

Chapter 2

Characterization and Quality Control of Antibodies Used in ChIP Assays

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Abstract

We present here the very robust characterization and quality control (QC) process that we have established for our polyclonal antibodies, which are mainly directed against targets relevant to the epigenetics field such as modified histones, modifying enzymes, and chromatin-interacting proteins. The final purpose of the characterization and QC is to label antibodies as chromatin immunoprecipitation (ChIP) grade. Indeed, the ChIP method is extensively used in epigenetics to study gene regulation and relies on the use of antibodies to select the protein of interest and then precipitate and identify the DNA associated to it. We have optimized in-house all protocols and reagents needed from the first to the last step of antibody characterization. First, following immunizations, the rabbit crude serum is tested for immune response. Whether or not the antibody is specific is determined in further characterizations. Then, only specific antibodies are tested in ChIP using an optimized method which is ideal for antibody screening. Once QC is established for one antibody, it is used to similarly characterize each antibody batch in order to supply researchers in a reproducible manner with validated antibodies. All in all, this demonstrates that we develop epigenetics research tools based on everyday's researcher's needs by providing batch-specific fully characterized ChIP-grade antibodies.

Key words: Antibody, characterization, quality control, specificity, chromatin immunoprecipitation.

1. Introduction

Extensive characterization of antibodies represents a real need in the research field (1–4). A defined quality control (QC) for each antibody is also of extreme importance due to possible batch to batch variation. Moreover, the use of chromatin immunoprecipitation (ChIP) grade antibodies is essential in any experiment aiming to study protein–DNA interactions. We present here the

very robust QC that we established for our antibodies, which are mainly directed against modified histones, chromatin-modifying enzymes, and chromatin-interacting proteins. As ChIP is a major method used to study gene regulation in epigenetics looking at *in vivo* protein–DNA interactions, we focus all our attention on the ChIP-grade antibody characterization using a variety of methods in sequentially ordered steps.

We first design immunogenic peptides in order to produce polyclonal antibodies directed against the target of interest, using preferentially N-terminal and C-terminal regions, including modifications when applicable (e.g., modified histone tails). We use the Lasergene software by DNASTAR (Madison, USA) to design the peptides, looking for large regions with high hydrophobicity. Selected regions are then checked for high surface probability and high antigenicity index. We choose peptides of about 16 amino acids or less, avoiding alpha helices and repeats. We use maximum two peptides for one target per rabbit immunization. Two rabbits are injected with the chosen peptide(s), which is conjugated to KLH to boost the antibody production (*see Note 2*). Although both crude sera and purified antibodies are submitted to a similar step-by-step QC, we focus first on crude sera before undertaking any purification (**Fig. 2.1A,B**).

Step 1: As soon as bleeds are available, the crude serum is first tested in ELISA side by side with the pre-immune for immune response assessment. Antibodies from crude sera can be affinity purified and tested in ELISA before and after purification (*see Section 3.1*). Step 2: Whether or not the antibody is specific is determined during further characterization. We use dot blot and western blot when applicable (note that at this stage, it is also possible to perform immunoprecipitations (IP) and immunofluorescence (IF) assays) (*see Sections 3.2 and 3.3*). Step 3: Then specific antibodies are tested in ChIP (*see Section 3.4*). Our LowCell# ChIP kit which was proven to give reproducible results is used for antibody screening. It is an ideal tool as it also ensures the use of low amount of reagents per reaction (not only cells but also antibodies, inhibitors, and buffers), the number of steps is greatly reduced, and handling is much easier. Finally, it is crucial to characterize each antibody batch with an established QC to validate the antibodies in a reproducible manner. An example of QC strategy is given below and results are shown for an antibody raised against one modified histone (H3K9me3; **Figs. 2.1, 2.2, and 2.3**).

