Imprint® Chromatin Immunoprecipitation Kit (CHP1) Protocol

Product Description

Protein-DNA interactions play critical roles in cellular functions including signal transduction, gene transcription, chromosome segregation, DNA replication/recombination, and epigenetic regulation. The Chromatin Immunoprecipitation (ChIP)[20] technology provides a rapid and reliable method to investigate these protein-DNA interactions *in vivo*.

Our Imprint Chromatin Immunoprecipitation Kit uses a plate-based system to allow rapid ChIP assays in a high-throughput format. The plate method limits the sensitivity of the process, and as such this kit is best used to characterize abundant protein-DNA events, e.g., histone or RNA polymerase II events. The protocol is optimized for chromatin isolated from 100,000-250,000 cells/ChIP reaction/well. Using more cells per well may increase non-specific background binding and reduce target enrichment.

The Imprint ChIP procedure is ideal for kinetic or drug screening studies of abundant, known protein-DNA interactions.[13, 14] It utilizes an abbreviated method starting with cross-linking of mammalian cells or tissue followed by chromatin extraction. The chromatin is then sheared and captured in antibody-coated microwells, the cross-links are reversed, and the DNA is purified by spin column cleanup.

The **CHP1** kit provides reagents for a one-day immunoprecipitation and purification of DNA from mammalian cells or tissue, faster than any other commercially available kit. The kit includes mouse IgG, anti-RNA Polymerase II, and human GAPDH primers for use as negative and positive controls. The resultant DNA is suitable for downstream applications ranging from individual target characterization to genome-wide profiling techniques. Additionally, an optimized GenomePlex Whole Genome Amplification (WGA) method is available for amplification of ChIP DNA.[15, 16, 18]

Kit Components

Reagent Description	Catalog Number	24 Rxn	96 Rxn
IP Wash Buffer	W4767	30 ml	120 ml
Antibody Buffer	A4356	17.5 ml	70 ml
Nuclei Preparation Buffer	N2663	6 ml	24 ml
Homogenization Buffer	H8039	10 ml	40 ml
Shearing Buffer	S3697	3 ml	12 ml
Dilution Buffer	D8943	3 ml	11 ml
Reversing Solution	R1655	1.2ml	4.5ml
DNA Release Buffer	D9068	1.2 ml	4.5 ml
Column Preparation Solution	**C2112**	15 ml	60 ml
Binding Solution	B7556	11 ml	50 ml
Wash Solution Concentrate	W3637	3 ml	12 ml
Elution Solution	E7777	1.5 ml	8 ml
GenElute™ Binding Column G	C6863	25 each	100 each
Collection Tube, 2 ml	T7813 or T5449	50 each	200 each
Protease Inhibitor Cocktail	**P8340**	0.05 ml	125 µL
Normal Mouse IgG (1 mg/ml)	M8695	15 µL	50 µL
Anti-RNA Polymerase II (1 mg/ml)	R1530	10 µL	40 µL
Proteinase K (>800 U/ml)	**P4850**	50 µL	125 µL
Forward Control Primer (GAPDH) (20 mM)	F3430	25 µL	100 µL
Reverse Control Primer (GAPDH) (20 mM)	R2280	25 µL	100 µL
Assay Stripwells	S6447	3 each	12 each
8-well Stripcaps	S6572	3 each	12 each

Storage/Stability

- Store the antibodies, protease inhibitor cocktail and primers at –20 °C.
- Store the assay Stripwells and Proteinase K at 2–8 °C.
- All other components may be stored at room

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- Purification
- Recombination
- Sample preparations
- Size-exclusion chromatography
- Sonication
- Transcription
- Transduction
- Whole genome amplification

Workflow

Preparation Instructions

Optimize shearing conditions for your sample:
The equipment and sample type used in preparing the sheared chromatin can have a significant impact on the success of the experiment. The **CHP1** kit was conceived for known protein-DNA events, and so a limited amount of extra reagents have been included for optimization. Additional Nuclei Preparation Buffer, Homogenization Buffer, and Shearing Buffer are provided for approximately 4 additional sample optimizations. See Troubleshooting Guide for more information.

