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A chromatin immunoprecipitation (ChIP) protocol for use in whole human adipose tissue

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Haim Y, Tarnovscki T, Bashari D, Rudich A. A chromatin immunoprecipitation (ChIP) protocol for use in whole human adipose tissue. *Am J Physiol Endocrinol Metab* 305: E1172–E1177, 2013. First published September 3, 2013; doi:10.1152/ajpendo.00598.2012.—Chromatin immunoprecipitation (ChIP) has become a central method when studying in vivo protein-DNA interactions, with the major challenge being the hope to capture "authentic" interactions. While ChIP protocols have been optimized for use with specific cell types and tissues including adipose tissue-derived cells, a working ChIP protocol addressing the challenges imposed by fresh whole human adipose tissue has not been described. Utilizing human paired omental and subcutaneous adipose tissue obtained during elective abdominal surgeries, we have carefully identified and optimized individual steps in the ChIP protocol employed directly on fresh tissue fragments. We describe a complete working protocol for using ChIP on whole adipose tissue fragments. Specific steps required adaptation of the ChIP protocol to human whole adipose tissue. In particular, a cross-linking step was performed directly on fresh small tissue fragments. Nuclei were isolated before releasing chromatin, allowing better management of fat content; a sonication protocol to obtain fragmented chromatin was optimized. We also demonstrate the high sensitivity of immunoprecipitated chromatin from adipose tissue to freezing. In conclusion, we describe the development of a ChIP protocol optimized for use in studying whole human adipose tissue, providing solutions for the unique challenges imposed by this tissue. Unraveling protein-DNA interaction in whole human adipose tissue will likely contribute to elucidating molecular pathways contributing to common human diseases such as obesity and type 2 diabetes.

transcriptional regulation; ChIP; whole human adipose tissue; endpoint PCR; protein-DNA interaction

CHROMATIN IMMUNOPRECIPITATION (ChIP) is a powerful and widely used tool for investigating DNA-protein interactions in vivo. This technique was developed in the mid 1990s and is based on the ability of formaldehyde to reversely cross-link proteins and DNA molecules that are within a maximal distance of 2Å. Such a distance is thought to capture mostly direct interactions and thus is suitable for exploring proteins that directly bind DNA (11). Breakage of genomic chromatin into appropriate size fragments (of 200–1,000 bp) is the next step. DNA-protein complexes are then immunoprecipitated using primary antibodies directed against the protein of interest. Subsequently, immunoprecipitated complexes are washed under stringent conditions to remove nonspecifically bound chromatin, the cross-link is then reversed, proteins are digested, and the precipitated ChIP-enriched DNA is purified. This purified DNA can be used in endpoint PCR, real-time PCR, labeling and hybridization to genomewide or tiling DNA microarrays (ChIP-on-chip), molecular cloning and sequencing, or direct high-throughput sequencing (ChIP-seq)(8, 14, 15, 25).

Originally, ChIP protocols were complicated, time-consuming, and required large amounts of fresh starting material, and each step had no alternatives. With time, improvements and modifications were introduced, enabling not only a reduction in the time required to perform the assay, but also the use of lower amounts of a starting material (4, 17, 18). Furthermore, for almost every step in the current standard protocol optional alternatives exist. Breakage of genomic chromatin, for example, can be done using either a mechanical method (sonicator) or by enzymatic digestion (with micrococcal nuclease). Additionally, magnetic beads are now commercially available for the precipitation of DNA-protein-antibody complexes as an alternative to the traditional agarose beads. Other examples include use of Chelex 100 (21) to reverse cross-linking instead of a highly concentrated salt or different DNA purification methods such as the "old school" phenol-chloroform method or spin-column technology with the selective binding properties of a uniquely designed silica membrane (commercially available).

An additional major development has been the optimization of ChIP protocols for specific cell types or tissues, such as the liver (1), primary pancreatic β-cells (10), monolayer cell lines (16), primary hippocampal cells, and whole hippocampus (24). Among these cell/tissue-specific ChIP protocols, one can also find protocols for primary isolated preadipocytes (16, 20) or adipocyte-like cell lines (16). Yet, a working ChIP protocol for whole adipose tissue (AT) has not been described.