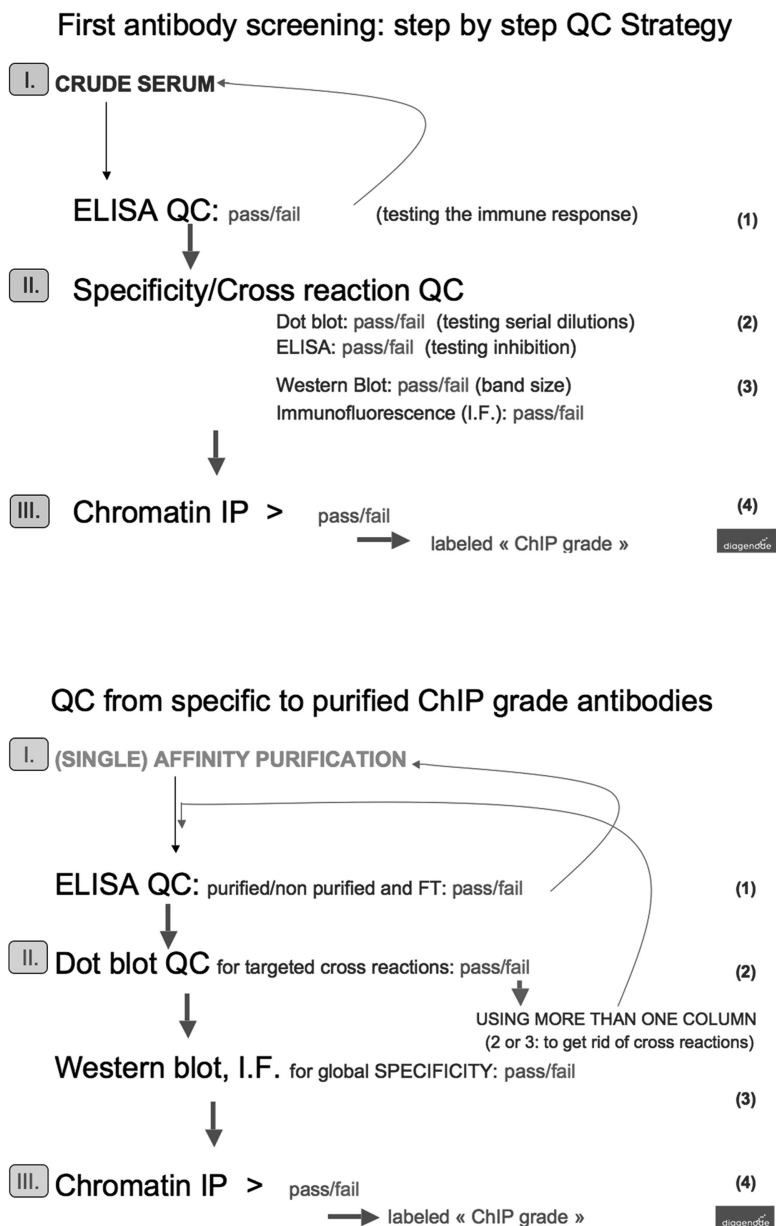


Fig. 2.1. In order to validate our antibodies, we go step by step. We start by designing immunogenic peptides. After immunization, we analyze the rabbit crude sera for immune response and antibody specificity (A), this corresponds to Steps 1 and 2, respectively. Affinity purified antibodies undergo a similar QC (B). The specific antibodies undergo ChIP validation (Step 3). Once ChIP graded, other tests can be performed such as ChIP-chip and ChIP-seq to validate the antibody further.

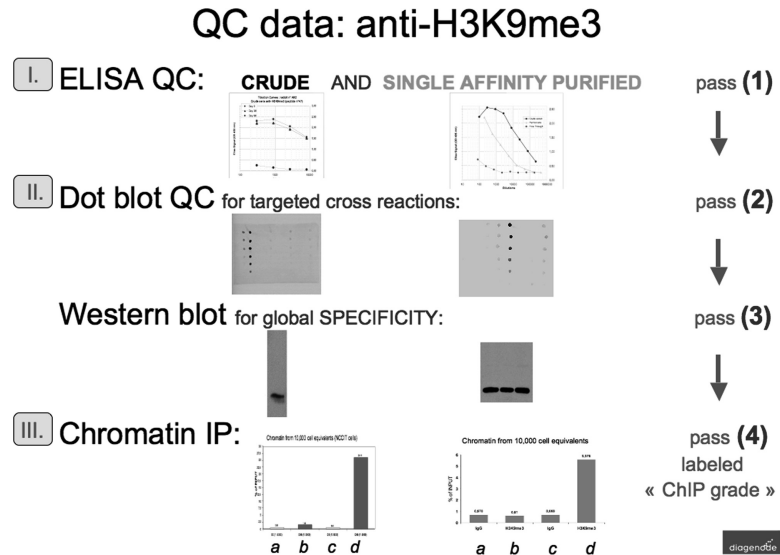


Fig. 2.2. Here is an example of antibody QC data obtained with crude serum and corresponding affinity purified antibody. In order to validate our antibodies directed against histone H3K9me3 (cat. no. CS-056 and pAb-056, Diagenode), we go step by step from Steps 1–3. After immunization, we analyze the rabbit crude sera for immune response and antibody specificity. Affinity purified antibodies undergo similar QC. In ELISA, pre-immune and flow-through after purification do not give any signal, while crude sera and purified antibody fraction give a positive signal. In dot blot, the specificity was tested using mono-, di-, and tri-methylated peptide sequences containing H3K9me1,2,3, H4K20me1,me2,me3, H3K27me1,me2,me3, and H3K36me1,me2,me3 (from *right to left*). Specific antibodies are then further validated in ChIP. We use the pre-serum as negative IP control (*a*, *c*), which gave no ChIP signal. We also use one positive (*a*, *b*) and one negative (*c*, *d*) PCR target for each antibody being tested. A good ChIP signal was obtained with the positive PCR target used after the IP of chromatin with the antibody anti-H3K9me3 (*d*). Note that optimal dilutions of both crude serum and purified antibodies to be used in each assay are determined by titration. Here, in dot blot, western blot, and ChIP, the dilutions are 1:10,000, 1:750, and 1:5,000, respectively, using crude serum and 1:1,000, 1:500, and 1 μ g/IP using purified antibody.

