Appendix E

Chromatin Immunoprecipitation Protocol for LNCaP Cells Used to Generate Initial ChIP-Seq Dataset

Chromatin Immunoprecipitation of Androgen Receptor in LNCaP Cells as Performed for Chapter 4 ChIP-Seq Experiment. (adapted from Wold lab ChIP protocol[1,2] and protocol used by Nicholas G. Nickols for AR ChIP[3])

Reagents/Equipment and Vendors Utilized

LNCaP cells (ATCC Cat. No. CRL-1740)

175 cm^2 Falcon Tissue Culture Plates (BD Biosciences Cat. No. 353112)

Trypsin (Invitrogen Cat. No. 25200056)

RPMI 1640 (Invitrogen Cat. No. 21870)

Fetal Bovine Serum (Omega Scientific Cat No. FB-01)

100X L-glutamine (Invitrogen Cat. No. 25030)

50 mL conical tubes (BD Biosciences Cat. No. 352098)

15 mL conical tubes (BD Biosciences Cat. No. 352097)

25 mL pipettes (VWR Cat. No. 101093-952)

10 mL pipettes (VWR Cat. No. 101093-962)

5 mL pipettes (VWR Cat. No. 101093-976)

Phosphate buffered saline pH 7.2 (Invitrogen Cat. No. 20012)

10X PBS pH 7.4 (make from salts and autoclave)

KCl (Biology stockroom)

NaCl (Biology stockroom)

Na_2HPO_4 (Biology stockroom)

KH_2PO_4 (Biology stockroom)

15 cm BD Falcon tissue culture plates (BD Biosciences Cat. No. 353025)

Charcoal-dextran treated FBS (Omega Scientific Cat No. FB-04, Lot No. 103365)

Dihydrotestosterone (Sigma-Aldrich Cat. No. A8380; this is a schedule III drug and requires extra time/steps/approval for ordering)

1.7 mL microcentrifuge tubes (Sorensen, DNase, RNase-free, Cat. No. 11700)

Absolute Ethanol (can be purchased in the Chemistry stockroom)

16% formaldehyde (Ted Pella Product No. 18505)

Dynabeads M-280, Sheep anti-Rabbit IgG, (Invitrogen Cat. No. 112-03D for 2 mL bottle and 112-04D for 10 mL bottle)

Bovine serum albumin, fraction V (Amresco Cat. No. 0903)

Magnet (Invitrogen Cat No. 123-21D is currently manufactured)

Santa Cruz Biotech SC-816 (N-20) polyclonal antibody (1 mL vials)

Glycine (Fluka Cat. No. 50046) – use to make 2.5 M stock of glycine; store at -20 ºC

PMSF (Roche Cat. No. 10236608001)

LiCl (Sigma-Aldrich Cat. No. L9650)

$NaHCO_3$ (can be purchased Biology stockroom)

SDS (Fluka Cat. No. 71727)

Sodium deoxycholate (Sigma-Aldrich Cat. No. D6750)

Isopropanol

Razor blades (can purchase on campus at VWR stock room)

Protease inhibitor cocktail tablets: Complete, EDTA-free, Roche Cat No. 11873580001 for tablets that make 50 mL 1X and Roche Cat No. 11836170001 for tablets that make 10 mL 1X PIC (Complete, mini, EDTA-free)

Farnham Lysis Buffer

RIPA Buffer

LiCl IP Wash Buffer

Elution buffer

Tris base (Biology stockroom)

0.5M EDTA pH 8.0 (Invitrogen Cat. No. 15575-020)

Branson Sonifier S-450D

Cary UV-Vis

Disposable clear plastic cuvettes (Biology stockroom)

BSA concentration standards (Bio-Rad Cat. No. 500-0207)

1X Bradford Reagent (Bio-Rad Cat. No. 500-0205)

0.15M NaCl (make using crystalline NaCl and Millipore water)

end-over-end rotator (VWR Cat. No. 13916-822)

O-ring screw-cap microcentrifuge tubes

1X TE buffer pH 8.0 (make from Tris base and EDTA)

phenol/$CHCl_3$/isoamyl alcohol (Invitrogen Cat. No. 15593-031)

