ChIP-IT® FFPE
Chromatin Preparation

(version A4)

Catalog No. 53030

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

Revision History

Revision	Date	Description of Change
A4	Jan 2019	Updated recommendations to include second-generation FFPE ChIP Kits.

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

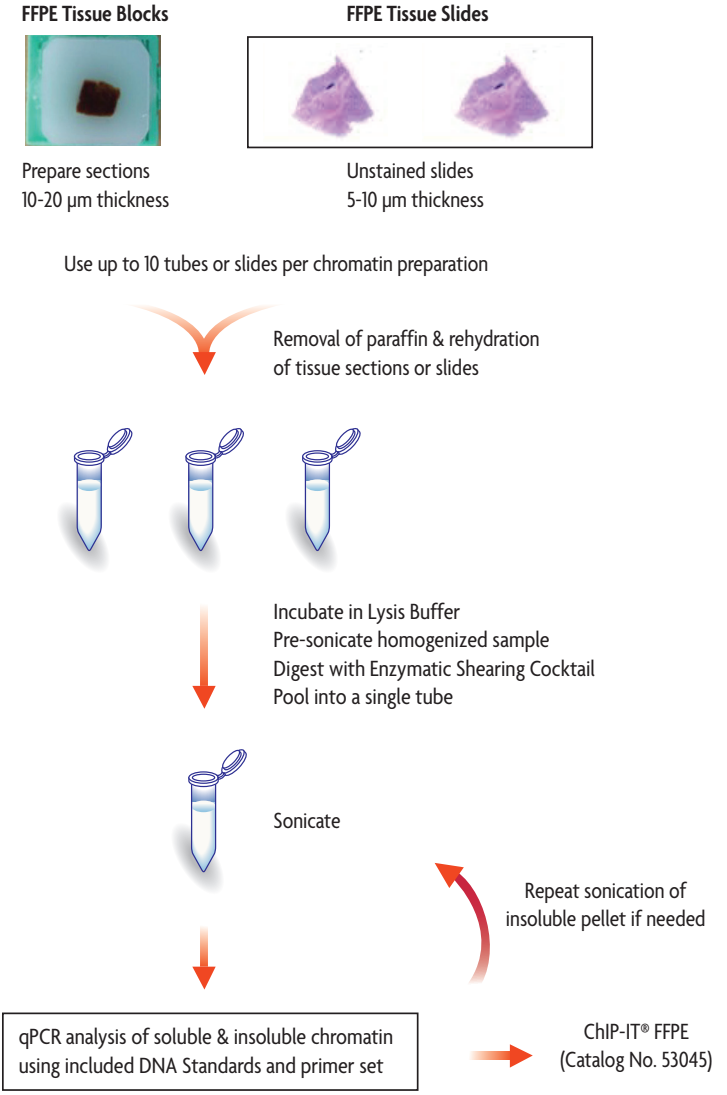
Formalin-fixed paraffin-embedded (FFPE) tissue blocks and histology slides are a valuable resource for retrospective research on clinical samples. Clinical information, treatments and outcomes are often available for these sample types and large collections of FFPE material is commercially available. 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 difficult to extract FFPE samples (for high quality FFPE samples, use the ChIP-IT FFPE Chromatin Preparation II Kit, Cat. No. .53031). This chromatin is then used in the ChIP-IT FFPE II Kit, which 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 Chromatin Preparation Kit contains sufficient reagents for 5 chromatin preparations using up to 10 unstained tissue slides or FFPE block sections per chromatin preparation. It is necessary to use Active Motif's ChIP-IT FFPE II Kit for the downstream ChIP reactions due to the limited yields obtained from FFPE samples and the ChIP-IT FFPE II Kit's high level of sensitivity. 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 Chromatin Preparation II Kit	5 rxns	53031
ChIP-IT® FFPE II Kit	16 rxns	53047

Flow Chart of Process



Flow Chart of the ChIP-IT FFPE Chromatin Preparation Kit.

In ChIP-IT FFPE Chromatin Preparation, 10-20 µm sections from FFPE tissue blocks, or 5-10 µm sections from unstained FFPE slides are used as the starting material. Up to ten tubes or slides can be used for each chromatin preparation in order to obtain sufficient yield for downstream ChIP analysis. The paraffin is removed and the tissue is rehydrated from each slide/tube. The tissue then undergoes an incubation in lysis buffer, tissue homogenization, a 30 second sonication followed by digestion with Enzymatic Shearing Cocktail. After a centrifugation step, all remaining sample is pooled into a single tube for sonication. A final centrifugation is performed and the soluble and insoluble chromatin is analyzed and quantified by qPCR.

