In vitro reconstitution of nucleosome arrays with a stoichiometric content of histone octamer and linker histone (PROT42)

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Introduction

Whilst great insights into the structure and properties of chromatin have been gained using chromatin samples extracted from native sources, analyses of such material have limitations because of their inherent heterogeneity. Native chromatin samples contain an ensemble of different core histones, linker histones and their variants, and other chromatin-associated proteins, all of which are adorned by a plethora of post-translational modifications. The DNA from these sources is also highly variable, prescribing varying and irregular nucleosome repeat lengths. Indeed, this heterogeneity is a consequence of the critical role that chromatin plays in the regulation of DNA transcription and replication, where local chromatin environments are tailored to suit the particular needs of a given DNA locus.

To overcome the limitations of using native chromatin samples, we have developed an in vitro nucleosome array reconstitution system that produces very long, highly regular and soluble nucleosome arrays, or folded ?30nm? chromatin fibres, with a stoichiometry of one histone octamer and up to one linker histone per nucleosome. The production of long nucleosome arrays of high homogeneity, with both a prescribed histone content and nucleosome repeat length is critical for obtaining biochemical, biophysical and structural data of high quality and reproducibility and permits the investigation of the oftentimes subtle determinants of chromatin compaction and function.

Our reconstitution system is based on DNA arrays constructed from the strong nucleosome positioning 601 DNA sequence and purified histones. The 601 DNA sequence was identified in a SELEX experiment by Widom which set out to find a DNA sequence that binds the histone octamer with high affinity and with a unique position (Lowary & Widom, 1998). The high affinity of the 601 DNA nucleosome positioning sequence for histones permits the use of Competitor DNA (crDNA) that controls nucleosome assembly, allowing the saturation of the 601 DNA array with the Histone octamer and Linker histones whilst preventing oversaturation (Huynh et al, 2005; Robinson et al, 2006; Routh et al, 2008). The assembly of the nucleosome arrays is by the classical and simple salt reconstitution system in which histones and DNA are mixed together in high salt followed by a slow dialysis to reduce the salt and to permit nucleosome formation.

Nucleosome array assembly

Basic principles of the reconstitution protocol:

1. DNA arrays are constructed using the Widom 601 nucleosome positioning DNA sequence. DNA arrays containing between 12 and 80 tandem 601 DNA repeats with different nucleosome repeat lengths (NRLs) (167 to 237bp) have been constructed (Huynh et al, 2005; Robinson et al, 2006; Routh et al, 2008).
2. Competitor DNA (crDNA) 147 bp in length is included in the reconstitution to prevent the super-saturation of the 601 DNA arrays with excess histone octamer or linker histone.
3. Histone octamers and linker histones: Histone octamers can be purified from native sources (Thomas & Butler, 1977) or assembled from recombinant histones (Luger et al, 1999). The latter method allows the incorporation of modified histones, or histone variants. Linker histones H1 and H5 isolated from native sources or recombinant linker histones can be used (Wellman et al, 1997), again permitting incorporation of a specified linker histone.

The starting point for the successful reconstitution of nucleosome arrays is the production of DNA arrays containing the 601 nucleosome positioning DNA sequence arranged in tandem. The use of a unique sequence DNA confers the advantage of specifying both the number of nucleosomes in the array and the NRL (Routh et al, 2008). The key to the reconstitution protocol is that the 601 DNA binds histone octamers with very high affinity (Lowary & Widom, 1998) so that any histone octamers that are added to the reconstitution mixture bind to the 601 DNA in preference to the weakly-binding Competitor DNA. The histone octamers will only bind to the crDNA once all the 601 DNA positions have been occupied, resulting in the saturation of the 601 DNA but not super-saturation. Without the crDNA, excess histone octamers would bind non-specifically to the 601 nucleosome arrays causing them to aggregate and to precipitate. This creates a ?window? in which the 601 DNA array is saturated but the crDNA is not, which can easily be determined by native gel electrophoresis. Other DNA arrays, such as the ones based on the 5S rDNA gene are available, but these are short (contain only 12 repeats) and produce less homogeneous nucleosome arrays because of multiple nucleosome positions (Panetta et al, 1998).