Qiagen Buffer PM (Cat. No. 19083)

Qiagen QIAquick PCR Purification Kit (Cat. No. 28104 for 50 tubes, and 28106 for 250 tubes)

FastStart Universal SYBR Green Master (Rox) (Roche Cat. No. 04913914001 for 10 x 5 mL tubes)

IDT DNA primers

USB Ultrapure water, RNase-Free, DEPC Treated (USB Cat. No. 70783)

Buffer Compositions

<u>10X PBS pH 7.4</u>

Dissolve the following into 800 mL deionized Millipore H_2O:

80 g of NaCl

2.0 g of KCl

14.4 g of Na_2HPO_4

2.4 g of KH_2PO_4

Adjust pH to 7.4.

Adjust volume to 1L in volumetric flask with additional deionized Millipore H_2O.

Sterilize by autoclaving.

<u>Farnham Lysis Buffer</u> (make in 100 mL volumetric flask)

5 mM PIPES pH 8.0 (from 100 mM PIPES pH 8.0 stock in Millipore water; sterile filter

with 0.2 μm filter)

85 mM KCl (from 850 mM KCl stock in Millipore water; sterile filter with 0.2 μm filter)

0.5% NP-40 (from 10% NP-40 stock)

Roche Complete, EDTA-free (added immediately prior to use)

RIPA Buffer (make in 100 mL volumetric flask)

1X PBS (from 10X PBS stock)

1% NP-40 (from 10% NP-40 stock in Millipore water)

0.5% Sodium Deoxycholate (from powder or from 10% stock in Millipore water; powder was used exclusively for these studies)

0.1% SDS (from 10% SDS stock in Millipore water)

Roche Complete, EDTA-free (added immediately prior to use)

LiCl IP Wash Buffer (make in 100 mL volumetric flask)

100 mM Tris (from 1 M Tris base in Millipore water; do not adjust pH after making Tris; must autoclave 1 M Tris base prior to use)

500 mM LiCl (from 5M LiCl stock in Millipore water stored at -20 ºC)

1% NP-40 (from 10% NP-40 stock in Millipore water)

1% Sodium Deoxycholate (from powder or from 10% stock in Millipore water; powder was used exclusively for these studies)

Elution buffer

1% SDS (from 10% stock in Millipore water)

0.1 M $NaHCO_3$ (from 1 M stock in Millipore water)

1X TE Buffer pH 8.0

1 mL 1M Tris-HCl pH 8.0

0.2 mL 0.5 M EDTA pH 8.0

998.8 mL Millipore water

Autoclave

Plating LNCaP Cells **Day 1**

1. 85–90% Confluent LNCaP cells grown in five 175 cm^2 Falcon plates were trypsinized by (1) decanting the RPMI 1640 from each plate, (2) rinsing each plate with 37 ºC PBS, (3) adding 5 mL trypsin, (4) decanting the trypsin from each plate, (5) incubating the plates at 37 ºC for five minutes, and (6) adding fresh, complete RPMI 1640 (Invitrogen Product No. 21870; supplemented with 10% FBS (Omega Scientific Cat No. FB-01) and 2 mM L-glutamine (Invitrogen Product No. 25030)) to collect the detached cells.
2. Detached LNCaP cells were collected in a 50 mL conical tube to give 50 mL total volume of cells. The cell suspension was aspirated in a 25 mL pipette to ensure even distribution. A 5 mL pipet is useful for disrupting clumped LNCaP cells. 10 mL of the 50 mL cell suspension was used to seed five new 175-cm^2 Falcon plates in 30 mL total volume per plate (complete RPMI 1640 used). An additional 10 mL complete RPMI 1640 was added to the 50 mL conical tube to yield a 50 mL volume once again.
3. 1 mL of the 50 mL volume was diluted to 10 mL using PBS in a separate 15 mL conical tube. The 1:10 dilution was counted by hemocytometer, and the cell concentration was noted.
4. 42 million LNCaP cells (sufficient for ~21 15-cm plates) were added to a solution of complete RPMI 1640 (defined in step 1) to give a total volume of 525 mL.
5. LNCaP cells, each at 2 million cells per plate in 25 mL complete RPMI 1640 were seeded into 15 cm tissue culture plates. A total of 20 plates were made.
6. After adding cells to each cells were dispersed by a horizontal sliding motion made along a single vector while standing.
7. Cell culture plates were placed in the 37 ºC incubator containing 5 % CO_2 and were allowed to grow for 72 hours.

