

**This protocol has been optimized from Baroni *et al.* [1] for immunopurification of endogenous human PUMILIO 1 and PUMILIO 2 proteins from HeLa S3 cells, isolation of associated mRNAs and consequent cDNA microarray analysis [2].**

**MATERIALS:**

1. RNase-free tips and tubes and reagent bottles
2. ultraPURE distilled, DNase-free, RNase-free water (GIBCO, Invitrogen, cat. # 10977-035) to prepare buffers and solutions
3. Cells of interest (HelaS3, ATCC)
4. 1X PBS
5. Antibody to RNA binding protein of interest ( $\alpha$ -PUM1 and  $\alpha$ -PUM2 antibody, Bethyl, AbVantage Pack cat. # A310-056A)
6. Protein G Sepharose 4 Fast Flow beads (Amersham, cat. # 17-0648-01) or Protein A Sepharose CL-4B (Amersham, cat. # 17-0780-01). Store at 4°C.
7. BSA, protease, DNase, Rnase-free (Gerber from USA). Store at 4°C.
8. 1M dithiothreitol (-20°C aliquots)
9. Complete, EDTA-free tablets, proteinase inhibitor (Roche, cat. #11-873-580-001). Store at 4°C.
10. RNase OUT™ (Invitrogen, cat. #10777-019, 40 U/ $\mu$ L). Store at -20°C.
11. Superase•IN™ (Ambion cat. #2696, 20U/ $\mu$ L). Store at -20°C.
12. **POLYSOME LYSIS BUFFER:** 10 mM HEPES pH 7, 100 mM Potassium Chloride, 5 mM Magnesium Chloride, 25 mM EDTA, 0.5% IGEPAL, one tablet of complete proteinase inhibitor (for 50 ml buffer), 2 mM DTT, 50 U/ml RNase OUT, 50 U/ml Superase IN, Heparin 0.2 mg/ml.

Stock solutions:**- HEPES 0.5 M pH 7**

HEPES MW = 238.3. Weight 5.95 g and add 50 ml UltraPure water

**- Potassium Chloride 1 M**

Potassium Chloride MW = 74.55. Weight 3.72 g Potassium Chloride and add 50 ml UltraPure water

**- 1 M Magnesium Chloride**

MgCl<sub>2</sub> MW = 203.43. Weight 10.17 g MgCl<sub>2</sub> and add 50 ml UltraPure water

**- 0.5 M EDTA**

EDTA MW= 292.25. Weight 7.31 g EDTA and add 50 ml UltraPure water

Store stock solution at 4°C up to 6 months

**- IGEPAL 20%**

2 ml IGEPAL 100% + 8 ml UltraPure Water. Store at room temperature

**- Heparin 20 mg/ml.** stored at -20°C.

To prepare 50 ml Polysome lysis buffer, add:

- 1 ml HEPES 0.5 M pH 7
- 5 ml Potassium Chloride 1 M
- 250 µl 1 M Magnesium Chloride
- 2.5 ml 0.5 M EDTA
- 1.25 ml IGEPAL 20%
- 1 one tablet of complete proteinase inhibitor

This solution can be stored at 4°C. Then, add DTT, RNase OUT and Superase•IN™, Heparin according to the buffer amount you need to prepare the cell extract.

13a. **NT2 buffer:** 50 mM Tris-HCl pH 7.5, 150 mM Sodium Chloride, 1 mM Magnesium Chloride, 0.05% IGEPAL

Stock solutions:

**- 1 M Tris-HCl pH 7.5**

Tris MW = 121.14. Weight 6.05 g Tris and add 50 ml UltraPure water

**- 1 M Sodium Chloride**

NaCl MW = 58.44. Weight 2.922 g NaCl and add 50 ml UltraPure water

**- 1 M Magnesium Chloride**

MgCl<sub>2</sub> = 203.43. Weight 10.17 g MgCl<sub>2</sub> and add 50 ml UltraPure water

Store stock solution at 4°C up to 6 months.

**- IGEPAL 20%**

2 ml IGEPAL 100% + 8 ml UltraPure Water. Store at room temperature

To prepare 50 ml NT2 buffer add:

- 2.5 ml 1 M Tris-HCl pH 7.5
- 7.5 ml 1 M Sodium Chloride
- 50 µL 1 M Magnesium Chloride
- 125 µL IGEPAL 20%

13b. **NT2 –Ab coupling buffer:** buffer supplemented with 5% BSA, 0.02% sodium azide, 0.02 mg/ml Heparin

To prepare 10 ml NT2 buffer supplemented add:

- 0.5 g BSA
- 20 µL 10% sodium azide
- 10 µL 20 mg/ml Heparin

13c. **NT2-RIP buffer:** NT2 buffer supplemented with 50 U/mL RNase OUT™, 50 U/mL Superase•IN™, 2 mM dithiothreitol, 30mM EDTA and Heparin 0.02 mg/ml.

