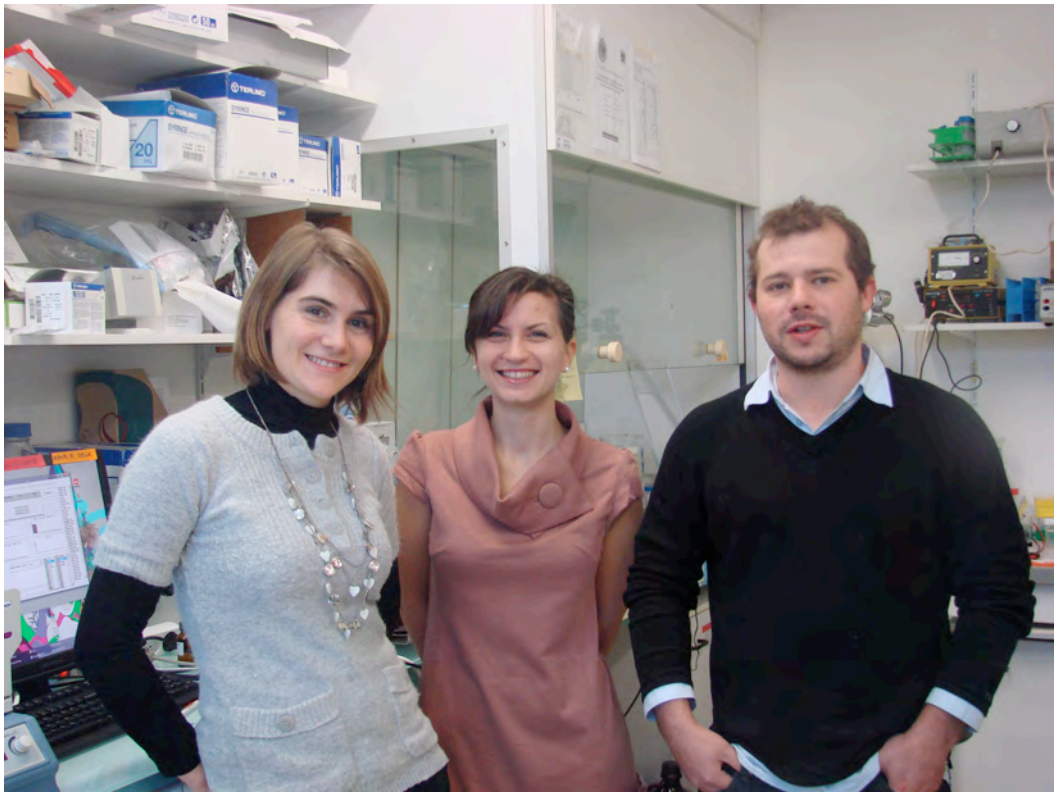
Proteomics of isolated chromatin segments (PICh) on human telomeres (Prot52)



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Introduction

Proteomics of **i**solated **ch**romatin segments (PICh) is a powerful method of repetitive chromatin isolation that allows identifying proteins associated to specific genomic loci.

Purification is based on nucleic acid probe hybridization to the DNA moieties present in formaldehyde-crosslinked chromatin. Hybridized chromatin is captured on magnetic beads, eluted and bound proteins can be identified by mass spectrometry. In order to increase the stability of the probe-chromatin interaction, **L**ocked **N**ucleid **A**cids (LNA) containing oligos are used. LNA probes have higher melting temperature than DNA probes of the same sequence. This facilitates strong interactions with DNA and stabilizes probe invasion. Between the LNA probe and the immobilization tag there is a long spacer that reduces steric hindrance effects. Desthiobiotin is used as the immobilization tag. Desthiobiotin has weaker affinity to streptavidin (Kd~10^{-12} M) which allows a competitive gentle elution from streptavidin beads with regular biotin (Kd~10^{-14} M).

The following protocol is optimized for 10^9 cells per pull down (which is approximately the number of adherent cells growing on 50 cell culture dishes of 150 mm diameter or 25 dishes of mouse stem cells cultured on gelatin).