Swapping Medium of LNCaP cells to CT-FBS **Day 4**

8. Cell culture plates were moved from the incubator to the cell culture hood and decanted. Each plate was carefully washed with 37 ºC PBS by angling the plate and adding PBS to the sidewall of the plate nearest the floor of the cell culture hood. The PBS was then decanted from each plate. A minimum of cells were detached resulting from the wash.
9. 25 mL RPMI 1640 (the RPMI 1640 used is Invitrogen Product No. 21870; supplemented with 10% CDT-FBS (Omega Scientific Cat No. FB-04, Lot No. 103365) and 2 mM L-glutamine (Invitrogen Product No. 25030)) was added to each plate as per the procedure in step 8, to minimize dislodging attached LNCaP cells.
10. The plates were returned to the 37 ºC, 5% CO_2 incubator for 48 hours.

Inducing LNCaP cells with Dihydrotestosterone **Day 6**

11. 500nM dihydrotestosterone (DHT) was prepared by aliquoting a 5-10 mg sample of DHT into a 1.5 mL microcentrifuge tube. 1 mL EtOH was added to the 1.5 mL microcentrifuge tube. A serial dilution ensued using absolute EtOH, with each dilution reducing DHT concentration 10-fold. The final three dilutions utilized are as follows: (1) 900 µL PBS + 100 µL previous dilution, (2) 900 µL PBS + 100 µL previous dilution, (3) (1000 – x) µL PBS + x µL previous dilution. A vehicle supplement was created as well by mimicking the final three dilutions but replacing step (1) with 900 µL PBS + 100 µL EtOH.
12. To each 25 mL plate of LNCaP cells was added 50 µL vehicle (10 plates) or 50 µL, 500 nM DHT (1 nM DHT final concentration to 10 plates).
13. Plates were allowed to incubate 16 hours with DHT treatment prior to formaldehyde fixation (step 22).

Coupling Antibody to Magnetic Beads (everything performed at 4 ºC) Day 6

14. Add 100 µL resuspended (read: VORTEX) magnetic bead slurry (Invitrogen Dynabeads M-280, Sheep anti-Rabbit IgG, Cat No. 112-03D for 2 mL bottle and 112-04D for 10 mL bottle) to 1.5 mL Eppendorf tube. (For other experiments, be sure to match the bead type to the organism of the antibody and try starting with 50 µL bead slurry).
15. Add 1 mL PBS + 5 mg/mL BSA (fraction V, prepare fresh + syringe filter; measure concentration of BSA in PBS using A_{280} = 6.61 for 10 mg / mL)
16. Place tube on magnet (Invitrogen Cat No. 123-21D is currently manufactured) for 5 minutes. Remove supernatant.
17. Add 1 mL PBS + 5 mg/mL BSA to beads. Mix on end-over-end rotator for 10 minutes.
18. Place tube on magnet for 5 minutes. Remove supernatant.
19. Repeat steps 17–18 two more times.
20. Add 10 µg of Santa Cruz Biotech SC-816 (N-20) polyclonal antibody (50 µL of 200 µg / mL) (If other antibodies, try 5 µg polyclonal antibody (25 µL of 200 µg / mL) or 1 µg of monoclonal antibody (5 uL of 200 ug / mL) to AR; inevitably titration of antibody will be necessary).
21. Allow beads to incubate with antibody for 24 hours while mixed on end-over-end rotator (16–24 hours have been utilized with good results).

