

Isolation of nuclei for chromatin analysis in fission yeast

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ABSTRACT

The methods available for analysis of the chromatin of *Schizosaccharomyces pombe* are time consuming (>8 h) and/or result in some degradation of the chromatin. Here we report an optimised method for the preparation of spheroplasts and the isolation of nuclei which takes <25 min and is suitable for analysis of chromatin structure by micrococcal nuclease, restriction endonuclease or by immunoprecipitation.

Analysis of chromatin structure in yeasts requires the preparation of spheroplasts by removal of the cell wall using enzymes in preparations such as Novozym (Nova Labs, Denmark or Calbiochem) or Zymolyase 20T (Yeast Lytic Enzyme; ICN) prior to further manipulations and treatment with enzymes that digest the chromatin (1).

Godde and Widom have evaluated all previously published work on the preparation of nuclei or broken cells from the fission yeast and examined the nucleosome repeat length (156 ± 2 bp) in these preparations (2). Many of the methods for preparing nuclei or broken cells from *Schizosaccharomyces pombe* are time consuming (2,3) and some lead to a progressive degradation of the chromatin structure, 'nucleosome sliding' and degradation of the DNA within the nucleosome (2). Here we describe a reliable and rapid method for the analysis of chromatin structure in *S.pombe* strain 557 (*h⁻*, *ade6-M210*, *ura4D-18*, *leu1-32*). To assay for the integrity of the chromatin in our preparations, we measured the nucleosome repeat length in bulk chromatin. To assay for the action of non-specific nucleases, we probed Southern blots for the central core region in the centromere of chromosome II which has an open structure and is very sensitive to degradation (3–5).

We encountered two major problems in developing rapid techniques. The first was in the preparation of spheroplasts and the second was the activity of endogenous nucleases (1,2). We observed batch to batch variation with preparations of Novozym. Some batches consistently yielded degraded genomic DNA in spheroplasts (data not shown). Successful production of spheroplasts using Zymolyase was achieved after pre-treatment of 1 ml of cells, harvested at between 1 and 2×10^7 cells/ml and resuspended in 1.2 M sorbitol in a 2 ml microcentrifuge tube at 1×10^9 cells/ml, for 5 min at 20°C with 14 mM 2-mercaptoethanol and 2.5 mM EDTA followed by the addition of 25 mg Zymolyase.

This mixture was incubated for 5 min at 30°C. For larger preparations, up to 10^{10} cells at 10^9 cells/ml, incubation with Zymolyase should be increased to ~15 min. The optimum time is before the cells show total lysis when made 5% with SDS. At this stage no gross morphological changes have occurred. However, the cells do bulge out very slightly at the edges and ends and the black refringence typically seen around the cell wall in light microscopy is just starting to disappear. For small preparations, the cells were harvested at 20°C in a microfuge by spinning at full speed for 8 s. The cells were washed twice in 1 ml of 1.2 M sorbitol by inverting the microfuge tube twice and then spinning the residual pellet from one side of the tube to the other at full speed for 8 s. For larger preparations, the cells were harvested at 2.5 kr.p.m. for 5 min at 4°C and washed twice in 1.2 M sorbitol. Under-digestion of the cells leads to poor nuclei/DNA yields whereas over-digestion of the cells leads to the activation of endogenous nuclease(s) and DNA degradation.

In budding yeast, permeabilization of spheroplasts is a rapid and effective way of introducing nuclease to the chromatin (6,7). Treatment of *S.pombe* spheroplasts with 0.075% NP-40 in micrococcal nuclease (MNase) digestion buffer (MNDB; 1.2 M sorbitol, 10 mM CaCl₂; 100 mM NaCl, 1 mM EDTA, 14 mM 2-mercaptoethanol, 50 mM Tris-HCl pH 8.0) for 5 min resulted in smeared total genomic DNA suggesting the detergent permeabilization is releasing/activating an endogenous nuclease (data not shown). In an attempt to remove endogenous DNase activity we prepared nuclei from spheroplasts using Ficoll.

