ChIP-IT® FFPE

(version A1)

Catalog No. 53045

Active Motif North America
1914 Palomar Oaks Way, Suite 150
Carlsbad, California 92008, USA
Toll free: 877 222 9543
Telephone: 760 431 1263
Fax: 760 431 1351

Active Motif Europe
Avenue Reine Astrid, 92
B-1310 La Hulpe, Belgium
UK Free Phone: 0800 169 31 47
France Free Phone: 0800 90 99 79
Germany Free Phone: 0800 181 99 10
Telephone: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050

Active Motif Japan
Azuma Bldg, 7th Floor
2-21 Ageba-Cho, Shinjuku-Ku
Tokyo, 162-0824, Japan
Telephone: +81 3 5225 3638
Fax: +81 3 5261 8733

Copyright 2013 Active Motif, Inc.

Information in this manual is subject to change without notice and does not constitute a commitment on the part of Active Motif, Inc. It is supplied on an "as is" basis without any warranty of any kind, either explicit or implied. Information may be changed or updated in this manual at any time.

This documentation may not be copied, transferred, reproduced, disclosed, or duplicated, in whole or in part, without the prior written consent of Active Motif, Inc. This documentation is proprietary information and protected by the copyright laws of the United States and international treaties.

The manufacturer of this documentation is Active Motif, Inc.

© 2013 Active Motif, Inc., 1914 Palomar Oaks Way, Suite 150; Carlsbad, CA 92008. All rights reserved.

All trademarks, trade names, service marks or logos referenced herein belong to their respective companies.

TABLE OF CONTENTS

	Page
Overview	1
Flow Chart of Process	2
Introduction	3
References	3
Kit Performance and Benefits	4
ChIP-IT FFPE	5
Kit Components and Storage	
ChIP-IT FFPE Kit	6
Additional Materials Required	7
Protocol Overview and Time Table	7
Protocols – Experimental Set Up	
Buffer Preparation	8
Recommendations	9
Protocols – Chromatin Immunoprecipitation	
Section A. Immunoprecipitation	10
Protocols – ChIP DNA Purification	
Section B. Reversal of Cross-links and DNA Purification	12
Protocols – ChIP DNA Analysis	
Section C. Quantitative PCR (qPCR)	13
Section D. ChIP-Seq	14
Appendix	
Section E. qPCR Primer Design and Data Analysis	16
Section F. Troubleshooting Guide	18
Section G. Related Products	19
Technical Services	20

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 or ChIP-chip, insights are possible into gene regulation, gene expression, mechanisms of chromatin modification and pathway analysis.

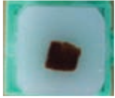
Formalin-fixed paraffin-embedded (FFPE) tissue blocks and histology slides are a valuable resource for retrospective research on clinical samples. Clinical information, treatments and outcomes are often available for these sample types and large collections of FFPE material is commercially available. 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 and ChIP-IT® FFPE Kits are designed to overcome these obstacles and provide the highest quality ChIP-enriched DNA for analysis by qPCR or Next Generation sequencing. The ChIP-IT FFPE Chromatin Preparation Kit provides specially formulated reagents and protocol guidelines to extract high quality chromatin from FFPE samples. This chromatin is then used in the ChIP-IT FFPE Kit, which is the only ChIP Kit available that has the sensitivity required to work with extremely limited starting material, while producing minimal background signal, thereby enabling specific detection of the target protein of interest.

The ChIP-IT FFPE Kit contains sufficient reagents to perform 16 immunoprecipitation reactions. It is necessary to use Active Motif's ChIP-IT FFPE Chromatin Preparation Kit to extract chromatin from the FFPE samples prior to starting the ChIP-IT FFPE Kit. The ChIP-IT® qPCR Analysis Kit can be used following ChIP for complete data analysis. To learn about available ChIP-IT® Control Kits, control qPCR primer sets, ChIP-Seq validated antibodies, or Active Motif's EpiShear™ sonication devices, please visit our website at **www.activemotif.com/chip**.

