

# **Histone Extraction Kit**

(version A2)

Catalog No. 40028

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## Overview

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Active Motif's Histone Extraction Kit isolates core histone proteins from tissues and cell lines using an acid extraction protocol that preserves histone post-translational modifications (e.g. acetylation, methylation, and phosphorylation). Histones isolated by this method are suitable substrates for downstream analysis in Western blots, or Active Motif's Histone ELISA Kits and Histone H3 PTM Multiplex assays.

The addition or removal of modifications such as phosphorylation, acetylation and methylation at specific amino acid residues on the tails of core histone proteins H2A, H2B, H3 and H4 can have a profound effect on cell signaling and human diseases. Many of these specific histone modifications are conserved throughout eukaryotes. Histone modifications function either by altering chromatin structure and accessibility to transcriptional machinery and/or they create binding sites for 'reader' proteins which either deposit 'write' or remove 'erase' these marks.

The Histone Extraction Kit provides enough reagents for two 96-well cell culture plates (192 wells), or 100 extraction reactions from cultured cell lines and tissues. A supplemental protocol is available for working with primary cells (e.g. T-cells).

<b>product</b>	<b>format</b>	<b>catalog no.</b>
Histone Extraction Kit	192 rxns	40028

# Introduction

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## Histone Overview

The basic structural unit of chromatin is the nucleosome, which consists of 147 base pairs (bp) of DNA wrapped around a histone octamer. The histone octamer consists of two copies of the core histone H2A-H2B dimers and a tetramer of H3-H4. A linker histone, histone H1, binds chromatin outside the nucleosome unit to regulate chromatin structure.

Histone modifications such as phosphorylation, acetylation and methylation at specific amino acid residues on the N-terminal histone tails that extend beyond the core nucleosome have been found to influence and regulate transcription, chromosome packaging and DNA damage repair. While the biological significance of some histone modifications remains to be understood, some have been demonstrated to correlate very closely with specific cellular states like transcriptional activity<sup>1-6</sup>.

Histone acetylation is catalyzed by histone acetyltransferases (HATs) and has been shown to be important in the regulation of transcription, replication, DNA damage repair, and chromosomal condensation<sup>7-10</sup>. Acetylation of histones is believed to result in decondensation of heterochromatin into the more relaxed euchromatin structure which increases accessibility of regulatory proteins, such as transcription factors and DNA repair elements, to the underlying DNA. Thus histone acetylation plays an important role in the active processes associated with chromatin remodeling.

Histone methylation is a post-translational modification which takes place on the side chains of both lysine (K) and arginine (R) residues. Histone methylation is a reversible process which is catalyzed by histone methyltransferase enzymes, such as PRMT1 or Suv39H and is reversed by histone demethylases, such as LSD1 or Jumanji domain-containing proteins. The gene regulation consequences of histone methylation on the transcriptional state depends on the methylated residue and the degree of methylation. Lysine residues can undergo mono-, di- or trimethylation<sup>11-14</sup>. Additionally, arginine dimethylation can occur in either a symmetric or asymmetric state.

Histone phosphorylation occurs on serine and threonine residues and influences chromosome condensation, DNA repair and apoptosis. For example, phosphorylation of serine 28 on histone H3 has recently been shown to associate with destabilized nucleosomes in transcribed chromatin, making this an interesting indicator of both mitotic activity and transcriptional activation<sup>15-16</sup>.

Given the importance of histone post-translational modifications in development and disease, Active Motif has developed a quick and easy method to isolate cell lysates that are highly enriched in histone proteins, while still preserving post-translational modifications. The Histone Extraction Kit uses acid precipitation to isolate the highly basic histone proteins from cell culture, tissue and primary cells. The histone lysate is suitable for use in downstream applications such as Western blot, Active Motif's Histone ELISAs or the Histone H3 PTM Multiplex assay. The histone lysate will contain a mixture of protein samples. For a more purified histone preparation, we suggest using one of Active Motif's Histone Purification Kits (Catalog Nos. 40025, 40026 and 40027).

