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Accelerated nuclei preparation and methods for analysis of histone modifications in yeast.

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Abstract

The continuing identification of new histone post-translational modifications and ongoing discovery of their roles in nuclear processes has increased the demand for quick, efficient, and precise methods for their analysis. In the budding yeast *Saccharomyces cerevisiae*, a variety of methods exist for the characterization of histone modifications on a global scale. However, a wide gap in preparation time and histone abundance exists between the most widely used extraction methods, a simple whole cell extraction (WCE) and an intensive histone extraction. In this work we evaluate various published WCE buffers for their relative effectiveness in the detection of histone modifications by western blot analysis. We also present a precise, yet time-efficient method for the detection of subtle changes in histone modification levels. Lastly, we present a protocol for the rapid small-scale purification of nuclei that improves the performance of antibodies that do not work efficiently in WCE, and aids in the detection of histone modifications that are low in abundance. These new methods are ideal for the analysis of histone modifications and could be applied to the analysis and improved detection of other nuclear proteins.

Keywords

Histones; Nuclear proteins; Chromatin; Histone Modification; Methylation; Nuclei extraction

1. Introduction

The investigation of chromatin structure and function in eukaryotes continues to be a fast-paced field. The basic unit of the highly organized chromatin structure is the nucleosome, in which ~147 base pairs of DNA is wrapped around an octamer of the core histone proteins [1,2]. Chromatin is crucial for protection of the genome from environmental insult, as well as the regulation of all processes in eukaryotes using the DNA template, such as gene transcription, replication, and recombination. The N- and C-terminal “tail” domains of the histone proteins extend away from the nucleosome core particle and, along with the globular domains, are targeted for various post-translational modifications including methylation, acetylation and phosphorylation [3–5]. Studies have revealed that these modifications are involved in transcriptional regulation, and likely specify a ‘histone code’ by which the proper regulation of chromatin structure and gene expression is maintained [3,6,7].

Histone methylation, in particular, has recently been intensely studied. While lysines can be mono-, di-, or trimethylated, arginines can be either mono- or dimethylated (symmetric or asymmetric) [8–14]. Strikingly, recent studies show that the distinct methyl states of these residues can be independently regulated and are subject to demethylation [15–21]. Modest or

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dramatic changes (local or genome-wide) in histone methylation can result in significant effects on chromatin organization, the outcome of other histone modification patterns, and gene transcription [22–24].

Key to our understanding of chromatin function is the detection of even modest changes in the level of histone post-translational modifications. Most often, detection requires high-quality antibodies specific to the modification of choice and, in many cases, core histones extracted using a time-intensive procedure. Analyses of global changes in histone modifications in yeast commonly begin with a whole cell extraction (WCE), however much variability exists in the components of buffers that are widely used [10,25–27]. It is unclear whether the differences between these distinct extraction buffers (i.e. salt type and/or salt concentration) lead to changes in histone yield or purity.

Outside of rapid WCE production, labor-intensive histone acid-extraction protocols are often needed to detect rare or low abundance histone modifications in yeast, which otherwise may be missed by the use of WCE alone [28,29]. In addition, certain antibodies are difficult to use with WCE due to either low abundance of the antigen or a large relative amount of cytoplasmic proteins in the extract, resulting in detection of a large number of cross-reacting bands. In these cases, the histone acid-extraction method can provide purified histones that are suitable for analysis by western blotting. Although a variety of nuclear isolation/histone acid-extraction protocols over several decades have been described, the vast majority require large scale cultures and multiple time-consuming centrifugation and wash steps [28–34]. While a rapid method used to detect the histone ubiquitin moiety has been presented by others, an abbreviated method for the analysis of low-abundance nuclear proteins, histone methyl states, or for detecting small changes in methylation states would be beneficial [35].

In this report, we compare several widely used WCE buffers with the goal of identifying whether a particular salt type or concentration would preferentially enrich for histones. We also describe a titration loading method that maximizes the precision of histone modification analysis via WCE, an approach recently used to demonstrate changes in various histone modification states [15,36]. Finally, we present a protocol for the small-scale and streamlined preparation of crude nuclei suitable for histone modification analyses by western blot procedures. These crude nuclei are enriched in histones, and although the purity is not at the level of most histone purification methods that involve acid extraction, they provide an ideal starting material for the rapid analysis of histone modifications (and presumably other nuclear proteins) that are in low abundance or to which only low avidity antibodies are available.

