume of *1x PBS + 0.1% Triton X-100* to resin and vortex for 5 sec; spin at 2000 x g for 2 min and discard supernatant.

Repeat previous wash step two additional times. ☐ ☐

Add resin volume * 2 of *1x PBS + 0.1% Triton X-100* to resin and vortex for 5 sec; and aliquot into tubes, keep in buffer until ready to add lysate (spin down 2000g x 2min and remove supernatant prior to adding lysate)

6. CLAP capture

Take 50 uL of 1 mL lysate for input (**Input**) and add the rest to 50 uL of Prepared Halolink Resin

Incubate on end-over-end mixer at RT for 1.5hrs or overnight at 4C

Spin down samples (spin at 1000 x g for 2 min) and collect flow through (**Unbound**).

Repeat washes as described below :

Use Heat Block to keep buffers warm (use 70C setting on heat block)

Use 400 uL buffer for each wash

Incubate for 3 minute at appropriate temperature with shaking on thermomixer

Spin down at 1000xg for 30 sec in between washes

Remove supernatant and add next wash buffer

RIPA buffer (Room Temp) ☐ ☐

NLS Buffer (90*C, 2% NLS, 10 mm EDTA, 1X PBS) ☐ ☐ ☐

1M NaCl Buffer (90*C, 50 mM HEPES pH 7.4, 1M NaCL, 0.1% NP-40, 10 mM EDTA) ☐ ☐ ☐

8M Urea Buffer (90*C, 50 mM HEPES pH 7.4, 8M Urea, 0.1% NP-40, 10 mM EDTA) ▢ ▢ ▢

Tween 20 Buffer (90*C, 50 mM HEPES pH 7.4, 0.1% Tween, 0.1% NP-40, 10 mM EDTA) ▢ ▢ ▢ ▢

TEV Buffer (30*C , 50 mM HEPES pH 7.4, 1 mm EDTA, 0.1% NP-40, 10 mM EDTA) ▢ ▢ ▢

Add 400 ul of *TEV Buffer* to resin, split using 90/10 split into 1.5 mL tubes (90 = 360 uL and 10= 40 uL)

Spin at 2000 x g for 30 sec and discard supernatant

7. TEV Elution from HaloLink Resin

Use 10% resin fraction for TEV elution and protein capture detection

Prepare TEV elution Buffer (3 ul TEV in 20 ul TEV buffer+1 mM DTT), add TEV elution buffer to 10% fraction of resin

Incubate at 30 C for 30 minutes with shaking on thermomixder

Stop reaction by adding SDS loading buffer to 1X **[TEV]**.

Boil and prepare as usual for gel. (This fraction is used to check for protein capture not for RNA prep, load all on gel)

8. Proteinase K elution of RNA

To the 90% fraction of resin add 100 uL of 1X NLS buffer + 10 uL Proteinase K

Incubate with shaking at 50*C for 30 minutes

Transfer reaction to Pierce microspin cup, sping at 2000xg for 30secs

Purify RNA using Zymo RNA Clean and Concentrate 5 (>17nt protocol, 2X binding buffer, 1:1 ETOH, elute in 21 ul ddH20)

Library Preparation

1. DNAse

Incubate RNA eluted from RNA clean and concentrate with Turbo DNAse for 10 minutes at 37*C

Cleaned RNA	20
Turbo DNAse Buffer	3
Turbo DNASE	3
RNase Inhibitor	1
ddH20	3
Total	30

Clean Using Zymo RNA Clean and Concentrate, Elute in 17 uL

2. Dephosphorylation/End repair

For Input Samples (Fragment for 2 min 30 sec at 91*C in 1X Fast AP Buffer hold at 4C, then add enzymes on top)

Add DNAsed RNA to FastAP mix according to the table below.

Cleaned RNA	15		
10X Fast AP Buffer	2	14	6
Fast AP	1	7	3 (ddH20)
RNase Inhibitor	1	7	3
Turbo Dnase	1	7	3
Total	20		

Incubate for 10 min at 37C.

Add PNK mix to each reaction according to the table below.

10X PNK Buffer	3	21	9
PNK enzyme	3	21	9 (ddH20)
Turbo DNase	1	7	3
ddH20	3	21	9
Total	10	70	30

Incubate for an additional 10 min at 37C.

Clean Using Zymo RNA Clean and Concentrate, Elute in 11uL

If performing on many samples, you can perform SILANE clean

3. First RNA ligation

Add 10uL of cleaned RNA + 1.5uL DMSO + 1uL Ril19 adapter (20uM)

5' – /Phosphate/rArGrArUrCrGrGrArArGrArGrCrGrUrCrGrUrG<> – 3' = RIL19 Needs: 5'-P and 3' ddC (or 3'-C3 spacer)

Heat at 65C for 2 min, then hold at 4C.

Prepare Ligation Mastermix

10X T4 RNA Ligase Buffer	2	20
ATP (100 mM)	0.2	2
50% PEG 8000	6	60
RNase Inhibitor	0.3	3
T4 RNA ligase	0.3	3
Total	8.8	88

Add 8.8uL of master mix to each sample

Mix mastermix well by flicking and pop-spin repeatedly, use low retention tips for ligation reactions

Add an additional 1uL of T4 RNA Ligase High Concentration to each reaction.

Incubate for 1 hr 15 min at 24C, 1600rpm (1 min on, 5 min off).

