



ChIP-IT FFPE II

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Overview

Chromatin Immunoprecipitation (ChIP) is a powerful tool for studying protein/DNA interactions, including transcription factors, co-regulatory proteins, modified histones, chromatin-modifying enzymes and polymerases because it enables identification of the localization of proteins bound to specific DNA loci. When used in combination with whole-genome analysis such as ChIP-Seq, insights are possible into gene regulation, gene expression, mechanisms of chromatin modification and pathway analysis.

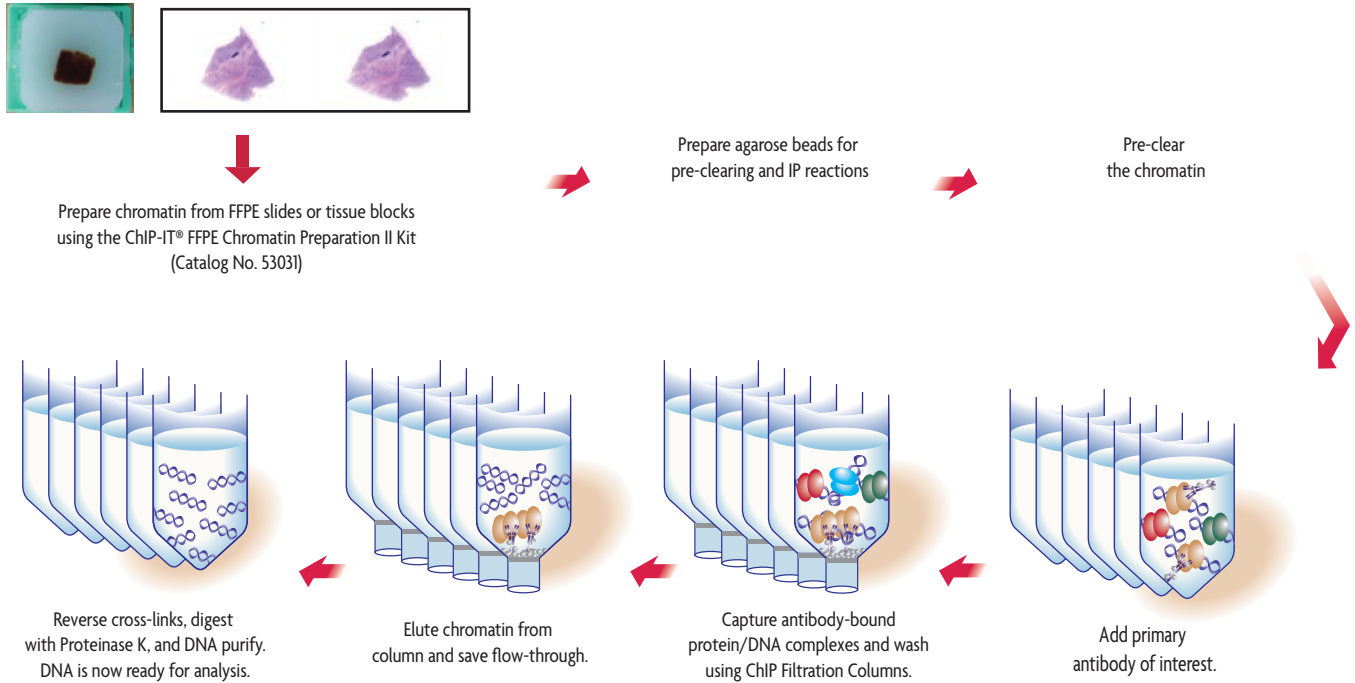
Formalin-fixed paraffin-embedded (FFPE) tissue blocks and histology slides are a valuable resource for retrospective research on clinical samples. Clinical information, treatments and outcomes are often available for these sample types and large collections of FFPE material is commercially available. The ability to study FFPE samples provides researchers with an opportunity to link FFPE data to disease, diagnosis and biomarker discovery. Traditionally, FFPE samples have not been useful in chromatin immunoprecipitation because of the limited size of the samples, and the fact that the formalin fixation process often causes degradation and loss of antigenicity.

Active Motif's ChIP-IT® FFPE Chromatin Preparation II and ChIP-IT® FFPE II Kits are our second generation FFPE ChIP kits in which the cumbersome de-paraffinization and dehydration procedure has been streamlined to enable the preparation of high quality ChIP-enriched DNA using less reagents and hands on time. This new chromatin preparation protocol is optimal when working with limited amounts of freshly prepared or high quality FFPE tissue (the first generation FFPE chromatin preparation protocol should be used for precious or highly degraded FFPE samples). The ChIP-IT FFPE II Kit has been optimized for use after either chromatin preparation using specially formulated reagents and protocol guidelines to increase sensitivity and enable analysis by both qPCR and Next Generation sequencing from extremely limited starting material.

The ChIP-IT FFPE II Kit contains sufficient reagents for 16 immunoprecipitation reactions. It is necessary to use one of Active Motif's ChIP-IT FFPE Chromatin Preparation Kits to extract chromatin from the FFPE samples prior to starting the ChIP-IT FFPE II Kit. The ChIP-IT® qPCR Analysis Kit (Catalog No. 53029) can be used following ChIP to examine site-specific ChIP enrichment. For the preparation of Next generation sequencing (NGS) libraries from the ChIP-enriched DNA we recommend that you use the DNA Library Prep Kit for Illumina® (Catalog No. 53220). To learn about available ChIP-IT® Control Kits, control qPCR primer sets, ChIP-Seq validated antibodies, or the EpiShear™ sonication devices, please visit our website at activemotif.com/chip.

