Chromatine associated RNA protocol (ChromRNA)

Adapted from Franziska’s protocol

**General view:**

1. Cell fractionation (~ 1/2 day)
2. RNA and protein isolation (~ 1/2 day)
3. WB on the different cell fractions to check purity (1 day)
4. RNAseq library and rRNA depletion (> 1 day)

PS: I estimated the time based on my experiment where I had 7 samples. It depends anyway on the number of samples that you have.

1. **Purification of chromRNA**
2. Pellet 3-5 M cells in a 1.5 mL tube (less can work but depends on the cell type).

1. Resuspend cell pellet in 1 mL cold PBS and centrifuge again to a pellet.
2. Resuspend cells in 300 μl cold Cytoplasmic Fraction Buffer
3. Vortex lysate for 2s and incubate on ice for 5 min.
4. Slowly layer the lysate onto 400 μl cold Sucrose Buffer
5. Centrifuge at 14,000 rpm for 10 min at 4°C.

1. The supernatant from this spin (800 μL) represents the **cytoplasmic fraction.**
	* 1. Add 80 μL of the supernatant volume to an equal volume of 2X sample buffer (2x SDS sample buffer + 5 % 2ME) for immunoblot analysis.
		2. Quickly add 250 µl of the supernatant to 750 µl of Trizol-LS. RNA purification from these and subsequent cellular fractions is described below. **This is to do only if you are interested in RNA from this fraction**
2. Wash once more in 200 µl Cytoplasmic Fraction Buffer, spin at 300 g for 3 min at 4 ˚C.
3. Resuspend the nuclear pellet in 200 μl cold Nuclear Lysis Buffer
4. Add an additional 200 μl of cold 2X NUN Buffer to the sample drop-by-drop while vortexing, incubation on ice for 20 min.
5. Spin the sample for 14,000 rpm for 30 min at 4°C. The supernatant from this spin represents the **nucleoplasmic fraction** (400 μl)
	* 1. Add 40 μl of the supernatant volume to an equal volume of 2X sample buffer for immunoblot analysis.
		2. Quickly add 250 µl of the supernatant to 750 µl of Trizol-LS. **This is to do only if you are interested in RNA from this fraction**
		3. Wash once again with 200 µl nuclear lysis buffer.
		4. Wash again with 1 ml cold PBS to eliminate any residual nucleoplasm.
6. The remaining pellet represents the **chromatin fraction**
	* 1. The chromatin fraction is an insoluble mass, in order to take a fraction for immunoblot analysis, protein must be extracted during the Trizol RNA extraction.
7. Add 1 mL Trizol to the chromatin pellet and heat to 65 ˚C for ~10 min (until no insoluble objects are visible).
8. Complete Trizol RNA extractions as desired and/or Western Blot to validate fractionation.
* Resuspend final RNA pellet in ~20 uL H2O
* Treat RNA with DNase (Ambion)
* RNA is ready for library preparation.

Buffer components:

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| --- | --- |
| Cytoplasmic Fraction Buffer  | 20mM Hepes-KOH ph 7.6, 2mM MgCl2, 10% glycerol, 0.1% NP40, 0.5mM DTT with protease inhibitor, phosphatase inhibitor and RNase inhibitor  |
| Sucrose Buffer  | 10mM Hepes-KOH pH7.6, 10 mM NaCl, 1.5 mM MgCl2, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 34% sucrose w/v with protease inhibitor, phosphatase inhibitor and RNase inhibitor |
| Nuclear Lysis Buffer | 10 mM Hepes-KOH pH 7.6, 100 mM NaCl, 0.5 mM EDTA, 50% glycerol, 0.5 mM DTT with protease inhibitor, phosphatase inhibitor and RNase inhibitor |
| 2X NUN Buffer  | 50 mM HEPES pH 7.6, 7.5 mM MgCl2, 0.2 mM EDTA, 0.6M NaCl, 2M urea, 2% NP-40, 2mM DTT |