Sonication has to be optimized for each cell line and the instrument. We recommend the Diagenode Biodisruptor (water based sonication) for reproducible sonication. A good starting point is 5, 10, and 15 minutes at High “H” setting with 30 seconds “ON” and 30 seconds “OFF” cycle.

Run a gel to check sonication:
i) Use 10 µL sample and add 40 µL H_2O
ii) Reverse cross-link by adding 2 µL of 5 M NaCl (Final concentration 0.2 M NaCl)
iii) Boil for 15 minutes
iv) After returning to room temperature, add 1 µL of 10 mg/ml RNase A at 37 °C for 10 mins
v) Clean and purify DNA with GenElute PCR Clean-Up Kit, Catalog Number NA1020
vi) Load 1 and 4 µL of sonicated DNA on gel and determine size of smear
vii) The sonication condition that gives a smear of DNA sizes from 200 bp to 1 kb with a peak around 500 bp (2-3 nucleosomes) should be used for ChIP reactions.

Thoroughly mix reagents. Examine the reagents for precipitation. If any reagent has formed a precipitate, warm at 55 °C until dissolved. Allow to equilibrate to room temperature before use.

Wash Solution: Dilute the Wash Solution Concentrate with 12 ml (24 Rxn) or 44 ml (96 Rxn) of ethanol, 200 proof, for molecular biology. After each use, tightly cap the diluted Wash Solution to prevent the evaporation of ethanol.

1.25 M Glycine: Prepare a 1.25 M glycine stock solution. Store at 2-8 °C when not in use. Remake fresh stock after 6 months.

65 °C incubator: Preheat a waterbath, incubator, or hybridization oven to 65 °C.

Procedure

I. Bind antibody to the assay wells

1. Determine the number of Stripwells required and leave these in the frame. Transfer unused Stripwells back into the bag, seal, and store at 2-8 °C. Wash Stripwells once with 150 µL of Antibody Buffer.
   Note: For highest sensitivity and lowest background, all incubations and washes should be allowed to proceed for the maximum value of the recommended time ranges. With some primary antibodies, background can be decreased by substituting Tris Buffered Saline, with BSA, pH 8.0, Catalog Number **T6789** (not supplied) for Antibody Buffer (A4356) throughout the protocol. This can result in improved sensitivity in some circumstances.
2. Add 100 µL of Antibody Buffer to each well. Then, add 1 µg of antibodies: 1 µL of Normal Mouse IgG as the negative control, 1 µL of Anti-RNA Polymerase II as the positive control, and 1-4 µg of each antibody of interest (up to 10 µL of antibody).
   Note: This protocol generally suggests the use of 1 µg of antibody per well. However, when working with antibodies that have not been validated for use in ChIP, one may need to optimize the amount of antibody used. Some antibodies may never be amenable to ChIP as the cross-linking may adversely affect the interaction of the Fab with its epitope.
3. Cover the Stripwells with Parafilm or an adhesive plate sealer and shake at 50-100 RPM on an orbital shaker for 60-90 min at room temperature. Meanwhile, prepare the sample extracts as described in the next steps. (II.A for cell culture or II.B for tissue).

