Estradiol Receptor (ER) Chromatin Immunoprecipitation in MCF-7 Cells

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[Abstract] Steroid hormone receptors, for example estradiol receptor, act like transcription factors. In the cell, steroids bind to a specific receptor. Upon ligand binding, many steroid receptors dimerize and enter nuclei where they bind specific DNA sequences called Hormone Responsive Elements (HRE) and regulate gene transcription. ER is able to bind DNA sites that are not Estrogen Responsive Elements (ERE) so regulating also the transcription of genes that are not classically controlled by estrogens.

Materials and Reagents

1. Fetal bovine serum (FBS) (Life Technologies, Gibco®, catalog number: 10270)
2. 200 mM L-Glutamine (100x) (Life Technologies, Gibco®, catalog number: 25030)
3. Penicillin-Streptomycin (100x) (Life Technologies, Gibco®, catalog number: 15140-122)
4. Hydrocortisone (Sigma-Aldrich, catalog number: H-0888)
5. Insulin (Human, recombinant) (F. Hoffmann-La Roche, catalog number: 11376497001)
6. Estradiol (Beta-estradiol) (Sigma-Aldrich, catalog number: E8875)
7. Protease inhibitors cocktail tablets (LAP tablets) (F. Hoffmann-La Roche, catalog number: 11836153001)
8. Protein A-agarose fast flow (Sigma-Aldrich, catalog number: P3476)
9. Formaldeyde (Sigma-Aldrich)
10. Salmon sperm DNA
11. DPBS (Sigma-Aldrich, catalog number: D5652)
12. Proteinase K (F. Hoffmann-La Roche, catalog number: 03115879001)
13. Qiagen PCR purification kit
14. Immunoprecipitating antibody (ER-alpha) (Sigma-Aldrich, catalog number: E1396)
15. Primers CCDN1 locus (-1039 to 770 bp): Fw (−1039) (AACAAAACCAATTAGGAACCTT), Rv (−770) (ATTTCCTTCATCTTGTCCTTCT)
16. Primers CCDN1 promoter (-235 to -53 bp): Fw (−235) (TATGAAAACCGGACTACAGG), and Rv(−53) (CTGTTGTTAAGCAAAGATCAAAG)
17. Charcoal dextran stripped serum (CSS) (see Recipes)

18. Phenol Red-free Dulbecco's Modified Medium (DMEM with PR) (Life Technologies, Gibco®, catalog number: 31885) (for MCF-7 cells) (see Recipes)
19. Phenol Red-free Dulbecco's Modified Medium (DMEM w/o PR) (Life Technologies, Gibco®, catalog number: 11880) supplemented with Charcoal (for MCF-7 cells) (see Recipes)
20. 50x LAP (Protease inhibitor cocktail) (see Recipes)
21. DTT solution (see Recipes)
22. Buffer I (see Recipes)
23. Buffer II (see Recipes)
24. Buffer III or cell lysis buffer (see Recipes)
25. Buffer IV (see Recipes)
26. TSE I buffer (see Recipes)
27. TSE II buffer (see Recipes)
28. TSE III buffer (see Recipes)
29. TE buffer (see Recipes)
30. Elution buffer (see Recipes)
31. PCR buffer (see Recipes)

Equipment

1. Sonicator (equipped with a 3 mm diameter tip) (Sonics & Materials, model: Vibracell VC 130PB)
2. 100 mm dishes
3. Centrifuge (Eppendorf centrifuge, model: 5417R)
4. Shaker
5. PCR thermal cycler

Procedure

1. The MCF-7 cells are plated in 100 mm dishes approximately at 40-50% cell confluence in Phenol red Dulbecco's modified medium. After12-18 h, the Red phenol medium is substituted with Phenol red-free Dulbecco's modified medium supplemented with CSS. Cells are maintained in this medium for 3 or 4 days (80% confluence in 100 mm dishes).
2. Stimulate the cells with 10 nM estradiol for various times from 15 to 75 min (the maximal ER binding to chromatin is usually observed after 30-40 min of hormone treatment). Use un-stimulated cells as negative control.
3. After hormone stimulation, wash the cells twice with cold PBS (5-10 ml).

4. Cross-link the cells with 10 ml 1% formaldehyde solution in PBS at room temperature for 10 min. Add the formaldehyde to PBS immediately prior to use.
5. Rinse cells twice with cold PBS (5-10 ml).
6. Add 1 ml DTT solution and collect the cells in a 1.5 ml tube using a cell scraper.
7. After collecting, incubate the cells for 15 min at 30 °C and centrifuge for 4 or 5 min at 3,000 RCF.
8. Wash the pellet three times with 1 ml PBS and after every wash centrifuge at 3,000 RCF for 3 min.
9. Wash sequentially the pellet with 1 ml Buffer I with 50x LAP, 1 ml Buffer II with LAP.
10. Suspend the cellular pellet in 300 µl Buffer III with 50x LAP and incubate 10 min on ice.
11. Sonicate three times at 30-40% amplitude and 0.4 W intensity for 35 sec each to obtain 200 bp DNA fragments. During the sonication, keep the samples on ice.
12. To define amplitude, intensity and duration of sonication, you can use a sample of MCF-7 cells, sonicate at different intensity, amplitude and duration and after check the DNA fragment size running the sonicated DNA on an agarose gel. The right conditions will be those that will allow you to obtain a DNA smear with the center on around 200 bp.
13. Centrifuge the cellular lysates at 20,000 RCF for 10 min at 4 °C, collect the supernatants and dilute ten fold using the Buffer IV with 50x LAP. Store in a rack and keep at 4 °C for 3 or 4 h or overnight (O.N).
14. For preparing the protein A-agarose (40 µl of 50% for each sample), wash twice the required amount of resin with TE solution (ten fold the protein A-agarose volume). Collect the protein A-agarose by brief centrifugation. After the last wash, discard the TE solution and add fresh TE solution and salmon sperm DNA (20 µl TE solution and 2 µg salmon sperm DNA for each sample). Keep on a rotating platform at 4 °C for 3 or 4 h or O.N.
15. Clear the samples at 20,000 RCF for 10 sec. Store at -80 °C or use immediately for immunoprecipitation and lysates. Save back 20% of the total supernatant as total input control and process with eluted IPs beginning with the cross-link reversal step.
16. Add the immunoprecipitating antibody (2 µl ER-alpha) to the 0.7 ml samples fraction in a new tube and precipitate for 6 h or overnight. For a negative control, incubate 0.7 ml samples fraction with 1 µl (2 µg) of rabbit IgG. Keep all the samples on a rotating platform at 4 °C.
17. Add 40 µl of blocked protein A-agarose beads at 4 °C with rotation O.N. if you have incubated the samples with the antibody for 6 h or for 2-3 h if you have incubated the samples with the antibody O.N.
18. Pellet beads by centrifugation (3,000 *x g*) at 4 °C and wash for 10 min sequentially with 1 ml of TSE I, 1 ml of TSE II and 500 µl of TSE III. Wash three times with 1 ml TE Buffer.