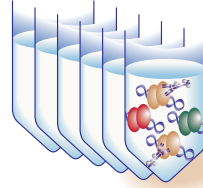
product	**format**	**catalog no.**
ChIP-IT® FFPE Chromatin Preparation Kit	5 rxns	53030
ChIP-IT® FFPE	16 rxns	53045
ChIP-IT® qPCR Analysis Kit	10 rxns	53029

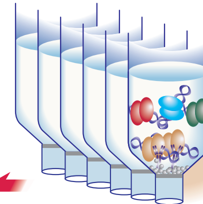
Flow Chart of Process



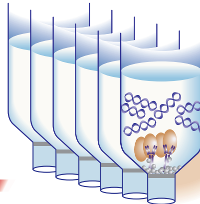
Prepare chromatin from FFPE slides or tissue blocks using the ChIP-IT® FFPE Chromatin Preparation Kit (Catalog No. 53030)

Add primary antibody of interest.

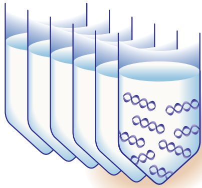




Capture antibody-bound protein/DNA complexes and wash using ChIP Filtration Columns.



Elute chromatin from column and save flow-through.



Reverse cross-links, digest with Proteinase K, and DNA purify. DNA is now ready for analysis.

Flow Chart of the ChIP-IT FFPE Assay.

The ChIP-IT FFPE Kit requires chromatin prepared from FFPE slides or tissue blocks using the ChIP-IT FFPE Chromatin Preparation Kit (Catalog No. 53030). Chromatin is then incubated 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 to better understand the disease, 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].

Active Motif has utilized our expertise with ChIP to develop the first commercially available kit for ChIP from FFPE samples for use in Next-generation sequencing. The ChIP-IT FFPE Chromatin Preparation Kit contains specially formulated reagents and protocols guidelines to extract high quality chromatin from histological slides or tissue sections. To perform ChIP analysis on the chromatin extracted from FFPE samples, it is necessary to use the ChIP-IT FFPE Kit. This is the only ChIP Kit available with the sensitivity required to work with extremely limited starting material while producing minimal background signal, thereby enabling specific detection of the target protein of interest. Both kits contain controls to help validate results at each step of the process.

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.

Kit Performance and Benefits

ChIP-IT FFPE 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 results

Detection limit: The ChIP-IT FFPE Kit requires a minimum of 200 ng chromatin extracted and validated using the ChIP-IT FFPE Chromatin Preparation Kit (Catalog No. 53030) 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 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. Some of the troubleshooting recommendations include heating the samples at an elevated temperature to improve solubility. This heat treatment may prematurely reverse cross-links from the chromatin, also resulting in reduced ChIP efficiency. A Histone H3K4me3 antibody (Catalog No. 39916) is included in the ChIP-IT FFPE 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, the affinity of the ChIP antibody and the abundance of the target protein.

ChIP-IT® FFPE

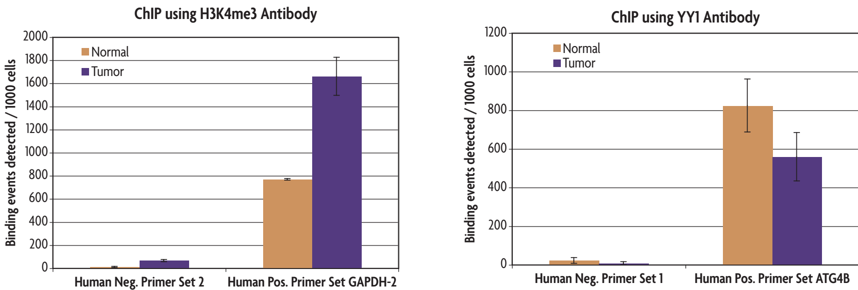


Figure 1: qPCR analysis of normal and tumor human colon samples assayed using the ChIP-IT FFPE Kit
Chromatin was extracted from 10-year old histological sections of a colon tumor and matched normal colon using the ChIP-IT FFPE Chromatin Preparation Kit. 250 ng and 185 ng of normal and tumor chromatin, respectively, were used per ChIP reaction in the ChIP-IT FFPE Kit. Antibodies for histone H3K4me3 or transcription factor YY1 were used for enrichment according to the recommendations in the manual. The quality of the ChIP-enriched DNA was then validated using the ChIP-IT qPCR Analysis Kit, which enables normalization of the data to account for differences in chromatin amounts, primer efficiency and ChIP elution volumes. The qPCR results for each ChIP antibody are shown above. The H3K4me3 results match observed data showing that GAPDH is up-regulated in certain cancers. The data represents triplicate values expressed as Binding events detected per 1,000 cells. To convert this scale to the percent of ChIP input recovered, divide the values by 1,000.

