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METHODS PAGE

Chromatin Immunoprecipitation (ChIP) from pancreatic acinar cells and whole pancreatic tissue

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1. Introduction

Over the past decade, chromatin immunoprecipitation (ChIP) has emerged as an important technique for determining the binding of proteins to specific DNA sequences. In particular, ChIP has been used to confirm whether transcription factors (TFs) work directly on target genes to promote gene activation or repression. ChIP has advantages over other techniques that examine the role of TFs in regulating gene expression. Promoter assays that use reporter genes such as *luciferase* or green fluorescent protein (GFP) can determine whether a TF stimulates the expression of a certain promoter, but do not assess DNA binding to determine whether the effects on expression are direct. Electrophoretic mobility shift assays (EMSA) can be used to determine TF binding but are relatively insensitive when compared to ChIP and require the ability of an antibody to supershift a protein/DNA complex to determine true interactions.

ChIP protocols have also been adapted to study epigenetic modifications that affect gene expression and are now being combined with Next-Generation sequencing (ChIP-Seq) as a data output revealing previously unknown binding targets of proteins or epigenetic marks of interest. The ability to obtain good quality starting material for ChIP analysis is crucial if scientists want to apply changes in gene transcription and epigenetic modifications to the study of human disease.

ChIP has become such a valuable and universally used tool that many companies have recognized a niche for ready-made ChIP kits, such as Millipore, Active Motif, Abcam and Pierce. While these kits are useful for some tissue or cell types, they are not especially cost-effective and have proven to be insufficient for use with adult pancreatic tissue or isolated acinar cells. After examining the efficiency and yield provided by a variety of ready-made ChIP kits, our laboratory has developed our own ChIP protocols for use with isolated acinar cells or whole pancreatic tissue.

One of the benefits for using isolated cells for

ChIP is that the acinar cells are purified from other pancreatic cell populations, thereby reducing any background that may be occurring with DNA targets of interest in non-acinar cells. The isolation of acinar cells also removes excess connective tissue that may interfere with efficient processing. However, the use of isolated acinar cells for ChIP can affect downstream analyses as a number of stress response signaling pathways are activated upon isolation, and may obscure relevant endogenous mechanisms. This becomes even more important if one is examining the transcriptional profile the tissue following the induction of cellular injury (i.e. pancreatitis). Acinar cell isolation may mask the effects of actual disease mechanisms on the disease process.

The following are protocols developed by our lab for ChIP analysis from either isolated acinar cells or from whole tissue. While the protocols for preparation of chromatin from tissue and cells are different, performance of ChIP itself is similar for both. One thing that is crucial for success is the existence of ChIP-grade antibodies and reliable primers that allow for efficient execution of these procedures. Using these protocols, we have obtained excellent results for quantifying protein-DNA interactions and have used the DNA for Next generation sequencing and bioinformatics analysis.

2. Materials

A. Preparation of Chromatin from Isolated Acinar Cells

Collagenase from *Clostridium hystolyticum* (Sigma, Cat # C7657)

DMEM

Penicillin/Streptomycin

Trypsin inhibitor (Invitrogen, Cat # 17075-029)

Oxygen tank

160 µM pore size mesh filters (Millipore, Cat # NY6H04700)

BSA fraction V (Hyclone, Cat # SH30574.03)

37.5% Formalin

2.5M Glycine

Refrigerated centrifuge (Eppendorf, Model # 5804R) and appropriate rotors (microcentrifuge F45-30-11, swinging bucket, A-4-44).

Potter-Elvehjem homogenizer, 3 mL capacity (VWR, Cat # KT886000-0020)

Sonic Dismembrator 100 (Fisher Scientific)

B. Preparation of Chromatin from Whole Tissue

Caerulein (Sigma, Cat # C9026)

37.5% Formalin

2.5M Glycine

Refrigerated centrifuge (Eppendorf, Model # 5804R) and appropriate rotors (microcentrifuge F45-30-11, swinging bucket, A-4-44).

