Nucleosome Preparation Kit

(version A3)

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Revision History

Revision	Date	Description of Change
A2	Dec 2018	Corrected Troubleshooting Guide recommendations

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Overview

Active Motif's Nucleosome Preparation Kit is designed to prepare mono- and oligonucleosomes from your samples. Nucleosomes comprise the smallest subunit of chromatin and consist of 147 base pairs of DNA wrapped around an octamer of core histone proteins. The histone octamer is composed of a central heterotetramer of histones H3 and H4, flanked by two heterodimers of histones H2A and H2B. Each nucleosome is separated by a linker of DNA.

In the Nucleosome Preparation Kit, an enzymatic cocktail is used to digest the linker DNA. By adjusting the digestion time, it is possible to modify the amount of mononucleosomes or oligonucleosomes extracted. Isolated nucleosomes are ideal for use as substrates in the analysis of histone post-translational modifications, enzyme kinetics or inhibitor screening studies as they represent more physiologically relevant substrates than histone proteins alone or synthetic peptides. The Nucleosome Preparation Kit includes enough reagent for 20 preparations from 10 cm plates and 5 sample optimizations.

product	format	catalog no.
Nucleosome Preparation Kit	20 rxns	53504

Kit Performance and Benefits

Nucleosome Advantages

- More physiologically relevant substrates than synthetic peptides
- Useful for the analysis of histone post-translational modifications, enzyme kinetics or inhibitor screening studies
- Easily adjust the level of mononucleosomes or oligonucleosomes by adjusting digestion time

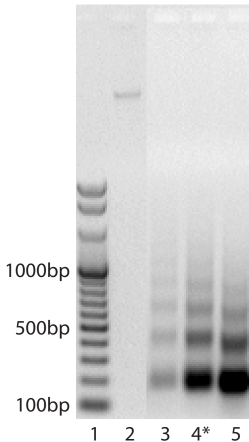

Figure 1: Gel analysis of Nucleosome digestion.
Nucleosomes were prepared from HeLa cells and digested with the Enzymatic Shearing Cocktail for 5, 10 & 15 minutes. Following digestion, the reactions were stopped with the addition of cold EDTA. The nucleosomes were then subjected to DNA clean up to assess the digestion efficiency. Samples were separated by electrophoresis through a 1.5% agarose gel.

Lane 1: 100 to 1000 bp ladder.
Lane 2: Unsheared HeLa DNA.
Lane 3: HeLa DNA treated for 5 minutes
Lane 4: HeLa DNA treated for 10 minutes
Lane 5: HeLa DNA treated for 15 minutes

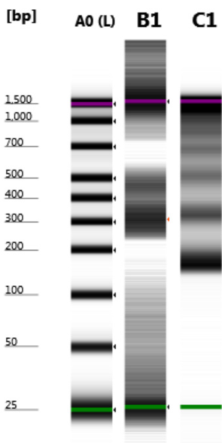

Figure 2: Gel shift seen with intact nucleosomes as compared to purified nucleosome DNA.
Nucleosomes were prepared from HeLa cells according the instructions in the Nucleosome Preparation Kit. A 50 µl aliquot of the extracted nucleosomes was then subjected to DNA clean up to assess the digestion efficiency. Both intact nucleosomes and purified DNA samples were analyzed by TapeStation. The results show an increase in molecular weight banding for the intact nucleosomes as compared to the purified DNA samples. This is predicted since an intact nucleosome complex contains both the DNA and histone proteins

Lane A: 25 to 1500 bp DNA ladder.
Lane B1: Intact nucleosome sample
Lane C1: Purified nucleosome DNA

Kit Components and Storage

Please store each component at the temperature indicated in the table below.

Reagents	Quantity	Storage / Stability
100 mM PMSF	500 µl	-20°C for 6 months
Protease Inhibitor Cocktail (PIC)	500 µl	-20°C for 6 months
1X Lysis Buffer	15 ml	-20°C for 6 months
Enzymatic Shearing Cocktail	8 µl	-20°C for 6 months
5 M NaCl	400 µl	-20°C for 6 months
RNase A (10 µg/µl)	40 µl	-20°C for 6 months
Proteinase K (10 µg/µl)	500 µl	-20°C for 6 months
10X PBS	100 ml	4°C for 6 months
Digestion Buffer	10 ml	4°C for 6 months
0.5 M EDTA	300 µl	4°C for 6 months
3 M Sodium Acetate	2 x 500 µl	RT for 6 months

