Preface

Significant advancements have been made in the study of chromatin structure and function over the past 50 years, but few as spectacular as those made in the last decade. Development of novel techniques and the ability to sequence large stretches of DNA has facilitated in-depth analysis of DNA structure and modifications as well as proteins associated with it. It has been well established that histones and non-histone proteins associated with DNA in chromatin modulate many vital cellular functions, including transcription, replication, repair and gene silencing. Moreover, the modifications of the proteins associated with chromatin have been correlated with many of these functions. Development of novel molecular techniques in the past decade has provided immense momentum to detailed analysis of chromatin, which can now be carried out with remarkable precision. The second edition of *Chromatin Protocols* compiles many of these techniques that facilitated the explosion of information on chromatin structure and function.

The first edition of *Chromatin Protocols* was published in 1999 and had become the staple of laboratories studying chromatin structure and function. Since its publication, the landscape of chromatin biology has changed immensely. The concept of 'histone code' was proposed during the earlier part of this decade and has found universal acceptance. Now it is possible not only to analyze specific histone modifications on each specific nucleosome but such analysis can also be carried out on a genome-wide scale. To a great extent, these advances in the analysis of histone modifications in relation to transcriptional regulation and gene expression were facilitated by the development of chromatin immunoprecipitation (ChIP) assays. Development and refinement of the ChIP assay have led to an understanding of molecular changes associated with histone modifications, transcription factor binding and gene expression in the cell at a level that could only be imagined a decade ago; the progress this technique and its variants have brought to the field of chromatin biology is similar to the progress electrophoretic mobility shift assays brought to transcription factor biology in the early 1980s. These are examples of techniques developed and fine-tuned in a handful of labs changing the landscape of an entire area of biology.

The second edition of *Chromatin Protocols* encompasses a wide array of techniques spanning from isolation of nucleosomes, assembly of nucleosomes and study of the basic chromatin structure to detailed analysis of histone modifications and chromatin function. Techniques to prepare chromatin assembly extracts, analyze chromatin decondensation and drug-induced premature chromatin condensation, study of reconstituted chromatin, assess DNA topology in chromatin, analyze unique mononucleosomes and a variety of other protocols to study chromatin structure form the initial part of this volume.

This section is followed by techniques to study DNA damage and repair in the context of chromatin; techniques for monitoring DNA breaks, detailed methods to study transcription-coupled repair in chromatin, techniques for analysis of H2AX phosphorylation to assess DNA damage as well as monitoring DNA breaks in chromatin are included here. A technique to study DNA damage induced by various drugs as well as radiation is included, in addition to a detailed protocol to study genomic aberrations by comparative genomic hybridization. These techniques to study DNA damage in chromatin are followed by an assay to study DNA replication in vitro using mammalian cell extracts. This technique and its variations allow the analysis of the role of different histone and non-histone proteins involved in the replication of DNA.

The above sections are followed by detailed protocols to study DNA methylation as well as histone modifications including methylation, acetylation, phosphorylation and ubiquitination. In addition to the analysis of histone modifications, protocols are also provided to study the activities of many of the enzymes involved, including histone demethylases and deacetylases. An elegant technique to study histone phosphorylation by immunostaining is provided, which can be modified to study other histone modifications and chromatin-associated proteins as well. Further, detailed methods to successfully carry out chromatin immunoprecipitation assays are presented. A protocol is presented which describes how genome-wide ChIP analysis can be conducted, using a ChIP-on-chip technique; success of such studies depends to a great extent on the analysis of the data generated and methods to analyze ChIP-on-chip tiling arrays are presented. Imaginative utilization of these ChIP based techniques has immense potential to generate information on various aspects of chromatin biology.

The role of telomeres in maintaining chromatin integrity and how alterations in telomerase activities lead to oncogenesis as well as other proliferative disorders have gained substantial interest in recent times. Two protocols are given in this broad context, one describing techniques to study telomerase activity and telomere length and one to study ATM-dependent chromatin modifications. These techniques, we believe, would be very useful for those working in these areas in the context of normal biology of chromatin as well as proliferative disorders associated with telomere function.

We believe that these protocols, as well as their creative modifications, will facilitate in-depth molecular analysis of various aspects of chromatin structure and function including transcriptional regulation, DNA repair and replication. As can be imagined, this volume would not have been made possible without valuable contributions from a truly international panel of authors, who are all experts in their fields. My sincere thanks to them for taking the time and effort to pen down the intricate details of their favorite techniques and for their willingness to share them with the readers. In addition, my thanks to Dr. John Walker, the series editor, without whose helpful suggestions and persistence this volume would not have been completed in its present form; it was a real pleasure working with John. It is our genuine belief that the second edition of Chromatin Protocols will be a valuable tool for studying various aspects of chromatin function and would contribute significantly to the development of new techniques as well as ideas in the field of chromatin biology.