Dounce homogenizer, 7 mL capacity (Wheaton, Cat # 357542)

Sodium sarcosine

Sonic Dismembrator 100 (Fisher Scientific)

C. Chromatin Immunoprecipitation

Protein A agarose (Millipore, Cat # 16-125)

Labquake rotator (Fisher Scientific, Model # 400110)

Antibodies to proteins of interest

Normal IgG negative control

GenElute PCR purification columns (Promega, Cat # A9282)

3. Preparation of Chromatin from Isolated Acinar Cells

A. Isolation of Pancreatic Acinar Cells

Acinar cells were isolated from pancreatic tissue using the previously described protocol developed

by Yule and Williams (1992). Collagenase (Sigma) diluted (1 mg/10 ml) in DMEM with 1% Pen/strep solution and trypsin inhibitor was repeatedly injected into the isolated pancreas. The tissue was minced into smaller pieces and oxygenated in collagenase containing media for 1 minute and incubated in a shaking water bath at 37^{o}C for 10 minutes. The media was exchanged with fresh collagenase-containing media and the tissue was oxygenated for 1 minute and incubated at 37^{o}C in a shaking water bath for a further 40 minutes. This tissue slurry was then further dissociated using a 5 ml pipette and passed through 160 mm nylon net filter (Millipore). The isolated acini were washed first with 4% BSA in DMEM and then with 1% BSA in DMEM. Finally, the cells were resuspended in 10 ml DMEM without serum or antibiotics.

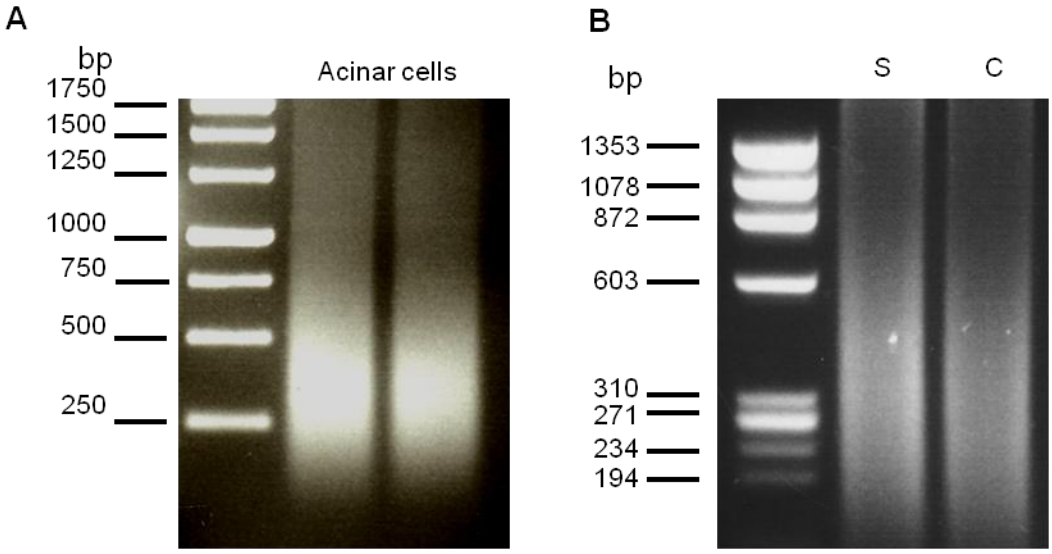

Figure 1. Efficient shearing of chromatin from (A) isolated acinar cells or (B) whole pancreatic tissue following cerulein (C) or saline (S) injection. Chromatin is sheared, crosslinks reversed and DNA run on a 1.5% agarose gel. Proper shearing is indicated by a smear with highest intensity in the region of 200-600bp.

B. DNA-Protein Cross-Linking

To cross-link protein to the genomic DNA, 270 µl of formalin was added to 10 ml of DMEM containing acini to obtain a final solution of 1% formaldehyde. Cells were agitated for 10 minutes (Note 1) on a shaker table. Following the addition of 514 µl of 2.5 M glycine (125 mM final) for 5 minutes to quench the formaldehyde and terminate the crosslinking reaction, cells were centrifuged at 2000 rpm for 3 minutes at 4^{0}C. The pellet of acinar cells was washed twice with ice cold PBS.