## References

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1. Kirmizis, A., *et al.* (2004) *Genes & Dev*, 18: 1592-1605.
2. Squazzo, S., *et al.* (2006) *Genome Res*, 16: 890-900.
3. Kouzarides T. (2007) *Cell*, 128 (4): 693-705.
4. Lee J.S., *et al.* (2010) *Cell*, 142 (5): 682-685.
5. Berger. S.L., *et al.* (2007) *Nature*, 447 (7143): 407-412.
6. Sims, R.J. and Reinberg, D. (2008) *Nat Rev Mol. Cell Biol*, 9 (10): 815-820.
7. Millar, C.B., *et al.* (2006) *Genes & Dev*, 20: 711-722.
8. Doyon, Y., *et al.* (2006) *Mol Cell*, 21: 51-64.
9. Qin, S., Parthun, M.R. (2006) *Mol Cell Biol*, 26: 3649-3658.
10. Shrogen-Knaak, M., *et al.* (2006) *Science*, 311: 844-847.
11. Berger, S.L (2002) *Curr Opin Genet Dev*, 12: 142-148.
12. Zhang, K., *et al.* (2002) *Annal Biochem*, 306: 259-269.
13. Zhang, L., *et al.* (2003) *Chromosoma*, 112: 77-86.
14. Marmorstein, R. and Trievel, R.C. (2009) *Biochim Biophys Acta*, 1789: 58-68.
15. Cruickshank, M.N., *et al.* (2010) *Amino Acids*, 39(5): 1087-1105.
16. Sun, J-M., *et al.* (2007) *Nucleic Acids Res*, 35(19): 6640-6647.

## Kit Performance and Benefits

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The Histone Extraction Kit can be used to prepare lysates for evaluation of histone post-translational modifications in the analysis of normal and diseased tissues, or to screen the effects of drug compounds in cultured cells.

**Input amounts:** The amount of input material recommended per reaction will depend on the sample type. Guidelines are provided below.

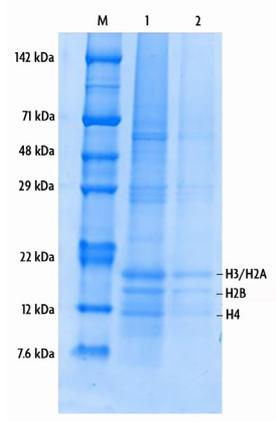
Sample Type	Input range
Cultured Cell Lines	100,000 cells – 2 million cells per reaction
96-well culture plate	5,000 – 50,000 cells per well
Tissue Samples	5 mg – 50 mg per reaction

**Expected yield:** The yield will vary depending on the cell or tissue sample used. Below are some guidelines of expected yields.

Sample Type	Input Amount	Protein Yield	Concentration (mg/ml)
Mouse Brain	51 mg	0.75 – 1.25 mg	3.0 – 4.5 mg/ml
Mouse Lung	62 mg	0.6 – 0.75 mg	2.0 – 3.0 mg/ml
Mouse Kidney	51 mg	0.6 – 0.9 mg	2.4 – 3.7 mg/ml
Mouse Spleen	58 mg	1.4 – 1.6 mg	2.4 – 5.8 mg/ml
Mouse Liver	58 mg	1.5 – 2.0 mg	6.5 – 7.6 mg/ml
Mouse Heart	52 mg	0.75 – 1.0 mg	3.5 – 4.0 mg/ml
Mouse Colon	52 mg	0.75 – 1.0 mg	2.5 – 3.5 mg/ml
6-well plate	300,000 cells/well	~220 µg	–
12-well plate	100,000 cells/well	~75 µg	–
24-well plate	50,000 cells/well	~37.5 µg	–

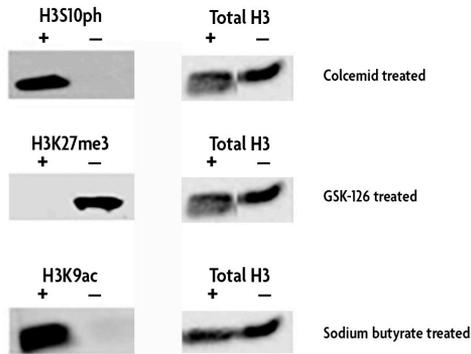
**Assay time:** As little as 2.5 hours.

# Histone Extraction Kit



**Figure 1:** SDS-PAGE gel of histones extracted from mouse kidney.

The Histone Extraction tissue sample protocol was used to prepare lysates from mouse kidney tissue. Twenty microgram mouse kidney lysate (Lane 1) or 10  $\mu$ g mouse kidney lysate (Lane 2) were loaded onto a 16% Tris-Glycine gel to show the purity of histone lysate.



**Figure 2:** Western blot confirms histone post-translational modifications are preserved during extraction.

The Histone Extraction Kit was used to isolate histone lysates from untreated (-) and treated (+) HeLa cells that underwent the one-step extraction protocol. Twenty micrograms of each lysate were run per well and probed with a 1:500 dilution H3S10ph mAb (Cat. No. 39636), 1:250 dilution H3K27me3 pAb (Cat. No. 39155), 1:500 dilution H3K9ac mAb (Cat. No. 61251), and 1  $\mu$ g/ml Histone H3 mAb (Cat. No. 61475). Results show the preservation of histone phosphorylation, methylation and acetylation with lysates extracted using the Histone Extraction Kit.

