Chapter 25

Salt Fractionation of Nucleosomes for Genome-Wide Profiling

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Abstract

Salt fractionation of nucleosomes, a classical method for defining "active" chromatin based on nucleosome solubility, has recently been adapted for genome-scale profiling. This method has several advantages for profiling chromatin dynamics, including general applicability to cell lines and tissues, quantitative recovery of chromatin, base-pair resolution of nucleosomes, and overall simplicity both in concept and execution. This chapter provides detailed protocols for nuclear isolation, chromatin fragmentation by micrococcal nuclease digestion, successive solubilization of chromatin fractions by addition of increasing concentrations of salt, and genome-wide analyses through microarray hybridization and next-generation sequencing.

Key words: Salt extraction, Nucleosome solubility, Chromatin organization

1. Introduction

Dynamic chromatin organization maintains DNA compaction while allowing for accessibility during active processes, such as transcription (1). These active processes are regulated through the action of nucleosome remodeling, histone modifications and variants, and chromatin-associated proteins. A variety of methods have been developed to study chromatin dynamics. Traditional methods utilize DNA cleavage systems coupled with chromatin probing, such as DNase I hypersensitivity (2–4) and micrococcal nuclease (MNase) (5, 6) mapping assays, which, respectively, measure chromatin accessibility and nucleosome occupancy using nuclease digestion. Other methods rely on chromatin solubility or partitioning differences that occur after formaldehyde cross-linking (FAIRE, Sono-Seq) (7, 8). In addition, there are methods that measure chromatin dynamics directly by mea-

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suring nucleosome turnover, either using protein-encoded tags (9) or metabolic labeling of histones (10). Protein-related information can be obtained using either chromatin immunoprecipitation (ChIP), which relies on affinity capture of the protein of interest (11–13), or DamID, which relies on DNA methylation by tethered Dam methyltransferase (14). All of these methods for chromatin characterization have been adapted for genome-wide profiling, taking advantage of the extraordinary improvements in microarray and sequencing technologies that have occurred over the past several years.

Another traditional method for assaying chromatin is salt fractionation (15). Chromatin digested with an enzyme, such as MNase, can be separated into soluble and insoluble fractions in the presence of physiological Mg^{2+} and low Na^{+} concentrations (16). Subsequently, increasing Na^{+} concentrations allow for separation of the insoluble fraction into high-salt soluble and insoluble fractions (15). Low-salt concentrations solubilize about 5–10% of chromatin composed primarily of mononucleosomes, whereas high salt solubilizes the majority of the nucleosomes (Fig. 1). Genome-wide profiling of low-salt soluble, high-salt soluble, and high-salt insoluble fractions versus total MNase-treated nuclei reveals that salt fractionation can differentially extract chromatin based on distinct physical properties (16). Highly accessible chromatin is enriched in the low-salt soluble fraction while

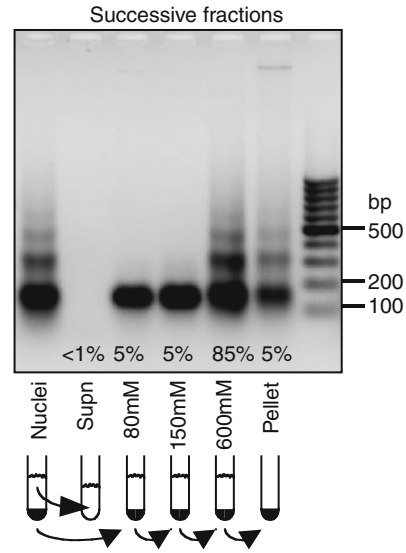

Fig. 1. Size distribution of salt fractions by agarose gel electrophoresis. Drosophila S2 cells were subjected to salt fractionation as described (16). DNA was extracted from each fraction and electrophoresed on a 1.5% agarose gel. *Lane 1* (Nuclei) corresponds to MNase-treated total chromatin. *Lane 2* (Supn) corresponds to the supernatant after the MNase-treated nuclei are pelleted. *Lane 3, 4, 5,* and *6* are the 80, 150, 600 mM, and insoluble pellet fractions, respectively. The percentage shown indicates the amount of chromatin solubilized in each fraction. The diagram below depicts the process of successive solubilization of chromatin with increasing amounts of salt (Reproduced from (16), with permission).

high salt solubilizes the majority of condensed chromatin, revealing insights into chromatin structure. The insoluble fraction is enriched in transcriptionally active chromatin, rendered insoluble presumably due to its association with large multiprotein complexes. Salt fractionation can, therefore, be used to map differences in physical properties and organization of chromatin. Furthermore, affinity capture of histones from each fraction can reveal differences in composition and modification of nucleosomes in their respective fraction. Although originally developed for studying nucleosomes, salt fractionation has recently been used to map paused RNA polymerase (17).

