

Video Article

Detection of Post-translational Modifications on Native Intact Nucleosomes by ELISA

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Abstract

The genome of eukaryotes exists as chromatin which contains both DNA and proteins. The fundamental unit of chromatin is the nucleosome, which contains 146 base pairs of DNA associated with two each of histones H2A, H2B, H3, and H4¹. The N-terminal tails of histones are rich in lysine and arginine and are modified post-transcriptionally by acetylation, methylation, and other post-translational modifications (PTMs). The PTM configuration of nucleosomes can affect the transcriptional activity of associated DNA, thus providing a mode of gene regulation that is epigenetic in nature^{2,3}. We developed a method called nucleosome ELISA (NU-ELISA) to quantitatively determine global PTM signatures of nucleosomes extracted from cells. NU-ELISA is more sensitive and quantitative than western blotting, and is useful to interrogate the epiproteomic state of specific cell types. This video journal article shows detailed procedures to perform NU-ELISA analysis.

Protocol

1. Mammalian Cell Culture

NU-ELISA can be performed on any mammalian cell type that can be grown in culture. We prefer to prepare moderate to large batches of cells so that nucleosomes can be isolated in sufficient quantity to prepare several identically loaded ELISA plates, thus allowing assays with several different antibodies (Abs). The following culture scales provide ample material:

For mouse embryonic stem cells, grow one or two 15 cm plates of cells without feeder cells. For fibroblasts, grow 5 to 10 15 cm plates to confluence.

2. Isolation of Nuclei

Note: All the steps are on ice with pre-chilled buffers, except as indicated.

1. Trypsinize cells with 3 ml trypsin per plate, combine, and add 20 ml of ice-cold PBS/butyrate. Centrifuge at 1000 rpm for 5 min.
2. Resuspend the cells in 10 ml PBS/butyrate and centrifuge at 1000 rpm for 5 min.
3. Resuspend in 4 ml lysis buffer with protease inhibitors (Sigma-Aldrich P-8340). Dounce homogenize 20 strokes with type B pestle on ice.
4. Centrifuge at 2000g for 10 min at 4°C. (The supernatant contains cytoplasm, which is not needed for this protocol, but it can be saved for other uses if desired).
5. Resuspend the pellet in 2 ml ice cold wash buffer C (with protease inhibitors).
6. Layer the resuspended material over 5 ml 30% sucrose cushion, then centrifuge at 2400g for 5 min in a swinging bucket rotor. Nuclei will migrate through cushion, and debris remains at the interface.
7. Remove all liquid volume carefully and resuspend the nuclei in 250 µl ice cold wash buffer C + protease inhibitors.

3. Isolation of Nucleosomes by *in situ* Micrococcal Nuclease (MNase) Digestion

We use a procedure in which chromatin is digested *in situ* within nuclei by infusing them with MNase, followed by a hypotonic treatment to drive free intact nucleosomes into the supernatant.

1. Add 3 µl 0.1 M CaCl₂ to the sample. Put in 37°C heat block. Allow to assume 37°C temperature.
2. Add 2 units MNase (Micrococcal Nuclease, Sigma-Aldrich, dissolved at 2 units/10µl in MNase buffer), then incubate for at 37°C for 12 min, with frequent mixing using a pipet tip.
3. Add 6 µl of 0.5 M Na-EDTA, pH 8.0 to stop the reaction. Put on ice.
4. Centrifuge at 2000g for 4 min, discard supernatant. Resuspend the pellet in 300 µl 0.2 mM Na-EDTA. Store on ice for 1 h with occasional gentle pipeting. (These hypotonic conditions liberate free nucleosomes from nuclei).
5. Centrifuge at 3000 g for 4 min at 4°C. Save the supernatant, which contains free nucleosomes, on ice.
6. Resuspend the pellet again with 300 µl 0.2 mM Na-EDTA. Store on ice for 1 h with occasional gentle pipeting.
7. Centrifuge at 3000g for 4min at 4°C. Retain the supernatant and combine with the first supernatant from step 5. The resulting mononucleosome preparations can be aliquoted and stored at -80°C.

Note: The amount of chromatin can be crudely quantitated by measuring absorbance at 260 nm of a 10 µl sample added to 990 µl of water. A₂₆₀ =10 (after adjusting for the 1/100 dilution) corresponds to about 1mg/ml of chromatin. Also, during the above procedure, small samples may be

retained and later analyzed for their DNA content to monitor the quality and extent of MNase digestions, which should predominantly contain DNA of 146 bp in length. Laddering is indicative of incomplete MNase digestion.

Note: Fig.1 contains a diagrammed summary of nuclear isolation and MNase digestions steps.

