

Nucleosome mobilization assay (PROT24)



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Introduction

The organization of eukaryotic genomes into nucleosome arrays restricts DNA sequence accessibility to many nuclear factors. Thus most DNA-based processes require opening (or "re-closing") of these arrays. One major class of enzymes, the "chromatin/nucleosome remodeling" factors, uses ATP hydrolysis to alter the canonical histone-DNA contacts. The term "nucleosome remodeling" can be defined and monitored in different ways (Flaus and Owen-Hughes, 2004). The simplest configuration to study one aspect of nucleosome remodeling is to use a purely reconstituted system consisting of mononucleosomes and an ATP-dependent nucleosome remodeler in the so-called "nucleosome sliding" or "nucleosome mobilization" assay. This technique was initially developed by Carl Wu and Peter Becker laboratories (Hamiche *et al.*, 1999; Langst *et al.*, 1999) by taking advantage of two nucleosome properties: Nucleosomal histones can moderately move on DNA under rather mild temperature and salt conditions (Beard, 1978; Meersseman *et al.*, 1991; Pennings *et al.*, 1991) and nucleosomes reconstituted on a short DNA fragment can adopt multiple positions that can be separated by native gel electrophoresis (Linxweiler and Horz, 1984; Pennings *et al.*, 1991).

In fact, the sliding assay monitors alterations in nucleosomes electrophoretic mobility in native gel that are caused by remodeling factors in an ATP-dependent manner. Repositioning of the histone octamer along a DNA fragment usually accounts for these mobility shifts. However, changes in electrophoretic mobility can also result from an altered (non-canonical) nucleosomal DNA path (Kassabov *et al.*, 2003; Narlikar *et al.*, 2001). Hence, actual repositioning of the histone octamer may need to be confirmed by mapping of the new positions for uncharacterized remodelers. It is also noteworthy that mononucleosomes do not recapitulate all chromatin properties (Hansen, 2002). Consequently, all conclusions derived from using this substrate may not always apply to nucleosome arrays (even not to other mononucleosomes using a different [DNA template](#)). Despite these caveats, the sliding assay is still a powerful tool that has greatly contributed to our understanding of how chromatin remodeling factors work.

Acknowledgements: I am grateful to Gernot Längst and Anton Eberharter for introducing me to this great technique.

Procedure

Preparation of the nucleosome substrate

Nucleosomes can be assembled using various methods (Chromatin Protocols, 1999 and ref. therein). The central theme to all these methods is mixing histones and DNA together, roughly around a 1:1 ratio (w/w).

Before getting into the details of nucleosome assembly, it is important to prepare the two reagents (DNA and histones) with special cares as follows:

Preparation of the [DNA template](#)

See [note 1](#)

As mentioned above, the nucleosome sliding assay relies on the fact that the location of a histone octamer on a DNA fragment

affects its electrophoretic mobility in native polyacrylamide (PAA) gels. Centrally positioned nucleosomes migrate slower than nucleosomes positioned at one end of a DNA fragment.

In this assay, DNA templates between 200 bp and 300 bp will be preferred for the following reasons: DNA fragments in this size range should allow the formation of essentially one nucleosome per template. Using longer DNA fragments would require performing the assembly at lower histone: DNA ratios (around 0.6:1) to avoid assembly of more than one nucleosome per [DNA template](#). This would result in lower mononucleosome assembly yields. More importantly, having a limited number of nucleosome positions (ideally two positions) greatly simplifies the readout of this assay (Note that a very strong positioning sequence on a long DNA fragment may lead to having one position after assembly and a weak positioning sequence may still lead to getting several positions on a small [DNA template](#)).

DNA fragments shorter than 200 bp can also be used however some remodelers require a minimal length of DNA protruding from the nucleosome core to perform the remodeling reaction (typically, ISWI subfamily members, (Aalfs *et al.*, 2001; Zofall *et al.*, 2004)) and separation of the various nucleosome positions is less apparent with such fragments. It is also worth mentioning that not all DNA sequences can be efficiently assembled into nucleosome (Cao *et al.*, 1998).

For all these reasons, it is convenient to use the 247 bp (or 248 bp) murine rDNA promoter fragment.

