RFP-Trap Applications Note for Chromatin Immunoprecipitation (ChIP)

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Introduction

Chromatin Immunoprecipitation (ChIP) is a widely used and well established technique to study the interaction between DNA-binding proteins and genomic regions. This applications note describes a ChIP protocol comprising formaldehyde cross-linking, sonication of chromatin, immunoprecipitation, and purification of captured DNA. The protocol described here is based on the ChIP protocols that were originally developed by the Farnham laboratory of UC Davis (see references).

Here we have investigated a hormone receptor that acts as an intracellular ligand-activated transcription factor. This receptor changes its confirmation in the presence of its hormone ligand and then binds to DNA. In addition, the receptor influences gene expression by recruitment of other factors including co-factors, histone modifying enzymes and chromatin remodeling complexes. The DNA binding site, which has been selected in this experiment, is a recognition site for the receptor, which has been extensively shown in both microscopy and biochemical approaches including EMSA gel-shift assay and nuclease foot-printing.

In absence of suitable antibodies, which recognize the intact hormone receptor, we fused mCherry to the N-terminus of the receptor in order to pull down the mCherry fusion protein DNA complex using the ChromoTek RFP-Trap. This ChIP-experiment confirmed the presence of the hormone receptor at the DNA.

Materials and Methods

Adherent cells were plated on 15 cm plates in 30 ml media at 8 million cells per plate. The cells were hormone-treated according to the required experimental conditions. Cross-linking with formaldehyde (final concentration 1%) was performed by adding 1.62 ml of freshly prepared 18.5% formaldehyde directly to the media. Cells were incubated at room temperature (RT) for 10 min on a rotating platform. Then cross-linking was stopped by quenching the remaining formaldehyde with 3 ml glycine (1.25 M in H2O, RT) for 5 min with rotation at RT. The media was poured off and the cells were rinsed 3 times with 25 ml ice-cold PBS. Residual PBS was thoroughly removed before lysis with 0.75 ml SDS lysis buffer containing freshly added protease and phosphatase inhibitors. Cell lysates were harvested into tubes, placed on ice, and passed 5 times through a needle (25G) to ensure efficient lysis.

Sonication was performed using a Branson Sonifier 450 (400 W, 20 kHz) on 10% output for 4 x 10 s pulses. Tubes should be placed on ice for 30 s between pulses. Sonication levels must be determined empirically for different sample types with an optimal chromatin size range of 100 to 600 bp. After

sonication, samples were centrifuged for 10 min at 13,000 rpm and at 4°C. Next, the supernatant was collected and quantified on a spectrophotometer, and frozen in aliquots at -80°C.

For immunoprecipitation, aliquots of chromatin were thawed on ice. 70 µg of chromatin samples were added to 100 µl SDS lysis buffer and 900 µl ChIP dilution buffer supplemented with protease inhibitors. In order to pre-clear the lysates, 25 µl binding control agarose beads (bab-20, ChromoTek) were added and the tubes were mixed end over end for 2h at 4°C. Samples were centrifuged gently (3 min, 4000 rpm, 4°C) to pellet the beads and the supernatant was transferred to a fresh tube. 10 µl were removed for a 1% input sample. For immunoprecipitation, either 25 µl ChromoTek RFP-Trap (rta-20) or bab-20 (no antibody control) were added to each sample and subsequently incubated overnight at 4°C with rotation. Beads were pelleted by centrifugation (2 min, 4000 rpm, 4°C) and washed in turn with 1 ml of the following ice-cold buffers followed by 3 min rotation and centrifugation to pellet the beads again:

- Low salt immune wash buffer
- High salt immune wash (x2 washes)
- LiCl immune wash buffer
- TE buffer (x2 washes)

Residual TE buffer was removed and 120 µl elution buffer (SDS 1%, $NaHCO_3$ 0.1 M) were added, shaken for 15 min at RT, and the supernatant collected. This elution step was repeated once. Both aliquots of eluate were combined, re-centrifuged and 200 µl bead-free solution was mixed with 8 µl 5 M NaCl and incubated at 65°C overnight to reverse the cross-links. The 1% input samples were combined with 190 µl elution buffer together with 8 µl 5 M NaCl and also incubated at 65°C overnight.

To clean and purify DNA fragments, samples were first treated with RNase (1 µl DNase-free RNase was added to each sample before incubation for 30 min at 37°C). This was followed by proteinase K digestion (4 µl EDTA 0.5 M, 8 µl Tris-HCl 1 M pH 6.5 and 1 µl proteinase K were added before incubation for 2 h at 45°C). phenol:chloroform extraction and ethanol precipitation recovered DNA fragments. Sample volumes were adjusted to 500 µl with TE buffer (pH 8), then mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), pH 8.0. The aqueous phase was extracted again with chloroform:isoamyl alcohol (24:1). 300 µl purified sample was precipitated with 30 µl NaCl 5 M, 20 µg glycogen and 750 µl ethanol 100% at -20°C overnight. DNA was pelleted by centrifugation at 13,000 rpm for 20 min at 4°C, the supernatant removed, the pellet was washed with 70% ethanol (add 500 µl ethanol 70%, re-centrifuge and pipette off ethanol) and air-dried before being re-suspended in 30 µl nuclease-free H_2O. Dissolving pellets were left for 30 min at RT, then 30 min at 65°C to aid resuspension before storage at -20°C. The re-suspended samples were tested by real time PCR using standard procedures and primer sets to relevant genomic DNA regions.