2. Methods

2.1. Detection of histone modifications in budding yeast using whole cell extraction

Whole cell extraction (WCE) by glass bead disruption is the most common method of lysing yeast cells for histone modification analysis. This method is rapid and effective for analyzing most histone modifications from a large number of yeast strains simultaneously. Interestingly, buffers have been used which contain a wide variety of salt types and concentrations, as well as other components [10,25–27]. A recent publication thoroughly analyzed sample preparation methods and buffers for their effectiveness in global metabolite extraction, and reported that some buffer components enrich for certain metabolites [37]. Therefore, will altering WCE buffer components such as salt type or concentration increase the relative yield of histone proteins extracted, and therefore result in clearer analysis with antibodies directed against histone modifications? We investigated these questions using the following method.

2.1.1. Yeast strains, antibodies, and buffers—The wild-type (WT), *set2Δ*, *rtf1Δ*, and *bur2Δ* strains in the BY4741 background used in this and following sections were obtained

from Research Genetics. The extraction buffers evaluated for effectiveness in detecting histone modifications are described in Table 1. The antibodies used to detect histone modifications were from Upstate Biotechnology and used at the following concentrations for western blotting: H3 lysine 36 di-methyl (H3K36me2, catalog 07–274) used at 1:3000 dilution, general H3 C-terminal (H3, catalog 05–928) for loading controls used at 1:10000 dilution, H3 lysine 4 di-methyl (H3K4Me2, catalog 07–030) used at 1:25000, H3 lysine 79 di-methyl (H3K79me2, catalog 08–835) used at 1:5000 dilution, and H3 lysine 4 tri-methyl (H3K4me3, catalog 07–473) used at 1:5000. Additional antibodies obtained from Abcam were used as follows: H3 lysine 36 tri-methyl (H3K36me3, catalog 9050) used at 1:3000 dilution, and H3 lysine 79 tri-methyl (H3K79me3, catalog 2621) used at 1:3000 dilution. Secondary antibodies used were sheep anti-mouse IgG Horseradish peroxidase and anti-rabbit IgG peroxidase linked (GE Healthcare), each used at 1:5000 dilution.

2.1.2. Comparison of various extraction buffers for effectiveness in detection of histone modifications—Described below is the method utilized for comparison of various WCE buffers and their effectiveness in histone modification analysis. The approach follows a common WCE preparation using four unique published buffers, differing primarily in their salt type and salt concentration [10,25–27]. Complete descriptions of the buffer components are presented in Table 1.

1. Yeast WT, *set2Δ*, and *rtf1Δ* strains were grown overnight and each was inoculated into fresh 100 ml YPD at a starting $O.D._{600}$ of 0.1. Cells were grown to an $O.D._{600}$ of 1.1, then each 100 ml culture was separated into four identical 25 ml portions, pelleted, and stored at −80 °C.
2. The cell pellets were thawed on ice, washed, and then re-suspended in 400 μl extraction buffer. A unique extraction buffer (see Table 1) was used in the lysis of one pellet from each of the three strains: WT, *set2Δ*, *rtf1Δ*.
3. WCE was performed for each sample according to a published method, differing in the extraction buffers and loading amounts [10]. The extraction method consisted of cell cultures (5 ml) grown overnight, diluted in fresh 100 ml YPD culture to a starting $O.D._{600}$ of 0.1, and grown to a final $O.D._{600}$ of 1.0. Pelleted cells were resuspended in extraction buffer and then disrupted by acid-washed glass beads using a mini-beadbeater (Biospec Products) for 3 x 30 sec pulses with 1 min on ice between each step. Tube bottoms were punctured, and cell extracts were separated by brief centrifugation. Lysates were clarified by centrifugation at 16,000g for 15 min, after which the supernatant (WCE) was transferred to a fresh tube for protein analysis.
4. WCE protein concentrations were determined using Coomassie Plus reagent (Pierce Biochem) according to the manufacturer's directions and assayed using the Bradford method.
5. Following addition of 10 μl 2 x Laemmli SDS-PAGE loading buffer to 10 μl of each WCE, the samples were boiled for 5 min.
6. Samples (30 μg) were resolved by SDS-PAGE using a 15% acrylamide gel.
7. Resolved proteins were transferred to PVDF (Millipore Immobilon-P) using a Hoefer TE-77 semi-dry transfer unit at 45 mA per 8 x 7 cm membrane for 90 min total.
8. Membranes were each blocked in 10 ml of a 5% non-fat dry milk solution and TBS (50 mM Tris pH 8.0, 138 mM NaCl, 2.7 mM KCl) at room temperature for 1 h. Histone modifications were detected by incubation of primary antibody overnight at 4 °C (see Section 2.1.1.). Membranes were then washed 2 x 5 min in TBS-Tween (TBS + 0.1% Tween 20), and secondary antibodies were added to a fresh 10 ml solution of 5% milk