2. Materials

2.1. ELISA

1. Strips F8 BioOne, High Binding (cat. no. 762.061, Greiner) or 96-well microplate BioOne, High Binding (cat. no. 655.081, Greiner).
2. Peptide solution stock: 10 mg/mL in 50 mM Tris-HCl, pH 8.0.
3. Coating buffer: 0.1 M carbonate-bicarbonate, pH 9.6.
4. Phosphate-buffered saline with Tween (PBS-T): 0.05% Tween 20 (v/v) in PBS.

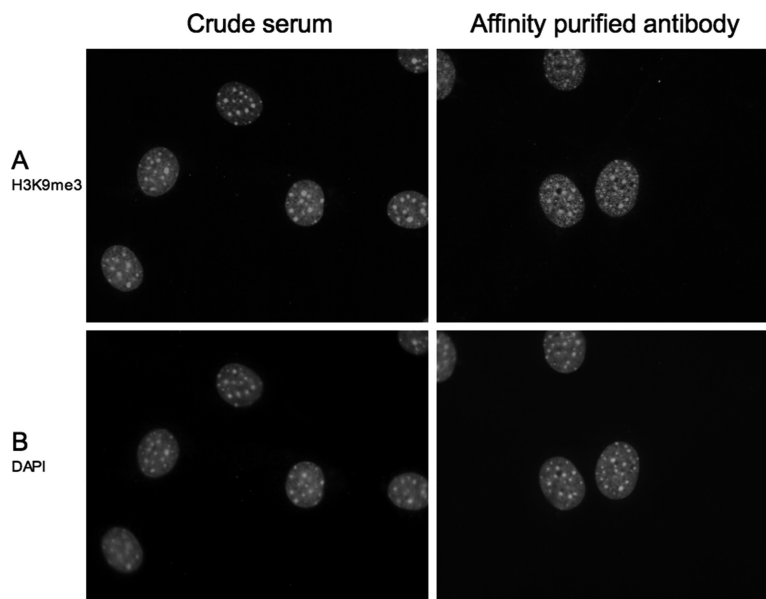


Fig. 2.3. Here is an additional antibody characterization that has been done to show antibody-specific binding to its target localized in the nucleus. Indirect immunofluorescence results obtained with the antibody anti-H3K9me3 (cat. no. CS-056 and pAb-056, Diagenode). NIH3T3 cells are stained with the antibody directed against H3K9me3 and with DAPI. Cells are formaldehyde fixed, permeabilized with Triton X-100, and then blocked with BSA containing PBS. **(A)** Cells are immunofluorescently labeled with the rabbit polyclonal antibody anti-H3K9me3 (both pAb-056 and CS-056 at dilution 1:200, and incubated for 1 h at RT) followed by goat anti-rabbit antibody conjugated to FITC. **(B)** Nuclei were DAPI stained to label specifically the DNA. Note the presence of more intense spots showing the distribution pattern of this modified histone. Both, antibody and DAPI staining are restricted to the nucleus.

5. ELISA saturation buffer: 3% (w/v) BSA in PBS-T.
6. ELISA dilution buffer: 1% (w/v) BSA in PBS-T.
7. ProClin 300 (Sigma).
8. ELISA wash buffer: 0.01% (v/v) ProClin 300 in PBS-T.
9. Primary antibody (rabbit pre-immune and crude serum, reference antibody).
10. HRP-conjugated goat antibody anti-rabbit IgG.
11. ELISA substrate: tetramethyl benzidine (TMB).
12. ELISA stop solution: 1 M H₂SO₄ (3X, 3 M Rectapur).
13. Keyhole limpet hemocyanin (KLH).
14. Microplate reader.

2.2. Dot Blot

1. Plate of 96-wells F (None or low binding; cat. no. 269620, NUNC).

2. PVDF membrane (cat. no. 162-0176, Bio-Rad).
3. Aliquot of 10 μ L of 5 mM peptide stock.
4. Dot blot buffers: 50 mM Tris-HCl, pH 7.5 (sterile, filtered on 0.2 μ m); TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl); TBS-T (0.05% (v/v) Tween in TBS); DB blocking buffer (2% (w/v) BSA in TBS-T); primary antibody dilution buffer (3% (w/v) BSA in TBS-T); secondary antibody dilution buffer (5% (w/v) low-fat dry milk in TBS-T).
5. Ponceau S solution (cat. no. 33427, Serva) used to double-check spotting efficiency.
6. Primary antibody (crude serum, pre-immune, and/or purified antibody).
7. Secondary antibody (enhanced chemiluminescent (ECL) peroxidase labeled anti-rabbit; cat. no. NA934VS, GE Healthcare).
8. Peroxidase substrate (ECL Advance western blotting detection kit; cat. no. RPN2135, GE Healthcare).
9. Imaging system (chemiluminescence detection; Kodak Gel Logic 1500).

