RiboTag Immunoprecipitation

FRIDAY, 7/7/2023

- Pierce™ Protein A/G Magnetic Beads Thermo Scientific 88802 (+4C Albana Box, bench fridge)
- Prepare 2x CHX stock, 5mg/ml for washing intestine (+4C, bench fridge)
- Anti-HA (abcam ab9110) (-20C Albana box 2, bench fridge)

Homogenization buffer (HB)

	A	Final Concentration	Stock	10 ml	Location
1	NP-40	1%	10%	1 ml	Chemical cabinet
2	KCl	0.1M	2M	500 ul	
3	TRIS pH 7.4	50mM	1M	500 ul	Chemical cabinet
4	MgCl2	120mM	1M	120 ul	Library room
5	water			7800 ul	

Supplemented homogenization buffer (HB-S)

	A	Final Concentration	Stock	Final Volume (1.5ml)	Location
1	Homonogenization buffer (HB)			1.40 ml	
2	Cyclohexamide (Protein inhibitor)*	100 µg/mL	5mg/ml	30 ul	Chemical Powder
3	Protease inhibitors*	5x	100x	75 ul	Deli 4C. Aliquots -20C Albana Box
4	Heparin*	3mg/ml	100mg/ml	45ul	RNA pol inhibitor. Powder in Deli 4C. Aliquots -20C Albana Box
5	SUPERaseIN*	500 U/ml	40U/ul	25 ul	-20C Albana Box or -20C old freezer
6	DTT*	1 mM	1M	1.5 ul	Powder in Deli 4C. Aliquots -20C Albana Box
7					

**add fresh*

High Salt buffer

	A	Final Concentration	Initial concentration	10 ml (volume)	30 ml (volume) 6 samples/4 washes	F
1	2M KCl	300mM	2M	1.5 ml	4.5 ml	
2	1M MgCl2	120mM	1M	120 ul	360 ul	
3	NP-40 Igepal CA-630	1%	10%	1 ml	3 ml	
4	1M Tris, pH 7.4	50mM	1M	500 ul	1.5 ml	
5	Cyclohexamide (CHX)*	100 µg/mL	5mg/ml	200ul	600 ul	
6	DTT*	1 mM	1M	10 ul	30 ul	
7	EDTA-free protease inhibitors*	1X	100x = 1 tablet in 500ul	100ul	300 ul	
8	Superase In Promgega*	150 U/mL	50U/ul	50 ul	150 ul	
9						

*add fresh

10x Wash Buffer I

	Chemical	Final Concentration of 10x	Stock	for 50ml Buffer	1X 10 mL
1	Tris-HCl pH7.5	500mM	1M	25mL	500 ul
2	EDTA	100mM	0.5M	10mL	200 ul
3	NaCl	1.25M	5M	12.5mL	250 ul
4	NP-40 Igepal CA-630	1%	10%	1.25mL	125 ul
5	*EDTA-free protease inhibitors		100x = 1 tablet in 500ul		100 ul

*add fresh

DAY 1

A. Tissue prep:

1. Dissect intestine on ice and rinse 6 times in a 6 well plate in cold 1x PBS + Cyclohexamide (translation inhibitors. Stalls ribosomes and entraps them over a region of mRNA) (2x CHX stock, 5mg/ml)
    a. For allergy assay, I gavage one mouse every 30 mins with OVA to give enough time to dissect duodenum and ileum
2. Cut 3-5cm for each segment of interest and remove all mesenteric fat
3. Separate muscularis layer and wash 6X in 1X PBS+CHX (2.5mg/ml)

B. Homogenization:

1. Add tissue and HB-S (+ EDTA-free PI 1:10, DTT 1:1000, CHX 1:50, Heparin 1:100, SuperaseIN 1:200) in **1 ml glass homogenizer** dounce ~2-3 times with loose pestle then leave on ice until all samples are prepped
2. Homogenize tissue with 4x loose and 2x tigh pestle appropriate volume of **ice-cold HB-S** (approximately **300 ul per sample**) until no clumps of cells persist (if they do, add 1x pestle)
3. Spin cells down, **10000g 10 mins at 4C, ribosomes will be in the supernatant**
4. Collect 25ul for INPUT. Add **Extraction Buffer from Pico Pure Kit (mantain ratio 1:1) and mix thoroughly (or vortex 10-30 sec). Freeze at -80C.**
    a. Alternatevely use 350ul of RLT lysis buffer from *Qiagen RNAeasy Kit*
    b. *Note: It is safe to freeze here because RLT buffer from Qiagen RNAeasy Kit or Extraction Buffer from Pico Pure kit contain guanidinium thiocyanate which functions as Trizol (stabilizes RNA by denaturing proteins such as nucleases)*
5. Collect as much supernatant as possible without perturbing pellet (~300ul for IP Sample)
6. Add 5ul of antibody (anti-HA) per IP and **incubate at 4C rotating for 1.5 hrs**

