bio-protocol

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HaloChIP-seq for Antibody-Independent Mapping of Mouse Transcription Factor Cistromes *in vivo*

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Abstract

Chromatin immunoprecipitation (ChIP) maps, on a genome-wide scale, transcription factor binding sites, and the distribution of other chromatin-associated proteins and their modifications. As such, it provides valuable insights into mechanisms of gene regulation. However, successful ChIP experiments are dependent on the availability of a high-quality antibody against the target of interest. Using antibodies with poor sensitivity and specificity can yield misleading results. This can be partly circumvented by using epitope-tagged systems (*e.g.*, HA, Myc, His), but these approaches are still antibody-dependent. HaloTag® is a modified dehalogenase enzyme, which covalently binds synthetic ligands. This system can be used for imaging and purification of HaloTag® fusion proteins, and has been used for ChIP *in vitro*. Here, we present a protocol for using the HaloTag® system for ChIP *in vivo*, to map, with sensitivity and specificity, the cistrome of a dynamic mouse transcription factor expressed at its endogenous locus.

Keywords: HaloTag®, ChIP, Nuclear receptor, Tag, Fusion protein, NR1D1

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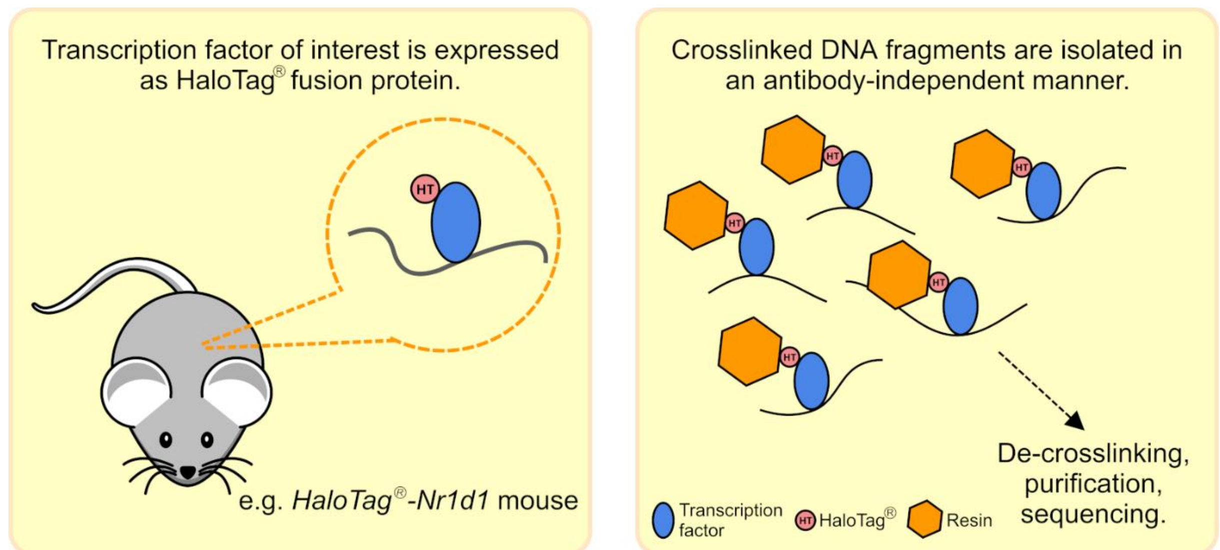
Graphical abstract:



Background

Transcription factors (TFs), such as nuclear receptors, are critical regulators of gene expression. They can be highly dynamic, low abundance proteins. By mapping the locations of TF binding genome-wide (the cistrome), mechanisms of gene regulation, and how these vary by cell type and state, can be examined. Chromatin immunoprecipitation (ChIP) makes use of reversible formaldehyde cross-linking, to capture DNA-protein interactions (Jackson, 1978). The protein-DNA complex can then be immunoprecipitated with an antibody against the protein of interest, and an antibody-binding protein (*e.g.*, Protein A, Protein G) coupled to agarose beads. DNA not bound by the protein of interest, *i.e.*, non-specific signal, is removed by washing. Cross-links are reversed by heat, proteins are digested (*e.g.*, with Proteinase K), and the released DNA is purified for analysis by quantitative polymerase chain reaction (qPCR) (Orlando, 2000), microarray (Ren and Dynlacht, 2003), or next generation sequencing (ChIP-seq) (Wei *et al.*, 2006).

Antibody-based ChIP is dependent upon a highly sensitive and highly specific antibody against the target protein of interest. Recent work in our group has focused on the action of circadian clock protein and nuclear receptor NR1D1 (REVERBα) in mouse adipose tissue and liver. Unfortunately, high-quality NR1D1 antibodies are lacking, and overlap between published NR1D1 cistromes in mouse liver is limited (Hunter *et al.*, 2020). Epitope-tagging, in which a recombinant version of the protein of interest carries a small peptide tag (against which antibodies are available), can partly circumvent this. Indeed, this approach has been taken successfully by another group in the study of NR1D1, using haemagglutinin (HA)-tagged NR1D1 (Adlanmerini *et al.*, 2019). However, epitope tag ChIP remains dependent on antibody binding affinity (Encell *et al.*, 2012), and this may limit yield when the protein of interest is expressed at low levels. We chose to use the HaloTag® system to map the NR1D1 cistrome (Hunter *et al.*, 2020, 2021). This approach is distinct, as it relies on covalent and irreversible binding between the 34-kDa HaloTag®, which is a modified haloalkane dehalogenase (Los *et al.*, 2008; Encell *et al.*, 2012), and synthetic haloalkane ligands. A ligand can be attached to a resin, allowing highly specific pull-down of HaloTag® proteins. The binding is sufficiently stable to permit the stringent wash steps needed to remove unbound DNA from the ChIP reaction. HaloChIP™ (Daniels and Urh, 2013) has been used *in vitro* to examine TF binding in transfected cell lines (Du *et al.*, 2009; Hartzell *et al.*, 2009; Deplus *et al.*, 2013). Here, we describe our method for HaloChIP-seq, to map the cistrome of an endogenously expressed mouse TF *in vivo*. The method, as presented, was developed for studying NR1D1 in mouse adipose tissue and liver. However, with careful adaptation and optimisation, the method should be adaptable to other TFs in other tissues.

Central to performing *in vivo* HaloChIP is a system for expressing the HaloTag® fusion protein of interest in a model organism. This requires gene editing expertise. Here, we provide a detailed description of the approach taken to generate the *HaloTag®-Nr1d1* mouse, but this is intended as a guide for a gene editing team with CRISPR-Cas9 experience. Thereafter follows a detailed protocol for the HaloChIP wet lab process, which is intended for use by readers with some general molecular biology experience.