1. **RNA and protein isolation from the chromRNA fraction**

Start with phenol-chloroform extraction followed by the use of the RNeasy Qiagen Kit (Zymoresearch kit might work too but not tested for this protocol)

**Phase separation**

1. Incubate the homogenized sample (see Homogenizing samples) for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.
2. Add 0.2 ml of chloroform per 1 ml of TRIzol®Reagent used for homogenization. Cap the tube securely.
3. Shake tube vigorously by hand for 15 seconds.
4. Incubate for 2–3 minutes at room temperature.
5. Centrifuge the sample at 12,000 × g for 15 minutes at 4°C. Note: The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is ~50% of the total volume.
6. Remove the aqueous phase of the sample by angling the tube at 45° and pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.
7. Place the aqueous phase into a new tube. Add same volume of 70 % EtOH and continue RNA isolation using Qiagen RNAeasy column.
8. Save the interphase and organic phenol-chloroform for Protein Isolation. The organic phase can be stored at 4 °C overnight.

**Protein isolation**

1. Remove any remaining aqueous phase overlying the interphase.
2. Add 0.3 ml of 100 % ethanol per of 1ml TRIzol®Reagent used for the initial homogenization.
3. Cap the tube and invert the sample several times to mix.
4. Incubate samples for 2–3 minutes at room temperature.
5. Centrifuge the tube at 2000 ×g for 5 minutes at 4 °C to pellet the DNA.
6. Remove the phenol-ethanol supernatant and save it in a new 2ml eppendorf tube for protein isolation. The supernatant can be stored at –70°C for several months.
7. Add 1.5 mL of isopropanol to the phenol-ethanol supernatant per of 1 ml TRIzol®Reagent used for the initial homogenization.
8. Incubate samples for 10 minutes at room temperature.
9. Centrifuge at 12,000 ×g for 10 minutes at 4 °C to pellet the protein. Remove and discard the supernatant.
10. Proceed to the Protein wash step with the remaining protein pellet.

**Protein wash**

1. Wash the pellet with 2 ml 100 % Isopropanol.
2. Centrifuge at 12,000 ×g for 10 minutes at 4 °C. Remove and discard Isopropanol wash.
3. Wash the pellet with 2 ml 100 % Ethanol and vortex.
4. Incubate for 10 min at room temperature.
5. Centrifuge at 7500 ×g for 5 minutes at 4 °C. Remove and discard Ethanol wash.
6. Air dry the protein pellet for 5-10 minutes. Do not allow the pellet to dry out.
7. Proceed to Protein resuspension.

**Protein resuspension**

1. Add 100 µl 2x Loading Buffer (+ 0.5 M DTT (1:1)).
2. Incubate for 30 min at 65 ˚C, with 1200 rpm shaking.
3. Incubate for 5 min at 95 ˚C.
4. Store samples at -20 ˚C.
5. **WB to check fractions purity**
6. **Sample preparation**
* Snap-freeze (liquid nitrogen) protein solution
* Store at -80C
* Thaw on ice
* Prepare samples for gel electrophoresis
* Load 20µl, 20 µg (if possible)