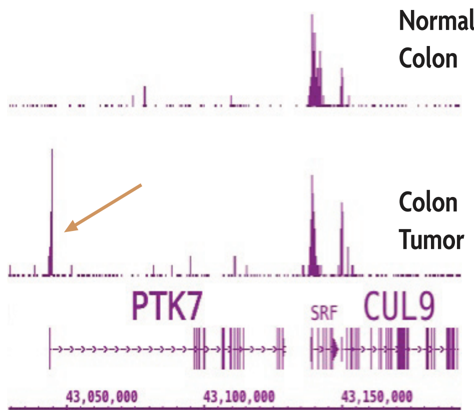


Figure 2: H3K4me3 ChIP-Seq on normal and tumor human colon FFPE samples.
Chromatin from matched normal and tumor human FFPE colon samples were used in H3K4me3 ChIP-Seq to generate genome-wide profiles of this histone modification. The expected promoter enrichment at > 12,000 genes was detected. A portion of that data is presented above. It shows nearly equal H3K4me3 occupancy at the SRF and CUL9 promoters. However, H3K4me3 is present at the promoter of the PTK7 gene only in the tumor sample. PTK7 is a gene known to be up-regulated in colon cancer.

Kit Components and Storage

Please store each component at the temperature indicated in the table below. **Do not re-freeze the Protein G Agarose Beads after you have received this kit**.

Reagents	Quantity	Storage
Protein G Agarose Beads*	500 µl	4°C
Proteinase K (10 µg/µl)	80 µl	-20°C
Blocker	100 µl	-20°C
Protease Inhibitor Cocktail (PIC)	100 µl	-20°C
Histone H3K4me3 pAb	2 x 10 µl	-20°C
Human Positive Control Primer Set GAPDH-2	400 µl	20°C
Human Negative Control Primer Set 2	400 µl	20°C
TE pH 8.0	1.5 ml	RT
ChIP Filtration Columns	16 ea	RT
ChIP Buffer	35 ml	RT
Wash Buffer AM1	100 ml	RT
Elution Buffer AM4	2 x 1.5 ml	RT
DNA Purification Binding Buffer	50 ml	RT
3 M Sodium Acetate	500 µl	RT
DNA Purification Wash Buffer**	10 ml	RT
DNA Purification Elution Buffer	5 ml	RT
DNA Purification Columns	16 ea	RT

* The Protein G Agarose Beads are shipped on dry ice, but **should not be re-frozen** by the customer. Upon receipt of this kit, the beads should be stored at 4°C.

**Requires the addition of ethanol before use.

Additional materials required

- A ChIP-validated antibody directed against the protein of interest
- 100% ethanol (absolute)
- DNase-free H_2O
- 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 & Thermocycler
- 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)

Protocol Overview and Time Table

	Required Time
Immunoprecipitation	Overnight incubation
Binding to Protein G agarose Beads	3 hours
Wash Immune Complexes	20 minutes
Reversal of Cross-links	2.5 hours
DNA Purification	15 minutes
qPCR Analysis	2 hours

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Buffer Preparation

Protease Inhibitor Cocktail (PIC)

Thaw 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.

Wash Buffer AM1

Is supplied ready to use.

Elution Buffer AM4

Is supplied ready to use.

DNA Purification Wash Buffer

The DNA Purification Wash Buffer requires the addition of ethanol before use. The final concentration of ethanol should be 80%. Add 40 ml of fresh 100% ethanol to the DNA Purification Wash Buffer bottle for a final volume of 50 ml. Invert repeatedly. The buffer can be stored at room temperature after the addition of ethanol. The ethanol only needs to be added before the first use, after that the Wash Buffer is ready for use.

3M Sodium Acetate

It is important to check the sodium acetate before use to ensure that the salts have not precipitated out of solution. Once the sodium acetate is in solution it should be stored at room temperature.