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 difficult to extract 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 II Kit. This second generation ChIP Kit 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. 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 Chromatin Preparation Advantages:

- Obtain quality chromatin using difficult to extract histological slides or tissue blocks as the starting material
- Optimized reagents help preserve the quality of the chromatin during extraction
- 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 DNA and PCR primers to quantify the chromatin and confirm the shearing efficiency prior to use in ChIP

Detection limit: The limits of the assay will depend on the size and tissue type and may require optimization. See Table 1 below for some examples of FFPE samples and quantities used in chromatin preparation. Our protocol offers guidelines and troubleshooting tips for processing samples to obtain the required 200 ng minimum of chromatin per ChIP reaction. Chromatin should be validated prior to use in the ChIP-IT FFPE II Kit (Catalog No. 53047).

Tissue Type	Sample used per chromatin preparation
Human Colon	Tissue block – five 20 µm sections
Human Kidney	Tissue block – twenty-five 20 µm sections
Human Lung	Tissue block – two 20 µm sections
Rat whole brain	5 slides – two 5 µm sections per slide
Rat hippocampus	25 slides – two 5 µm sections per slide

Table 1: Examples of FFPE samples successfully used in ChIP-IT FFPE.

Product Performance: The ChIP-IT FFPE Chromatin Preparation Kit assumes that FFPE tissues are already available as standard sections. 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. The ChIP-IT FFPE Chromatin Preparation Kit provides recommendations and guidelines to assist in the extraction process, but Active Motif cannot guarantee successful chromatin preparations for variables that fall outside of our assay kit.

If preparing samples for FFPE preservation, it is important to minimize the time between the resection of the tissue and the fixation in formalin to generate high quality FFPE tissues. Samples should be fixed immediately and stored in a cool, dry location. With human samples, due to the need for surgical removal, there is often variability in the time from resection to fixation. For animal models, *in vivo* perfusion can be used to generate high quality FFPE tissues.

ChIP-IT® FFPE Chromatin Preparation

Extraction using FFPE slides (5-10 µm thickness) or tissue sections (10-20 µm thickness)

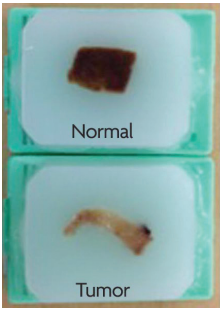


Figure 1: FFPE tissue blocks of normal and tumor sample from human colon stored for over 10 years.

Images of normal and tumor human colon FFPE blocks used in the ChIP-IT FFPE Chromatin Preparation Kit. These samples had been stored in an uncontrolled environment for more than 10 years. Five 20 µm sections from each sample were pooled and processed to obtain enough chromatin for use in the ChIP-IT FFPE Kit.

Evaluating chromatin quantity and quality using qPCR

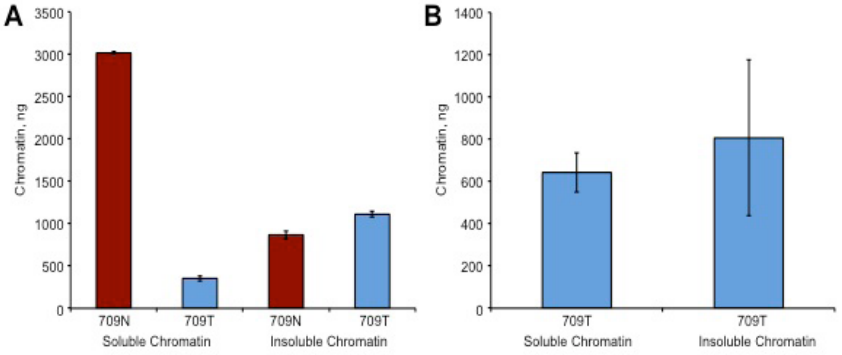


Figure 2: Analysis of chromatin quality and quantity using qPCR.

FIve 20 µm sections each of normal and tumor human colon samples were processed using the ChIP-IT FFPE Chromatin Preparation Kit. A sample of both the soluble chromatin and the insoluble pellet 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. Image A shows the results of the initial chromatin preparation. Chromatin from the normal sample was primarily in the soluble fraction and based on the DNA quantification using the provided DNA standards was of a sufficient quantity to move forward with ChIP. Chromatin from the tumor sample was still primarily in the insoluble pellet. The pellet was then treated to another round of sonication and samples of the secondary sonication soluble fraction and pellet were reverse cross-linked, proteinase K treated and purified for qPCR analysis. Image B shows the results of the secondary sonication which increased the amount of soluble chromatin. The soluble chromatin from the first and second round of sonication were combined, quantified and used for ChIP.

