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## METHODS IN MOLECULAR BIOLOGY: ASSAYING CHROMATIN SIRTUINS

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### Summary

Most of the sirtuins' nuclear substrates identified so far are histones or other chromatin-associated proteins and, thus, it is of special relevance the development of good biochemical techniques to analyze the biology of these proteins in the context of chromatin. Here, we describe several of the chromatin-based techniques to identify sirtuins' substrates, including a chromatin immunoprecipitation (ChIP) protocol, an acid-extraction protocol, and a nucleosomal immunoprecipitation protocol to analyze putative sirtuin chromatin interactors.

### Keywords

sirtuins; chromatin; chromatin immunoprecipitation (ChIP); acid-extraction; nucleosomal immunoprecipitation

### 1. Introduction

Four of the seven mammalian sirtuins have nuclear localization. SIRT1, SIRT6 and SIRT7 are mostly nuclear, whereas SIRT2, a cytoplasmic sirtuin, can be found also in the nucleus during mitosis. Most of the sirtuins' nuclear substrates identified so far are histones or other chromatin-associated proteins and, thus, it is of special relevance the development of good biochemical techniques to analyze the biology of these proteins in the context of chromatin. Here, we describe several of the chromatin-based techniques to identify sirtuins' substrates that we have been routinely using in our laboratory. Those include a chromatin immunoprecipitation (ChIP) protocol to analyze sirtuin-bound DNA regions as well as histone modifications that might be affected by sirtuins, an acid-extraction protocol to purify chromatin from cells (a method that allow us to analyze changes in protein chromatin localization and histone modifications in bulk chromatin), and a nucleosomal immunoprecipitation protocol to analyze putative sirtuin chromatin interactors. Finally, we detail a cell-based approach to analyze sirtuins' activity qualitatively and quantitatively.

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## 2. Materials

### 2.1. ChIP

1. Formaldehyde: 36.5%
2. 1xPBS
3. 2.5 M glycine: for 1 L, dissolve 75 g glycine in 1 L of distilled water. Filter to sterilize. Store at room temperature.
4. Lysis Buffer: 150 mM NaCl, 1% v/v Nonidet P-40, 0.5% w/v Sodium deoxycholate, 0.1% w/v SDS, 50 mM Tris-Cl pH 8.0, and 5 mM EDTA.
5. Protein A agarose beads
6. Salmon sperm DNA
7. BSA: For 10 mg/ml stock solution, dissolve 100 mg in 8 ml of distilled water. Adjust the volume to 10 ml with distilled water. Aliquot and store at  $-20^{\circ}\text{C}$ .
8. SIRT6 antibody (See Note 1)
9. High Salt Buffer. 100 mM Tris-Cl pH 8.5, 500 mM LiCl, 1% Nonidet P-40 and 1% w/v Sodium deoxycholate. For 500 ml, add: 50 ml of 1 M Tris-Cl pH 8.5, 50 ml of 5 M LiCl (For 5 M LiCl, dissolve 21.2 g LiCl in 90 ml distilled water. Adjust the volume to 100 ml. Store at  $4^{\circ}\text{C}$ ), 5 ml of 100% Nonidet P-40, and 5 g of Sodium deoxycholate. Adjust the volume to 500 ml with distilled water. Store at  $4^{\circ}\text{C}$ .
10. Low Salt Buffer. 150 mM NaCl, 0.5% w/v Sodium deoxycholate, 0.1% w/v SDS, 1% Nonidet P-40, 1 mM EDTA pH 8.0, Tris-Cl pH 8.0. For 500 ml, add: 15 ml 5 M NaCl (Final concentration: 150 mM), 2.5 g Sodium deoxycholate, 2.5 ml 20% SDS, 5 ml 100% Nonidet P-40, 1 ml 0.5 M EDTA, pH 8.0, 25 ml 1M Tris-Cl, pH 8.0. Adjust the volume to 500 ml with distilled water. Store at  $4^{\circ}\text{C}$ .
11. Tris-EDTA (TE, pH 8.0). 100 mM Tris-Cl pH 8.0 and 10 mM EDTA pH 8.0. For 500 ml, mix 50 ml 1M Tris-Cl pH 8.0 and 10 ml 0.5 M EDTA pH 8.0. Adjust the volume to 500 ml with distilled water.
12. Talianidis Elution Buffer. 70 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 1.5% w/v SDS. For 50 ml, mix 3.5 ml 1M Tris HCl pH 8.0, 100 ul 0.5 M EDTA pH 8.0, and 3.75 ml 20% w/v SDS. Adjust the volume to 50 ml with distilled water. Store at room temperature.
13. DNA purification Kit
14. Real-time PCR kit

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<sup>1</sup>The quality of the Sirt6 antibody is critical to the success of the ChIP. A desirable Sirt6 antibody would give one single clear band on a whole cell lysate western blot. It should also work well in an immunoprecipitation experiment. If such Sirt6 antibody is not available, proper controls are necessary to differentiate real Sirt6 binding signals from non-specific binding. Always include an IgG control when planning the ChIP experiment. Another important control is to use Sirt6 deficient cells or Sirt6 knockdown cells (Fig. 3).

## 2.2. Chromatin extraction

1. Lysis buffer: 10 mM HEPES pH 7.4, 10 mM KCl, 0.05% NP-40. For 50 ml, mix 500 ml of 1 M HEPES pH 7.4, 500 ml of 1 M KCl and 25 ml of NP-40 (See Note 2). Make up to 50 ml with water and store at 4°C. Prior to use, add protease, phosphatase (1 mM sodium ortovanadate) and deacetylase (5 mM TSA) inhibitors.
2. Low salt buffer: 10 mM Tris-HCl pH 7.4, 0.2 mM MgCl<sub>2</sub>. For 50 ml, mix 500 ml of 1 M Tris-HCl pH 7.4 and 100 ml of 1 M MgCl<sub>2</sub> and make up to 50 ml (See Note 3). Store at 4°C. Prior to use, add protease, phosphatase (1 mM sodium ortovanadate) and deacetylase (5 mM TSA) inhibitors.
3. 0.2 N HCl: for 10 ml, dilute 165.3 µl of 12.1 N HCl in 10 ml of water.
4. 1 M Tris-HCl pH 8: for 10 ml, dissolve 1.21 g of Tris in 5 ml of water and adjust the pH to 8. Make up to 10 ml with distilled water.