Product	Format	Catalog No.
ChIP-IT FFPE Chromatin Preparation II Kit	5 rxns	53031
ChIP-IT FFPE II	16 rxns	53047
DNA Library Prep Kit for Illumina®	48 rxns	53220

Flow Chart of Process



Flow Chart of the ChIP-IT FFPE II Assay.

The ChIP-IT FFPE II Kit requires chromatin prepared from FFPE slides or tissue blocks using ChIP-IT FFPE Chromatin Preparation II Kit (Catalog No. 53031). Chromatin is pre-cleared followed by an incubation with an antibody directed against the DNA-binding protein of interest. The antibody-bound protein/DNA complexes are immunoprecipitated through the use of Protein G agarose beads and washed via gravity filtration. Following immunoprecipitation, cross-links are reversed, the proteins are removed by Proteinase K, and the DNA is recovered and purified. ChIP enriched DNA can be used for either gene-specific or whole-genome analysis.

Introduction

Formalin-fixed paraffin embedded (FFPE) samples represent an opportunity for researchers to study clinical outcomes of disease and/or treatment conditions in the search of a better disease understanding, or as a mechanism to identify biomarkers for screening purposes. FFPE samples serve as the “gold standard” for pathology sample preservation and large collections of these tissues are available.

There are many challenges associated with working with FFPE samples. The samples are often limited in size and require the use of multiple histological slides or tissue sections to extract sufficient quantities of material for downstream analysis. Another challenge is the lack of consistency in the methodologies used for formalin fixation. Some treatments tend to be harsh, causing degradation of the sample, loss of antigenicity, or they create “overfixed” chromatin which is difficult to efficiently shear. Although FFPE samples have been used for high-throughput DNA and RNA analysis^{1,2}, the challenges explained above have prevented FFPE material from being used in chromatin immunoprecipitation (ChIP).

ChIP itself can be technically demanding. ChIP requires high-quality antibodies to recognize the fixed, target-bound proteins of interest, and an efficient means to precipitate the antibody/chromatin complex (usually protein A or G beads). In addition, specialized buffers, inhibitor cocktails and blocking reagents are required to minimize non-specific enrichment and reduce protein degradation.

Researchers have started to address the need for a methodology to study the influences of epigenetics on normal and tumor samples beyond the traditional immunohistochemistry (IHC) analysis. Pathology tissue chromatin immunoprecipitation (PAT-ChIP) was the first method to extract and analyze FFPE chromatin for use in high-throughput analysis, such as ChIP-Seq^{3,4}. Subsequently, many laboratories have attempted to prepare high quality chromatin from FFPE samples⁵, however many are still reporting issues in generating quality chromatin from ChIP-seq analysis.

Active Motif has utilized our expertise with ChIP to develop a suite of kits for performing ChIP on FFPE samples for use in Next-generation sequencing. Coupled with our newly developed and most sensitive ChIP kit, ChIP-IT FFPE II, you have everything you need to generate high quality ChIP-Seq data from extremely limited starting material while producing minimal background signal, thereby enabling specific detection of the target protein of interest. All Active Motif FFPE ChIP kits contain controls to help validate results at each step of the process.

Kit Performance and Benefits

ChIP-IT FFPE II Advantages

- Sensitive enrichment of DNA from nanogram quantities of FFPE chromatin
- Optimized reagents reduce background levels caused by non-specific binding events
- Works with both transcription factor and histone ChIP-validated antibodies
- Filtration based washes are the easiest wash method available and result in increased consistency in multi-sample experiments
- Highly robust procedure has been validated using FFPE chromatin from both normal and tumor samples with proven performance in both qPCR and ChIP-Seq analysis
- Includes positive control antibody to help confirm chromatin quality and successful IP

Detection limit

The ChIP-IT FFPE II Kit requires a minimum of 200 ng chromatin extracted and validated using the ChIP-IT FFPE Chromatin Preparation or ChIP-IT FFPE Chromatin Preparation II Kits (Catalog No. 53030/53031) for use in each ChIP reaction. Using larger quantities of chromatin is recommended when available in order to ensure good enrichment efficiency, but due to the limited size and yield of FFPE material, it may not be possible to obtain more than 200 ng of chromatin for use per ChIP reaction.

Product Performance

The ChIP-IT FFPE II Kit relies on the use of chromatin extracted from FFPE samples. It is important to understand the caveats of working with FFPE chromatin and the potential pitfalls. Due to the variability that exists in the formalin fixation process and the storage conditions of the sample, not all FFPE material may yield high quality chromatin. Some fixation treatments tend to be harsh, causing degradation of the sample or loss of antigenicity. This loss of antigenicity may result in reduced ChIP efficiency. Additionally, certain FFPE samples may be more difficult to physically homogenize and sonicate during chromatin preparation. A Histone H3K9ac antibody (Catalog No. 91104) is included in the ChIP-IT FFPE II Kit as a positive control antibody to help confirm success of the ChIP reactions. Successful chromatin immunoprecipitation depends on the quality of the chromatin preparation, retained antigenicity, the affinity of the ChIP antibody and the abundance of the target protein.