The reconstitution starts with the mixing of histones and DNA in high salt. Here, the exact molar input of histone octamer required to saturate a DNA array must be determined empirically by a histone octamer titration. Several samples are reconstituted in parallel with increasing concentrations of histone octamer. A straight forward calculation to determine the

correct stoichiometric input based upon mass is usually inaccurate. This is due to difficulty in estimations of histone octamer sample concentrations, the loss of histone octamer through aggregation and due to the ?sticking? ([comment 1](#)) of the highly charged histone octamers onto the reconstitution vessels. This is why the empirically determined input ratios of histone octamer to DNA template may vary greatly between seemingly identical reconstitutions if they are carried out in different vessels, reaction volumes, temperatures, etc. Once the saturation point of the histone octamer has been established, a similar titration must also be carried out (perhaps even more critically) to find the correct molar input of linker histone required to saturate the nucleosome core array.

The protocol given below describes the reconstitution using a DNA array containing 25 tandem repeats of 197bp [601 DNA](#) (197bp x 25). The protocol is essentially split into two sections, a histone octamer titration and a linker histone titration, performed in three overnight steps.

Procedure

Day 1:

Histone octamer titration

1. A series of reactions are set up side-by-side, encompassing a molar input ratio from 0.5:1 to 3:1 of histone octamer: DNA (for example, see table 1). The histone octamer and DNA are mixed together in high salt (2M NaCl) in RNase-free, non-stick Eppendorf tubes and placed on ice. Typically, the DNA array is at a concentration of 25µg/ml (0.2 10^{-6} M [601 DNA](#) repeat) but should not exceed ~250µg/ml (2.0 10^{-6} M [601 DNA](#) repeat). For optimal reconstitutions, the crDNA is included at a mass ratio of 1:1 crDNA: [601 DNA](#) array. All manipulations are carried out at +4ºC. Note: It is usually more practical and reproducible to make up a stock of the 197bp x 25 [601 DNA](#) array and crDNA aliquot the mixture.
2. Each reaction is placed into a dialysis vessel. We prefer to use small glass tubes (siliconized to prevent sticking), which are sealed at one end with a silicone bung, and at the other with a section of dialysis membrane (7?000 MWCO).
3. All the dialysis vessels are then placed inside another dialysis bag, filled with ~20ml reaction buffer (2M NaCl, 10mM TEA-HCl pH7.4, 1mM EDTA). This is then placed into a large volume (e.g. 4 litres) of low-salt buffer (10mM TEA-HCl ([comment 2](#)) pH 7.4, 1mM EDTA) and left to dialyse overnight at +4ºC. The TEA ?HCl buffer is used in the reconstitution to permit glutaraldehyde cross-linking required in later analyses.

Such ?double bag? dialysis slows the dialysis to provide sufficient time for the histones to ?find? their [601 DNA](#) sequence before the salt concentration drops too low forcing the proteins to stick non-specifically to any available DNA. Dialysis that is too quick can produce material that either precipitates, or creates smeary bands when run on a native agarose gel ([comment 3](#)).

Table 1: Histone Octamer Titration								
Sample	Conc (mg/ml)	1	2	3	4	5	6	7
Histone Octamer	0.5	2	4	6	8	10	12	14

197bp x 25	0.5	4	4	4	4	4	4	4
Competitor DNA	0.5	4	4	4	4	4	4	4
2M NaCl, 10mM TEA - CHl pH7.4, 1mM EDTA	-	70	68	66	64	62	60	58

(volumes expressed in µl)

Day 2:

4. After high to low salt dialysis, the reconstituted samples are analysed by native agarose gel (comment 4) electrophoresis. 5µl of each reconstitution mixture is mixed with 5µl running buffer (0.2X TBE) and 2µl gel loading buffer (20% glycerol, 20mM Tris pH 7.4, 1mM EDTA, 0.1% bromophenol blue) and loaded onto a 0.8% agarose gel (normally 10x10cm) in 0.2X TBE buffer. The gel is run at 15mA for approximately 1h20mins. **Note:** The percentage agarose and the length of the gel can be adjusted to improve resolution as required.
5. The DNA and nucleosomal complexes can be visualised either by ethidium bromide staining or standard phosphorimaging techniques if a radionucleotide has been incorporated into the DNA array prior to the reconstitution.