## 2.3. Nucleosome immunoprecipitation

1. Lysis buffer: 10 mM HEPES pH 7.4, 10 mM KCl, 0.05% NP-40, 0.5 mM DTT and 1 mM PMSF. For 50 ml, mix 500 ml of 1 M HEPES pH 7.4, 500 ml of 1 M KCl, 25 ml of NP-40, 25 µl of 1M DTT and 100 µl of 0.5 M PMSF (See Note 2 and <sup>4</sup>). Make up to 50 ml with water and store at 4°C.
2. Wash buffer: 20 mM HEPES pH 7.4, 20 mM KCl, 0.5 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF. For 50 ml, mix 1 ml of 1M HEPES pH 7.4, 1 ml of KCl, 50 µl of 0.5 M EDTA, 25 µl of 1M DTT and 50 µl of 0.5 M PMSF (See Note 4). Make up to 50 ml with water and store at 4°C.
3. Buffer A: 20 mM HEPES pH 7.4, 20 mM KCl, 0.4 mM EDTA, 0.4 mM DTT. For 50 ml, mix 1 ml of 1M HEPES pH 7.4, 1 ml of KCl, 40 µl of 0.5 M EDTA and 20 µl of 1M DTT.
4. 5 M NaCl. For 50 ml, dissolve 14.62 g of NaCl in 50 ml of water.
5. 2 M CaCl<sub>2</sub>. For 50 ml, dissolve 11.1 g of CaCl<sub>2</sub> in 50 ml of water.
6. Micrococcal nuclease
7. 0.5 M EGTA. For 50 ml, dissolve 9.5 g of EGTA in 50 ml of water.
8. 10% NP-40. For 50 ml, dilute 5 ml of NP-40 in 50 ml of water.
9. Agarose beads
10. Laemmli buffer: 200 mM Tris-Cl pH 6.8, 8% SDS, 40% Glycerol, 4% β-mercaptoethanol, and 0.08% bromphenol blue. For 20 ml, add 4 ml of 1M Tris-Cl,

<sup>2</sup>To prepare 1 liter of 1M HEPES pH 7.4, dissolve 238.3 g of HEPES in 800 ml of water, adjust pH at 7.4 and make up to 1 liter with water. To prepare 1 liter of 1M KCl, dissolve 74.55 g of KCl and make up to 1 liter with water.

<sup>3</sup>Prepare 1 liter of 1M Tris-HCl pH 7.4 by dissolving 121.12 g of Tris base in water, adjust pH at 7.4 and make up to 1 liter. To prepare 1 liter of 1M MgCl<sub>2</sub>, weigh 95.21 g of MgCl<sub>2</sub> and dissolve with water to make 1 liter of solution.

<sup>4</sup>To prepare 1M solution of DTT, weigh and dissolve in 10 ml of water 1.54 g of DTT. Make aliquots of 0.5 ml and store at -20°C. To prepare 10 ml of 0.5 M PMSF, dissolve 0.87 g of PMSF in methanol and make up to 10ml. Make aliquots of 0.5 ml and store at -20°C. To prepare 1 liter of 0.5M EDTA, dissolve 146.12 g of EDTA and make up to 1 liter with water.

8 ml 20% SDS, 8 ml Glycerol, 0.8 ml  $\beta$ -mercaptoethanol and 16 mg of bromphenol blue.

#### 2.4. In vivo SIRT6 deacetylation activity assay by immunofluorescence

1. Gelatin coated 96 well plates: Prepare a 0.2% gelatin solution by weighting 1 g of gelatin (Sigma) in 500 ml of distilled water. Solution can be stored at room temperature. Use enough volume to cover the bottom of each well and let stand for 30 min at room temperature. Remove the gelatin before plating the cells.
2. Histone Deacetylase (HDACs) inhibitors: Suberoylanilide hydroxamic acid (SAHA, Cayman Chemical). For maximum solubility in aqueous buffers, dissolve first in dimethyl sulfoxide (DMSO) and then dilute in the aqueous buffer of choice. For a 1M stock solution, add 189  $\mu$ l of DMSO to a 50 mg vial and store at  $-20^{\circ}\text{C}$ . Trichostatin A (TSA): To make 5 mM TSA stock solution, dissolve 5 mg in 3.3 ml DMSO.
3. Fixing solution: Paraformaldehyde (PFA) 4%. Make sure to use gloves when working with PFA. Use Paraformaldehyde 16% solution, EM grade (Electron Microscopy Sciences) and make a 1:4 dilution to get a working solution (E.g. for 20 ml, take 5 ml and add 15 ml of distilled water). Store at room temperature.
4. Permeabilization solution: 0.2% Triton-X 100 (v/v) in PBS. For 100 ml of PBS, add 0.2 ml of Triton-X 100. Store at room temperature.
5. Blocking solution: 1% BSA (w/v), 0.1% Tween 20 (v/v) in PBS. Prepare freshly by weighting 0.5 g of bovine serum albumin (BSA) and diluting in 45 ml of PBS. Add 50 ml of Tween 20 and adjust to a final volume of 50 ml.
6. Antibodies: Prepare the working solutions diluting the antibody in blocking solution. Make sure to keep secondary antibody in the dark.
7. Wash buffer: PBS, 0.1% Tween 20 (v/v). For 100 ml of PBS use 0.1 ml of Tween 20. Store at room temperature.
8. Nuclei counterstaining: DAPI 100 ng/ml in PBS. Use DAPI (Sigma D9542) to prepare a stock solution (5mg/ml) by weighting 10 mg of DAPI and dissolving in 2 ml of dimethylformamide (DMF). Aliquot and store at  $-20^{\circ}\text{C}$ . Make a working solution (100 ng/ml) by adding 4 ml of the stock solution in 50 ml of PBS. Store at  $4^{\circ}\text{C}$  (use a brown bottle or wrap with aluminum foil to protect from light).
9. Fluorescent microscope with a 20X or 40X (dry) objective with suitable excitation and emission filters for DAPI and two additional fluorophores. For automated acquisition of images, a fluorescence high-throughput microscope with the same characteristics and automated stage and focus must be used. Captured images in a variety of formats (.bmp, .jpg, .jpeg, .tif, .tiff, .zvi, among others), can be read by the image analysis software (*CellProfiler*).
10. *CellProfiler* software: The software can be downloaded for free at [www.cellprofiler.org](http://www.cellprofiler.org)