Additional materials required

- Phase contrast/tissue culture microscope and hemocytometer
- Cell scraper (rubber policeman)
- Microcentrifuge (table top centrifuge 4°C) and microcentrifuge tubes
- Pipettors and tips (filter tips are recommended)
- 50% Glycerol in dH_2O
- (Optional) Dounce homogenizer with a small clearance pestle (Active Motif Catalog Nos. 40401 & 40415)
- Phenol/chloroform (1:1) TE saturated pH 8 (DNA Purification, Molecular Biology Grade)
- 100% ethanol
- 70% ethanol
- DNase-free H_2O
- Spectrophotometer for DNA quantitation
- Agarose gel electrophoresis apparatus or Bioanalyzer or TapeStation Instruments

Nucleosome Preparation Experimental Design

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Points to consider:

- **Cell growth and nucleosome preparation.** When planning an experiment, calculate the number of preparations you will require. Note that if you wish to analyze the effect of particular compounds or culturing conditions, you should prepare nucleosomes from control (untreated) cells as a reference sample.

	1 well of a 24-well plate	10 cm plate	15 cm plate
Number of Cells	130,000	0.66 x 10^7	1.5x 10^7
1X PBS Washes	2 x 1 ml	2 x 5 ml	2 x 10 ml
1X PBS + PMSF	500 µl + 2.5 µl PMSF	1 ml + 5 µl PMSF	5 ml + 30 µl PMSF
Lysis Buffer + PIC + PMSF	200 µl + 1 µl PIC + 1 µl PMSF	500 µl + 2.5 µl PIC + 2.5 µl PMSF	1 ml + 5 µl PIC + 5 µl PMSF
Digestion Buffer + PIC + PMSF	50 µl + 0.25 µl PIC + 0.25 µl PMSF	175 µl + 1 µl PIC + 1 µl PMSF	350 µl + 1.75 µl PIC + 1.75 µl PMSF
Enzymatic Shearing Cocktail, diluted	2.5 µl	8 µl	17 µl
0.5 M EDTA	1 µl	3.5 µl	7 µl

- **Resuspend solutions completely.** Thaw the PMSF at room temperature until fully dissolved, which takes about 30 minutes. Vortex gently and spin down briefly before use.
- **Safety precautions.** PMSF is a highly toxic chemical. Appropriate safety precautions (*i.e.* safety glasses, gloves and lab coat) should be used. Cell culturing should be performed in a biosafety hood if the nucleosomes are extracted from biohazardous or infectious materials.
- **Use of a dounce homogenizer.** The most critical aspect of nucleosome preparation is efficient lysis of the cells so that the enzymatic cocktail can access the chromatin. If your cells do not lyse efficiently in the Lysis Buffer, we strongly recommend a dounce homogenizer with a small clearance size pestle to mechanically shear the plasma membrane without disrupting the nuclei. Monitoring the cell lysis using a microscope can be very helpful. It is important to not add the Enzymatic Cocktail until the cells are lysed.

Section A. Optimization of Enzymatic Digestion

Prior to performing nucleosome extraction, it is recommended to optimize the digestion conditions for your cell line and treatment conditions. Modification of the digestion time may be utilized to increase or decrease the abundance of mononucleosomes or oligonucleosomes. For the optimization protocol, nucleosomes are prepared from one 15 cm plate (approximately 1.5 x 10^7 cells). Note that although ~350 µl of sample is prepared, only 200 µl is used to analyze digestion efficiency. Enough reagents are included to perform the optimization protocol with 5 samples.

1. Grow cells to 70-80% confluency in one 15 cm plate. Stimulate cells as desired to activate the pathway of interest.
2. When cells are ready to harvest, wash twice with 10 ml 1X PBS.
3. Just before use, add 30 µl 100 mM PMSF to 5 ml of ice-cold 1X PBS to each plate and scrape cells with a rubber policeman. Hold the plate at an angle and scrape cells down to collect them at the bottom edge of the plate. Use a 1 ml pipette to transfer the cells to a 15 ml conical tube on ice.
4. Pellet the cells by centrifugation for 10 minutes at 2,500 rpm (720 RCF) at 4°C.
5. Remove the supernatant and discard. At this point the protocol can be continued or the pellet can be frozen. If freezing the pellet, add 1 µl 100 mM PMSF and 1 µl PIC and freeze at -80°C.
6. Thaw pellet (if necessary) on ice and resuspend cells in 1 ml ice-cold Lysis Buffer supplemented with 5 µl PIC + 5 µl 100 mM PMSF. Pipette gently and vortex briefly to resuspend. Transfer to a 1.5 ml microcentrifuge tube. Incubate on ice for 30 minutes.