PCR cycles (see [comment 1](#) and [note 2](#))

94°C for 2 minutes / (94°C for 15 seconds / 60°C for 30 seconds / 72°C for 30 seconds) x30 cycles / 72°C for 5 minutes.

DNA precipitation

1. Precipitate PCR product by adding 1/10 volume of 3M Na-Acetate pH 5.2 and 2.5 volumes of ice-cold ethanol;
2. Leave on ice for 20 minutes;
3. Collect DNA by centrifugation at 13000Xg for 30 minutes at 4°C;
4. Wash pellet with ice-cold 70% ethanol;
5. Dry (partially) and resuspend DNA in 100µl volume of TE.

Gel purification of the DNA (see [comment 2](#))

1. Pour a 4.5% PAA (0.5X TBE) mini-gel (classic protein gel dimensions) with at least 2 wells. A large one that allows loading of the PCR ($\geq 110\mu\text{l}$) and an additional well to load running dyes (orange G, bromophenol blue and xylene cyanol) to follow the migration;
2. Rinse wells (with a syringe) and pre-run gel at 80-100V for about 45 minutes;
3. Add 10% glycerol (v/v) to the PCR product, mix and load on the 4.5% PAA gel;
4. Run until orange G reaches the bottom of the gel. Using TBE, there is no need for buffer recirculation like for TE gels;
5. From now on, handle the gel behind a Plexiglas screen. Open gel (leave on one plate) and cover with Saran™ wrap;
6. Stick phosphorescent position markers on 2 opposite sides of the gel, place a film on the gel and put a small glass-plate on top to maintain film against the gel for 10-15 minutes (in a dark room);
7. Develop film and make a hole around the band of interest BEFORE overlaying the film on the gel;
8. Place the film according to the position markers and cut through the Saran™ wrap into the gel following the outline of the hole using a clean scalpel;
9. Place the gel slice in 1.5ml tube containing 1ml of TE (10mM TRIS-HCl pH 8.0, 1mM EDTA) and allow diffusion of the DNA out of the gel for about 2 hours;
10. Recover the supernatant and repeat DNA elution with 1ml of TE for at least 2 hours (preferably overnight);
11. Combine supernatants and roughly monitor elution efficiency with a Geiger counter by comparing supernatants to gel slice (take into consideration that the aqueous solution reduces the radioactive signal);
12. Optional: If a 'significant' amount of DNA is left behind in gel slice, repeat DNA elution with another 1ml of TE overnight;
13. Combine all eluates and precipitate the DNA in 15ml tube as described above (in the DNA precipitation section);
14. Resuspend DNA in about 100µl volume of TE containing 150mM NaCl (TE150);
15. Determine DNA concentration by using a spectrophotometer or by running 1µl and 2µl of DNA on a 1.5% agarose gel along with a cold DNA fragment of known concentration (e.g. DNA size markers);

Depending on the efficiency of all the previous steps and the option you choose, the final DNA concentration may range from 100-800ng/µl. (see [note 3](#))

Preparation of the Histone-PGA (HP)-mix

As mentioned above, mononucleosomes can be assembled in different ways. However, when handling radioactive DNA and/or small amounts of material, it is very convenient to use the following method developed by Stein and colleagues (Stein *et al.*, 1979).

Poly-L-glutamic acid (PGA) is a negatively charged polymer, which can force histones to form octamers in low salt conditions. It can also prevent histones from precipitating onto DNA when both are mixed under these ionic conditions. The PGA polymers then progressively exchange with DNA molecules allowing nucleosome assembly (see [note 4](#)).

HP-mix

1. Prepare a 10mg/ml PGA (Sigma P4886; MW: 50,000-100,000) stock solution in water (keep aliquots at -20°C);
2. Add a 2-fold weight excess of PGA to histones. Flick tube about 6-10 times;
3. Adjust salt concentration to 150mM NaCl with TE (pH 8.0); gradually fill up with TE150 to a final histone concentration of 50-100ng/μl.
Typical reaction
25 μl histones (2μg/μl, in 1M NaCl/50% glycerol) = 50μg histones
10μl PGA (10μg/μl) = 100μg PGA
132μl TE
833μl TE150;
4. GENTLY pipette up and down 3-4 times and leave at room temperature for 1 hour;
5. Spin down possible aggregates at 13000Xg for 10 minutes and transfer into fresh tubes;
6. Aliquot supernatant (200μl each) and store at -20°C (see [note 5](#))

