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SHORT COMMUNICATION
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A protein isolation method for western blot to study histones with an internal control protein

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ABSTRACT: Histone modification is one of the attractive targets for epigenetic studies. However, current methods to extract chromatin-associated proteins for western blot of histone modification have some weaknesses such as the loss of housekeeping proteins. In this study, we are presenting a simple method to isolate nuclear protein for studying histone modification by immunoblotting with housekeeping proteins. This method provided high protein concentration from minute tissue samples and importantly, it allowed us to detect acetylated histones together with internal control proteins such as β actin.

KEYWORDS: Protein isolation; histone modification; housekeeping protein

Epigenetics has been emerging as an attractive field in biomedical sciences. Somatic changes within an individual or between individuals, which are not caused by changes in DNA sequence such as mutation, are promising targets for epigenetic studies, where DNA methylation and histone modifications are mostly considered. DNA methylation, one of the most important mechanisms controlling the gene expression, is established early in development [1]. Histone modifications regulating the manipulation and expression of DNA in most biological processes [2] which may occur throughout the life under different conditions.

To study the epigenetic regulation of gene expression, we have some good techniques such as western blot, DNA methylation assay, and chromatin immunoprecipitation (ChiP) assay. Western blot seems to be method considered as a simple and cheap technique to investigate global histone modifications applied in several epigenetic studies [3-5]. However, the traditional methods of nuclear protein isolation and histone extraction for immunoblotting of global histone modifications have some weaknesses including the low yield of isolated proteins and do not allow to have internal controls [3, 5]. Therefore, some additional steps and costs are needed to spend on preparing samples and analyzing the global histone modifications.

In this study, we have established a simple method (Fig. 1) to isolate nuclear proteins from lesser amounts of frozen tissues. This is a very simple method that produced not only the highest percentage of histones but also carried significantly high-level housekeeping proteins.

Concentrated protein isolated by our method and control method (acid extraction), was measured and compared by Bradford reagent as presented in Table 1. From the same amount of tissues (150mg/sample) and equal volume of dilution buffer (200ul/sample), our method yielded higher protein concentration than the acid extraction around 13 times or 29 times for inguinal fat (ING) or liver, respectively. But, the protein concentration was almost the same in the soluble (So) and nuclear (Nu) part of a homogenized sample isolated using our method. In our method, total proteins were collected with enriched histones in the nuclear part, whereas acid extraction tends to get pure nuclear proteins. However, in order to test the global histone modifications by western blot, the acid extraction method shows some weaknesses such as 1) it requires large amount of tissues for getting enough histones, 2) cannot run western blots with internal

controls, and 3) isolated histones cannot be stored for a long period of time because they were extracted by 0.2N HCl. With such high protein concentration, our method showing low expressed acetylated histone 3. This result indicated that chromatins were abundant in the nucleus allowed us to run several western blots to check global modifications for all interested histones.