   Monitor Cell Lysis: To ensure cell lysis, take 10 µl of the cell lysate and look at it under a phase contrast microscope using a hemocytometer to verify that the nuclei have been released. It is often helpful to look at the cells before and after the lysis step as this makes it easier to identify the nuclei versus whole cells. Intact cells should have a dark central region (nucleus) surrounded by a halo of less dense cytoplasm. In lysed cells, the nuclei will appear as dots surrounded by asymmetric debris. If the cells are not lysed, then we recommend using a dounce homogenizer with a small clearance size pestle to mechanically shear the plasma membrane without disrupting the nuclei. Dounce on ice with 10 strokes, or until the cells are lysed.
7. During this incubation, prepare a working stock of Enzymatic Shearing Cocktail (200 U/ml) by diluting the supplied Enzymatic Shearing Cocktail (2 x 10^4 U/ml) 1:100 with 50% glycerol in dH_2O (not provided). The 200 U/ml working stock will be used in step 11 below and is stable at 4°C for 1-2 weeks.

Reagent	5 rxns
Stock Enzymatic Shearing Cocktail (2 x 10^4 U/ml)	0.5 µl
50% glycerol	49.5 µl

8. Centrifuge lysed cells for 10 minutes at 5,000 rpm (2,400 RCF) in a 4°C microcentrifuge to pellet the nuclei.
9. Carefully remove the supernatant and discard. Resuspend the nuclei pellet in 350 µl Digestion Buffer supplemented with 1.75 µl PIC and 1.75 µl 100 mM PMSF and incubate for 5 minutes at 37°C.
10. Transfer 50 µl of the chromatin in Digestion Buffer to each of 4 fresh microcentrifuge tubes and incubate the tubes at 37°C for 2 minutes. The remaining ~150 µl chromatin can be stored at -80°C.
11. To optimize enzymatic digestion conditions, set up 4 reactions as indicated below. Vortex the tubes on a low setting to mix components. Vortex the tube briefly approximately every 2 minutes during the incubation to increase the digestion efficiency.
    a. 50 µl chromatin plus 2.5 µl dH_20 (No Enzyme) – incubate for 10 minutes at 37°C
    b. 50 µl chromatin plus 2.5 µl working stock Enzyme – incubate for 5 minutes at 37°C
    c. 50 µl chromatin plus 2.5 µl working stock Enzyme – incubate for 10 minutes at 37°C
    d. 50 µl chromatin plus 2.5 µl working stock Enzyme – incubate for 15 minutes at 37°C
12. Stop the reactions by adding 1 µl ice-cold 0.5 M EDTA to each tube. Chill on ice 10 minutes.
13. Centrifuge the digested nucleosome samples for 10 minutes at 15,000 rpm (18,000 RCF) in a
    4°C microcentrifuge. Collect the supernatant. Nucleosomes can be stored at -80°C. Or, continue immediately with Section B below to purify the DNA for gel analysis and quantification.

Section B. DNA Clean Up to Assess Digestion Efficiency and DNA Concentration

1. If necessary, thaw the 50 µl aliquots of each digested nucleosome sample.
2. Add 150 µl dH_2O, then 10 µl 5 M NaCl to each tube.
3. Add 1 µl RNase A to each sample and incubate at 37°C for 15 minutes.
4. Add 10 µl Proteinase K to each sample and incubate at 42°C for 1.5 hours.

    Note: If you intend to use a spectrophotometer to determine the DNA concentration, the DNA must first be cleaned up by phenol/chloroform extraction and precipitation using the following protocol:

    a. Add 200 µl 1:1 phenol/chloroform TE saturated pH 8 to the sample, vortex to mix completely and centrifuge for 5 minutes at maximum speed in a microcentrifuge.
    b. Transfer the aqueous phase to a fresh microcentrifuge tube, then add 20 µl 3 M Sodium Acetate pH 5.2 and 500 µl 100% ethanol. Vortex to mix completely and place at -80°C for at least 1 hour. Alternatively, the sample can be left at -20°C overnight.
    c. Centrifuge at maximum speed for 10 minutes in a microcentrifuge at 4°C.
    d. Carefully remove and discard supernatant. Do not disturb the pellet.
    e. Add 500 µl 70% ice cold ethanol without disturbing the pellet and spin for 5 minutes at

maximum speed in a 4°C microcentrifuge.

f. Carefully remove and discard supernatant. Do not disturb pellet. Allow pellet to air-dry.

g. Resuspend pellet in 30 µl dH_2O and use a spectrophotometer to measure the absorbance at 260 nm to determine the DNA concentration (1.0 A_{260} unit = 50 µg/ml).