and TBS-Tween with incubation at room temperature for 2 h, then washed as before. Membranes were developed using the ECL Plus Western Blotting Detection Kit (GE Healthcare) according to the manufacturer's directions.

The relative effectiveness of each buffer tested is presented in Fig. 1. Although we initially predicted that changes in salt type or concentration of the WCE buffers would result in a significantly greater elution of proteins and/or histones in the lysate, we surprisingly found that analysis of total protein concentration revealed that the concentration of all WCEs examined were nearly the same - within 10% (data not shown). In addition, we found that the relative concentration of histones and the background binding of the antibodies to cellular proteins were nearly identical regardless of the buffer components tested (data not shown). These data indicate that changes in the salt types or concentrations (i.e. ionic strength) we tested have little to no effect on the global extraction of protein in the WCE method, nor does it affect the purity of histones extracted.

2.2. Titration and stripping approach for precise detection of changes in specific histone modifications

To determine whether a gene deletion or mutation affects a particular histone modification, histones from the wild type and deletion strain must be equally loaded for comparison. In most cases, especially when a deletion completely abolishes a histone modification, the histone loading amount may not be critical. However, when a deletion only partially reduces or increases a given histone modification, loading too much WCE (or using too high an antibody concentration) can cause an over or underestimate of the amount of modified histone. In addition, loading too little WCE could appear as though a factor abolishes a particular modification when it may only moderately reduce that modification. To address this issue, we have combined a titration assay and membrane stripping procedure. The membrane stripping approach we present here ensures proper comparison between modification levels and equal histone loading, as variations in sample loading or transfer efficiency are common and can make for difficult interpretations. This method is particularly useful when more than one histone modification is being investigated. In our experience, a single membrane can be stripped and reused up to four times if the membrane has not dried after each immunoblotting assay. The titration and stripping approach is described below.

1. Prepare cells and WCE using a general method such as that described in 2.1.2.
2. For each mutant or strain to be analyzed, load at least three different amount of protein in adjacent gel lanes. Suggested ranges should begin near 10 μg of WCE and increase at two or three-fold increments when using the Hoefer Mighty Small gel apparatus (1.0 mm spacers, 10 well combs).
3. Analyze the extracts using a standard western blotting approach for the first antibody of choice.
4. Begin the stripping procedure by placing the membrane in a plastic bag filled with ~10 ml stripping buffer (65 mM Tris-HCl pH 6.8, 2% SDS, 0.75% β-mercaptoethanol).
5. Seal plastic bag and put it in water bath set at 55 °C for 30 min. Invert bag 3 times every 10 min.
6. Decant stripping buffer, and transfer membrane to a box.
7. Wash membrane with 1 x TBS at room temperature for 5 min, repeat 3 times.
8. Verify that the membrane is stripped of the first antibody by re-probing with the matching secondary antibody and ECL.

9. Proceed to standard immunoblotting assay with the next antibody.

The effectiveness of this approach is demonstrated in Fig. 2. The use of at least three WCE concentrations per strain in adjacent lanes makes it likely that at least one lane will not produce a saturated signal when blotted with the desired antibody. This, in combination with the stripping approach, allows for effective screening of defects in histone modification levels that may otherwise be missed in a cursory screening. We note that while a stripping approach is recommended, a non-stripping approach that involves examining parallel blots can also be effective, although it may require multiple independent repeats to quantitatively detect a subtle change in a particular histone modification.

2.3. Detection of histone modifications when only low-avidity antibodies are available or the modification is in low abundance

For detecting histone modifications in yeast by western analysis, two types of histone preparations are generally used: the WCE method and histone acid-extraction method. The choice depends on the quality of antibody and/or the relative abundance of the particular histone modification of interest. In the first method, all cellular proteins are extracted with one simple buffer, which allows rapid preparation of histones along with other cellular proteins. However, since the histone abundance is generally low as compared to a histone acid-extraction procedure, the WCE method is most useful when antibody avidity is high. The second method is designed to first isolate nuclei from cells followed by detergent washes and acid extraction; therefore, its histone yield is much higher than in the first method. However, existing versions of this method in the literature require significant time and effort [28,30–34]. For this reason, a histone purification method is only used when antibody avidity is low, or if the modification of interest is rare.