Chapter 2

Analysis of Reconstituted Chromatin Using a Solid-Phase Approach

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Abstract

Complex experimental strategies involving in vitro reconstituted chromatin or simple chromatin interaction studies are much facilitated by immobilizing the nucleosomal arrays to paramagnetic beads. Chromatin-containing beads can be retrieved from a reaction mix solution on a magnet fast and quantitatively, effectively separating bound, loosely attached and unbound components efficiently. This chapter details a convenient strategy for immobilization of linear plasmid DNA on streptavidin-coated beads, the reconstitution of chromatin on such beads and some fundamental handling procedures.

Key words: Biotin-streptavidin, Dynabeads, solid phase, chromatin assembly, chromatin constituents.

1. Introduction

The value of a solid support was recognised early in history by the ancient Greek engineer Archimedes who, amazed by the power of the leverage machines that he invented, exclaimed that he could even move the entire planet had he only a suitable solid support to rely on. In biochemistry, sophisticated multi-step experimental procedures require that a substrate is purified and processed through a sequence of reactions under different optimal conditions. Solid-phase techniques are invaluable because they allow instant and quantitative purification of reaction intermediates and readjustment of new reaction conditions. Here we describe a method for chromatin reconstitution on a solid support and present how solid-phase chromatin can be analysed or prepared as a substrate in subsequent reactions.

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Chromatin reconstitution in crude extracts from *Xenopus* oocytes or eggs or *Drosophila* embryos provides a powerful means to study structure/function relationships in chromatin organisation *(1–5)*. For many of those analyses, e.g. the evaluation of the transcriptional potential of a chromatin template, the chromatin must be purified from the complex reconstitution reaction. The most common method for chromatin purification is its centrifugation through a sucrose gradient. Although efficient, this method is time consuming, does not allow parallel processing of many samples and many

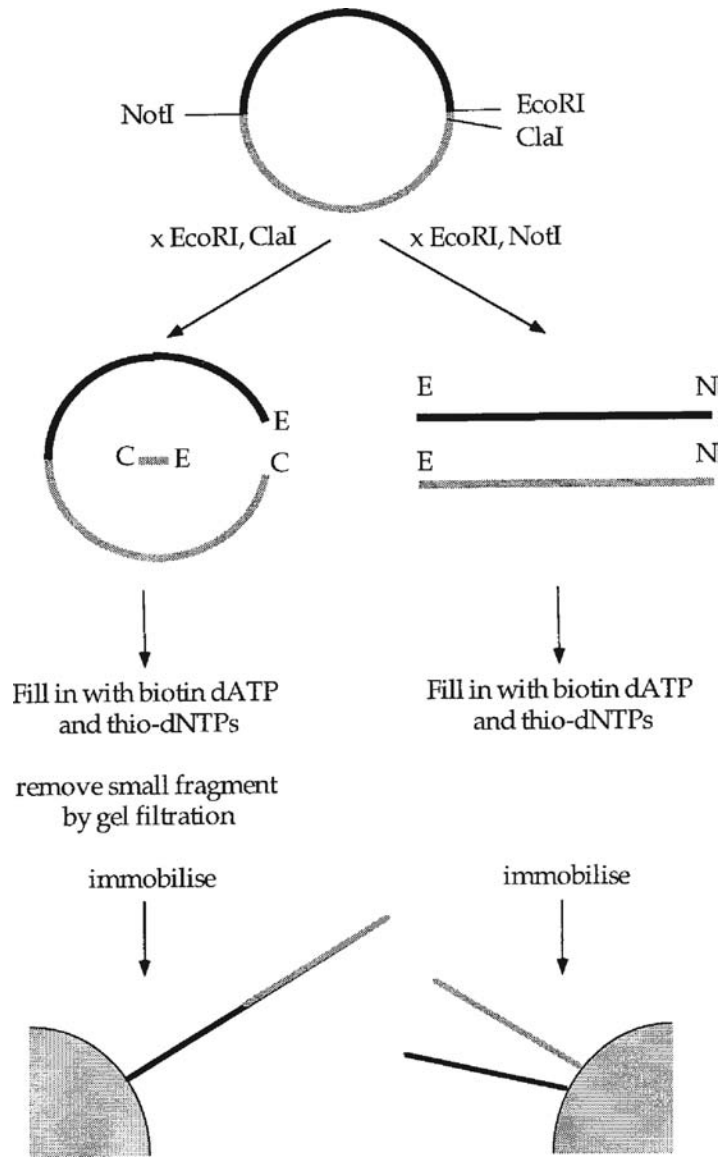

Fig. 2.1. DNA immobilisation strategies (*see* **Note 1**).

loosely associated chromatin components that may be of pivotal importance, e.g. for chromatin dynamics, may be lost during the long centrifugation.