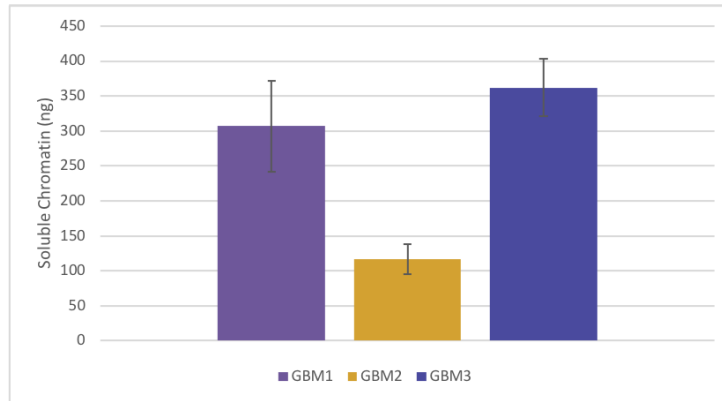


Figure 1: qPCR analysis of Glioblastoma samples assayed using the ChIP-IT FFPE II Kit

Samples of glioblastoma patient-derived xenografts were processed using the ChIP-IT FFPE Chromatin Preparation II Kit. Three 5 µm curls were used for Sample GBM1, and cell pellets from dissociated tumor were used for Samples GBM2 and GBM3. Samples were reverse cross-linked, proteinase K treated and purified for analysis by qPCR using the included DNA standards and qPCR primer sets to determine the quantity and quality of the extracted chromatin. If sufficient quantities are not obtained after a single sonication, additional rounds of sonication can be performed (as specified in Section F).

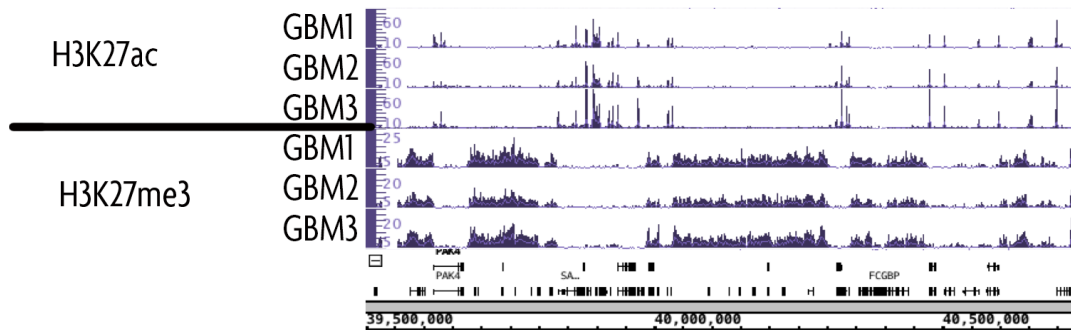


Figure 2: H3K27ac and H3K27me3 ChIP-Seq on Glioblastoma samples

Samples of glioblastoma patient-derived xenografts were used in H3K27ac and H3K27me3 ChIP-Seq to generate genome-wide profiles of this histone modification. Libraries were constructed using the DNA Library Prep Kit for Illumina® (Catalog No. 53220). A portion of that data is presented above.

Kit Components and Storage

The ChIP-IT FFPE II Kit is for research use only. Not for use in diagnostic procedures. All components can be stored at -20°C prior to first use. Then we recommend storing each component at the temperatures indicated in the tables below.

All components are guaranteed for 6 months after receipt when stored properly.

Reagents	Quantity	Storage
Proteinase K (10 µg/µL)	75 µL	-20°C
10X PBS	450 µL	-20°C
100 mM PMSF	160 µL	-20°C
Protease Inhibitor Cocktail (PIC)	80 µL	-20°C
Carrier	32 µL	-20°C
Blocker	80 µL	-20°C
Blocking Reagent AM1	112 µL	-20°C
BSA (10 mg/mL)	112 µL	-20°C
Protein G Agarose Beads*	1.12 mL	4°C
Histone H3K9ac rAb	8 µL	-20°C
Human Positive Control Primer Set GAPDH-2	350 µL	-20°C
Human Negative Control Primer Set 2	350 µL	-20°C
TE pH 8.0	33.6 mL	RT
5 M NaCl	350 µL	RT
ChIP Filtration Columns	16 ea	RT
ChIP Buffer	45 mL	RT
Wash Buffer AM1	29 mL	RT
LiCl Buffer	29 mL	RT
Elution Buffer AM4	1.6 mL	RT
1.7 mL siliconized tubes	32 ea	RT
Low EDTA TE	1 mL	RT

* The Protein G Agarose Beads are shipped on dry ice and can be stored frozen until their first use. Once thawed, the Protein G beads should not be re-frozen by the customer. Protein G Agarose Beads should be stored at 4°C.

Additional Materials Required

- A ChIP-Seq-validated antibody directed against the protein of interest
- Phenol, saturated (DNA Purification, Molecular Biology Grade, Amresco Catalog No. 0945)
- Chloroform/isoamyl alcohol (24:1) (DNA Purification, Molecular Biology Grade)
- 100% ethanol (absolute)
- 70% ethanol
- DNase-free H₂O
- Apparatus to rotate tubes end-to-end at 4°C (e.g. a Labquake from Barnstead/Thermolyne with a tube holder for 1.5 mL microcentrifuge tubes)
- Microcentrifuge (table top centrifuge 4°C) and microcentrifuge tubes
- 250 µL PCR tubes
- Thermal cycler
- Pipettors and tips (filter tips are recommended)
- (Optional) ChIP-IT® qPCR Analysis Kit (Catalog No. 53029)
- (Optional) SYBR Green qPCR master mix (Bio-Rad Catalog No. 170-8882)
- (Optional) DNA Library Prep Kit for Illumina® (Catalog No. 53220)

Protocols – Experimental Set Up

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Protocol Overview and Time Table

Step	Required Time
Protein G Agarose Bead Preparation*	3 - 6 hours (Pre-clearing beads) to overnight (IP beads)
Pre-clearing the Chromatin*	2 - 3 hours
Immunoprecipitation	Overnight incubation
Binding to Protein G agarose Beads	3-4 hours
Wash Immune Complexes & Elute DNA	45 minutes
Reversal of Cross-links	Overnight incubation
DNA Purification	1.5 hours
qPCR Analysis	2 hours

Buffer Preparation

100 mM PMSF and Protease Inhibitor Cocktail (PIC)

Thaw the PMSF and the PIC at room temperature until fully dissolved, which takes about 30 minutes. Vortex gently and spin down briefly before use, then add to the buffers immediately before use.