Nucleosome assembly

The optimal nucleosome assembly is usually obtained around a histone:DNA ratio of 0.9:1 (w/w). However, the affinity of DNA sequences for the histone octamer can vary significantly (Thastrom *et al.*, 2004; Wu and Travers, 2005). Furthermore, histone preparations of slightly different quality may also affect optimal assembly concentrations. Therefore it is important to carefully determine the most advantageous histone:DNA ratio experimentally (for every new DNA or new histone/HP-mix preparation) before setting up a large assembly for the best results. A meticulous titration is especially important here since preparative nucleosome reconstitutions often require to scale up test assemblies about 200 folds.

Test assembly

This "test" consists of adding increasing amounts of HP-mix to about 50ng of radiolabeled-DNA (gel-purified PCR).

Typical titration

1. Dilute HP-mix 1:8 in TE150 containing 0.4μg/μl BSA (prepare a 12μl dilution);
2. Make a DNA master mix for 10 reactions as follow:
5μl of DNA (i.e. if DNA around 100ng/μl)
1.8μl BSA (20μg/μl)
83.2μl TE150;
3. Distribute 9μl of the DNA mix into 9 tubes;
4. Add 0.4μl / 0.8μl / 1.2μl / 1.6μl / 2.4μl / 3.2μl of 1:8-diluted or 0.5μl and 1μl of undiluted HP-mix to one tube of DNA mix (Keep one control tube without HP-mix);
5. Incubate for at least 1 hour 30 minutes at room temperature (see [note 6](#));
6. Pour a 4.5% PAA 0.4X TBE gel, 14x16x0.15cm (doing it before setting up assembly saves time);
7. Rinse wells (with a syringe) and pre-run gel at 110V for about 1-2 hours (during incubation time);
8. Add about 5-10% glycerol (v/v) to each test assembly tube (i.e. 2μl of 50% glycerol) and mix by slowly pipetting up and down 2-3 times;
9. Rinse wells of the gel again, load the test assembly on the gel and run until the orange G reaches the last third of the gel (around 2 hours 30 minutes);
10. Dry and expose the gel for 10 minutes against a Phosphorimager screen (or to film for 1 hour at -80°C against an intensifying screen).
11. Develop screen/autoradiography ([see figure 1](#)).

Interpretation of the titration

As increasing amounts of histones are added to the DNA mix ([figure 1a](#) lane 2 to 9 and [figure 1b](#) lane 2 to 7), a doublet (the 2 positioned nucleosomes) appears above the free DNA and it gradually gets stronger in intensity. At elevated histone concentrations higher-molecular weight histone-DNA complexes become visible ([figure 1a](#) lane 7 to 9 and [figure 1b](#) lane 5 to 7, *). These complexes have a histone-DNA ratio that is too high (note that end-positioned nucleosomes get preferentially up-shifted as they offer longer stretches of "free" DNA for the histones to bind). Ultimately, if more histones would be added (or if the titration would be off range) the DNA would end up engaged in larger histone-DNA complexes (a.k.a. aggregates) that would hardly enter the gel.

In this titration, the optimal DNA:HP-mix ratio is probably between 0.4μl (3.2μl of 1:8-diluted, [figure 1a](#) lane 7) and 0.5μl of HP-mix ([figure 1a](#) lane 8). At this ratio, nucleosomes are assembled with moderate formation of "aggregates". However, in these assemblies a small proportion of histones sticks to the surface of the tube and will not be used to assemble nucleosomes. In a larger volume, this effect will be less pronounced as a proportionally smaller volume of the reaction will be in contact with the tube. Therefore when scaling up, take this surface/volume effect into accounts and use about 10-15% less histones than in the test assemblies.

Preparative nucleosome assembly

(Optimal test assembly scaled up x160):

0.5µl DNA x160 = 80µl

0.4µl x160 = 64µl HP-mix

2.9µl BSA (20µg/µl)

1. Pipette first BSA and DNA, mix and then add HP-mix progressively;
2. Incubate at RT for 3 hours (or overnight).

Nucleosome purification

Preparative gel

1. Pour a 4.5% PAA 0.4X TBE gel (14x16x0.15cm) with a large well that will allow loading of the whole nucleosome assembly (about 2x2x0.15cm) and an additional well for loading of the running dyes;
2. Rinse wells and pre-run gel for 2 hours;
3. Add 5-10% glycerol to the nucleosome assembly prior to loading it on the gel;
4. Rinse wells, load the assembly and run at 110V until the orange dye runs off + 1 extra-hour (or overnight at 60V).