By contrast, the solid-phase approach enables the rapid, non-disruptive and quantitative purification of chromatin. A linear fragment of DNA that bears the sequences of interest (i.e. enhancer/promoter and gene coding regions) is biotinylated at one end and then immobilised on streptavidin-coated superparamagnetic beads (**Section 3.1** and **Fig. 2.1**). The bead-coupled template is then subjected to chromatin assembly (**Section 3.2.1**). Chromatin assembled on immobilised DNA resembles that of soluble DNA with respect to the optimal reconstitution conditions, the kinetics of chromatin assembly, the nucleosomal repeat length, the histone stoichiometry, the association of histone H1, the inhibition of transcription and the association of many non-histone proteins. The immobilised chromatin can then be purified in a magnetic field, washed as desired and then used to purify and analyse chromatin-associated proteins (**Section 3.3.1**) or to develop assays for putative chromatin-binding proteins. The rapid isolation of immobilised chromatin in a magnetic field facilitates quick buffer exchanges and the efficient removal of soluble components, such as nucleotides or unbound proteins. Therefore, multi-step reconstitutions are facilitated, i.e. reactions in which the chromatin reconstitution must be separated from other steps, such as the interaction of transcription factors prior to chromatin assembly or subsequent chromatin "remodelling" reactions [for examples of applications, *see* **refs**. *(6–13)*].

We also describe how nucleosomes can be reconstituted from pure histones by a salt gradient dialysis procedure on immobilised DNA (**Section 3.2.2**).

2. Materials

2.1. Immobilisation of DNA

1. Streptavidin-coated paramagnetic beads (Dynabeads M-280, Invitrogen-Dynal).
2. Magnetic particle concentrator (MPC-6, Invitrogen-Dynal).
3. 0.5 mM or 10 mM biotin-21-dUTP in 50 mM Tris–HCl pH 7.5 (Clontech) or 0.4 mM biotin-14-dATP in 100 mM Tris–HCl pH 7.5, 0.1 mM EDTA (Invitrogen-Gibco). Biotin-dCTP is available from Invitrogen-Gibco, but we have not tested it yet.
4. 10 mM a-thio-deoxyribonucleotides, pH 8.0.

5. Restriction enzymes and 10 × digestion buffers (according to the supplier's recommendation).
6. Klenow (exo$^-$) polymerase.
7. 10 × polymerase buffer: 0.1 M Tris–HCl pH 7.5, 50 mM $MgCl_2$, 75 mM DTT).
8. Vent (Exo$^-$) DNA polymerase (New England Biolabs).
9. 10 mg/ml glycogen (Roche Applied Science).
10. Ethanol.
11. Quick Spin columns TE Sephadex G-50 fine (Roche Applied Science) or ChromaSpin column 50-TE (Clontech) or equivalent home-made spin columns.
12. PBS-BSA-NP40: 1.7 mM KH_2PO_4, 5 mM Na_2HPO_4, 150 mM NaCl pH 7.4, 0.05% (w/v) BSA, 0.05% v/v nonidet P40 (IGEPAL CA-630).
13. Wash and binding buffer (WB buffer): 2 M NaCl, 50 mM Tris–HCl, pH 8.0, 1 mM EDTA.
14. Lambda DNA (Promega).
15. Kilobase binder reagent (Invitrogen-Dynal).
16. Shaker with regulated speed and temperature (e.g. thermomixer, Eppendorf).
17. TE: 10 mM Tris–HCl pH 7.5, 0.1 mM EDTA.
18. Rotating wheel with regulatable speed.

2.2. Chromatin Reconstitution on Immobilised DNA

1. Chromatin assembly extract *(4)*
2. 0.5 M $MgCl_2$.
3. McNAP mix *(4)*: 100 mM creatine phosphate, 30 mM ATP pH 8.0, 10 mM DTT, 100 mg/ml creatine phosphokinase.

2.2.1. Reconstitution in *Drosophila* Embryo Extracts

4. Extract buffer (EX): 10 mM Hepes–KOH pH 7.6, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10% v/v glycerol, 10 mM b-glycerophosphate, 1 mM dithiothreitol, 0.2 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride, Sigma-Aldrich).
5. Temperature-regulated chamber with integrated rotating wheel (e.g. hybridization oven).

2.2.2. Nucleosome Reconstitution by Salt Gradient Dialysis

1. Core histones.
2. Two peristaltic pumps.
3. Magnetic stirrer and stirrer bars.
4. DB-1: 2 M NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.05% nonidet P40, 1 mM b-mercaptoethanol (freshly added).
5. DB-2: 50 mM NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.05% nonidet P 40, 1 mM b-mercaptoethanol (freshly added).
6. Dialysis tubing Spectra/Por 2, MWCO 12-14,000, 2 ml/cm.