B. Block beads for IP (equilibrate beads to buffer solution):

1. Add volume of beads (40 ul of protein AG Pierce Beads / IP)
2. Pull down the beads, remove sup, and resuspend beads in 1 ml **Blocking Buffer** (make fresh): 100 ul BSA (20mg/ml) + 200 ul yeast tRNA (10mg/ml) in **1x Wash Buffer (+PI)**
3. **Rotate o/n at 4C**
4. Pull down blocked beads with a magnet, wash once with 1ml Wash buffer I, then resuspend in **HB buffer (100ul/IP) and add to new tube**
5. Resuspend in cold room and rotate for 5 min
6. Aliquot beads per sample and remove wash buffer
    a. Do each tube one at the time to avoid excessive drying of beads
7. Add to each bead aliquot the antibody-tissue homogenate samples
8. **Incubate o/n rotating at 4C (from 6 to 12 hours max)**

DAY 2

A. Extraction of RNA from bead-antibody-protein Sample

1. Place eppendorf containing beads in magnetic rack on ice
2. Transfer supernatant in a new tube. Alternatevely discard.
    a. *This is RNA not bound to anti-HA. Add 60 ul of Extraction Buffer to 40 ul of supernatant. Freeze -80C.*
3. Wash beads with **800ul of High Salt Buffer (+ PI 1:100, DTT 1:1000, CHX 1:50, SuperaseIN 1:200)** 3 times 10 mins at 4C in the cold room on rotator
4. Add 50 ul of **Extraction Buffe**r to each sample and resuspend carefully. Incubate 5 mins at 4C, then vortex 5x for 10 seconds at 4C to break apart antibody-bead-protein bond
5. Place samples on magnetic stand, wait 3 mins and draw off volume of Extraction Buffer. **This is your IP**

B. RNA purification with Picopure RNA kit

Note: Take INPUT out for purification from -80C in BOX Albana RNA, 4 shelf, first rack on the left

Note: Take non HA bound RNA for purification

Note: Prepare DNAseI and buffer solution while equilibrating columns

6. Add 250ul of **Conditioning Buffer** into columns and incubate 5 minutes at RT
7. Spin down columns at 16,000g for 1 min at RT
8. Add 50ul of 70% EtOH (RNase Free) to 50uL of eluted sample
9. Pipette up and down at least 20 times and transfer to column
10. Spin at 100g for 2 minutes, and immediately followed by 16,000g for 30sec at RT to remove flowthrough
11. Add 100ul of **Wash Buffer 1 (W1)** and sping for 1 minute at 8000g at RT
12. DNA may be removed by DNAse treatment from the preparationn (From Zymogen kit):
    b. Pipette 5 ul DNAse I stock solution to 35ul Buffer RDD
    c. Mix gently inverting
    d. Add 40ul DNseI+RDD to the column and incubate for 15 minutes at RT
    e. Add 40ul **Wash Buffer 1 (W1)** to the column adn spin at 8,000g for 15 sec
13. Add 100 ul of **wash buffer 2 (W2)** to the column and sping at 8000g per 1 minute at RT
14. Repeat wash with **W2** with 16000g
    f. *Check the purification column for any residual wash buffer. If wash buffer remains re-centrifuge at 16,000 x g for one minute*
15. Put the purification column tops into RNA/DNAse free 1.5 ml eppendorf tube or 0.5 mL microcentrifuge tube provided in the kit
16. Add 12ul of Elution Buffer (EB) for IP (for input add 24 ul) and incubate for 3 minutes at RT
    g. *Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of EB into the membrane*
17. Spin at 1000g for 1 minute at RT and then 16000g for 1 min at RT
18. Remove top, put samples on ice, and transfer to -80C freezer for long term storage