Protein G Agarose Beads

The supplied agarose beads require washing prior to use. Follow the instructions in the manual to wash the beads for use in the ChIP reactions. There is no need to pre-block the beads or pre-clear the sample. 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, but should not be re-frozen by the customer. Upon receipt, the beads should be stored at 4°C.**

Histone H3K4me3 pAb

Active Motif's Histone H3K4me3 pAb (Catalog No. 39916) 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 H3K4me3 is a mark of transcriptional activity and should be highly abundant in all sample types. Use 3 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 www.activemotif.com/chipprimers. The primers are provided at a concentration of 2.5 µM. Use 4 µl of each primer set per qPCR reaction.

Recommendations

ChIP-validated Antibody

We recommend using 4 µg antibody per ChIP reaction in a maximum volume of 30 µl. 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-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 www.activemotif.com/chipabs.

Chromatin Quantity

It is recommended to use a minimum of 200 ng chromatin prepared using Active Motif's ChIP-IT FFPE Chromatin Preparation Kit (Catalog No. 53030) for each ChIP reaction in a total ChIP reaction volume of 200 µl. 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 µl ChIP reaction volume, we suggest preparing multiple ChIP reactions at 200 µl 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.

Section A. Immunoprecipitation

Successful chromatin immunoprecipitation depends on the quality of the chromatin preparation, the affinity of the ChIP antibody and the abundance of the target protein. Chromatin should be prepared and validated using Active Motif's ChIP-IT FFPE Chromatin Preparation Kit (Catalog No. 53030). A minimum of 200 ng per ChIP reaction is required for successful enrichment, but using a larger quantity of chromatin is recommended when possible.

1. Thaw sonicated chromatin on ice. Spin chromatin at 4°C in a microcentrifuge at maximum speed for 2 minutes.
2. Set up the ChIP reactions by adding the components in the order shown in Table 1 below to 1.5 ml microcentrifuge tubes. Be sure to use the DNA concentration that was determined for your sonicated chromatin sample to calculate the volume to use. We recommend using a minimum of 200 ng chromatin per ChIP reaction.
3. In a 1.5 ml microcentrifuge tube prepare the antibodies to be used in the ChIP reactions. Use a separate tube for each antibody. To the tube add 5 µl Blocker and 4 µg ChIP antibody. (Antibody volume should not exceed 30 µl per reaction). Incubate Antibody/Blocker mix for 1 minute at room temperature and then add to the ChIP reactions.

Table 1

Reagent	1 reaction
Sheared Chromatin (200 ng - 10 µg)	X µl
ChIP Buffer	adjust up to 200 µl
Protease Inhibitor Cocktail (PIC)	5 µl
Antibody/Blocker mix (from Step 3)	not to exceed 35 µl
Maximum Volume Allowed	**240 µl**

4. Cap tubes and incubate on an end-to-end rotator overnight at 4°C.
5. The Protein G agarose beads require washing before use. Transfer 30 µl Protein G agarose beads for each IP reaction to a 1.5 ml microcentrifuge tube. Add an equal volume of TE, pH 8.0 and invert to mix. Spin at 1250 x g in a microcentrifuge for 1 minute. Remove the supernatant equivalent to the volume of TE added to the agarose beads.

   Note: Before pipetting the Protein G agarose beads, they should be fully resuspended by inverting the tube. When pipetting the beads, cut 2 mm from the end of a pipet tip to prevent the tip from becoming clogged.

6. Wash the beads a second time with the same volume of TE, pH 8.0. Invert to mix. Spin at 1250 x g for 1 minute in a microcentrifuge. Remove the supernatant equivalent to the volume

of TE added to the agarose beads. The beads are now ready to use.