If the reconstitution has been successful, then a clear bandshift in the DNA array should be observed (Figure 1). The retardation in migration rate is a result of the DNA array becoming ?heavier? as the histone octamers are loaded onto the DNA. The point of histone octamer saturation (i.e. one histone octamer per 601 DNA repeat) is taken to be the histone octamer concentration at which the mobility of the 601 DNA array does not increase further and reaches a plateau (Figure 1, Lane 7, marked Red in Table 1). After this point, the histone octamer will start to bind the crDNA forming additional nucleosome cores (Figure 1, Lanes 7-8). Once the crDNA has been depleted, histone octamers will bind non-specifically to any available DNA. At this point, the nucleosome arrays become insoluble, precipitate, and hence do not migrate into the agarose gel (Figure 1, Lane 9).

At the end of the histone octamer titration the researcher will have produced two or three samples of soluble nucleosome core arrays with a histone octamer: 601 DNA repeat ratio of 1:1. The position and loading of histone octamer can further be checked by digesting the reconstituted array with AvaI and analysis in native agarose gels. Fully reconstituted samples should release only mono-nucleosome cores (Huynh et al, 2005)). Each sample will now be in 10mM NaCl, 10mM TEA-HCl pH7.4 and 1mM EDTA. In these salt conditions, the chromatin arrays will be unfolded and hence only suitable for experiments requiring chromatin lacking the linker histone (comment 5).

Linker histone titration

To produce nucleosome arrays (rather than nucleosome core arrays) that can form fully folded ?30nm? chromatin fibres ? say for studies of higher-order chromatin structure - the incorporation of the linker histone is required (Routh et al, 2008).

Similarly to the histone octamer titration, a reconstitution must be performed using increasing concentrations of linker histone in order to determine the optimal point of linker histone binding. This step is often troublesome due to difficulties in handling this insoluble and poorly behaved protein. The degree of this will vary depending upon what linker histone

sample is used (i.e. different variants, different preparations, different storage buffers, different pairs of socks, etc). For this reason, it is critical to establish empirically the optimal input of linker histone to obtain saturation in binding rather than simply adding the ?correct? concentration based upon mass.

6. Using the concentration of histone octamer required to saturate the 601 DNA array established in **Step 5** (Marked Red in Table 1), a series of reconstitution reactions are set up side-by-side with increasing concentrations of linker histone, encompassing molar input ratios of 0.5: to 5:1 (for examples, see table 2). The histones and DNA are mixed together in high salt (2M NaCl) in RNase-free, non-stick Eppendorf tubes and placed on ice. **Note:** It is usually more practical and reproducible to make up a stock of the 197bp x 25 601 DNA array, crDNA and histone octamer, and make aliquots of the mixture.
7. **Steps 2** and **3** described above are repeated using the reconstitution mixture containing the linker histone shown in Table 2.

Table 2: Linker Histone Titration											
Sample	Conc (mg/ml)	1'	2'	3'	4'	5'	6'	7'	8'	9'	10'
Histone Octamer	0.5	10	10	10	10	10	10	10	10	10	10
197bp x 25	0.5	4	4	4	4	4	4	4	4	4	4
Competitor DNA	0.5	4	4	4	4	4	4	4	4	4	4
H5 Linker Histone	0.1	0	1.6	3.2	4.8	6.4	8.0	9.6	11.2	12.8	16.0
2M NaCl, 10mM TEA-HCL pH 7.4, 1mM EDTA	-	62	60.4	58.8	57.2	55.6	54	52.4	50.8	49.2	46
(Apparent) H5: Nucleosome	-	0	0.5	1	1.5	2	2.5	3	3.5	4	5
Corrected H5: Nucleosome	-	0	0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	2

(volumes expressed in µl)

Day 3:

As before, after overnight double-bag dialysis, each sample will now be in 10mM NaCl, 10mM TEA-HCl pH7.4 and 1mM EDTA. Although the binding of the linker histone can be observed directly by gel electrophoresis, we have found it more reproducible to analyse the histone content of the folded nucleosome arrays. This is especially important when using very long arrays (>25 repeats). Therefore, a second overnight dialysis into a ?Folding buffer? is now performed. The folding buffer should contain enough mono- or divalent cations (or both) to direct the higher order folding of the chromatin fibre. The folding buffer may be chosen to suit a particular experiment, or to reflect the physiological concentration of ions found in the nuclear milieu. **Note:** An appropriate choice of folding buffer is critical. A discussion of these can be found in (Huynh et al, 2005; Robinson et al, 2006; Routh et al, 2008)

8. Each tube is removed the low salt buffer ? still capped with a dialysis membrane ? and transferred into another beaker containing 2 litres folding buffer (e.g. 1mM $MgCl_2$ 10mM TEA-HCl pH 7.4) and dialysed overnight. **Note:** The second, outer dialysis bag is not required at this point.