Each experiment should consist of at least two pulldowns: one using a scrambled and the other a telomere specific probe. In both conditions, chromatin should be processed in the same way and split just before adding LNA probes.

Procedure

Preparation of the chromatin sample:

1. Crosslinking
   -for adherent cells:
   - Discard the medium and incubate for 30 minutes in crosslinking solution at room temperature
   - Wash twice in PBS-PMSF to remove formaldehyde by dilution (see: Note 1), dispose of washes containing formaldehyde properly.
   - Scrap cells with polyethylene cell lifter (Biologix #70-2180) and collect them in scrapping solution. 3 ml of scrapping solution is sufficient for one 150mm plate. Transfer scrapped cells into a 50 ml tube (1 tube for about 10-11 plates).
   - Spin down the crosslinked cells at 3200g for 10 minutes
   - Pool the pellets in one 50 ml tube (see: Note 2)
   - Wash the pellet 2-4 times in PBS-PMSF (spin down at 3200g for 10 mins)

   -For suspension cells:
   - Spin down cells at for 5 minutes at 400g
   - Discard the growing medium

- Resuspend pellet in crosslinking solution and incubate for 30 minutes at room temperature on a nutator (volume of crosslinking solution must not be below 50 times the volume of pelleted cells: for a pellet of 4 ml cells, use at least 200ml of crosslinking solution).
- Spin down cells for 10 minutes at 3200g.
- Discard the crosslinking solution properly.
- Wash the pellet twice in PBS-PMSF (see: Note 1)

The pellet can be stored at -80°C up to one month (see: Note 3)

2. Resuspend the pellet by vortexing in 10 ml of sucrose solution and bring volume to 50 ml (see: Note 4)
3. Spin down at 3200g for 10 minutes at 4°C
4. Discard the supernatant, resuspend the pellet in 10ml of sucrose solution and bring the volume to 20 ml with sucrose solution
5. Transfer this mixture to a 40 ml dounce homogenizer
6. Dounce on ice approximately 20 times with a tight pestle
7. Transfer the dounced mixture back to a new 50 ml tube, wash the homogenizer 2 times with 15 ml of sucrose solution to minimize loosing material and pool the washes with the dounced mixture in the 50ml tube.
8. Spin down at 3200g for 10 minutes at 4°C
9. Discard the supernatant
10. Resuspend the pellet by vortexing in glycerol buffer and bring up volume to 50 ml
11. Spin down at 3200g for 10 minutes at 4°C
12. Discard the supernatant

Steps 13 and 14 are optional if one wants to store the chromatin sample

13. Resuspend the pellet into the same pelleted volume of glycerol buffer (i.e. 5 ml pellet in 5 ml of glycerol buffer)
14. Snap-freeze into liquid nitrogen and store in -80°C or proceed to the next step (see: Note 3)
15. Resuspend the pellet in the same pelleted volume of triton solution
16. Add 15µl of concentrated RNaseA (100mg/ml) per 1 ml of chromatin (Qiagen #1007885)
17. Incubate for 60 minutes at room temperature on a nutator than overnight at 4°C. Alternatively the RNaseA treatment can be performed 2 hours at room temperature (see: Note 5)
18. Transfer the mixture into a 50 ml falcon tube and bring the volume to 50 ml with PBS-PMSF
19. Spin down at 3200g for 10 min at 4°C and discard the supernatant
20. Repeat 3 -6 times washing in PBS-PMSF to dilute RNaseA away. Number of washes depends on quality of centrifuging. Number of washes should be reduced, if chromatin fails to be pelleted (an effect growing with the number of washes),

21. Resuspend the pellet by vortexing in 50 ml of freshly prepared high salt lysis buffer (see: Note 6)
22. Spin down at 3200g for 10' and discard supernatant
23. Sonication:
    -Dilute the pellet twice in high salt lysis buffer. Resuspend well by pipetting with 1 ml micropipette and split into 3-3.5 ml aliquots in 15 ml falcon tubes (use all the time a same tip to minimize chromatin sample loss). DO NOT VORTEX, as vortexing leads to excessive foaming of the sample which inhibits efficient sonication.
    -Sonicate samples on ice

    Sonication parameters are (Misonix S-4000 sonicator with a high power probe #419):