5. We recommended loading two different quantities of each digested sample on the gel to ensure one falls within an acceptable range. Add 4 µl of a 6X Loading Buffer to 16 µl of sample, then load 5 µl & 10 µl of each sample on a 1.5% TAE agarose gel. Run the gel at 100V until the loading dye reaches 3/4 of the way to the end of the gel.

   Note: Alternatively an instrument such as the Bioanalyzer or TapeStation may be used to analyze the purified DNA and assess the digestion efficiency.

   The enzymes used in the Enzymatic Cocktail are random cleavers (sequence independent). However, they prefer open regions that are easily accessible, such as between nucleosomes. Therefore, digestion produces a specific DNA fragment size banding pattern: ~150 bp (1 nucleosome), ~300 (2 nucleosomes), ~450 bp (3 nucleosomes) *etc.*, with an ever decreasing band intensity as the molecular weights increase.

6. After you have determined the condition that provides the desired nucleosome pattern, proceed with Section C to prepare nucleosomes.

   Note: The remaining chromatin sample stored in Step A.10 (~150 µl) can be digested using 7.5 µl working stock Enzyme. Incubate at 37°C for the time that yielded suitable digestion. Stop the reaction with 3 µl ice-cold 0.5 M EDTA. Chill on ice 10 minutes. Centrifuge the sample for 10 minutes at 15,000 rpm (18,000 RCF) in a 4°C microcentrifuge. Collect the supernatant. This extracted nucleosomes can be stored at -80°C for up to a year or used immediately.

Section C. Nucleosome Preparation

This protocol describes nucleosome preparation under optimized conditions (as determined in Sections A-B) from one 10 cm plate (approximately 6.6 x 10^6 cells). To scale the protocol for other cell numbers, please use the table on page 7 as a guide. Enough reagents are provided to perform 20 nucleosome preparations from 10 cm plates.

1. Grow cells to 70-80% confluency in one 10 cm plate. Stimulate cells as desired to activate the pathway of interest.
2. When cells are ready to harvest, wash twice with 5 ml 1X PBS.
3. Just before use, add 5 µl 100 mM PMSF to 1 ml of ice-cold 1X PBS to each plate and scrape cells with a rubber policeman. Hold the plate at an angle and scrape cells down to collect them at the bottom edge of the plate. Use a 1 ml pipette to transfer the cells to a 1.5 ml microcentrifuge tube on ice.
4. Pellet the cells by centrifugation for 10 minutes at 2,500 rpm (720 RCF) at 4°C.

5. Remove the supernatant and discard. At this point the protocol can be continued or the pellet can be frozen. If freezing the pellet, add 1 µl 100 mM PMSF and 1 µl PIC and freeze at -80°C.
6. Thaw pellet (if necessary) on ice and resuspend cells in 500 µl ice-cold Lysis Buffer supplemented with 2.5 µl PIC + 2.5 µl 100 mM PMSF. Pipette gently and vortex briefly to resuspend. Incubate on ice for 30 minutes.

   Monitor Cell Lysis: To ensure cell lysis, take 10 µl of the cell lysate and look at it under a phase contrast microscope using a hemocytometer to verify that the nuclei have been released. It is often helpful to look at the cells before and after the lysis step as this makes it easier to identify the nuclei versus whole cells. Intact cells should have a dark central region (nucleus) surrounded by a halo of less dense cytoplasm. In lysed cells, the nuclei will appear as dots surrounded by asymmetric debris. If the cells are not lysed, then we recommend using a dounce homogenizer with a small clearance size pestle to mechanically shear the plasma membrane without disrupting the nuclei. Dounce on ice with 10 strokes, or until the cells are lysed.
7. During this incubation, prepare a working stock of Enzymatic Shearing Cocktail (200 U/ml) by diluting the supplied Enzymatic Shearing Cocktail (2 x 10^4 U/ml) 1:100 with 50% glycerol in dH_2O (not provided). The 200 U/ml working stock will be used in step 10 below and is stable at 4°C for 1-2 weeks.