2.3. Analysis of Reconstituted Chromatin

1. EX-S, (EX, *see* **Section 2.2.1**), where S represents the concentration of KCl in mM.
2. EX-50-NP40: extract buffer supplemented with 50 mM KCl and 0.05% v/v nonidet P40 (IGEPAL CA-630).

2.3.1. Analysis of Chromatin Proteins

3. 4 × SDS-loading buffer: 200 mM Tris–HCl pH 6.8, 40% (v/v) glycerol, 400 mM b-mercaptoethanol, 4% (w/v) SDS, 0.002% (w/v) bromophenol blue.
4. Equipment for PAGE.

2.3.2. Micrococcus Nuclease Digestion

1. Micrococcal nuclease (Roche Applied Science), 50 units/ml in EX buffer).
2. 5 × nuclease stop mix: 2.5% v/v sarkosyl, 100 mM EDTA pH 8.0.
3. TE: 10 mM Tris–HCl, 1 mM EDTA pH 8.0.
4. 5 × Orange loading buffer: 50% (v/v) glycerol, 5 mM EDTA pH 8.0, 0.3% w/v Orange G.
5. Equipment for agarose gel electrophoresis.

3. Methods

3.1. Immobilisation of DNA

3.1.1. Digestion of DNA

1. Cleave plasmid DNA with an appropriate pair of restriction enzymes (example is given for *Cla*I and *Eco*RI; *see* **Note 1** and **Fig. 2.1**) as follows: Mix 40 µl of supercoiled plasmid (1 µg/µl = 40 µg), 5 µl 10 × digestion buffer, and 5 µl *Cla*I (10 u/µl). Incubate for 3 h at 37°C.
2. Assure complete linearisation by analysing 0.2 µl of the digest by electrophoresis on an 0.8% agarose gel and staining with ethidium bromide (*see* **Note 2**).
3. Add 50 µl H_2O, 11 µl 10 × digestion buffer, and 10 µl *Eco*RI (10 u/µl). Adjust the final volume to 160 µl with H_2O. Incubate for 3 h at 37°C.
4. Precipitate DNA: add 16 µl 3 M sodium acetate pH 5.3. Mix. Add 480 µl ethanol, mix. Incubate for 10 min on ice.
5. Spin 15 min at top speed in a tabletop centrifuge. Discard supernatant.
6. Wash pellet with 800 µl 80% ethanol. Dry pellet 2 min in speed vac without heating.
7. Dissolve pellet thoroughly in 40 µl TE.

3.1.2. Biotinylation of DNA

1. Add 7.5 µl 0.4 mM biotin-14-dATP, 1.2 µl each of 10 mM a-thio-dTTP, 10 mM a-thio-dCTP and 10 mM a-thio-dGTP

(*see* **Note 3**), 6.0 µl 10 × polymerase buffer and 3.5 µl of 5 units/ml Klenow (exo$^-$).

2. Incubate for 1 h at 37°C (*see* **Note 4**).

3.1.3. Removal of Free Biotin (*see* Note 5).

1. Resuspend the matrix of a Quick spin sephadex G-50 TE spin column. Uncap the top then the bottom of the column.
2. Place in a reaction tube provided (without lid) and let drain in a vertical position (about 5 min).
3. Empty the reaction tube and put the column (together with the reaction tube) in a 15 ml Falcon tube.
4. Spin for 1 min at 1100 *g*.
5. Discard flow-through and spin at 1100 *g* for 2 min.
6. Replace the collection tube by a fresh one. Apply the biotinylation reaction slowly at the centre of the resin without touching the resin.
7. Spin for 2 min at 1100 *g* and collect flow-through. The volume of your sample should stay constant (approx. 60 µl). Measure optical density at 260 nm to define DNA concentration in order to account for the losses during gel filtration. Usually the losses are between 10 and 30%.
8. Add 200 µl 2 × WB buffer and 140 µl H_2O. This is the coupling mix that is ready to be added to the beads. The final NaCl concentration must be 2 M. Save 1 µl for testing immobilisation efficiency.

3.1.4. Coupling of DNA to Dynabeads

1. Resuspend beads well.
2. Remove appropriate amount of bead suspension from the vial. About 1 mg of beads (100 µl) is required for the immobilisation of 1 pmol of DNA (*see* **Note 6**). For example, since 1 pmol of a 5 Kb DNA fragment is 3.3 mg, 1.210 µl (40/3.3 times 100) of bead suspension is needed to immobilise 40 µg of fragment.
3. Place tube on MPC (magnetic particle concentrator) for 1 min.
4. Discard supernatant.
5. Wash beads in 300 µl PBS-BSA-NP40.
6. Wash beads twice with 300 µl WB.
7. Resuspend beads in coupling mix (*see* **Section 3.1.3**, step 8).
8. Rotate at room temperature for at least 3 h or overnight.
9. Concentrate beads and remove supernatant.
10. Check 10 µl of supernatant (equivalent to originally 300 ng) on 0.8% agarose gel alongside the uncoupled aliquot (*see* **Note 7**).