## Histone Extraction Kit Components and Storage

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Histone Extraction Kits are for research use only. Not for use in diagnostic procedures. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
Neutralization Buffer	5 ml	RT
10X PBS	2 x 100 ml	4°C
Lysis Buffer AM8	25 ml	4°C
Extraction Buffer	25 ml	4°C
Deacetylase Inhibitor	1 ml	-20°C
Protease Inhibitor Cocktail (PIC)	2 x 500 µl	-20°C
1 M DTT	100 µl	-20°C
Phosphatase Inhibitors	1 ml	RT to -20°C

### Additional materials required

- 15 ml or 50 ml conical tube
- 1.5 ml low retention microcentrifuge tubes (e.g. USA Scientific, Cat. No. 1415-2600)
- Refrigerated centrifuge and microcentrifuge
- Multichannel pipette and tips
- Scalpel, scissors or razor blades for tissue extraction
- Mortar and pestle or 1.5 ml plastic pestle (VWR, Cat. No. 47747-370) for tissue extraction
- Liquid nitrogen for tissue extraction
- Ice bucket
- Dry ice
- Methanol
- Orbital shaker (e.g. LabNet Orbit P4)
- Vortex
- DNase-free sterile water
- 96-well clear, flat bottom microtiter plate (e.g. Dynex, Cat. No. 9205)
- BSA Stock Solution (10 mg/ml)
- Bio-Rad Protein Assay Dye Solution (e.g. Bio-Rad, Cat. No. 500-0006)

## Protocols

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### Buffer Preparation and Recommendations

#### 1X PBS (See the Quick Chart for Preparing Buffers)

The table below shows the amount of PBS needed per reaction/well based on the sample type used for extraction. Calculate the amount of 1X PBS needed based on your experiment and the number of samples to be tested. Prepare a 1X PBS solution by diluting 10X PBS in sterile water. For every 10 ml of 1X PBS needed, add 1 ml 10X PBS to 9 ml sterile water. Vortex to mix. Chill on ice.

	24-well plate (per rxn)	12-well plate (per rxn)	6-well plate (per rxn)	T-25 flask (per rxn)	96-well plate (per well)
Adherent cells	4 ml	8 ml	12 ml	20 ml	200 µl
Suspension cells	2 ml	4 ml	6 ml	10 ml	N/A

#### Complete Lysis Buffer AM8 (See the Quick Chart for Preparing Buffers)

Lysis Buffer AM8 is only needed for the two-step extraction protocol for cultured cell lines and for tissue extraction. Before use, add Deacetylase Inhibitor, Phosphatase Inhibitors and Protease Inhibitor Cocktail as described in the Quick Chart. Prepare fresh buffer each time and keep on ice.

#### Extraction Buffer

Is provided ready to use. We suggest pre-chilling buffer on ice.

**WARNING:** The Extraction Buffer is corrosive. Wear personal protective equipment when handling, *i.e.* safety glasses, gloves and labcoat.

#### Protease Inhibitor Cocktail (PIC)

Is provided as a 100X stock solution. Thaw the PIC at room temperature until fully dissolved, which takes about 30 minutes. Vortex gently and spin down briefly before use, then add to the buffers immediately before use.

#### Deacetylase Inhibitor

Is provided as a 100X stock solution. Thaw on ice.

#### Phosphatase Inhibitors

Is provided as a 100X stock solution. Inhibitors can be stored at a range of temperatures from room temperature to -20°C. For long-term use we suggest storing at -20°C and thawing on ice prior to use.

#### 0.1 M DTT (See the Quick Chart for Preparing Buffers)

Prepare a 10-fold dilution of 1 M DTT in sterile water and keep on ice. 0.1 M DTT should be prepared fresh for each experiment and discarded after use. We suggest making aliquots of the 1 M DTT stock to minimize repeated freeze thaws.

## Complete Neutralization Buffer (See the Quick Chart for Preparing Buffers)

Before use, add 0.1 M DTT, Deacetylase Inhibitor, Phosphatase Inhibitors and Protease Inhibitor Cocktail to the Neutralization Buffer as described in the Quick Chart below. This should always be prepared fresh for each extraction and discarded after use. Keep buffer on ice.