19. Elute with 300 µl of freshly prepared elution buffer. Prepare buffer fresh each time. Vortex briefly to mix and shake gently on the vortex shaker for 10 min.
20. Centrifuge at 20,000 rpm for 10 min, transfer supernatants to clean tube and add 200 µl elution buffer in 100 µl of the frozen input control.
21. Reverse formaldehyde cross-linking by heating at 65 °C O.N.
22. Add 18 µl 1 M Tris-HCl (pH 6.5) 0.5 M EDTA and 5 µg Proteinase K to each sample and heat at 48 °C for 90 min.
23. Isolate DNA by using either the Qiagen PCR purification kit.
24. Another method for isolating DNA is the 1x phenol/chloroform extraction, 1x chloroform extraction, O.N. precipitation using 2.5 volumes absolute ethanol with 30 µl potassium acetate 3 M pH 5.7 and 5 µg glycogen. Wash pellets twice with 500 µl 70% ethanol and air dried.
25. Resuspend pellets in 35 µl TE with 0.1 mg/ml RNase A.
26. Perform the conventional PCR using 2 µl DNA per reaction. For example, for CCDN1 locus (-1039 to -770 bp) or for CCDN1 promoter (-235 to -53 bp) PCR, use 20 µl H_2O solution with 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.25 µM PRIMER F, 0.25 µM PRIMER R, 0.5 U/µl TAQ Pol, 2 µl DNA and 2 µl PCR buffer. Use 33-35 cycles of amplification (denaturation 96 °C for 20 sec, annealing 50 °C for 90 sec, elongation 69 °C for 60 sec). Run in a 2% agarose gel in TBE 0.5%.

Recipes

1. Charcoal dextran stripped serum
   Dextran coated charcoal is used to strip steroid hormones from serum. Charcoal/dextran stripped serum is commercially available, but we prepare the serum as described in Migliaccio *et al*. (2011).
2. Phenol red-free Dulbecco's modified medium for MCF-7 cells (DMEM with PR)
   5% FBS
   2 mM L-glutamine
   100 U/ml penicillin-streptomycin
   3.75 ng/ml Hydrocortisone
   6 ng/ml Insulin
3. Phenol red-free Dulbecco's modified medium supplemented with Charcoal for MCF-7 cells
   (DMEM w/o PR)
   5% CSS
   2 mM L-glutamine

100 U/ml penicillin-streptomycin

3.75 ng/ml Hydrocortisone

6 ng/ml Insulin

4. 50x LAP (Protease inhibitor cocktail)

   Dissolve a tablet in 1 ml distilled H_2O and use 50x

5. DTT solution

   10 mM DTT

   100 mM Tris-HCl

   pH 9.5

6. Buffer I

   0.25% Triton X-100

   10 mM EDTA

   0.5 mM EGTA

   10 mM Hepes

   pH 6.5

7. Buffer II

   200 mM NaCl

   1 mM EDTA

   0.5 mM EGTA

   10 mM Hepes

   pH 6.5

8. Buffer III or cell lysis buffer

   1% SDS

   10 mM EDTA

   50 mM Tris-HCl (pH 8.1)

   50x protease inhibitor cocktail

9. Buffer IV

   1% Triton X-100

   2 mM EDTA

   150 mM NaCl

   20 mM Tris-HCl (pH 8.1)

10. TSE I buffer

    0.1% SDS

    1% Triton X-100

    2 mM EDTA

    20 mM Tris-HCl (pH 8.1)

    150 mM NaCl

11. TSE II buffer
    0.1% SDS
    1% Triton X-100
    2 mM EDTA
    20 mM Tris-HCl (pH 8.1)
    500 mM NaCl
12. TSE III buffer
    0.25 M LiCl
    1% NP40
    1% Sodium Deoxycholate
    1 mM EDTA
    10 mM Tris-HCl (pH 8.1)
13. TE buffer
    20 mM Tris-HCl (pH 8.1)
    1 mM EDTA (pH 8.0)
14. Elution buffer
    1% SDS
    0.1 M $NaHCO_3$
15. PCR buffer
    300 mM Tris-Base
    100 mM Hepes
    250 mM Potassium Chloride
    200 mM Potassium Glutamate
    20 mM DTT
    50% Glycerol
    Sterilize by filtration

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