7. Spin the ChIP reactions at 1250 x g for 1 minute to collect liquid from the inside of the cap.
8. Using a cut pipet tip, add 30 µl washed Protein G agarose beads to each immunoprecipitation reaction. Cap tubes and incubate on an end-to-end rotator at 4°C for 3 hours.
9. 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 (see Figure 3 below).
10. Remove ChIP reactions from rotator and spin at 1250 x g for 1 minute to collect liquid from inside of the cap.
11. Add 600 µl ChIP Buffer to each ChIP reaction then transfer the entire reaction (including the protein G agarose beads) to its labeled column. Allow flow-through to occur by gravity.
12. During the gravity flow, transfer 100 µl per ChIP reaction of Elution Buffer AM4 to a 1.5 ml microcentrifuge tube and allow to pre-warm at 37°C during the wash steps.
13. Wash each column with 900 µl Wash Buffer AM1. Let stand for 3 min.
14. Repeat Step 13 four more times for a total of five washes.
15. Transfer columns to a new 1.5 ml microcentrifuge tube and spin in a room temperature microcentrifuge at 1250 x g for 3 minutes to remove residual Wash Buffer.
16. Following the spin, transfer the ChIP Filtration Columns to new 1.5 ml microcentrifuge tubes. Add 50 µl 37°C Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes. Spin in a room temperature microcentrifuge at 1250 x g for 3 minutes.
17. With columns remaining in the same microcentrifuge tube, add another 50 µl 37°C Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes and spin in a room temperature microcentrifuge at 1250 x g for 3 minutes.
18. Discard the ChIP Filtration Columns. The flow-through (~100 µl volume) contains the ChIP DNA. Proceed to Section B: Reversal of Cross-links and DNA purification.

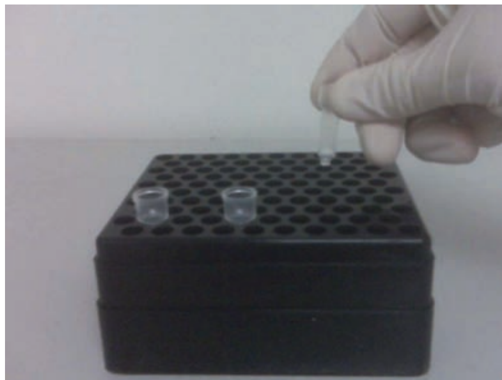


Figure 3: Using the ChIP Filtration Columns.
Remove the tab from the bottom of the ChIP Filtration Columns and place columns in an empty 1 ml pipet tip box to perform the wash steps.

Protocols – ChIP DNA Purification

Section B. Reversal of Cross-links and DNA Purification

1. Transfer each eluted ChIP DNA to a 250 µl PCR tube and add 2 µl Proteinase K. Vortex to mix and heat in a thermocycler at 55°C for 30 minutes and then increase the temperature to 80°C for 2 hours.
2. Transfer the DNA to a 1.5 ml microcentrifuge tube and add 5 volumes (500 µl) DNA Purification Binding Buffer to each tube and vortex to mix. Adjust the pH with 5 µl 3M Sodium Acetate. The sample should be bright yellow in color to indicate a proper pH. If your sample is not bright yellow, please refer to the Troubleshooting guide in the Appendix on page 17 for details to adjust pH prior to loading the sample into the purification column.
3. For each sample, place a DNA purification column (AM #103928) in the collection tube and add each pH adjusted sample to its own column. Close the cap on each column, place them with the collection tubes in a microcentrifuge and spin them at 14,000 rpm for 1 minute.
4. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
5. Prepare DNA Purification Wash Buffer (AM #103497) before the first use. Follow the instruction on page 8 for the addition of ethanol prior to using the solution. Add 750 µl DNA Purification Wash Buffer to each column and cap the column.
6. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
7. For ChIP-Seq applications, we recommend pre-warming the required DNA Purification Elution Buffer (AM #103498) volume (see Step 10b below) at 37°C for 5 minutes prior to use.
8. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
9. With the column cap open, spin at 14,000 rpm for 2 minutes in a microcentrifuge to remove any residual Wash Buffer from the column.
10. Transfer the column to a clean microcentrifuge tube. Depending on the method to be used for downstream analysis of the ChIP enriched DNA, use the appropriate elution volume.
    a. **For qPCR analysis:** add 50 µl of DNA Purification Elution Buffer (AM #103498) to the center of the column matrix and incubate for 1 minute at room temperature. Spin at 14,000 rpm for 1 minute in a microcentrifuge. Add an additional 50 µl DNA Purification Elution Buffer to the column and incubate for 1 minute at room temperature. Spin at 14,000 rpm for 1 minute in a microcentrifuge. Total elution volume is 200 µl.
    b. **For ChIP-Seq analysis:** add 36 µl of 37°C DNA Purification Elution Buffer (AM #103498) to the center of the column matrix and incubate for 1 minute at room temperature. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
11. Discard column. Purified DNA may be stored at -20°C for future use.