Small-scale accelerated nuclei preparation—Here we present a modified nuclear extraction method to detect histone modifications. Typical large-scale histone purification methods require approximately seven hours of preparation time once the cell pellet is obtained. This small-scale method requires less than half the time of a histone purification method (3 h versus 7 h), yet is superior to WCE for the detection of low-abundance histone modifications or when an antibody of low avidity is utilized. In addition, the common nuclei cushion step of most histone purification methods is omitted. The required culture volume is also decreased to 200 ml, from the 1 L volume commonly used in histone purifications. The protocol for this approach is described below.

1. Yeast strains are grown overnight and inoculated into fresh 200 ml YPD at a starting $O.D._{600}$ of 0.1. Inoculated cultures are grown at 30 °C with shaking to an $O.D._{600}$ of 1.1.
2. Centrifuge cells at 4000 x g for 15 min at 4 °C, then wash once with 40 ml ice-cold H_2O, and spin as before. For convenience, pellets can be frozen at −80 °C at this step.
3. Suspend each pellet in 3 ml of spheroplasting buffer (1 M Sorbitol, 50 mM K_2HPO_4 pH 6.5 buffer, 0.018% β-mercaptoethanol). Cells pellets should be mixed gently in this and subsequent steps.
4. Centrifuge cells 3500 x g for 10 min at 4 °C.
5. Suspend each pellet in 3 ml of spheroplasting buffer containing 40 units/ml Zymolase 100T (MP Biomedicals).
6. Incubate mixture at 30 °C for 30 min (when ~90% of the cells should be spheroplasted). Complete spheroplasting can be analyzed by measuring the $O.D._{600}$ in a 1:100 dilution of spheroplasts with 1% SDS. The reaction should not proceed longer than 45 min due to the risk of shearing nuclei.

7. Centrifuge the spheroplasted cells at 4000 x g for 5 min.
8. Wash spheroplasted pellets in 3 ml of spheroplasting buffer (without Zymolase).
9. Gently pellet again as in step 7.
10. Suspend each pellet in 8 ml lysis buffer (18% Ficoll 400, 20 mM $K_2(HPO_4)$ pH 6.8 buffer, 1 mM $MgCl_2$, 0.5 mM EDTA pH 8.0, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin.
11. Lyse cells with 20 strokes of a small Dounce homogenizer with pestle A (we find that pestle A is easier to use yet just as effective as pestle B). This step is performed on ice. The homogenizer should be rinsed as follows:
    a. Pour lysed cells into a sterile tube and wipe the homogenizer handle along the tube interior.
    b. Pour 4 ml of fresh lysis buffer over the handle into homogenizer.
    c. Use a pipette to rinse inside of homogenizer with the remaining lysis buffer.
    d. Pipette the buffer rinse into the conical tube, resulting in 12 ml final volume of homogenized solution.
12. Spin samples at 3500 x g for 10 min to remove cell debris (nuclei in supernatant, debris is in pellet).
13. Transfer supernatant into (16 x 76 mm) tubes and pellet nuclei in Type 50 Ti rotor at 50,000 x g for 35 min
14. Suspend nuclei in 200 μl NP buffer (0.34 M sucrose, 20 mM Tris-HCl pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, with protease inhibitors added as in step 10. Vigorous pipetting may be required to suspend the nuclei pellet. Aliquot and store nuclei at −80 °C.

Using the above protocol, nuclei were prepared from the WT, *set2Δ* and *rtf1Δ* strains and western blot analysis was performed as in section 2.1.2. As shown in Fig. 3, this method is effective for a variety of difficult antibodies such as the H3K36me2 or H3K79me3. However, when comparing WCE versus nuclei, it is evident that nuclei provide no additional benefit when using antibodies that perform well in WCE (Fig. 3, compare H3K4me3 and H3K36me3 blots). While one might predict that increasing the WCE load could allow for an increased signal for difficult antibodies (see Fig. 3 H3K79me3 and H3K36me2 blots), we note that the maximum amount of WCE that can be loaded is near 100 μg when using our gel apparatus (see section 2.2). Beyond this WCE maximum load, the extract lodges in the well and the high protein concentration results in significant smearing and insufficient resolution of bands. In our experience, dramatic increases in WCE loading do not significantly improve western results with poor performing antibodies. Importantly, equivalent protein concentrations were used between WCE and nuclei (WCE: 60 μg and nuclei: 15, 30, and 60 μg), thus confirming that the nuclei preparation method enriches for the histone proteins and is superior to WCE for the detection of histone modifications. In addition to the analysis of histone modifications, the purified nuclei could be useful for the analysis of any nuclear protein that may otherwise be undetectable in WCE due to low antibody avidity or a low relative abundance of the protein in total cellular extract.

