RIP using F2 FLAG

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RNA -Immunoprecipitation (RIP) using Dm cell extract and M2-FLAG 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 40 ul of gel suspension per reaction (~20 ul of packed gel volume). Smaller amounts of resin (~10 ul of packed gel volume, which binds >1 mg FLAG fusion protein) can be used.
Note: control reactions are recommended for the procedure. An important control is immunoprecipitation with FLAG-peptide in the reaction to compete for the FLAG binding sites at the fusion protein

RESIN PREPARATION:

1) Thoroughly re-suspend the ANTI-FLAG M2 affinity gel in the vial, in order to make a uniform suspension. Immediately transfer 40 ul of the gel suspension to a fresh tube. For resin transfer, use a clean cut off yellow tip.
2) Centrifuge the resin at 5,000-8,200 X *g* for 30 seconds. In order to let the resin settle in the tube, wait for 1-2 minutes before handling the samples. Remove the supernatant with a narrow-end pipette tip, being careful not to transfer any resin.
3) Wash the packed gel 2 x 5' with 0.5 ml steril PBS @ RT. Be sure that most of the wash buffer is removed and no resin is discarded.
4) Equilibrate resin by washing it 1 x with 0.5 ml of **appropriate buffer.** 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.

After the final wash, resuspend beads in x 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. (based on 1 ml rxn volume)

Blocking M2 resin as control:
An important control is immunoprecipitation with FLAG-peptide in the reaction to compete for the FLAG binding sites at the fusion protein.

5) To step 4) add FLAG peptide from 5 ug/ul stock to a final concentration of 150 ng/ul and mark tube as control.

EXTRACT PREPARATION (Drosophila cells):

6) 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 homogenized extract 20 x. Spin high speed for 2x 30' @ 4 C.

7) Remove sup avoiding the turbid layers.

8) Measure protein concentration.

9) 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 Immediate freezing of the lysate is essential to complete the lysis process as well as preventing adventitious binding. Additional freeze-thaw cycles should be avoided to prevent protein and RNA degradation

Immunoprecipitation reaction and RNA precipitation

10) Thaw lysate on ice and centrifuge at 15,000g for 15' to clear lysate of large particles.

11) Transfer cleared supernatant to (siliconized) microfuge tube and store on ice.

▲ CRITICAL STEP Additionally, pre-clearing of lysate with beads may be used to reduce background, if necessary. This may, however, reduce signal.

12) Add x µl of cleared lysate to M2 resin prepared in **Step 4** and fill tube to 1 ml with appropriate buffer.

▲ CRITICAL STEP This dilution of lysate is important to reduce adventitious binding.

13) Incubate for 60' @ 4 °C tumbling end over end.

14) Centrifuge the resin at 5,000-8,200 X *g* for 30 seconds. Remove sup and save for later analysis.Supernatant may be stored at –20 °C for several months.

15) Wash beads 4−5 times with 1 ml of ice-cold **buffer** by pulsing in an ultracentrifuge and removing supernatant with a hand pipettor or an aspirator.

▲ CRITICAL STEP Thorough washing is critical for reducing background. 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.

16) 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' @ 55 °C, shaking with 700 rpm.

17) Release the RNP components and isolate the RNA from the immunoprecipitated pellet by adding 200 ul phenol-chloroform-isoamyl alcohol directly to the beads. Use 200 ul chloroform to remove traces of Phenol.

18) Precipitate RNA by adding 1/10 volume 3 M NaOAc pH 5.2 and 3 volumes 100 % EtOH (Addition of glycogen (20 µg) 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' @ RT. Do not overdry. Resuspend in 10-20 ul H_20.

▲ 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 70% ETOH.

RT reaction

If testing for tRNA asp (Dnmt2 pull down), use 1 ul of ppt material and perform stem loop primed cDNA synthesis (see protocol).

If testing for other RNAs, use QuantiTect kit from Qiagen. 1 ul of ppt RNA per reaction. Do genomic WipeOUT for 5 min at 42 C. Add RT mix for 20 min at 42 C. Inactivate RT 3 min at 95 C. Store cDNA at 4 C.

Use 1 ul in a PCR.

Haifan Lin buffer

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	

NT5 buffer:

Stock solution	final	To 50 ml	
5 M NaCl	0,15 M	1,5 ml	
1 M Tris pH 8,0	0,05 M	2,5 ml	
10 % Tx-100	1 %	1 ml	
10 % SDS	0,1 %	0,1 ml	
1 M $MgCl_2$	0,001 M	0,05 ml	
2,5 % Na-deoxycholate	0,1 %	0,4 ml	Add before IP
1 M DTT	0,002 M	0,1 ml	Add before IP
VRC		2 ul per ml	Add before IP
RNAse Inhibitor			
H_2O		44,75 ml	