AT is increasingly considered a major endocrine organ that contributes to whole body metabolic homeostasis. AT dysfunction is observed in obesity and diabetes mellitus (6, 7, 23), two health problems that are reaching epidemic proportions. To better understand the molecular mechanisms underling AT dysfunction, there is a need to explore AT regulation also at the transcriptional dimension, in which ChIP is currently a leading technique. Many ChIP analyses were used to investigate transcriptional regulation of either adipogenesis or adipocyte function; however, none of them was conducted in human whole AT. Several problems make it uniquely difficult to work with AT using a standard ChIP protocol. First, uniquely, small pieces of AT do not precipitate but rather float and make an upper dense phase (due to their fat content), complicating wash-aspirate-resuspend steps. Moreover, the standard ChIP protocol requires resuspending cells/tissues in SDS lysis buffer followed by sonication. Adipose fat content may reduce the sonication efficiency, resulting in DNA fragments unsuitable

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for the immunoprecipitation step. Therefore, a working protocol for ChIP using human AT is highly desired. Here, we describe a modified standard ChIP protocol adjusted for work with human AT fragments.

MATERIALS AND METHODS

Materials. Formaldehyde solution 37% (vol/vol) and protease inhibitors (PI) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS) was purchased from Biological Industries (Beit-Haeemek, Israel). Salmon sperm DNA/protein A-agarose beads and Magna ChIP protein A magnetic beads were purchased from Merck-Millipore (Billerica, MA). Antibodies were as follows: anti-E2F1 (sc-191x; ChIP-grade), anti-PPARγ (sc-7273x; ChIP-grade) anti-RNA pol-II (sc-21750x; ChIP-grade), and anti-ICAM-1 (sc-7891), obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Chelex was purchased from Bio-Rad Laboratories (Hercules, CA). Proteinase K solution was obtained from Invitrogen-Life Technologies (Grand Island, NY). Phusion High Fidelity master mix with HF buffer was purchased from Finnzymes Thermo Scientific (Vantaa, Finland).

Tissue preparation. Fresh paired human AT biopsies [subcutaneous and omental (visceral) fat] were obtained from female patients who underwent elective cholecystectomy or bariatric surgeries as part of a setup established at Soroka Medical Center and Ben-Gurion University of the Negev, Beer-Sheva, Israel (2, 3, 9, 13). All patients signed a written informed consent form approved in advance by the Institutional Ethics Committee for conducting studies in humans. Immediately after tissue excision, samples were transferred on ice (in sterile polyethylene cups) to the laboratory. One gram of each tissue was taken for further analysis. Each sample was rinsed once with sterile 1× PBS supplemented with PI (1:1,000 dilution) in a 50-ml conical tube. Afterward, each sample was resuspended in 10 ml of sterile 1× PBS supplemented with PI and minced into small pieces (1–3 mm^3) using two pairs of sterile surgical scissors. Formaldehyde was added to a final concentration of 1%, and samples were incubated for 10 min at 37°C with moderate shaking (85 rpm).

Cross-linking reaction was quenched (without aspirating the formaldehyde) by adding glycine to a final concentration of 125 mM for additional incubation (8 min, 37°C, moderate shaking). After incubation, samples were centrifuged [5 min, 2,500 rpm, room temperature (RT)] and placed on ice until the end of the procedure. Three distinct phases could be readily visualized at this point: tissue aggregates of higher density (pellet, tissue debris), buffer containing the PBS, formaldehyde and glycine (intermediate liquid phase), and lipid-rich (low-density) tissue pieces and fat (upper layer). By use of a sterile Pasteur pipette the pellet and intermediate liquid phase were aspirated, and the upper phase was washed twice with ice-cold 1× PBS supplemented with PI followed by centrifugation (5 min, 2,500 rpm, 4°C). After removal of the liquid phase from the second wash, small AT pieces were resuspended in 3 ml of adipocyte lysis buffer (500 mM PIPES, 80 mM KCl, and 1% Igepal) supplemented with PI, homogenized using a Dounce homogenizer (20 strokes; Wheaton, Millville, NJ). Sample incubation on ice for 15 min (vortexed every 3 min) was required to ensure rupture of the cell membranes and release of the nuclei. Twenty additional strokes were carried out followed by larger particle removal using 250-μm mesh. Then, samples were centrifuged (5 min, 2,500 rpm, 4°C), the pellet of the nuclei was resuspended in 500 μl of SDS lysis buffer supplemented with PI (1% SDS, 10 mM EDTA, pH 8.1, and 50 mM Tris·HCl, pH 8.1), transferred to a sterile 1.5-ml Eppendorf tube and incubated on ice for 20 min prior to sonication.