Salt fractionation has several advantages over other methods for characterizing chromatin dynamics. No antibodies, transgenes, or special treatments are needed so that salt fractionation can be applied to essentially any eukaryotic cell type, whether from cell lines or tissues. By assaying the low-salt-soluble (active) fraction, the high-salt fraction, and the insoluble ("nuclear matrix") pellet, essentially 100% of native chromatin is characterized. An important advantage of salt fractionation over other methods such as X-ChIP, FAIRE, Sono-Seq, and DamID, is that mononucleosome resolution is achieved, which allows for mapping of active chromatin at single base-pair level using massively parallel sequencing (Fig. 2). Furthermore, the simplicity of the salt fractionation process makes it an attractive method for characterizing epigenomes.

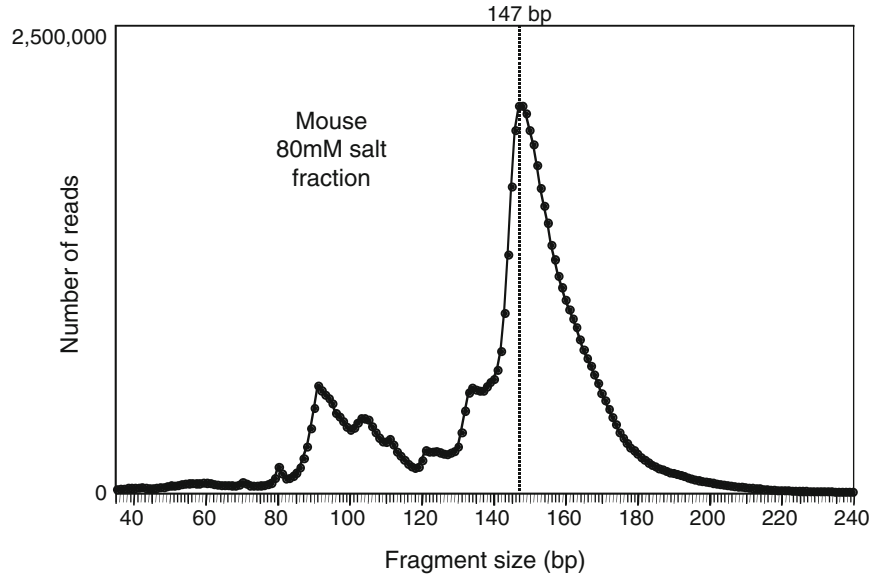

Fig. 2. Size distribution of a low-salt fraction by paired-end Solexa sequencing. Immortalized mouse pre/pro B cells PD31A were subjected to salt fractionation as described here. A paired-end Solexa library was generated from the 80-mM fraction following Subheading 3.3.2. Paired-end sequencing was performed in a single lane of an Illumina Hi-Seq 2000 instrument following the Illumina protocol, and reads were mapped to the mouse genome using the Bowtie alignment program (http://www.bowtie-bio.sourceforge.net/index.shtml). The length distribution of all 84 million mapped paired-end reads is shown at base pair resolution. Note that the dominant peak of nucleosomal DNA is centered around 147 bp, with small-sized fragments indicative of MNase cleavages within the nucleosome.

We divide the salt fractionation procedure into three stages: (1) preparation of nuclei and MNase digestion, (2) chromatin extraction and DNA isolation, and (3) preparation for genome-scale assays. The use of EGTA, instead of EDTA, retains free Mg^{2+} ions, which are critical for nuclear and chromatin integrity (15), allowing for ease of nuclear isolation with mild nonionic detergents. Protein analysis of each fraction can be performed using SDS-polyacrylamide gel electrophoresis and immunoblotting. Affinity capture and subsequent DNA isolation can be used to identify changes in nucleosome composition within each fraction. Finally, DNA isolated from each fraction can be used to generate genome-wide profiles of chromatin structure and physical properties using microarray hybridization or next-generation sequencing.