11. Example images and their corresponding *CellProfiler* pipelines can be downloaded from the Mostoslavsky laboratory webpage <http://mostoslavskylab.mgh.harvard.edu>

### 3. Methods

#### 3.1 Prepare Buffers

1. To prepare 500 ml of lysis buffer, mix 15 mL of 5 M NaCl (For 1 L of 5 M NaCl, dissolve 292 g of NaCl in 800 ml of distilled water. Adjust the volume to 1 L with distilled water)
2. Add 5 ml of 100% Nonidet P-40
3. Add 2.5 g Sodium deoxycholate
4. Add 2.5 ml of 20% SDS (For 1 L of 20% SDS, dissolve 200 g of SDS in 900 ml of distilled water. Heat to 68°C with constant stir. Adjust the volume to 1 L with distilled water)
5. Add 5 ml of 1 M Tris-Cl pH 8.0 (For 1 L of 1 M Tris-Cl pH 8.0, dissolve 121.1 g of Tris base in 800 ml distilled water. Adjust the pH to 8.0 with concentrated HCl. Adjust the volume to 1 L with distilled water)
6. Add 5 ml of 0.5 M EDTA (For 1 L of 0.5 M EDTA, add 186.1 g of EDTA.2H<sub>2</sub>O to 800 ml distilled water. Stir constantly. Adjust the pH to 8.0 with NaOH. Adjust the volume to 1 L with distilled water).
7. Adjust the volume to 500 ml with distilled water. Store at 4°C.
8. Right before use, add: Protease Inhibitors Cocktail (Roche Applied Science), Phosphatase Inhibitors: 50 mM NaF (For 5 ml of 500 mM NaF, dissolve 0.1 g in 5 ml distilled water. Aliquot and store at -20°C.), 0.2 mM sodium orthovanadate (For 5 ml of 200 mM sodium orthovanadate, dissolve 0.18 g in 5 ml distilled water. Aliquot and store at -20°C), HDAC inhibitors: 5 μM trichostatin A (For 5 mM TSA, dissolve 5 mg in 3.3 ml DMSO), 5 mM sodium butyrate (For 5 M, dissolve 2.75 g in 5 ml of distilled water. Aliquot and store at -20°C), 0.5 mM PMSF (For 100 mM PMSF, dissolve 87.09 mg in 5 ml ethanol. Aliquot and store at -20°C).

#### 3.2 ChIP

This Sirt6 ChIP protocol is adapted with modifications from Joaquin Espinosa's laboratory (1).

1. Grow Cells in 150 mm plates until cells are 50-70% confluent.
2. Cross-linking: Remove media from the plates completely. Add 19 ml of 1% Formaldehyde in 1xPBS. Incubate on a shaking platform at room temperature for 15 minutes (See Note 5).
3. Quench formaldehyde with 125 mM glycine (1 ml of 2.5 M glycine per 19 ml of 1% formaldehyde in 1xPBS) on a shaking platform at room temperature for 5 minutes.

4. Remove the formaldehyde solution completely from the plates.
5. Wash the plates twice with ice-cold 1×PBS. After the second wash, place the plates vertically on ice for 1 minute to collect the leftover PBS at the bottom of the plates. Remove PBS by aspiration.
6. Put the plates on ice or at 4°C. Add lysis buffer (1 ml per plate) to the first plate (See Note 6). Scrape the plate and transfer the lysate to the next plate, until all the plates are collected (See Note 7).
7. Sonicate the lysate to shear chromatin (See Note 8).
8. Centrifuge at 12,000 rpm for 20 minutes at 4°C to clear the lysate. Transfer the supernatant to a fresh tube. The lysate can be snap frozen and stored at –80°C (See Note 7).
9. Measure protein concentration and dilute the lysate to 2 mg/ml with complete lysis buffer. Aliquot ~1.1 ml/tube and snap freeze the lysate if it is not used immediately. Store aliquots at –80°C.
10. Wash 2 sets of protein A agarose beads (50 µl per IP sample) 3 times with lysis buffer to remove ethanol. A wash consists of resuspending the beads with 1 ml lysis buffer, centrifuging at 4,000 rpm for 1 minute at 4°C, and removing the supernatant. One set of protein A agarose beads will be used to pre-clear the lysate. The other set will be blocked and used in the IP.
11. Pre-clear lysate with 50 µl of 50% slurry protein A agarose beads previously washed in lysis buffer, not blocked with salmon sperm DNA nor BSA. Rotate at 4°C for 2 hours. Centrifuge 1 minute at 4,000 rpm at 4°C. Transfer supernatant to a fresh tube.
12. During step 11, block the other set of washed agarose beads with 1 mg/ml BSA and 0.3 mg/ml Salmon Sperm DNA. Rotate for 2 hours at 4°C. Wash 3 times with lysis buffer.
13. Transfer 10 µl of pre-cleared lysate to a new tube and store at –20°C for later use. This is the Input sample.
14. Transfer 1 ml of pre-cleared lysate to a new tube and add Sirt6 antibody or equivalent amount of non-specific serum or IgGs. The amount of Sirt6 antibody added should be determined empirically (See Note 1). Add 50 µl of blocked protein A agarose beads from Step 12. Rotate at 4°C overnight.

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<sup>5</sup>The cross-linking time and the formaldehyde concentration are the key factors that affect cross-linking efficiency. Longer cross-linking time or higher formaldehyde concentration is more efficient, but may cause detection of non-specific binding. On the other hand, shorter cross-linking time or lower formaldehyde concentration may increase chromatin shearing efficiency, but this may decrease the yield of precipitated chromatin for certain proteins, especially those that do not directly bind to DNA.

<sup>6</sup>The protease/HDAC/phosphatase inhibitors should be added right before the harvest.

<sup>7</sup>The lysate can be snap-frozen by dropping the tubes into liquid nitrogen and stored at –80°C until ready to proceed. To thaw, put tubes in room temperature water bath, then put on ice.

<sup>8</sup>The sonication conditions should be determined empirically for each cell type or sonicator model. To check the sonication efficiency, take an aliquot of 50 µl from the sonicated chromatin, add 3 µl of 5 M NaCl and put on a 95°C heatblock for 5 minutes. Run the mixture on a 1.5% agarose gel. The fragment should be between 300-1000 bp.

