

Locus specific chromatin purification protocol (optimized for HeLa S3 telomeres).

### **1. Preparation of chromatin sample that is suitable for hybridization:**

-From suspension cells in spinner flasks (20 liters culture at a density of  $0.5-1 \times 10^6$  cells/ml): cells are pelleted (by centrifugation at 2500g for 10 minutes at room temperature) then immediately resuspended in cross-linking solution (200ml for  $10^{10}$  cells).

-From adherent cells: media is discarded and crosslinking solution is immediately added to the plates (10ml/15cm plate).

-Incubate cells in crosslinking solution for 30 minutes at room temperature.

-For suspension cells: Spin down cells at 3200g for 10 minutes at 4C. Properly dispose of the supernatant solution. The material is aliquoted into 4 falcon tubes (usually 8 ml pellet/tube).

For adherent cells: Properly dispose of the crosslinking solution and rinse plates twice with 1X PBS solution. Add 3ml of cell scrapping solution/plate and pool the cells into Falcon 50 tubes on ice.

**DON'T 'QUENCH' FORMALDEHYDE WITH GLYCINE SOLUTIONS (AS IS USUALLY PERFORMED DURING TYPICAL CHROMATIN IMMUNOPRECIPITATION EXPERIMENTS, AS THIS MAY RESULT IN NON SPECIFIC CROSSLINKING OF GLYCINE TO PROTEINS, WHICH MAY ULTIMATELY PREVENT PEPTIDE MASS ATTRIBUTION DURING THE MASS SPEC ANALYSIS). INSTEAD, UNREACTED FORMALDEHYDE IS DILUTED OUT BY WASHES.**

-Wash cells 4 times in PBS:

-Resuspend cell pellet in 1X-PBS solution (bring the volume to 50 ml/tube)

-Spin down at 3200g for 10 minutes at 4C

-Discard the supernatant

-Resuspend cell pellet in Sucrose solution (bring the volume to 50 ml/tube)

-Spin down at 3200g for 10 minutes at 4C

-Discard supernatant and bring the volume to 40ml with Sucrose solution

-Transfer this mixture to a 40ml dounce homogenizer on ice

-Dounce 20 times with a 'tight' pestle

-Transfer the dounced mixture back to a new falcon tube.

-Spin down at 3200g for 10 minutes at 4C

-Discard the supernatant.

-Resuspend the pellet into 50 ml of Glycerol Buffer

-Spin down at 3200g for 10 minutes at 4C

-Discard the supernatant.

-Resuspend the pellet into the same pelleted volume of Glycerol Buffer (that is, if the pellet volume is 7.5 ml, add 7.5ml of glycerol buffer).

-Aliquot in 1.5ml volumes (that is  $\sim 0.5 \times 10^9$  cell equivalent per Eppendorf tube)

-Snap freeze into liquid nitrogen and store at -80C or proceed to the next step with 6 tubes for the telomere pull-down (that is  $\sim 3 \cdot 10^9$  cell equivalent or 3 liters for HeLaS3 telomere purification) and 6 tubes for the scramble pull-down.

The following volumes and numbers are given for one pull-down:

- Using a table top centrifuge, spin-down the nuclear prep at 2000g for 2 minutes at room temperature.
- Resuspend the pellet into 1.5ml/aliquot of 1X PBS-Triton solution.
- Add 15  $\mu$ l of Concentrated RNaseA (Qiagen 100mg/ml)/tube.
- Incubate for 60 minutes at room temperature with shaking (I am using an Eppendorf Thermomixer at 1400rpm) then at 4C (without shaking) for 12-16 h.

THIS RNase STEP IS TO OPTIMIZE THE SUBSEQUENT LNA PROBE/CHROMATIN HYBRIDIZATION STEP. THIS STEP MIGHT BE OMITTED IF THE GOAL IS TO PURIFY RIBONUCLEOPROTEIN COMPLEXES THAT ARE OR ARE NOT CHROMATIN ASSOCIATED (E.G. XIST COATED CHROMATIN DOMAINS ON THE INACTIVE X CHROMOSOME OR ANY RNA-PROTEIN COMPLEX OF INTEREST).

- Pool the mixture into a 50ml Flacon tube and bring the volume to 50ml with 1X PBS.
- Spin down at 3200g for 10 minutes at 4C and discard the supernatant.
- Repeat this washing step 6 times to dilute out traces of RNaseA
- Resuspend the pellet in 50ml of fresh LB3JD at room temperature.
- Spin down at 3200g for 10 minutes at 4C
- Dilute pellet twice. Resuspend well and split into 2X3.5ml aliquots into 2 Falcon 15ml tubes. The mixture should be very viscous.
- Sonicate samples on ice in the cold room:  
I am using a Misonix 3000 sonicator with a micro-tip. The tube is held by a ring clamp system, immersed at about 90% into an ice-water mix into a beaker. The tip is immersed down 2-3 mm for the bottom of the Falcon 15 tube.