    -power setting 7 (Amplitude 70%)
    -15 seconds constant pulse
    -45 seconds pause
    -7 minutes total process time

    This program corresponds an energy of 8 500 Joules

    -After 14 minutes (in the middle of the program) mix the chromatin with the sonicator tip to ensure all the chromatin is evenly sonicated.
24. Soluble chromatin should be handled at room temperature to prevent SDS precipitation
25. Aliquot sonicated chromatin into 1.5 ml microcentrifuge tubes and spin it down at 16000g for 15 minutes at room temperature.
26. Pool the supernatants into 15 ml tube
27. Incubate at 58°C for 5 minutes (see: Note 7)
28. Allow the sample to cool down at room temperature.
29. Equilibrate 0.5 ml of High Capacity Streptavidin Agarose Resin slurry into 10 ml of high salt lysis buffer for 5 minutes at room temperature
30. Spin down at 3200g for 2 minutes and discard the supernatant
31. Bring the volume to 10 ml with high salt lysis buffer
32. Spin down at 3200g for 2 minutes and take off the supernatant
33. Add streptavidin beads to the chromatin sample
34. Incubate 2 hours at room temperature on a nutator (incubation can be extended to overnight) (see: Note 8)
35. Spin down at 3200g for 10 minutes at room temperature
36. Save the supernatant, this is the precleared chromatin
37. Prepare GE Sephacryl S-400 HR column. Use the same volume of resin as that of the chromatin sample (i.e. for 10 ml of chromatin use 10 ml of resin which is 20 ml of GE Sephacryl mixture). Put appropriate volume of well resuspended sephacryl mixture into PIERCE empty 10 ml centrifuge column and place the column in an empty 50 ml tube (see: Note 9)
38. Spin down at 800g for 2 minutes

39. Add chromatin to the dried column, place it in a clean 50 ml tube and centrifuge for 2 minutes
40. Aliquot chromatin into 1.5 ml microcentrifuge tubes and spin down at 16000g for 15 minutes at room temperature
41. Pool the aliquots together
42. Check the OD of precleared chromatin using low salt lysis buffer as a blank solution. It should have the following characteristics:
    -OD260: 2-2.5 mg/ml
    -OD260/OD280: 1.3-1.45
    Higher values of the 260/280 ratio would mean that RNaseA treatment was not effective and that chromatin is contaminated with RNA-protein compexes. This will strongly affect the outcome of the PICh procedure as this usually results in the non specific capture of those RNP complexes. In such a case both scrambled and telomere pull downs contain many hnRNPs ribosomal proteins.

43. Add 20% SDS to 0.02% (1/100 final vol.)

Hybrydization and chromatin capture:

44. Add 50µl of LNA probe (100 µM stock solution). One tube should contain telomere specific LNA probe, the other one the LNA probe containing scramble sequence)
45. Aliquot into PCR tubes. Distribute 150µl-170µl of chromatin-LNA mixture per tube.
46. Hybridize in a thermocycler using the following program (see: Note 10):

    -25°C for 3 minutes
    -71°C for 7 minutes
    -37°C for 3 hours
    -25°C final temperature

47. Pool back samples from PCR tubes and spin down at 16000g for 15 minutes at room temperature in order to pellet any precipitate that could have formed during the hybridization step.
48. Pool the supernatants into 15 ml tubes
49. Prepare 1.5 ml of MyONE C1 magnetic streptavidin beads (750µl of beads for 50µl of used LNA probe) and bring up the volume to 10ml with low salt lysis buffer
50. Immobilize the beads on the magnetic stand and discard the supernatant
51. Add 10 ml of low salt lysis buffer and mix gently
52. Split the beads solution into two 15 ml tubes, 5 ml of beads solution in each tube
53. Immobilize the beads on the magnetic stand and discard the supernatant
54. Keeping beads on the stand add Milli-Q water to the beads (same volume as chromatin)
55. Still keeping beads on the stand add chromatin to previously added MILIQ water. **The order of adding water and chromatin to the beads is crucial to prevent chromatin precipitation on the beads.**