15. Wash 2 times with lysis buffer, 4 times with high salt buffer (See Note 9), 2 times with lysis buffer, and 2 times with TE.
16. Aspirate supernatant from the last wash down to the 100  $\mu$ l mark in the microcentrifuge tube (Take special care to be consistent between samples).
17. Add 200  $\mu$ l of Talianidis Elution Buffer.
18. Elute immunocomplexes by incubating 10 minutes at 65°C, vortex samples occasionally.
19. Transfer 240  $\mu$ l to a fresh tube and add 10  $\mu$ l of 5M NaCl (200 mM final).
20. Take out the Input samples from -20°C (from step 13). Add 10  $\mu$ l of 5M NaCl and 230  $\mu$ l of Talianidis Elution Buffer.
21. Reverse-crosslink all the samples (including Inputs) by incubating them in a 65°C water bath for 5 hours. Spin occasionally to bring down liquid condensation formed on the lids of the tubes.
22. Purify DNA with commercially available DNA purification kit of your choice. Purified DNA can be stored at -20°C.
23. Setup real time PCR with the primer pairs designed to amplify genomic region of interest. Follow the PCR protocol of the PCR kit of your choice. For data analysis, follow standard procedure (for example: <http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/epigenetics-noncoding-rna-research/Chromatin-Remodeling/Chromatin-Immunoprecipitation-ChIP/chip-analysis.html>)

### 3.3. Chromatin extraction

This protocol is adapted with modifications from Junjie Chen's laboratory (2).

1. Collect cells and wash them in 1X PBS.
2. Resuspend cell pellet in 2-5 volumes of lysis buffer.
3. Incubate 20 min on ice.
4. Centrifuge at 14,000 rpm at 4°C, 10 min. The **supernatant** contains the **cytoplasmatic proteins** and the pellet contains the nuclei (Fig. 1, lanes 1 and 2).
5. Wash the nuclei once with lysis buffer and centrifuge at 14,000 rpm at 4°C, 10 min. If nucleoplasmatic proteins are desired go to step 6, otherwise go to step 8.
6. OPTIONAL: Resuspend the nuclei with 2-5 volumes of Low Salt Buffer+1% Triton-X 100 and incubate 15 min on ice.
7. Centrifuge at 14,000 rpm at 4°C, 10 min. The **supernatant** contains the **nucleoplasmatic proteins**, and the pellet contains the chromatin.
8. Resuspend chromatin with 2-5 volumes of HCl 0.2N and incubate 20 min on ice.

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<sup>9</sup>If the Sirt6 antibody is not strong, substitute High Salt Buffer with Low Salt Buffer.

9. Centrifuge at 14,000 rpm at 4°C, 10 min.
10. Keep and neutralize SN (contains the acid soluble proteins) with the same volume of 1M Tris-HCl pH 8. This would be the chromatin fraction (Fig. 1, lanes 3 and 4).

### 3.4. Nucleosome immunoprecipitation

This protocol is adapted with modifications from Don W. Cleveland's laboratory (3).

1. Collect cells ( $15\text{-}30 \times 10^6$  per IP) and wash them with 1 ml of 1X PBS.
2. Resuspend cell pellet in 5 volumes of lysis buffer.
3. Incubate 20 min on ice.
4. Pellet nuclei at  $300\times g$  at 4°C, 10 min.
5. Wash the nuclei once in 1 ml of wash buffer and centrifuge at  $1000\times g$  at 4°C, 5 min.
6. Wash the nuclei once in 1 ml of wash buffer containing 300 mM NaCl and centrifuge at  $1000\times g$  at 4°C, 5 min.
7. Resuspend nuclei in 1 ml of wash buffer containing 300 mM NaCl and 3 mM  $\text{CaCl}_2$ . Digest chromatin for 1h at room temperature using 140 units/ml of micrococcal nuclease (see Note 10).
8. Stop reaction by adding 10  $\mu\text{l}$  of 0.5 M EGTA (5 mM final) and 5  $\mu\text{l}$  of 10 % NP-40 (0.05 % final) and centrifuge at  $10,000\times g$  for 15 min at 4°C. Keep the supernatant, which contains the soluble chromatin (mainly mononucleosomes) (see Note 11).
9. Incubate chromatin extract with the antibody overnight at 4°C in an orbital rotator.
10. Add 40  $\mu\text{l}$  of agarose beads (50 % slurry) and incubate 4h at 4°C in an orbital rotator.
11. Pellet the beads at  $3000\times g$  for 2 min at 4°C.
12. Wash the beads sequentially with 1 ml of buffer A, 1 ml of buffer A with 300 mM KCl and 1 ml of buffer A with 300 mM KCl, 1 mM DTT and 0.1 % Tween-20. Centrifuge at  $3000\times g$  for 2 min between washes to pellet the beads.
13. Elute the immunocomplexes by boiling the beads with 50  $\mu\text{l}$  of Laemmli buffer for 10 min.

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<sup>10</sup>The time of digestion and the units of micrococcal nuclease used might be different among several enzymes. Testing and optimizing the right conditions to get all DNA in the mononucleosomal fraction is strongly recommended.

<sup>11</sup>To confirm that most of the DNA is the mononucleosomal fraction, take an aliquot and purify the DNA as follows: add 1 volume of phenol/chloroform, mix well and centrifuge at 14,000 rpm for 3 min. Take aqueous phase (upper phase) and precipitate DNA with 1 volume of isopropanol. Centrifuge at 14,000 rpm for 5 min and discard the supernatant. Wash the pellet with 70% ethanol, centrifuge at 14,000 rpm for 3 min and air-dry the pellet. Resuspend DNA in water and run the samples in a 1.2 % agarose gel. The DNA present in mononucleosomes is about 147 bp in size (Fig. 4).