C. Isolation of Nuclei from Isolated Acinar Cells

To isolate nuclei, acinar cells were incubated in Cytoplasmic Extract buffer (CE Buffer; 10 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.1% Triton-X100, 0.5 mM DTT and protease inhibitors [5 µg/mL Aprotinin, 5 µg/mL Leupeptin, 5 µg/mL Pepstatin, 75 µg/mL PMSF]) followed by 5 strokes of Potter-Elvehjem homogenizer. The cells were set on ice for 15 minutes. To pellet the nuclei, the samples were centrifuged at 4^{o}C and 5000 g for 10 minutes. The supernatant can be discarded or used as a cytoplasmic fraction, and the pellet contains intact nuclei.

D. Preparation of Chromatin for Immunoprecipitation

Pelleted nuclei were thoroughly dispensed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, and protease inhibitors) incubated for 30 minutes and sonicated to generate 200-600 bp

fragments (Figure 1A). The lysates were centrifuged at 13000 rpm for 25 minutes. The supernatant, which is the chromatin, can be used for ChIP. Sonication parameters for pancreatic acinar cells need to be determined empirically for each cell disruptor. We used a sonicator with a setting at level 4. Each sample went through 15 cycles of sonication, each lasting 10 seconds with 20 seconds between each cycle. Sonicated chromatin should be kept -80^{o}C until use.

4. Preparation of Chromatin from Whole Pancreas

A. Preparation of Nuclei from Whole Tissue

We have been able to obtain high quality ChIP DNA from pancreatic tissue that is either untreated, or four hours following initiation of cerulein induced pancreatitis (CIP). CIP was initiated as described in Kowalik et al (2007) and is a variation of the CIP model described in Niederau et al. (1990). The pancreas was dissected and finely minced (Note 2). To cross-link protein to DNA, the minced pancreatic tissue was incubated in 40 mL of freshly made 1% formaldehyde in DMEM for 10 minutes at room temperature with rocking. To quench the formaldehyde, glycine was added to a final concentration of 0.125 M and tissue was incubated for 5 minutes with rocking. The tissue was pelleted by centrifugation at 95 x g for three minutes and washed with ice cold PBS containing protease inhibitors (Note 3). The pellet was resuspended in 1 mL of cold cytoplasmic lysis buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP40, 1 mM DTT, 1 mM PMSF and protease inhibitors, the final pH was adjusted to 7.6) and homogenized with a loose pestle in a 7 mL glass Dounce homogenizer (Wheaton, Millville, NJ) until uniform. The resulting lysate was centrifuged at 95 x g at 4°C for 3 minutes to pellet nuclei. The cytoplasmic supernatant was removed, and the nuclear pellet resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, and protease inhibitors) and incubated on ice for a minimum of 30 minutes. The lysate was further homogenized using the tight pestle of the Dounce homogenizer and placed in a 1.5 mL centrifuge tube.

B. Preparation of Chromatin for IP

Sodium sarcosine was added to increase sonication efficiency (1) and was added to a final concentration of 0.5% (w/v) and the lysate split into 250 µL fractions (Note 4). To shear DNA into 200-600 base pair fragments, samples were sonicated on ice once at level 4 for 20 seconds and four times at level 4 for 10 seconds using the Sonic Dismembrator 100 (Note 5). Between sonication, samples were left to incubate on ice. The resulting lysates were centrifuged at 4°C at 14,000 rpm for 10 minutes to remove debris. Sheared DNA samples should be stored at -80°C until use.

It is best to assess shearing efficiency before proceeding with immunoprecipitation. To do so, 20 µl of the sonicated chromatin was combined with 6 µl of 5 M NaCl and incubated overnight at 65^{o}C, to reverse the cross-linking. 1 µl of proteinase K (5 mg/ml) was added to the reaction and incubated at 45^{o}C for 1-2 hours. Following phenol chloroform extraction and ethanol precipitation, the DNA was resuspended in 20 µl H_2O and 1 µl of RNase A (1 mg/ml). Following resolution on a 1.5% agarose gel, a DNA smear running from 200-600 bp was observed representing chromatin fragments (Figure 1B).