## Quick Chart for Preparing Buffers

Reagents to prepare	Components	Example volumes for buffer preparation					
1X PBS	Sterile water	9 ml	45 ml	90 ml	900 ml	1800 ml	
	10X PBS	1 ml	5 ml	10 ml	100 ml	200 ml	
	<b>TOTAL REQUIRED</b>	<b>10 ml</b>	<b>50 ml</b>	<b>100 ml</b>	<b>1 L</b>	<b>2 L</b>	
0.1 M DTT	Sterile water	4.5 $\mu$ l	9 $\mu$ l	18 $\mu$ l	45 $\mu$ l	180 $\mu$ l	
	1 M DTT	0.5 $\mu$ l	1 $\mu$ l	2 $\mu$ l	5 $\mu$ l	20 $\mu$ l	
	<b>TOTAL REQUIRED</b>	<b>5 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>	<b>20 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>	<b>200 <math>\mu</math>l</b>	
Complete Lysis Buffer	Lysis Buffer AM8	242.5 $\mu$ l	970 $\mu$ l	4.85 ml	12.125 ml	24.25 ml	
	Deacetylase Inhibitor	2.5 $\mu$ l	10 $\mu$ l	50 $\mu$ l	125 $\mu$ l	250 $\mu$ l	
	Phosphatase Inhibitors	2.5 $\mu$ l	10 $\mu$ l	50 $\mu$ l	125 $\mu$ l	250 $\mu$ l	
	Protease Inhibitor Cocktail	2.5 $\mu$ l	10 $\mu$ l	50 $\mu$ l	125 $\mu$ l	250 $\mu$ l	
	<b>TOTAL REQUIRED</b>	<b>250 <math>\mu</math>l</b>	<b>1 ml</b>	<b>5 ml</b>	<b>12.5 ml</b>	<b>25 ml</b>	
Complete Neutralization Buffer	Neutralization Buffer	10 $\mu$ l	50 $\mu$ l	100 $\mu$ l	250 $\mu$ l	1 ml	
	0.1 M DTT	1.5 $\mu$ l	7.5 $\mu$ l	15 $\mu$ l	37.5 $\mu$ l	150 $\mu$ l	
	Deacetylase Inhibitor	1 $\mu$ l	5 $\mu$ l	10 $\mu$ l	25 $\mu$ l	100 $\mu$ l	
	Phosphatase Inhibitors	1 $\mu$ l	5 $\mu$ l	10 $\mu$ l	25 $\mu$ l	100 $\mu$ l	
	Protease Inhibitor Cocktail	1 $\mu$ l	5 $\mu$ l	10 $\mu$ l	25 $\mu$ l	100 $\mu$ l	
	<b>TOTAL REQUIRED</b>	<b>14.5 <math>\mu</math>l</b>	<b>72.5 <math>\mu</math>l</b>	<b>145 <math>\mu</math>l</b>	<b>362.5 <math>\mu</math>l</b>	<b>1.45 ml</b>	

# Assay Protocol

## Read the entire protocol before use.

The Histone Extraction Kit provides multiple protocols based on sample type: adherent cultured cells, suspension cell lines, 96-well cell culture and tissue samples. A supplemental protocol is provided in the appendix for working with primary cells. Select the protocol to match your sample and follow the instructions provided.

### Section A: Cultured Cell Lines

The following section describes the preparation of histone containing extracts from a minimum of 100,000 cells. Protocols are provided for adherent and suspension cell culture. Please follow the instructions corresponding to your sample type.

For the majority of cell lines, a one-step extraction method can be used for isolation of histone proteins for downstream analysis in WB, ELISA or Histone H3 PTM Multiplex assay. However, some cell types are more difficult to work with and may require the optional two-step extraction method. When working with a new sample type, we suggest performing a small scale pilot experiment comparing the two extraction methods to identify the best extraction protocol for your sample.

#### Adherent Cell Culture:

	24-well plate	12-well plate	6-well plate	T-25 flask
Seeding Density	0.05 x 10 <sup>6</sup>	0.1 x 10 <sup>6</sup>	0.3 x 10 <sup>6</sup>	0.7 x 10 <sup>6</sup>
Cells at 70-80% Confluency*	0.15 x 10 <sup>6</sup>	0.3 x 10 <sup>6</sup>	0.9 x 10 <sup>6</sup>	2.1 x 10 <sup>6</sup>
Growth Medium volume to culture cells	1 ml	2 ml	3 ml	5 ml
Trypsin	500 µl	1 ml	2 ml	3 ml
Growth medium	500 µl	1 ml	2 ml	3 ml
1 X PBS (volume per wash)	1 ml	2 ml	3 ml	5 ml

Table 1: Cell culture recommendations.

\*The number of cells on a confluent plate or dish will vary with cell type. For this table, HeLa cells were used. Please adjust as needed based on your particular cell type.

1. Follow the recommendations for cell culture seeding as shown in Table 1.
2. Treat cells as desired. Grow cells to 70-80% confluency.
3. Aspirate growth medium and wash the cells twice with the recommended volume of 1X PBS.
4. Detach cells from plate using trypsin. Transfer to a 15 ml conical tube.
5. Add an equal volume of growth medium the 15 ml conical tube. Pipet to mix.
6. Perform a cell count and determine cell viability. Record the number of viable cells.  
# viable cells per pellet: \_\_\_\_\_
7. Pellet the cells by centrifugation in a pre-chilled (4°C) centrifuge at 200 x g for 5 minutes.