### 3.5 In vivo SIRT6 deacetylation activity assay by immunofluorescence

As observed for many proteins, recombinant purified SIRT6 exhibit low activity in vitro, likely due to either post-translational modifications that are missing or else co-factors needed for its activity. In this regard, overexpressing SIRT6 in mammalian cells is a highly useful approach to assess its deacetylase activity *in vivo*. Indeed, commercially available antibodies specific for the acetylated form of the protein of interest make immunofluorescence a powerful tool that can be used to directly examine potential inhibiting or activating compounds. By using a FLAG tagged SIRT6 expression system, double staining can be performed to determine both levels of expression of SIRT6 (anti-FLAG antibody) and levels of acetylation of a target protein (anti-acetyl-protein antibody). In this assay, fluorescence intensity of the acetyl-histone mark is inversely correlated with SIRT6 expression levels. We have developed a method where automated-microscopy based analysis allows for a cell-based quantitative analysis of SIRT6 activity, which could be adapted for other HDACs as well (Fig. 2).

All procedures should be carried at room temperature unless otherwise specified.

1. Use FLAG tagged SIRT6 fusion protein subcloned in the pCMV vector to transfect HeLa cells (5 $\mu$ g of DNA in 10 cm plates). See Note 12.
2. Plate FLAG tagged SIRT6 expressing HeLa cells (100  $\mu$ l,  $2 \times 10^4$  cells/well) in 0.2% gelatin coated 96-well plates and allow to adhere overnight.
3. In order to increase basal levels of histone acetylation, pre-incubate transfected attached cells with HDACs inhibitors [suberoylanilide hydroxamic acid (SAHA, 1 $\mu$ M) or trichostatin A (TSA, 200nM)] for 12-24 h. This pre-treatment can be used for determining the potential effect of different compounds. For SAHA, prepare a fresh dilution for every experiment by making a 1:100 dilution (10 mM) in the culturing media and use 10  $\mu$ l/well (1 $\mu$ M). For TSA, make a 1:1000 dilution of the stock solution and add 10  $\mu$ l per well.
4. Fix with 100  $\mu$ l/well of 4% paraformaldehyde (v/v) in PBS for 10 minutes.
5. Permeabilize with 100  $\mu$ l/well of 0.2% Triton-X 100 (v/v) in PBS for 10 minutes.
6. Block with 100  $\mu$ l/well of 1% BSA (w/v), 0.1% Tween 20 (v/v) in PBS for 30 minutes with gentle shaking.
7. Stain by adding 50  $\mu$ l/well of anti-Flag antibody and an antibody specific for the acetylated form of the protein of interest in blocking buffer (in the case of SIRT6, anti-H3K9Ac or anti-H3K56Ac antibody). Incubate for 1 hour at room temperature (or overnight at 4°C) with gentle shaking. See Note 13.
8. Wash 3 times with 100  $\mu$ l/well of PBS, 0.1% Tween 20 (v/v) for 5 minutes.

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<sup>12</sup>The amount of DNA can be modified according to the size of the plates used (E.g. 2.5 mg of DNA for 6 cm plates). HEK 293T cells can be used but they easily detach, so especial care should be taken during washes. Volumes described in this protocol can be adapted for 384 well plates.

<sup>13</sup>Make sure that different hosts were used to generate the primary antibodies.

9. Incubate with fluorophore-conjugated secondary antibodies diluted in blocking buffer for 1 hour at room temperature protected from light.
10. Wash 2 times with 100  $\mu$ l/well of PBS, 0.1% Tween 20 (v/v) for 5 minutes and
11. Counterstain nuclei with 100  $\mu$ l/well of DAPI 100 ng/ml in PBS for 2 minutes at room temperature.
12. Wash with 100  $\mu$ l/well of PBS and keep the cells in PBS at 4°C until visualization with a fluorescence microscope.
13. Images from each well must be acquired using a fluorescent microscope with a 20X or 40X (air) objective using the suitable excitation and emission filters for the fluorophores that were used in the staining of Sirt6-Flag and the acetylated-protein of interest. It is recommended to acquire images from at least 9 different fields per well when using a 40X objective. Alternatively, images can be acquired with a high-throughput microscope with automated stage and focus.
14. Cell image analysis is then performed using *CellProfiler* analysis software (4). First, download and install *CellProfiler* analysis software from [www.cellprofiler.org](http://www.cellprofiler.org).
15. Download the file called *Cellular Measurement of Sirt6 activity* from Mostoslavsky lab webpage (<http://mostoslavskylab.mgh.harvard.edu>). After downloading the file, make sure that it is decompressed. The file contains example images and a pipeline made of different analysis modules placed in order.
16. Run the pipeline *Measurement of Sirt6 activity* on the example images you downloaded. To do this, follow the instructions *loading an existing pipeline* clicking in Help/Using CellProfiler/How to build a pipeline.
17. Adjust the main pipeline for your images using test images.
18. First, create a test input folder in your computer where you copy several test images into it and a test output folder. In the CellProfiler, set the default input folder and the default output folder to be your test input folder and test output folder, respectively.
19. Some adjustments might be done in the module LoadImages. This module loads an image set, which is a group of related images (in our case, the multiple channels at the same image location) and gives each image a name for further processing. Our image set is comprised of three images: DAPI, Sirt6-Flag and acetyl-protein. We use text matching to define the difference between images in a set: in the example images, all the images stained for nuclei have the text DAPI in the name, Sirt6-Flag images have the text Alexa555 and acetyl-protein images have Alexa488. Some adjustments might be done to load your images by matching files in the folder against the unique text pattern for each stain.
20. Additional modules will require some adjustments; the parameters that can be adjusted to run your images appear on the right after clicking on a module. In

general, the modules that might require some adjustment are IdentifyPrimaryObjects and ClassifyObjects (See Notes 14 and <sup>15</sup>)

21. Run the pipeline on your images.
22. Perform data acquisition (See Note 16)
23. Perform downstream data analysis (See Note 17).