11. Resuspend DNA beads in WB buffer at a concentration of 30 ng of immobilised DNA per µl of buffer and store at 4°C (under these conditions, they can be stored for several months).

3.1.5. Efficient Immobilisation of Very-Long DNA (*see* Note 6).

1. Mix: 300 µl lambda DNA (100 mg, 40 µl 10 × Vent polymerase buffer, 8 µl 10 mM a-thio dGTP, 8 µl a-thio 10 mM dCTP, 8 µl 10 mM a-thio dATP, 4 µl 10 mM biotin-21-dUTP, 5 µl Vent (exo$^-$) DNA Polymerase (2 units/µl) and 27 µl H_2O (total volume is 400 µl).
2. Incubate for 30 min at 76°C
3. Add 40 µl 3 M sodium acetate, pH 5.3 and mix gently.
4. Add 1100 µl absolute ethanol. Mix and incubate for 5 min on ice.
5. Spin for 10 min at top speed in a tabletop centrifuge.
6. Wash pellet twice with 70% ethanol.
7. Dry and resuspend in 300 µl H_2O (approximately 1 pmol/100 µl) (*see* **Note 8**).
8. Add an equal volume of 2 × WB buffer and transfer to equilibrated beads (steps 1–7 of 3.1.4). Then add 1/4 of this volume of kilobase binder reagent (*see* **Note 9**). Mix gently.
9. Rotate at room temperature for overnight.
10. Check immobilisation efficiency and store DNA beads as in **Section 3.1.4,** steps 9–11 (*see* **Note 8**).

3.2. Chromatin Reconstitution on Immobilised DNA

3.2.1. Chromatin Reconstitution Using *Drosophila* Embryo Extracts

1. Resuspend stock of immobilised template. Pipette out appropriate amount of bead-DNA. About 900 ng of DNA is sufficient for a MNase assay or analysis of bound histones by silver staining.
2. Concentrate on the MPC. Remove supernatant and wash once with 300 µl of PBS-BSA-NP40.
3. Wash again with 300 µl EX-NP40.
4. Prepare chromatin assembly reaction by mixing 70 µl chromatin assembly extract, 12 µl McNAP and 38 µl EX buffer for each 900 ng of DNA (*see* **Note 10**).
5. Concentrate bead DNA, remove supernatant and resuspend beads in complete chromatin assembly reaction.
6. Transfer to 250 µl micro test tubes (*see* **Note 11**).
7. Rotate at 26°C for up to 6 h, the rotation axis being perpendicular to the longitudinal axis of the tube.
8. Check occasionally for aggregation of beads. If necessary disperse clumps by gently tapping the tube. Some clumping

may occur during the first 1–2 h of the assembly reaction. If beads are redispersed once, they usually do not clump again.

3.2.2. Chromatin Reconstitution Using Purified Histones

1. During a salt gradient dialysis reconstitution, histones and DNA are first dialyzed into high salt buffer DB-1 (*see* **Note 12**). During overnight dialysis the salt concentration is reduced by diluting the dialysis buffer with low salt buffer while keeping the volume of the dialysis constant.
2. Set up a beaker with 600 ml DB-1 buffer at 4°C and prepare a larger container with 3 L DB-2. Use two peristaltic pumps and appropriate tubing to pump DB-2 at a rate of 3 ml/min into the dialysis beaker containing DB-1 while at the same time pumping the equivalent volume out of the diluted dialysis buffer into a waste container. This set-up assures that the volume of the dialysis reaction remains constant while the salt concentration is reduced.
3. Prepare the samples. Mix 60 µl 5 *M* NaCl, 82.25 µl TE, 3.75 µl 20 mg/ml BSA, and 4 µl (0.375 mg/ml) purified core histones (*see* **Note 13**).
4. Prepare the mini-dialysis chamber (**Fig. 2.2**). Detach the cap of a 1.5-ml reaction tube by cutting the connecting hinge with a pair of scissors. Cut the remaining tube at the 1.5 ml mark. The cut-off ring will serve as membrane clamp. Cut dialysis membrane (12-14,000 MWCO) to 2 cm × 3 cm pieces. Equilibrate membrane pieces to DB-1 for 30 min.
5. Pipet 50 µl of bead-DNA suspension (30 ng DNA/µl) into a reaction tube. Concentrate the beads on an MPC. Discard supernatant and wash once with 200 µl PBS-BSA-NP40.