Clean using Silane beads

Add 12 uL Silane beads in 60 uL RLT per sample

Incubate for 1 minute at RT

Add 50 uL 100% EtOH

Incubate for 2 minutes at RT

Place on Magnet, discard supernatant

Wash 3X with 80% EtOH

Dry at RT and elute in 13 uL ddH20

4. Reverse Transcription

Add 1uL of AR17 (5uM) to 11uL of each sample.

5′ – ACACGACGCTCTTCCGA – 3′ = AR17 (no modifications, standard desalting)

Heat at 65C for 2 min, then hold at 4C.

Add 8.5uL of RT mastermix to each sample, according to the table below.

5X RT Buffer	4	40
Maxima RT H-	1	10
dNTP	2	20
Ribolock	1	10
Total	8	

Incubate at 50C for 20 min-1.5 hours then hold at 4C.

DO NOT HEAT INACTIVATE AFTER RT JUST GO STRAIGHT TO 4C

Add 4uL of Exo-SAP IT (or EXO1 enzyme).

Incubate for 15 min at 37C.

Add 1uL of 0.5M EDTA.

Place on ice for 3 min.

Add 2.5uL of 1M NaOH.

Heat at 80C for 6 min, then hold at 4C.

Add 2.5uL of 1M HCl to neutralize base.

Clean using Silane beads

Add 12 uL Silane beads in 90 uL RLT per sample

Incubate for 1 minute at RT

Add 75 uL 100% EtOH

Incubate for 2 minutes at RT

Place on Magnet, discard supernatant

Wash 3X with 80% EtOH

Dry at RT and elute in 7.5 uL ddH20

5. cDNA ligation

Add 1uL of DMSO + 0.6uL of p38 or 3TR3 linker (80uM) to 7.5uL of cDNA.

/Phosphate/AGATCGGAAGAGCACACGTCTG<> — 3Tr3 Needs: 5'-P and 3' ddC (or 3'-C3 spacer)

Heat at 80C for 2 min, then hold at 4C.

Add 11.8uL of cDNA ligation master mix to each sample, according to the table below.

10X T4 RNA Ligase Buffer	2
ATP (100 mM)	0.2
50% PEG 8000	9
T4 RNA ligase	0.3
Total	11.5

Add an additional 1uL of T4 RNA Ligase High Concentration to each reaction.

Incubate overnight at 24C, 1600rpm (1 min on, 5 min off).

This ligation can go as short as 4 hours.

Clean using Silane beads

Add 12 uL Silane beads in 60 uL RLT per sample

Incubate for 1 minute at RT

Add 50 uL 100% EtOH

Incubate for 2 minutes at RT

Place on Magnet, discard supernatant

Wash 3X with 80% EtOH

Dry at RT and elute in 35 uL ddH20

6. PCR amplification of cDNA

To a new strip of PCR tubes, add 23uL sample + 2uL primers (indices) + 25uL of 2X Q5 Master Mix.

Save the remaining ligated cDNA as pre-PCR sample and freeze at -20C (can be used for reamplification)

PCR enrichment primers: Use one primer from file “IndexedPrimersBARCODES.xls”, second primer is 2P_universal 5’-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3’

PCR conditions:

98	**40**	**1**
98	15	4
69	15	"
72	30	"
98	15	10 for Elutions / 3 for Inputs
72	40	"

Clean with SPRI (1.4x)

Add 1.4x SPRI beads (70uL) to each sample

Mix well by pipetting and incubate at room temp for 10 minutes (mix by pipetting every 3 min).

Place on magnet for 3 minutes and discard supernatant.

Keeping beads on magnet, wash 2X with 100uL of 80% EtOH.

Let dry for 4-5 minutes at room temp (beads will start to crack)

Elute in 13 uL of ddH20

Run samples on DNA D1000 TapeStation, measure and gel extract sizes (>150nt -- 700nts)

CLAP Fugene Transfection

Fugene Transfection 10cm plate

Bring Fugene bottle and OPTI-MEM (1mL per 10cm plate; 500uL into 2 – 1.5mL tubes) to room temperature

LET SIT FOR 15 MINUTES

Mix the Fugene(25uL per 10cm plate) with half of the OptiMem (500uL) and in a separate tube DNA (12.5 ugrams per 10cm plate) with other half of OptiMem (500uL)

LET SIT FOR 10 MINUTES

Mix the Fugene+Opti w/ DNA+Opti (mix thoroughly by pipetting or vortexing briefly)

LET SIT FOR 10 MINUTES

Add 1mL of Fugene+Opti+DNA mixture from step 6 to each plate, swirl and place plate back in incubator

Fugene Transfection 1 well in 6 well plate

Bring Fugene bottle and OPTI-MEM (200uL per well; 100uL into 2) to room temperature

LET SIT FOR 15 MINUTES

Mix the Fugene(2.5 * 2 = 5 uL Fugene) with half of the OptiMem (100uL) and in a separate tube DNA (2.5 ugram per well) with other half of OptiMem (100uL)

LET SIT FOR 15 MINUTES

Mix the Fugene+Opti w/ DNA+Opti (mix thoroughly by pipetting or vortexing briefly)

LET SIT FOR 10 MINUTES

Add 200 uL of Fugene+Opti+DNA mixture from step 12 to each plate, swirl and place plate back in incubator