Isolation of the positioned mononucleosomes

It is very important not to let the gel dry out during the following steps. And again, handle gel behind a Plexiglas screen.

1. Open gel (leave on one plate) and quickly pour 4-5ml of cold [Nucleosome elution buffer](#) (without BSA) onto the gel (where you expect the nucleosomes to be) and wrap with Saran™ film;
2. Stick phosphorescent position markers on 2 opposite sides of the gel; place a film on the gel and put a small glass-plate on top to maintain the film against the gel for 10-15 minutes (in a dark room);
3. Develop film and make a hole around the nucleosome bands (and DNA if you like) BEFORE overlaying film on gel;
4. Place film according to the position markers and cut through Saran™ into the gel following the outlines of the holes using a clean scalpel;
5. Remove the autoradiography and let 200-300µl of [Nucleosome elution buffer](#) drop onto the Saran™ film around the cuts (this will make sure that the nucleosomes do not dry out and this will facilitate handling of the gel slices as they'll become less sticky);
6. Take away the pieces of cut Saran™ wrap and place (using a scalpel) the gel slices into a 1.5ml tube containing 250-300µl of [Nucleosome elution buffer](#) for about 5 minutes (to wash away the TBE) (see [comment 3](#) and [note 7](#));
7. Discard elution buffer and replace by the same volume of fresh buffer;
8. Put the tubes in the fridge and allow diffusion of the nucleosomes for about 2-3 hours (see [comment 4](#));
9. Recover the supernatant and repeat the nucleosome elution for at least 3-4 hours (preferably overnight);
10. Spin down (potential) gel particles at 13000Xg for 5 minutes, at 4°C, and transfer into a fresh tube;
11. Check the quality of the eluates on a 4.5% PAA 0.5X TBE gel ([figure 2](#), lane 2) and store nucleosomes at 4°C (do not freeze!) (see [note 8](#)).

Nucleosome mobilization ("sliding") assay

Typical reaction (10µl)

1-3µl of positioned nucleosome (=about 50-100 fmol)

0.2µl BSA (20µg/µl) -> 0.4µg/µl final

0.1µl ATP (100mM) ->1mM final

Fill up with [Nucleosome elution buffer](#) to 9µl

1. Prepare a master mix for the number of desired reactions +1, (it is always good to include a minus ATP control);
2. Prepare serial dilutions of the remodeler (i.e. from 45 to 450 fmol) (see [note 9](#));
3. Add 1µl of enzyme dilutions to the nucleosome mix and incubate for 90 minutes at 26°C (This temperature is optimal for a *Drosophila* enzyme like dMi-2, a different temperature may be used for other enzymes);
4. Stop reaction by addition of about 500ng-1µg of unlabeled plasmid DNA, flick tube 6-10 times and incubate on ice for 10 minutes (to compete the remodeler off the nucleosome);
5. Load directly onto 4.5% PAA 0.4X TBE gel (already pre-run and with rinsed wells as described above);
6. Run at 100-120V for about 2.5-3 hours (until Orange G reaches the last third or the bottom of the gel);
7. Dry gel prior to expose to film overnight at -80°C (against intensifying screen);
8. Develop film and enjoy the result (optional: while eating a piece of chocolate) (see [note 10](#)).

Materials & Reagents

DNA template	Sequence of the mouse rDNA promoter fragment, 247 bp (between -232 and +16 relative to the transcription start site):
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5'-**GAAAGCTATGGGCGCGGTTTTCTTTTCATTGACCTGTCGGTCTTATCAGTTC**
TCCGGGTGTGTCAGGGTCGACCAAGTTGTTCCCTTTGAGGTCCGGTCTTTTCGTTA
TGGGGTCATTTTTGGGCCACCTCCCCAGGTATGACTTCCAGGCGTCGTTGCTC
GCCTGTCACTTTCCTCCCTGTCTCTTTTATGCTTGTGATCTTTTCTATCTGTTC
TATTGGACCTGGAGATAGGTA**CTGACACGCTGTC**-3'