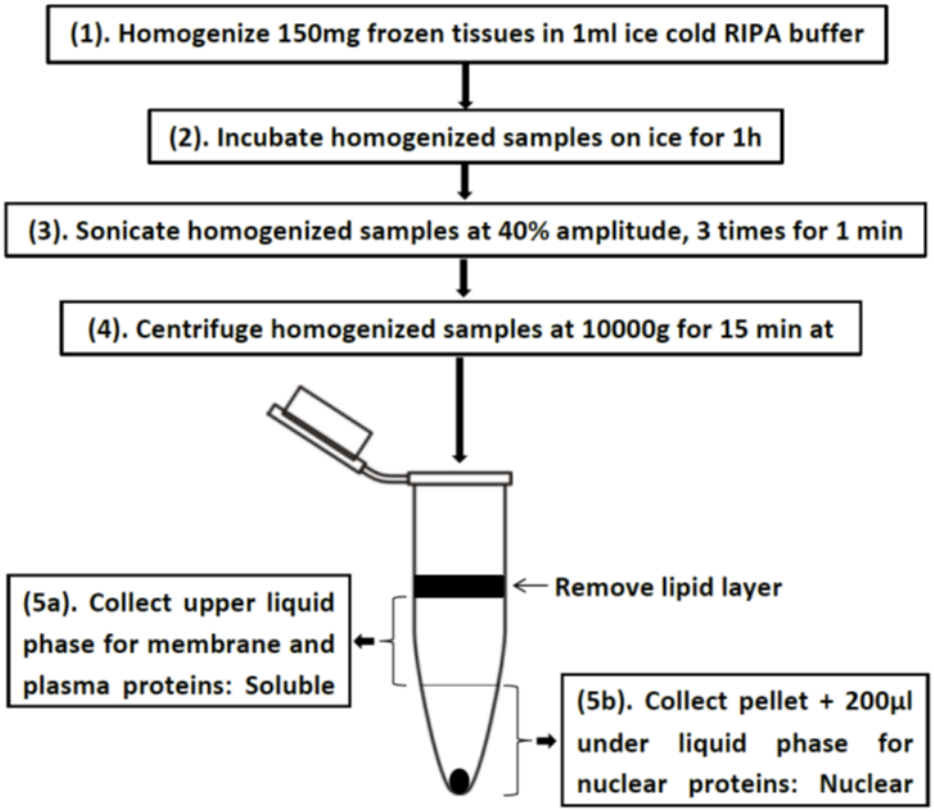

Figure 1. Our method to isolate soluble and nuclear proteins. Soluble and nuclear proteins were isolated using ice-cold radioimmune precipitation assay buffer (RIPA) modified from a protocol we had done [6-8]. RIPA buffer includes 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Nonodet P-40 (NP40), 0.25% sodium deoxycholate, and 0.02% sodium azide in ddH2O. 150mg of frozen mouse inguinal fat tissues (ING) or frozen liver was homogenized in 1ml of ice-cold RIPA supplemented with phenylmethylsulfonyl fluoride (PMSF – Sigma, P7626), protease inhibitor cocktail (Sigma, P8340) and PhosStop (PhosStop - Roche, 4906837001) (1). Homogenized samples were incubated on ice for 1h (2), and sonicated by VibraCell Sonicator at 40% of the sonicator's amplitude with 3 times for 1 min (3), and then centrifuged at 10000g for 15 min at 4ºC (4). Upper liquid phase (Soluble - So) was transferred into a new 1.5ml Eppendorf for membrane and plasma proteins (5a). Pellet plus around 200µl under liquid phase (Nuclear - Nu) was kept for nuclear proteins (5b). In case of fat tissues, the fat fraction on the top of samples was carefully removed after centrifuging. Pellet was dissolved in under liquid phase by pipetting. In the control method, histone extraction was done from 150mg of frozen ING and liver of mice according to the Abcam's protocol (http://www.abcam.com/protocols/histone-extraction-protocol-for-western-blot) using Triton Extraction Buffer, this buffer contains PBS 1X, 0.5% Triton X100 (V/V), 2mM PMSF, 0.02% Sodium azide (W/v) (NaN3). Histones were extracted by 0.2N HCl over night at 4°C (Acid). Protein concentration in both methods was measured using Bradford reagent (Sigma, B6916).

In order to confirm the enrichment of nuclear proteins are isolated by our method, we performed the western blot using a specific antibody against acetylated histone 3, has been considered as one of the most common chromatins. As shown in the Fig.2, the nuclear parts (Nu) of both inguinal fat (ING) (lane 2) and liver (lane 4) showing strong acetylated histone 3 expression, whereas the soluble parts (So) (lane 1 or 3) from same samples Parts (Nu) isolated by our method, even we had similar protein concentrations in the Nu and So parts of the samples (Table 1). The expression of acetylatedhistone 3 was also found from the pure histones (lane 5, 6 and 7) purified by acid extraction method, because of low protein concentration was given by this method. We were unable to load more than 20µg protein/gel-well to get

stronger signals, this is one of the weaknesses of acid extraction method.

Table 1. Protein concentration of from different samples

Isolation methods	Sample ID	Tissue weight (mg)	Isolated proteins	Concentration (µg/µl)	Total amount (µg/200µl)
Our method	ING_1	150	Soluble (So)	6.13	1225.47
	ING_2		Nuclear (Nu)	6.36	1272.73
	Liver_1	150	Soluble (So)	20.77	4154.10
	Liver_2		Nuclear (Nu)	19.94	3987.70
Acid extraction	ING_1	150	Nuclear (Nu)	0.47	94.50
	Liver_2	150	Nuclear (Nu)	0.68	136.20

ING: Inguinal fat tissues; Liver: Liver tissues

In the immunoblotting assay, it is better to have internal controls to confirm the equal amount of loaded proteins to normalize the quantified data. Internal controls (normalization controls) are the proteins of

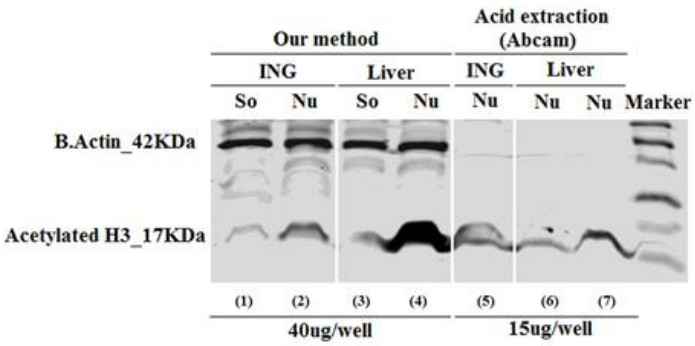

Figure 2. Our protein isolation method gave strong signals of acetylated histone 3 and internal control (β-actin). Acetylated histone 3 (H3) and β-actin (B. Actin) in protein isolated by our and control methods were detected by western blot as described [9, 10, 6]. Blots were incubated with antibodies against acetyl histone 3 (rabbit anti acetyl H3 – Millipore, Cat. # 06-599) (1:1000), β actin (mouse anti β actin –Abcam, Ab6276) (1:10000). Specific antibody-antigen complexes were detected using fluorescent-labeled secondary antibodies (goat anti-rabbit IRDye 800, Rockland, 611-132-122; goat anti-mouse IRDye 800, Rockland, 610-730-124). Bands were visualized and quantified using the Odyssey imaging system (Licor Bioscience).

housekeeping genes such as β actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypozanthine phosphoribosyltransferase 1 (HPRT1), and ribosomal protein large P1 (RPLP1) [11, 12]. Westernblots are used to study histones or their modifications use coomassie blue-stained gels to demonstrate equal loading [3, 5]. In our protein isolation method, we collected the part of samples having high chromatins, so our samples still had nuclear proteins as well as proteins of housekeeping genes. Therefore, this method allowed us to have the internal controls, such as β-actin in the blot (Fig. 2). As shown in Fig. 2, from lane (1) to (4); the signals of β-actin were strong and equal in both So and Nu parts of one tissue, and in both fat (ING) and liver samples. As expected, acid extraction methods did not give any signal of β-actin in both fat and liver samples (lane 5 to 7 of Fig. 2).

The results of the current study indicate that this alternative protein isolation method is a better choice to study chromatins and other proteins as well. Moreover, this method offers several advantages including small amount of tissue samples giving high protein concentration, one gel for both targeted and control proteins, and it was simple. However, this method is only suitable for studying global expressions of proteins by western blot not by other methods such as ChiP assay as it does not yield pure chromatins.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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