Materials and Reagents

1. *0.5 M EDTA (*e.g.*, Thermo Scientific™, catalog number: J15694-AE), store at room temperature (RT)
2. *Nonidet® P40 (Substitute) (*e.g.*, PanReac AppliChem, catalog number: A1694,0250), store at RT
   **Note: Only needed for making adipose tissue lysis buffer.*
3. Ultra-High Recovery 1.5 mL microcentrifuge tubes (*e.g.*, Star Lab, catalog number: E1415-2600), store at RT
4. 15 mL centrifuge tubes (*e.g.*, Corning, catalog number: 430791), store at RT
5. Phase Lock Light 2 mL gel tubes (5 PRIME, catalog number: 2302820), store at RT
6. Surgical scalpel blade No.22, sterile (Swann-Morton, catalog number: 0308), store at RT
7. Sterile 10 cm dishes (*e.g.*, Corning, catalog number: 430167), store at RT
8. **Glass Pasteur pipettes (*e.g.*, 230 mm, Fisher Scientific, catalog number: 1156-6963), store at RT
   ***Note: Only needed for working with adipose tissue, or other tissue likely to float on water.*
9. Dimethylsulfoxide (DMSO) (*e.g.,* Thermo Scientific™, catalog number: 20688)
10. Disuccinimidyl glutarate (DSG) (*e.g.,* Thermo Scientific™, catalog number: 20593)
    Prepare a 0.5 M DSG solution, by adding 306 µL of DMSO to 50 mg DSG. Invert several times to mix, and store 40-µL aliquots at -20°C.
11. Formaldehyde solution for molecular biology 36.5–38% in H_2O (Sigma-Aldrich, catalog number: F8775), store at RT.
12. $RNase A 10 mg/mL (*e.g.*, Thermo Scientific™, catalog number: EN0531), store at -20°C.
13. $Proteinase K 20 mg/mL (*e.g.*, Thermo Scientific™, catalog number: 26160), store at -20°C.
14. $Molecular biology grade glycogen 20 mg/mL (*e.g.*, Thermo Scientific™, catalog number: R0561), store at -20°C.
15. $Glycine, ≥99%, Molecular Biology Grade, Ultrapure (*e.g.*, Thermo Scientific™, catalog number: 15815158), store at RT
16. $PBS, 10× solution, molecular biology grade (*e.g.*, Thermo Scientific™, catalog number: 15825418), store at RT
17. $NaCl (5 M), RNase-free (*e.g.*, Invitrogen™, catalog number: AM9760G), store at RT
    $Note: Alternatively, the High Sensitivity Chromatin Prep Kit (Active Motif, catalog number: 53046) includes the equivalent of these reagents (note that the Proteinase K supplied in the Active Motif kit is 10 µg/µL). Purchasing reagents as a kit has the advantages of avoiding reagent contamination and degradation, and can be more convenient. Cost-effectiveness may depend on your sample numbers.
18. A lysis buffer in which you will homogenise your tissue. For liver tissue, we use the Chromatin Prep Buffer in the Active Motif High Sensitivity Chromatin Prep Kit (see above). An alternative is the Mammalian Lysis Buffer in the Promega HaloCHIP™ System. For adipose tissue, we use a homemade lysis buffer (see Recipes), store at 4°C.
19. HaloCHIP™ System (Promega, catalog number: G9410). Mixed storage—see manufacturer's instructions.
    Comprises HaloLink™ Resin, Mammalian Lysis Buffer, High Salt Wash Buffer, Reversal Buffer, HaloCHIP™ Blocking Ligand, nuclease-free water, TE buffer 1× molecular biology grade.
20. Protease Inhibitor Cocktail, 50× (Promega, catalog number: G6521)
    Reconstitute protease inhibitor cocktail (PIC) by adding 1 mL of 100% ethanol to the vial. Store reconstituted PIC at 4°C.
21. #Universal tracrRNA (IDT, Coralville, USA, catalog number: 1072533)
22. #EnGen Cas9 protein (New England Biolabs, catalog number: M0646M)
23. #KOD Hot start DNA polymerase (Merck, catalog number: 71086)
24. #Zero Blunt PCR cloning kit (ThermoFisher, catalog number: K270020)

25. #REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich, catalog number: XNAT)
    #Note: Only needed in the mouse generation process.
26. ChIP DNA Clean & Concentrator kit (Zymo Research, catalog number: D5205); an alternative is the MinElute PCR Purification Kit (Qiagen, catalog number: 28004), store at RT
27. Water for molecular biology (*e.g.*, Lonza AccuGene, catalog number: BE51200), store at RT
28. Phenol–chloroform–isoamyl alcohol mixture, BioUltra for molecular biology, 25:24:1 (Sigma-Aldrich, catalog number: 77617), store at 4°C
29. IGEPAL® CA-630 (*e.g.*, Sigma-Aldrich, catalog number: 18896), store at RT
30. 1 M Tris-HCl (pH 8.0) (*e.g.*, Sigma-Aldrich, catalog number: T3038), store at RT
31. Lithium chloride (anhydrous) (*e.g.*, Scientific Laboratory Supplies Ltd., catalog number: CHE2356), store at RT
32. Sodium deoxycholate 10% (*e.g.*, BioWORLD, catalog number: 40430018-1), store at RT
33. Wet ice
34. Ethanol absolute (100%), store at RT
35. 1× PBS (see Recipes)
36. 1% formaldehyde-PBS solution (see Recipes)
37. 2.5 M glycine solution (see Recipes)
38. Adipose tissue lysis buffer (see Recipes)
39. 1 M lithium chloride (LiCl) solution (see Recipes)
40. Lithium chloride wash buffer (Promega recipe) (make fresh) (see Recipes)

Equipment

Specialist equipment:

1. Handheld tissue homogeniser (*e.g.,* Qiagen TissueRuptor 220V)
2. 15 mL glass dounce homogeniser (*e.g.,* Active Motif, catalog number: 40415)
3. Sonicator with cooling system (*e.g.,* Active Motif EpiShear™ Probe Sonicator 230V, catalog number: 53052). *See ChIP protocol section D.*
4. System for accurate quantification of ChIP DNA mass. We use Qubit™ dsDNA HS and BR Assay Kits (Invitrogen™, catalog number: 10616763) with the Qubit™ fluorometer.
5. Electrophoresis system for assessment of DNA shearing quality. We use the Agilent TapeStation 2200 system; it is also possible to perform traditional agarose gel electrophoresis, but this does not offer as good a resolution as the TapeStation, Bioanalyzer, and equivalents.

General equipment:

You will also need temperature-controlled benchtop centrifuges capable of holding 15-mL and 1.5-mL tubes, a benchtop tube rotator, a benchtop vortexer, a thermocycler or heat block, Parafilm or similar, micropipettes (*e.g.*, Gilson) and tips, and a pipette boy and stripettes. You will need access to a fume hood to work safely with formaldehyde and phenol. Access to a phase-contrast microscope is useful for checking nuclei release after douncing.

Procedure

Description of mouse generation

We routinely use CRISPR-Cas9 to tag endogenous genes with genetically encoded tags, for a range of downstream applications. Here, the intention was to fuse the HaloTag® to the endogenous *Nr1d1* gene. The process involves the following steps: (i) *in silico* design of the gene editing, (ii) generation of the gene editing reagents, (iii) manipulation of mouse embryos and embryo transfer to pseudopregnant mothers, and (iv) screening of pups for correct gene integration. The following protocol describes the process for this particular allele, but can serve as a blueprint for similar tagging strategies for other genes.

Note: Why CRISPR-Cas9? *CRISPR-Cas9 is a gene editing tool that allows us to make changes to a genome. By designing a single guide RNA (sgRNA) specific for the genomic target, and coupling it with Cas9, we can generate a double strand break (DSB) at that site, and exploit the natural DNA repair mechanisms of the cell to direct a desired change. By altering the endogenous genes in this way, we avoid many of the issues associated with random integration of exogenous constructs.*

A. Design of the gene editing

1. Where to tag

   When tagging genes with functional fusions, we take into account a number of factors, to ensure both preservation of endogenous function, and the functionality of the tag itself. With respect to the *Nr1d1* gene, evidence from the literature suggests that tagging at the N-terminus with fluorescent genes, and expression from exogenous vectors results in functional, visible proteins (Ka *et al.*, 2017). However, CRISPR-Cas9 results in the manipulation of endogenous sequences, and one must consider collateral genomic disruption that may have unintended consequences and effects. The mouse *Nr1d1* gene is found on chromosome 11, and the 3' sequences of this gene overlap with the 3' sequences of the *Thra* (thyroid hormone receptor alpha) gene on the antisense strand (**Figure 1**). Thus, integration of the HaloTag® fusion gene directed at the C-terminus would result in perturbation of the *Thra* gene. As a result, we targeted the HaloTag® integration at the N-terminus of *Nr1d1*. These considerations should be taken when integrating tags on endogenous genes.