2. Materials

2.1. Preparation of Nuclei and MNase Digestion

1. Mid-log-phase cultured cells.
2. 14-mL polypropylene tubes.
3. Phosphate-buffered saline (PBS): 11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl.
4. Protease-inhibitor tablets, EDTA-free (Roche).
5. *TM2 buffer: 10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 0.5 mM phenylmethylsufonyl fluoride (PMSF).
6. 10% Nonidet P-40.
7. 0.2 M $CaCl_2$.
8. MNase 200 U resuspended to 0.2 U/μL.
9. 0.2 M EGTA.

2.2. Chromatin Extraction and DNA Isolation

1. *80 mM Triton buffer: 70 mM NaCl, 10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 2 mM EGTA, 0.1% Triton X-100, 0.5 mM PMSF.
2. *150 mM Triton buffer: 140 mM NaCl, 10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 2 mM EGTA, 0.1% Triton X-100, 0.5 mM PMSF.
3. *600 mM Triton buffer: 585 mM NaCl, 10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 2 mM EGTA, 0.1% Triton X-100, 0.5 mM PMSF.
4. *TNE buffer: 10 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA.
5. 5 M NaCl.
6. 0.5 M EDTA.
7. RNase, DNase-free 500 μg/mL solution.

8. Proteinase K 20 mg/mL RNA grade.
9. Phenol–chloroform–isoamyl alcohol (25:24:1 v/v).
10. 200 proof ethanol.
11. TE, pH 8: 10 mM Tris–HCl, 1 mM EDTA.
12. Phase-lock gel tubes (Heavy 1.5 mL – 200 tubes) (5 Prime).
13. Glycogen, 20 mg/mL.

2.3. Genome-Scale Profiling

1. 5′ Cy labeled NimbleGen Validated Random 7-mer (Tri-Link).
2. 40 mM dNTPs (10 mM each).
3. Nuclease-free H_2O.
4. Klenow fragment 3′→5′ exo- (50 U/μL).
5. Isopropanol.
6. Paired-end Sample Preparation Kit (Illumina).
7. Qiagen gel purification kit or similar substitute.
8. Qiagen MinElute PCR purification kit or similar substitute.

2.4. General Equipment

1. Refrigerated tabletop centrifuge.
2. Non-refrigerated centrifuge for 15-mL conical tubes (e.g., IEC Centra CL2).
3. End over end Eppendorf tube rotator (e.g., Labquake Shaker – Thermo Scientific).
4. PCR thermocycler.
5. Vortexer.
6. Water bath at 37°C.
7. Nanodrop spectrometer.

* Buffers are supplemented with 1× Protease inhibitor (Roche).

3. Methods

Briefly, cells are harvested and lysed with mild NP-40 to release nuclei. Washed nuclei are subjected to limited MNase digestion to fractionate the chromatin. Successive incubation with buffers containing increasing salt concentrations differentially solubilizes the chromatin. DNA can then be purified from each fraction for genome-wide analysis using microarray hybridization or massively parallel sequencing, such as the Illumina platform.

3.1. Preparation of Nuclei and MNase Digestion

1. Grow Drosophila Schneider 2 (S2) cell line cells in preferred media to exponential growth phase and 90% confluency (~2×10^6 cells per cm^2; see Note 1).