ChIP Buffer

Is supplied ready to use.

Protein G Agarose Beads

For best results, gently shake and invert the tube to resuspend the agarose beads. The beads settle quickly, and therefore should be resuspended just before pipetting. We recommend cutting 2 mm from the end of a pipet tip prior to pipetting to prevent the tip from becoming clogged. Protein G Agarose Beads are shipped on dry ice and can be stored frozen until their first use. Once thawed, beads should not be re-frozen by the customer. Protein G Agarose Beads should be stored at 4°C.

1X PBS Buffer

Prepare a 1X PBS solution by adding 1 mL 10X PBS to 9 mL sterile water. Mix by inverting and place on ice to chill. 1X PBS Buffer can be prepared in large quantities and stored at 4°C for 6 months.

Histone H3K9ac rAb

Active Motif's Histone H3K9ac rAb (Catalog No. 91104) is included as a ChIP-validated positive control antibody. This antibody can be used to confirm the ChIP reactions are working with your FFPE chromatin material. Histone H3K9ac is a mark of transcriptional activity and should be highly abundant in all sample types. Use 2-4 µL of antibody per ChIP reaction.

Human Positive and Negative Control qPCR Primer Sets

Active Motif's Human Positive Control Primer Set GAPDH-2 (Catalog No. 71006) and Human Negative Control Primer Set 2 (Catalog No. 71002) are included to confirm the enrichment of the positive control Histone H3K4me3 pAb from human chromatin samples. To confirm Histone H3K4me3 enrichment using a different species, please see Active Motif's list of available Control qPCR primer sets at activemotif.com/chipprimers. The primers are provided at a concentration of 2.5 μ M. Use 4 μ L of each primer set per qPCR reaction.

Recommendations

ChIP-Seq-validated Antibody

We recommend using 2-4 μ g antibody per ChIP reaction in a maximum volume of 30 μ L. However, this will vary according to the affinity of the antibody and the quality of the chromatin; you may need to use more of a particular antibody. ChIP antibodies must recognize fixed, native protein that is bound to DNA and/or complexed with other proteins. Many antibodies that perform well in other applications do not perform in ChIP. Thus, ChIP performed with an antibody that has not been ChIP-Seq-validated must include appropriate controls (such as Active Motif's ChIP-IT Control qPCR Kits, Catalog Nos. 53026, 53027 and 53028) to validate the chromatin preparation and the ChIP methodology. To see a list of available ChIP-validated antibodies available from Active Motif, please visit activemotif.com/chipabs.

Chromatin Quantity

It is recommended to use a minimum of 200 ng chromatin prepared using Active Motif's ChIP-IT FFPE or ChIP-IT FFPE II Chromatin Preparation Kit (Catalog No. 53030/53031) for each ChIP reaction in a total ChIP reaction volume of 200 μ L. When possible, using larger quantities of chromatin is recommended in order to ensure good enrichment efficiency. However, due to the limited size and yield of FFPE material, for some sample types it may not be feasible to obtain more than 200 ng. We suggest using qPCR quantitation, or a Qubit™ Fluorometric Quantitation Method to determine the concentration of the FFPE chromatin. These methods are more sensitive at detecting low DNA quantities and will provide more accurate results than a spectrophotometer such as Nanodrop. If the chromatin concentration is too dilute to utilize 200 ng in a 200 μ L ChIP reaction volume, we suggest preparing multiple ChIP reactions at 200 μ L and then pooling the enriched DNA together during the DNA purification step. It is not recommended to perform ChIP reactions in larger volumes as the increased volume of the reaction reduces the enrichment efficiency.

Protocols

Section A. Protein G Agarose Bead Preparation

1. Set up two 1.5 mL microcentrifuge tubes to prepare the Protein G Agarose beads for the IP and pre-clearing. We recommend cutting 2 mm from the end of a pipet tip prior to pipetting Protein G agarose beads to prevent the tip from becoming clogged. If more than 12 IP reactions will be performed, we suggest setting up multiple tubes to ensure sufficient room in the tube for the addition of all the required components. Volumes to add will be based on the number of IP reactions performed. Include negative and positive control IPs into the calculation for the number of IP reactions. Volumes shown below include excess for pipetting.

Reagents	IP Reactions			Pre-clearing Reactions		
	4 rxns	8 rxns	12 rxns	4 rxns	8 rxns	12 rxns
Protein G Agarose	140 µL	270 µL	400 µL	140 µL	270 µL	400 µL

Note: We do not recommend more than 12 IP reactions in a single tube. If more than 12 IPs are to be performed in a single experiment, set up multiple tubes.