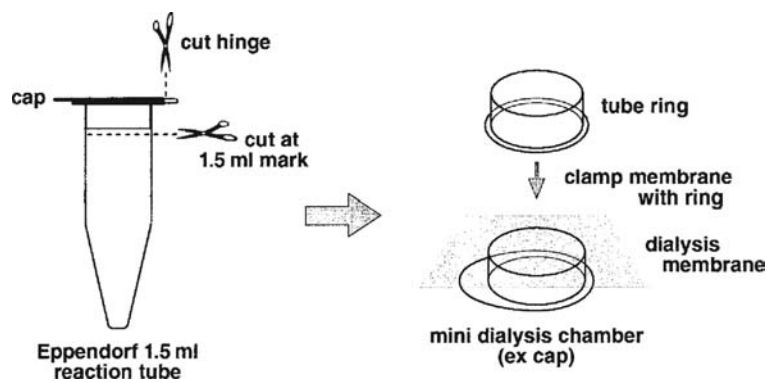

Fig. 2.2. The preparation of a mini-dialysis chamber (*see* **Section 3.2.2**). The entire dialysis chamber containing the nucleosome reconstitution is thrown into the beaker with dialysis buffer. The tumbling of the chamber due to the vigorous stirring will assure that the beads remain suspended during the 16 h dialysis. We acknowledge the help of Udo Ringeisen in preparing this figure.

Wash again with 200 µl of WB buffer (*see* **Section 2.1**). Concentrate beads, discard supernatant, and resuspend beads into the reaction mix prepared at **step 2**.

6. Transfer the suspension into empty, inverted Eppendorf tube cap. Place a piece of dialysis membrane on top and clamp membrane with the tube ring (*see* **Fig.** 2.2). A reaction volume of 150 µl should essentially fill the cap. Avoid trapping air in the cap which will interfere with dialysis.
7. Throw the closed dialysis cap(s) into the dialysis container and start stirring very fast. Vigorous stirring is essential for maintaining beads in suspension.
8. Dialyze for 45 min before you turn on the pumps to dilute the salt concentration. Dialyze until most of DB-2 has been pumped into the dialysis container (15–16 h).
9. Recover bead suspension by puncturing the membrane with a pipet tip. Transfer to a fresh reaction tube and process as desired.

3.3. Analysis of Reconstituted Chromatin

Reconstituted, immobilised chromatin can be purified from the reaction mix and analysed in various ways. Chromatin proteins can be separated by SDS-PAGE and visualised by Western blotting, silver staining, or even Coomassie staining, depending on the scale of the reaction. The quality of the reconstituted chromatin can be tested by visualisation of the correct histone stoichiometry and a regular nucleosomal array by micrococcal nuclease digestion. Chromatin association of proteins of interest can be tested and the correct stoichiometry of core histones verified.

3.3.1. Analysis of Chromatin Proteins (*see* Note 14)

1. If a smaller tube was used for chromatin assembly reaction transfer all liquid to Eppendorf tubes that fit into the MPC. The small tube may be rinsed with 100 µl EX-50-NP40 to ensure complete recovery. Concentrate on an MPC for 1 min (not longer!) and remove supernatant completely. Be careful not to touch the pellet with the tip. This may lead to losses.
2. Wash twice with 200 µl EX-50-NP40. Resuspend well each time by gently tapping the tubes. Do not pipette to resuspend. Remove all supernatant each time. If droplets are dispersed on the tube walls spin for 15 s at 1000 rpm in a benchtop minifuge if necessary.
3. Suspend beads in 7.5 µl of EX-Y1-NP40 for elution (*see* **Note 15**). Concentrate beads and save supernatant. Repeat and pool supernatant for PAGE (total volume 15 µl).
4. Wash beads with 200 µl of EX-Y2-NP40. Discard wash.
5. Proceed to the next salt concentration. Each elution is done by extracting twice in 7.5 µl (save for gel) and a large 200 µl wash (for completeness).

6. Place all samples for PAGE on the MPC for 2–3 min to remove any trapped beads. Recover supernatant into new tubes containing 5 µl 4 × SDS loading buffer.
7. Resuspend beads in 20 µl 1 × SDS-loading buffer. Incubate for 10 min at 37°C. Do not boil (*see* **Note 16**). Concentrate the beads and save supernatant. This sample represents the proteins that are not eluted even with the most stringent wash applied.
8. Denature all samples for 5 min at 95°C, separate by 15% SDS-PAGE.
9. Stain gel with silver or transfer to membrane for Western blotting.