II.A. Cell culture sample preparation

1. Between 1-25 x 10^4 cells are recommended for each ChIP sample assay well, e.g., for 8 ChIP assay wells with 2 x 10^5 cells per assay, 1.6 x 10^6 cells should be prepared.
   For monolayer or adherent cells:
   Briefly trypsinize the cells and collect into a 15 ml conical tube. Alternatively, fix the cells directly in the flask using the cross-link solution described in step 3 below. Quench with glycine as described in step 4 below. Use a cell scraper to collect your cells on ice. Count the cells using a hemacytometer.
   For suspension cells:
   Collect cells into a 15 ml conical tube. Count cells using a hemacytometer.
2. Centrifuge the cells at ~180 x *g* for 5 min, and discard the supernatant. Wash the cells once with 10 ml of PBS by centrifugation.
3. Resuspend the cells in 9 ml fresh culture medium. Add 270 µL of 37% formaldehyde to the sample, inverting the tube several times immediately after formaldehyde addition. The final cross-linking solution is 1% formaldeheyde. Incubate for 10 min at room temperature on a rocking platform.
4. Quench by adding 1 ml of 1.25 M glycine for every 9 ml cross-linking solution, mix and centrifuge at ~180 x *g* for 5 min. Remove medium and wash cells three times with 10 ml ice-cold PBS by centrifugation.
5. Add Nuclei Preparation Buffer to resuspend the cell pellet (200 µL/10^6 cells, 50 µL minimum for adherent and 100 µL/10^6 cells for suspension). Transfer cell suspension to a 1.5 ml vial and incubate on ice for 10 min. Vortex vigorously for 10 sec and centrifuge at ~180 x *g* for 5 min.
6. Carefully remove the supernatant and resuspend the nuclear pellet in Shearing Buffer (100 µL/106 cells) containing Protease Inhibitor Cocktail (PIC)
   (10 µL PIC/1 ml Shearing Buffer). 50 µL minimum and 500 µL maximum per vial to be sonicated. Incubate the sample on ice for 10 min, vortexing occasionally.
7. Shear DNA by sonication on ice. Sonication must be optimized based on the specific cells, volume and equipment used. Additional Shearing Buffer is supplied for ~4 samples in optimization. Analyze 5 µL of reverse cross-linked DNA by standard agarose electrophoresis. Sheared DNA should be approximately 200-1000 bp. See Troubleshooting Guide for additional information.
8. Pellet cell debris by centrifuging at 14,000 rpm for 10 min.
9. Transfer clarified supernatant to a 1.5 ml vial Supernatant can be stored at –70 °C at this step. Proceed with Protein/DNA Immunoprecipitation section III.

II.B. Tissue Sample Preparation

1. Put the tissue sample into a 60 mm or 100 mm plate. Remove unwanted tissue such as fat and necrotic material from the sample. Weigh the sample and dice into smallest pieces possible with a clean scalpel or razor blade. Use 10-20 mg of tissue for each assay well (ChIP for RNA Pol II is successful down to 1 mg/well).
2. Transfer tissue pieces to a 15 ml conical tube. Prepare cross-link solution by adding formaldehyde to culture medium (270 µL of 37% per 9 ml, final concentration of 1%). Add 1 ml of cross-link solution per every 40 mg tissue. Incubate at room temperature on a rocking platform for 15-20 min.
3. Add 1 ml of 1.25 M glycine per 9 ml cross-link solution, mix and centrifuge at ~220 x *g* for 5 min. Discard the supernatant. Wash tissue with 10 ml of ice-cold PBS 3X by centrifugation at ~220 x *g* for 5 min. Discard the supernatant.
4. Transfer tissue pieces to a Dounce homogenizer. Add 1 ml Homogenization Buffer per every 200 mg tissue and disaggregate tissue pieces with 10-20 strokes.
5. Transfer homogenized mixture to a 1.5 ml tube (500 µL maximum for each vial) and centrifuge at ~960 x *g* for 5 min at 4 °C. Remove supernatant.
6. Add Shearing Buffer containing Protease Inhibitor Cocktail (PIC) using 100 µL/20 mg tissue. (10 µL PIC/1 ml Shearing Buffer). Incubate 10 min on ice, vortexing occasionally.
7. Shear DNA by sonication. Sonication conditions must be optimized based on the specific tissue and equipment used. Additional Shearing Buffer is supplied for ~4 samples in optimization. 5 µL of sonicated lysate may be removed, reverse cross-linked and purified for agarose gel electrophoresis. Sheared DNA should be ~200-1000 bp. See Troubleshooting Guide.
8. Pellet cell debris by centrifuging at 21,000 x *g* for 10 min at 4 °C.
9. Transfer clarified supernatant to a 1.5 ml vial Supernatant can be stored at –70 °C at this step. Proceed with Protein/DNA Immunoprecipitation, Section III.