Reagent	1-2 rxns	3-5 rxns	6-10 rxns
Stock Enzymatic Shearing Cocktail (2 x 10^4 U/ml)	0.5 µl	1 µl	2 µl
50% glycerol	49.5 µl	99 µl	198 µl

8. Centrifuge for 10 minutes at 5,000 rpm (2,400 RCF) in a 4°C microcentrifuge to pellet the nuclei.
9. Carefully remove the supernatant and discard. Resuspend the nuclei pellet in 175 µl Digestion Buffer supplemented with 1 µl PIC and 1 µl PMSF and incubate for 5 minutes at 37°C.
10. Add 8 µl of the working stock of Enzymatic Shearing Cocktail (200 U/ml) to the pre-warmed nuclei and vortex to mix.
11. Incubate the tube at 37°C for the amount of time that you determined to be optimal for your cell line. Vortex the tube briefly approximately every 2 minutes during the incubation to increase the digestion efficiency.
12. Stop the reaction by adding 3.5 µl ice-cold 0.5 M EDTA to each tube; chill on ice 10 minutes.
13. Centrifuge the digested nucleosome samples for 10 minutes at 15,000 rpm (18,000 RCF) in a 4°C microcentrifuge. Carefully transfer supernatant to a fresh 1.7 ml microcentrifuge tube. This contains your nucleosomes. Nucleosomes should be aliquoted before freezing to minimize freeze-thaw cycles and stored at -80°C. Before freezing, remove 50 µl for use in assessing the efficiency of your digestion and determining the DNA concentration.

Section D. Nucleosome Assessment

1. To assess the efficiency of the enzymatic digestion or to quantify the nucleosome DNA, use the 50 µl aliquot and follow the steps in Section B: DNA Clean Up to Assess Digestion Efficiency and DNA concentration.
2. To quantify nucleosome protein content, measure the absorbance at 230 nm. An OD of 0.42 from a sample diluted 1:10 indicates a protein concentration of 1 mg/ml.

   Undiluted samples may be outside the linear range of your spectrophotometer. Therefore, we recommend preparing a 1/10 dilution of your samples before quantifying.

OD of 0.42 = 1 mg / ml

$$\frac{\text{OD } 0.42}{1\text{ mg/ml}} = \frac{\text{Your OD}}{x\text{ mg / ml}}$$

Solve for x = Concentration of diluted stock
Multiply by 10 = Actual histone concentration

Section E. Troubleshooting Guide

Problem/question	Recommendation
Poor yield of nucleosomes	Nuclei not released. It may be necessary to perform dounce homogenization. Use a dounce homogenizer with a small clearance pestle (see reference in Optional materials on page 3). Monitor cell lysis under a microscope as described in the procedures. Generally, the more cells that are lysed, the higher the yield.
Digestion efficiency is not clear from gel analysis.	Material is stuck in the wells, and smears or streaks are seen from the top to bottom of the lane. The sheared chromatin needs to have protein removed (Proteinase K) and RNA removed (RNase), followed by DNA purification using phenol/chloroform. Follow the DNA Clean Up protocol in Appendix – Section C. DNA purification columns are not recommended, as the high protein content may clog the columns.
	Lost DNA during the purification step. Phenol should be saturated with TE pH 8. Lower pH solutions will degrade the DNA. Column purification is not recommended due to the high protein content of the sample, which may clog the column.
	High molecular weight products. You must repeat the chromatin preparation using longer digestion times. Be sure to vortex the sample every 2 minutes during the digestion.
The ladder-like banding pattern is not seen on the gel	A single ~100-200 bp band is present, and its appearance is not altered by the different digestion times. This usually indicates that the cells were not completely lysed. Follow the instructions for douncing the sample.
	No DNA. This is normal for the undigested DNA lane. The DNA is present, but in such a wide range of sizes it is not visible on the gel. No DNA in a digested sample lane indicates that the sample was too small or the DNA was lost during DNA purification.
	Low molecular weight smear. The digestion time was too long or the Enzymatic Shearing Cocktail was not properly diluted. A large band/smear at bottom of gel may indicate RNA was not removed using the provided RNase.
	No specific bands, but a smear that decreases in size in lanes with longer digestion time. This indicates degraded DNA. Process the samples (particularly tissue) more quickly, be sure to keep on ice as directed and to add the appropriate proteinase inhibitors.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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