For small preparations (~ 10^9 cells), 0.05 ml of 18% Ficoll solution [Ficoll 400 (Pharmacia), 20 mM HEPES pH 8.0, 0.1 mM EDTA] at 4°C was added to the spheroplast pellet. The spheroplasts were lysed by gently rubbing the pellet with a yellow tip until the slurry was a uniform consistency. A further 0.05 ml was added and mixed using the yellow tip. Ficoll solution (9%, 1 ml) was added and mixed by gentle inversion. The tubes were spun at full speed in a microfuge for ~12 s. The pellet was washed in cold 1 M sorbitol and the nuclei recovered in a pellet by spinning at full speed for 8 s. For larger preparations (> 10^9 cells), nuclei were prepared in round bottomed polycarbonate centrifuge tubes using 0.2 ml of 18% Ficoll solution and a rounded 4 mm thick glass rod. A further 1 ml of 18% Ficoll solution was added to the cell slurry, then 4.8 ml of 9% Ficoll solution. The slurry was mixed into the 9% Ficoll by gentle inversion. The tubes were centrifuged at 20 g at 4°C for 20 min,

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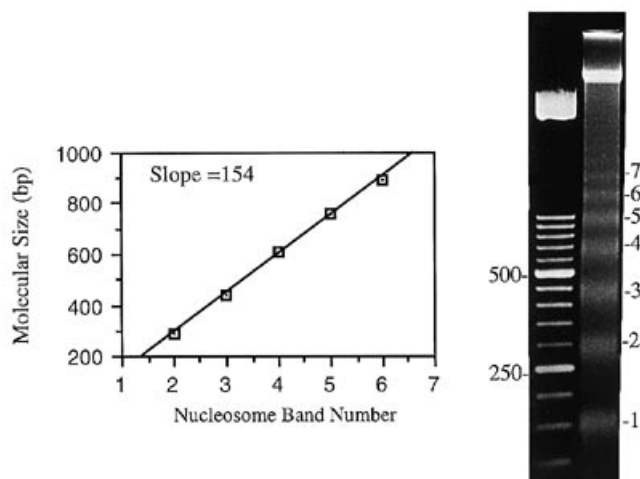


Figure 1. Estimation of nucleosome repeat length. Ethidium bromide stained DNA extracted from nuclei digested with 300 U/ml micrococcal nuclease for 4 min at 37°C. DNA was separated on a 2.5% gel in 1× TBE with a 50 bp ladder. The nucleosome repeat length was calculated from the slope of the size at the midpoint of the nucleosome bands plotted versus the band number. The slope was calculated using a least squares computer programme and is equal to 154 bp.

the pelleted nuclei washed in cold 1 M sorbitol and re-pelleted at 9 g for 10 min.

After removal of the supernatant, the nuclei were resuspended in buffer [MNDB or restriction endonuclease buffer (REB); 1.2 M sorbitol, 2 mM MgCl₂, 10 mM CaCl₂, 1 mM EDTA, 14 mM 2-mercaptoethanol; 500 mM Tris pH 8.0 plus 100 mM NaCl for *EcoRI*, *BamHI* and *NcoI* or 50 mM NaCl for *HindIII*] to a density of 1–2 × 10⁹ nuclei/ml. Successful lysis is indicated by the white nuclei enriched pellet; unlysed cells and membrane debris will appear creamy coloured. Light microscopy of a DAPI stained nuclei enriched pellet revealed a high proportion of discrete nuclei, membrane debris, ghosts and some unlysed cells. The unlysed cells may account for the residual high molecular weight DNA present after nuclease digestion of some samples prepared using this method.

Aliquots (0.1 ml) of nuclei enriched preparations were added to 0.1 ml of MNDB containing between 1.5 and 120 U MNase (Pharmacia) and incubated for 4–64 min at either 30 or 37°C. Reactions were stopped by adding 20 µl of 250 mM EDTA/5%

SDS followed by one phenol:chloroform extraction. The supernatant was incubated for 30 min at 37°C with 0.004 ml of 10 mg/ml RNase A, DNA extracted with phenol:chloroform and precipitated with isopropanol. The precipitate was collected using a 10 min spin at full speed in a microfuge and washed with ice cold 80% ethanol and resuspended in 0.02 ml of TE. Half the DNA was mixed with 0.002 ml of 50 bp ladder (Boehringer Mannheim) to aid in the accurate sizing of the nucleosome ladders. A nucleosome repeat length of 154 was calculated from the slope in a plot of average DNA size in *n*-mer oligonucleosomes versus *n* (*n* = 2–6) excluding the mononucleosome for the preparation in Figure 1. The nucleosome repeat length did not change with time of incubation (7.5 U/ml for up to 66 min) or with increasing amounts of micrococcal nuclease (up to 300 U/ml). No degradation of the total genomic DNA or the centromeric central core DNA was observed in nuclei prepared using this method after 15 min incubation at 37°C (data submitted but not shown). This method can be used successfully to study chromatin dynamics in synchronised cultures of *S.pombe* (after release from a hydroxyurea block) in real time.

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