5. Chromatin Immunoprecipitation (ChIP)

Each ChIP reaction requires approximately 50 µg of chromatin, but can be varied based on target protein abundance. The appropriate amount of chromatin was combined with dilution buffer (20 mM Tris pH8.1, 1% Triton X-100, 1 mM EDTA, 167 mM NaCl, protease inhibitors) to dilute chromatin 6-fold. The diluted chromatin was pre-cleared by incubating with protein A agarose (Millipore, Billerica, MA) for 1.5 hours at 4°C with

rotating. Beads were pelleted at 95 x g at 4°C and the supernatant placed in a clean tube. At this point, 12% of total chromatin used per IP was set aside as an input control (Note 6). One to 5 µg of antibody (Note 7) was added and IP reactions incubated overnight at 4°C with rotating. As a negative control, an IP reaction using either similar IgG or an irrelevant antibody was used. Inclusion of a positive ChIP control may be beneficial in preliminary experiments to determine if the protocol is working. We have successfully used rabbit anti-acetylated histone H3 (AcH3; Millipore, Billerica, MA; Figure 2A) and antibodies against histone 3 trimethylated at lysines 4 (H3K4Me3) and 27 (H3K27Me) (Figure 2B). After antibody incubation, protein A agarose beads were added and the reaction incubated for another two hours at 4°C while rotating to capture antibody:protein complexes. Reactions were centrifuged at 95 x g at 4°C for 3 minutes to pellet beads and the supernatant was removed using thin bore pipet tips. Beads were run through successive washes for 10 minute intervals at 4°C with rotating, using the following buffers containing protease inhibitors: a low salt wash (20 mM Tris pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), a high salt wash (20 mM Tris pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), a LiCl wash (10 mM Tris pH 8.1, 0.25M LiCl, 1% NP40, 1% deoxycholate Na, 1 mM EDTA), and, finally, two washes in Tris-EDTA (TE). The resulting beads were incubated in 75 µl of elution buffer (1% SDS, 100 mM $NaHCO_3$) with rotating at room temperature for 15 minutes. Beads were pelleted and the eluent transferred to a new tube before repeating the elution procedure. The two eluents were pooled together and prepared for reversal of crosslinks. To the corresponding input samples, 150 µl of elution buffer was added and reversal of crosslinks carried out by adding 6 µl of 5 M NaCl at 65°C for 4 hours to overnight. 1µl RNase was added to the reaction at 37°C for 30 minutes, and the mix was incubated with 3 µl of 0.5 M EDTA, 6 µl of 1 M tris-HCl (pH 6.5), and 1 µl of Proteinase K (5 mg/ml) at 45°C for 1-2 hours.

The DNA can be recovered by using either a PCR purification kit or by using phenol/chloroform extraction. We have successfully used Promega GenElute PCR purification columns (Fisher Scientific, Ottawa, ON) as per manufacturer specifications. After purification, chromatin was eluted to 80 µL for use in downstream applications. If the preferred method of DNA extraction is phenol/chloroform, following extraction and precipitation (Note 8), the DNA is resuspended in 80 µl of H_2O.

The isolated DNA now can be used for ChIP-PCR, ChIP-chip or ChIP-seq. Depending on the recovery rate, the purified DNA is sufficient for library construction for Next generation sequencing. If not being immediately used, isolated DNA can be stored at -20°C.

6. Analysis of DNA isolated from ChIP

PCR of ChIP DNA

PCR with the ChIP DNA was carried using 1.5 µl of the final eluted DNA and conditions for typical qRT-PCR. After an initial incubation of 5 minutes at 95°C, 40 cycles of denaturing (95°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 30 s), was followed by a final extension run at 72°C for 7 minutes. q-PCR was performed using the Roche LightCycler 1.5 carousel based system (Roche Diagnostics, Laval, Canada; Figure 2C). The formulas for quantification were adapted from Mukhopadhyay et al (2008) and Kernohan et al. (2010).

We have also analyzed ChIP DNA using the GoTaq PCR Mastermix system (Promega, Madison WI). Briefly, individual primers were added to the GoTaq mastermix followed by addition of 2 µL of eluted DNA. Using similar cycling conditions as listed above, samples were evaluated using an ABI Prism 7900HT Sequence

Detection System and corresponding SDS 2.2.1 software (Applied Biosystems, Foster City, CA). Average C_t values for individual IPs and IgG controls are expressed as a percent of input (2).

The fidelity of the ChIP procedure was confirmed by amplifying developmentally regulated genes that had previously been identified as targets of epigenetic regulation (6).