ChIP-IT FFPE qPCR and ChIP-Seq Results

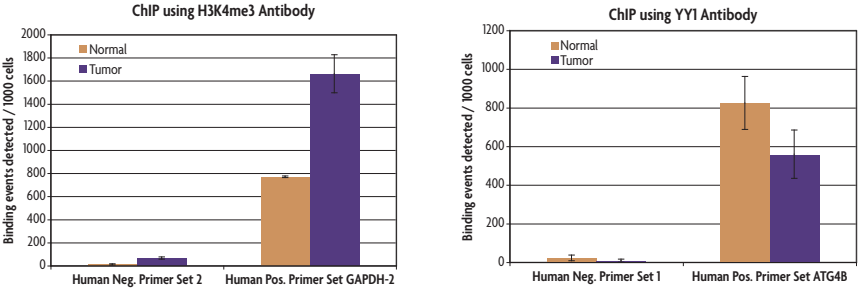


Figure 3: qPCR analysis of normal and tumor human colon samples assayed using the ChIP-IT FFPE Kit

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.

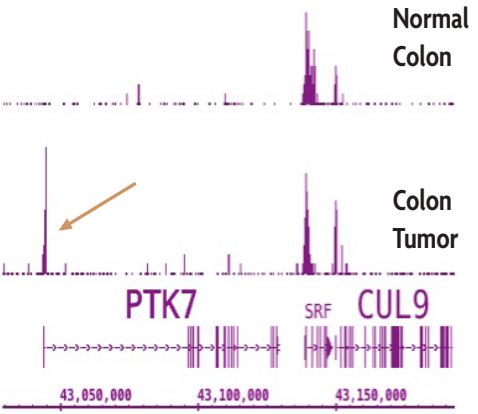


Figure 4: H3K4me3 ChIP-Seq on normal and tumor human colon FFPE samples.

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. Reagents are for research use only.

Reagents	Quantity	Storage
Paraffin Removal Solution	10 x 25 ml	RT
ChIP Buffer	30 ml	RT
5M NaCl	400 µl	RT
TE, pH 8.0	3 x 1.5 ml	RT
DNA Purification Elution Buffer	5 ml	RT
Lysis Buffer AM5	25 ml	4°C
Digestion Buffer AM2	26 ml	4°C
RNase A (10 µg/µl)	3 x 45 µl	-20°C
Proteinase K (10 µg/µl)	200 µl	-20°C
Protease Inhibitor Cocktail	500 µl & 100 µl	-20°C
Precipitation Buffer	2 x 1.5 ml	-20°C
Carrier	2 x 35 µl	-20°C
Enzymatic Shearing Cocktail	10 µl	-20°C
DNA Standard AM1	100 µl	-20°C
DNA Standard AM2	100 µl	-20°C
DNA Standard AM3	100 µl	-20°C
Human Positive Control Primer Set GAPDH-2	400 µl	-20°C

Additional materials required

- Dounce homogenizer (*e.g.* Active Motif Catalog Nos. 40401 & 40415) with both tight and loose fitting pestles. Use of a homogenizer is necessary to homogenize samples and aide in the lysis of cells and nuclei.
- Razor blades
- 100% ethanol (absolute)
- Milli-Q H_2O
- Vortex
- Heat block
- 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
- 15 ml conical tubes
- Phenol and chloroform/isoamyl alcohol (24:1) or Phenol/chloroform/isoamyl alcohol (25:24:1) (DNA Purification, Molecular Biology Grade)
- Fluorometer and/or qPCR instrument for DNA quantitation
- Pipettors and tips (filter tips are recommended)
- Sonicator (*e.g.* Active Motif's EpiShear™ Sonicator with a 1/8" probe (Catalog No. 53051) with the EpiShear™ Cooled Sonication Platform (Catalog No. 53080))
- Hand-held homogenizer (*e.g.* Biospec Products Tissue-Tearor, or Kontes Microtube Pellet Pestle®)
- (Optional) ChIP-IT® qPCR Analysis Kit (Catalog No. 53029)
- (Optional) SYBR Green qPCR master mix (USB Catalog No. 75762)

Protocol Overview and Time Table

	Required Time
Deparaffinization and rehydration of FFPE material	2.5 hours
Tissue homogenization & pre-sonication	1.5 - 2.5 hours
Enzymatic Digestion	5 minutes
Sonication	40 minutes per sample
Input Preparation	5 hours
qPCR Analysis of Input	2 hours

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Buffer Preparation

Paraffin Removal Solution

Is supplied ready to use. Appropriate safety precautions (*i.e.* safety glasses, gloves and lab coat) should be used when working with the Paraffin Removal Solution as it is a skin irritant. It is recommended to work with the paraffin removal solution in a biosafety hood to avoid inhalation. Please discard the paraffin removal solution into a glass container for organic solvents and dispose of in accordance with local and federal regulations. **Do not discard down the sink.**

Lysis Buffer AM5

Is provided ready to use.