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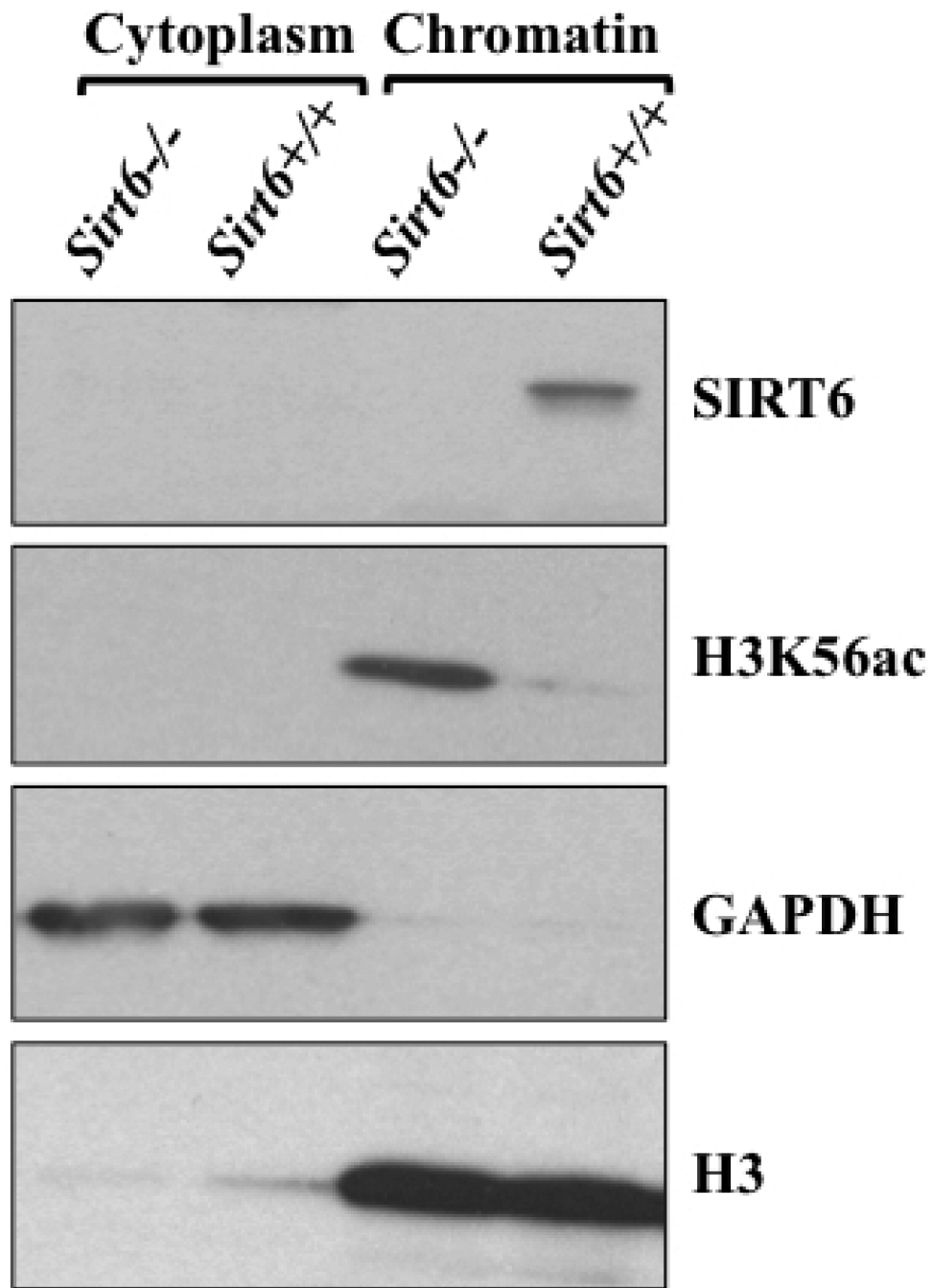
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<sup>14</sup>In general, the IdentifyPrimaryObjects module often needs adjustment. IdentifyPrimaryObjects: the *typical diameter of objects* (will depend on the cell type and the magnification of the objective used for the acquisition), the *thresholding method*, *threshold correction*, the *lower and upper bounds on the threshold*, the *size of smoothing filter* and *suppress local maxima*.

<sup>15</sup>In general, the ClassifyObjects module often needs adjustment. ClassifyObjects: Enter the custom threshold separating the values between bins. The pipeline allows you to classify nuclei according to Sirt6-Flag staining into positive or negative for Sirt6-Flag. After measuring the intensity of nuclear Sirt6-Flag staining, the values are shown in the corresponding image (in the module DisplayDataOnImage) in order to visually determine a cutoff. The empirically determined cutoff value must be introduced in the setting Enter the custom threshold separating the values between bins.