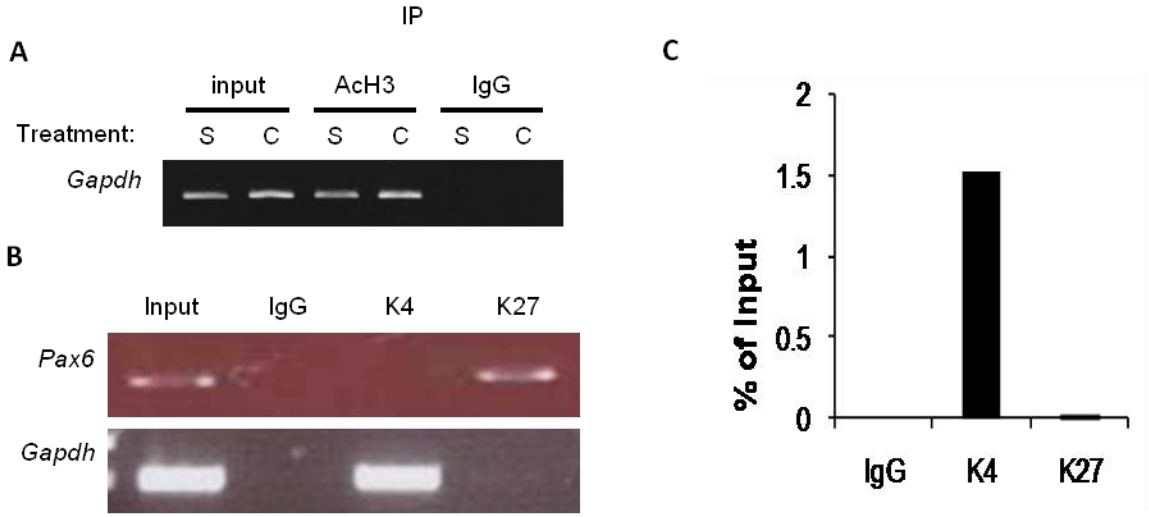

Figure 2. PCR of ChIP results. (A) Antibodies against acetylated histone 3 (AcH3) or rabbit IgG (negative control) were used to determine the fidelity of the ChIP protocol on whole tissue. PCR was performed using primers specific for the *Gapdh* promoter. S = saline, C = cerulein induced pancreatitis. **(B)** Similar ChIP analysis using antibodies against H3K4Me3 (K4) or H3K27Me3 (K27) on isolated acinar cells. PCR was performed using primers specific for the *Gapdh* (active) and *Pax6* (silenced) promoters. **(C)** Quantitative PCR comparing the enrichment of H3K4Me3 and H3K27Me3 at the *Gapdh* promoter in primary acinar cells.

7. Notes

1. Do not exceed 13 minutes of crosslinking since this decreases overall ChIP efficiency.

2. Fine mincing is crucial for proper processing as a rougher mince proved to be unsuccessful for crosslinking and subsequent ChIP.

3. We use Aprotinin (5 µg/mL), Leupeptin (5 µg/mL), Pepstatin (5 µg/mL), and PMSF (75 µg/mL)

4. Suboptimal volumes for sonication can result in excessive sample frothing (decreasing sonication efficiency) or sample loss (as seen when sonicating too large a volume)

5. Efficient sonication parameters must be empirically determined for individual sonic dismembrators. Our sonicator has a metal probe with a 3mm tip and is optimal for shearing small volumes (0.1 to 50 mL).

6. Input samples are not required for each individual IP (per antibody) but are required per biological treatment or genotype (i.e. Saline vs. Cerulein).

7. The amount of antibody required for each IP varies depending on antibody quality and specificity. We have successfully used ChIP grade H3K27Me3 (Millipore, Billerica, MA), H3K4Me3 (Millipore, Billerica, MA) for ChIP from isolated cells, and rabbit anti ATF3 (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-spliced XBP1 (Biolegend, San Diego, CA) for ChIP from whole tissue. Rabbit anti-AcH3 (Millipore, Billerica, MA) has been used as a positive ChIP control and Normal rabbit IgG (Millipore) as a negative control in both preparations.

8. Precipitate using ethanol and 10 μl of glycogen (5 mg/ml). Glycogen is required as a carrier since there may be only a small amount of DNA recovered.

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