Primers (bold above)
rDNA_fw: GAAAGCTATGGGCGCGGTT
rDNA_rev: GACAGCGTGTGTCAGTACCTATCT

Standard PCR reaction **Standard PCR reaction (1ml) (see comment 1)**

dNTP: 200μM of each
Primers (fw and rev): 0.6μM of each
DNA template: 100ng
10X AmpliTaq Buffer (Roche): 100μl
Fill up to 990μl with sterile, bidistilled H₂O.
Add 3μl (=60μCi) of [α -³²P]dCTP (6000Ci/mmol, PerkinElmer)
Taq polymerase: 7.5μl (of 5u/μl)

Nucleosome elution buffer 10mM Tris-HCl (pH 7.5)
50mM KCl
10% glycerol
0.4μg/μl BSA

Authors Notes

1. In this protocol, the DNA fragment is body-labeled and generated by PCR. However, DNA fragments can also be generated by restriction enzyme digestion and subsequently end-labeled.
2. Be careful it's quite hot! Handle with all precautions conform to working with radioactive material.
3. It appears to me as if gel purification of this DNA fragment is important to get "good" nucleosomes (that slide). I suspect that traces of single-stranded DNA resulting from the PCR (which actually comigrates and copurifies with end-positioned nucleosome after assembly, see below) inhibit sliding.
4. Use siliconized/low-retention tubes when handling histones or nucleosomes, as they tend to be sticky. This will also minimize surface/volume effects when scaling up nucleosome assembly.
5. Avoid numerous freeze-thaw cycles as this may result in aggregates formation.
6. You may want to do a much broader titration to make sure you cover the optimal assembly histone:DNA ratio.
7. Since you are dealing with valuable material you may want to be on the safe side by also cutting 3mm above and 3mm below the bands you just cut out and place these gel slices into a tube containing [Nucleosome elution buffer](#) as well.
8. Like for DNA, one can roughly monitor elution efficiency with a Geiger counter by comparing the supernatants to the remaining gel slices. The second (and if enough material left) the third elutions can actually be nicer (less free DNA if any) than the first one. Using this DNA fragment, the centrally-positioned nucleosome can also be purified away from the free DNA by ultracentrifugation on a 10 to 30% glycerol gradient ([figure 2](#)).
9. It is necessary to initially titrate the remodeler over a very broad range to determine its optimal concentration in the assay (for each protein preparation). Both not enough remodeler and an excess of remodeler can result in poor remodeling. While the first case is self-explained, it is likely that an excess of enzyme presumably "coats" the nucleosome substrate and inhibits sliding by other remodeler molecules.
10. Remodeling by some enzymes can lead to partial mononucleosomes disassembly, which results in the appearance of free DNA (e.g. [figure 3 lane 4](#)) in an ATP-dependent manner. However, if the nucleosome concentration used in this assay is too low, nucleosomes may fall apart and free DNA may also appear in the absence of remodeler.

The sliding assay is essentially a qualitative assay. However, by doing careful titrations and time courses, semi-quantitative assessments may be made by comparing lanes that did not reach the end-point of the reaction.

Nucleosome EMSA ("bandshift assays") can be performed in the same conditions as the sliding assay by omitting the addition of competitor DNA (and ATP) and by simply incubating of the reaction on ice for 10 minutes prior to loading.

Reviewer Comments

Reviewed by: [Patrick Varga-Weisz](#), Babraham Institute, Cambridge, United Kingdom.

1. We use the ROCHE Expand enzyme for amplifying the DNA fragment.
2. An especially important point is that it is essential to gel purify the DNA after PCR, for obtaining pure center positioned nucleosomes, at least when using the 247bp (or 248bp) murine rDNA promoter fragment for nucleosome assembly. This

is, as stated by Karim, because single stranded DNA, a by-product from the PCR reaction, co-migrates with this nucleosome species and seems to inhibit the sliding reaction. We made the same observation.

3. We do not bother to keep the gel slices moist by adding elution buffer during or after "gel surgery".
4. We elute the nucleosome from the gels at room temperature - rather than at 4°C - over several hours up to overnight.