Sonication, dilution, and preclearing of the fixed chromatin. To ensure that sonication would result in 200- to 1,000-bp DNA fragments (PCR/qPCR applications) or 200- to 300-bp fragments (sequencing application), a 2-mm probe of VibraCell VCX-130 sonicator (Sonics, Newtown, CT) or Bioruptor Sonication System UCD-300 (Diagenode, Denville, NJ) was used, respectively, as follows. For generation of 200- to 1,000-bp DNA fragments, a probe sonicator was set on 15 pulses, each pulse 15 s "ON" and 30 s "OFF" at 40% of the sonicator's amplitude (see Fig. 2*A*). To generate the smaller (200- to 300-bp fragments), we obtained optimal results with a Bioruptor UCD-300 set at 20 pulses, each pulse 20 s "ON" and 30 s "OFF", using the "low density mode" of the sonicator (see Fig. 2*B*). Samples were maintained on ice during sonication to minimize foaming and avoid overheating. After sonication, samples were cenrifuged (10 min, 14,000 rpm, RT). Fifty microliters of sonicated chromatin (undiluted) were taken aside to evaluate sonication efficiency by running samples on a 1% agarose gel and validating the size of the chromatin fragments (200–1,000 or 200–300 bp).

Two hundred microliters of supernatant (sonicated chromatin) was taken per one immunoprecipitation and diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1 mM EDTA, pH 8.1, 16.7 mM Tris·HCl, pH 8.1, and 167 mM NaCl) supplemented with PI in a sterile 2-ml tube. It is possible to generate another 2-ml tube with sonicated and diluted chromatin and snap-freeze it in liquid nitrogen for another ChIP assay; however, as presented later, we did not succeed in reproducing our results in frozen samples. To enable comparison of results between different samples, the input of each sample, i.e., the amount of starting chromatin prior to immunoprecipitation, needs to be assessed and later to be used for normalization of the immunoprecipitation results. For this, 100 μl of previously diluted and sonicated chromatin was transferred to a new sterile 1.5-ml tube and frozen until DNA purification.

Immunoprecipitation. There are two commercially available types of beads that one can choose from: magnetic or agarose.

When agarose beads were chosen, ChIP protocols required a preclearing step. For this, 60 μl of DNA/protein A-agarose 50% beads was added to each sample for 60 min at 4°C with agitation. Then agarose beads were precipitated by centrifugation (5 min, 1,200 rpm, 4°C), and supernatant was transferred to a new 2-ml tube. Following this step, two μg of primary ChIP-grade antibody (anti-E2F1 or -PPARγ used here, or anti-RNA pol II for positive control and anti-ICAM-1 Ab for the negative control) were added to the precleared chromatin, and samples were incubated overnight at 4°C with constant rotation. Then, 70 μl of DNA/protein A-agarose 50% beads was added to each sample (in case of agarose beads) followed by 90-min incubation at 4°C with rotation. Agarose beads were then precipitated by centrifugation (5 min, 1,200 rpm, 4°C) followed by careful removal of the supernatant.

When magnetic beads were used, chromatin was directly (without a preclear step) incubated overnight at 4°C with constant rotation after addition of the same amounts of primary ChIP-grade antibodies as described above and 40 μl of Magna ChIP protein A magnetic beads. Thereafter, protein A magnetic beads were captured with magnetic separator, and supernatant was completely removed.

Washing and DNA recovery. Protein A-agarose beads/antibody/chromatin or protein A magnetic beads/antibody/chromatin complexes were washed for 5 min with rotation at 4°C and then precipitated (5 min, 1,200 rpm, 4°C in case of agarose beads or magnetic separator in case of magnetic beads), and each wash buffer was discarded between steps. The first wash was with 1 ml of low-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.1, 20 mM Tris·HCl, pH 8.1, and 150 mM NaCl). The second wash was with 1 ml of high-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.1, 20 mM Tris·HCl, pH 8.1, and 500 mM NaCl) followed by a third wash with 1 ml of LiCl immune complex wash buffer (250 mM LiCl, 1% deoxycholic acid, 1 mM EDTA, pH 8.1, 10 mM Tris·HCl, pH 8.1, and 1% Igepal). The last two washes were with a TE buffer (1 mM EDTA, pH 8.1, and 10 mM Tris·HCl, pH 8.1) at RT.