III. Protein/DNA Immunoprecipitation

1. Dilute the clarified supernatant from step 9 above 1:1 with Dilution Buffer, i.e. 50 µL sonicated chromatin + 50 µL Dilution buffer.
2. Remove 5 µL of diluted supernatant and set this aside on ice as Input (5%) control vial.
3. Remove the incubated antibody solution.
4. Wash the wells 3 times with 150 µL of Antibody Buffer by pipetting up and down. Add 100 µL of diluted supernatant (or desired amount in a final volume of 100 µL) to each well. Cover the wells with Parafilm or plate sealer and incubate at room temperature for 60-90 min on an orbital shaker (50-100 rpm).
5. Remove supernatant.
6. Wash the wells 6 times with 150 µL of IP Wash Buffer, allowing 2 min for each wash. For optimal results, remove these washes by inverting wells and striking onto an absorbent material.
7. Wash the wells once with 150 µL of 1x Tris-EDTA Buffer. Proceed with cross-link reversal Section IV

IV. Cross-Link Reversal

1. Add 40 µL of DNA Release Buffer containing Proteinase K to the input vial and each sample. well (1 µL Proteinase K/40 µL DNA Release Buffer). Mix by pipetting.
2. Cover the sample wells with Stripcaps. Incubate the wells and input vial at 65 °C in a waterbath or hybridization oven for 15 min.
3. Add 40 µL of Reversing Solution to the samples (input vial also). Mix, recover, and incubate at 65 °C for 90 min. Proceed with DNA purification Section V

V. DNA Purification

1. Place a GenElute Binding Column G in a Collection Tube. Equilibrate column by adding 500 µL of Column Preparation Solution. Centrifuge the column for 1 min at ~12,000 x *g* and remove the flow through. All subsequent centrifugations should be ≥12,000 x *g*.
2. In a separate vessel, add 400 µL of Binding Solution and the ChIP lysate, and briefly vortex. Transfer the mixed solution to an equilibrated binding column. Centrifuge for 1 min and remove the flow through.
3. Add 500 µL of diluted Wash Solution Concentrate to each spin column. Spin for 1 min and remove the flowthrough. Centrifuge the column(s) for an additional 2 min to thoroughly dry the column.
4. Transfer the spin column to a new collection tube. Add 15-50 µL Elution Solution directly to the membrane in the column; incubate for at least 1 minute.
5. Centrifuge 1 minute. For complete recovery, transfer the eluate back to the column, and spin down again. The DNA is now ready for downstream applications or storage at –20 °C.
   Note: If you are planning to amplify your sample with using the available GenomePlex methodology, a 15-20 µL elution is recommended to maximize the eluate concentration.

Analysis

Quantification of ChIP DNA

The amount of template pulled down from a ChIP reaction is dependent on many factors: number and type of cells, treatment, template length due to sonication, etc. Often the concentration of the sample cannot be accurately quantified via a simple spectrophotometric (A_{260}) reading. The following two methods are suggested as possibilities to this minor technical dilemma.

1. Fluorometric Quantitation:
   The Quant-iT™ dsDNA Assay Kit, High Sensitivity kit (Invitrogen) using a standard curve of 0.2-10 ng per well provides accurate quantitation for most ChIP samples. However, some targets may still be too dilute to be accurately quantified via this method.
2. Quantitative PCR (qPCR):
   This method requires knowledge of a genomic location that interacts with, and a locus that is completely disassociated with, the targeted immunoprecipitated protein. Using specific primers to these two loci, relative and/or absolute (standard curve with known amounts of sonicated gDNA) quantitation can be achieved by comparing cycle threshold (Ct) values for both primers across samples.

PCR Conditions (provided primer set)

If conventional PCR or qPCR is performed, control human GAPDH primers included in the kit can be used as a positive control for human source material
(246 bp amplicon). If using SYBR® Green JumpStart Taq ReadyMix™, Catalog Number **S4438**, use these cycling parameters:

hGAPDH For caattccccatctcagtcgt
hGAPDH Rev tagtagccgggccctacttt

1 cycle	95 °C	2 min
40 cycles	95 °C	30 sec
	58 °C	30 sec
	72 °C	30 sec

Other sources of ChIP material will require control primers designed by the user. The number of cycles for conventional PCR will also need to be optimized by the user.

Amplification (Optional)

After ChIP, only a few nanograms of template are available for downstream use. Amplification of this template expands the number, and scope of genome-wide analyses.