Day 4:

Provided that the samples have been dialysed into a folding buffer that promotes higher-order folding of the chromatin arrays into folding buffer, linker histone binding can then be analysed by native agarose gel electrophoresis. This exploits the observation that folded nucleosome arrays migrate faster in agarose gels and the fact that the degree of compaction is directly related to the amount of bound linker histone (Routh et al, 2008) However, the reconstituted nucleosome array must first be fixed gently with glutaraldehyde prior to gel electrophoresis to preserve linker histone binding and to trap the chromatin arrays in their folded states. Without glutaraldehyde fixation a band shift cannot be observed, regardless of whether linker histone is bound or not, because the chromatin arrays will decompact in the agarose milieu.

9. Glutaraldehyde fixation is carried out by diluting 2µl of 25% glutaraldehyde solution into 98µl of the folding buffer to give a 0.5% glutaraldehyde fixing solution. 10µl of each reconstitution reaction is mixed with 2.5µl of the fixing solution, thus giving a final concentration of 0.1% glutaraldehyde. These samples are then placed on ice and incubated for 30mins.
10. Similarly to **Step 4**, 6.25µl of each reconstitution sample is mixed with 3.75µl running buffer (0.2X TBE) and 2µl gel loading buffer (20% glycerol, 20mM Tris-HCl pH7.4, 1mM EDTA, 0.1% bromophenol blue) and loaded onto a 0.8% agarose gel (normally 10x10cm) in 0.2X TBE buffer. The gel is run at 15mA for approximately 1h20mins. Note: The percentage agarose and the length of the gel can be adjusted to improve resolution if required.
11. The DNA and nucleosomal complexes can be visualised either by ethidium bromide staining or phosphorimaging techniques if a radionucleotide has been incorporated into the DNA array prior to the reconstitution.

If the reconstitution, folding and fixing has been successful, then a clear downward shift in the migration of the nucleosome arrays should be observed (Figure 2). Note: A band shift will not be observed unless the samples had been dialysed into a folding buffer that promotes the formation higher-order chromatin structure (Step 8). The point of linker histone saturation is again established when the mobility of the band reaches a plateau (Figure 2, Lanes 7-9) At this point in a linker histone titration, it has been shown that these in vitro reconstituted chromatin arrays contain native-like stoichiometries of linker histone (Huynh et al, 2005).(comment 6)

The point of linker histone saturation can also be established by visualizing the fibres by electron microscopy (EM) or by analysing their degree of compaction by sedimentation velocity analysis (Routh et al, 2008). For EM, negatively stained samples can be prepared using standard techniques. The presence of higher-order structure - i.e. ?30nm? chromatin fibres - indicates that the samples are saturated with linker histone (Routh et al, 2008). Sedimentation velocity analysis provides a quantitative means of determining linker histone saturation, and thus chromatin fibre compaction. However, this method consumes a large amount of material, and thus is often not practical.(comment 7)

In some circumstances, a thorough analysis of protein content may be necessary ? see (Huynh et al, 2005). This may be the case when working with unusual histone modifications, histone variants or with unusual DNA templates. For example, linker histone binding has been shown to be reduced on DNA templates with a short nucleosome repeat length 167bp ? as found in Saccharomyces cerevisiae (Routh et al, 2008).

Conclusions

By following this reconstitution protocol, the researcher should now have prepared nucleosome arrays that have one histone octamer and one linker histone bound per 601 DNA sequence in the array. The use of native agarose gel electrophoresis described here can also be used to obtain a measure of the degree of compaction and so provides a quick method for analysing (for example) the effects of histone tail post-translational modifications on nucleosome array compaction (Robinson et al, 2008).