2. Centrifuge the tubes at 1250 x *g* for 3 minutes. Carefully remove and discard supernatant, taking care to avoid the beads.
3. Add the calculated volume of TE, pH 8.0 to the each tube of beads:

Reagents	IP Reactions			Pre-clearing Reactions		
	4 rxns	8 rxns	12 rxns	4 rxns	8 rxns	12 rxns
TE, pH 8.0	460 µL	900 µL	1330 µL	460 µL	900 µL	1330 µL

4. Centrifuge the tubes at 1250 x *g* for 3 minutes. Carefully remove and discard supernatant, taking care to avoid the beads.
5. Set up bead blocking reactions according to the table below:

Reagents	IP Reactions			Pre-clearing Reactions		
	4 rxns	8 rxns	12 rxns	4 rxns	8 rxns	12 rxns
TE, pH 8.0	126 µL	243 µL	360 µL	140 µL	270 µL	400 µL
Blocking Reagent AM1	14 µL	27 µL	40 µL	14 µL	27 µL	40 µL
BSA	14 µL	27 µL	40 µL	14 µL	27 µL	40 µL
Blocker	14 µL	27 µL	40 µL	–	–	–
Incubated at 4°C with rotation	Overnight			3 - 6 hours		

6. Cap tubes and incubate on an end-to-end rotator at 4°C. Follow the recommendations in the chart above for the incubation time.
7. Following the incubation, proceed to Section B with the pre-clearing bead reactions.

Section B. Pre-clearing the Chromatin

1. If chromatin is stored at -80°C , place on ice to thaw.
2. Remove the pre-clearing reactions from the rotator and quick spin to collect contents to the bottom of the tube. Allow the IP beads to continue to incubate on the rotator.
3. Centrifuge the tubes at $1250 \times g$ for 3 minutes. Carefully remove and discard supernatant, taking care to avoid the beads.
4. Add the calculated volume of ChIP Buffer to the pre-clearing reaction. Mix by inverting.

Reagents	IP Reactions			Pre-clearing Reactions		
	4 rxns	8 rxns	12 rxns	4 rxns	8 rxns	12 rxns
ChIP Buffer	–	–	–	460 μL	900 μL	1330 μL

5. Centrifuge the tubes at $1250 \times g$ for 3 minutes. Carefully remove and discard supernatant, taking care to avoid the beads.
6. Perform a second wash with ChIP Buffer using the volumes below. Mix by inverting

Reagents	IP Reactions			Pre-clearing Reactions		
	4 rxns	8 rxns	12 rxns	4 rxns	8 rxns	12 rxns
ChIP Buffer	–	–	–	160 μL	315 μL	465 μL

7. Cut off 2 mm from the end of pipet tip and transfer 50 μL of the pre-cleared Protein G agarose bead slurry to the 1.7 mL siliconized tube containing the sonicated chromatin from Section C, Step 3.
8. Add 5 μL Protease Inhibitor Cocktail and 5 μL PMSF to each tube.
9. If using less than 200 μL chromatin, adjust the final volume of the reaction to a final volume of 260 μL using ChIP Buffer.
10. Cap tubes and incubate on an end-to-end rotator for 2-3 hours at 4°C .

Section C. Immunoprecipitation

1. Prepare separate, labeled 1.5 mL microcentrifuge tubes for each ChIP reaction, even if the same antibody will be used for more than one sample. Prepare the antibody mixture according to the table below and place on ice.

Reagent	Antibody Mixture
Antibody	2-4 μg
1X PBS	Up to 30 μL final volume

2. Centrifuge the tubes containing the pre-clearing reactions (Section B, Step 10) in a microcentrifuge at 3500 rpm for 3 minutes. Carefully transfer the supernatant, avoiding the bead pellet, to the microcentrifuge tube containing the appropriate antibody mixture.
3. Cap tubes and incubate on an end-to-end rotator overnight at 4°C.
4. The next day, remove the IP reactions (from Section A, Step 5) from the rotator and quick spin to collect contents to the bottom of the tube.
5. Add 50 μL of the IP Reaction bead slurry to each immunoprecipitation reaction. We recommend cutting off 2 mm from the end of a pipet tip to make it easier to pipet the bead slurry.
6. Cap tubes and incubate on an end-to-end rotator for 3-4 hours at 4°C.

Section D. Washing & Elution of IP reactions

1. Label a ChIP Filtration column for each ChIP reaction. Remove the tab from the bottom of the column and place in an empty 1 mL pipet tip box as a holder.
2. Remove the immunoprecipitation reactions from the rotator and quick spin to collect contents to the bottom of the tube.
3. Add 600 μL ChIP Buffer to each immunoprecipitation reaction to wash any remaining beads off the sides of the tube and transfer the entire volume (including the Protein G agarose beads) to its labeled column. Allow flow-through to occur by gravity.
4. During the gravity flow, transfer 100 μL Elution Buffer AM4 per ChIP reaction to a new 1.5 mL microcentrifuge tube. Pre-warm at 37°C. (DO NOT add to columns until Step 10 below.)
5. Wash each column with 900 μL ChIP Buffer. Allow a minute for the buffer to flow through the column. Wash a second time with an additional 900 μL ChIP Buffer.
6. Wash each column with 900 μL Wash Buffer AM1. Incubate for 3 minutes on the column. Allow the buffer to flow through the column. Wash a second time with an additional 900 μL Wash Buffer AM1.
7. Wash each column with 900 μL LiCl Buffer. Incubate for 3 minutes on the column. Allow the buffer to flow through the column. Wash a second time with an additional 900 μL LiCl Buffer.
8. Wash each column with 900 μL TE Buffer pH 8.0. Allow a minute for the buffer to flow through the column. Wash a second time with an additional 900 μL TE Buffer pH 8.0.
9. Transfer columns to a new 1.5 mL microcentrifuge tube. Centrifuge at 1250 x *g* for 3 minutes at room temperature to remove residual wash buffer.
10. Label new 1.5 mL microcentrifuge tubes. Transfer the ChIP Filtration Columns to the labeled tubes. Add 50 μL pre-warmed (37°C) Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes. Centrifuge at 1250 x *g* for 3 minutes at room temperature to collect ChIP DNA.
11. Keeping the columns in the same microcentrifuge tube, add an additional 50 μL pre-warmed (37°C) Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes. Centrifuge at 1250 x *g* for 3 minutes at room temperature to collect ChIP DNA.
12. Discard the ChIP Filtration columns. The flow through contains the ChIP DNA.