Section C. 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. The ChIP-IT qPCR Analysis Kit provides consistency in data analysis and allows direct comparison across samples and experiments. If not using the ChIP-IT qPCR Analysis Kit, qPCR data normalization and graphing can be done using the methods described in Section E.

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 14 to dilute the ChIP DNA for use in qPCR. Input DNA that was prepared in the ChIP-IT FFPE Chromatin Preparation Kit (Catalog No. 53030) 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**

* 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 www.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 I.

Section D. ChIP-Seq

The ChIP-IT FFPE Kit has been validated for whole genome analysis using ChIP-Seq. This process involves the preparation of libraries from ChIP DNA by the addition of adapter sequences to the ends of the DNA fragments. The library is then PCR amplified and validated prior to sequencing.

Commercial kits are available to prepare the ChIP DNA libraries for sequencing such as Illumina® ChIP-Seq DNA Sample Prep Kit (Catalog No. IP-102-1001) which is compatible with sequencing on HiSeq 2000, HiSeq 1000, HiScanSQ or Genome Analyzer. For 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 C: 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 6 µl of ChIP DNA in 94 µl DNA Purification Elution Buffer (AM #103498). Use 5 µl of the diluted ChIP DNA per qPCR reaction.
- Library generation usually requires 10 ng of ChIP enriched DNA. However, by using the ChIP-IT FFPE Kit the enriched DNA is of such a high quality that sequencing libraries can be generated from sub-nanogram levels of ChIP DNA. 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 (See Figure 4 on page 17). If the qPCR enrichments are satisfactory then the remaining 30 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 E: qPCR Primer Design and Data Analysis

A. Design of the primers

- Design and analyze your potential primer pairs using an *in silico* PCR program (*i.e.* Primer3 at http://frodo.wi.mit.edu/ or the UCSC Genome Browser at http://genome.cse.ucsc.edu/cgi-bin/hgPcr).
- Primers that dimerize should be avoided, as they will be bound by SYBR Green, which will compromise accurate quantitation. You can test your primers for self-complementarity and secondary structure at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.
- 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 www.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 µl and used 5 µl 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 x 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 µg 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%.

Example of ChIP enrichment analysis using Active Motif's qPCR Analysis Kit (Catalog No. 53029)

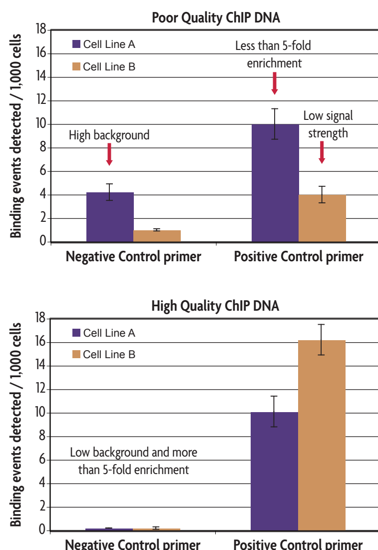


Figure 4: Comparison of qPCR results showing good versus poor enrichment over negative control primers.
Data shows qPCR results analyzed using the ChIP-IT qPCR Analysis Kit with the Human Negative Control Primer as a reference and a gene-specific positive control primer. In the top image the negative control primer set for Cell Line A gives high background levels with Binding events detected per 1,000 cells above a value of 2, while Cell Line B has positive control signal levels below 5 Binding events detected per 1,000 cells. The bottom image shows low background in the negative control primer set with Binding events detected per 1,000 cells below a value of 2. The fold enrichment of the positive control primer set exceeds 5-fold. Only the ChIP DNA from the bottom sample is recommended for use in ChIP-Seq.