- Discard supernatant and wash cells two times with ice-cold 1X PBS. Centrifuge at 200 x g for 5 minutes at 4°C for each wash.
- After the second wash aspirate any remaining PBS without disturbing the pellet. Use a P200 pipette to remove any traces of PBS.
- Cell pellet can be stored at -80°C, or proceed directly into the one-step or two-step extraction protocols in Section B or Section C.

### Suspension Cell Culture:

- Follow the recommendations for cell culture seeding as shown in Table 1.
- Treat cells as desired.
- Transfer cells into a 15 ml or 50 ml conical tube.
- Perform a cell count and determine cell viability. Record the number of viable cells.  
# viable cells per pellet: \_\_\_\_\_
- Pellet the cells by centrifugation in a pre-chilled (4°C) centrifuge at 200 x g for 5 minutes.
- Discard supernatant and wash cells two times with the recommended volume of ice-cold 1X PBS. Centrifuge at 200 x g for 5 minutes at 4°C for each wash.
- After the second wash aspirate any remaining PBS without disturbing the pellet. Use a P200 pipette to remove any traces of PBS.
- Cell pellet can be stored at -80°C, or proceed directly into the one-step or two-step extraction protocols in Section B or Section C.

## Section B: One-Step Extraction for Cell Lines

- Using the calculations for cell viability, add 100 µl per one million cells of ice-cold Extraction Buffer to each cell pellet. Pipet up and down to resuspend the cells. Transfer cells to a low-bind 1.5 ml microcentrifuge tube.

$$[(\# \text{ viable cells per pellet}) / (1,000,000 \text{ cells})] \times 100 \mu\text{l} = \text{_____} \mu\text{l Extraction Buffer}$$

- Incubate the cells on an end-to-end rotator for 2 hours at 4°C.
- Label new low-bind 1.5 ml microcentrifuge tubes for each sample and chill on ice.
- Following the incubation, place the tubes in a microcentrifuge at 20,800 x g for 10 minutes at 4°C to pellet the acid insoluble material.
- During the spin, prepare a dry ice/methanol bath. Place a microcentrifuge rack in an ice bucket. Add dry ice and methanol.
- Transfer supernatant containing histone proteins into the pre-chilled microcentrifuge tube. If the sample will be used multiple times we suggest making aliquots to avoid freeze thaw cycles. Record the volume per tube. **Histone lysate volume:** \_\_\_\_\_ µl
- Snap-freeze the samples by placing them in the dry ice/methanol bath.

**Note:** Once frozen, histone samples can be stored at -80°C for long-term or short-term

storage. It is better to store the histones under acidic conditions to prevent protein aggregation that may cause the histones to fall out of solution. Histones will need to be neutralized prior to use in downstream assays. When ready to use the histone samples in downstream analysis, proceed with the protocol.

- Take the frozen histone samples and allow them to thaw on ice. While the samples are thawing, prepare the Complete Neutralization Buffer as described on page 8.
- Add Complete Neutralization Buffer to the histone lysate and vortex to mix. Calculate the amount needed per sample:

$$(\text{Histone lysate volume}) \times 0.161 = \text{_____ } \mu\text{l Complete Neutralization Buffer}$$

- Determine protein concentration using Bradford Quantification (see Appendix Section G).
- Proceed directly with downstream analysis.

## Section C: Two-Step Extraction for Cell Lines

- Add 250  $\mu\text{l}$  of ice-cold Lysis Buffer AM8 supplemented with inhibitors to each cell pellet. The volume added is not dependent upon pellet size. The same amount of Lysis Buffer is added to each sample. Pipet up and down until solution is homogenous.
- Incubate on ice for 30 minutes.
- Centrifuge at 2,644 x g for 2 minutes at 4°C to isolate the nuclei.
- Add 100  $\mu\text{l}$  per one million cells of ice-cold Extraction Buffer to each nuclear pellet. Pipet up and down to resuspend. Transfer nuclei to a low-bind 1.5 ml microcentrifuge tube.

$$[(\# \text{ viable cells per pellet}) / (1,000,000 \text{ cells})] \times 100 \mu\text{l} = \text{_____ } \mu\text{l Extraction Buffer}$$

- Incubate on an end-to-end rotator for 2 hours at 4°C.
- Label new low-bind 1.5 ml microcentrifuge tubes for each sample and chill on ice.
- Following the incubation, place the tubes in a microcentrifuge at 20,800 x g for 10 minutes at 4°C to pellet the acid insoluble material.
- During the spin, prepare a dry ice/methanol bath. Place a microcentrifuge rack in an ice bucket. Add dry ice and methanol.
- Transfer supernatant containing histone proteins into the pre-chilled microcentrifuge tube. If the sample will be used multiple times we suggest making aliquots to avoid freeze thaw cycles. Record the volume per tube. **Histone lysate volume:** \_\_\_\_\_  $\mu\text{l}$
- Snap-freeze the samples by placing them in the dry ice/methanol bath.