Materials & Reagents

601 DNA	The DNA arrays were constructed using an AvaI asymmetric restriction site between each repeat to achieve a tandem, head-to-tail arrangement. A monomeric 601 DNA repeat, containing the nucleosome positioning sequence at its centre, was amplified by PCR. Primers were designed containing AvaI, EcoRV and either EcoRI/XbaI restriction sites at either end of the 601 DNA. The EcoRI/XbaI-cut PCR product was cloned into EcoRI/XbaI cut pUC18. For multimerisation, the plasmid was grown in DH5a E. coli, purified, and the 601 DNA fragment excised by digestion with AvaI. The 601 DNA fragments were multimerised in a 100µl ligation reaction containing 4µg of purified monomer fragment and 4000 units of T4 DNA ligase for two hours at 16 °C. The ligation products were fractionated on a 1.2% (w/v) native agarose gel and the DNA arrays of interest cut out and extracted. The DNA arrays were then ligated into AvaI-cut pUC18 containing flanking EcoRI/XbaI and EcoRV sites. For blunted-ended release, the DNA arrays were excised using EcoRV. DNA arrays that are very long (e.g. 4925bp for the 197bp x 25), are purified by first digesting the plasmid DNA into fragments of less than 1000bp using DraI/HaeII and removing the plasmid DNA fragments by precipitation with polyethylene glycol (PEG), which permits the selective precipitation of long DNA fragments. The differential precipitation was carried out using 5-8% PEG 6000 and 0.5 M NaCl. Purified DNA arrays were precipitated in ethanol, dried and resuspended in 2M NaCl, 10mM TEA/HCl pH 7.4, 1mM EDTA at 0.5 mg/ml and stored at -20°C.
Competitor DNA	The Competitor DNA (crDNA) of about 147bp in length can be the mixed sequence DNA extracted from nucleosome core preparation (Huynh et al, 2005), but it is often more practical to amplify a fragment from pUC18. Primers were designed to PCR amplify the following 147bp region of random sequence DNA from a region outside the multiple cloning site of the pUC18 vector: attcattaat gcagctggca cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaatg tgagttagct cactcattag gcacccagg ctttacactt tatgcttccg gctcgtatgt tgtgtggaat tgtgagc Forward Primer: ATTCATTAATGCAGCTGGCACGACAGG Reverse Primer: GCTCACAATTCCACACAACATACGCGCC

	At the end of the PCR reaction the crDNA phenol extracted and precipitated with ethanol and resuspended in 10mM TEA/HCl pH 7.4, 10mM EDTA at 1-5mg/ml and stored at -20°C ([comment 8](#))
[Histone octamer and Linker histones](#)	The histone octamer and the linker histones H1 and H5 were purified from native sources such as chicken erythrocytes, as described (Huynh et al, 2005; Thomas & Butler, 1977). Recombinant histone octamers assembled from purified H2A, H2B, H3 and H4 histones produced in E. coli were produced as described (Luger et al, 1999). The latter method allows the incorporation of modified histones, or histone deletion mutants or histone variants (Robinson et al, 2008). Similarly, specific linker histones can be expressed in E. coli (Wellman et al, 1997), again permitting incorporation of a specified linker histone. Purified histones were stored at 0.1-5.0 mg/ml in 2.0 M NaCl, 10mM TEA/HCl and 1mM EDTA at +4°C

Reviewer Comments

Reviewed by: [Verena Maier](#), Laboratory of Peter B. Becker, Adolf-Butenandt Institut, University of München, Germany

1. Nonspecific protein binding and therefore loss of material can be reduced by preincubating the dialysis chambers with 2mg/ml BSA in 10 mM Tris-HCl, 1 mM EDTA, 500 mM NaCl for two hours.
2. TEA can be substituted with Tris-HCl.
3. As an alternative to the ?double-bag? system, the salt concentration of the dialysis buffer can also be lowered gradually as described in Lee KM, Narlikar G (2001) Assembly of nucleosomal templates by salt dialysis. Curr Protoc Mol Biol Chapter 21:Unit 21.6. In this case, dialysis chambers (commercial, e. g. Pierce Slide-A-Lyzer, or home-made according to Sandaltzopoulos and Becker, Methods in Molecular Biology 119, 195-206, 1999, in Chromatin Protocols, PB Becker, ed, Humana Press) can be placed in a floating rack.
4. Highly pure agarose such as Biozym LE GP Agarose greatly improves the quality of the EMSA.
5. If required, nucleosome arrays can be purified from unbound proteins and crDNA by $MgCl_2$-precipitation. For this purpose, $MgCl_2$ is added to 5 mM, the mixture is incubated on ice for 15 min and nucleosome arrays are pelleted by a 15 min centrifugation in a tabletop centrifuge at 13,000 rpm (see Schwarz PM, Felthauser A, Fletcher TM, Hansen JC (1996) Reversible oligonucleosome self-association: dependence on divalent cations and core histone tail domains. Biochemistry 35, 4009-4015; Huynh et al, 2005).
6. We observed a downward shift upon addition of H5 to 12mer nucleosome arrays also in TE buffer and without glutaraldehyde fixation. Under these conditions, addition of Drosophila H1 results in an upward shift of the 12mer array (see Maier VK, Chioda M, Rhodes D, Becker PB (2008) ACF catalyses chromatosome movements in chromatin fibres. EMBO J 27, 817-26).
7. As a further control for stoichiometric incorporation of the linker histone, H1-, but not H5-containining arrays can be digested to chromatosomes (nucleosome + linker histone) by AvaI in 10 mM HEPES-KOH, pH 7.6, 50 mM KCl, 1.5 mM MgCl2, 0.5 mM EGTA at 26°C. Nucleosome arrays saturated with H1 should release mostly chromatosomes, which can be distinguished from nucleosomes by native agarose gel electrophoresis (see Maier et al, 2008).