In case of agarose beads, after removal of the last wash, 100 μl of 10% (wt/vol) Chelex 100 was added to each sample and input (inputs were thawed on ice), followed by vortex and incubation for 13 min at

95°C. Samples and inputs were then cooled on ice, followed by addition of 2 μl of 20 μg/ml proteinase K and vortex and incubation for 45 min at 55°C. Additional incubation for 10 min at 95°C was performed to inactivate the proteinase K. Samples and inputs were then centrifuged for 5 min at 14,000 rpm at RT, and supernatant (containing purified DNA suitable for further analysis) was transferred to a new sterile 1.5-ml Eppendorf tube, avoiding carry-over of Chelex beads. One hundred microliters of PCR-suitable water was then added to the Chelex beads, followed by vortex and centrifugation for 5 min at 14,000 rpm at RT. Supernatant was transferred to the same 1.5-ml tube.

Note that the required boiling step when using Chelex yields single-stranded DNA. As an alternative, particularly if double-stranded DNA is desired, the DNA can be eluted from the DNA/protein A-agarose 50% beads by adding to the samples after the final wash 250 μl of a fresh elution buffer (SDS 0.1% and $NaHCO_3$ 100 mM) and incubation for 15 min at RT with rotation. Centrifugation for 5 min at 1,200 rpm was needed to precipitate the agarose beads, followed by a transfer of the supernatant to a new sterile 1.5-ml tube. The elution step was then repeated, and eluates were combined (total volume ~500 μl). Cross-linking was reversed by adding 20 μl of 5 M NaCl to the combined eluates and incubation for 4 h at 65°C. For protein digestion, 2 μl of 20 μg/ml proteinase K, 10 μl of 500 mM EDTA, and 20 μl of 1 M Tris·HCl were added, and eluates were incubated for 1 h at 55°C. DNA recovery can be performed using the phenol-chloroform method or commercially available kits.

In case of magnetic beads, after removal of the last wash, 250 μl of fresh elution buffer (SDS 0.1% and $NaHCO_3$ 100 mM, 20 mM Tris·HCl, 5 mM EDTA, 50 mM NaCl) containing 2 μl of 20 μg/ml proteinase K was added to the samples. Samples were incubated for