2.3. Western Blot

2.3.1. Histone Extraction

1. Cultured cells and tissue-culture grade PBS (cat. no. 14190, Gibco).
2. Triton extraction buffer (TEB; 0.5% (v/v) Triton X-100 in PBS).
3. Protease inhibitors (100X solution; P8340, Sigma). Add to TEB before use.
4. 0.2 N HCl.
5. Bradford reagent (Sigma).

2.3.2. Nuclear Extract Preparation

1. Cultured cells, tissue-culture grade PBS, and tissue culture scrapers.
2. Igepal-CA630. Prepare 10% (w/v) Igepal-CA630 in H₂O.
3. Protease inhibitors (100X solution; P8340, Sigma) to be added to buffers before use.
4. Membrane lysis buffer: 10 mM Hepes, pH 8.0, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT.
5. Nuclear envelope lysis buffer: 20 mM Hepes, pH 8.0, 1.5 mM MgCl₂, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT.

2.3.3. Western Blot

1. SDS-PAGE: 40% acrylamide solution and 2% bis solution; SDS-PAGE migration buffer (10X) and broad range protein molecular weight marker.

2. Laemmli sample buffer (2X); beta-mercaptoethanol. Complete Laemmli sample buffer (Laemmli sample buffer supplemented with 5% beta-mercaptoethanol).
3. Transfer buffers: 10X Tris/glycine/SDS, 10X Tris/glycine, and methanol for transfer from gel to PVDF 0.45 μ m membrane. Mini-trans blot electrophoretic transfer cell.
4. Ponceau S solution used to double-check transfer efficiency.
5. Western blot buffers: TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl); TBS-T (0.05% (v/v) Tween in TBS); WB buffer (5% (w/v) low-fat dry milk in TBS-T).
6. Streptavidin peroxidase polymer used to detect the molecular weight marker.
7. Primary antibody (crude serum, pre-immune, and/or purified antibody).
8. Secondary antibody (enhanced chemiluminescent (ECL) peroxidase labeled anti-rabbit).
9. ECL Western blotting detection kit.
10. Gel imaging system.

2.4. Chromatin Immunoprecipitation

1. Cultured cells. Trypsin-EDTA. Formaldehyde to fix the cells. Consider that you need chromatin from 10,000 cells per IP.
2. BioruptorTM (cat. no. UCD-200, Diagenode) to prepare sheared chromatin.
3. LowCell# ChIP kit (cat. no. kch-maglow-016, Diagenode).
4. Magnetic rack (cat. no. kch-816-001, Diagenode).
5. Antibody (crude serum, pre-immune, and/or purified antibody).
6. Phosphate buffered saline (PBS).
7. 1 M sodium butyrate (1 M NaBu).
8. RNase/DNase-free 1.5 mL tubes.
9. Galaxy Mini with strip rotor.
10. Centrifuge for 1.5 mL tubes (4°C), rotating wheel (4°C), and vortex.
11. Floating rack for 1.5 mL tubes, tube claps, and boiling water.
12. Thermomixer (50 and 65°C).
13. Quantitative PCR facilities and reagents.

3. Methods

3.1. ELISA Test (Characterization and QC Step 1)

As soon as bleeds are available, the crude serum is first tested for immune response assessment (*see Note 1*). The crude and pre-immune sera are tested side by side in ELISA, the pre-immune being used as negative control (*see Note 2*). The peptide that has been used for the rabbit immunizations to raise the antibody is coated on a 96-well plate. When recognition of the peptide by the crude serum is observed, the serum can be tested further (**Fig. 2.2**). Antibodies from crude sera can also be affinity purified and the ELISA method is then used again to compare purified antibody fractions to initial crude serum (*see Note 7*; **Fig. 2.2**). We also include in our standardized protocol the use of a reference antibody to enable comparison of data from experiment to experiment.

1. Prepare solutions of peptide and KLH in carbonate buffer (100 ng/100 μ L).
2. Coat the wells in duplicate; adding 100 ng/100 μ L of peptide per well in two eight-well strips (total of 16 wells); and 100 ng/100 μ L of KLH per well in another two eight-well strips. In addition, in another eight-well strip, add the ELISA negative control (carbonate buffer alone, in four wells) and the ELISA peptide positive control (peptide to be tested with the serum of reference or ELISA antibody positive control, in four wells) (*see Note 3*).
3. Incubate overnight at 4°C.
4. Wash twice with ELISA wash buffer and dry on paper.
5. Add ELISA saturation buffer (125 μ L/well) and incubate 1 h at room temperature.
6. Wash once with ELISA wash buffer and dry on paper.
7. Each antibody sample is tested in duplicate (in two eight-well strips) and at different dilutions (in eight wells, from wells A to G). Using ELISA dilution buffer, prepare serial dilutions of both crude serum and pre-immune (for two strips each, prepare 250 μ L of each diluted antibody sample). From wells A to G, dilutions are: 1:50; 1:150; 1:450; 1:1,350; 1:4,050; 1:12,150, and 1:36,450.
8. Add 100 μ L of each dilution of antibody in duplicate and incubate overnight at 4°C. Add 100 μ L of ELISA dilution buffer in two wells as negative ELISA control. Also, add 100 μ L positive antibody control in another two wells.
9. Wash four times with deionized water and dry on paper.
10. Dilute the HRP-conjugated goat antibody anti-rabbit IgG (1:100,000) in ELISA dilution buffer.