Section E. Reversal of Cross-links and DNA Purification

1. Transfer each eluted ChIP DNA to a 250 μ L PCR tube. Add 2 μ L Proteinase K and 5 μ L 5 M NaCl. Vortex to mix. Heat in a thermocycler at 65°C overnight.
2. Following reversal of cross-links, transfer DNA to a new 1.5 mL microcentrifuge tube. Add 125 μ L phenol and 64 μ L chloroform/isoamyl alcohol (24:1). Shake vigorously for 15 seconds to mix (do not vortex). Incubate at room temperature for 5 minutes.
3. Centrifuge in a microcentrifuge at maximum speed for 2 minutes. Transfer the aqueous layer (top layer) to a new 1.5 mL microcentrifuge tube. A hazy white layer at interface may still be present.
4. Add 125 μ L chloroform/isoamyl alcohol (24:1) to the aqueous layer in the new tube. Shake vigorously for 15 seconds to mix (do not vortex). Incubate at room temperature for 5 minutes.
5. During the incubation, set up new 1.5 mL microcentrifuge tubes for each ChIP reaction and add 2 μ L Carrier to each tube.
6. Centrifuge the DNA purifications in a microcentrifuge at maximum speed ($> 18,000 \times g$) for 2 minutes. Transfer the aqueous layer (top layer) to microcentrifuge tube containing Carrier.
7. Add 300 μ L 100% ethanol to the DNA solution and briefly vortex to mix.
8. Place samples at -80°C for 30 minutes to precipitate the DNA.
9. Centrifuge in a microcentrifuge at maximum speed ($> 18,000 \times g$) for 15 minutes at 4°C. Mark the tube where you expect the pellet to form as it may not be visible.
10. Carefully remove the supernatant with a pipet. Avoid disturbing the location of the DNA pellet.
11. Add 500 μ L 70% ethanol to each tube and invert to mix.
12. Centrifuge in a microcentrifuge at maximum speed ($> 18,000 \times g$) for 5 minutes at 4°C. Mark the tube where you expect the pellet to form as it may not be visible.
13. Remove 400 μ L supernatant and centrifuge a second time at maximum speed ($> 18,000 \times g$) for 2 minutes at 4°C. Carefully remove as much residual ethanol as possible with a P200 pipet taking care not to disturb the pellet.
14. Air-dry the pellet for 10-15 minutes (until residual ethanol has evaporated). Resuspend the pellet in 40 μ L Low-EDTA TE Buffer. ChIP DNA is ready for analysis by qPCR or NGS library preparation, or may be stored at -20°C for future use.

Protocols – ChIP DNA Analysis

Section F. Quantitative PCR (qPCR)

ChIP DNA can be analyzed on a gene-specific basis using qPCR. Positive control and negative control PCR primer pairs should be included in every analysis to determine the fold enrichment. Negative control primers will amplify a region of the genome not bound by the antibody target of interest. Active Motif recommends the use of its ChIP-IT® qPCR Analysis Kit (Catalog No. 53029), which contains positive and negative control primer pairs, standard curve DNA, standard curve primers and a qPCR Analysis spreadsheet to perform the qPCR analysis calculations. Active Motif's analysis strategy determines primer efficiencies and the ChIP sample values are normalized according to input, primer efficiency, chromatin amount used in the ChIP reaction and resuspension volume. If not using the ChIP-IT qPCR Analysis Kit, qPCR data normalization and graphing can be done using the methods described in Section H.

1. Below is an example qPCR reaction. Please follow the specific instructions for your qPCR instrument. We recommend using a commercially available SYBR Green qPCR master mix (e.g. Bio-Rad Cat # 170-8882) and preparing triplicate reactions. If the ChIP elution volume was reduced for ChIP-Seq analysis, please see the notes under General Recommendations on page 16 to dilute the ChIP DNA for use in qPCR. Input DNA that was prepared in the ChIP-IT FFPE Chromatin Preparation or ChIP-IT FFPE Chromatin Preparation II Kit (Catalog No. 53030/53031) and used to quantify the chromatin extracted from the FFPE samples should also be included.

Reagent	20 µL PCR reactions
2X SYBR Green master mix	10 µL
Primer mix (2.5 µM each primer)*	4 µL
Sterile water	1 µL
DNA sample (ChIP or Input)	5 µL
Total volume	20 µL

Note: We recommend designing primers to perform at an annealing temperature of 58°C so that all qPCR reactions can be performed under identical conditions. An amplicon length of 75-150 bp is recommended. Active Motif offers validated species-specific qPCR primers designed according to these recommendations at activemotif.com/chipprimers.

2. Place tubes in a real time PCR instrument and program as below:
95°C for 2 minutes
(95°C for 15 seconds, 58°C for 20 seconds, 72°C for 20 seconds) for 40 cycles
3. Include and inspect the melt curve based on the protocols recommended by the qPCR instrument manufacturer to ensure that primer pairs amplify only a single product.
4. If using the ChIP-IT qPCR Analysis Kit, please refer to the product manual for analysis instructions. Otherwise, follow the recommendations in Section H.