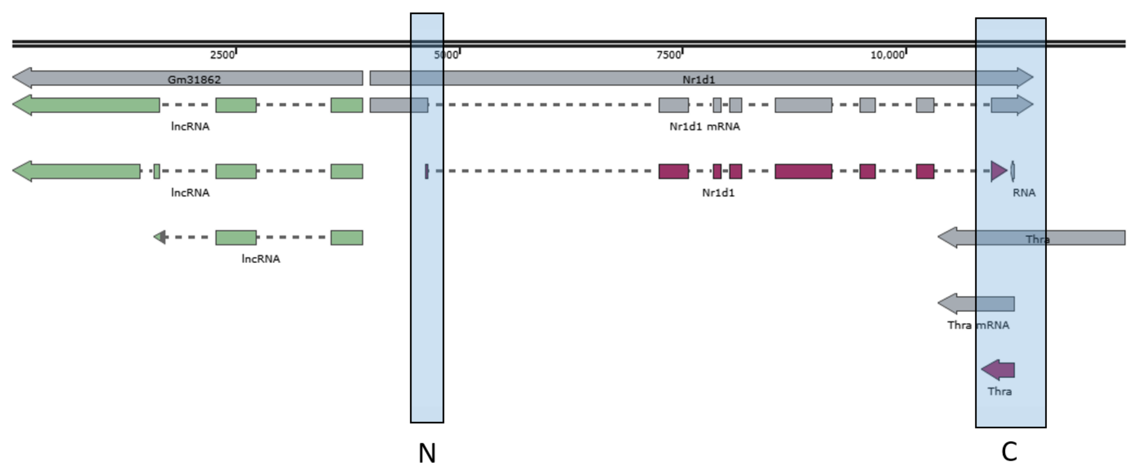


Figure 1. Targeting *Nr1d1*.
Region of mouse chromosome 11 where *Nr1d1* is situated. Blue shaded boxes correspond to the N- and C-terminus coding sequences. For CRISPR-mediated HaloTag® knock in, we targeted exon 1 of *Nr1d1* (corresponding to the N-terminus) (Hunter *et al.*, 2020). Note the overlap of the C-terminus with the *Thra* gene, negating C-terminal tagging as an option. lncRNA = long non-coding RNA.

2. Design of sgRNA

   When designing guide RNAs for CRISPR, we modify our design rules according to the desired outcome. For gene knock out or disruption via InDel (insertion and deletion) formation, following repair of the Cas9-induced DSB by non-homologous end joining (NHEJ), we can target different exons of the target gene to achieve the same result. Thus, one usually has a number of potential sgRNA to choose from, and can stratify the selection according to low off-targeting potential, and perhaps factor in predicted on-target efficiency (Doench *et al.*, 2016). With respect to precise genetic changes mediated by homology directed repair (HDR), the breadth of sgRNA choice is more limited; one must design sgRNA for the DSB to be induced proximally to the insertion site (usually, 10–15 bp, or so). Thus, one may have to compromise on

using sgRNA with a less than ideal off-targeting score. In such circumstances, it may be advisable to screen for off-target editing in positive founders. Should undesirable co-edits be identified, if they are located on a different chromosome, then they can be segregated through breeding and screening the following generations.

Note: What are off-target effects*? Partial sgRNA recognition may occur elsewhere in the genome. On these sites, it is possible, though less likely, that the sgRNA:Cas9 complex can generate DSB, and result in unwanted collateral DNA editing.*

Off-target effects are probably not as great an issue as first feared, especially in non-transformed cells, and can be largely mitigated through improved sgRNA design (Thomas *et al.*, 2019; Haeussler, 2020), and transient delivery of the sgRNA and Cas9 (Kim *et al.*, 2014). In gene-edited animals, it is also possible to segregate unwanted edits through breeding. Nevertheless, we factor in off-targeting potential in our design.

A number of webtools have been developed that will predict guide RNA target sites, searching by either genes, chromosomal positions, or inputting a sequence for targeting. Here, we used the Sanger WTSI site, http://www.sanger.ac.uk/htgt/wge/ (Hodgkins *et al.*, 2015), and selected the sgRNA sequences shown in **Table 1**, positioned close to the ATG start site of the *Nr1d1* gene. Both of these sgRNA have low off-targeting potential.

3. Design of the Homology Flanked Donor

For fusion tags, the aim is to integrate the coding sequence of the tag plus linker to be produced in frame with the endogenous gene, and homology arms are designed to achieve this precisely. We use a long single strand DNA (lssDNA) donor strategy, which requires far shorter homology arms (here, 96 nt and 101 nt) than classic double stranded DNA (dsDNA) donors (~800 bp), and has been demonstrated to be more effective than dsDNA donors (Quadros *et al.*, 2017). We also integrated synonymous changes into these homology arms, intended to ensure the same amino acid is produced by the altered codon, but the genetic sequence of the sgRNA PAM site (protospacer adjacent motif) is disrupted. These so-called 'shield mutations' insulate the correctly repaired gene sequence from further binding of the sgRNA:Cas9 complex, and undesired editing.

The seamless insertion of the donor at the ATG site will ensure the preservation of all critical endogenous regulatory regions and sequences, which is vital for a dynamically regulated gene, such as *Nr1d1*.

B. Generation of editing reagents and embryo injection mixture

In addition to the description below, we refer the reader to our recent publication (Bennett *et al.*, 2021) for this step.

1. The sgRNA and Cas9 complex

We inject mouse embryos with CRISPR RiboNucleoProtein (RNP) complexes, using commercially sourced reagents. For the sgRNA in this study, we used dual oligos supplied by Sigma (part of Merck KGaA Darmstadt), which require the crRNA (crispr RNA) with the specific targeting sequence to be annealed with a universal tracrRNA (trans-activating crispr RNA). The simple protocol for this is provided below, but it should be noted that many suppliers now offer the synthesis of full length sgRNA for Cas9-related applications. We routinely use these full sgRNA (supplied by either Sigma or Integrated DNA Technologies), as it streamlines the process of making the embryo injection mix.

a. Resuspend universal tracrRNA in sterile RNase-free injection buffer (1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) to a concentration of 1 µg/µL. Prepare aliquots of 5-µL volume, and freeze at -80°C for long term storage.

b. Resuspend the crRNA oligo in sterile RNase-free injection buffer, to a concentration of 1 µg/µL. We typically hope to generate the desired mouse model at the first attempt, but the resuspended crRNA can be aliquoted and stored at -20°C for future use, should a re-attempt be required.

c. Combine 2.5 µg crRNA with 5 µg universal tracrRNA (IDT, Coralville, USA, 1072533), and heat to 95°C in a thermal cycler, before allowing to cool slowly to RT.

d. Combine the annealed sgRNA with EnGen Cas9 protein (New England Biolabs, M0646M), by adding 1 µg sgRNA to 0.3 µL (equivalent to 1 µg, or 0.24 µM) Cas9 diluted to 5 µL in injection buffer, and incubating at room temperature for 10 min.
Note: There are many commercial suppliers of Cas9 protein. We have used Sigma, IDT, and Synthego Cas9, as well as NEB EnGen Cas9, and have been satisfied with all. We recommend screening batch activity of Cas9 in blastocyst assays (Scavizzi et al., 2015).

2. The lssDNA donor purification
The workflow consists of (i) Gibson assembly (Gibson *et al*., 2009) of a Homology-HaloTag®-Homology donor vector, (ii) use of this vector as a template for Biotinylated PCR, (iii) on column denaturation and purification of the lssDNA. We have recently published our method for generating ultra-pure lssDNA templates for mouse embryo injection (Bennett *et al*., 2021). We have applied this method successfully on >20 mouse knock in alleles, and the detailed protocol is available in the above reference. The lssDNA sequence for the *HaloTag®-Nr1d1* mouse is provided in **Table 1**.