11. Add 100 μL /well of diluted HRP-conjugated secondary antibody.
12. Incubate 1.5 h at room temperature.
13. Wash four times with deionized water and dry on paper.
14. Add 100 μL /well of TMB.
15. Incubate 30 min at room temperature.
16. Add 100 μL /well of ELISA stop solution.
17. Read at 450 nm on an ELISA plate reader.

3.2. Dot Blot (Characterization and QC Step 2)

When a crude serum is shown by ELISA to recognize the peptide used for immunizations, the crude serum undergoes more characterization. The antibody cross-reactivity can be tested against several other peptides. The crude serum directed against a determined histone modification is tested against other histone modifications by dot blot using corresponding peptides spotted on membrane (e.g., for H3K9me3, other histone modifications include mono- and di-methylation of the same lysine and mono-, di-, and tri-methylation of other lysines in the same and other histones). It should be pointed out that some lysines are contained in very similar amino acid sequence, e.g., H3K9 and H3K27 (2). Dot blot analysis to check antibody specificity was reported earlier (2–3). Based on previous publications and optimization in-house of our protocols, we set up a standardized QC method. A good antibody only recognizes the peptide used to generate the immune response (**Fig. 2.2**).

3.2.1. Peptide Dilution in an Eight-Well Strip

1. Prepare aliquots of 10 μL of 5 mM peptide stock.
2. Add 990 μL of 50 mM Tris-HCl, pH 7.5, in each 10 μL of 5 mM peptide stock to obtain a peptide concentrated at 50 pmol/ μL (peptide solutions can be aliquoted and kept at -20°C).
3. In a 96-well plate, per eight-well strip, add 50 mM Tris-HCl, pH 7.5, in the successive wells as follows: B (100 μL), C (100 μL), D (240 μL), E (240 μL), F (240 μL), and G (100 μL). Prepare one row per peptide.
4. Add 200 μL of each diluted peptide in the well A of one row.
5. Make a serial dilution of the peptide as follows: transfer 100 μL of peptide solution from well A to B, then from well B to C. Transfer 60 μL from well C to D, D to E, and then E to F.

3.2.2. Spotting Membranes with Serially Diluted Peptides

1. Cut a PVDF membrane (size: $X\text{ cm}/7\text{ cm}$ – X is the number of peptides to spot).
2. Wet a filter paper with TBS.
3. Re-hydrate the PVDF membrane 1 min in methanol 100%.

4. Wash the membrane 5 min in deionized or distilled water.
5. Wash the membrane 10 s in TBS.
6. Place the wet filter paper on a plane surface.
7. Place PVDF membrane on the wet filter paper.
8. Spot each dilution of peptide on the membrane in drops of 2 μL : spot 1 (100 pM), spot 2 (50 pM), spot 3 (25 pM), spot 4 (5 pM), spot 5 (1 pM), spot 6 (0.2 pM), and spot 6 (50 mM Tris) (*see Note 4*).

3.2.3. Incubation of Peptide Blots with the Antibody and Detection

1. Incubate the membrane 1 h at room temperature with DB blocking buffer.
2. Incubate the membrane overnight at 4°C with primary antibody diluted in primary antibody dilution buffer (*see Note 5*).
3. Wash the membrane four times 10 min with TBS-T.
4. Incubate the membrane 1 h at room temperature with the secondary antibody at the dilution 1:20,000 in secondary antibody dilution buffer.
5. Wash the membrane four times 10 min with TBS-T.
6. Proceed to detection by incubating the membrane with the appropriate substrate as follows. Prepare the detection solution (ECL Advance western blotting detection kit: 750 μL solution A and 750 μL solution B gives 1.5 mL for two membranes).
7. Incubate the membrane for 5 min with the freshly prepared detection solution.
8. Visualize and take pictures.

