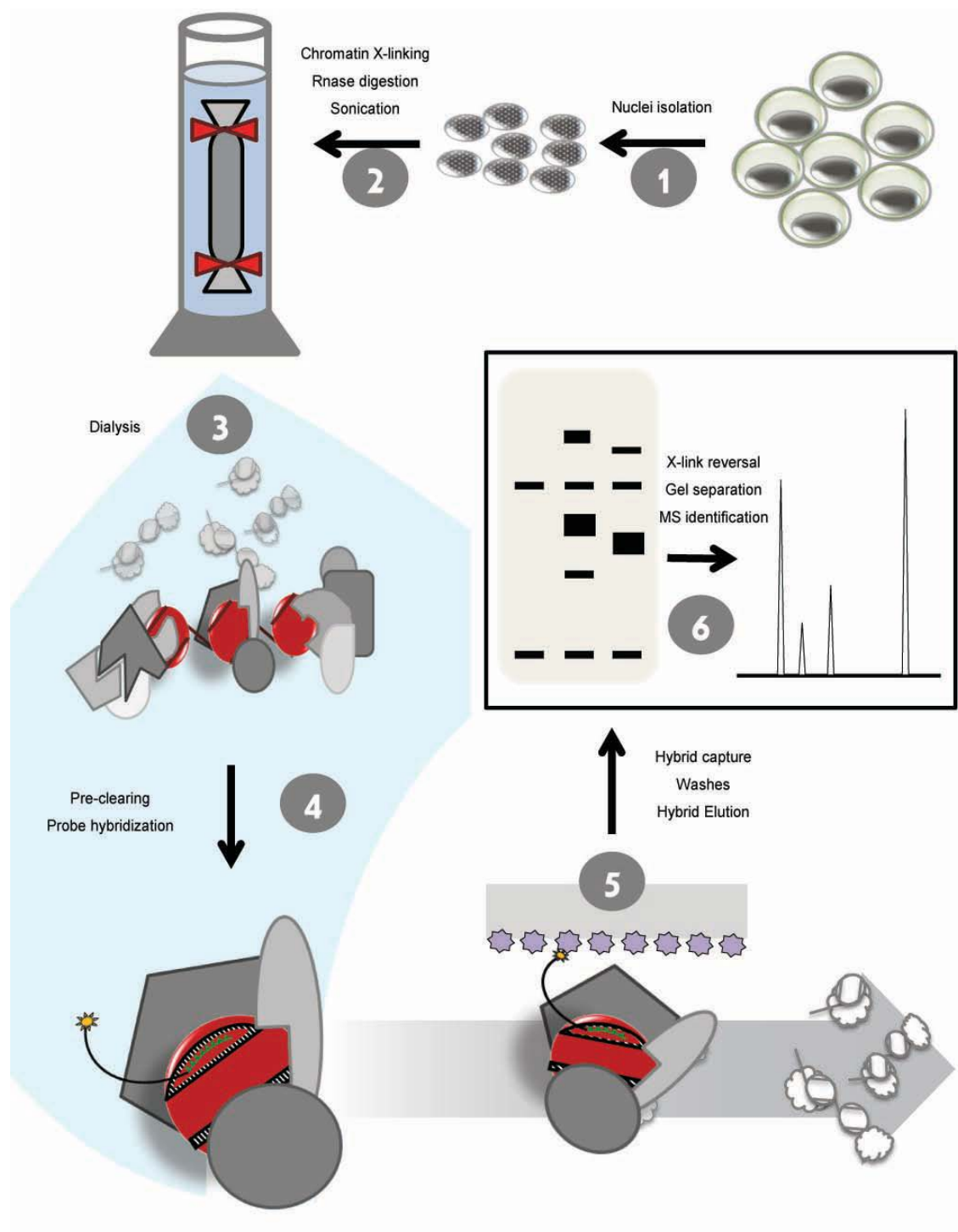


## Proteomics of Isolated Chromatin Segments

### Purification of chromatin from *Drosophila* TAS sequences

José Antão, November 2011



## **Protocol**

*Drosophila* S3 and Kc cells were grown in suspension in CCM3 medium (Hyclone), supplemented with Pen/Strep (Gibco), at 27°C, 100rpm, in 2.8L culture flasks, up to a density of  $1\text{-}2 \times 10^7/\text{ml}$ . For PICH experiments, typically  $10^{11}$  cells were spun down at 4000rpm, room temperature (RT), in a Beckman J6 centrifuge, washed with 400ml 1X PBS (137mM NaCl; 2.7mM KCl; 10mM  $\text{Na}_2\text{HPO}_4$ ; 2mM  $\text{KH}_2\text{PO}_4$ ; pH 7.4), spun down 4000g, RT; washed once with 5pcv Hypotonic Buffer (10mM HEPES, pH 7.9; 1.5mM  $\text{MgCl}_2$ ; 10mM KCl); resuspended with 3pcv Hypotonic Buffer and swelled 10 minutes on ice in two 100ml dounce homogenizers (Kontes); added 37% formaldehyde (Fisher) to a final concentration of 3% and immediately homogenized with 15 strokes of a tight pestle; spun down 10 minutes, RT, 5000g; disposed of supernatant and resuspended pellets with a total of 400ml crosslinking solution (3% formaldehyde; 1X PBS); incubated 30 minutes at RT, in a shaking platform; pellets were washed 3 times with PBS, then once with Sucrose Buffer (0.3M Sucrose; 10mM HEPES, pH 7.9; 1% Triton X-100; 2mM  $\text{MgOAc}$ ), resuspended with 3pvn Sucrose Buffer and homogenized with 20 strokes of tight pestle in an 100ml homogenizer; pellets were kept after spinning chromatin down at 5000g, 10 minutes.