**Note:** Once frozen, histone samples can be stored at -80°C for long-term or short-term storage. It is better to store the histones under acidic conditions to prevent protein aggregation that may cause the histones to fall out of solution. Histones will need to be neutralized prior to use in downstream assays. When ready to use the histone samples in downstream analysis, proceed with the protocol.

- Take the frozen histone samples and allow them to thaw on ice. While the samples are thawing, prepare the Complete Neutralization Buffer as described on page 8.

9. Add Complete Neutralization Buffer to the histone lysate and vortex to mix. Calculate the amount needed per sample:  
$$(\text{Histone lysate volume}) \times 0.161 = \text{_____ } \mu\text{l Complete Neutralization Buffer}$$
10. Determine protein concentration using Bradford Quantification (see Appendix Section G).
11. Proceed directly with downstream analysis.

## Section D: 96-well Cell Culture Plate Extraction

The following section describes the preparation of histone containing extracts from 96-well cell culture plates (5,000 to 50,000 cells per well). Given the limited starting material from a 96-well plate, samples should be processed for immediate use in downstream assays. Storage of the histone lysate is not recommended and will result in a significant loss of signal. Plan your experiments accordingly to prepare fresh samples the day of the assay.

1. Culture cells as appropriate in a 96-well cell culture plate using 100  $\mu\text{l}$  growth medium.
2. Treat cells as desired. Grow cells to 70-80% confluency.
3. Aspirate cell growth medium and wash the cells twice with 200  $\mu\text{l}$  1X PBS.
4. Remove excess PBS by quickly inverting plate and blotting onto paper towels to remove residual traces of PBS.
5. Add 100  $\mu\text{l}$  per well of Extraction Buffer. Seal plate with adhesive film and place the cell culture plate on a plate shaker at 1,000 rpm for 1 hour at 4°C. While the samples are incubating, prepare the Complete Neutralization Buffer as described on page 8.
6. Add 16.1  $\mu\text{l}$  Complete Neutralization Buffer to each well. Pipet up and down to mix.
7. Samples are now ready for downstream analysis. The amount of histones isolated from each 96-well is usually too low to quantify. We recommend using a specific volume in downstream Histone ELISAs or Histone H3 PTM Multiplex kit and normalizing samples based on total H3 levels.

## Section E: Tissue Sample Extraction

The following section describes the preparation of histone lysates from tissue samples. We recommend using 5-50 mg tissue per extraction with 50 mg as the optimal amount. Isolated histone proteins can be used for downstream analysis in WB, ELISA or Histone H3 PTM Multiplex assay.

1. Weigh out 50 mg of tissue and place into a conical tube on dry ice.
2. Remove a pre-chilled mortar and pestle from -80°C freezer.
3. Transfer tissue to a mortar and add 1-2 ml liquid nitrogen. Homogenize by grinding the tissue with a pestle until a fine powder is achieved. Carefully transfer the tissue to a microcentrifuge tube. If using small tissue samples that fit within a 1.5 ml microcentrifuge tube, use a battery powered pestle (VWR, Catalog No. 47747-370) to crush the tissue within the tube.
4. Resuspend the tissue homogenate in 250 µl of ice-cold Lysis Buffer AM8 supplemented with inhibitors to each cell pellet. Pipet up and down until solution is homogenous.

**Note:** If working with large tissue sections that are not resuspended in 250 µl volume, you can increase the amount of Lysis Buffer AM8 to a final volume of 1 ml to completely resuspend the tissue. Please note that excess lysis buffer is not provided to perform all 100 reactions of the kit using this larger volume.

2. Incubate on ice for 30 minutes.

**Note:** We recommended monitoring the cell lysis. Take 10 µl of the tissue homogenate 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, continue to incubate on ice for an additional 10 minutes on ice. Refer to the Troubleshooting notes in the Appendix for additional tips.

3. Centrifuge at 2,644 x g for 2 minutes at 4°C to isolate the nuclei.
4. Add 250 µl of ice-cold Extraction Buffer per 50 mg tissue. Pipet up and down to resuspend. Transfer nuclei to a low-bind 1.5 ml microcentrifuge tube.
5. Incubate on an end-to-end rotator overnight at 4°C.
6. Label new low-bind 1.5 ml microcentrifuge tubes for each sample and chill on ice.
7. Pellet acid insoluble material in a microcentrifuge at 20,800 x g for 10 minutes at 4°C.
8. During the spin, prepare a dry ice/methanol bath. Place a microcentrifuge rack in an ice bucket. Add dry ice and methanol.
9. Transfer supernatant containing histone proteins into the pre-chilled microcentrifuge tube. If the sample will be used multiple times we suggest making aliquots to avoid freeze thaw cycles. Record the volume per tube. **Histone lysate volume:** \_\_\_\_\_ µl
10. Snap-freeze the samples by placing them in the dry ice/methanol bath.