C. Generation and screening of mice

The injection mix developed in section B is microinjected in single cell mouse zygotes, according to standard protocols (Nagy *et al*., 2006; Pu *et al*., 2019). Once pups are born, we follow a standard genotyping strategy, detailed in Bennett *et al.* (2021) for gene tags, that involves a series of PCR reactions (**Figure 2**), followed by Sanger sequencing. Reaction 1 uses primers (here, AL04 Cut Test F, and AL04 Cut Test R—see **Table 1**) that amplify over the sgRNA target site, from outside the homology arms, and produce a product of ~200–400 bp on WT sequences, and can also detect size changes as a result of NHEJ and InDel formation (a useful quality control, to confirm sgRNA were active), and at times can detect the integration of the full transgene.
Notes:

1. *We currently use microinjection, but others have demonstrated electroporation to be a viable delivery method of lssDNA and RNP complexes into mouse embryos https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5930688/.*
2. *NHEJ and InDel formation when tagging a gene at the N terminus can result in coding frameshifts and potential gene KO. This has some implications for reducing and refining animal use: 1. We regularly ask if a KO mouse allele would be of any use to the research group, and can genotype by sequencing if desired, to prevent animal wastage. 2. It should also be noted that some gene KO can be detrimental to animal health, and this should be carefully considered from the outset of the project. If gene KO is predicted to be detrimental, then pup health should be monitored as appropriate.*

This is followed by two targeted integration PCRs, where the above AL04 Cut Test F primer from outside the 5' homology arm is used in combination with an internal Reverse HaloTag primer (AL04 Geno HaloR1b), and, similarly, an internal Forward HaloTag primer (AL04 Geno Halo F1b) is used with the Cut Test R primer designed outside the 3' homology arm. Primer sequences can be found in **Table 1**. Mice that are positive for these assays can have their amplicons sequenced, to confirm precise integration of the tag.

1. Genomic DNA (gDNA) extraction, PCR amplification, and sequencing
There are several commercially available kits to extract mouse genomic DNA from tissues, such as ear punches. We use the Sigma Aldrich REDExtract-N-Amp™ Tissue PCR Kit as detailed in Bennett *et al.* (2021). From the master stock of gDNA, we set up 96 well PCR plates with 1/10 dilutions (in ddH_2O) ready for assay. The above array of PCR reactions, using the RedEx polymerase provided with the kit, are performed and run on either an agarose gel or automated gel system (*e.g.*, Qiaxcel Advanced system Qiagen, #9002123). As we described in Hunter *et al.* (2020), three *HaloTag®-Nr1d1* mice were candidates for further analysis from these assays.
2. Sequencing of the edited gDNA
Focusing on potential positive pups from the initial PCR screen, use High Fidelity polymerase, such as KOD Hot start DNA polymerase (Merck, #71086) (Bennett *et al*., 2021), to re-amplify the products using the flanking primer pair, and gel extract the larger band, which corresponds to the insert. This product can be sequenced directly using primers giving full coverage of the product. For higher quality sequencing,

one can blunt clone the amplicon using Zero Blunt PCR cloning kit (ThermoFisher, K270020), transform, and miniprep colonies to instead sequence purified plasmid DNA, using the M13F and M13R primers. Align the sequencing data against the predicted post-CRISPR genomic DNA sequence in an appropriate software (*e.g.*, Snapgene, GSL Biotech). This sequencing step is necessary to ensure sequence fidelity, especially when using lssDNA, as imperfect or illegitimate repairs can give false conclusions from the PCR (Codner *et al.*, 2018) (**Figure 2**).

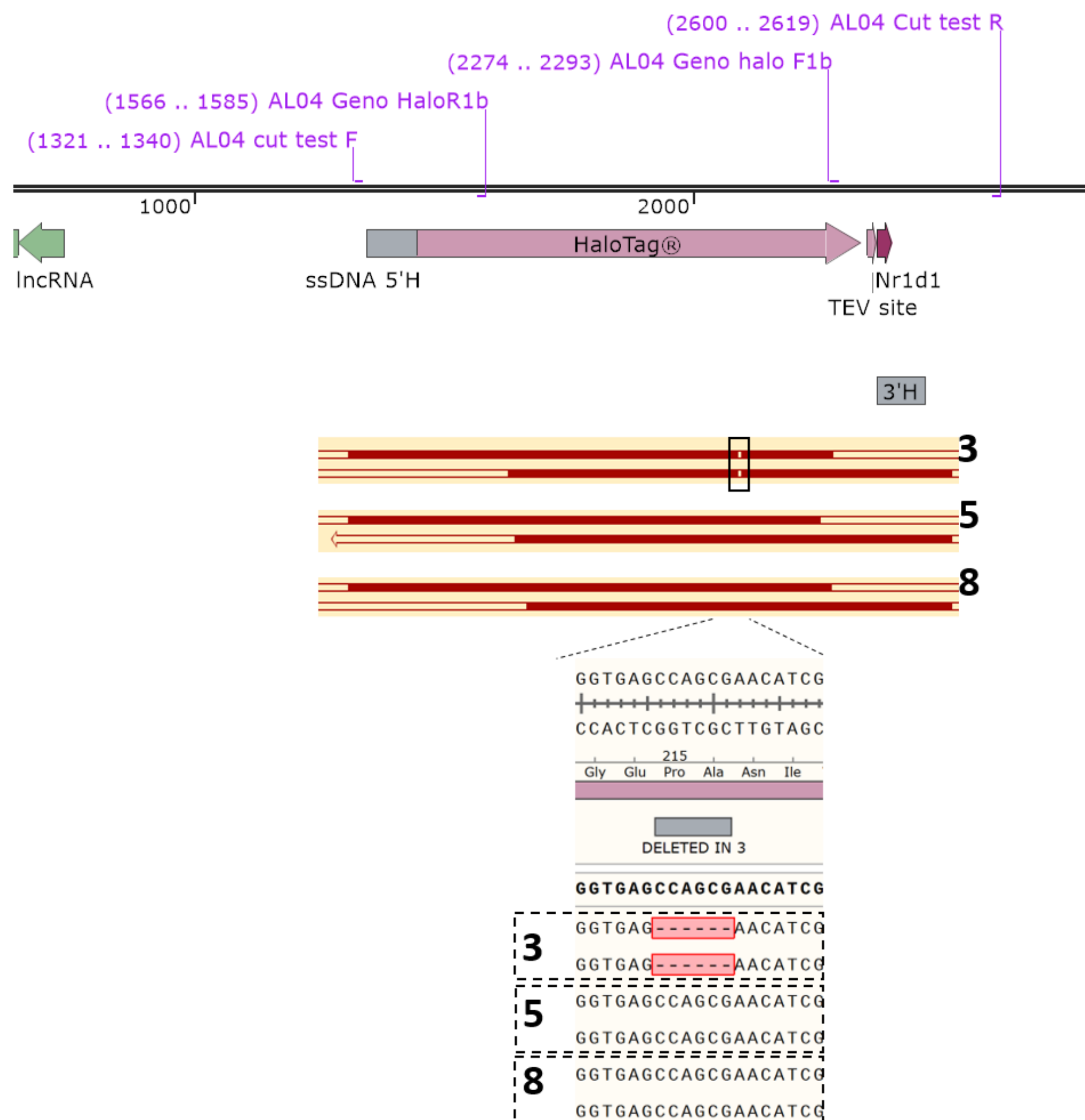


Figure 2. Genotyping and screening.
Genotyping primer design (upper panel) and alignment (filled red bars) of Sanger sequences from 3 pups with predicted knock in sequence (middle panel). Note the small non-filled section of Pup 3, which corresponds to a 6 bp loss of sequence (lower panel). lncRNA = long non-coding RNA; ssDNA = single stranded DNA; 5'H = 5' homology arm; 3'H = 3' homology arm; TEV site = Tobacco etch virus (TEV) protease cleavage site.