Chromatin was washed once with RNase buffer (0.5% Triton X-100; 1X PBS) and resuspended with 5pvn RNase buffer; added 0.01pvn RNase A (Sigma) and incubated 5 hours, RT, on a rotating wheel; kept overnight at 4°C. Washed pellet twice with 6pvn PBS, once with 6pvn LB3JD buffer (10mM HEPES, pH7.9; 0.1M NaCl; 2mM EDTA, pH 8.0; 1mM EGTA, pH 8.0; 0.2% SDS; 0.1% Na-Laurosylsarkosine), and resuspended with 3pvn LB3JD; split into 5ml aliquots in 15ml polystyrene tubes and sonicated each on ice, for a total processing time of 7 minutes, 15" ON pulses, 45" OFF pulses, in a Misonix sonicator, with the power level set to 7.0 (39-42W output); pooled the aliquots and spun down 25,000g, 1hour, RT; dialyzed chromatin against 30 volumes Buffer Y (5% Glycerol; 20mM HEPES, pH7.9; 50mM NaCl; 0.05% SDS; 0.05% Na-Laurosylsarkosine; 0.02% Triton X-100; 1mM EDTA, pH 8.0; 0.5mM EGTA, pH8.0), through a CE-MWCO 1,000,000 dialysis membrane (Spectra/Por), for 4 hours.

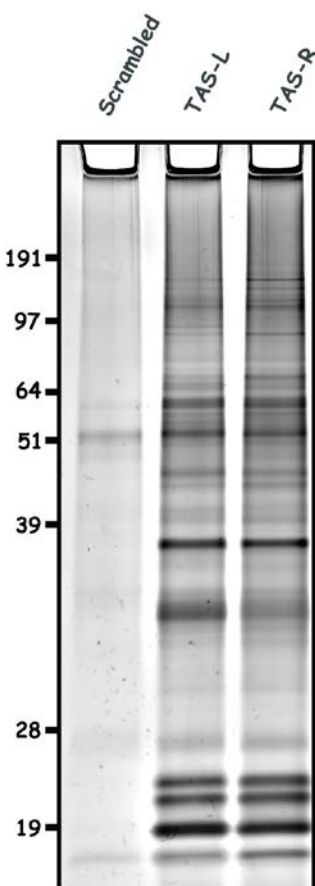
For pre-clearing biotinylated molecules from the mixtures, chromatin was incubated 5 minutes in a water bath at 60°C, in 50ml falcon tubes, mixing regularly to transfer heat uniformly; removed from water bath and added 1:100 (v:v) Ultralink Plus Streptavidin beads slurry (Thermo Scientific); incubated on rotating wheel, RT, 2 hours and collected flow-through from Econo-pac column (Bio-Rad).