56. Resuspend the beads very gently and slowly. Nutate at room temperature overnight.
57. Bring the volume to 10 ml with high salt lysis buffer
58. Immobilize on magnetic stand and collect the supernatant. This is the unbound fraction

Washes:

59. Wash the beads six times with high salt lysis buffer. Resuspend them gently between washes (avoid vortexing!) Do the additional wash with low salt lysis buffer (see: Note 7)
60. Resuspend the beads into 1.2 ml high salt lysis buffer/tube and transfer to low binding microtubes
61. Immobilize the beads on the magnetic stand
62. Discard the supernatant
63. Resuspend in 1 ml of high salt lysis buffer
64. Incubate for 5 minutes at 42°C in the thermomixer (shaking at 1000 rpm)
65. Immobilize the beads on the magnetic stand
66. Discard the supernatant and resuspend the beads in 1 ml of high salt lysis buffer
67. Incubate for 5 minutes at 42°C in the thermomixer (shaking at 1000 rpm)
68. Immobilize the beads on the magnetic stand

Elution and proteins precipitation:

69. Resuspend the beads in 1 ml of elution buffer
70. Incubate for 60 minutes at room temperature in the thermomixer shaking at 1000 rpm and additional 10 minutes at 65°C without shaking.
    Alternatively elution can be performed for 2 hours at room temperature shaking at 1000 rpm
71. Immobilize the beads on the magnetic stand
72. Transfer the eluates to new microcentrifuge tubes and keep these tubes on magnetic stand
73. Transfer eluates again to fresh microcentrifuge tubes. **This step is extremely important to eliminate all the beads from the eluates.**
74. Check the OD260 of the eluates (should be 15-25 ng/µl) (see: Note 11)
75. Add 230 µl of 100% TCA (not more than 15-20% final)
76. Incubate on ice for 10 minutes
77. Spin down at 16000g for 15 minutes at 4°C
78. Remove 1 ml by pipetting (leave about 200ml)
79. Bring volume to 1.5 ml with -20°C Acetone 100%
80. Vortex for 10 seconds
81. Spin down at 16000g for 10 minutes at 4°C
82. Remove the supernatant (now a small white pellet should be visible)
83. Bring the volume to 1.5 ml with -20°C cold Acetone
84. Vortex for 10 seconds
85. Spin down at 1600g for 10 minutes at 4°C

86. Remove the supernatant
87. Air-dry the pellet
88. Resuspend into 50µl of crosslinking reversal solution
89. Incubate for 12 minutes at 99°C, spin down to retrieve condensation from the cap back to the bottom of the tube and incubate another 13 min at 99°C
90. Load on a 12% Bis-tris gel or store at -80°C
91. Gel staining:

    Stain the gel with SilverQuest Staining Kit (Invitrogen#LC6070). If 1/6 of eluted material was used after 3 minutes of developing there should be visible proteins bands. If the bands do not appear after 5 minutes it means there is not enough proteins for MS analysis (Fig.1).

Authors Notes:

Note 1:

Do not quench formaldehyde with glycine solutions as usually performed during classical chromatin immunoprecipitation experiments. It may result in non specific crosslinking of glycine to proteins and prevent peptide mass attribution during the mass spec analysis. Instead, dilute and wash out unreacted formaldehyde with PBS washes.

Note 2:

The cell pellet sufficient for the two pulldowns (scramble and sequence- specific), spinned at 3200g should be 4-5 ml volume.

Note 3:

The crosslinked material should be stored at -80^{o}C to minimize crosslinking reversal of proteins from chromatin. It is not recommended to use material stored longer than one month as we observed significant decrosslinking and much lower efficiency of PICh. The best results are obtained when fresh material is used without any storage time.

Note 4:

Always resuspend a pellet by vortexing till the step before sonication. Using pipette results in loosing plenty of chromatin. Pay attention to minimize material loss at each step, as half of the sample can easily be lost during manipulations.

Note 5:

This RNaseA step is to optimize the subsequent LNA probe/chromatin hybridization step and avoid contamination with non chromatin RNA-protein complexes.

Note 6:

High salt lysis buffer and low salt lysis buffer should not be older than one week.