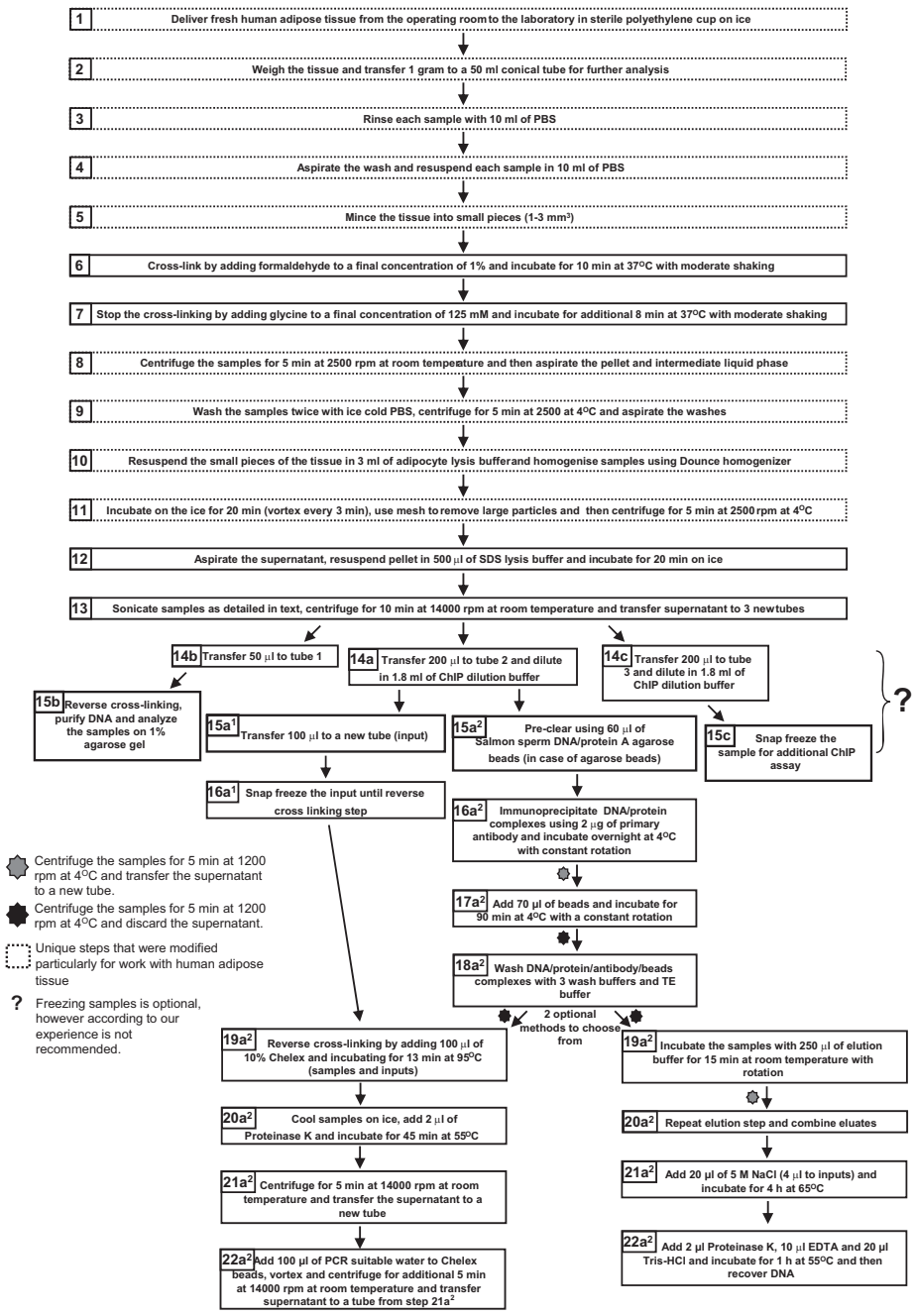
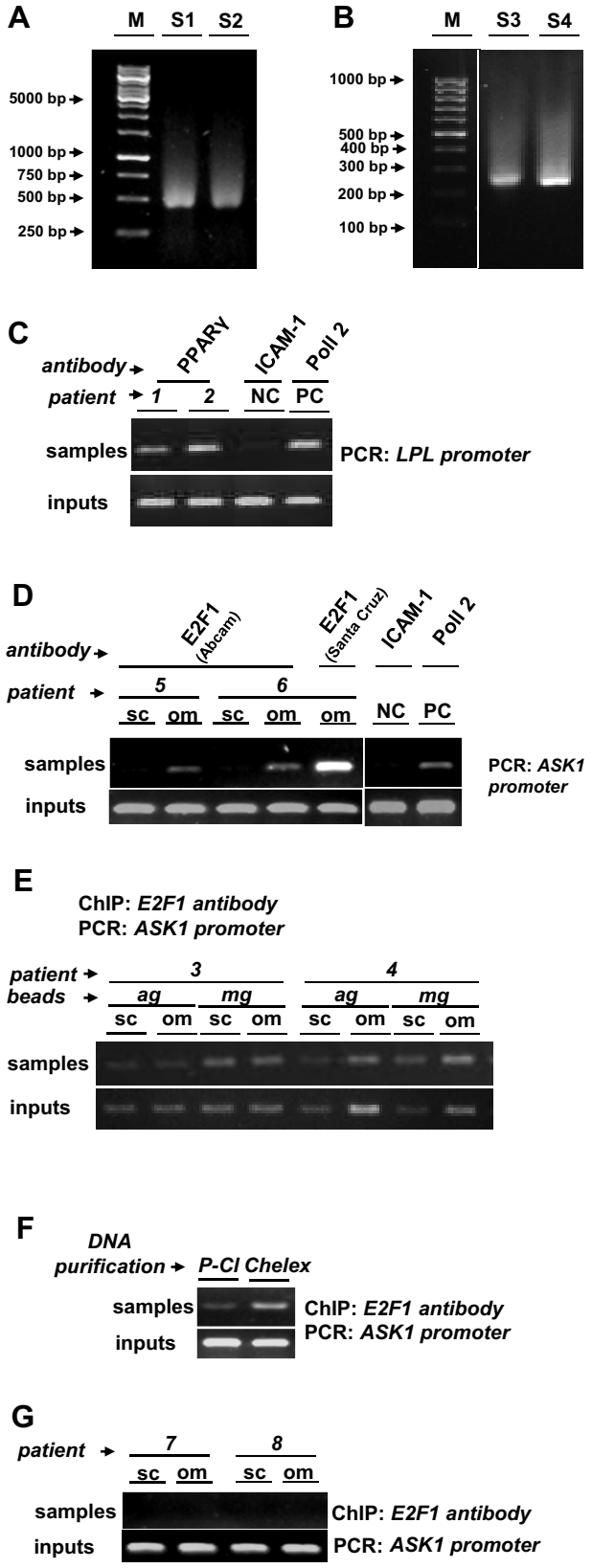


Fig. 1. Detailed flowchart of a modified ChIP protocol optimized for application for human adipose tissue. Please refer to text for changes in this protocol (from step 15 and on) when using magnetic beads instead of agarose beads for the protein-DNA complex precipitation.