3.3. Western Blot (Characterization and QC Step 3)

When a crude serum is shown by ELISA to recognize the peptide used for immunizations, the crude serum undergoes more characterization. For antibody directed against modified histones, the antibody cross-reactivity is assessed by dot blot as described above and by western blot using histone extracts. For any other antibody, cross-reactivity and specificity are observed by using the western blot method on nuclear extracts. Use cellular extracts, if the protein target is strictly cytoplasmic. By western blot, the specific antibody detects a single protein band of expected molecular weight (**Fig. 2.2**). At this stage, it is also possible to perform immunoprecipitations (IP) and immunofluorescence (IF) assays to determine further antibody specificity (*see Fig. 2.3*).

3.3.1. Histone Extraction

1. Harvest 10 million cells and wash with PBS.
2. Resuspend cells in TEB freshly supplemented with protease inhibitors at a cell density of 10 million cells per milliliter.
3. Lyse cells on ice for 10 min with gentle stirring.
4. Centrifuge at 380g for 10 min at 4°C. Discard the supernatant.

5. Wash the cells in half the volume of TEB (0.5 mL) and centrifuge as above.
6. Resuspend the pellet in 250 μ L of 0.2 N HCl (cell density of 4×10^6 cells per mL).
7. Incubate 1 h at 4°C. This is the step for acid extraction of histones.
8. Centrifuge samples at 380*g* for 10 min at 4°C.
9. Removed the supernatant and determine protein concentration using the Bradford assay reagents. The protein content should be about 500–1000 μ g of protein/mL.
10. Dilute histones to 0.5 μ g/ μ L, add equal volume of 2X complete Laemmli sample buffer (final histone concentration: 0.25 μ g/ μ L) and store at –20°C or directly load on gel.

3.3.2. Nuclear Extract Preparation

1. Aspirate culture medium and wash the cells twice with ice-cold PBS.
2. Add 3 mL ice-cold PBS and scrape cells gently into a 15 mL tube.
3. Centrifuge for 5 min at 380*g* at 4°C.
4. Carefully aspirate supernatant and keep the pellet.
5. For each culture flask resuspend the pellet in 4 mL of ice-cold membrane lysis buffer freshly supplemented with protease inhibitors.
6. Transfer to 1.5 mL tubes, and add 1 mL of cell suspension per tube.
7. Incubate 15 min on ice to allow cells to swell.
8. Add 100 μ L of 10% Igpal-CA630 per tube and vortex for 10 s.
9. Centrifuge 2–3 min at 14,000*g*.
10. Carefully aspirate supernatant; this is the cytoplasmic fraction. Keep the pellet.
11. Resuspend the pellet in 200 μ L ice-cold nuclear envelope lysis buffer freshly supplemented with protease inhibitors.
12. Vortex 30 s; rotate vigorously for 30 min at 4°C.
13. Centrifuge 15 min at maximum speed. Keep the supernatants, and transfer all the supernatant fractions (see Step 6 above) in a single new ice-cold tube.
14. Aliquot and store at –80°C until use. Do not freeze/thaw.
15. Determine protein concentration using the Bradford reagent.

3.3.3. Immunoblotting

1. Perform an SDS-PAGE electrophoresis using a standard protocol and instructions from the buffer supplier (Bio-Rad). For histone analysis, we use a stacking gel of 4% acrylamide and

running gel of 12% polyacrylamide. For nuclear extracts analysis, use a running gel according to the expected molecular weight of the target of interest.

2. Cut and treat a piece of PVDF membrane as described in **Section 3.2.2** (Steps 1–5).
3. Transfer the proteins from gel to membrane using a standard protocol and instructions from the buffer and apparatus supplier (*see Note 4*). For histone analysis, the transfer buffer 1X contains 0.05% SDS and 20% methanol final (mix both transfer buffers: Tris/glycine/SDS and Tris/glycine, and add methanol). For nuclear extracts analysis, the Tris/glycine transfer buffer is used supplemented with 20% methanol. Transfer for 1 h at 100 V.
4. Incubate the PVDF membrane in WB buffer during 1 h at room temperature.
5. Dilute the primary antibody in WB buffer (for dilutions to use and titration to perform, *see Note 5*).
6. Add the diluted antibody solution to the membrane and incubate overnight at 4°C.
7. Wash the membrane in WB buffer 5 min twice, and wash 10 min twice again.
8. Add to WB buffer both secondary antibody (1:50,000) and S-HRP (1:3,000).
9. Incubate the membrane 1 h in WB buffer supplemented with secondary antibody and S-HRP.
10. Wash the membrane in TBS-T 5 min twice, and wash 10 min twice again.
11. Prepare the detection solution (ECL Advance western blotting detection kit: 750 μ L solution A and 750 μ L solution B gives you 1.5 mL for two membranes).
12. Incubate the membrane 5 min with the freshly prepared detection solution.
13. Visualize and take pictures.