3. Concluding Remarks

The methods and approaches outlined in this article provide for time-saving and precise global analysis of even modest changes in histone modifications. We have provided three key points concerning the analysis of histone modifications in yeast: (i) altering the salt type or salt concentration (ionic strength) in WCE buffers results in little to no improvement in the ability

to detect histone modifications; (ii) a titration and membrane stripping approach is superior in gauging quantitative changes in histone modification levels; and (iii) our abbreviated nuclei preparation method is beneficial in the detection of rare histone modifications or if an antibody's avidity is low.

Interestingly, changes in the ionic strength or salt concentration of WCE buffers do not lead to an appreciable change in the total protein concentration extracted or enrichment of histones (Fig. 1). Empirically, there is greater freedom in buffer components than may have been predicted. Also of importance is the observation that modest changes in histone modification levels could be missed in a cursory screen of WCEs. The results shown in Fig. 2 demonstrate that a careful, yet rapid analysis of yeast extracts using a titration and stripping approach aids in identifying genes, that when deleted, may have a minor, yet biologically relevant change in specific histone modification levels. This approach has been utilized in several recent reports to demonstrate selective changes in specific histone modification states [15,36]. Without the use of this titration approach, a factor with genuine effects on a specific histone modification could appear to have no effect (Fig. 2A–B compare lanes 1,4,7 and 3,6,9). Also presented here is an abbreviated nuclei preparation procedure, which provides a midpoint in quality and time expenditure between the rapid WCE and a time-consuming histone acid-extraction (Fig. 3). Previously, use of antibodies with low avidity often resulted in low-quality blots and required large-scale preparation of core-histones or nuclei for clear detection [15,17]. The enriched histone procedure provided through the abbreviated nuclei preparation presented in this report is also applicable for use with antibodies that have low avidity or when there is a low abundance of the target modification/protein in the cell that would be difficult to analyze using WCE (see Fig. 3 WCE vs. nuclei). In a similar manner, the enriched nuclei could also be of use for the analysis of low-abundance nuclear proteins. Collectively, the applications discussed in this article allow for clear and reproducible detection of nuclear proteins and/or global changes in histone modification levels.

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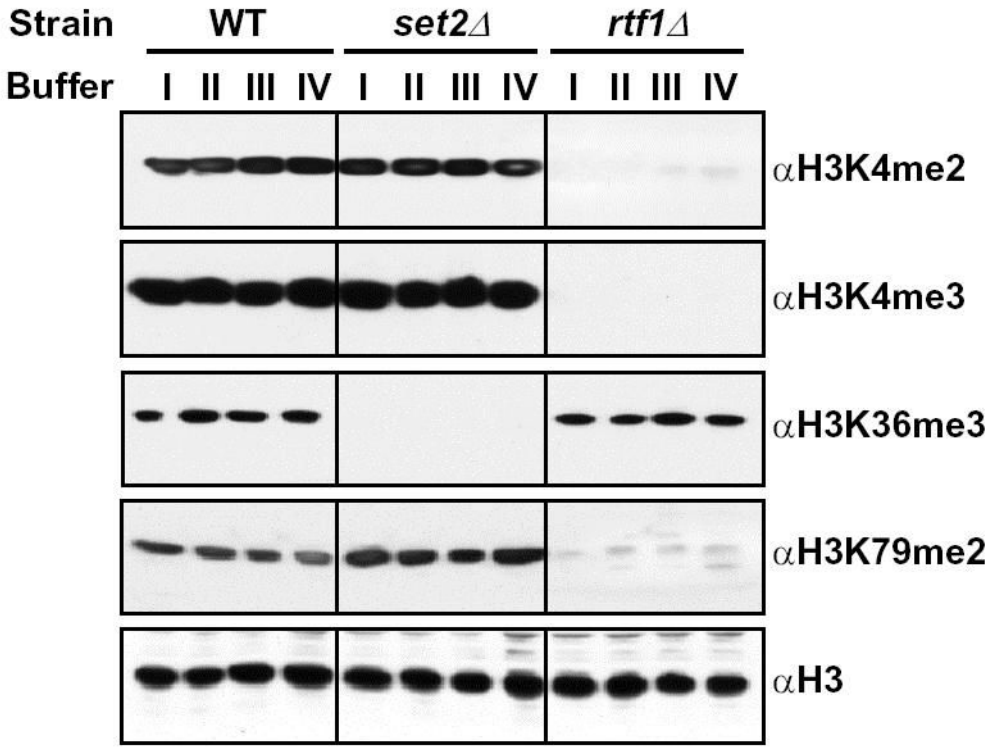