Formaldehyde Cell Fixation Day 7

22. Decant medium from 15 cm plates.
23. Wash each plate with ~20 mL 37 ºC PBS.
24. To each plate add 20 mL 1% formaldehyde in RPMI 1640 (no L-glut, no FBS, no P/S, warmed to room temperature)
25. Fix at room temperature for 10 minutes on rocker.

26. Stop cross-linking reaction by adding 1 mL 2.5 M glycine (final concentration ~ 0.125 M; RPMI 1640 solution turns yellow in color)
27. Rock for 5 minutes.
28. Decant medium from plates.
29. Wash plates with ~20 mL 4 ºC PBS.
30. Add 2 mL 4 ºC PBS supplemented with 1/200 vol. 100 mM PMSF in isopropanol (PMSF added immediately before use) to each plate of cells.
31. Scrape cells off plate with razor blade, and transfer them to a conical Falcon tube on an ice-water bath (4 ºC).
32. Pellet cells at 2,000 RPM for 5 minutes (4 ºC). Decant supernatant.
33. Resuspend cell pellet in 5 mL / plate Farnham Lysis Buffer (add PIC (Roche, Protease inhibitor cocktail: Complete, EDTA-free, Cat No. 11873580001 for tablets that make 50 mL 1X and Cat No. 11836170001 for tablets that make 10 mL 1X PIC) fresh prior to use). Mix gently on rocker for 15 minutes at 4 ºC (cold room).
34. Centrifuge crude nuclear prep at 2000 RPM for 5 minutes (4 ºC). Decant. (if necessary, cell pellet can be frozen in LN_2 and stored at -80 ºC prior to use)

Sonication of Isolated Cell Pellets **Day 7**

From here down, everything is done in the Cold Room (4 ºC)

35. Resuspend pellet in 1.0 mL RIPA Buffer (add PIC fresh prior to use) and transfer to 1.7 mL Eppendorf tube.
36. Sonicate on -80 ºC EtOH bath (50 mL conical tube with EtOH stored on dry ice or in freezer). Sonicate 30 times 30 sec. (30 secs on, 30 secs off; be consistent with total number of cycles) at 25% amplitude. The EtOH bath should be switched for a fresh, cold EtOH bath every 5 sonication cycles (a cycle = 30 secs on, 30 secs off). Be careful that you do not froth the solution. Work quickly so that the initial solution does not freeze.

37. Microcentrifuge sheared solution at 14,000 RPM for 15 mins at 4 ºC. Decant supernatant to a new tube. If any of the pellet transfers to the new Eppendorf tube, repeat the centrifugation, and transfer the supernatant to a new Eppendorf tube.
38. If sheared chromatin is not to be used immediately, the samples may be frozen in LN_2 and stored at -80 ºC prior to use. To thaw frozen samples, allow them to warm in the cold room on ice. If time is short, samples may be centrifuged at 14,000 RPM for 10 mins at 4 ºC. The thawed sample should be aspirated several times to ensure it is evenly mixed.

Bradford Assay to Determine Protein Concentration (at room temperature) Day 7

39. Turn on Cary UV-Vis at least 1 h prior to use.
40. For each sample of chromatin to be measured, make a stock solution (30 μL), a 1:5 dilution (50 μL) in RIPA buffer and a 1:10 dilution (100 μL) in RIPA buffer. Make a 1 mL tube of RIPA buffer to use in the blank.
41. Use disposable clear plastic cuvettes (2 cuvettes per measured condition). For the blanks, add 10 μL RIPA buffer, 90 μL 0.15M NaCl. For the standard curve (2.0 mg/mL BSA, 1.5 mg/mL BSA, 1.0 mg/mL BSA, 0.75 mg/mL BSA, and 0.5 mg/mL BSA, all available in Bio-Rad Cat. No. 500-0207), add 10 μL standard, 10 μL RIPA buffer, and 80 μL 0.15M NaCl. For the samples to be measured, add 10 μL sample, 90 μL 0.15M NaCl. When all cuvettes are ready, add 1 mL 1X Bradford Reagent to each tube, in the order they are to be scanned (blanks, then standards, then samples). Wait at least 5 but no more than 50 minutes prior to scanning samples in the UV-Vis.
42. Open the Simple Reads application. Click setup and set the wavelength to 595 nm. Add the blank to the UV-Vis and click zero. Use the second blank to ensure the machine reads close to 0.0. Measure the standard curve first. Then measure the individual samples. If the absorbance for an individual sample does not fit within

the range of measured absorbances for the standard curve, the sample cannot be interpolated.