Section F. Troubleshooting Guide

Problem/question	Recommendation
Performing ChIP with a large volume of chromatin.	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.
ChIP DNA does not turn bright yellow following the addition of 3 M sodium acetate	If the color is light orange or violet, this indicates the pH is too high. Add more 3 M sodium acetate 5 µl at a time, mixing after addition until the color is bright yellow. This step is crucial to the success of DNA binding and purification. For a full color image please see the manual for Active Motif's Chromatin IP DNA Purification Kit Catalog No. 58002 available online at our website www.activemotif.com.
High background.	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.
	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.
Poor or no enrichment with target antibody.	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.
	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 H3K4me3 (Catalog No. 39915) 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).
	Low-affinity antibody. Use a different antibody.
	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.
	Problems with PCR. Confirm the amplified sequence for the positive control primer set is bound by the antibody target. Identify other binding sites.
No PCR products for the ChIP'd samples (but the Input DNA yields the correct PCR product)	Increase the amount of chromatin used in the ChIP reaction, the amount of antibody used, or both.
	Use a different antibody.

Problem/question	Recommendation
No PCR products with real-time PCR	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.
	No ethanol in DNA Purification Wash Buffer. Make sure that ethanol has been added to the DNA Purification Wash Buffer prior to first use.

Section G. Related Products

ChIP-IT® Kits	Format	Catalog No.
ChIP-IT® High Sensitivity	16 rxns	53040
ChIP-IT® Express	25 rxns	53008
ChIP-IT® Express Enzymatic	25 rxns	53009
ChIP-IT® Express Shearing Kit	10 rxns	53032
ChIP-IT® Express Enzymatic Shearing Kit	10 rxns	53035
ChIP-IT® Express HT	96 rxns	53018
Re-ChIP-IT®	25 rxns	53016
RNA ChIP-IT®	25 rxns	53024
Chromatin IP DNA Purification Kit	50 rxns	58002
EpiShear™ Multi-Sample Sonicator	110 V	53062
EpiShear™ Probe Sonicator	110 V	53051
ChIP-IT® Protein G Magnetic Beads	25 rxns	53014
Siliconized Tubes, 1.7 ml	25 tubes	53036
ChIP-IT® qPCR Analysis Kit	10 rxns	53029
ChIP-IT® Control qPCR Kit – Human	5 rxns	53026
ChIP-IT® Control qPCR Kit – Mouse	5 rxns	53027
ChIP-IT® Control qPCR Kit – Rat	5 rxns	53028
ChIP-IT® Control Kit – Human	5 rxns	53010
ChIP-IT® Control Kit – Mouse	5 rxns	53011
ChIP-IT® Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015
Ready-to-ChIP Hep G2 Chromatin	10 rxns	53019
Ready-to-ChIP K-562 Chromatin	10 rxns	53020
Ready-to-ChIP NIH/3T3 Chromatin	10 rxns	53021
Bridging Antibody for Mouse IgG	500 µg	53017
Dounce Homogenizer	1 ml	40401
Dounce Homogenizer	15 ml	40415

ChIP-validated Antibodies

For an up-to-date list of over 125 ChIP-validated antibodies, please visit **www.activemotif.com/chipabs**.

Whole Genome Amplification	Format	Catalog No.
GenoMatrix™ Whole Genome Amplification Kit	1 kit	58001

Co-Immunoprecipitation	Format	Catalog No.
Nuclear Complex Co-IP Kit	50 rxns	54001
Universal Magnetic Co-IP Kit	25 rxns	54002

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

Active Motif North America
1914 Palomar Oaks Way, Suite 150
Carlsbad, CA 92008
USA
Toll Free: 877 222 9543
Telephone: 760 431 1263
Fax: 760 431 1351
E-mail: tech_service@activemotif.com

Active Motif Europe
Avenue Reine Astrid, 92
B-1310 La Hulpe, Belgium
UK Free Phone: 0800 169 31 47
France Free Phone: 0800 90 99 79
Germany Free Phone: 0800 181 99 10
Telephone: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050
E-mail: eurotech@activemotif.com

Active Motif Japan
Azuma Bldg, 7th Floor
2-21 Ageba-Cho, Shinjuku-Ku
Tokyo, 162-0824, Japan
Telephone: +81 3 5225 3638
Fax: +81 3 5261 8733
E-mail: japantech@activemotif.com

Visit Active Motif on the worldwide web at **http://www.activemotif.com**

At this site:

- Read about who we are, where we are, and what we do
- Review data supporting our products and the latest updates
- Enter your name into our mailing list to receive our catalog, *MotifVations* newsletter and notification of our upcoming products
- Share your ideas and results with us
- View our job opportunities

Don’t forget to bookmark our site for easy reference!