3.3.2. Micrococcal Nuclease Treatment (*see* Note 17)

1. Assemble 900 ng of immobilised DNA into chromatin as described in **Section 3.2.1.** Concentrate chromatin on MPC and remove supernatant.
2. Wash chromatin twice with 100 µl of EX-Y-NP40 (*see* **Note 15**).
3. Wash beads with 50 µl EX-50-NP40.
4. Resuspend in 120 µl EX-50-NP40 containing 5 mM $MgCl_2$, prewarmed at 26°C.
5. Add 180 ml of MNase premix (168 µl EX-50, 9 µl $CaCl_2$, 3 µl MNase (5 u/µl) prewarmed at 26 °C (*see* **Note 18**).
6. After 30 s, 1 min and 8 min recover 100 µl into a tube containing 25 µl of nuclease stop mix and vortex briefly.
7. When all samples are processed, add 1 µl RNase (10 mg/ml) and incubate for 5 min at 37°C.
8. Add 2 µl 20% SDS and 5 µl proteinase K (10 mg/ml) and digest overnight at 37°C.
9. Concentrate beads on MPC and recover supernatant.
10. Add 90 µl 7.5 M ammonium acetate, pH 5.3 and 0.5 µl glycogen 20 mg/ml. Mix and add two volumes ethanol.
11. Leave on ice for 5 min and spin for 15 min at top speed in a benchtop centrifuge at 4°C.
12. Wash pellet carefully with 800 µl of 75% ethanol and air dry on the bench. Do not dry pellet in the speed vac as this may cause DNA denaturation!
13. Take pellet up in 8 µl TE and add 2 µl Orange loading buffer (5 ×).
14. Electrophorese on a 1.3% agarose gel in Tris–glycine buffer (*(4)*; *see* **Note 19**).

4. Notes

1. In order to immobilise a plasmid two restriction enzymes must be selected as follows (*see* also **Fig.** 2.1): The plasmid must be linearised with an enzyme leaving a 5′ overhang that can be filled in with biotin-21-dUTP or biotin-14-dATP with Klenow polymerase. In order to prevent the coupling via both ends (which may result in the shearing of the DNA) the linearised DNA must be restricted with a second enzyme leaving a site where no biotin will be incorporated during the fill-in reaction (e.g. blunt ends, 3′ overhangs or 5′ overhangs with GC-rich sequences). If the secondary cut results in two large fragments, a mixture of both fragments will be immobilised. If the secondary enzyme is chosen such that one large fragment and one very small fragment are produced, this fragment may be removed during the subsequent gel filtration step (**Fig.** 2.1). Ideally, the biotinylated residue should not be the last nucleotide to be incorporated during the fill-in reaction so that it can be protected against exonuclease activity by sealing the ends with a-thio-dNTPs (*see* **Note 3**). Some enzymes that we have used to create an end suitable for biotinylation are *Eco*R I, *Spe* I, *Afl* II, *Hin*d III, and *Sal* I. *Not* I and *Cla* I can be used for the other end. These enzymes produce 5′ overhang sequences lacking A or T residues which are not filled in with biotin-14-dATP or biotin-21-dUTP.
2. Incomplete restriction enzyme digestion may lead to low coupling efficiency. We routinely check completeness of digestion at each step. Therefore, even when two compatible restriction enzymes are utilised, we prefer to perform the digestions in two steps rather than in one step, in order to monitor digestion efficiency. Digest first with the enzyme that creates the end that will not be biotinylated and assure complete linearisation.
3. In order to protect the ends from exonuclease invasion that may occur in some experimental systems, we use a-thio-dNTPs in addition to the biotinylated dNTP to fill in the ends which increase the half-life of the ends in crude exonuclease-containing extracts considerably. Ideally, the biotinylated dNTPs should be shielded by 1–2 a-thio-dNTPs. In principle, other modified nucleotides (e.g. aminoallyl–dNTPs) which are easier to find could also be used instead of α-thio-dNTPs but we have not compared their efficiency.
4. Poor filling-in by Klenow DNA polymerase affects immobilisation. Avoid using ammonium acetate for DNA precipitation as it may inhibit the polymerase. Klenow Exo– is better

suited for this application than ordinary Klenow DNA polymerase.

5. Incomplete removal of unincorporated biotin is a common reason for inefficient coupling. Biotin reacts with streptavidin readily and may outcompete the immobilisation of DNA. Spin columns from different suppliers have diverse specifications which should be followed precisely.
6. Coupling efficiency drops drastically with increasing length of DNA to be immobilised. For some applications *(6)* long chromatin templates may be particularly useful. We describe here a protocol for efficient immobilisation of lambda DNA (50 kb) using the kilobase binder reagent from Dynal. Approximately 1 pmol of lambda DNA can be immobilized per 100 µl of Dynabeads.
7. If coupling was efficient the supernatant from the coupling reaction should be free of DNA (missing band test). In the case of incomplete immobilisation, comparison of band intensities serves to accurately estimate the percentage of immobilised template. Efficiencies higher than 95% are routinely obtained.
8. It is essential to dissolve the pellet completely at this step. Do not vortex to avoid shear. Allow a long time, if possible overnight, to dissolve DNA pellet. In general, minimise manipulations such as extensive pipetting that may shear the concentrated, viscous lambda DNA. We cut the end of the pipette tips with scissors to widen the tip opening. Avoid pipetting up and down in order to resuspend lDNA after its precipitation.
9. See Dynal's instructions for up-to-date effective concentration.
10. The amount of chromatin assembly extract to be added has to be determined empirically on soluble plasmid DNA. For each amount of extract used, chromatin assembly efficiency is monitored by MNase digestion and agarose gel electrophoresis in order to define the optimum *(4)*. In general 50–90 µl of extract is required for 900 ng of template in a 120 µl reaction. Once the optimal conditions are determined scaling up or down is feasible. If a small amount of immobilised DNA is to be assembled into chromatin it is advisable to fill the reaction up with soluble carrier DNA to keep reaction volume conveniently high rather than scaling down.
11. Reaction tubes of different sizes are used in order to match the volume of chromatin assembly reactions. If there is too much empty space in the tube, the reaction mixture spreads all over the surface of the rotating tube. When possible scale up the chromatin assembly reaction to fill up most of the tube. A small air bubble trapped in the tube will help to