43. Use a linear regression to fit the standards to a line. Use the resulting least-squares fitted line to interpolate the quantity of the individual samples. Make sure to multiply the appropriate dilution factor by the resulting concentration. Each sample's concentration will be determined.

Addition of Chromatin to Antibody-incubated Beads (in 4 ºC room) Day 7

44. Wash magnetic beads from Day 6 three times (steps 17–18). Prior to pelleting on the magnet each time, briefly spin the tube(s) in the microcentrifgue (short spin to no more than 2000 RPMs).
45. To fully decanted beads, add 100 μL PBS+BSA (fresh) to beads. Add 1 mg total protein concentration (determined from steps 39-43). Add RIPA to make the total volume in the tube 1 mL.
46. Save some of the material from step 37 (50 uL is sufficient) for use as input DNA for qPCR.
47. Incubate step 45 on the end-over-end rotator at 4 ºC for 24 h (16–24 h is sufficient).

LiCl IP Wash of Chromatin-incubated Beads Day 8

Still in the cold room (4 ºC)

48. Briefly spin the tube to remove any liquid / beads from the cap of the microcentrifuge tube. Pellet on magnet for 5 minutes. Decant all of the liquid from the tube.
49. Wash pellet 5 times with 1 mL LiCl IP Wash Buffer with 10 min mixing between washes and 5 minute pellet against magnet. Prior to each pelleting, briefly centrifuge the tube to remove any liquid / beads from the cap of the microcentrifuge tube. Decant all of the liquid from the tube.

50. Wash pellet once with 1 mL TE. Spin briefly. Pellet on magnet 5 minutes. Decant all of the liquid from the tube. **Outside of cold room**
51. Resuspend beads in 200 μL IP Elution buffer. (To 50 μL input from step 46, add 150 μL IP Elution buffer)
52. Elute immune complexes by incubating in 65 ºC water bath for 1 h, vortexing every 15 mins to resuspend magnetic beads.
53. Spin at 14,000 RPM for 3 mins to pellet beads. Save the supernatant as the IP.
54. Continue cross-link reversal by incubating IP in O-ring screw-cap microcentrifuge tubes at 65 ºC on the heated shaker (1400 RPM). Allow to proceed overnight (~16 h should be sufficient).

Workup / purification of IP **Day 9**

55. Extract once with 200 μL phenol/$CHCl_3$/isoamyl alcohol, vortex thoroughly, and centrifuge at 14,000 RPM for 3 minutes.
56. Save aqueous phase. Back extract organic phase once (for IP only, for input it is unnecessary) with 100 μL elution buffer and pool both aqueous phases.
57. Add three volumes of Qiagen Buffer PM.
58. Add half of the solution (if IP, otherwise all if input) to Qiagen PCR Cleanup Kit spin column. Spin 30 secs at 14,000 RPM. Repeat with second half of solution. Discard all flow through.
59. Wash column with 750 μL Buffer PE. Spin 30 secs. Discard flow through. Spin 1 minute at 14,000 RPM.
60. Elute IP columns with 100 μL warmed Buffer EB (~55 ºC) and input DNA columns with 200 μL warmed Buffer EB. For IP columns, run eluate through column again for second elution.

qPCR Verification of Enriched Loci **Day 9**

All conditions are run in technical quadruplicate. Each PCR reaction consists of:

5 μL template DNA

5 μL Forward + Reverse primers (each at 1.8 μM concentration)

10 μL Roche FastStart Universal SYBR Green Master (Rox)

A standard curve must be run for each set of primers and for each unique input sample (i.e., vehicle treated, 1 nM DHT treated, etc.). If there are more than 20 experimental samples to analyze for a single primer pair (more than a single plate is required for a single primer set), the standard curve must be repeated on each plate. The standard curve consists of wells containing 50 ng, 5 ng, 500 pg, and 50 pg input DNA (i.e., the concentrations must be 10 ng/μL, 1 ng/μL, 100 pg/μL, and 10 pg/μL; adjust to these concentrations by using the NanoDrop UV/Vis to sample the concentration of your input samples).