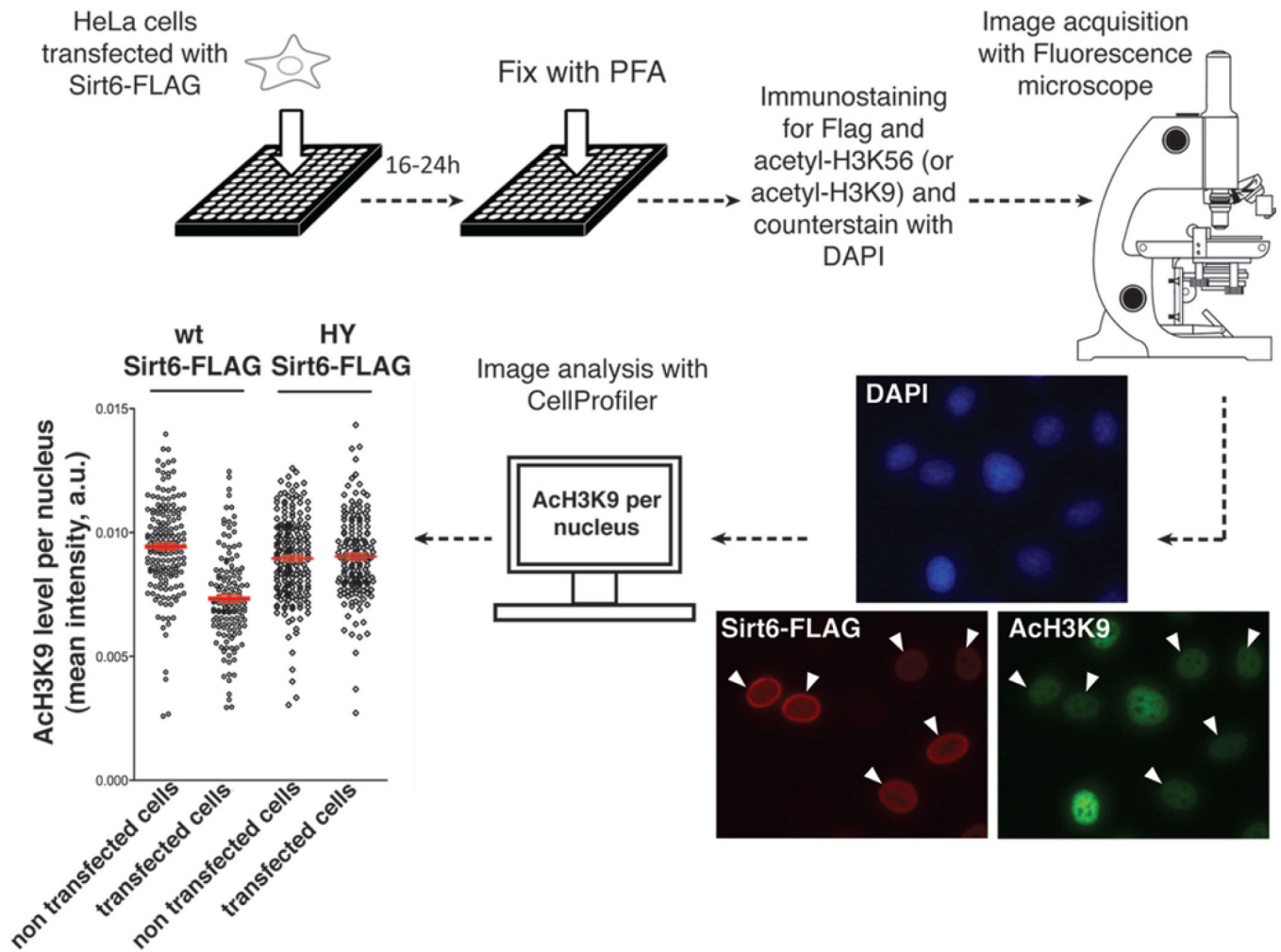
<sup>16</sup>The data files are exported to the designed output folder as .csv files that can be opened using Excel. For example, the DefaultOUT\_Image.csv file contains per-image data. Each row represents one image and the parameters measured in each image are displayed in columns: total number of nuclei per image, number of Sirt6-Flag positive nuclei per image, number of Sirt6-Flag negative nuclei per image and Sirt6-Flag positive and negative nuclei as percentage of total nuclei in the image. Also, DefaultOUT\_Sirt6PosNuclei.csv and DefaultOUT\_Sirt6NegNuclei.csv files contain per-cell data. Each row represents one nucleus and the parameters measured in each nucleus are displayed in columns. The columns *Intensity\_MedianIntensity\_OrigGreen* and *Intensity\_MeanIntensity\_OrigGreen* display the median and mean intensity of the acetyl-protein staining respectively, and correlate with the acetylation levels of the target protein per nucleus. The *ImageNumber* column relates each nucleus to the image where it belongs to.

<sup>17</sup>Data analysis and plotting can be performed in programs like Excel or Prism. This cell-based image analysis allows us to detect changes in the whole population (if the median or mean intensity of acetylated-protein in Sirt6-Flag positive cells is used) but also allows us to detect small changes in a percentage of cells (if the data per individual cell is used). For robustness, it is strongly recommended a minimum of 200 cells/condition acquired, if the transfection efficiency was at least 30% (Fig. 2).



**Figure 1.**

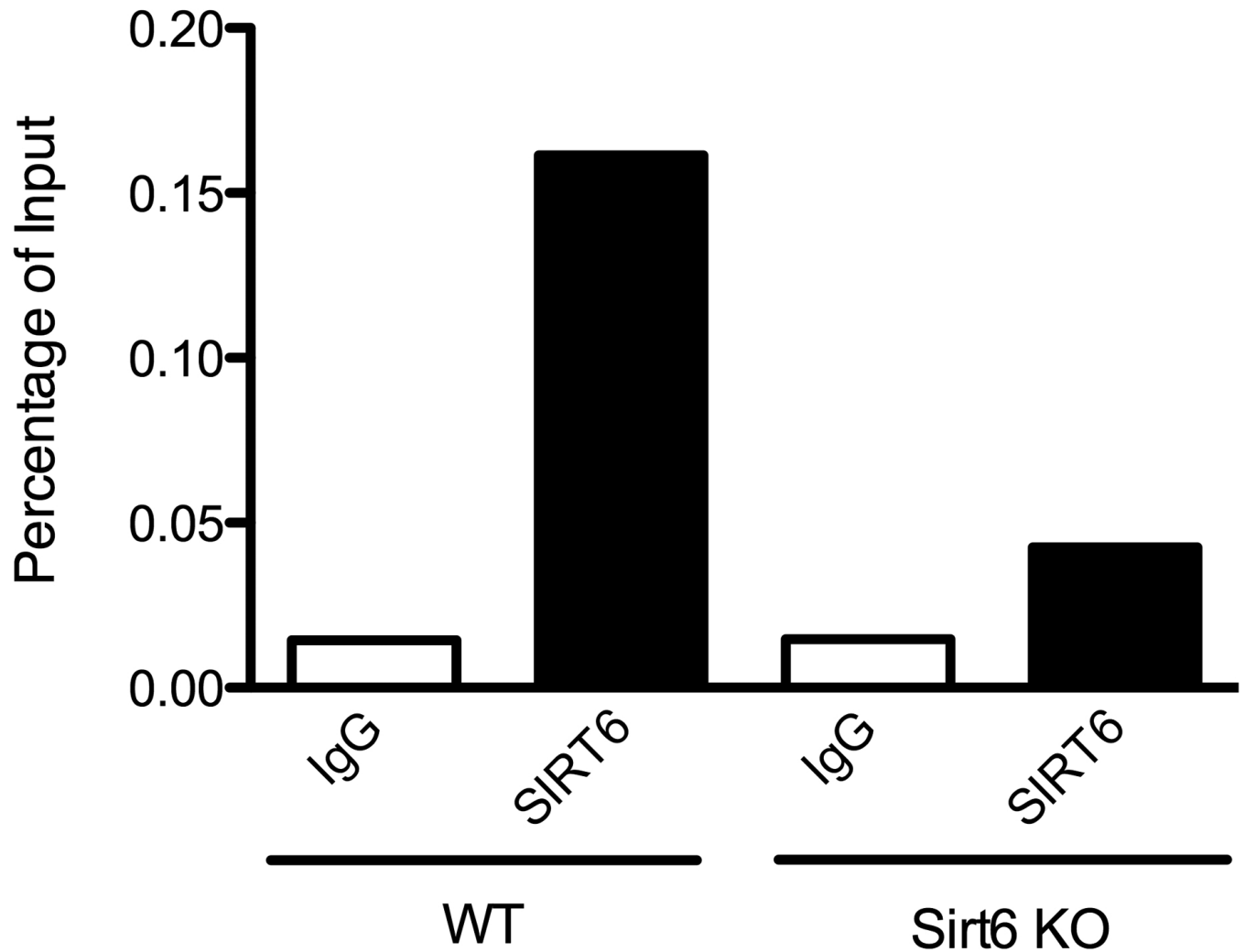
Chromatin purification from *Sirt6* WT and KO ES cells. The supernatant (containing the cytoplasmic proteins, 50  $\mu$ g) and chromatin (20  $\mu$ g) fractions were used to analyze by western blot the localization of SIRT6 (mostly chromatin-bound). Note that acetylation levels of H3K56 (a main substrate for SIRT6) are increased in *Sirt6* KO ES cells. GAPDH and H3 were used to confirm the purity of the fractions.