4. Nucleosome-ELISA (NU-ELISA)

We detect PTMs on fractions containing native intact nucleosomes that have been immobilized on 96 well ELISA microtiter plates. For each sample, we make a series of 2-fold dilutions, and these are coated onto wells in triplicate.

1. Coat MaxiSorp plates overnight at 4°C with 50 µl/well of nucleosomes diluted in coating buffer. Suggested serial twofold dilutions of nucleosomes are prepared from top row to the bottom row by adding 0.1 µg, 0.05 µg, 0.025 µg, 0.0125 µg, 0.00625 µg, 0.00313 µg, 0.00156 µg, with coating buffer only (0 µg chromatin) in the bottom row. (Note: plate covers are needed for this step.)
2. In the morning, wash the plates 4 times with 200 µl/well PBS/0.5% Tween-20 at room temperature (RT) for a combined total of 10 min with a plate washer.
3. Block 1 hr at RT with 100 µl/well PBS/0.05% Tween-20/5% BSA.
4. Remove the blocking buffer by shaking the inverted plate briskly over a sink. Cover and store the plates at -20°C, or go directly to the next step.
5. Add 1° Abs in a volume of 50 µl/well diluted 1:1000 (or as needed) in PBS/0.05% Tween-20/5% BSA, incubate at RT for 1 hour on a rotator.
6. Wash plates 4 times with 200 µl/well PBS/0.5% Tween-20 at RT and for a combined total of 10 min in a plate washer.
7. Add horse radish peroxidase (HRP)-conjugated 2° Abs, diluted 1:5000 (or as needed) in PBS/0.05% Tween-20/5% BSA at RT for an hour on a rotator.
8. Wash plates 4 times with 200 µl/well PBS/0.5% Tween-20. Each wash is at RT and for a total of 10 minutes with a plate washer.
9. Develop the plates by adding 50 µl of TMB substrate to each well for 10 min at RT. Stop the reaction by adding 50 µl/well of 2N H₂SO₄ and read the plates 450 nm. (Tip: Centrifuge at 1500 rpm for 2 min using a plate rotor to dissipate any air bubbles in the wells prior to reading absorbances).
10. Export the readings to spreadsheet files for quantitative and statistical analyses.

Reagents

PBS/butyrate

135 mM NaCl
2.5 mM KCl
8 mM Na₂HPO₄
1.5 mM KH₂PO₄
10 mM Na-butyrate

Lysis Buffer

250 mM sucrose
10 mM Tris-HCl, pH 7.4
10 mM Na-butyrate
4 mM MgCl₂
0.1% Triton X-100

Wash buffer C

250 mM sucrose
10 mM Tris-HCl, pH 7.4
10 mM Na-butyrate
4 mM MgCl₂

Sucrose Cushion

30% (w/v) sucrose in wash buffer C

Micrococcal Nuclease Buffer

5 mM NaPO₄ Buffer, pH 7.0
0.025 mM CaCl₂

Coating buffer

80ml Solution A + 170ml Solution B + 250ml dH₂O
Solution A: 0.2 M Na₂CO₃
Solution B: 0.2 M NaHCO₃

5. Representative Results

Using the NU-ELISA method, a series of several identical plates are prepared which can be loaded with chromatin prepared in the form of mononucleosomes. We typically prepare series of identically-loaded plates so that each can be probed with different anti-PTM specific antibodies (Abs). We also always prepare a plate that can be probed with an Ab that detects histones without regard to their modification state total

chromatin loading control. This control is essential in order to later quantitatively compare PTM content of nucleosomes prepared from different samples. To quantitate the levels of PTMs within a given chromatin sample we correct the raw absorbance values using this approach: First, we subtract any background signal using values obtained from control wells containing no chromatin (background is usually negligible). We then standardize PTM-specific signals to NU-ELISA results obtained from an identically loaded plate that was assayed with an Ab that detects nucleosomes independent of their modification state. (We have used polyclonal Abs specific for histones H2A or H2B for this purpose). We then determine means and variances for each dilution of chromatin, and use data obtained from the linear portion of the ELISA assay. Detailed examples of NU-ELISA mathematical and statistical analyses have been reported previously⁴