Table 1. Nucleotide sequences used in *HaloTag®-Nr1d1* mouse generation

Name	Sequence
sgRNA	atgtcttcaccagctgaaag-tgg
sgRNA	ctcagtacctgtgttgttat-cgg
lssDNA	ctcgtctccctcagccattgcccagggggcgagagaggccatcacaacctccagtttgtgtcaaggtcca gtttgaatgacggctttcagctggtgaagacATGGCAGAAATCGGTACTGGCTTTCC ATTCGACCCCCATTATGTGGAAGTCCTGGGCGAGCGCATGCACTA CGTCGATGTTGGTCCGCGCGATGGCACCCCTGTGCTGTTCCTGCA CGGTAACCCGACCTCCTCCTACGTGTGGCGCAACATCATCCCGCA TGTTGCACCGACCCATCGCTGCATTGCTCCAGACCTGATCGGTAT GGGCAAATCCGACAAACCAGACCTGGGTTATTTCTTCGACGACC ACGTCCGCTTCATGGATGCCTTCATCGAAGCCCTGGGTCTGGAA GAGGTCGTCCTGGTCATTCACGACTGGGGCTCCGCTCTGGGTTT CCACTGGGCCAAGCGCAATCCAGAGCGCGTCAAAGGTATTGCAT TTATGGAGTTCATCCGCCCTATCCCGACCTGGGACGAATGGCCAG AATTTGCCCGCGAGACCTTCCAGGCCTTCCGCACCACCGACGTC GGCCGCAAGCTGATCATCGATCAGAACGTTTTTATCGAGGGTACG CTGCCGATGGGTGTCGTCCGCCCGCTGACTGAAGTCGAGATGGA CCATTACCGCGAGCCGTTCCTGAATCCTGTTGACCGCGAGCCACT GTGGCGCTTCCCAAACGAGCTGCCAATCGCCGGTGAGCCAGCGA ACATCGTCGCGCTGGTCGAAGAATACATGGACTGGCTGCACCAG TCCCCTGTCCCGAAGCTGCTGTTCTGGGGCACCCCAGGCGTTCT GATCCCACCGGCCGAAGCCGCTCGCCTGGCCAAAAGCCTGCCTA ACTGCAAGGCTGTGGACATCGGCCCGGGTCTGAATCTGCTGCAA GAAGACAACCCGGACCTGATCGGCAGCGAGATCGCGCGCTGGC TGTCGACGCTCGAGATTTCCGGCGAGCCAACCACTGAGGATCTG TACTTTCAGAGCatgacgaccctggacagcaataacaacacaggtactgagattcttatctttgct ctgtcactgtctccatttccatccaaggcttatgagaggaac *Homology arms in lower case (red lower case indicates shield mutations); Halotag and TEV linker insert in upper case.*
AL04 Cut Test F primer	atcctgattgcgaactgcgg
AL04 Cut Test R primer	gttctcggactgaggggaac
AL04 Geno HaloR1b primer	GTAGGAGGAGGTCGGGTTAC
AL04 Geno Halo F1b primer	AAGACAACCCGGACCTGATC

HaloChIP

Our HaloChIP protocol is an adaptation of the Promega HaloCHIP™ manufacturer's instructions (indeed, for some steps, we recommend that the manufacturer's instructions are followed unchanged), the Active Motif High Sensitivity Chromatin Prep kit protocol (notably, the tissue fixation and chromatin preparation steps), and is indebted (for adipose tissue work) to the published methods of other groups (Haim *et al.*, 2013; Castellano-Castillo *et al.*, 2018). Reagent volumes are given for a single sample. We recommend that, if working alone, you do not try to process large numbers of samples simultaneously, as many steps are time- and temperature-sensitive. Processing 4–8 samples simultaneously is usually manageable. Also, bear in mind that ChIP DNA yields are in the picogram-nanogram range; thus, it is imperative to minimise reagent and sample contamination as much as possible. We strongly recommend using low binding plasticware to minimise sample loss (we use 1.5 mL ultra-high recovery tubes throughout the protocol).

A. Tissue collection

We have applied this protocol to both fresh and flash-frozen tissue. We have found fresh tissue to yield superior results, but have still produced sequencing-quality data from flash-frozen samples. No adjustments to the protocol are needed. We recommend that you test the protocol in both fresh and flash-frozen tissue, if possible. The starting mass required will vary between tissues. We find 100 mg of liver tissue yields sufficient chromatin for HaloChIP-seq, whilst we have needed >1,000 mg of white adipose tissue. As NR1D1 is a highly rhythmic clock protein, we collected tissue at the peak [zeitgeber time (ZT) 8 (8 hours after lights on)] and trough (ZT20) of NR1D1 recruitment to the genome, using the ZT20 samples as negative controls.

B. Fixation

We adopt a dual cross-linking approach (Papachristou *et al.*, 2018), using both disuccinimidyl glutarate (DSG) and formaldehyde fixation, to maximise yield. Before starting, ensure that you have chilled your benchtop centrifuges down to 4°C, and have made and chilled the buffers needed for washing and tissue lysis. Ensure your dounce homogeniser is cold (store at 4°C, or chill on wet ice).

1. Add 40 µL of 0.5 M DSG to 10 mL 1× PBS (to make a 2 mM DSG solution). The PBS should be at room temperature (RT) to avoid DSG precipitation. Mix well, and warm a little between your hands if precipitate appears.
2. With two No.22 scalpel blades, finely mince the tissue on a 10 cm dish kept cold on wet ice, in 10 mL of 2 mM DSG, aiming for <1 mm pieces (**Figure 3A**).
3. Using a 1-mL pipette tip (with the end of the tip cut off), or a stripette, transfer the tissue and DSG solution to a clean 15-mL tube.
4. Rotate at 10 rpm and RT for 15 min.
   Note: During this step, freshly prepare a 1% formaldehyde-PBS solution (see Recipes below).
5. Centrifuge the minced tissue suspension at 1,000 × *g* and 4°C for 3 min.
6. For most tissues, the tissue pieces will collect at the bottom of the tube. Remove the liquid layer above. For lipid-rich tissues (*e.g.*, white adipose), the tissue pieces will float on top (**Figure 3B**). In this case, we use fine glass Pasteur pipettes (to which the tissue pieces do not stick) to remove the liquid from beneath the tissue.
7. Resuspend the tissue pieces in 10 mL of 1% formaldehyde-PBS, and rotate at 10 rpm and RT for 15 min.
8. Quench the formaldehyde by adding glycine to a final concentration of 0.125 M (*e.g.*, add 0.5 mL of 2.5 M glycine solution, recipe below), or by using Stop Solution as directed in Section C of the Active Motif High Sensitivity Chromatin Prep kit manual, and rotate at 10 rpm and RT for 5 min.
9. Remove the liquid, and add 10 mL of ice-cold 1× PBS. Invert 2–3 times to mix. Centrifuge the tissue suspension at 1,000 × *g* and 4°C for 3 min. Repeat this wash step once more. Remove the liquid.