8. Alternatively, the pUC18 vector can be cut with DraI and the resulting DNA fragments (between 692 and 1113 bp) can serve as [Competitor DNA](). Since also the [601 DNA]() arrays are subcloned into the pUC18 vector, purification of 601 repeats and the production of crDNA can easily be done in one step of enzymatic digestion.
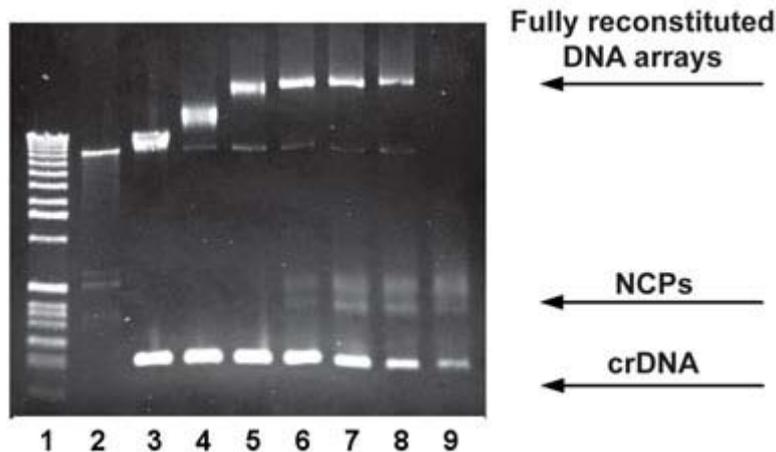
Figures

Figure 1: Histone octamer titration analysed by a native gel electrophoresis (0.8% agarose, post-stained with Ethidium Bromide).
- **Lane 1:** 1kb Mw Marker
- **Lanes 2:** 197bp x 25 DNA array
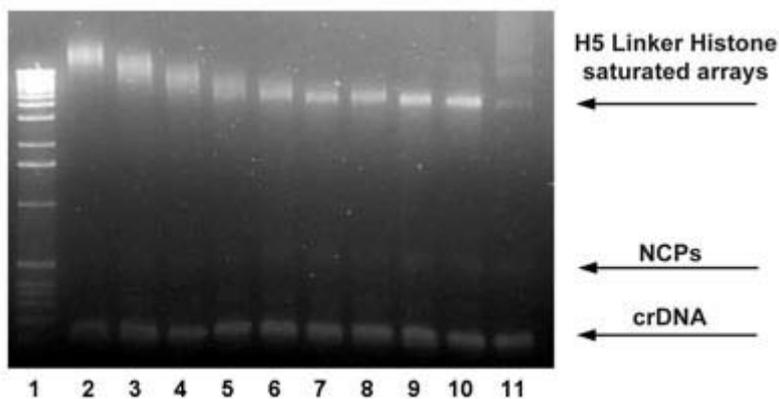- **Lanes 3 ? 9:** Reconstitution samples 1-8 from Table 1

Figure 2: Linker histone titration analysed by a native 0.8% agarose gel, post-stained with Ethidium Bromide.
- **Lane 1:** 1kb Mw Marker
- **Lanes 2-11:** Reconstitution samples 1-10 from Table 2

References

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