Figures

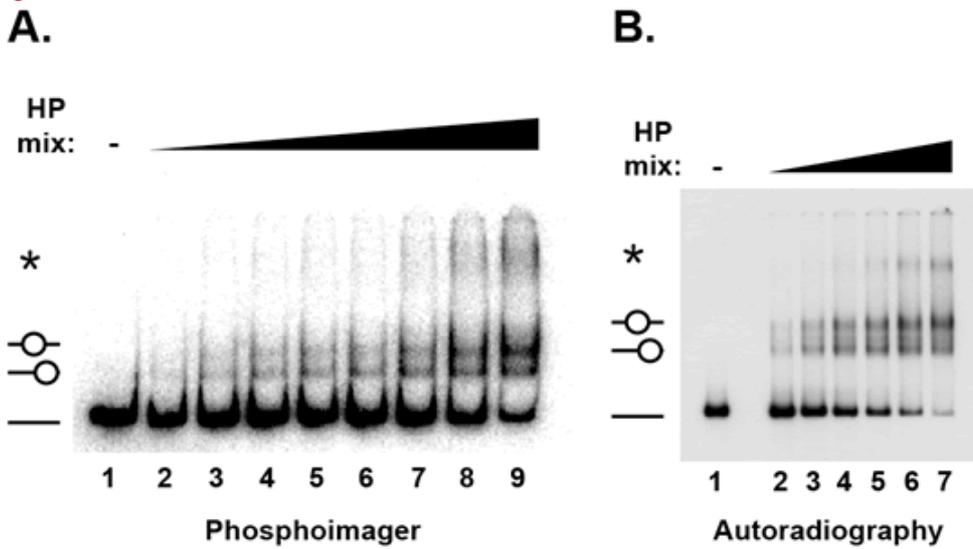


Figure 1: Typical nucleosome test assemblies.

(A): The typical test assembly described above was exposed to a Phosphoimager screen. Lane 1, free DNA (control tube without HP-mix), lane 2 / 3 / 4 / 5 / 6 / 7 / 8 and 9, DNA +0.4 μ l / 0.8 μ l / 1.2 μ l / 1.6 μ l / 2.4 μ l / 3.2 μ l of 1:8-diluted / 0.5 μ l and 1 μ l of undiluted HP-mix, respectively. (B): An example of a different test assembly exposed to film is shown. The asterisks denote histone-DNA complexes with an excess of histones and a schematic representation of free DNA (line) and positioned nucleosomes (lines with circle) indicates their positions on the left of the gels.

Purified positioned nucleosomes

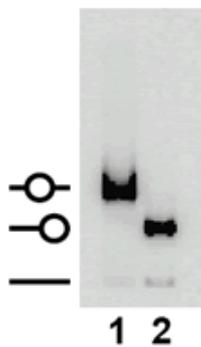


Figure 2: Purified positioned nucleosomes.

Lane 1: example of a glycerol gradient-purified centrally-positioned mononucleosome. Lane 2: example of a gel-purified end-positioned mononucleosome.

Nucleosome mobilization assay

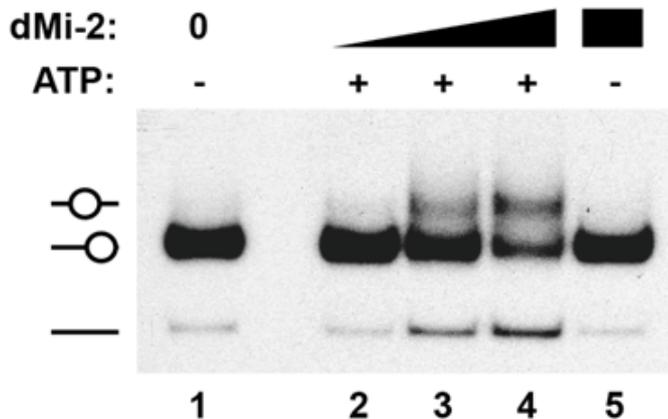


Figure 3: Nucleosome mobilization assay.

Lane 1: Purified end-positioned nucleosome. Lane 2 to 5: 90 fmol (lane 2), 180 fmol (lane 3), or 450 fmol (lane 4 and 5) of dMi-2 were incubated with end-positioned mononucleosomes in the presence or absence of ATP as indicated at the top. Positions of mononucleosomes (line with circle) and free DNA (line) are shown on the left.

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Chromosome mobilization

