January 7, 2014

Immunoprecipitation

Basic good technique when handling the RiboTag samples:

Cold is good. Washes should be done in the cold room. O/N incubations are in the cold room, etc.
Use barrier pipet tips to prevent RNase contamination
Wipe down surfaces and items with RNase Zap.
Store samples at -80 for long-term storage.

PLEASE NOTE: IP must be performed immediately following homogenization! In other words, your tissue samples must be processed fresh directly to RNA yield OR tissue samples may be snap frozen whole and processed to RNA yield at a later time.

Homogenization volume	
Volume of anti-HA per sample	
Volume of beads per sample	
IP volume:	
Input volume:	
Elution volume:	

Groups:

Notes:

Homogenization buffer (HB)

Ingredient	Location	20 ml	10 ml	5 ml
RNase-free H2O	Sterile, RT	15.09 ml	7.55 ml	3.78 ml
10% NP-40	4C in hall	2000	1000	500
KCl 1M	4C in hall	2000	1000	500
Tris 1.5M, pH 7.4	4C in hall	666	333	167
MgCl2 1M	4C in hall	240	120	60

Store at 4C. Good for 2 months at most.

Supplemented homogenization buffer (HB-S)

Ingredient	Location	20 ml	10 ml	5 ml	2.5 ml
Homogenization buffer (HB)	From stock (recipe above)	19080	9549	4.775	2.39
Cyclohexamide (5mg/ml)	-20 (horiz) or 4C stock (K554)	400	200	100	50
Protease inhibitors	-20 (horiz)	200	100	50	25
Heparin (100mg/ml)	4C outside K561	200	100	50	25
RNAsin	-20 (outside K562)	100	50	25	12.5
DTT 1M	-80	20	10	5	2.5

Store at 4C. Make supplemented fresh no more than day before from homogenization buffer.

High Salt buffer

Ingredient	Location	20 ml	10 ml
RNase-free H2O	Room temp	10.67	5.34
KCl 1M	4C	6000	3000
10% NP-40	4C	2000	1000
Tris 1.5M, pH 7.4	4C	666	333
MgCl2 1M	4C	240	120
Cyclohexamide	--20 (horiz) or 4C stock (K554)	400	200
DTT 1M	-80 or -20	10	5

Make fresh day of use. Keep on ice.

Procedure-**Indirect Conjugation**

In the indirect conjugation method, the antibody is allowed to incubate in solution with the lysate for 4 hours at 4C prior to the introduction of beads. We have found that this "indirect" method greatly enhances our yield.

DAY ONE

Clean dounce homogenizer:

- Gather 7 or 2 ml dounce homogenizer and A and B pestles
- Rinse 3xs with RNase-free water then spray with RNase Zap
- Rinse several more times with RNase-free water
- Shake off water
- Place on ice, one pestle in homogenizer (pestle A), the other (pestle B) in a 15 ml conical
- Add desired volume of **supplemented homogenization buffer (HB-S)** to the dounce-add slowly to prevent bubbles
- TURN ON 4C centrifuge (in Mike's bay)

Tissue preparation:

- Sacrifice and dissect tissue of interest (speed necessary)
  - Note: dissection may be done on ice on a metal block to improve RNA yield.
  - Snap-freeze samples following dissection and store at -80C until ready to progress to homogenization.

Homogenization:

- Homogenize the samples **at 2 or 10% w/v** first with pestle A and then with pestle B 15-50 times each.
  - Determination of w/v concentration is based upon the total amount of tissue mass added to the homogenization buffer during the homogenization. For example, an entire brain in 10 ml of homogenization buffer gives you between 3-10% w/v (mouse brain wt: 0.3 g/10 ml of HB-S = 3%)
- Label eppendorf tubes for each sample:
  - SPIN, +HA, +BEADS, INPUT, SUPERNATANT, IP
- Transfer each homogenate to its SPIN eppendorf.
- Centrifuge 4C/10K/10min-COLD IS IMPORTANT
- Collect a 40-80µl from SPIN for your INPUT sample and add 350 of **lysis buffer** (RLT buffer from RNeasy kit + bME), vortex 10-30 sec, flash freeze and store at -80C.
- Collect 800 µl from SPIN without drawing up tissue pellet from bottom of tube. This spun-down lysate will become your IP sample. Transfer it to the +HA labeled tube and add anti-HA antibody, usually 5µl.
- Place in cold room on gentle spinner.
- Incubate for 4 hours.