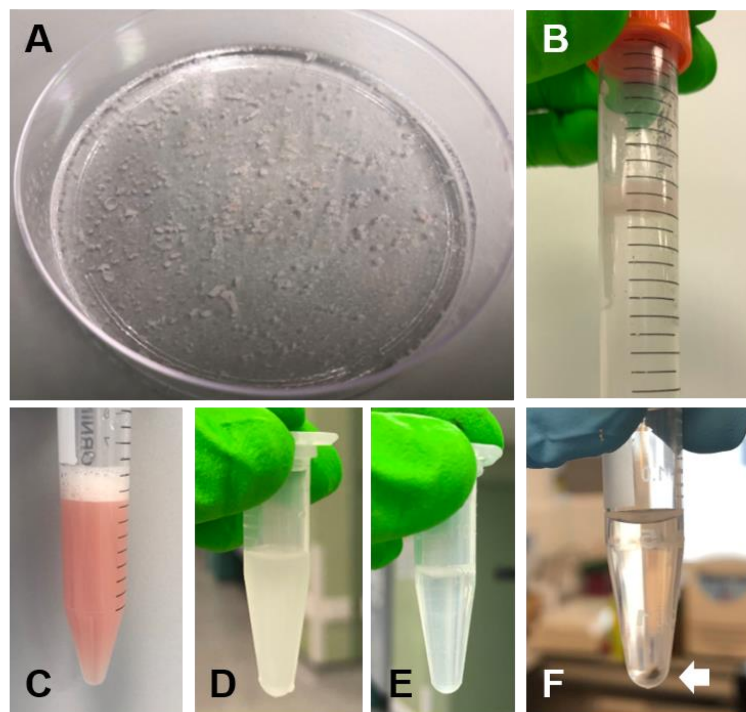


Figure 3. Tissue processing and chromatin preparation.
A. Tissue should be minced very finely, so pieces are <1 mm in size. **B.** Before homogenisation, adipose tissue pieces float on fixation/wash buffer. **C.** After homogenisation, the suspension should have a uniform appearance (this image shows liver tissue). **D.** Before sonication, the nuclei suspension is milky in appearance. **E.** After sonication, the chromatin suspension clears. **F.** After centrifugation, the suspension clears further, with a black and white pellet of debris (arrow) visible at the bottom of the tube.

C. Preparing the chromatin suspension

CRITICAL STEP! Chromatin preparation must be optimised for your tissue type, using the sonicator available to you. The sonication step of HaloChIP must be adjusted, so that the chromatin is adequately sheared, but the suspension does not reach too high a temperature (so risking denaturation of the HaloTag®).

1. Resuspend the tissue pellet in 5 mL of ice-cold lysis buffer (Active Motif Chromatin Prep Buffer, Promega Mammalian Lysis Buffer, or adipose tissue lysis buffer), with 50 µL of proteinase inhibitor cocktail (PIC) (Promega) added.
2. On ice, disrupt the tissue with a handheld homogeniser, to produce a uniform suspension (**Figure 3C**), whilst avoiding foaming and overheating.
   Note: You will need to optimise this step for your tissue type. For adipose tissue, we homogenise for 15 s with the Qiagen TissueRuptor, on setting 6 (medium-high). For liver tissue, we homogenise for 45 s.
3. Incubate on wet ice for 10 min.
4. Nuclei release is performed mechanically, using a chilled dounce homogenizer. Using a stripette, transfer your sample to the douncer, and homogenise with the tight pestle until nuclei release is evident on microscopy (free nuclei are visible as small dense bodies, separate from cell debris).
   Note: In our experience, this typically requires 30–70 strokes of the pestle, depending on tissue type. Be sure to optimise this for your samples. If you are processing multiple samples, use the same homogenisation conditions for each sample.
5. Transfer the sample to a clean 15-mL tube. Centrifuge at 4°C for 3 min to pellet nuclei. For adipose tissue, we centrifuge at 2,780 × *g*; for liver, at 1,000 × *g*. Again, this may require adjustment depending on your

sample type.

6. Carefully remove the supernatant (and overlying lipid layer, if adipose). Removing the lipid layer cleanly requires practice. We recommend using a 1 mL pipette tip with the end of the tip cut off, to produce a wide bore.
7. Resuspend the nuclei pellet in 650 µL of Mammalian Lysis Buffer (Promega HaloCHIP™ System), and transfer to a clean 1.5-mL tube.
8. Ensure the nuclei pellet is fully resuspended by pipetting (**Figure 3D**), and add 13 µL of PIC (Promega).
9. Incubate on wet ice for 15 min.
10. Proceed to sonication.
    The aim is to shear chromatin to 200–1200 bp fragments, without introducing excessive heat or foaming (**Figure 3E**). See the Active Motif High Sensitivity Chromatin Prep Kit manual for useful advice. We use a 3.5 mm probe sonicator with cooling blocks, keeping samples on wet ice between sonication rounds. We have previously found eight rounds of sonication (30 s on, and 30 s off, for a total of 2 min 'on' time per round), at 37% amplitude, to produce optimal chromatin shearing in liver and adipose tissue (**Figure 4A**). However, when tested on HEK293 cells overexpressing HaloTag®-Nr1d1, we found that these sonication conditions rendered the tag undetectable (SDS-PAGE analysis of cell lysate incubated with HaloTag® fluorescent ligand). The tag remains detectable when samples are sonicated at 20% amplitude, with some sacrifice of shearing quality (**Figure 4B**).
11. Centrifuge at top speed (*e.g.*, 17,000 × *g*) and 4°C for 3 min. The black and white pellet at the bottom of the tube represents debris (**Figure 3F**).
12. Reserve a 25-µL aliquot of the supernatant (chromatin suspension), for quantification of DNA concentration, and analysis of shearing quality (section D). Transfer the remainder of the chromatin suspension to a clean 1.5-mL tube (or divide between two or more tubes, if concentrated), and store at -80°C until the pull-down reaction.

PAUSE POINT Prepared chromatin suspension can be stored at -80°C.

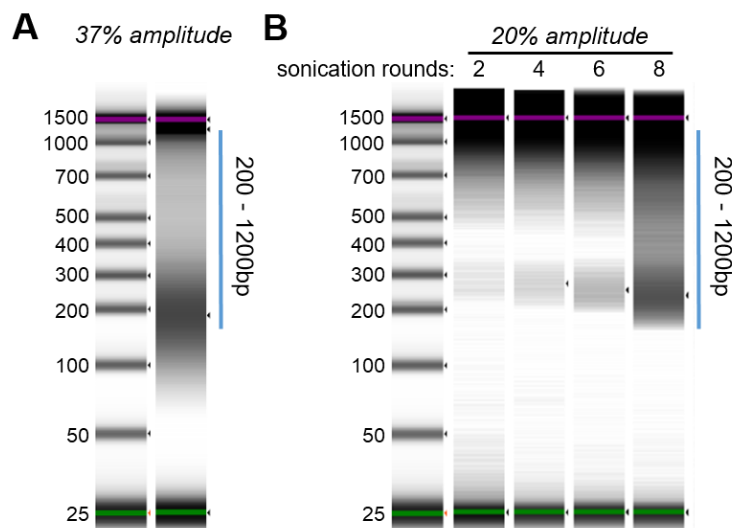


Figure 4. Chromatin shearing.
A. TapeStation image showing optimal shearing of liver chromatin following eight rounds of sonication at 37% amplitude (1 round = 30 s on, 30 s off, cycling for 2 min). **B.** TapeStation image showing chromatin shearing at 20% amplitude, after increasing rounds of sonication. Purple and green bands indicate upper and lower markers, respectively; arrowheads indicate peaks of fragment distribution.

D. Input quantification and analysis

Before proceeding to the Halo pull-down, it is necessary to quantify your chromatin and assess shearing quality, using the 25-µL aliquot of chromatin suspension reserved in the previous step.