3.4. Chromatin Immunoprecipitation (Characterization and QC Step 4)

Antibodies that have been shown to be specific in the previous two steps of the characterization and QC are submitted to the ChIP assay. It is essential to use a standardized protocol such as in a kit, including IP controls and to analyze by qPCR the isolated DNA looking at two loci: a locus that is positive for the target of interest and a locus that is negative (**Fig. 2.2**). We use the LowCell# ChIP method, which enables the immunoprecipitation of up to 14 parallel histone ChIP reactions plus two controls from a total of as few as 16,000 cells in a day's work. It requires low amounts of reagents per assay, the number of steps is reduced, and rapid

handling at constant temperature is enabled by the use of our magnetic rack (*see Note 8*). It is, therefore, a valuable tool for antibody characterization and QC, which involves titration and batch testing.

3.4.1. Binding Antibodies to Magnetic Beads

1. Wash twice the protein A-coated paramagnetic beads with ice-cold Buffer A as follows: add Buffer A, suspend the beads in Buffer A, then centrifuge for 5 min at 1,300 rpm, discard the supernatant, and keep the bead pellet. 10 μ L of beads are needed per IP. Scale accordingly.
2. After washing, resuspend in Buffer A to the same bead concentration as the stock.
3. Aliquot 90 μ L of Buffer A per 200- μ L PCR tube for each magnetic ChIP reaction.
4. Add 10 μ L of pre-washed protein A-beads per IP tube.
5. Add the specific antibody and positive and negative control antibodies (*see Note 6*).
6. Incubate the IP tubes at 40 rpm on a rotating wheel for at least 2 h at 4°C.

3.4.2. Cell Collection and Protein–DNA Cross-Linking

1. Immediately before harvesting the cells, add inhibitors, if needed, to the culture medium and mix gently.
2. Prepare cells as described in section “4. Kit Assay Protocol”. Count the cells.
3. Label new 1.5 mL tube(s), add PBS (including inhibitors) to a final volume of 500 μ L after cells have been added. Transfer cells and wash the pipette tip thoroughly.
4. Add 13.5 μ L of 36.6% formaldehyde per 500 μ L sample.
5. Mix by gentle vortexing. Incubate for 8 min at room temperature to allow fixation to take place.
6. Add 57 μ L of 1.25 M glycine to the sample.
7. Mix by gentle vortexing. Incubate for 5 min at room temperature. This is to stop the fixation.
8. Centrifuge at 470*g* for 10 min at 4°C.
9. Aspirate the supernatant. Take care not to remove the cells. Aspirate slowly and leave approximately 30 μ L of the solution behind.

3.4.3. Cell Lysis and Bioruptor™ Chromatin Shearing

1. Wash the cross-linked cells twice with 0.5 mL ice-cold PBS (adding NaBu and/or any other inhibitor of choice). Add the solution, gently vortex, and centrifuge at 470*g* (in a swing-out rotor with soft settings for deceleration) for 10 min at 4°C.

2. After the last wash, aspirate the supernatant. Leave about 10–20 μL behind.
3. Add protease inhibitor and NaBu to Buffer B at RT. This is the complete Buffer B. Keep the buffer at room temperature until use, discard what is not used during the day.
4. Add 130 μL of complete Buffer B (RT) to the cells. Vortex until resuspension. Incubate for 5 min on ice.
5. Submit the samples to sonication to shear the chromatin using the BioruptorTM for 12 cycles of 30s “ON”, 30s “OFF” each.
6. Use the sheared chromatin directly in ChIP.
7. Add 5 μL of protease inhibitor mix per milliliter of Buffer A. Add NaBu (20 mM final) or any other inhibitor to Buffer A.
8. Add 870 μL complete Buffer A to the 130 μL of sheared chromatin.
9. Once shearing efficiency is assessed, proceed to the next step.

3.4.4. Magnetic Immunoprecipitation

1. Briefly spin the 0.2 mL tubes containing the antibody-coated beads to bring down liquid caught in the lid.
2. Place tubes in the ice-cold magnetic rack (cooled by placing on ice), and wait for 1 min.
3. Discard the supernatant. Keep the pellet of antibody-coated beads.
4. Use 100 μL of diluted sheared chromatin per IP. Transfer 100 μL to each 0.2 mL IP tube. Keep 100 μL as input sample; keep at 4°C.
5. Close the tube caps and remove tubes from magnetic field.
6. Incubate under constant rotation on a rotator at 40 rpm for 2 h up to overnight, at 4°C.

3.4.5. Washes After Magnetic Immunoprecipitation

1. Wash three times using 100 μL ice-cold Buffer A. Each wash is done as follows: add buffer, invert to mix, incubate for 4 min at 4°C on a rotating wheel (40 rpm), spin, place in the magnetic rack, wait for 1 min, and discard the buffer. Keep the captured beads.
2. Wash one time with Buffer C. Add 100 μL Buffer C to the beads and invert to mix. Incubate on a rotating wheel for 4 min at 4°C (40 rpm). Spin and place the clean tubes now containing the beads in the magnetic rack after washing; capture the beads and remove Buffer C.