GenomePlex Whole Genome Amplification (WGA) has proven to be an effective method to amplify ChIP DNA.[15, 16, 18] The kit utilizes a proprietary amplification technology based upon initial nicking of genomic DNA to relax the template for the subsequent hybridization of a unique dual-function primer. One function of the primer is to representatively anneal roughly every 500 bp throughout the entire genome. After annealing, the adjacent template is extended to create PCR amplifiable OmniPlex® fragments flanked by universal priming sites, creating a library, which represents the entire genome. The OmniPlex library is then PCR amplified using universal oligonucleotide primers complementary to the initial primer sequence; hence, the second function of the WGA primer.

The GenomePlex Complete Whole Genome Amplification (WGA) Kit is recommended[18] for amplification of immunoprecipitated DNA with the following modifications.

1. Add 10 µL of your ChIP eluate to 1 µL of the Fragmentation Buffer, Catalog Number F4304, but skip the 4 min 95 °C heat. Continue on to Library Preparation.
2. Add 6 additional cycles during the amplification for a total of 20 cycles of 94 °C for 15 sec and 65 °C for 5 min.
3. If the total WGA yield, after purification, is below 3 µg, take 10 µL of your purified WGA product and reamplify using the GenomePlex WGA Reamplification Kit, catalog number **WGA3**.[15]

Troubleshooting Guide

Observation	Cause	Recommended Solution
Little or no PCR product	Insufficient cell number or tissue amounts.	Amplify ChIP'ed DNA (**WGA2**/**WGA4** kits) before q-PCR or use an alternate larger scale bead based ChIP assay.
	Suboptimal cross-linking	Titrate the length of time cross-linking is performed from 5-20 min.
	Insufficient cell or tissue lysis	Check lysis efficiency by observing a 5 µL aliquot of the lysate under a microscope.

	Sub optimal sonication	Optimize sonication conditions by varying number and length of sonication bursts. Avoid foaming and keep sample on ice during sonication. Large volumes, and/or too much sample in one tube, yields inefficient DNA fragmentation.
	Antibody does not bind protein of interest	Check if the subclass or isotype of the antibody is correct. Choose an antibody that is qualified for use in ChIP. Antibodies recognizing native protein conformations have the highest chance for success, i.e., immunoprecipitation qualified.
	Incorrect temperature or insufficient DNA release and cross-link reversal	Longer or shorter times used in cross-linking may require different incubation times for the DNA release and cross-link reversal steps.
	Incorrect PCR conditions	Optimize PCR conditions using purified genomic DNA from a like sample.
	Poor primer design	Ensure the primers are specific to the desired target sequence
	DNA eluate contains ethanol	Make sure the spin column is thoroughly dried after the wash step during DNA purification before adding Elution Solution.
	Harsh sonication conditions	Sonication must be optimized for appropriate shearing conditions. Sonicating in warm conditions, for too long, or at too high a setting may disrupt the protein-DNA complex. Always sonicate on ice, at the lowest setting and the shortest amount of time possible that still yields appropriately fragmented DNA (~200-1000 bp)
Little or no amplification difference between positive and negative controls	Insufficient wash at each step	Follow the protocol to ensure sufficient removal of nonspecific DNA-protein complexes.
	Too many PCR cycles	Titrate the number of PCR cycles by removing a small amount of the reaction approximately every 3 cycles and analyze by gel electrophoresis. Cycle numbers still in the exponential phase will yield the best results.
	High non-specific DNA binding	Follow the protocol for “highest sensitivity and lowest background” as described in Protocol I., Step 1, Note, using Tris Buffered Saline, with BSA, pH 8.0 (T6789, not supplied).
	Protein-DNA target is too rare for detection.	This kit is designed for abundant events that can be detected using 10^4-10^5 cells. Rare events usually need more capture for detection; in such cases a bead-based system is recommended.

Frequently asked questions

Can the number of cells be increased? What is the maximum and minimum number of cells that can be used with this kit?

The range of cells that can be used with this kit is 0.2–2.5 x 10^5/well/ChIP sample. Using more than 0.5 million cells per well will increase the non-specific binding and reduce the yield and specificity of the ChIP reaction. If higher yield of ChIP DNA is desired for downstream applications (e.g. ChIP-Seq., ChIP-chip): a) DNA could be pooled by eluting consecutively (with 50 µl of elution buffer) from multiple individual ChIP reactions; or b) DNA may be amplified using one of our Whole Genome Amplification kits, Catalog Numbers **WGA2** or **WGA4**.