maintain the beads dispersed in suspension. Since the magnetic field is much stronger close to the base of the tube, we avoid using relatively big volumes (greater than 600–700 μl) per tube as this would increase the duration of the concentration (in a viscous milieu this can lead to incomplete recovery). Thus when it is necessary to concentrate a greater volume (e.g. when conditioning a great volume of bead suspension for coupling reaction) split the reaction into aliquots and concentrate them successively. After the first aliquot of beads is concentrated and the supernatant discarded, the second aliquot is added to the tube and so on. If the reaction volume is very low, use small (250 μl), elongated tubes. In this case apply the reaction mixture to the bottom of the tube avoiding contact with its walls. The droplet of the reaction mixture will remain at the bottom of the tube due to surface tension.

12. The nucleosome assembly by salt dialysis is a modification of the one described by Neugebauer and Hörz *(14)*. For further descriptions of salt gradient dialysis procedures *see (15, 16)*. Here we concentrate on those modifications to the procedure required when working with immobilised DNA.
13. A ratio of purified core histones to DNA of 1:1 reproducibly results in efficient nucleosome assembly. However, an empirical titration of core histones using soluble DNA may be required. As an internal control in the assembly reactions a short, radioactively labelled and gel purified PCR fragment may be added in the same dialysis chamber with the immobilised DNA. This will serve to determine the efficiency of nucleosome assembly by a band shift assay. Complete nucleosome assembly results in a shift of the probe from free to mononucleosome band.
14. A background of proteins sticking non-specifically to the bead matrix itself is anticipated. Consider the following parameters to optimise the signal-to-noise ratio. First, maximise the amount of DNA per bead by adding an excess of biotinylated DNA in the coupling reaction. Second, pre-adsorb the beads by washing them a couple of times in a buffer containing 0.01% w/v BSA and 0.05% v/v nonidet P 40. This decreases background and also enables easier handling of the beads by reducing their stickiness. Third, different suppliers provide beads with different matrix characteristics. In our experience, Dynabeads gave a low background when used with *Drosophila* embryo extracts.
15. You have the option to elute proteins sequentially with washes of increasing salt to determine how tightly a protein interacts with chromatin. In general, a buffer containing 400 mM KCl strips off most of the chromatin-associated

proteins while core histones require high salt (2 M) for their elution. In the following protocol substitute salt concentration (Y1= salt 1 in mM) in the buffers according to your application.

16. Many proteins that interact with the bead matrix per se are not eluted in SDS-loading buffer unless the beads are boiled. By contrast, chromatin proteins (including histones) are stripped from DNA without boiling. Therefore it is very important to omit boiling of the beads.
17. MNase digestion can be performed with or without prior purification of the template. Here we describe a protocol for MNase digestion of purified chromatin that has been washed. In the case of nuclease treatment without isolation of the DNA from the assembly reaction, approximately ten times more MNase units are required. Conversely, the more stringent the washings of chromatin are the less the nuclease is needed.
18. Upon addition the MNase mix, pipette up and down a couple of times to suspend beads. During longer incubation times resuspend beads once by tapping the tube. Alternatively use the Eppendorf Thermomixer at setting 10.
19. The appearance of the characteristic, ladder-like pattern of DNA fragments generated by MNase analysis and subsequent agarose gel electrophoresis is slightly compromised because only those fragments that are cleaved off the beads (by a double-stranded cut) are recovered for electrophoresis. Since underdigestion may result in only very little DNA on the gel, fine-tuning of the MNase digestion may be required.

    The nucleosome repeat length of immobilised chromatin assembled in *Drosophila* extracts is a bit shorter compared to chromatin assembled on plasmid template under identical conditions. This difference is not due to the immobilisation, but rather reflects a difference between linear and supercoiled DNA *(17)*.

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