Stock solution:

- RNase OUT (Invitrogen) 40U/  $\mu$ L
- SuperscriptIN (Ambion) 20U/  $\mu$ L 1 M DTT
- 0.5 M EDTA
- Heparin 20 mg/ml

14. **SDS-EDTA ELUTION BUFFER:** 50 mM Tris pH 8, 100 mM Sodium Chloride, 10 mM EDTA, 1% SDS

Stock solutions:**- 1 M Tris pH 8**

Tris MW = 121.14. Weight 6.05 g Tris and add 50 ml UltraPure water

**- 1 M Sodium Chloride**

NaCl MW = 58.44. Weight 2.922 g NaCl and add 50 ml UltraPure water

**- 0.5 M EDTA**

EDTA MW= 292.25. Weight 7.31 g EDTA and add 50 ml UltraPure water

**- 5% SDS.** Weigh 5 g SDS and add 100 ml ml UltraPure water

15. **mirVana™ PARIS™ KIT** (Ambion, cat. #1556) for RNA extraction  
(see manufacturer instruction)

16. **Amino Allyl MessageAmp™ II aRNA Amplification** (Ambion, cat. # 1753) to amplify and label RNA for microarray experiment (see manufacturer instruction)

17. for oligo array ONLY: **RNA fragmentation Reagents** (Ambion, cat. # AM8740)

18. **cDNA or oligo-array** (Stanford Genomic Facility). See microarray hybridization protocol.

## A. RIP

### 1. Preparation of whole cell RNP lysates from cultured cells

1. Grow 10 x 150mm HeLaS3 dishes until they are around 90% confluent.
2. Wash twice with ice-cold PBS (8 ml), harvest using a scraper (5 ml PBS), and pellet the cells by centrifugation at 3000g for five minutes at 4°C. Snap-freeze the cell pellet in liquid nitrogen and store it at -80°C until use.
3. Thaw the cell pellet on ice. Estimate the pellet volume and add approximately 1.5 volumes of **POLYSOME LYSIS BUFFER**.
4. Pipette several times until the extract looks uniform, transfer it in 2 ml centrifuge tubes, and spin in a microcentrifuge at 14,000g for ten minutes at 4°C.
5. Remove the supernatant, transfer it in new tubes and spin it again two times (or more) to remove the lipid layer on top of the supernatant (the lipid layer sticks to the beads!)
6. Resuspend the pellet in one volume of **POLYSOME LYSIS BUFFER**, repeat the pipetting and centrifugation and combine with the supernatant from step 5 (above).
7. Determine the protein concentration by Bradford.
8. Prepare **20 mg cell extract** aliquots. The extracts are now ready to proceed to mRNP isolation. Snap-freeze the extract in liquid nitrogen and store it at -80°C until use (TIP: prepare the extract the day before RIP)
9. Keep 100 µL cell extract for total RNA isolation (miRVANA Paris kit, Ambion).

## 2. Antibody coating of bead matrix

### Use Protein G Sepharose beads for monoclonal antibodies or protein A Sepharose beads for rabbit serum or rabbit polyclonal antibodies

**A. Protein G Sepharose beads** (Amersham) are stored in 20% EtOH.

1. Use 50  $\mu$ L of drained beads (beads only) for each IP.
2. Remove EtOH by spinning at 10,000 rpm for 3 minutes, at 4°C.
3. Resuspend beads in 8 volumes (400  $\mu$ L) **NT2 buffer containing 5% BSA, 0.02% sodium azide and heparin 0.02 mg/ml** and wash three times by spinning as described in point 2.
4. Add the immunoprecipitating antibody (check the datasheet of the antibody for the recommend Ab amount to use. For PUM1 IP, 20  $\mu$ g  $\alpha$ PUM1 Ab are used) and incubate O/N (around 12 hours) on a rotating device at 4°C.
5. For **MOCK RIP**, incubate the beads **only** with NT2 buffer supplemented as described at point 3.