**Note:** Once frozen, histone samples can be stored at -80°C for long-term or short-term storage. It is better to store the histones under acidic conditions to prevent protein aggregation that may cause the histones to fall out of solution. Histones will need to be neutralized prior to use in downstream assays. When ready to use the histone samples in downstream analysis, proceed with the protocol.

11. Take the frozen histone samples and allow them to thaw on ice. While the samples are thawing, prepare the Complete Neutralization Buffer as described on page 8.
12. Add Complete Neutralization Buffer to the histone lysate and vortex to mix. Calculate the amount needed per sample:

$$(\text{Histone lysate volume}) \times 0.161 = \text{_____ } \mu\text{l Complete Neutralization Buffer}$$

13. Determine protein concentration using Bradford Quantification (see Appendix Section G).
14. Proceed directly with downstream analysis.

## Appendix

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### Section F: Extraction from Primary Cells (e.g. T-cells)

The following section is a supplemental protocol that describes extraction modifications required when working with primary cells. Please note this protocol is provided as a guideline only and optimization may be required. This protocol is based on the use of 500,000 cells per reaction.

1. Isolate primary cells according to standard protocols. Aliquot 500,000 cells per microcentrifuge tube and snap freeze cells in a dry ice/methanol bath.
2. Resuspend the frozen cell pellet in 200  $\mu$ l ice-cold **T-cell Buffer** (see below) supplemented with DTT and protease inhibitor cocktail. Pipet up and down to mix.
3. Incubate the cells on ice for 30 minutes. The cells should swell during this incubation.
4. Add 12.5  $\mu$ l of a **10% IGEPAL Solution** (see below) and vortex on high speed for 10 seconds.
5. Centrifuge the homogenate for 1 minute at 14,000 rpm in a microcentrifuge at 4°C.
6. Carefully remove supernatant and discard.
7. Resuspend the nuclear pellet in 100  $\mu$ l ice-cold Extraction Buffer. Pipet up and down to mix. Transfer cells to a low bind tube and vortex on high for 30 seconds.
8. Incubate overnight on ice.
9. Spin the sample at maximum speed in a microcentrifuge at 4°C for 5 minutes.
10. Transfer supernatant containing histones to a new low bind tube.
11. Determine protein concentration using Bradford Quantification (see Appendix Section G).
12. Proceed directly with downstream analysis.

#### Additional materials required:

- **T-Cell Buffer:**

- 10 mM HEPES, pH 7.9

- 10 mM KCl

- 0.1 mM EGTA

- 0.1 mM EDTA

- 1 mM DTT

- 1X Protease Inhibitor Cocktail (PIC)

- Add DTT and PIC just before use. Discard any unused solution.

- **10% IGEPAL Solution:**

- IGEPAL CA-630 (Sigma, Cat # I8896) diluted 1:10 in sterile water

## Section G: Bradford Quantification

The following protocol is a suggested guideline for quantification of histone lysates. Other quantification methods suitable with your downstream analysis may also be used. We suggest using a BSA standard to determine protein concentration. Standards should be prepared in the same buffer as the lysate samples (including addition of inhibitors) for accurate quantification.

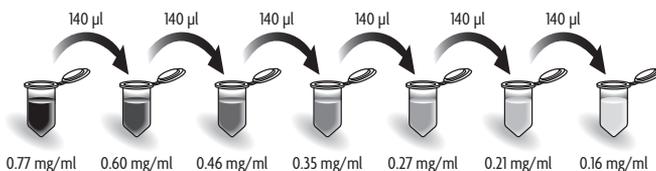
### Materials required

- 96-well clear, flat bottom microtiter plate (e.g. Dynex, Cat. No. 9205)
- BSA Stock (10 mg/ml)
- Bio-Rad Protein Assay Dye Solution (e.g. Bio-Rad, Cat. No. 500-0006)
- Extraction Buffer
- Complete Neutralization Buffer (with inhibitors)
- 1.5 ml microcentrifuge tubes & 15 ml conical tube
- Sterile water

1. In a 15 ml conical tube, prepare the Protein Assay Dye solution according to manufacturer's recommendations by adding 2 ml Dye Reagent Concentrate to 8 ml sterile water. Filter through Whatman #1 filter (or equivalent) to remove particulates. Keep at room temperature.
2. Prepare Neutralized Extraction Buffer as follows:

Reagent	Volume
Extraction Buffer	600 $\mu$ l
Complete Neutralization Buffer (See page 8)	96.6 $\mu$ l
<b>Final Volume</b>	<b>697 <math>\mu</math>l</b>

3. Prepare a 1 mg/ml BSA standard in 140  $\mu$ l Neutralized Extraction Buffer. (e.g. Add 14  $\mu$ l BSA stock (10 mg/ml) to 126  $\mu$ l Neutralized Extraction Buffer.)
4. Set up 7 microcentrifuge tubes. Add 42  $\mu$ l Neutralized Extraction Buffer to each tube. Next, pipette 140  $\mu$ l of the 1 mg/ml BSA standard to the first microcentrifuge tube. Vortex to mix. Prepare a dilution series as indicated below by transferring 140  $\mu$ l of each diluted standard to the next tube. Mix tubes thoroughly before each transfer.



5. We suggest testing the sample neat and at a 1:2 dilution. Prepare histone lysate dilutions in Neutralized Extraction Buffer. If your sample falls outside the linear range of the BSA standard, we recommend preparing alternate dilutions.

**Neat:** Read the sample directly in the assay without further dilution.

**1:2 dilution:** Add 5  $\mu$ l histone lysate to 5  $\mu$ l Neutralized Extraction Buffer for each quantification reaction. Scale values as needed based on the number of quantification reactions. Vortex to mix.

6. In a clear, flat bottomed microtiter plate, set up your standard and samples in triplicate using the template shown below. Add 10  $\mu$ l of each standard or sample per well. For the blank wells, add 10  $\mu$ l Neutralized Extraction Buffer.

**Note:** For some samples there may not be enough material to perform sample quantification in triplicates. Adjust plate layout as needed.

	BSA Standard			Lysates								
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.77 mg/ml		Neat Lysate 1			-	-	-	-	-	-	-
B	0.60 mg/ml		1:2 Lysate 1			-	-	-	-	-	-	-
C	0.46 mg/ml		Neat Lysate 2			-	-	-	-	-	-	-
D	0.35 mg/ml		1:2 Lysate 2			-	-	-	-	-	-	-
E	0.27 mg/ml		Neat Lysate 3			-	-	-	-	-	-	-
F	0.21 mg/ml		1:2 Lysate 3			-	-	-	-	-	-	-
G	0.16 mg/ml		Neat Lysate 4			-	-	-	-	-	-	-
H	Blank			1:2 Lysate 4			-	-	-	-	-	-

7. Add 200  $\mu$ l diluted dye reagent to each well. Mix the sample and reagent thoroughly by pipetting up and down. Use clean tips with each well.
8. Cover plate and incubate at room temperature for 5-10 minutes.
9. Measure absorbance at 595nm using a microplate reader.

## Section H: Troubleshooting Guide

Problem/question	Possible cause	Recommendation
When using a metal pestle freshly ground tissue is sticking to the tip	After cleaning the pestle the excess liquid was not dry prior to placing in the freezer. Excess liquid will form ice and stick to freshly homogenized tissue	Dry all tools thoroughly prior to cold storage
Incomplete homogenization of tissue	Some tissue types (e.g. heart, skeletal muscle and uterus) will generate a fibrous strand that is unable to be homogenized easily	Add more liquid nitrogen to keep the tissue brittle. When working with fibrous tissues, crush tissue samples very quickly to avoid partial thawing. This will help to avoid the formation of the fibrous strand.
How should I quantify my histone lysate?	Because histones are small positively charged proteins, methods for determining protein concentration often give differing results	We recommend to use a Bradford assay with a BSA standard to quantify the histone lysate. It is important to note that core histone proteins react poorly with Coomassie dye in solution, but due to the presence of other proteins within the lysate, Bradford will still provide a reliable protein concentration. However, alternative methods such as A280nm can also be used to quantify proteins, including histones, within the lysate. Concentration can be compared to a BSA standard prepared in the same buffer.
Low yield	Incomplete cleaning of fresh tissue prior to storage will add weight to final tissue sample giving a higher starting mass than actual tissue.	Clean tissue thoroughly prior to use or storage to avoid calculating extra mass.
	Sample loss due to protein sticking to the walls of the plastic tube.	We recommend using low retention microcentrifuge tubes for all steps of the extraction process. If working with culture cell lines of less than 100,000 cells or precious tissue samples we strongly recommend low retention tubes to avoid sample loss.
	Incomplete lysis	If working with cell culture samples in excess of 1 million cells per reaction, you may need to add additional Lysis Buffer AM8 to fully resuspend the cell pellet for complete cell lysis if using the two-step extraction protocol. We suggest adding Lysis Buffer AM8 proportionally at the ratio of 250 $\mu$ l per 1 million cells.

## Technical Services

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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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