2. Scrape cells off one 75-cm² flask and collect them in 14-mL polypropylene, round-bottom tubes. Pellet the cells from the media in a room-temperature tabletop centrifuge (IEC Centra CL2) for 3 min at 1500 rcf.
3. Discard the media and wash cells in cold 1× PBS and pellet cells as above.
4. Resuspend cells in 1 mL TM2 buffer and cool on ice for 3 min.
5. To lyse the cells while keeping the nuclei intact, slowly add 60 μL 10% NP-40 while gently vortexing the tube. Incubate the cells on ice for 3 min with 5-s gentle vortexing every minute (see Note 2).
6. Separate the nuclei from cellular debris by gentle centrifugation (100 × *g*) for 10 min in a refrigerated tabletop centrifuge. The nuclear pellet appears as a white, loose pellet that is easily disrupted. Carefully remove the supernatant to prevent disrupting the nuclear pellet. Wash the nuclei with 1 mL of TM2 buffer by gently pipetting the buffer into the tube. The pipetting action easily disrupts most of the nuclei pellet and the rest can be fully resuspended by gentle flicking of the tube (see Note 3). Pellet the nuclei for 10 min at 100 × *g* and remove the supernatant as before.
7. Resuspend nuclei in 400 μL TM2 and warm to 37°C in a water bath for 5 min.
8. To fractionate the chromatin, add 2 μL of 0.2 M $CaCl_2$ to final concentration of 1 mM and 2.5 μL of MNase (final concentration of 1.25 U/mL). Return to 37°C in a water bath for 10 min with intermittent mixing to prevent aggregation of nuclei at the bottom of the tube (see Note 4).
9. Addition of 4 μL of 0.2 M EGTA to final concentration of 2 mM stops the MNase reaction. Remove 40 μL (10% of reaction) and label as "Nuc" for total MNase-treated chromatin, and an additional 40 μL for protein analysis. To remove the MNase, pellet the nuclei for 10 min at 100 × *g* and carefully remove the supernatant, and save as "Supn" fraction. Remove 30 μL for protein analysis of the Supn fraction. Wash the nuclei carefully with 1 mL of TM2 buffer, pellet, and remove the supernatant as above. Proceed with the nuclei pellet to the salt fractionation step (Subheading 3.2).

3.2. Salt Fractionation and DNA Isolation

1. From step 9 of Subheading 3.1, resuspend the nuclear pellet in 700 μL of 80 mM Triton buffer (see Note 5) and incubate in constant agitation by placing the tube in a rotator (e.g., Labquake Shaker) at 4°C for 2 h. This releases nucleosomes soluble in 80 mM salt concentration into the supernatant.

2. To extract the low-salt-soluble nucleosomes, pellet the nuclei at 100×*g* for 10 min at 4°C and save the supernatant labeled as "80 mM" fraction. The loose nuclear pellet is often slightly disrupted during this process, causing some of the nuclei to be aspirated with the supernatant. To clear the 80-mM fraction, respin the supernatant for 2 min at maximum centrifugation speed and transfer the cleared 80-mM fraction to a new tube. Remove 30 μL for protein analysis.
3. Optional: Resuspend the nuclei from the 80 mM salt extraction with 700 μL 150 mM Triton buffer and incubate with constant agitation at 4°C for 2 h. Extract and clear the supernatant as in Subheading 3.2, step 2, to release 150 mM soluble nucleosomes and remove 30 μL for protein analysis. The 150-mM fraction consists of primarily mononucleosomes (Fig. 1) and is enriched at the 5′ ends of active genes (16). This fraction is very similar to the 80-mM fraction, but with lower resolution.
4. The low-salt buffers solubilize ~5–10% of total chromatin. To solublize the majority of nucleosomes, resuspend the nuclei in 600 mM Triton buffer and incubate at 4°C in constant agitation in a rotator for 2 h to overnight. Extract and clear the supernatant labeled as "600 mM" fraction as in step 2 of Subheading 3.2. Remove 30 μL for protein analysis.
5. The remaining pellet fraction corresponds to ~5–10% of chromatin. Resuspend the pellet in 700 μL of TNE buffer and label as "Pel" fraction. Hold all fractions on ice prior to DNA extraction.
6. Aliquots of each fraction can be electrophoresed on an SDS gel to visualize histones and probe for the presence of specific proteins.
7. ChIP assays can be performed on each salt fraction using standard native ChIP protocols. Save an aliquot of the salt fraction for "input" DNA and use the remainder for affinity purification.
8. For each fraction, add 1/50th volume of 0.5 M EDTA (14 μL). For the nuclei, Supn, 80 mM and the optional 150-mM fractions, add 1/50th volume (14 μL) of 5 M NaCl. The 600 mM and pellet fractions contain sufficient amounts of NaCl for DNA precipitation purposes.
9. Prepare 1:10 dilution of RNase enzyme in H_2O and add 5 μL of diluted RNase to each fraction. Allow for RNA digestion to proceed for 10 min in a 37°C water bath.
10. To remove proteins, add 1/16th volume of 10% SDS (44 μL) for a final concentration of 0.63%. Then, add 2.5 μL of proteinase K and incubate at 75°C for 10 min.
11. Extract DNA by adding one volume of phenol–chloroform–isoamyl alcohol. Transfer the samples to phase-lock gel tubes and vortex for 2 min. Centrifuge the samples at maximum

speed in a refrigerated microfuge for 10 min. Transfer the supernatant into a new phase-lock tube and repeat the extraction one more time. Transfer the aqueous solution into Eppendorf tubes. Alternatively, a standard phenol–chloroform–isoamyl alcohol extraction may be performed, provided the aqueous solution is carefully extracted from the interphase and organic phase.