Prepare beads (equilibrate beads to buffer solution):
About 30 minutes before you add the antibody-lysate solution to the beads, the beads must be equilibrated to the homogenization buffer.

- Add volume of beads to RNase/DNase-free eppendorfs according to your desired prep (usually 200µl of Pierce beads or 400µl of Dynabeads).
- Place in magnet
- Suction off the storage buffer
- Add 800-1000µl of **HB** or HB-S
- Resuspend on cold room gentle rotator for 10 min
- Place in magnet
- Suction off the buffer
- Place on ice
- Quickly add your antibody-tissue homogenate sample to the beads according to your desired preparation.
- Allow them to incubate O/N in cold room on gentle spinner/rotator.

DAY TWO

Equipment/Supplies:
RNeasy Micro kit
Ice
Samples (should have been stored in -80C prior to work or directly from IP on ice)

Solutions:
RNA only bottle of 100% etOH for making solutions-freshly opened is best
70% etOH made with RNase-free water
80% etOH made with RNase-free water
Buffers from RNeasy kit:

Lysis buffer
Recipe: 10µl bME/ml of RLT lysis buffer from RNeasy Micro kit
Make up 500µl of lysis buffer/sample.

Extraction of RNA from bead-antibody-protein sample

- Place eppendorfs containing bead-antibody-tissue homogenate in magnetic rack on ice
- Remove supernatant and place in supernatant labeled tube. Store on ice or move to -80C with input sample.
- Wash beads (now bound to our desired antibody-protein conjugation) with 800µl of **high salt buffer** per sample eppendorf**,** 3 times,10 min at 4C in cold room on rotator.
- Prepare **lysis buffer** (RLT lysis buffer + bME) during washes.
- Add 350µl of **lysis buffer** to each sample *immediately* after removing final high salt wash buffer.
- Vortex 30 seconds (you are trying to break apart the antibody-bead-protein bond).
- Place sample back in magnetic stand.

- Draw off volume (RLT lysis buffer and beads in Eppendorf) and place in IP labeled tube.

Prepare input sample

- Add 350µl of lysis buffer to the 40-80µl of frozen input sample
- Vortex 10-30 sec
- Follow steps in RNeasy Micro handbook starting at the step where you add equal volume of 70% Ethanol to each sample.
- *Note:* If spinning while cap is open, remember to label your collection tube.
- For elution volume, you may add up to 20µl of RNase-free water, if desired.
- Immediately store samples at -80C following isolation or proceed to RNA quantification (Ribogreen assay).

Quantification of RNA by Ribogreen assay

Basic Introduction:
The Ribogreen assay is able to quantify the total RNA content of your sample. Our yield is usually under 5 ng/µl for the IP samples and can be between 20 and 100 for the input sample. Considering this, remember to dilute your input sample in RNase-free water to allow it to fall solidly within your standard curve. Usually a 1:10 dilution is effective. However, during initial runs, test multiple dilutions and use for comparison.

Equipment/Supplies:
Quant-It Ribogreen Kit
RNase-free water
Microplate reader
96 well plates made for Microplate reader

Solutions (made from Ribogreen kit):
TE 1X-prepare from 20X using RNase-free water
Component A (Ribogreen) dilute as desired considering the range of standard curve your sample might fall into (low range or high range).
RNA standard stock
RNA standard dilutions

Procedure:

Total RNA (ng)	Final [ ] (ng/ml)	Vol. **TE 1X**	Vol. **100ng/ml diluted RNA standard**	Vol. **Ribogreen**
0	0	100	0	100
0.5	2.5	95	5	100
1	5	90	10	100
2.5	12.5	75	25	100
5	25	50	50	100
7.5	37.5	25	75	100
10	50	0	100	100
SAMPLE	**Unknown**	**99**	**1 (of sample)**	**100**

- Prepare TE 1X using TE 20X provided and diluting with RNase free H2O.
- Dilute component A (ribogreen) for a total volume of 4 mls.
- Prepare 100ng/ml RNA standard
  - 4µl 100µg/ml (provided) into 196µl of TE 1X = 2µg/ml
  - 50µl of 2µg/ml into 950 µl of TE 1X
- Dilute RNA standard to create a 7-point standard curve including the blank (zero).
- Total Reaction volume= 200µl.
- Add 100µl of diluted Ribogreen to each standard and sample *quickly*-a repeat pipettor is helpful.
- Tap plate to make sure that all of the liquid is in the bottom and mixed.
- Incubate in dark at RT for 5 min.
- Read on Microplate reader at 488 excite and 520 emission.