ChIP Buffer

Is provided ready to use.

Digestion Buffer AM2

Is provided ready to use.

Enzymatic Shearing Cocktail

The Enzymatic Shearing Cocktail is provided at a concentration of 20 U/µl. Immediately before use, the enzymatic shearing cocktail should be diluted 1:200 into Digestion Buffer AM2 by adding 1 µl Enzymatic Shearing Cocktail to 200 µl Digestion Buffer AM2. Use 20 µl (2 units) of diluted enzymatic shearing cocktail per tube.

DNA Purification Elution Buffer

Is provided ready to use.

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.

DNA Standards AM1, AM2 and AM3

Due to the low quantities of chromatin recovered from FFPE samples, it is necessary to quantify the chromatin using qPCR analysis against known quantities of DNA, such as those provided in DNA Standards AM1, AM2 and AM3. In the qPCR instrument, set the values for DNA Standard AM1 to 2.5 ng/µl, DNA Standard AM2 to 0.25 ng/µl and DNA Standard AM3 to 0.025 ng/µl. Use 2 µl of each DNA standard in the qPCR reactions.

Alternatively, a fluorometric method, such as Qubit™ Fluorometric Quantitation, can be used to quantify the chromatin input. Nanodrop readings are not sensitive enough to detect the low quantities of DNA and, therefore, use of the Nanodrop for DNA quantification is not recommended.

Human Positive Control Primer Set GAPDH-2

The Human Positive Control Primer Set GAPDH-2 is provided for use in the qPCR quantification of the chromatin input. This primer set can be used to amplify DNA Standards AM1, AM2, AM3 and chromatin input samples. The primer is provided at 2.5 µM. Use 1 µl per qPCR reaction. Alternatively, a positive control primer set specific for a human gene-of-interest can be used to amplify the DNA Standards and chromatin samples for quantification.

If not using human material, you will need to design appropriate qPCR primer sets to match your species. 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. To see a list of validated species-specific qPCR primers designed according to these recommendations, visit www.activemotif.com/chipprimers.

Recommendations

Chromatin Shearing Tips

ChIP experiments require chromatin that has been sheared to a size of 200-1200 bp. Due to the limited sample size and yield, there is usually insufficient chromatin available for analysis of shearing efficiency by agarose gel electrophoresis. Instead, we suggest analyzing the quantity and quality of the chromatin preparation through qPCR. The included DNA Standards and qPCR Primer set enables quantification of the chromatin input. Both the soluble chromatin fraction and the insoluble pellet should be analyzed to determine the efficiency of the chromatin preparation. If a majority of the chromatin is still found in the insoluble fraction, the pellet should undergo additional sonication and extraction preparation. The soluble chromatin that is obtained from the additional processing can be combined with the original soluble fraction and quantified.

In general, shearing efficiency is improved through the use of a small shearing volume and a V-bottom tube rather than a round-bottom tube. Also, note that shearing is inefficient if the chromatin sample becomes emulsified with air bubbles. To determine the appropriate shearing level for your sample, set up a "practice" tube containing only ChIP Buffer. Slowly increase the sonication amplitude until foaming starts to occur. Reduce the amplitude setting down slightly and mark this as the highest possible intensity to use without foaming. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample at maximum speed for 4 minutes at 4°C in a microcentrifuge to remove trapped air. Finally, to prevent overheating and denaturation of chromatin, samples should be kept on ice as much as possible during shearing, and shearing should be performed discontinuously (*i.e.* sonicate for 30 seconds, then place on ice/water for 30 seconds, sonicate again for 30 seconds, *etc.*). If possible, shear while on ice or use Active Motif's EpiShear Cooled Sonication Platform (Catalog No. 53080) to help regulate sample temperature.

Chromatin Quantity

A minimum of 200 ng chromatin is needed per ChIP reaction in Active Motif's ChIP-IT FFPE Kit. The volume of the ChIP reaction should not exceed 200 µl. If more chromatin is available, it is recommended to use larger quantities per ChIP in order to improve the efficiency of the ChIP reaction. If insufficient chromatin is obtained, we suggest evaluating the insoluble chromatin pellet or processing additional tissue sections or slides to obtain the required chromatin quantity.

Safety Precautions

Paraffin Removal Solution is a skin irritant. Appropriate safety precautions (*i.e.* safety glasses, gloves and lab coat) should be used. It is recommended to work with the paraffin removal solution in a biosafety hood to avoid inhalation. Please discard the paraffin removal solution into a glass container for organic solvents and dispose of in accordance with local and federal regulations. **Do not discard down the sink.** Also, chromatin sonication should be performed in a biosafety hood if the chromatin is extracted from biohazardous or infectious materials.