12. To precipitate the DNA, add 2 μL of glycogen and 2.5 volumes of ice-cold 100% ethanol. Incubate on ice for 20 min and centrifuge at maximum speed for 15 min in a refrigerated microfuge. Remove the supernatant and wash the pellet with 1 mL of ice-cold 80% ethanol. Centrifuge the samples for 5 min at maximum speed, remove the supernatant, and allow the pellet to dry.
13. Once fully dried, resuspend the DNA with 0.1× TE, pH 8. Determine the concentration of DNA using Nanodrop. Electrophorese an aliquot in a 1.5% agarose gel with ethidium bromide.

3.3. Genome-Scale Profiling

3.3.1. Microarray Analysis

1. The following protocol is a modified version of Nimblegen labeling methods specifically adapted to Drosophila S2 cells. Bring 0.2–1 μg of DNA from each fraction to 20 μL with H_2O and add 20 μL of Cy5 dye in 0.6-μL thin-walled PCR tubes. Use the same amount of DNA for Nuc and add 20 μL of Cy3 dye (see Notes 6 and 7).
2. Incubate each sample at 95°C for 10 min on a thermocycler. Immediately place the samples on ice water for 2 min (see Note 8).
3. After instant chill, add 5 μL of 50 mM dNTPs, 4 μL of nuclease-free H_2O, and 1 μL of Klenow fragment. Allow the labeling reaction to proceed for 4.5 h at 37°C in a thermocycler (see Note 9).
4. To stop the labeling reaction, add 5 μL of 0.5 M EDTA. Precipitate the labeled DNA by adding 5.75 μL of 5 M NaCl and 55 μL of isopropanol. Incubate at room temperature for 10 min and pellet the DNA at maximum speed for 10 min in a room-temperature microfuge. Wash the colored pellet with ice-cold 80% ethanol and recentrifuge for 2 min at maximum speed at room temperature. Discard the supernatant carefully and speed-vac dry the samples for at most 5 min to prevent overdrying.
5. Resuspend the pellet in 20 μL of nuclease-free H_2O. To quantify the labeled DNA, dilute 0.5 μL of sample in 4.5 μL H_2O and use 1.5 μL for nanodrop measurement.
6. Combine 34 μg of Cy5-labeled salt fraction with 34 μg of Cy3-labeled Nuc and concentrate the volume to 12.5 μL using

a speed-vac. Proceed with Nimblegen hybridization protocol with high-density Drosophila microarrays.

3.3.2. Paired-End Solexa Library Preparation

1. Library preparation for sequencing of salt fractions follows closely the Illumina protocol provided with its Paired-End Sample Preparation Kit. Use 500 ng of DNA and follow the Illumina protocol for end repair, 3′ adenylation, and adapter ligation.
2. After adapter ligation, samples must be size selected and purified from free adapters. Electrophorese the samples on a 2% agarose gel and excise DNA in the 100- to 600-bp range (see Note 10). This isolates double-stranded DNA derived from mono- to trinucleosomes. Extract DNA from the agarose gel following the Qiagen gel extraction kit protocol using MinElute columns and elute the DNA with 30 μL of EB buffer.
3. To amplify the library, follow Illumina's protocol on PCR amplification and clean up. For salt fractionation, it is not necessary to perform a secondary size selection process after amplification. Quality control analysis varies depending on the sequencing facility's specification, but may include PicoGreen quantification, Bioanalyzer (Agilent) analysis, and qPCR quantification. The resulting paired-end library can be sequenced using the Illumina Genome Analyzer platform.