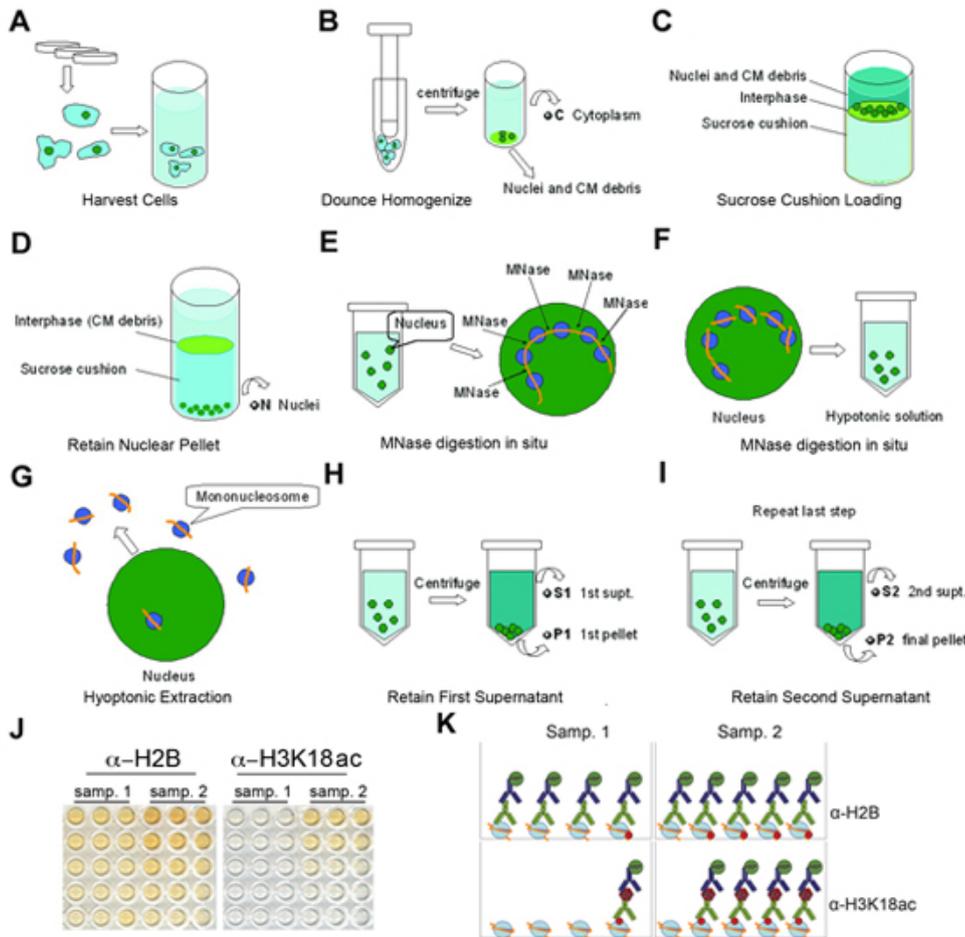


Figure 1. Schematic diagram of NU-ELISA steps. **A.** Mammalian Cells are harvested; **B.** Nuclei are separated from cells by Dounce homogenization, **C.** Disrupted cells are loaded on top of a 30% w/v sucrose cushion, **D.** After centrifugation, nuclei are deposited in the pellet; **E,** **F.** Chromatin are digested *in situ* to predominantly mononucleosomes by MNase; **G,** **H,** **I.** Soluble mononucleosomes are extracted by hypotonic treatment and repeated centrifugation of residual nuclear material. **J.** Mononucleosomes from different samples (labeled samp. 1 and 2 in this case) are coated on microtiter wells in series of identically-loaded plates and interrogated with PTM-specific Abs and PTM-independent Abs to determine total chromatin loading. **K.** Depiction of differential signal detection levels in two samples that differ in their PTM content, yet have similar overall chromatin content, as judged by anti-H2B immunoreactivity.

Discussion

NU-ELISA provides a method to ascertain the global status of nucleosome PTMs present within a particular cell type. NU-ELISA studies have shown that global nucleosome modification states differ in comparisons of divergent cell types⁴. In addition, NU-ELISA PTM profiles change when cells are exposed to epigenetic modulatory agents such as trichostatin-A or when mouse embryonic stem cells are differentiated⁴. The method has also been applied successfully to study the chromatin of human ES cells⁵. It is important to note the NU-ELISA, in its present form, can detect only the composite signature of PTMs present within the sum total of the cellular epigenome. This differs markedly from genome-wide chromatin immunoprecipitation, which can determine the distribution of a specific PTM across specific genetic loci.

The initial steps of the NU-ELISA procedures are adapted from previous methods to isolate mononucleosomes from mammalian nuclei^{6,7}. These adapted methods provide an expedient way to obtain high-quality intact mononucleosomes from mammalian cells, and the resulting fractions are comprehensive in their chromatin content, but contain a great deal of additional nuclear material. Since specific Abs are used, contaminating non-nucleosomal material is tolerated well in the NU-ELISA assay, unlike mass-spec approaches that require greater purity. However, NU-ELISA is more sensitive and quantitative than western blotting⁴, thus providing good alternatives to westerns and mass spec for the detection of nucleosomal PTMs.

Disclosures

No conflicts of interest declared.

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