Section G. ChIP-Seq

The ChIP-IT FFPE II Kit has been validated for whole genome analysis using the DNA Library Prep Kit for Illumina® (Catalog No. 53220), designed to prepare libraries from double-stranded input DNA for use with Illumina® sequencing platforms. Libraries can be generated from as little as 10 pg high quality genomic DNA, ChIP DNA, FFPE DNA or cell-free DNA (cfDNA), which is 500-fold more sensitive than other library preparation kits. Additionally, libraries can be prepared from 100 ng DNA if preparing PCR-free libraries. The Next Gen DNA Library Kit offers the advantage of including molecular identifiers (MIDs) during library generation to enable removal of true PCR duplicates from the sequencing analysis. This helps to distinguish PCR duplicates from fragmentation duplicates, thereby, increasing the number of unique alignments for more accurate data analysis.

We strongly recommend that you use the DNA Library Prep Kit for Illumina® (Catalog No. 53220) to prepare your libraries for sequencing due to its high sensitivity. However, if using alternative sequencing platforms, please select an appropriate library preparation method compatible with the sequencing apparatus to be used.

A. General Recommendations

- Verification of the quality of the ChIP-enriched DNA should be performed prior to proceeding with downstream sequencing. Follow the instructions in Section F: Quantitative PCR (qPCR) with the following modification: the ChIP DNA will need to be diluted prior to use in qPCR due to the reduced elution volume. Dilute 5 µL of ChIP DNA in 45 µL sterile water. Use 5 µL of the diluted ChIP DNA per qPCR reaction.
- For good ChIP-Seq data, quality enrichments with low background is more important than the total quantity of DNA recovered. Therefore, we recommend performing qPCR on known binding sites to verify enrichment levels against a negative control primer set using Active Motif's ChIP-IT qPCR Analysis Kit (Catalog No. 53029) to confirm DNA quality rather than quantifying the DNA. Quality DNA that is suitable for use in ChIP-Seq should show enrichment of known binding sites over negative control primer sets of 5-fold. If the qPCR enrichments are satisfactory then the remaining 35 µL of ChIP DNA can be used for library generation.
- A typical high quality ChIP-Seq reaction will yield 10-20 million unique alignments. The number of unique alignments is highly correlated with the amount of starting ChIP DNA that goes into the library preparation reaction. Therefore, as the amount of ChIP DNA decreases, the number of unique alignments in the ChIP-Seq data set will decrease. Due to the limited size of FFPE samples, there is usually a low amount of starting material available for ChIP and therefore, subsequent low amounts of ChIP-enriched DNA. This means the library complexity can be less than in a typical ChIP-Seq data set where >5 million cells may have been used per ChIP reaction. FFPE yields will be in the range of 2 million to 10 million unique alignments. Although these results are not as robust as a typical ChIP-Seq data set, these data sets can still be reliably interpreted. The interpretation will be easier for targets that yield tight peaks as compared with targets that produce broad peaks. Data sets may be improved by increasing the amount of FFPE material used in ChIP, by pooling multiple ChIP reactions together for library generation or by increasing the sequencing depth. Despite the reduction in the number

of unique alignments, FFPE ChIP-Seq is enabling the generation of data sets that were not possible to obtain in the past.

- 36 bp single end reads are sufficient for unique mapping and good ChIP-Seq data, although longer reads can be used.
- Input DNA should be sequenced as a control reaction in order to identify false “peaks” and also to reveal regions of the genome that have been duplicated. Subtracting the input peaks from the experimental peaks will help to eliminate false data. Use 50 ng Input DNA for library generation.

Section H: qPCR Primer Design and Data Analysis

A. Design of the primers

- Design and analyze your potential primer pairs using an in silico PCR program
- Primers that dimerize should be avoided, as they will be bound by SYBR Green, which will compromise accurate quantitation.
- Ideally, the amplicons should be 75-150 bp in length.
- For use with the ChIP-IT qPCR Analysis Kit, primers should be designed to anneal optimally at 58°C with a recommended length of 18-22 bp.
- Active Motif offers ChIP Control qPCR primer sets validated to work in our ChIP-IT qPCR Analysis Kit. To see a list of the available species-specific primers, please visit activemotif.com/chipprimers.

B. Data Analysis

If the data analysis will not be performed using Active Motif's ChIP-IT qPCR Analysis Kit, two other simplified methods of analysis are provided below. Both methods listed require the generation of a standard curve, containing known amounts of DNA, for each primer pair being used in the experiment.

Method 1: Fold enrichment of positive primers relative to negative control primers

1. Produce a standard curve by performing qPCR with your primer set on known DNA quantities of Input DNA in triplicate. Run three to five samples that are 10-fold dilutions, e.g. 0.005 ng, 0.05 ng, 0.5 ng, 5 ng and 50 ng.
2. Run the ChIP and IgG samples along with the dilution series of the Input DNA standards using both positive control primers (known binding sites) and negative control primers (regions of the genome not bound by your protein of interest).
3. Your qPCR instrument will assign values to each qPCR reaction based on the standard curve. If your machine does not average your triplicate reactions automatically, you will need to calculate these averages.
4. Divide the average value from the positive control primer set by the average value of the negative control primer set to obtain your fold enrichment.