**B. Protein A Sepharose beads** (Amersham) are stored dried.

1. Dissolve 0.5 g Protein A Sepharose beads in 5 ml **NT2 buffer containing 5% BSA, 0.02% sodium azide and heparin 0.02 mg/ml** and swell them O/N (around 12 hours) on a rotating device at 4°C. (this procedure gives you enough beads for many RIPs)
2. Store swollen beads at 4°C.
2. Use 50  $\mu$ L of drained beads (beads only) for each IP.
3. Resuspend beads in 8 volumes (400  $\mu$ L) **NT2 buffer containing 5% BSA, 0.02% sodium azide and heparin 0.02 mg/ml**.
4. Add the immunoprecipitating antibody (check the datasheet of the antibody for the recommend Ab amount to use. For PUM2 IP, 50 $\mu$ g  $\alpha$ PUM1 Ab are used) and incubate O/N (around 12 hours) on a rotating device at 4°C.

5. **For MOCK RIP**, incubate the beads **only** with NT2 buffer supplemented as described at point 3.

### 3. Immunoprecipitation of mRNPs: whole cell mRNP isolation

1. Thaw the lysate on ice and centrifuge it in a microcentrifuge at 14,000g for ten minutes at 4°C, then transfer the supernatant to a new tube on ice.
2. Wash the beads three times at room temperature with **NT2 buffer**, spinning at 10,000 rpm for 3 minutes, then transfer the beads in 14 ml tubes and repeat the washing once more, and spin down the beads at 2,000 rpm for 2 minutes and 4°C and keep the beads on ice until use.
3. Resuspend the antibody-coated beads in NT2 buffer supplemented with 50 U/mL RNase OUT™, 50 U/mL Superase•IN™, 1mM dithiothreitol, and 30mM EDTA.
4. The volume of resuspended beads in NT2 buffer correspond to ten times the volume of the RNP lysate being used because performing the immunoprecipitation reactions in larger volumes can decrease background problems. Perform the RIP in 14 ml tubes.
5. Mix the resuspended antibody-coated beads several times by inversion, add the RNP lysate and tumble the immunoprecipitation reactions on a rotation device for 6 hours at 4°C.
6. Collect a sample of the supernatant at the beginning of the incubation, then after 2h, 4 h and 6h to check the efficiency of the IP during the time (spin at 2000 rpm for 2 minutes at 4°C)
7. After the incubation, spin the beads down as described at point 6, remove the supernatant, add 1.0 ml ice-cold **NT2 buffer** and transfer the beads to a 1.5 ml tube, mix, then spin down the beads at 10,000 rpm for 3 minutes at 4°C.

8. Repeat the washing step three more times.
9. Save samples from each washing step
10. Resuspend the washed beads in 500µL SDS-EDTA solution and incubate at 65°C for 10 minutes mixing every two minutes by inverting the tubes.
11. Spin down the beads by spinning at 10,000 rpm. 3 minutes at room temperature
- 12.** Save the supernatant as **1<sup>st</sup> ELUTION** in a 2 ml tube and keep it at room temperature.
13. Resuspend the beads again in in 500µL SDS-EDTA solution and incubate at 65°C for 10 minutes mixing every two minutes by inverting the tubes.
14. Spin down the beads by spinning at 10,000 rpm. 3 minutes at room temperature
- 15.** Save the supernatant as **2<sup>nd</sup> ELUTION** in a 2 ml tube and keep it at room temperature.
- 16.** Save samples from 1<sup>st</sup> and 2<sup>nd</sup> elution for SDS-PAGE before proceeding to RNA extraction.  
Save samples from beads after elution, then store the beads at -80°C.

#### **4. IP-RNA extraction**

RNA isolation is performed by using *mirVana*<sup>TM</sup> *PARIS*<sup>TM</sup> KIT according to the manufacturer's instruction

NOTE: elute RNA in 50 µL nuclease-free water, incubate 1 minute at RT and then elute by centrifugation.

## **B. Chip**

### **1. mRNA amplification and labelling**

mRNA is amplified by using **Amino Alkyl MessageAmp™ II aRNA**

**Amplification KIT** according to the manufacturer's instruction.

**NOTE:** use 25 µL of RNA sample for amplification (half elution volume). Use 10 µg aa-RNA for each labeling.

### **2. Microarray hybridization**

Is performed according to the standard hybridization procedure either for cDNA or oligo arrays (<http://cmgm.stanford.edu/pbrown/protocols/index.html>.)

## References

1. Baroni TE, Chittur SV, George AD, Tenenbaum SA (2008) Advances in RIP-chip analysis: RNA-binding protein immunoprecipitation-microarray profiling. *Methods Mol Biol* 419: 93-108.
2. Galgano A, Forrer M, Jaskiewicz L, Kanitz A, Zavolan M, et al. (2008) Comparative analysis of mRNA targets for human PUF-family proteins suggests extensive interaction with the miRNA regulatory system. *PLoS ONE* 3: e3164.