1. Add 175 µL of TE buffer and 20 µg RNase A to the 25 µL of chromatin. Mix. Using a heat block or thermocycler, incubate at 37°C for 30 min.
2. Add 50 µg Proteinase K, and 10 µL of 5 M NaCl. Incubate at 65°C for 6 h, or overnight.
3. Add 375 µL of phenol–chloroform–isoamyl alcohol. Vortex to mix.
4. Prepare the Phase Lock tubes by centrifuging at top speed (*e.g.*, 17,000 × *g*) and RT for 1 min.
5. Transfer each sample to a Phase Lock tube, on top of the gel layer.
6. Centrifuge at top speed and RT for 2 min. Remove the aqueous (top) layer, and transfer to a clean 1.5-mL tube. Add 40 µg glycogen and 900 µL of 100% ethanol. Vortex vigorously and leave at -80°C for at least 30 min.
7. Centrifuge at top speed and 4°C for 15 min. A small white/translucent pellet should be visible at the base of the tube. Pipetting slowly, carefully remove the supernatant from the pellet. Discard the supernatant.
8. Add 500 µL of 70% ethanol, and centrifuge at top speed and 4°C for 5 min.
9. Again, carefully remove and discard the supernatant, leaving just the pellet.
10. Allow residual ethanol to evaporate from the tube for 10–15 min.
11. Resuspend the pellet in 25 µL of water for molecular biology (at room temperature).
12. Quantify DNA, by fluorometric (*e.g.*, Qubit™), or spectrophotometric (*e.g.*, Nanodrop) methods, following the manufacturer's instructions.
13. Check shearing quality, by analysing an aliquot of DNA by electrophoresis (*e.g.*, TapeStation). Adequately sheared DNA will appear as a smear of fragments 200–1,200 bp in length (**Figure 4**). If DNA fragments are under- or over-sheared (majority of fragments >1,200 bp or <200 bp in length), your sonication conditions will require adjustment.

E. HaloLink™ resin preparation

This step exactly follows the manufacturer protocol (HaloCHIP™ System Technical Manual) (section 5.A. Phase 1. Resin Equilibration). Take care when removing the supernatant from the resin, to avoid removing any of the resin (**Figure 5A–5C**). Pipette slowly, lowering your tip down the tube as the supernatant is removed.

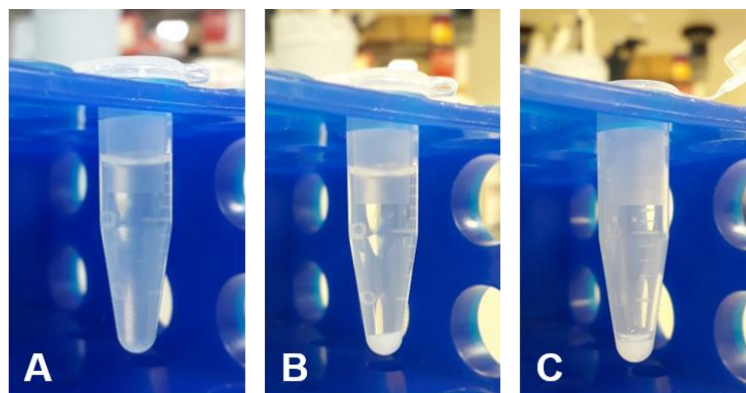


Figure 5. Handling the HaloLink resin.
A. Resin in suspension, dispersed throughout buffer. **B.** After centrifugation, resin collects at the bottom of the tube. **C.** The supernatant is removed carefully to avoid loss of resin.

F. Pull-down

1. If frozen, gently thaw your chromatin suspension on ice. Centrifuge at top speed and 4°C for 3 min.
2. We recommend that you use the same mass of chromatin across all your samples. For liver tissue, we

have found a single pull-down with 100 µg chromatin to yield sufficient DNA for sequencing; for adipose tissue (where chromatin yield is much less), we have performed multiple (*e.g.*, 3–5) pull-down reactions of 30–50 µg, and pooled DNA at the elution stage. Prepare chromatin suspension with your desired mass of chromatin, making up a total volume of 600 µL with Mammalian Lysis Buffer (Promega).

3. Add 600 µL of prepared chromatin suspension to the prepared HaloLink Resin.
4. Rotate at RT for 3 h.
5. Centrifuge at 800 × *g* and RT for 2 min, then carefully discard the supernatant.

G. Wash steps and de-crosslinking

1. Following the HaloCHIP™ System Technical Manual, wash the resin in the order described in section 5.A. Phase 3. Capture and Release of DNA, steps 6–12, including the Lithium Chloride Wash Buffer step (see recipes below), to reduce non-specific DNA binding. For each wash, mix well, spin down (800 × *g* at RT for 2 min), and then remove the supernatant. Take care to avoid pipetting up any of the resin. Note that, as the detergent is washed away in the final water steps, the resin will stick to the sides of the tube.
2. Following the next step of the HaloCHIP™ System Technical Manual, add 300 µL of Reversal Buffer, and mix as directed. If you have resin on the sides of the tube, carefully pipette the Reversal Buffer down the side,s to ensure all the resin is mixed with the buffer.
3. Incubate at 65°C for 6–16 h (this is the cross-link reversal step—the heat and the high salt content of the Reversal Buffer serve to reverse the protein-DNA cross-links).

H. DNA elution

1. Centrifuge resin and Reversal Buffer at 800 × *g* and RT for 2 min.
2. Carefully remove the supernatant and split between two clean 1.5-mL tubes (*i.e.*, 150 µL in each) (**Figure 6A**).
3. Clean up the DNA with the Zymo ChIP DNA Clean & Concentrator kit, following the manufacturer's instructions. As the ChIP DNA Binding Buffer is added to the sample in a volume ratio of 5:1 (*i.e.*, 750 µL added to 150 µL supernatant), the sample is split between two 1.5-mL tubes, but can be recombined at the column step (*i.e.*, running each aliquot successively through one column) (**Figure 6B**). At the elution step, perform two successive elutions, with 11 µL of buffer each time (final volume ~20 µL).
4. You now have your purified ChIP DNA, which can be taken forward for further analysis. Use 1 µL for quantification (see section K), and set aside an aliquot of 1–2 µL for the PCR enrichment check.
5. ****PAUSE POINT**** Purified ChIP DNA can be stored at -20°C.

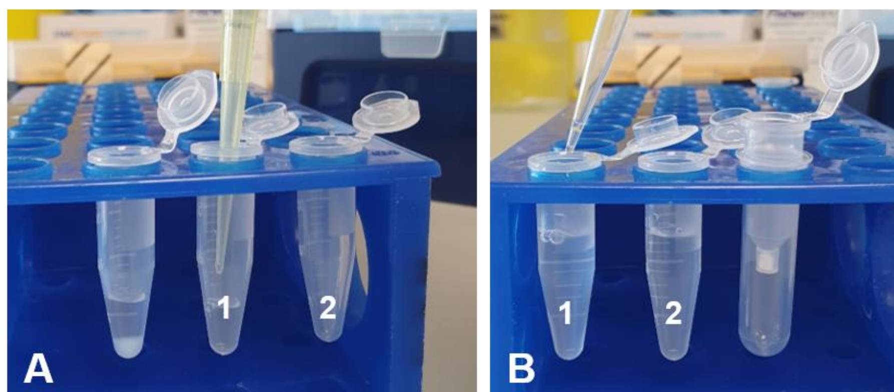


Figure 6. DNA elution.

A. The de-crosslinked supernatant (containing the ChIP DNA) is removed from the resin, and divided between two clean tubes (1 and 2). **B.** After adding Binding Buffer, the contents of tubes 1 and 2 can be run through the DNA column successively, so that all the ChIP DNA is collected with one column.

I. Quality control—PCR enrichment check

CRITICAL STEP! To avoid sequencing poor quality samples, we strongly recommend that you check that each sample shows expected enrichment of one or more known TF binding sites, compared to one or more unbound regions. We use droplet digital PCR (ddPCR) to do this, because of its high sensitivity (Hunter *et al.*, 2019); qPCR is an alternative.