4. Notes

1. This protocol is specifically designed for S2 cells. However, it can be adapted to any cultured cells from any species, provided that the cells are undergoing exponential growth. Changes in the growth phase lead to changes in the transcriptional program, which may also lead to changes in chromatin structure. As such, salt fractionation methods are most reproducible and reliable for cells in the same log-phase growth. For adherent cells, follow established trypsin conditions for cell harvest and proceed to step 3 of Subheading 3.1.
2. The conditions for cell lysis must be empirically determined for each cell type and for each species. This can be done by altering the final concentration of NP-40 from 0.08 to 0.8% in TM2 solution. Using the lysis protocol described in step 5 of Subheading 3.1, check for complete lysis of the cellular membrane while maintaining nuclei integrity by removing an aliquot and examining the nuclei under a microscope in comparison to intact cells. Alternatively, one can use Trypan-blue exclusion.

Remove an aliquot of the lysis, add an equal volume of Trypan-blue solution, and visualize the nuclei under a microscope.

3. The nuclear membrane is sensitive to mechanical disruption, which can lead to lysis and subsequent release of chromatin into the solution. This results in a nuclear pellet that is difficult to resuspend in solution. Formation of clumps in resuspended nuclei is a telltale sign of nuclear lysis. Discard samples and repeat nuclei preparation using less NP-40 in the lysis buffer or gentler handling of nuclei.
4. MNase conditions must be determined empirically for consistent digestion. The optimal conditions yield mostly mononucleosomes with decreasing amounts of di- and trinucleosomes (Fig. 1). To determine MNase conditions, prepare nuclei from 150×10^6 cells as described in Subheading 3.1 and divide the nuclei into 5–10 aliquots, depending on the number of MNase conditions to be tested. Add increasing amounts of MNase starting with 0.5 U per reaction and incubate each sample in a 37°C water bath for 10 min. Isolate DNA as described in Subheading 3.3 and electrophorese an aliquot in a 1.5% agarose gel. Increasing amounts of MNase should yield increasing intensity of the mononucleosome band at 150 bp. Determine which amount of MNase yields the distinct ladder of mono- to trinucleosomes and repeat the conditions to ensure replicability. Furthermore, intermittent mixing of the MNase reaction is important as nuclei can pellet in the span of the 10-min digestion, which can lead to unequal and incomplete digestion.
5. The concentration of nuclei in the salt buffers is about 2×10^5 nuclei per μL of buffer. This ratio is critical for the maintenance of nuclear integrity through the interaction of Mg^{2+} ions with the chromatin and nuclear complex. When adapting this protocol for using less starting number of cells, different cell types, or different species, this ratio must be maintained for proper nuclear integrity.
6. For control purposes, dye swaps may be necessary to determine dye-labeling biases. In these cases, the Nuc fraction can be labeled with Cy5 and salt fractions with Cy3. Alternatively, the Nuc fraction can be labeled with Cy5 and Cy3 so that hybridization should result in zero enrichment and depletion in the microarray profile. Labeling biases can be identified by lack of inverse correlation with the dye swap or nonzero profiles in the nuclei hybridization.
7. The labeling reactions described are half of the total volume of the Nimblegen dye-labeling protocol. This is sufficient for Drosophila samples because of their smaller genome size, but for mammalian systems, such as mouse, a full reaction is optimal with 1 μg of starting material. In this case, follow the Nimblegen dye-labeling protocol closely.

8. It is important that samples are chilled in ice water for faster and more uniform cooling. Otherwise, efficiency of the labeling reaction is decreased.
9. The Nimblegen protocol calls for 2-h labeling reactions. However, a single labeling reaction does not usually produce enough material needed for hybridization. For the smaller Drosophila genome, a longer labeling reaction produces sufficient material for hybridization without introducing labeling biases. However, for larger and more complex mammalian genomes, labeling biases become more pertinent. Therefore, set up 2 or more full-volume reactions (Nimblegen protocol) and limit the length to 2 h.
10. The standard Illumina protocol for size selection of libraries calls for extraction of a relatively small range of sizes for sequencing. However, one advantage of paired-end sequencing is the ability to measure sizes of the sequenced population. This allows for mapping of nucleosomes at base-pair resolution. It should be noted that breaks caused by MNase result in primarily ~147-bp fragments from gel-purified mono-, di-, and trinucleosomes (17), with smaller species indicative of internal cleavages (Fig. 2).

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