Section A: Removal of Paraffin and Rehydration of FFPE Tissue Sections Mounted on Slides

This protocol describes processing FFPE tissue sections mounted on slides. Depending on the size and tissue type, it may be necessary to process multiple slides together within a single chromatin preparation in order to obtain enough chromatin for DNA quantification and downstream ChIP analysis using the ChIP-IT FFPE Kit (Catalog No. 53045). This protocol is designed for a maximum of 10 slides to be combined per chromatin preparation. The kit contains enough material for 5 chromatin preparations and the analysis of 25 Input samples.

1. Prepare appropriate number of slides (5-10 µm thickness) required to obtain sufficient soluble chromatin for DNA quantification and ChIP analysis. A minimum of 200 ng chromatin is required for each ChIP reaction in the ChIP-IT FFPE Kit. We suggest starting with at least 5 slides per chromatin preparation. Enough reagent is provided to process 10 slides per chromatin preparation.
2. Start the deparaffinization process by applying 1 ml Paraffin Removal Solution to each slide to be processed. Incubate for 10 minutes at room temperature.
3. Carefully discard the solution using the appropriate glass disposal container. Do not discard down the sink. Repeat Step 2 three more times for a total of 4 treatments.
4. Transfer the deparaffinized tissue into a coupling jar containing absolute (100%) ethanol and incubate 10 minutes at room temperature.
5. To start rehydration of the tissue, transfer the samples into a coupling jar containing 95% (vol/vol) ethanol solution and incubate 10 minutes at room temperature.
6. Continue the rehydration process of Step 5 by progressively increasing the percentage of water to obtain 70%, 50% and 20% ethanol (vol/vol) solutions, respectively, for each wash step. The final rehydration incubation should be in Milli-Q water only.
7. Carefully transfer the rehydrated tissue sections to a 1.5 ml microcentrifuge tube by scraping the tissue off the slides using a razor blade. If using multiple slides, you will want to start combining the sample material into one or two tubes as soon as possible. If there is too much material to properly combine at this stage, continue to proceed with multiple tubes.
8. Proceed immediately to Section C: Tissue Homogenization and Chromatin Isolation.

Section B: Removal of Paraffin and Rehydration of FFPE Tissue Sections From FFPE Blocks

This protocol describes processing FFPE tissue sections from FFPE blocks. Depending on the size and tissue type, it may be necessary to process multiple sections together within a single chromatin preparation in order to obtain enough chromatin for DNA quantification and downstream ChIP analysis using the ChIP-IT FFPE Kit (Catalog No. 53045). This protocol is designed for a maximum of 10 tubes to be combined per chromatin preparation. Each tube may contain between 1-5 tissue sections each depending on the sample size. The kit contains enough material for 5 chromatin preparations and the analysis of 25 Input samples.

1. Prepare appropriate number of tissue blocks (10-20 µm thickness) required to obtain sufficient soluble chromatin for DNA quantification and ChIP analysis. A minimum of 200 ng chromatin is required for each ChIP reaction in the ChIP-IT FFPE Kit. We suggest starting with at least 5 sections per chromatin preparation. Enough reagent is provided to process 10 tubes per chromatin preparation. Place 1-5 tissue sections into a 1.5 ml microcentrifuge tube. The number of sections per tube will depend on the size of the sample and how much tissue material will fit into a single tube.
2. Start the deparaffinization process by applying 1 ml Paraffin Removal Solution to each tube to be processed. Incubate for 10 minutes at room temperature on a rotating platform.
3. Centrifuge at 18,000 x g for 5 minutes at room temperature. Carefully aspirate and discard the supernatant using the appropriate glass disposal container. Do not discard down the sink.
4. Repeat Steps 2 and 3 three more times for a total of 4 treatments.
5. After the last incubation and centrifugation, carefully remove and discard the supernatant. Add 1 ml absolute (100%) ethanol to the sample and incubate 10 minutes at room temperature on a rotating platform.
6. Centrifuge at 18,000 x g for 5 minutes at 4°C. The tissue may be very difficult to pellet following rehydration. Carefully aspirate and discard the supernatant taking care not to disturb the sample pellet. Add 1 ml 95% (vol/vol) ethanol solution and incubate 10 minutes at room temperature on a rotating platform.
7. Repeat Step 6 four more times, progressively increasing the percentage of water to obtain 70%, 50% and 20% ethanol (vol/vol) solutions, respectively, for each wash step. The final rehydration incubation should be in Milli-Q water only.
8. Centrifuge at 18,000 x g for 5 minutes at 4°C. The tissue may be very difficult to pellet following rehydration. Carefully aspirate and discard the supernatant taking care not to disturb the sample pellet.
9. Proceed immediately to Section C: Tissue Homogenization and Chromatin Isolation.