Fig. 1. Evaluation of WCE buffer components reveals equal effectiveness in the detection of histone methyl modifications

A 100 ml culture of the indicated strains was separated into four identical pellets, and WCE were prepared for each pellet using one of four distinct buffers (see Table 1 for a complete list of buffer components). WCEs (30 μg) were resolved by SDS-PAGE, transferred to PVDF, and probed with antibodies directed against H3 di-methyl lysine 4 (αH3K4me2), tri-methyl lysine 4 (αH3K4me3), tri-methyl lysine 36 (αH3K36me3), and di-methyl lysine 79 (αH3K79me2). An antibody directed against the C-terminus of H3 (αH3) was used as a loading control.

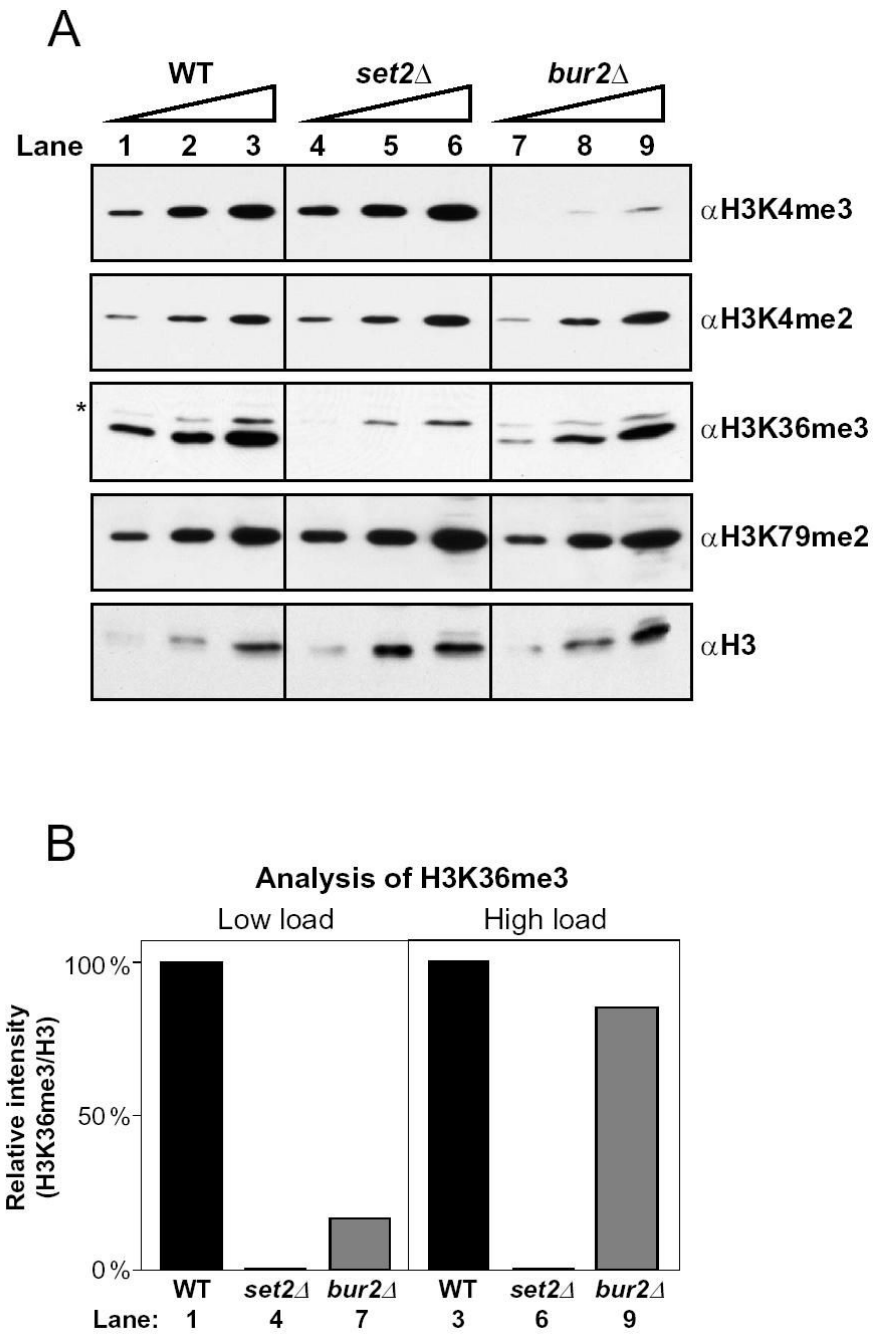

Fig. 2. Titration and stripping approach is helpful for the precise detection of changes in histone modification levels

(A) Following the standard WCE protocol (see section 2.1.2.), extracts were prepared from WT, *set2Δ* and *bur2Δ* strains using Buffer II described in Table 1. The titration approach consisted of three concentrations of extract from each strain resolved by SDS-PAGE and transferred to PVDF (generally a range between 10 and 90 μg). Each membrane probed for a histone modification was stripped and re-probed for the H3 loading control. We note that the use of the same membrane to blot for the loading control and modification of interest avoids the complications that arise from variations in gel loading and transfer efficiency which occur between gels. However, we also find that comparisons between two independent membranes

(one probed for the modification of interest and one probed for the histone loading control) generally results in a similar conclusion, but is best confirmed with multiple independent repeats. Asterisks denote non-specific bands. *(B)* Quantification of band intensities for the H3K36me3 blot is displayed as a ratio of H3K36me3 band intensity to the corresponding H3 loading control. Band intensities are plotted relative to the WT level (set to 100%) in each lane. Important to note is that at the highest concentration loaded, *bur2Δ* appears to effect a negligible change in H3K36me3 (compare lanes 3 and 9 of the H3K36me3 blot), yet the lowest concentration (lanes 1 and 7) reveals the decreased H3K36me3 found to occur in the absence of Bur2 [38]. This observation is consistent for these blots regardless of exposure time.