Note 7:
This step helps unmasking biotin from endogenously biotynylated proteins, which have to be removed prior hybridization.

Note 8:
Preclearing is necessary to remove most of endogenously biotynylated proteins. Otherwise they will compete with desthiobitynylated probe for binding to streptavidin beads.
If one performed overnight preclearing step and then observed weak signal on silver-stained gel it is recommended to reduce preclearing to 2 hours only.

Note 9:
This step is very important for the quality of the purification. The ultra fast gel filtration reduces the salt concentration in sample by about 2/3rd leaving roughly 30 mM NaCl. The less salt there is the more specific the hybridization and the less non-specific precipitation will occur on the beads later-on.

Note 10:
The hybridization program was optimized so that LNA probe can invade DNA sequence into chromatin without observable protein de-crosslinking.
Denaturation temperatures, higher than 75^{o}C should be avoided as they result in significant crosslinking reversal and proteins loss from the target chromatin. At temperatures lower than 70^{o}C hybridization is much less efficient, and at temperatures lower than 65^{o}C no proteins could be detected in purified material.

Note 11:
This value is a good indication that the LNA probe has been eluted from the beads. Usually at the same concentration, the scramble LNA probe absorbs more than the telomere LNA probe in the eluates. The reason for this discrepancy is unknown.

Materials and Reagents

Crosslinking solution:
-3.6% formaldehyde-1xPBS

1xPBS:
-Standard Phosphate buffered Saline
-1 mM PMSF

Scrapping solution:
-1xPBS
-0.05% Tween-20.

Triton solution:
-0.5% Triton-X100
-1xPBS

Sucrose solution:
-0.3 M Sucrose
-10 mM HEPES-NaOH pH 7.9
-1% Triton-X100
-2 mM MgOAc (Magnesium Acetate)

Glycerol buffer:
-25% glycerol
-10mM HEPES-NaOH pH 7.9
-0.1 mM EDTA
-5 mM MgOAc (magnesium acetate)

High salt lysis buffer:
-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 1 mM concentration

Low salt lysis buffer:
-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 1 mM concentration

Elution buffer:
-25% biotin (12.5mM final from a 50mM stock solution)
-75% high salt lysis buffer

Crosslinking reversal solution:
-250 mM Tris pH 8.8
-2% SDS
-0.5 M 2-Mercaptoethanol

Materials:

36% formaldehyde stabilized with 10% methanol (AnalR NORMAPUR cat#20909.290)
DPBS (Lonza cat#BE17-512F)
PMSF (phenylmethylsulfonyl fluoride) (Sigma cat#P7626)
Tween (Sigma cat#P7949)
Triton-X 100 (Sigma cat#T8787)
Sucrose (AnalR NORMAPUR cat#27480.294)
Magnesium acetate (MERCK cat#A712619 715)
Glycerol (Sigma cat#G5516)
EDTA (ethylenediaminetetraacetic acid) (Sigma cat#E5134)
EGTA (ethylene glycol tetraacetic acid) (Sigma cat#E3889)
N-lauroylsarcosine sodium salt (sodium sarcosyl) (Sigma cat#L9150)
Sephacryl S-400 High Resolution (GE Healthcare cat#17-0609-01)
RNaseA 100 mg/ml (Qiagen cat#1007885)
High Capacity Streptavidin Agarose Resin (Thermo Scientific cat#20361)
SilverQuest Staining Kit (Invitrogen cat#LC6070)
Pierce Centrifuge Columns 10ml (Thermo Scientific cat#89898)
Dynabeads MyONE Streptavidin C1 (Invitrogen cat#650.02)
Biotin (Invitrogen cat#B20656)

About the capture probes:

The probes are constituted of a mixture of LNA and DNA residues (25nt 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 the efficiency of chromatin immobilization on beads is influenced by steric hindrance.

The telomere probe sequence is:

TtAgGgTtAgGgTtAgGgTtAgGgt

The scramble probe sequence is:

GaTgTgGaTgTggAtGtGgAtgTgg

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

The design is thus:

Telomere:

Desthiobiotin-Spacer-5' TtAgGgTtAgGgTtAgGgTtAgGgt-3'

Scramble:

Desthiobiotin-Spacer-5' GaTgTgGaTgTggAtGtGgAtgTgg-3'

Desthiobiotin is used instead of biotin to provide gentle elution conditions. Probes with lower LNA content were also tested (6 and 3 LNA residues); their use resulted in very low/no protein recovery.