Section C. Tissue Homogenization and Chromatin Isolation

The section below describes the fragmentation of chromatin using a combination of enzymatic digestion and sonication. Sonication results may vary depending on the quality of the FFPE sample and the sonication device being used. This protocol has been validated using Active Motif's EpiShear™ Probe Sonicator in combination with an EpiShear™ Cooled Sonication Platform to maintain probe height and temperature consistency between samples.

1. If using multiple tubes, you will want to start combining the sample material into one or two tubes as soon as possible. If there is too much material to properly combine at this stage, continue to proceed with multiple tubes. Resuspend the rehydrated tissue in 500 µl Lysis Buffer AM5 containing 5 µl PIC and 1 µl RNase A per tube. Incubate on a rotating platform for 1 hour at room temperature. (Alternatively, the samples can be stored at 4°C overnight.)
2. Centrifuge at 18,000 x g for 5 minutes at room temperature. Carefully aspirate and discard the supernatant taking care not to disturb the sample pellet.
3. Resuspend each pellet in 500 µl Digestion Buffer AM2 containing 5 µl PIC.
4. Homogenize the tissue. If working with one or two tubes of 500 µl volume, use a hand held microtube pellet pestle for 30 seconds. If you are working with multiple tubes, or if the tissue is difficult to homogenize, pool all tubes from the same sample together and homogenize together using a tissue tearor for 40 seconds (Use a volume of 5 ml with the tissue tearor. Add additional Digestion Buffer containing PIC to achieve this volume if needed)
5. Transfer the chromatin to a dounce homogenizer and homogenize with the tight fitting pestle for 30 strokes. (You may have to start with a loose pestle and then proceed to the tight pestle once the tissue begins to homogenize.) If tissue was combined into one pool for homogenization, transfer the tissue to microcentrifuge tubes containing 0.5-1 ml sample per tube. Try to consolidate the material to one or two tubes if possible.
6. Sonicate the sample using the EpiShear Cooled Sonication Platform or an ice bath for 30 seconds with a probe tip sonicator at 42% amplitude for a 500 µl volume and at 63% amplitude for a 1 ml volume.
7. Prepare Enzymatic Shearing Cocktail according to the recommendations in the Buffer Preparation and Recommendation Section on page 9.
8. Pre-warm chromatin from step 6 at 37°C for one minute. Add 20 µl diluted Enzymatic Shearing Cocktail to each chromatin preparation tube. Vortex briefly to mix. Incubate at 37°C for 1-3 minutes.
9. Centrifuge at 18,000 x g for 5 minutes at room temperature. Carefully aspirate and discard the supernatant. If working with multiple tubes, you must combine the material into a single tube at this stage. Resuspend the pellet in 500 µl ChIP Buffer plus 5 µl PIC (maximum acceptable amount for resuspension is 1 ml ChIP Buffer containing 10 µl PIC) for the chromatin sonication.

   Note: See Troubleshooting guide in the Appendix for notes to increase solubility.

10. Sonicate the samples using a probe sonicator at 42% amplitude for a 500 µl volume and at 63% amplitude for a 1 ml volume. Sonicate the samples using the EpiShear Cooled Sonication Platform or an ice bath for 30 seconds on and 30 seconds off for 40 pulses (approximate sonication time is 40 minutes per sample).
11. Carefully remove cellular debris by centrifugation at maximum speed for 2 minutes at 4°C. Collect the supernatant in a new microcentrifuge tube. Resuspend the pellet in 200 µl ChIP Buffer. Transfer 25 µl of both the soluble (supernatant) and insoluble (pellet) fractions to a 250 µl PCR tube. Label each tube as soluble Input and insoluble Input respectively. Store the remainder of each chromatin fraction at -80°C.
12. Process the Input fractions according to Section D. The Input DNA will be used to quantify the chromatin and evaluate the shearing efficiency.

Section D. Input Preparation

1. To each 25 µl Input preparation (both soluble and insoluble) from Step 12 above, add 175 µl TE pH 8.0 and 2 µl RNAse A. Cap the PCR tubes and vortex to mix.
2. Incubate in a thermocycler at 37°C for 30 minutes
3. Add 5 µl Proteinase K to each tube, vortex and incubate in a thermocycler at 55°C for 30 minutes.
4. Add 10 µl 5 M NaCl, vortex and incubate at 80°C for 2 hours to reverse cross-links.
5. Remove tubes from the thermocycler and add 250 µl phenol and 125 µl chloroform:isoamyl alcohol (24:1). Vortex vigorously and spin tubes in a room temperature microcentrifuge at maximum speed for 2 minutes.