For capture probe hybridization and purification, a 500-fold molar excess (to target copies) capture probe was added to 10mg chromatin (as determined by  $A_{260}$ ), in the case of TAS repeats, ~3nmol capture probe/10mg chromatin; the capture probe hybridization was carried out in 15ml polystyrene tubes: 6' at 70°C; 60' at 37°C; 2.5' at 60°C; 60' at 37°C; 2.5' at 60°C; 120' at 37°C; the mixture was transferred into 1.5ml tubes and centrifuged 15', maximum speed, RT; supernatant was transferred into a new 15ml Falcon tube, added NaCl to 100mM and 300μl MyOne Magnetic Streptavidin beads (Invitrogen) in LB3JD buffer; incubated on rotating wheel, RT, 2 hours; beads were immobilized on magnetic stand, washed 7 times by gently resuspending with 8ml LB3JD buffer, transferred into a low-binding 1.5ml tube, washed

twice for 5' with 1ml LB3JD buffer at 42°C, 1000rpm in a Thermomixer (Eppendorf), then 1hr, RT, 1000rpm; elution was done for each sample with 1ml Elution Buffer (12.5mM Biotin; 7.5mM HEPES, pH 7.9; 75mM NaCl; 1.5mM EDTA, pH 8.0; 0.75mM EGTA, pH 8.0; 0.15% SDS; 0.075% Na-Lauroylsarkosine) at RT, 1000rpm, for 2hr; eluates were collected into a clean tube, centrifuged 1 minute to remove any magnetic beads that might have been carried over, and supernatants transferred to new tubes.

Proteins were precipitated by adding 100% cold TCA to a final concentration of 20%. Samples were incubated 10' on ice, spun down 15', RT, and supernatant carefully removed; pellet was washed twice with -20°C acetone, by vortexing a few seconds and centrifuging between washes; pellets were briefly air-dried and resuspended with 40µl crosslink reversal buffer (0.25M Tris, pH 8.8; 2% SDS; 0.5M β-Mercaptoethanol); crosslinks were reversed by incubating samples at 99°C, 25'.

Samples were separated using SDS-PAGE gel electrophoresis (or stored at -20°C); gels were stained with the SilverQuest kit (Invitrogen) or with Colloidal Blue (Invitrogen), according to the manufacturer's instructions, and relevant regions of the gel were cut out of the TAS-specific and corresponding regions of the negative control lanes and sent for analysis at the Taplin Mass Spectrometry Facility at the Harvard Medical School.



Our decision to isolate large sections of the gel for mass spectrometry analysis was done to the detriment of deeper coverage, but with the advantage of providing an overview of the protein composition at reduced cost. The clean appearance of the negative control lane relative to the sample lanes indicates the extent to which we enriched for proteins with the specific capture probe. The number of peptides detected by mass spectrometry does not reflect the total amount of protein isolated by these two probes, given the mechanics of the LC/MS/MS tandem mass spectrometry apparatus, which discards a fraction of the injected peptides above its resolution power. For example, compare the number of bands detected by silver stain using specific vs. non-specific probes to the total number of proteins identified in each sample the protein enrichment with the specific capture probes. This technical consideration means that more dilute samples (such as the negative control) will have a deeper coverage than the more concentrated samples (such as the specific purifications), thus leading to a reduction in the difference between the number of proteins identified in these samples. Increased coverage from the material enriched with a specific probe might be obtained by performing mass spectrometry analysis using greater numbers of smaller slices from the gel.

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