1. Dilute a small volume (*e.g.*, 1–2 µL) ChIP DNA 1:10 in nuclease-free water.
2. Perform ddPCR or qPCR with primer sets directed to one or more known (or expected) sites of TF binding, and one or more sites where the TF is not expected to bind (*e.g.*, a gene desert). Use 2 µL of diluted ChIP DNA per PCR reaction. 'Positive control' and 'Negative control' ChIP primer sets are commercially available (*e.g.,* from Active Motif). Signal from the unbound site(s) reflects non-specific DNA signal ('background') in your ChIP DNA. Primer efficiency can be compared, by quantifying signal in input DNA (diluted at least 1:10) (**Figure 7A**).
3. Calculate enrichment of the positive over negative signal(s), by simply dividing the positive signal by the negative signal. For sequencing, we typically take forward samples where enrichment is at least 5-fold (for a robust TF binding site, much higher enrichment can be seen in good quality samples).
4. Poor enrichment may be seen in samples where the HaloTag® protein is not expressed (*e.g.*, wildtype or negative controls) (**Figure 7B**), or in instances where the ChIP has failed.

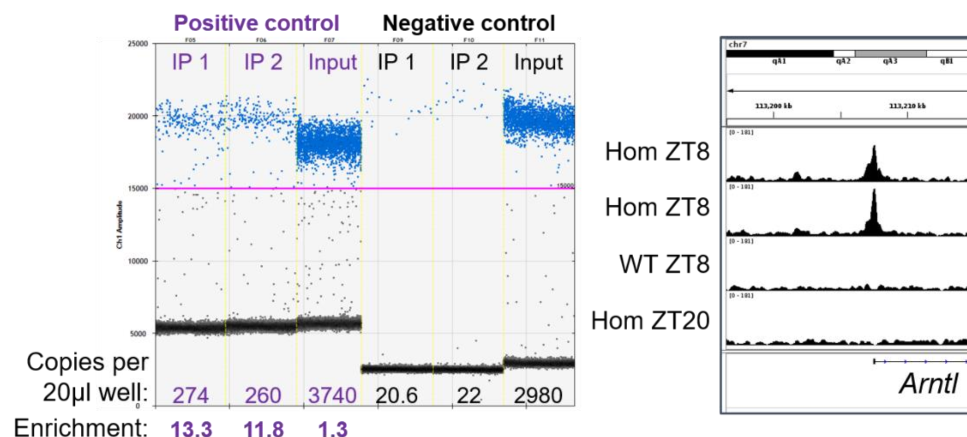


Figure 7. HaloChIP results.
A. ddPCR quantification (copies per 20 µL well) of a positive control region (*Arntl* promoter) and a negative control region (gene desert) in two HaloChIP samples (IP1, and IP2), and in input DNA. Enrichment of the positive region over the negative region is calculated by dividing the positive signal by the negative signal. Positive and negative signals should be comparable in the input DNA. **B.** Visualised ChIP-seq signal at the *Arntl* promoter in mouse adipose tissue in animals homozygous (Hom) wildtype (WT) for *HaloTag®-Nr1d1* at zeitgeber time (ZT) 8, the zenith of NR1D1 recruitment to the genome, or ZT20, the nadir.

J. Quality control – DNA quantification

1. Measure the concentration of your ChIP DNA, using 1 µL of your sample, following the manufacturer's instructions [*e.g.*, for the Qubit™ dsDNA HS (high sensitivity) kit].
2. Calculate the total mass of ChIP DNA. Our sequencing core uses the Illumina TruSeq ChIP Libary Preparation Kit, which is designed for 5–10 ng ChIP DNA in a 50-µL volume.

K. ChIP-seq—library preparation and sequencing

1. Liaise with your sequencing core or service provider regarding library preparation and sequencing. We recommend that this is done prior to starting any experiment. They will also be able to advise on expected number of reads that their sequencing platform should produce, and make recommendations about read depth. The ENCODE (Encyclopedia of DNA Elements) guidelines (https://www.encodeproject.org/chip-seq/transcription_factor/) are also helpful here.

Data analysis

A detailed overview of ChIP-seq data analysis is beyond the scope of this protocol, but broadly speaking, ChIP-seq data is processed and analysed along the following lines:

1. Raw reads are trimmed, filtered, and aligned to a reference genome. Duplicate reads are marked and (usually) removed.
2. The genomic locations of transcription factor binding sites are called by identifying peaks of signal in the aligned reads. A negative control sample may be used as the 'background' against which peaks in the experimental sample(s) are called. Alternatively, sites where transcription factor binding differs between conditions may be identified by comparing ChIP-seq data from two or more conditions (differential binding analysis).
3. The properties of transcription factor binding sites are examined. For example, the underlying DNA sequences may be studied to look for enrichment of certain transcription factor binding motifs, or the proximity of sites to other genomic features may be examined.

A comprehensive overview of ChIP-seq data analysis has been published recently (Nakato and Sakata, 2021); readers may find Figure 1 of this review useful for illustrating the data analysis process. We outline our approach in the Methods sections of our publications (Hunter *et al.*, 2020, 2021).

Recipes

1. 1× PBS

Reagent	Final concentration	Amount
10× PBS	1×	10 mL
Water for molecular biology	n/a	90 mL
Total	**n/a**	**100 mL**

2. 1% formaldehyde-PBS solution

Reagent	Final concentration	Amount
10× PBS	1×	5 mL
Formaldehyde solution 36.5–38% in H_2O	1%	1.37 mL
Water for molecular biology	n/a	43.63 mL
Total	**n/a**	**50 mL**

3. 2.5 M glycine solution

Reagent	Final concentration	Amount
Glycine	2.5 M	9.38 g
Water for molecular biology	n/a	50 mL
Total	**n/a**	**50 mL**

4. **Adipose tissue lysis buffer (make fresh)**

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 8.0)	10 mM	1 mL
5 M NaCl	140 mM	2.8 mL
0.5M EDTA	5 mM	1 mL
10% Nonidet® P40 (Substitute)*	1%	10 mL
Water for molecular biology	n/a	85.2 mL
Total	**n/a**	**100 mL**

*Like most detergents, NP-40 is viscous. Make a fresh 10% solution first, to allow for more accurate pipetting, especially when working with small volumes.

5. **1 M lithium chloride (LiCl) solution**

Reagent	Final concentration	Amount
Lithium chloride, anhydrous	1 M	4.239 g
Water for molecular biology	n/a	100 mL
Total	**n/a**	**100 mL**

6. **Lithium chloride wash buffer (Promega recipe) (make fresh)**

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 8.0)	100 mM	1 mL
1 M LiCl	500 mM	5 mL
10% IGEPAL® CA-630*	1%	1 mL
10% sodium deoxycholate	1%	1 mL
Water for molecular biology	n/a	2 mL
Total	**n/a**	**10 mL**

*As above, we recommend that you make a fresh 10% solution first, to allow for more accurate pipetting, especially when working with small volumes.

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This protocol was derived from two original research papers (Hunter *et al.*, 2020, 2021), the Promega HaloCHIP™ manufacturer's instructions, and the Active Motif High Sensitivity Chromatin Prep kit protocol, with adaptations for adipose tissue work informed by the publications of other groups (Haim *et al.*, 2013; Castellano-Castillo *et al.*, 2018).
We acknowledge the core facilities at the University of Manchester: the Genome Editing Unit, the Genomic

Technologies Core Facility, and the Biological Services Unit.

Competing interests

ALH and TMP have received free merchandise from Active Motif. The other authors have no competing interests to declare.

Ethics

All animal procedures described in this protocol were approved by the University of Manchester Animal Welfare and Ethical Review Body and carried out under licence, according to the UK Animals (Scientific Procedures) Act 1986.

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