   Note: Alternatively, an equal sample volume of phenol/chloroform/isoamyl alcohol (25:24:1) can be added to the sample for DNA purification. Due to the large amount of debris, it is recommended to perform Step 5 twice if using the phenol/chloroform/isoamyl alcohol mixture. Proceed to Step 7 below.

6. Transfer each upper aqueous layer to a new 1.5 ml microcentrifuge tube and add 250 µl chloroform:isoamyl alcohol (24:1). Vortex vigorously and spin tubes in a room temperature microcentrifuge at maximum speed for 2 minutes.
7. Transfer the upper aqueous layer to a new 1.5 ml microcentrifuge tube. Add 83 µl Precipitation Buffer, 2 µl Carrier and 900 µl absolute ethanol. Vortex to mix and chill at -80°C for 30 minutes to overnight.
8. Spin at 4°C in a microcentrifuge at maximum speed for 15 minutes.
9. Carefully remove the supernatant taking care not to disturb the pellet. Wash the pellet with 500 µl 70% ethanol and spin at 4°C in a microcentrifuge at maximum speed for 5 minutes.
10. Carefully remove the supernatant taking care not to disturb the pellet. Remove residual ethanol with a pipet tip. Leave the tubes uncapped and air dry for 10-15 minutes.
11. When the pellets are dry, add 25 µl DNA Purification Elution Buffer to each tube. Incubate

at room temperature for 10 minutes. If the pellet is difficult to resuspend, you can heat the pellet to 65°C for 10 minutes to help with solubilization. Then, vortex to ensure the pellet is completely resuspended. This solution contains your Input DNA.

12. Due to the low DNA concentration of the Input material from FFPE chromatin extractions, we do not recommend analysis of the shearing efficiency by agarose gel electrophoresis. Instead, we recommend evaluating the quality and quantity of the chromatin by qPCR analysis. Alternatively, DNA quantity can also be determined with use of a fluorometer, such as Qubit. The Nanodrop is not recommended for quantification as the DNA quantities are usually below the threshold that the Nanodrop can accurately quantify.

Section E. Analysis of Input DNA and Chromatin Quality

1. Set up a qPCR reaction to quantify the chromatin Input and to evaluate the solubility of the chromatin. Use the provided DNA Standards and Human Positive Control GAPDH-2 PCR Primer Set to analyze the Input DNA. 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.* USB Cat # 75762) and preparing triplicate reactions.

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

* If not using human samples, see notes in Buffer Preparation on page 10.

2. Place the PCR tubes in a real time PCR instrument. Using the software for your qPCR machine, assign a value of 2.5 ng/µl to DNA Standard AM1, a value of 0.25 ng/µl to DNA Standard AM2 and a value of 0.025 ng/µl to DNA Standard AM3. These values will be used to quantify your sample chromatin Input.

3. Place tubes in a real time PCR instrument and program as below:

   95°C for 2 minutes
   (95°C for 3 seconds, 58°C for 30 seconds, 72°C for 30 seconds) for 40 cycles

4. 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.

5. Your qPCR instrument will assign values (in ng/µl) to each qPCR reaction based on the values assigned to DNA Standards AM1, AM2 and AM3. If your machine does not average your triplicate reactions automatically, you will need to calculate these averages.

6. For each qPCR reaction you will have used a percentage of your total chromatin. In order to calculate the total amount in each chromatin fraction, multiply the recovered volume

of the soluble chromatin fraction (~500 µl) or insoluble chromatin fraction (~200 µl) by the calculated concentration.

7. Generate a graph to compare the total chromatin yield (ng) for both the soluble and insoluble fractions for each chromatin preparation (See Figure 5).

   Evaluate the concentration of the chromatin in the soluble fraction (supernatant).

   a. A minimum of 200 ng is required for each downstream ChIP reaction, although more is recommended if possible. Ensure your soluble fraction contains enough chromatin to perform ChIP reactions in duplicate with your ChIP antibody of interest. A positive control H3K4me3 antibody is included in the ChIP-IT® FFPE Kit (Catalog No. 53045) and one positive control ChIP reaction can be performed for each chromatin preparation.

   b. If enough chromatin is available in the soluble fraction to perform the desired ChIP reactions, the aliquots stored at -80°C from Section C, Step 11 can be used to perform the ChIP reactions using the ChIP-IT® FFPE Kit (Catalog No. 53045).

   Evaluate the concentration of the chromatin in the insoluble fraction (pellet)

   a. If the soluble fraction did not contain enough chromatin for ChIP, evaluate the amount of chromatin present in the insoluble fraction. If a majority of the chromatin is still in the pellet, it is recommended to perform additional sonication of the pellet to help fragment and solubilize the chromatin. Follow the instructions in Section F: Additional Sonication of Insoluble Pellet.