3.4.6. DNA Purification

1. Put water to boil.
2. Label new 1.5 mL tubes. IP# 1–8 (one row), IP# 1–8, and # 9–16 (two rows).

3. Add 100 μL of DNA purifying slurry directly to the washed beads and remove the eight-tube strips from the Diagenode Magnetic Rack. Mix by pipetting up and down and transfer the ChIP sample (beads and DNA purifying slurry) into the newly labeled 1.5 mL tubes.
4. Add 100 μL of input sample in a clean 1.5 mL tube and supplement with 100 μL of DNA purifying slurry.
5. Invert the tubes and lock the tubes with tube claps.
6. Incubate the samples for 10 min in boiling water.
7. Turn on the thermomixer, set the temperature at 55°C.
8. Thaw the provided proteinase K on ice.
9. Label new 1.5 mL tubes. IP#1–8 (one row), IP# 1–8, and # 9–16 (two rows).
10. Take the tubes out of the boiling water (boiling water will be needed again) and spin briefly to bring down the liquid caught in the lid.
11. Take off the tube claps. Wait for samples to cool down.
12. Add 1 μL of proteinase K to each sample and 2 μL for the input.
13. Vortex for 2s at medium power.
14. Shake all the samples for 30 min at 1,000 rpm in the thermomixer at 55°C.
15. Spin briefly and lock the tubes with tube claps before boiling.
16. Incubate the samples for 10 min in boiling water.
17. Centrifuge 1 min at 14,000*g* at 4°C.
18. Do not disturb the pellet. Transfer 50 μL of the IP sample supernatant and 150 μL of the input sample supernatant to the newly labeled 1.5 mL tubes. The pellet of the input sample can be discarded.
19. Add 100 μL of water to the pellet of the IP sample.
20. Vortex for 10 s at medium power.
21. Centrifuge for 1 min at 14,000*g* at 4°C.
22. Collect 100 μL of supernatant and pool with the previous supernatant; mix; the DNA sample can be tested in qPCR.

4. Notes

1. The ELISA is a quantitative method used to determine the concentration of a primary antibody using a series of dilutions of crude sera in antigen-coated wells. We plot the absorbance versus antibody dilution to estimate the antibody titer.

2. From starting rabbit immunization at day 0, we obtain bleeds at day 66, day 87, 4 months and then the final bleed at month 4.5. Volumes of bleeds are of 20, 20, 20 and 50 mL, respectively. We start by testing bleeds from day 66.
3. Keyhole limpet hemocyanin (KLH) is the most common protein carriers, and KLH is preferred since it is more antigenic in the majority of animals. Carrier molecule is critical since peptide molecules alone often fail to initiate an immune response. In ELISA, it is essential to test the sera against KLH. In addition, a known peptide is coated on the wells and used as peptide positive control; it is to be tested with a serum of reference (or ELISA antibody positive control), which was already shown to recognize the peptide.
4. Several dot blot membranes can be spotted and stored (dried between two filter papers) during several weeks (one aliquot of 10 μ L of 5 mM peptide stock is enough for about 200 membranes). For regular spotting use multichannel pipette and/or draw on the membrane a grid (1 cm²) with a pencil. You can use Red Ponceau to color and double-check the spotting, but do not use it to quantify between peptides of different sequences as they will be stained differently based on their sequences (2). Ponceau S solution can also be used to double-check SDS-PAGE transfer efficiency. Incubate membranes in Ponceau solution for 5 min and wash twice in deionized water.
5. In dot blots and western blots, the dilution of the primary antibody depends of antibody titer: 1:1,000 could be the starting dilution, but a titration should be done (depending on results) to determine an optimum concentration for each antibody.
6. In ChIP, the amount of the antibody to use is about 1–5 μ g/IP. It is advised to perform a titration of the antibody, e.g., use in ChIP: 1, 2, and 5 μ g of antibody to determine the best ChIP conditions. Crude serum dilutions depend on titration as well; dilute the crude serum at 1:1,000 and 1:5,000 if the corresponding titer is high in ELISA and dot blots. Dilute the crude sera 10 times less, if otherwise. Note that antibodies with high titers are the best (4).
7. Affinity purification must be performed with the antigen that was used for generating the immune response. Antibody purification method used is affinity chromatography with coupled peptide on a pre-packed HiTrapTMNHS-activated HP column (#17-07-01, GE HealthCare) followed by a buffer exchange by Gel Filtration on G-25 fine (HiPrepTMTM 26/10 Desalting, #17-5087-01, GE HealthCare) on Äkta-PrimeTM System (#11-0013-13, GE HealthCare). After

peptide affinity purification, the antibody specificity must be checked since the antibody preference substrate might have been altered as well as its titer greatly reduced.

8. A magnetic rack from Diagenode has been specially designed for simple sample handling with the LowCell# ChIP kit. It can hold up to 16X 0.2 mL tubes simultaneously in a chilled environment even on the bench top, and enables efficient and fast magnetic separation. Note that the LowCell# ChIP kit also allows immunoprecipitation of transcription factors as well as histones.

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