The sonication parameters are:

- Power setting 7
- 15 seconds constant pulse
- 45 seconds pause
- 7 minutes total process time.

That means 28 cycles of 15 seconds sonication/45 seconds pause. The power output reading is 36W (Watts) at the beginning of the sonication step. This power reading increases to 42-45W as the mixture becomes less viscous during sonication. I usually stir the mixture with the sonicator tip after 2 minutes of sonication (8 cycles). These conditions usually give telomere fragments that are about 2-4kb long, which is perfectly fine for telomere pull-down.

IF A HIGHER RESOLUTION IS DESIRED (I.E. SMALLER AVERAGE LENGTH OF CHROMATIN FRAGMENTS), LONGER AND/OR MORE INTENSE SONICATION

WILL NOT WORK, AS CHROMATIN IS EXTENSIVELY CROSS-LINKED WITH 3% FORMALDEHYDE FOR 30 MINUTES). I HAVE SUCCESSFULLY USED A MICROCOCCAL NUCLEASE DIGESTION STEP BEFORE SONICATION (SEE PART 3). I DID PERFORM THIS MNASE DIGESTION FOR THE ALT TELOMERE PULLDOWN, BRINGING THE AVERAGE CHROMATIN FRAGMENT DOWN TO 600 BASE PAIRS WITH NO MAJOR DIFFERENCE IN THE MS RESULTS.

Soluble chromatin should now be handled at room temperature, to prevent precipitation.

- Aliquot the sonicated mixture into 1ml size into Eppendorf tubes.
- Spin down at 16000g for 15 minutes at room temperature and pool the supernatants into a falcon 15 tube (should be ~6.5ml, 0.5ml being 'lost' during the sonication steps. Also, I am leaving some liquid above the pellets of each Eppendorf when I'm retrieving the supernatants).
- Prepare 16 S-400-HR spin columns:
  - vortex the columns
  - loosen the caps
  - snap off the bottom closure and place the column into an empty tube
  - spin down at 800g for 2 minutes at room temperature
  - place the dried column into new collection tubes.
- Add 400ul/dried column of the soluble chromatin mixture.
- Spin down at 800g for 2 minutes.

THIS STEP IS VERY IMPORTANT FOR THE QUALITY OF THE PURIFICATION. THIS ULTRA-FAST GEL-FILTRATION REDUCES THE SALT CONCENTRATION IN THE SAMPLE BY ABOUT  $2/3^{\text{rd}}$  LEAVING ROUGHLY 30mM of NaCl. THE LESS SALT THERE IS, THE MORE SPECIFIC THE HYBRIDIZATION AND THE LESS NON-SPECIFIC PRECIPITATION WILL OCCUR ON THE BEADS LATER-ON.

- Pool the eluates together into a Falcon 15ml tube (> 5ml).
- Incubate at 58C for 5 minutes.

I FOUND THIS STEP TO HELP UNMASKING BIOTIN FROM ENDOGENOUSLY BIOTINYLATED PROTEINS, WHICH HAVE TO GET REMOVED PRIOR HYBRIDIZATION.

- Cool down the sample to room temperature.
- Equilibrate 0.5ml of Ultralink Streptavidin slurry into 10 ml of LB3JDLS (30mM NaCl) for 5 minutes at room temperature.
- Spin down at 3200g for 2 minutes and take off the supernatant
- Bring the volume to 10 ml with LB3JDLS.
- Spin down at 3200g for 2 minutes and take off the supernatant
- Add the streptavidin beads (~0.5ml to the chromatin sample)
- Incubate for 2h at room temperature on a nutator.

- Spin down at 3200g for 10 minutes at room temperature.
- Save the supernatant, leaving about 100 µl of liquid above the beads surface.

THIS PRE-CLEARING IS NECESSARY TO REMOVE MOST OF THE ENDOGENOUSLY BIOTINYLATED PROTEINS THAT OTHERWISE WILL COMPETE WITH THE DESTHIOBIOTINYLATED PROBE FOR BINDING TO THE STREPTAVIDIN BEADS.