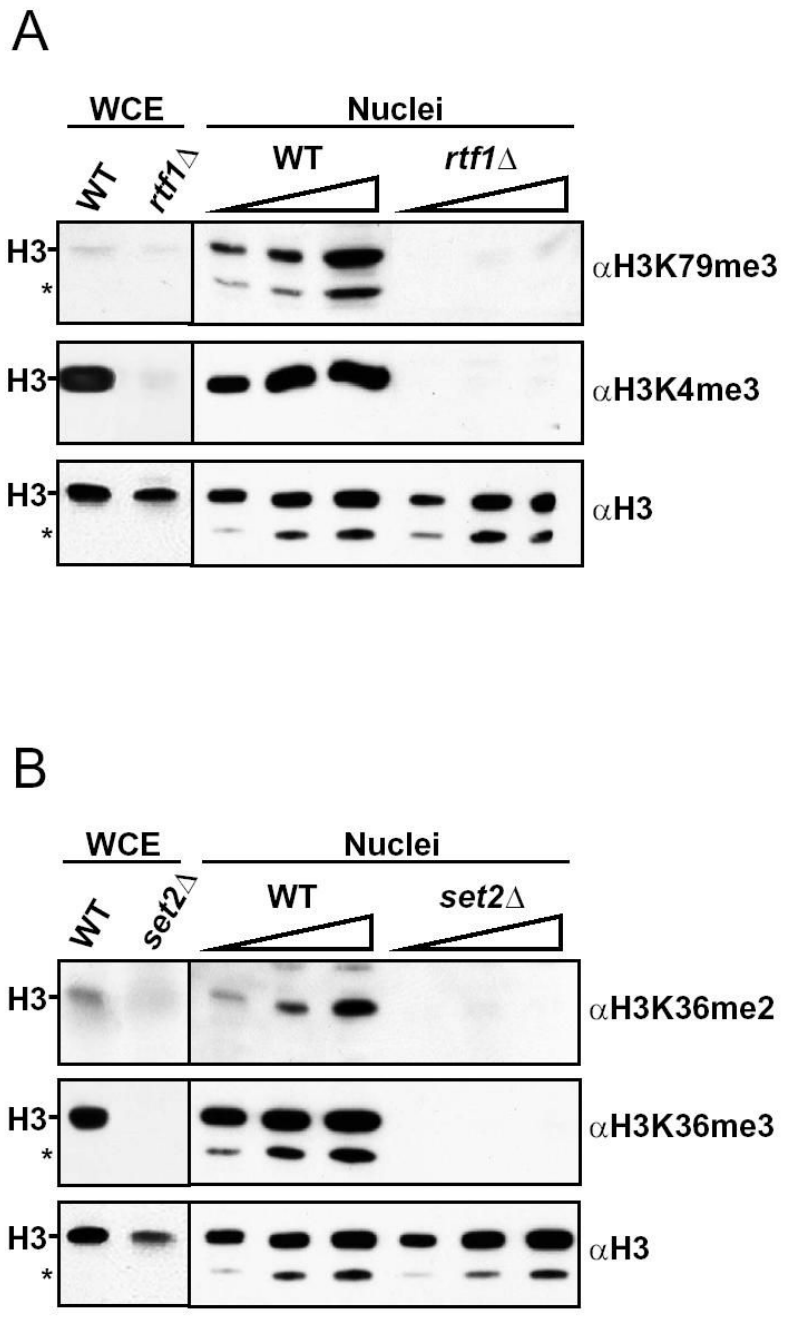

Fig. 3. The abbreviated nuclei preparation method allows for detection of histone modifications when antibody avidity or modification abundance is low

In each panel, nuclei were isolated from the indicated strains using the abbreviated nuclei method and compared to WCEs of identical strains. For comparison of antibody effectiveness, 60 μg WCE was compared to a range of nuclear extract at 15, 30 or 60 μg. The location of H3 is indicated and asterisks indicate a partial N-terminal H3 breakdown products detectable in the nuclear extract. Important to note is that more protease cleavage (breakdown product) of H3 is typically observed in nuclei preparations as compared to WCE preparations. *(A)* WCEs (left) and nuclei (right) were resolved by SDS-PAGE, transferred to PVDF, and probed with antibodies directed against H3 tri-methyl lysine 79 (αH3K79me3) and tri-methyl lysine 4

(αH3K4me3) while an antibody directed against the C-terminus of H3 (αH3) was used as a loading control. *(B)* WCEs (left) and nuclei (right) were resolved by SDS-PAGE, transferred to PVDF, and probed with antibodies directed against H3 di-methyl lysine 36 (αH3K36me2) and tri-methyl lysine 36 (αH3K36me3), with an antibody directed against