Lysate (diluted with H2O) 18 µl

Laemmli buffer (with 5% 2-ME) 6 µl

🡪 5 min 99 ˚C

* flip and vortex
1. **Run gel**
* Gel: Midigel (18 well comb, 30 µl, 1.0 mm, Bio-Rad # 567-1124, cold room)
* Tear off tape of gel cassette, remove comb
* Install gel cassette (plain side to the outer side, reservoir to the middle of the apparatus)
* Fill in running buffer (10x Buffer, Bio-Rad # 161-0732, Final concentrations: 25 mM Tris, 192mM Glycine, 0.1 % 2D2, diluted in nanopure H2O, pH 8.3, do not adjust pH) only on the side that's running plus the reservoir of the gel, first into gel cassette reservoir, then in apparatus until “FILL”
* Load gel (5 µl ladder, 20 µl sample, 2 more wells with 20 µl 4x Laemmli buffer)
* 100 V (= limit), approx. 1.5 h
* Wait until bromphenol blue front reaches the bottom (for small proteins)
1. **Transfer to membrane**
* Open semi‐dry transfer cassette (contains membrane and papers), use nitrocellulose for smaller proteins
* Lift the upper tab and place it in the transfer module base (anode = +). Use roller pin to remove any bubbles. Don’t let it dry out. Add PBS if necessary.
* Open gel cassette and cut off stack and bottom of gel (instruments used from Hanson’s group)
* Place gel on membrane. Cut off the part you don’t need/want using a razor blade. Straighten out and remove bubbles from it.
* Pick up bottom tab from the transfer cassette and place exactly like that on top of the gel. Use roller pin to smooth out.
* Close transfer module by adding the lid and insert into machine. Select port A or B for semi‐dry transfer and whether low, medium, high or mixed molecular weight molecules
* Press start
1. **Probe for protein of interest**
* Remove membrane from transfer module. Remember which side proteins are on
* Cut membrane using a ruler and a razor blade (keep hydrated using PBS, green band is 8kb)
* For nitrocellulose, immerse in 1x PBS for 2 minutes.
* For PVDF, immerse in 100% methanol for 2 minutes, then 1x PBS for 2 minutes.
* Block membrane with Odyssey blocking buffer for 1h at room temp or overnight at 4 ˚C on rocker.
* Add primary antibody (or both simultaneously) at correct dilution in Odyssey blocking buffer containing 0.1% tween 20

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| --- | --- | --- | --- |
| Beta-Tubulin | 50 kDa | Sigma, T8328, monoclonal, produced in mouse | 1:2000 |
| SNRP70 | 65 kDa | Sigma, SAB2102255, produced in rabbit | 1:500 |
| H3K27Ac | 15 kDa | Abcam, ab4729, produced in rabbit | 1:2000 |

* Wash 4 times for 5 min each with PBS + 0.1% tween
* Add secondary antibody at correct dilution, in Odyssey blocking buffer containing 0.1% tween. Add 0.01% SDS if you are using PVDF. Do not add SDS for nitrocellulose membranes. For Secondary antibodies, goat anti‐mouse IgG IRDye 800CW, at 1:8000 and goat anti-rabbit IgG IRDye 680, at 1:8000 should both work well. Note that the IRDye680 (red) is less bright than IRDye800CW (green), so try to use IgG IRDye 800CW for the least abundant protein (and IgG IRDye 680 at 1:5000)
* Incubate for 1h at room temp on rocker.
* Wash membrane 4 times for 5 min each with PBS + 0.1% tween
* Rinse once with PBS to remove all traces of tween.
1. **Scan membrane**
* Open Odyssey software
* New file
* Browse for correct path
* Name folder and save
* Get scan
* Benutzername: riveraj, Password: mcige
* Preset: membrane, Resolution: 84, Intensity: 5.0 and 5.0, Size: starting from x=1 and y=1 (look on the screen)
* Put membrane on screen, protein side down, ladder on the left
* Place rubber mat up on the membrane
* Remove air
* Start scan, press start button on the machine, choose membrane
1. **Odyssey – ImageStudio lite**
* Import folder
* Flip image
* Crop image
* Correct brightness for every channel
* Annotate ladder and samples
1. **RNAseq library and rRNA depletion**

Nanodrop extracted RNA

Use Nugen Ovation RNA-seq system for model organisms kit

* Deplete polyadenylated mRNA by incubating with oligo-dT beads (Sera Mag oligo dT or maybe from NEB mRNA polyA enrichment kit, 10ul beads for 1ug RNA), use unbound RNA fraction for library prep!
* Use either none or ½ amount of oligo-dT primers during library prep