SUCH CONTAMINATION IS OBVIOUS WHEN LOOKING AT THE MS RESULTS (MITOCHONDRIAL CARBOXYLASES ENZYMES ARE NATURALLY BIOTINYLATED PROTEINS, AND USUALLY FOUND IN LOW AMOUNTS IN THE PULL-DOWNS DESPITE THIS PRE-CLEARING)

The chromatin sample (the supernatant) is now ready for hybridization. It should have the following spectrometric characteristics:

- DNA Concentration (O.D.<sub>260</sub>): 2-2.5 mg/ml
- O.D.<sub>260</sub>/O.D.<sub>280</sub> = 1.30-1.45. Higher values would mean that the initial RNaseA treatment was not fully effective and that the purified material might be contaminated by non chromatin RNA-protein complexes. These values are obtained when LB3JDLS is used as the 'blank' solution.

This chromatin can be stored at 4C, if one cannot proceed to the next step.

## **2. Hybridization and chromatin capture:**

- Spin-down the chromatin samples for 15 minutes at 16 000g at room temperature.
  - Pool the aliquots together. This should give about 5ml of chromatin sample.
  - Add 20% SDS to 0.02% final (add 1/1000 vol.)
  - Add 30 µl of the desired LNA (100µM stock solution, see part 4 for technical details and sequence) (1 tube will have the telomere specific LNA, the other the LNA containing the scramble sequence).
  - Split into ~34 X 150 µl aliquots into PCR tubes
- I am using (with the same results) either a HYBAID Px2 or an MJ PTC-200 PCR machine for the hybridization step.

The program is as follows:

- 25C for 3 minutes
- 70C for 6 minutes
- 38C for 60 minutes
- 60C for 2 minutes
- 38C for 60 minutes
- 60C for 2 minutes
- 38C for 120 minutes
- 25C final temperature

THIS PART HAS BEEN OPTIMIZED SO THAT THE LNA PROBE CAN INVADE THE DNA SEQUENCE INTO CHROMATIN WITHOUT OBSERVABLE PROTEIN

DE-CROSSLINKING. DENATURATION TEMPERATURES HIGHER THAN 73C SHOULD BE AVOIDED. I HAVE TESTED 76, 80 AND 83C AS DENATURATION TEMPERATURES AND THESE RESULTED IN CROSSLINKING REVERSAL AND PROTEIN LOSS FROM THE TARGET CHROMATIN. 73C SEEMS OK, BUT NOT DIFFERENT FROM 70C. I THUS CHOSE THE LOWEST EFFECTIVE TEMPERATURE (70C). LOWER TEMPERATURES WERE ALSO TESTED (55→65C). HYBRIDIZATION WAS MUCH LESS EFFICIENT AT 65C THAN AT 70C. AT TEMPERATURES LOWER THAN 65C, NO PROTEINS COULD BE DETECTED IN THE PURIFIED MATERIAL.

-Samples from PCR tubes are pooled back to Eppendorf tubes  
Spin down at 16000g for 15 minutes at room temperature to get rid off any precipitate that have formed during the hybridization step.

-During the spin:

-Prepare 1.2 ml of MyONE C1 magnetic streptavidin beads into a Falcon 15 tube  
add 8.8 ml of LB3JD

-Immobilize the beads on the magnetic stand and discard the supernatant.

-Add 10 ml of LB3JD

-Mix gently

-Immobilize on the stand and discard the supernatant

-Resuspend into 1.2ml total volume with LB3JD. This is the C1 beads solution.

-Transfer the supernatant from the spun chromatin samples to 2 falcon 15 tubes

-Add 3.5 ml of milliQ water/tube.

-Add 0.6ml of C1 beads solution/tube.

-Nutate for 12h at room temperature.

-Bring the volume to 10 ml/tube with LB3JD

-Immobilize on the magnetic stand

-Save the supernatant (10ml) this is the unbound fraction.

Washes:

-7 times 10 ml washes with LB3JD. Gently resuspend the beads by nutation in between each wash (this takes about 1 minute).

-Resuspend the beads into 1.2mlLB3JD/tube and transfer to Eppendorf low binding tubes.

-Immobilize on the Eppendorf magnetic stand

-Discard the supernatant

-Resuspend in 1ml of LB3JD

-Incubate for 5 minutes at 42C in the thermomixer (shaking at 1000rpm)

-Immobilize on the Eppendorf magnetic stand.

-Discard the supernatant

-Resuspend in 1ml of LB3JD

-Incubate for another 5 minutes at 42C in the thermomixer (shaking at 1000rpm)

-Immobilize on the Eppendorf magnetic stand.

-Resuspend in 0.6 ml of LB3JD and pool the Telomere tubes together, the Scramble tubes together.

-Immobilize on the Eppendorf magnetic stand.