2 h at 65°C on a Thermomixer (Eppendorf, Hamburg, Germany) with constant vortexing (1,300 rpm). After capture of the beads using a magnetic separator, the supernatant was recovered, and ChIP material was reincubated in 250 μl of elution buffer-proteinase K for 15 min on the Thermomixer. Both supernatants were pooled. As before, DNA recovery can be performed using the phenol-chloroform method or commercially available kits.

The recovered DNA yield was ~200 ng per 1 gram of starting tissue material. Then, this recovered DNA was subjected to semiquantitative PCR (5 μl and 0.5 μl from samples and inputs, respectively) using specific primers and Phusion High Fidelity master mix with HF buffer. Alternatively, quantitative real-time PCR was successfully performed (not shown).

RESULTS AND DISCUSSION

We hereby report the optimization of standard ChIP protocols for application to study protein-DNA interaction in human whole AT. Unique modifications of the protocol to adopt for AT are highlighted in Fig. 1, and elaborated below.

Mincing the tissue into small pieces (1–3 mm^3) using surgical scissors and performing the cross-linking step directly on those tissue fragments (explants) is a modification that enables work with whole AT. While AT includes many cell types, suggesting that changes in ChIP may represent also alterations in the cellular composition of the tissue, the advantages over ChIP studies on isolated adipocytes include the higher chance of capturing "authentic" in vivo protein-DNA interactions in the tissue, avoiding the potential effects of the isolation procedure. Indeed, classical adipocyte isolation procedures with collagenase were criticized for causing substantial loss of adipocytes and/or altering the expression of multiple genes, including inflammatory and adipocyte-specific genes (19, 22). While some of these shortcomings have been improved, the theoretical risk of changing protein-DNA interaction by in vitro manipulations is still significant.

Isolation of nuclei prior to chromatin release enabled us to remove the fat, which might affect sonication efficiency and/or protein-antibody interactions.

To validate this modified ChIP protocol, we performed two important controls. For positive control, we precipitated RNA

Fig. 2. ChIP in human adipose tissue. Chromatin shearing of human adipose tissue. Cross-linked chromatin was sonicated to fragments of 200–1,000 bp (*A*) or 200–300 bp (*B*), using different sonication systems, as described in MATERIALS AND METHODS. Samples were then electrophoresed on 1% agarose gel. M denotes a 250- or 100-bp ladder; S1/2/3/4 denote the different sonicated samples. *C*: formaldehyde cross-linked chromatin from human omental adipose tissue (from 2 patients) was subjected to ChIP experiment. Immunoprecipitation of PPARγ containing complexes was performed using anti-PPARγ antibody. Anti-RNA Poll 2 was used as positive control (PC) and anti-ICAM-1 was used as negative control (NC). sc, subcutaneous; om, omental. After isolation of bound DNA, endpoint PCR was performed for a 450-bp region of the endogenous human LPL promoter. Inputs indicate PCR performed on DNA (diluted 1:300) without any immunoprecipitation. *D*: ChIP was performed on human adipose tissue by using two different ChIP-grade anti-E2F1 antibodies (one from Abcam and the other from Santa Cruz). PC and NC were as described in *C*. After isolation of bound DNA, PCR was performed for a 297-bp region of the endogenous human ASK1 promoter. *E*: ChIP of human adipose tissue was performed using anti-E2F1 (Santa Cruz) followed by comparison of agarose A beads (ag) to magnetic beads (mg) for the protein-DNA complex precipitation step. *F*: E2F1 ChIP was performed comparing two DNA purification methods: phenol-chloroform extraction (P-Cl) or Chelex. *G*: effect of freezing sonicated chromatin from human adipose tissue on results of E2F1 ChIP.

pol-II because of its robust interactions with the chromatin, while ICAM-1 was chosen as a negative control given the exclusive plasma membrane localization of this integrin (nonimmune isotype control antibody is an equally valuable alternative). As shown in Fig. 2, *C* and *D*, precipitation with anti-RNA pol-II resulted in a PCR signal, while precipitation with anti-ICAM-1 revealed no signal, as expected. Performing these two controls in each immunoprecipitation experiment provides the required reassurance for the reliability of the entire assay.

