RIP GFB

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RNA -Immunoprecipitation (RIP) using Dm cell extract and GFP-binder resin:

The procedure described below is an example of a single immunoprecipitation reaction. For multiple immunoprecipitation reactions, calculate the volume of reagents needed according to the number of samples to be processed. For easy performance of immunoprecipitation reactions, it is recommended to use 10 ul of GFP-binder (CHROMOTEK^{TM}) suspension per reaction (~ 5 ul of packed gel volume).

Note: control reactions are recommended for the procedure. An important control is immunoprecipitation with extract containing only GFP.

RESIN PREPARATION:

1. Before use, wash GFP binder-coated beads with 1 ml of PBS/0.1% Tx-100 for 5' at RT, followed by equilibrating in ice-cold buffer 2 times 5' @ 4 C (choice of buffer depends on experimental settings). To wash, spin down beads by pulsing in an ultracentrifuge at 4 °C, remove liquid with hand pipettor or aspirator and resuspend in ice-cold buffer by flicking the tube several times with a finger. This washing removes unbound antibody.
▲ CRITICAL STEP Use siliconized tubes to avoid loss of RNA.

2. After the final wash, resuspend beads in 850 µl of ice-cold buffer. Add 200 units of an RNase inhibitor (5 µl RNase Out), 2 µl (to final concentration of 400 µM) Vanadyl ribonucleoside complexes (NEB).

3. Add myc-peptide (1mg/ml stock to 0.5 ug/ml final) as unspecific protein block and incubate resin on wheel for at least 1 hr @ 4 C.

EXTRACT PREPARATION:

If embryonic tissue^{A} is being used, grind tissue in bubbling liquid nitrogen. Using cell culture cells^{B} start to resuspend cells in appropriate buffer. Using ovaries or testis^{C} (dissected in extraction buffer) thaw on ice. Incubate on ice for at least 10'. For **A** and **B**, transfer extract into douncer. Homogenize on ice using 20 strokes (loose pestle, followed by tight pestle). For **C**, use 23 G syringe needle and homogenize extract 20 x. Spin high speed for 2x 30'@ 4 C. Remove sup avoiding the turbid layers.
Measure protein concentration.
Use at least x mg total protein per RIP (0.3 mg for PCR based detection; > 1 mg for 3' end labeling detection).
Unused extract should be snap-frozen in liquid nitrogen and stored at -80 C.

▲ CRITICAL STEP Additional freeze-thaw cycles should be avoided to prevent protein and RNA degradation

Immunoprecipitation reaction and RNA precipitation

If using snap-frozen extract, centrifuge RNP lysates at 16,000g for 15' @ 4 C to clear lysate freeze/thaw related artifacts. Transfer cleared supernatant to (siliconized) microfuge tube and store on ice. Additionally, pre-clearing of lysate with agarose beads (RNAse free) may be used to reduce background, if necessary. This may, however, reduce signal.

Add x µl of cleared lysate to GFP binder mixture prepared in **Step 2**.
▲ CRITICAL STEP This dilution of lysate is important to reduce adventitious binding.

Immediately flick tube several times with a finger to mix, and centrifuge briefly at 8,000–10,000g to pellet beads. Remove 100 µl of supernatant to represent total cellular RNA (to be used in control PCRs).

Incubate for 60 min at 4 °C tumbling end over end.

Pellet beads (1 min, 5000 x g, 4°C) and save supernatant for later analysis if desired (remove of 10% of the supernatant as flowthrough/not bound + x µl 4x Laemmli). Supernatant may be stored at –20 °C for several months.

Wash beads 4–5 times 10' with 1 ml of ice-cold buffer @ 4 C by pulsing in an ultracentrifuge and removing supernatant with a hand pipettor or an aspirator.
▲ CRITICAL STEP Thorough washing is critical for reducing background. Buffer may be supplemented with urea, sodium deoxycholate or SDS to increase stringency depending upon the RNA-binding protein being investigated. Increase time of washes if necessary. All tubes should be kept on ice as much as possible while working quickly during the washing process to reduce degradation.

Resuspend the beads in 200 µl of PBS buffer. Supplement with 4 ul of Proteinase K solution (50 mg/ml). Incubate mixture for 30-60' at 55 °C, shaking with 700 rpm.

Release the RNP components and isolate the RNA from the immunoprecipitated pellet by adding phenol-chloroform-isoamyl alcohol (pH 4.5, AMBION) directly to the beads. Use chloroform twice to remove traces of Phenol. Precipitate RNA by adding 1/10 volume 3 M NaOAc pH 5.2 and 3 volumes 100 % EtOH (Addition of glycogen (20 µg) or linear acrylamid

to 0,25 ug/ml (AMBION) as a carrier to the precipitation reaction aids in making the RNA pellet more readily visible and aids in recovery of RNA). Put @ -80 C for at least 1 hr. Air dry for 1-5'. Do not overdry. Resuspend in 10-20 ul H_20 (DEPC). Store @ -80 C until end-labeling or cDNA synthesis.

▲ CRITICAL STEP Usually, more than 2 times Phenol are necessary to remove all of the protein, 2 times chloroform to remove phenol traces are recommended. DO NOT WASH WITH 75% ETOH.

Stock solution	**final**	**To 50 ml**	
3 M KCl	**0,1 M**	**1,7 ml**	
1 M Hepes pH 7,9	**0,02 M**	**1,0 ml**	
10 % Tx-100	**1 %**	**5 ml**	

100% glycerol	**5%**	**2,5 ml**	
1 M $MgCl_2$	**0,005 M**	**0,25 ml**	
2,5 % Na-deoxycholate	**0,1 %**	**2,0 ml**	**Add before IP**
1 M DTT	**0,01 M**	**0,5 ml**	**Add before IP**
VRC		**2 ul per ml**	**Add before IP**
RNAse Inhibitor			
H_2O		**36,95 ml**	