the C-terminus of H3 (αH3) used as a loading control. Although the abbreviated nuclei extraction method allows for better detection when using a poorly performing antibody, it does not appear to provide an advantage when the antibody performs sufficiently well in WCE (compare WCE vs. nuclei with the H3K36me3 and H3K4me3 antibodies).

Table 1

Extraction buffers evaluated for effectiveness in detecting histone modifications

	Buffer I[a]	Buffer II[b]	Buffer III[c]	Buffer IV[d]
Salt type and concentration	320 mM $(NH_4)_2SO_4$	300 mM NaCl	600 mM NaCl	200 mM $KC_2H_3O_2$
Other components[e]	200 mM TRIS-HCl pH 8.0 20 mM EDTA pH 8.0 10 mM EGTA pH 8.0 5 mM $MgCl_2$ 1 mM DTT 10% glycerol protease inhibitors	50 mM TRIS-HCl pH 8.0 1 mM EDTA 0.1% NP40 1 mM $Mg(C_2H_3O_2)_2$ 1 mM imidazole 10% glycerol protease inhibitors	10 mM TRIS-HCl pH 7.4 5 mM EDTA 300 mM sorbitol 5 mM $MgCl_2$ 10% glycerol protease inhibitors	20 mM HEPES 7.6 1 mM EDTA 10% glycerol protease inhibitors

[a] See reference [26]

[b] See reference [27]

[c] See reference [10]

[d] See reference [25]

[e] Protease inhibitors at 2 μg/ml each of pepstatin, leupeptin, aprotinin, 2 mM PMSF and 10% glycerol were prepared consistently for all buffers, regardless of original published recipe.