Quantification of IP'd DNA **Day 9**

The Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen Cat. No. P11496) was used as directed to quantitate concentration of immunoprecipitated chromatin. The Sternberg lab NanoDrop fluorometer was used to generate a standard curve and to measure fluorescence emission of the PicoGreen dye. Typical concentrations for a single IP experiment were ~1 ng IP'd DNA.

Preparation of DNA for ChIP-Seq Submission

Five IP'd experiments for 1 nM DHT-induced LNCaP cells were examined by qPCR. The three most strongly enriched samples were combined, and the sample was quantified by PicoGreen assay. The input DNA sample (≥ 500 ng material, as determined by NanoDrop UV-Vis) and 1 nM DHT-induced pooled IP sample were each SpeedVac'd to ≤ 75 μL. For submission, a paper including my name, reference to the Dervan

laboratory, my sample labels (JWP VII-171 1 nM DHT induced input, JWP VII-171 1 nM DHT induced IP), and sample concentrations / total amount (and how they were measured: 3 ng IP'd solution measured by PicoGreen assay)

Primers Used

PSA ARE III F: 5'-GGATTGAAAACAGACCTACTCTGG-3'

PSA ARE III R: 5'-CAACAGATTTGTTTACTGTCAAGGA-3'

PSA ARE II F: 5'-CTGGTCTCAGAGTGGTGCAG-3'

PSA ARE II R: 5'-AGACCCAGTGTGCCCTAAGA-3'

PSA ARE I F: 5'-TGCATCCAGGGTGATCTAGT-3'

PSA ARE I R: 5'-ACCCAGAGCTGTGGAAGG-3'

PSA HRE F: 5'-GGTGTTGCTGTCTTTGCTCA-3'

PSA HRE R: 5'-GAGGCAATTCTCCATGGTTC-3'

FKBP5 intron-L F: 5'-AGCAATTTTGTTTTGAAGAGCA-3'

FKBP5 intron-L R: 5'-CTGTCAGCACATCGAGTTCA-3'

Neg1 F: 5'-AAAGACAACAGTCCTGGAAACA-3'

Neg1 R: 5'-AAAAATTGCTCATTGGAGACC-3'

Neg2 F: 5'-CCAGAAAACTGGCTCCTTCTT-3'

Neg2 R: 5'-TAGAAGGGGGATAGGGGAAC-3'

Primers were designed using Primer3[4] and checked for specific amplification by BLAST,[5] and the UCSC *in silico* PCR[6] prior to ordering. Each primer pair has had its concentration optimized. Primers were screened for unique product formation by quantitative PCR melting temperature examination and by running the primers on a native 4.0% agarose gel and visualizing the products relative to a 25 bp ladder (Invitrogen TrackIt Cat. No. 10488022).

References

(1) Johnson, D. S.; Mortazavi, A.; Myers, R. M.; Wold, B. *Science* **2007**, *316*, 1497–502.

(2) Mortazavi, A.; Leeper Thompson, E. C.; Garcia, S. T.; Myers, R. M.; Wold, B. *Genome Res.* **2006**, *16*, 1208–21.

(3) Nickols, N. G.; Dervan, P. B. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 10418–23.

(4) Rozen, S.; Skaletsky, H. *Methods Mol. Biol.* **2000**, *132*, 365–86.

(5) Johnson, M.; Zaretskaya, I.; Raytselis, Y.; Merezhuk, Y.; McGinnis, S.; Madden, T. L. *Nucleic Acids Res.* **2008**, *36*, W5–9.

(6) Kent, W. J.; Sugnet, C. W.; Furey, T. S.; Roskin, K. M.; Pringle, T. H.; Zahler, A. M.; Haussler, D. *Genome Res.* **2002**, *12*, 996–1006.