Cross-linking Chromatin Immunoprecipitation (X-ChIP) Protocol
*From AbCam website

1. Cross-linking and cell harvesting
   1. Start with two confluent 15-cm dishes (1 X 10⁷ – 5 X 10⁷ cells/dish).
   2. Add formaldehyde drop-wise to a final conc. of 0.75%
   3. Rotate at room temperature gently for 10 min (preferably on belly dancer)
   4. Add glycine to final conc. of 125mM to media and incubate for 5 min at RT
   5. Wash cells 2X with 10 mL cold PBS
   6. Scrape cells into 5 mL of cold PBS and transfer to 50 mL conical.
   7. Add 3 mL cold PBS to dish and collect remainder of cells
   8. Centrifuge for 5 min at 1000 g
   9. Aspirate supernatant and resuspend pellet in FA lysis buffer (750 µL/1x10⁷)

2. Sonication
   1. Sonicate lysate to shear DNA to average fragment size of 500 – 1000 bp. You will need to optimize depending upon cell line. Remove samples over a time-course and check fragment size on 1.5% agarose gel. Ex. Optimal time observed is at 15 min in gel to the right.
   2. Pellet cell debris by centrifuging at 30 sec, 4°C, 8000 g.
   3. Transfer supernatant to new tube.
   4. Remove 50 µL of each sonicated sample. This is INPUT to quantify DNA concentration and as control in PCR.

3. Determination of DNA concentration (using INPUT ONLY)
   1. The INPUT samples are used to calculate DNA concentration. Clean up with a PCR purification kit (Add 70µL of elution buffer and proceed to step 3.2a) or phenol:chloroform extraction (Add 350 µL of elution buffer and proceed to step 3.2b)
   2. Purify by either method
      a. Add 2 µL RNase A (0.5 mg/mL). Heat with shaking at 65°C for 4 hr – O/N. Purify with PCR purification kit according to manufacturer’s instructions.
      b. Add 5 µL proteinase K (20 mg/mL). Heat with shaking at 65°C for 4 hr – O/N. Phenol:chloroform extract and EtOH precipitate in the presence of 10 µL glycogen (5 mg/mL). Resuspend in 100 µL dH₂O.
   3. Transfer 5 µL of the purified DNA into a tube containing 995 µL TE to give a 200-fold dilution and read the OD_{260}. The concentration of DNA in µg/mL is OD_{260} x 10,000. This is used to calculate the DNA concentration of the chromatin preparation
4. **Immunoprecipitation**

1. Use approximately 25 µg of DNA/IP. Do an experimental sample and beads only control.
2. Dilute 1:10 with RIPA buffer.
3. Add primary antibody to all samples except beads-only control. This must be determined empirically. Usually 1 – 10 µg antibody/25 µg DNA works well.
4. Add 20 µL protein A/G beads (pre-absorbed with sonicated single stranded herring sperm DNA and BSA, see step 4.4a) to all samples and rotate O/N at 4°C.
   a. If using both protein A and protein G beads, mix an equal volume of each and wash 3x with RIPA buffer. Aspirate RIPA and add single stranded herring sperm DNA to final concentration of 75 ng/µL beads and BSA to final conc. of 0.1 µg/µL beads. Add RIPA buffer to twice the bead volume and incubate for 30 min with rotation at RT. Wash once with RIPA buffer and add RIPA buffer to twice the bead volume.
5. After O/N incubation, centrifuge beads for 1 min, 2000 g and remove sup.
6. Wash 3X with 1 mL wash buffer. Centrifuge 1 min, 2000 g to remove sup.
7. Wash 1X with 1 mL Final Wash Buffer. Centrifuge 1 min, 2000 g to remove supernatant

5. **Elution and reverse cross-links**

1. Elute DNA by adding 120 µL of Elution buffer to protein A/G beads and rotate for 15 min at 30°C
2. Centrifuge for 1 minute, 2000 g and transfer the supernatant to fresh tube.
3. DNA can be purified by PCR purification kit (proceed with step 3.2a) or phenol:chloroform (add 280 µL elution buffer and proceed with step 3.2b)
4. DNA levels are quantitatively measured by real-time PCR

6. **Buffers**

   **FA Lysis buffer**
   50 mM HEPES-KOH (pH 7.5)
   140 mM NaCl
   1 mM EDTA
   1% Triton X-100
   0.1% Deoxycholate
   0.1% SDS
   Protease inhibitors

   **RIPA Buffer**
   50 mM Tris-HCl (pH 8.0)
   150 mM NaCl
   2 mM EDTA (pH 8.0)
   1% NP-40
   0.5% Sodium Deoxycholate
   0.1% SDS
   Protease inhibitors

   **Wash Buffer**
   0.1% SDS
   1% Triton X-100
   2 mM EDTA (pH 8.0)
   150 mM NaCl
   20 mM Tris-HCl (pH 8.0)

   **Final Wash Buffer**
   0.1% SDS
   1% Triton X-100
   2 mM EDTA (pH 8.0)
   500 mM NaCl
   20 mM Tris-HCl (pH 8.0)

   **Elution Buffer**
   1% SDS
   100 mM NaHCO₃