Reagents

Formaldehyde, 18.5%: combine 9.6 ml distilled H_2O, 1.85 g paraformaldehyde pellets and 70 µl 1N KOH in a small glass bottle. Stir on a 65°C hotplate in a fume hood until dissolved [approximately 30-60 min] and filter through a 0.45 µm syringe filter.
SDS lysis buffer: SDS 2%, EDTA 10 mM, Tris-HCl 50 mM pH 8.1 supplemented with protease and phosphatase inhibitors.
ChIP dilution buffer: 167 mM NaCl, 16.7 mM Tris-HCl pH 8.1, 1.1% Triton X-100, 1.2 mM EDTA, 0.01% SDS.
Low salt immune wash buffer: 159 mM NaCl, 20 mM Tris-HCl pH 8.1, 2 mM EDTA, 1% Triton X-100, 0.1% SDS.
High salt immune wash buffer: 300 mM NaCl, 20 mM Tris-HCl pH 8.1, 2 mM EDTA, 1% Triton X-100, 0.1% SDS.

LiCl immune wash buffer: 250mM LiCl, 10 mM Tris-HCl pH 8.1, 1 mM EDTA, 1% IGEPAL-CA630, 1% sodium deoxycholic acid.
Tris-EDTA (TE) buffer, pH 8.0: 10 mM Tris-HCl, 1 mM EDTA.
Protease inhibitors: Roche cOmplete™ EDTA-free Protease Inhibitor Cocktail Tablets (1 tablet dissolved in 1 ml H_2O and added at 1:50)
Phosphatase inhibitors: Sodium fluoride (stock 200 mM added at 1:100) and sodium ortho-vanadate (stock 200 mM added at 1:1000).

Results

ChIP using RFP-Trap confirmed a hormone-inducible enrichment of fluorescent protein-tagged hormone receptors at the expected DNA binding site. Experiments were performed on mammalian cells transiently transfected with mCherry-tagged hormone receptor cDNA and treated with 100 nM hormone or appropriate vehicle control (no hormone). On the contrary, there is no signal in the presence or absence of hormone if bab-20 control agarose beads are used for immunoprecipitation.

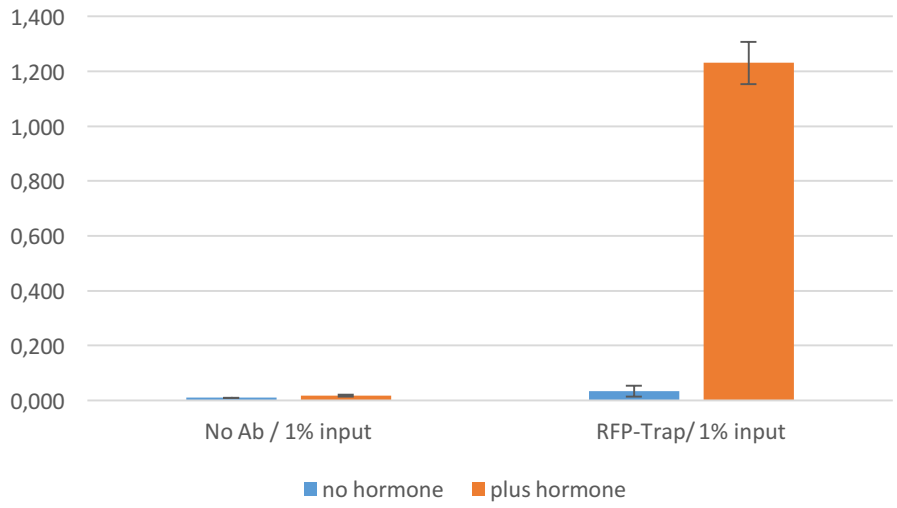

Figure 1. ChIP analysis with RFP-Trap. Mammalian cells were transiently transfected with mCherry-tagged hormone receptor (n=3) with/without 100 nM hormone. DNA binding of the hormone receptor was investigated by ChIP analysis as described. Binding of the hormone receptor to DNA should occur in the presence of hormone only. The y-axis describes recovery of PCR-amplifiable DNA containing the known location for hormone receptor binding. This is expressed in % of the input (DNA recovered from the experimental immunoprecipitation condition, divided by DNA recovered from 1% of total input material; both in nanogram). Binding events are portrayed as significant enrichments in recovered DNA relative to controls.

Conclusion

We demonstrated that the RFP-Trap can be used for ChIP analysis in order to detect enrichment of mCherry-tagged proteins at DNA binding sites.

References/Relevant Literature

Farnham Lab Chromatin Immunoprecipitation (ChIPs) Protocol for Tissues (2006 Revision): http://farnham.genomecenter.ucdavis.edu/protocols/tissues.html

Ray S and Das SK (2006): Chromatin immunoprecipitation assay detects Er alpha recruitment to gene specific promoters in uterus. Biological Procedures Online 8; 69-76.