To ensure that despite adipose tissue heterogeneity (adipocytes, immune cells, preadipocytes, endothelial cells, etc.) adipocyte fraction is represented in ChIP results, we conducted PPARγ ChIP given the known role of this transcription factor in adipogenesis and adipocytes. In vivo binding of PPARγ to the lipoprotein lipase (LPL) promoter, a well-established PPARγ target gene in humans, was clearly detected (Fig. 2*C*).

Several steps are particularly crucial for the success of this ChIP protocol. Optimizing the chromatin breakage using sonication is particularly crucial while working with limited amounts of human AT. Confirming sonication efficiency can be achieved as shown in Fig. 2, *A* and *B*. DNA fragment sizes should be defined depending on the desired final application. Here, we show optimized sonication using two different sonication systems and resulting in different DNA fragment sizes. The optimized protocol presented is the result of testing different numbers of pulses, pulse amplitudes, and durations of each pulse.

Choosing the right antibody (preferably "ChIP grade") is another critical step to ensure a successful ChIP assay. We tested four different ChIP-grade E2F1 antibodies, but only two produced any ChIP product (Fig. 2*D*). Clearly, a higher PCR product was noted with one of these antibodies, but overall the results obtained with the two were comparable (data not shown).

This protocol tested two types of commercially available beads for immunoprecipitation. As shown in Fig. 2*E*, it may be suggested that magnetic beads might increase the sensitivity of the assay, as we tended to see a stronger signal particularly in samples that resulted in low PCR product (or real-time PCR product, not shown) following ChIP.

There are three common methods for DNA purification. We tested phenol-chloroform extraction vs. Chelex (Fig. 2*F*). The Chelex-dependent method resulted in higher DNA yield, but, as mentioned earlier, this recovered DNA is single stranded and is thus suitable for PCR and qPCR but not necessarily for sequencing. To conduct sequencing application, phenol-chloroform extraction or DNA purification spin columns, both of which isolate double-stranded DNA, should be preferred. In our experience, phenol-chloroform resulted in a superior result to the spin columns (data not shown).

Choosing the right primers for the correct DNA promoter areas is another cricital step. We used known primers (5, 12) for the promoter area of interest, but designing new primers is also available using different software tools [Primer3 Input (frodo.wi.mit.edu/), Primer designing tool (www.ncbi.nlm.nih.gov/tools/primer-blast/), Primer3Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi)] or others. It is important to note that, in addition to E2F1 binding to the ASK1 promoter, we could readily detect binding to a different, "classical" E2F1 target gene, cyclin d1 (not shown). Along with the PPARγ ChIP (Fig. 2*C*), we believe the described ChIP protocol is not dependent on a specific primer.

Freezing samples postsonication (steps 14c, 15c) for future immunoprecipitations is widely used in ChIP procedures; however, we had a poor experience following this option: no PCR signal was generated from chromatin that was frozen for 2 wk in −80°C (Fig. 2*G*). Reducing or even harming in vivo interactions between E2F1 and chromatin during freezing might explain the differences between our and others' results. Alternatively, this may represent AT-specific sensitivity to freeze-thaw. Given this experience, we currently cannot recommend freezing sonicated chromatin, although other transcription factors or possibly using larger amounts of starting AT material may not be as sensitive.

In conclusion, we optimized the standard ChIP protocol for human whole AT. The most important advantage of this protocol is the ability to study transcriptional regulation in "authentic" human tissues and not in models such as adipocyte cell lines or nonhuman ATs. Theoretically, we believe this protocol can be used not only to investigate known promoter areas (using endpoint PCR or real-time PCR), but also to discover novel promoters regulated by a specific transcription factor of interest utilizing ChIP-seq.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Y.H. and A.R. conception and design of research; Y.H. and T.T. performed experiments; Y.H., T.T., D.B., and A.R. analyzed data; Y.H., D.B., and A.R. interpreted results of experiments; Y.H. and A.R. prepared figures; Y.H. and A.R. drafted manuscript; Y.H. and A.R. edited and revised manuscript; A.R. approved final version of manuscript.

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