-Resuspend into 1 ml of Elution buffer

- Incubate for 60 minutes at room temperature in the thermomixer (shaking at 1000rpm)
- Set the temperature to 65C. When the temperature reaches 65C (~5 minutes), incubate for 10 minutes. (Alternatively, elution can be performed for an extra 2h at room temperature).
- Immobilize on the Eppendorf magnetic stand.
- Transfer the eluates to new Eppendorf tubes, keep these new tubes on the magnetic stand
- Check the OD260 of the eluates (should be 15-25 ng/μl). This does not indicate that chromatin is present in the eluate fractions but it is a good indication that the LNA has been eluted from the beads. Usually at the same concentration, the scramble LNA absorbs more than the telomere LNA.
- Transfer the 1ml eluates to new Eppendorf tubes and add 230 μl of 100% TCA.
- incubate on ice for 10 minutes
- spin down at 16000g for 15 minutes at room temperature
- remove ~1100 ml by pipeting
- bring to 1.5ml with -20C cold acetone
- vortex for 10 seconds
- spin down at 16000g for 10 minutes at room temperature
- remove the supernatant (now a small white pellet should be visible)
- bring to 1.5ml with -20C cold acetone
- vortex for 10 seconds
- spin down at 16000g for 10 minutes at room temperature
- remove the supernatant (now a pellet should be visible)
- air-dry the pellet
- resuspend into 50μl of crosslinking reversal solution.
- incubate at 99C for 25 minutes and load on a gel or store at -80C.

### 3. Reagents, buffer recipes and product references

#### 1X PBS

Standard Phosphate Buffered Saline solution supplemented with 1mM PMSF

#### Crosslinking solution

3% Formaldehyde into 1X PBS (Formaldehyde 37%, Methanol stabilized solution from Fisher, cat#BP531500).

#### PBS-Triton solution:

1X-PBS

0.5% Triton-X100.

#### Sucrose Buffer:

Jerome Dejjardin- Kingston lab

0.3 M Sucrose; 10 mM HEPES-NaOH pH 7.9; 1% Triton X-100; (3 mM CaCl<sub>2</sub>:optional if one wants to MNase chromatin in addition to sonication); 2 mM MgOAc (Magnesium Acetate)

Glycerol Buffer:

25% Glycerol; 10 mM HEPES-NaOH pH 7.9; 0.1 mM EDTA; 0.1 mM EGTA; 5 mM MgOAc (Magnesium Acetate)

LB3JD

- 10 mM HEPES-NaOH pH 7.9
- 100 mM NaCl
- 2 mM EDTA pH 8
- 1 mM EGTA pH 8
- 0.2% SDS
- 0.1% Sodium Sarkosyl

Make fresh, keep at room temperature and add PMSF to 1mM final concentration.

LB3JDLS

- 10 mM HEPES-NaOH pH 7.9
- 30 mM NaCl
- 2 mM EDTA pH 8
- 1 mM EGTA pH 8
- 0.2% SDS
- 0.1% Sodium Sarkosyl

Make fresh, keep at room temperature and add PMSF to 1mM final concentration.

Elution buffer

- 12.5mM Biotin (Invitrogen cat#B20656)
- 7.5 mM HEPES-NaOH pH 7.9
- 75 mM NaCl
- 1.5 mM EDTA pH 8
- 0.75 mM EGTA pH 8
- 0.15 % SDS
- 0.075 % Sodium Sarkosyl

Cross-linking Reversal solution:

- 250mM Tris pH 8.8
- 2% SDS
- 0.5M 2-mercaptoethanol

About the capture probes:

The probes are constituted of a mixture of LNA and DNA residues (25 nt long), that contain an extra-long spacer between the 5' of the oligo and the biotin analog desthiobiotin. The extra-long spacer is of critical importance, since a lot of chromatin immobilization strategies are greatly impaired by steric hindrance problems

Jerome Dejardin- Kingston lab

The telomere probe sequence is:

TtAgGgTtAgGgTtAgGgTtAgGgt

The scramble probe sequence is:

GaTgTgGaTgTggAtGtGgAtgTgg

Where CAPITALIZED letters are LNA residues and small letters are DNA residues.

These probes are made 'custom' by Fidelity Systems.

I have designed the probes so that they are minimally self hybridizing using the Exiqon website (<http://lnatools.com/>). Finally, I use desthiobiotin (also called dethiobiotin) instead of biotin, so that I could use gentle elution conditions.

The design is thus:

Telomere:

Desthiobiotin-108Carbons-5'TtAgGgTtAgGgTtAgGgTtAgGgt-3'

Scramble:

Desthiobiotin-108Carbons- 5'GaTgTgTgGaTgTggAtGtGgAtgTgg-3'

Probes with lower LNA content were also tested (6 and 3 LNA residues); their use resulted in very low/no protein recovery.

#### **4. General comments:**

The formaldehyde concentration and time of crosslinking have been optimized. Using milder crosslinking conditions such as the ones used for normal ChIP results in very low protein recovery.