**Figure 2.**

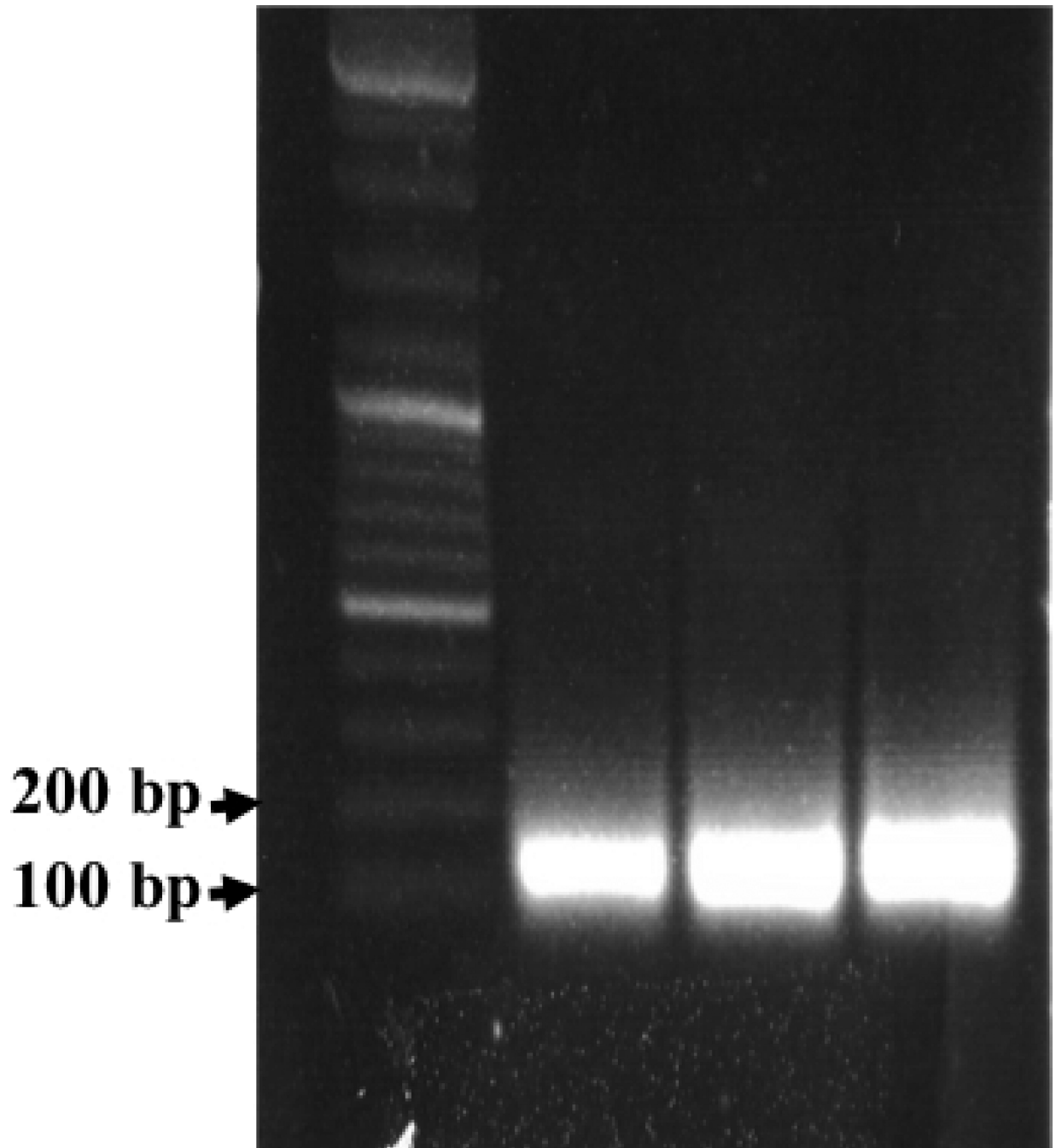
SIRT6 overexpression inversely correlates with the levels of H3K9 acetylation. HeLa cells were transfected with a SIRT6-FLAG expressing vector and double immuno-stained with anti-FLAG and anti-AcH3K9 antibodies. The scheme depicts a summary of the analysis performed after acquiring the images with a fluorescent microscope. Arrows indicate SIRT6 expressing cells. Images were analyzed using the *Cell Profiler* software. Quantitation shows lower levels of H3K9 acetylation in SIRT6-FLAG overexpressing cells. This difference is abolished when a catalytic mutant of SIRT6 is used (HY-SIRT6-FLAG).

## Sirt6 ChIP at LDHB promoter



**Figure 3.**

An example of Sirt6 ChIP at LDHB promoter in mES cells. Controls include Sirt6-deficient cells (Sirt6 KO) and IgGs for both WT and Sirt6 KO cells. Note that even in Sirt6 KO cells, Sirt6 ChIP signal is 4 fold higher than its IgG counterpart, possibly due to unspecific binding of Sirt6 antibody. This example highlights the necessity of a Sirt6 KO control in ChIP experiments.



**Figure 4.** Mononucleosome purification. Nuclei from  $15 \times 10^6$  293T cells (lanes 1-3) were digested in 1 ml of digestion buffer (see main text) and an aliquot (100  $\mu$ l) was taken to purify the DNA as described in the methods section. Note that almost all DNA is contained in the mononucleosomal fraction.