Is the kit compatible with lysing enzymes if sonication does not work? Yes, sheared chromatin can also be made by enzymatic (Micrococcal nuclease, MNase) digestion (reagents/protocol not provided in kit). The amount, temperature and duration of the MNase treatment will have to be optimized by the user depending on the cell line.

Can this kit be used with plants? Reptilian cells?

Yes, the kit is compatible with sonicated chromatin prepared from plants or reptilian cells, provided the user has optimized conditions for cross-linking and preparation of appropriately sized sonicated chromatin.

How is the qPCR data analyzed post ChIP analysis?
How is “% Input” and “Fold Enrichment” calculated?

ChIP-qPCR Data Analysis (ΔΔCt method)[17,19]

i. Normalize each ChIP DNA fractions’ Ct value to the Input DNA fraction Ct value for the same qPCR Assay (ΔCt) to account for chromatin sample preparation differences.

ΔCt [normalized ChIP] = (Ct [ChIP] - (Ct [Input] - Log_2 (Input Dilution Factor)))
Where, Input Dilution Factor = (fraction of the input chromatin saved)$^{-1}$ The default Input fraction is 1% which is a dilution factor of 100 or 6.644 cycles (i.e. log_2 of 100). Thus, subtract 6.644 from the Ct value of the 1% Input sample as mentioned in the equation above.

Average normalized ChIP Ct values for replicate samples.

ii. Calculate the % Input for each ChIP fraction (linear conversion of the normalized ChIP ΔCt).

% Input = $2^{(-\Delta Ct\ [normalized\ ChIP])}$

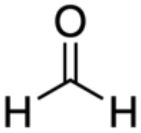
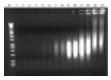
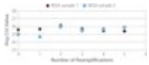
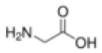
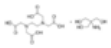
iii. Adjust the normalized ChIP fraction Ct value for the normalized background [non-specific (NS) Ab] fraction Ct value (first ΔΔCt).

ΔΔCt [ChIP/NS] = ΔCt [normalized ChIP] - ΔCt [normalized NS]

iv. Calculate Assay Site IP Fold Enrichment above the sample specific background (linear conversion of the first ΔΔCt).

Fold Enrichment = $2^{(-\Delta\Delta Ct\ [ChIP/NIS])}$

Materials

Product #	Image	Description	Molecular Formula	Add to Cart
F8775		Formaldehyde solution for molecular biology, 36.5-38% in H_2O	CH_2O	pricing
WGA2		GenomePlex® Complete Whole Genome Amplification (WGA) Kit Optimized kit with enzyme for amplifying a variety of DNA including FFPE tissue		pricing
WGA3		GenomePlex® WGA Reamplification Kit Reamplification of WGA product with minimal bias		pricing
WGA1		GenomePlex® Whole Genome Amplification (WGA) Kit Kit for whole genome amplification from a variety of DNA sources including FFPE tissue		pricing
G7403		Glycine BioXtra, ≥99% (titration)	$C_2H_5NO_2$	pricing
CHP1		Imprint® Chromatin Immunoprecipitation Kit Complete ChIP reaction in 6 hours in flexible strip well format		pricing
P5493		Phosphate buffered saline 10× concentrate, BioPerformance Certified, suitable for cell culture		pricing
SEQXE		SeqPlex DNA Amplification Kit For use with high throughput sequencing technologies, Whole Genome Amplification kit designed to facilitate Next Gen Sequencing.		pricing
T6789		Tris Buffered Saline, with BSA, pH 8.0 powder		pricing
T9285		Tris-EDTA buffer solution 100X concentrate for Northern and Southern blotting		pricing

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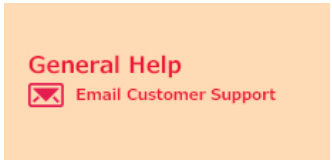
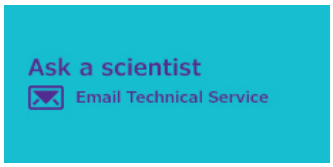
Poster & Application Note

Rapid and easy method for Chromatin IP (ChIP) analysis of metastatic cells (PDF 0.5 MB)

ChIP Protocol To Rapidly Characterize DNA-Protein Interactions





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