Figure:

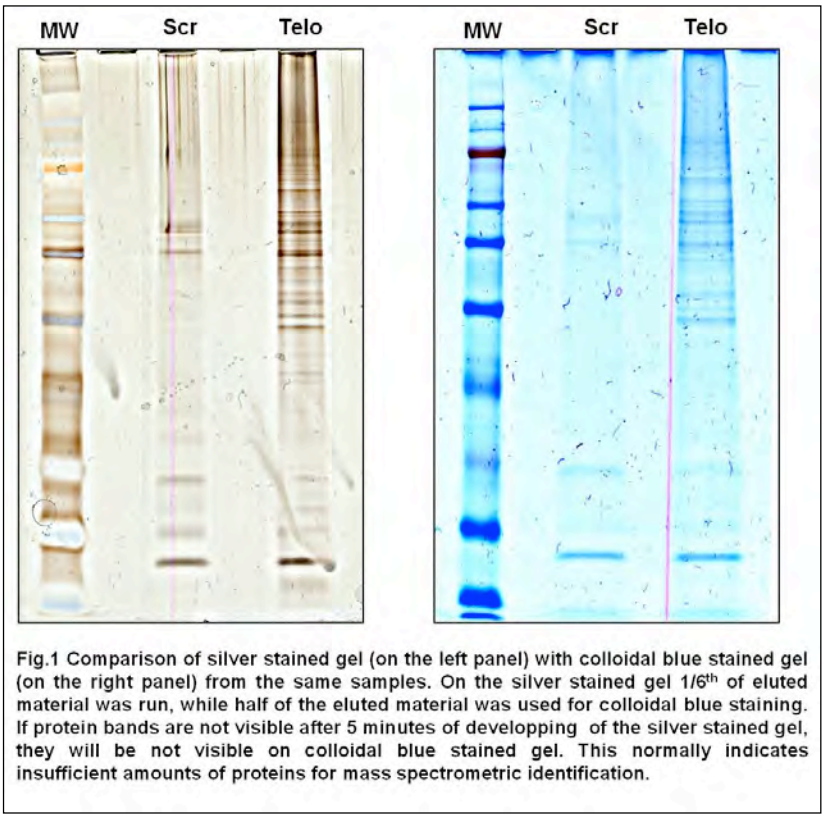

Fig.1 Comparison of silver stained gel (on the left panel) with colloidal blue stained gel (on the right panel) from the same samples. On the silver stained gel 1/6th of eluted material was run, while half of the eluted material was used for colloidal blue staining. If protein bands are not visible after 5 minutes of developping of the silver stained gel, they will be not visible on colloidal blue stained gel. This normally indicates insufficient amounts of proteins for mass spectrometric identification.

Reviewer comments:

Review by: Jose Antao

Department of Molecular Biology, Simches Research Center, Boston

The current protocol is optimized for the purification of chromatin from mammalian telomeres. Several characteristics of these genomic regions make them theoretically more accessible for

purification than less repetitive, less abundant or less accessible loci. This protocol should be used as a starting point for the purification of chromatin from any locus, but optimization steps should be considered, such as the pre-enrichment of the target chromatin, when it is less abundant than telomeres, or the use of multiple specific probes, when it is less repetitive.

The most critical points in the protocol are the thorough crosslinking of chromatin, the adequate pre-clearing with both RNase A and streptavidin beads (further pre-clearing/enrichment strategies may include dialysis or density gradients), and the maintenance of chromatin solubility throughout. The latter point will be better achieved by minimizing the storage steps and by carefully adhering to the centrifugation steps in the protocol, namely the one before adding magnetic beads. This protocol starts with a very large amount of chromatin and ends with very small quantities. Minimal non-specific binding to the probes or the beads, either by mis-hybridization or precipitation, will lead to a substantial level of noise that can compromise the experiment.