I. Formaldehyde Fixation

A. Adherent Cells

1. Grow adherent cells in a 100 mm dish to about 80– 90% confluency (~5×10^6 cells).
2. Remove media.
3. Add 10 ml of 1% formaldehyde in culture medium. Incubate plate for 5–10 min at room temperature.
4. Add 2.1 ml quenching solution to final 347 mM. Incubate for 5 min at room temperature.
5. Remove solution. Wash plate with 10 ml 1X PBS.
6. Remove PBS and add 1 ml 1X PBS.
7. Remove cells off plate using rubber scraper. Collect cells in a tube.
8. Centrifuge cells at 400 x g for 3 min at 4°C.
9. Remove supernatant. Add 1 µl of ProteoGuard™ Protease Inhibitor Cocktail.
10. Proceed to Section II or store at –80°C until use.

B. Suspension Cells

1. Grow floating cells in a 100 mm dish to about 80– 90% confluency (~5×10^6 cells).
2. Transfer cells to a tube.
3. Add 3.3 ml of 4% formaldehyde to final 1% concentration.
4. Incubate for 5–10 min at room temperature.
5. Add 2.8 ml quenching solution to final 347 mM. Incubate for 5 min at room temperature.
6. Centrifuge at 400 x g for 3 min at 4°C.
7. Remove supernatant. Wash with 10 ml of 1X PBS.
8. Centrifuge at 400 x g for 3 min at 4°C.
9. Add 1 µl of ProteoGuard Protease Inhibitor Cocktail.
10. Proceed to Section II or store at –80°C until use.

II. Cell Lysis

1. Resuspend cells in 1 ml of cytoplasmic lysis buffer with ProteoGuard Protease Inhibitor Cocktail.
2. Place on ice for 10 min. Gently vortex every 5 minutes.
3. Centrifuge cells at 2,400 × g for 10 min at 4°C to pellet nuclei.
4. Remove cytoplasmic supernatant and resuspend nuclei in 300 µl of RB1.

III. Cell Sonication

1. Sonicate chromatin on sonicator. If using the BIORUPTOR UCD-250 (COSMO BIO), 30 sec (sonication) - 30 sec (break) -12 cycles is best. Sonication step should be optimized for instrument of choice.
2. Centrifuge at 15,000 × g for 10 min at 4°C.
3. Remove and keep supernatant.
4. Aliquot sheared chromatin into 25 µl per tube and store at –80°C.

IV. Determination of Chromatin Shearing Efficiency after Sonication

1. Remove 10 µl sheared chromatin.
2. Incubate at 95°C for 15 min to reverse cross-link.
3. Add 1 µl of Proteinase K and 1 µl of 1 mg/ml RNase A. Incubate at 60°C for 15 min.
4. Run 10 µl of shearing DNA on 2% agarose gel to ensure that chromatin lengths are between 200–2000 bp.

V. Immunoprecipitation

1. Wash 200 µl of magnetic beads (2 mg/ml stock) 2 times with 200 µl of RB1. Resuspend beads in 20 µl of RB1.
2. Set up immunoprecipitation reaction in siliconized tubes:

20 µl	Magnetic Beads in RB1
25 µl	Sheared Chromatin
2 µl	ProteoGuard Protease Inhibitor Cocktail
20 µl	10X Easy Dilution Buffer
X µl	Antibody
Y µl	RB1, bring total volume up to 200 µl
200 µl	Total Volume

3. Incubate at 4°C with rotation for > 4 hours.
   * 10 µl sheared chromatin dispense as "Input DNA". Place on ice or at 4°C.
4. Using a magnetic stand, remove unbound chromatin.
5. Wash beads with 800 µl of RB1.
6. Wash beads with 800 µl of RB2.
7. Wash beads with 800 µl of RB3.
8. Resuspend in 800 µl of RB3 and transfer materials to a fresh tube.
9. Remove wash buffer. Proceed to Section VI.

VI. Elution

1. Add 100 µl of 10 % DNA Purifying Slurry to the beads and vortex.
2. Incubate at 95°C for 15 min to reverse crosslink.
3. Add 1 µl of Proteinase K and 1 µl of 1 mg/ml RNase A. Incubate at 60°C for 15 min.
4. [Optional]: Incubate at 95°C for 10 min to inactivate the enzyme.
5. Using a magnetic stand, collect supernatant into new tube.
6. Proceed to Section VII or VIII. Samples are compatible with direct PCR analysis. DNA purification is optional.

VII. [Optional] DNA Purification

1. Use the NucleoSpin Gel Extraction and PCR Clean-Up Kit (Cat. No. 740609.50) to purify DNA. Use Buffer NTB to bind DNA to the column (not Buffer NT). Elute in the Elution Buffer provided in the kit.
2. Phenol chloroform extraction or another DNA purification method can also be used.

VIII. PCR Analysis

1. To amplify, set up the PCR cocktail on ice as follows:

5 µl	10X PCR Buffer (Mg^{2+})
1 µl	10 mM dNTPs
4 µl	GAPDH Primer mix (5 µM each)
25 µl	Chromatin
0.25 µl	TaKaRa *Taq* HS (5U/µl)
14.75 µl	RNase-Free Water up to 50 µl
50 µl	Total Volume

2. Commence thermal cycling using the following program:
   - 95°C 3 min
   - 25–30 cycles:
     95°C 20 sec
     60°C 30 sec
     72°C 30 sec
   - 72°C 5 min
   - 4°C ∞

   Analysis of the PCR product by gel electrophoresis should reveal a ~200 bp band.

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