Method 2: Express data as a percent of input

1. Produce a standard curve by performing qPCR with your primer set on known DNA quantities of Input DNA in triplicate. Run three to five samples that are 10-fold dilutions, e.g. 0.005 ng, 0.05 ng, 0.5 ng, 5 ng and 50 ng.
2. Run the ChIP and IgG samples along with the dilution series of the Input DNA standards using both positive control primers (known binding sites) and negative control primers (regions of the genome not bound by your protein of interest).

3. Your qPCR instrument will assign values (in ng) to each qPCR reaction based on the standard curve. If your machine does not average your triplicate reactions automatically, you will need to calculate these averages.
4. For each qPCR reaction you will have used a percentage of your total ChIP DNA. In order to calculate the amount in the whole reaction, divide the elution volume of the entire ChIP reaction by the volume used in the qPCR reaction (e.g. if you eluted ChIP DNA in 100 μ L and used 5 μ L in the qPCR reaction the formula is $100/5 = 20$). Then, multiply the average qPCR quantity by this number (e.g. qPCR quantity in ng \times 20).
5. To express data as a percent of input, divide the adjusted values from Step 4 above by the amount of DNA that went into the ChIP reaction and then multiply by 100%. (e.g. if 20 μ g was used in the ChIP reaction this is equivalent to 20,000 ng of chromatin. The calculation would be the adjusted value from Step 4 divided by 20,000 ng and then multiplied by 100). Typical percent of input recovered values are 0.05% to 1%.

References

1. Weng, L. *et al.* (2010) *J Pathol.*, 222: 41-51.
2. Gu, H., *et al.* (2010) *Nat. Methods*, 7: 133-136.
3. Fanelli, M. *et al.* (2010) *PNAS*, 107(50): 21535-21540.
4. Fanelli, M. *et al.* (2011) *Nat. Protocols*, 6(12): 1905-1919.
5. Cejas, P. *et al.* (2016) *Nat. Med.*, 22(6): 685-91.

Troubleshooting Guide

Problem/Question	Recommendation
At what points in the protocol can I stop?	<p>The protocol may be stopped and samples stored at the times and temperatures below:</p> <ol style="list-style-type: none">1. During overnight incubation of pre-cleared chromatin and antibody, 4°C.2. During overnight reversal of cross-links, 65°C3. After DNA clean up, -20°C.
Performing ChIP with a large volume of chromatin.	<p>This is not recommended. It is better to set up several small ChIP reactions (240 µL each) and pool the samples at the end, rather than trying to ChIP a single large sample. Do not perform a single scaled-up reaction, as the capture efficiency is lower.</p>
High background.	<p>Chromatin not sheared enough. Shearing should produce DNA fragments that are small enough to exclude background from neighboring chromosomal sequences, but still large enough that there is a good possibility your amplicon remains intact. We recommend 200-1200 bp fragments. If the DNA fragments are too large, the background is increased. Consider increasing the number of pulses for sonication.</p> <p>Antibody issue. Too much antibody relative to the amount of chromatin in the ChIP reaction. Excess antibody will result in more non-specific binding, which will be detected as increased background. We recommend using 2-4 µg of antibody per IP.</p>

Problem/Question	Recommendation
<p>Poor or no enrichment with target antibody.</p>	<p>Too little chromatin. Generally, we recommend using greater than 200 ng of chromatin per ChIP reaction. If your ChIP antibody has low binding affinity and you have limited chromatin for the ChIP, you may obtain low enrichment. Try and repeat the ChIP reaction using more chromatin. Be sure to quantitate the concentration of the sheared chromatin sample(s) being ChIP'd to ensure both that adequate chromatin is used per sample, and that equal mass quantities of chromatin are used in each ChIP. Due to the low yields of chromatin from FFPE samples, using a Qubit® Fluorometric Quantitation or qPCR quantification method is recommended over a Nanodrop since the Nanodrop does not have the accuracy to read very low DNA quantities.</p> <hr/> <p>Antibody is not ChIP validated. The antibody does not efficiently recognize fixed proteins, either because the epitope is destroyed by fixation or because the epitope is masked by other proteins in a larger complex. To assist in ChIP validating an antibody, it is very useful to use a positive control antibody such as Histone H3K9ac (Catalog No. 91104) and a negative IgG from the same species, and primers that have been proven to work in the type of PCR being used. Active Motif offers species-specific ChIP-IT Control qPCR Kits for antibody validation (Catalog Nos. 53026, 53027 & 53028).</p> <hr/> <p>Low-affinity antibody. Use a different antibody.</p> <hr/> <p>Antibody affinity to protein G is weak. Individual monoclonals have variable binding affinities to protein G, which are pH dependent; the optimal pH may vary for each Ig. For those with low to medium affinity, capture efficiency by protein G can be dramatically improved through use of our Bridging Antibody (Catalog No. 53017). This antibody is a rabbit anti-mouse pAb that recognizes all subclasses of mouse immunoglobulins. If your IgG has a weak/medium affinity to protein A or G, the Bridging Antibody will increase antibody capture by the beads without increasing background.</p> <hr/> <p>Problems with PCR. Confirm the amplified sequence for the positive control primer set is bound by the antibody target. Identify other binding sites.</p>
<p>No PCR products for the ChIP'd samples (but the Input DNA yields the correct PCR product).</p>	<p>Increase the amount of chromatin used in the ChIP reaction, the amount of antibody used, or both.</p> <hr/> <p>Use a different antibody.</p>
<p>No PCR products with real-time PCR.</p>	<p>Confirm the species specificity and efficiency of your primers. You may need to redesign your primers. Primers that work in end point PCR do not always work in qPCR.</p>

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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