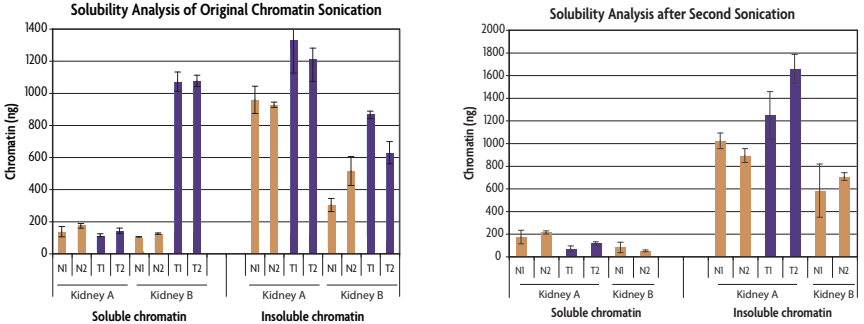


Figure 5: Solubility analysis of chromatin preparation from human kidney FFPE tissues.

Chromatin was extracted from two different case studies (A & B) from 10-year old histological sections of a human kidney with matched normal (N) and tumor (T) samples. Chromatin preparations were performed in duplicate. The soluble and insoluble chromatin fractions were analyzed by qPCR using the positive control GAPDH-2 primer set and DNA standards for quantification. The image on the left shows that only the tumor sample from case B had significant yields in the soluble fraction suitable for use in downstream ChIP analysis. The insoluble pellets of the normal sample from case B and both normal and tumor samples from case A were diluted to a 1 ml final volume with ChIP Buffer and subjected to a second round of sonication for 20 pulses at an amplitude of 63%. A 25 µl sample was collected from the new soluble and insoluble fractions and the input was reverse cross-linked, proteinase K treated and analyzed by qPCR using the GAPDH-2 primer set and DNA Standards (right image). Again, the solubility of the chromatin remained low. However, by combining the soluble fractions of the replicates, the chromatin yield was high enough to proceed with ChIP analysis.

Section F. Additional Sonication of Insoluble Pellet

Based on the results of the qPCR analysis in Section E, if there is a significant amount of chromatin remaining in the insoluble pellet, it is important to continue with additional processing of the pellet to release the chromatin. Additional processing of the pellet should also be performed if the yield of the soluble chromatin is too low to proceed with downstream ChIP reactions. A minimum of 200 ng chromatin is required for each ChIP reaction.

For further sonication, the soluble chromatin can be combined with the insoluble pellet and the entire mixture can undergo a second round of sonication and a second analysis by qPCR. This results in a single soluble and insoluble fraction and is ideal if the sample was overfixed or there was very little chromatin obtained in the original soluble fraction. One potential issue with this method is that further sonication of soluble chromatin may begin to affect the antigenicity of the chromatin, which could affect the quality of the chromatin for use in ChIP.

A second option would be to preserve the original soluble fraction and just process the insoluble chromatin pellet with a second round of sonication. Following sonication, the two soluble fractions can be combined and quantified by qPCR.

1. Dilute the insoluble chromatin pellet to a final volume of 500 µl - 1 ml using ChIP Buffer. Add 5 µl PIC.
2. Sonicate the samples using a probe sonicator at 42% amplitude for a 500 µl volume and at 63% amplitude for a 1 ml volume. Sonicate the samples using the EpiShear Cooled Sonication Platform or an ice bath for 30 seconds on and 30 seconds off for 20 - 40 pulses.
3. Carefully remove cellular debris by centrifugation at maximum speed for 2 minutes at 4°C. Collect the supernatant in a new microcentrifuge tube. Resuspend the pellet in 200 µl ChIP Buffer. Transfer 25 µl of both the soluble (supernatant) and insoluble (pellet) fractions to a 250 µl PCR tube. Label each tube as soluble Input and insoluble Input respectively. Store the remainder of each chromatin fraction at -80°C.
4. Prepare the Input DNA according to Section D. Then proceed to Section E for analysis of the soluble and insoluble fractions by qPCR.

Section G: qPCR Primer Design

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.

Section H. Troubleshooting Guide

Problem/question	Recommendation
At what points in the protocol can I stop?	The protocol may be stopped and samples stored at the times and temperatures below: 1. After rehydration of the tissue and resuspension in Lysis Buffer (C.1), 4°C 2. After enzymatic digestion and centrifugation (C.9) store the pellet, -80°C. 3. After sonication (C.11), -80°C. 4. During Input preparation DNA purification (D.7), -80°C
My sample does not pellet during tissue rehydration	Tissue may be difficult to pellet following rehydration. Carefully remove supernatant and discard. Continue to repeat centrifugation steps until the tissue is primarily left as a pellet with little to no liquid.
Difficulty with homogenization of tissue	Samples may behave differently during homogenization depending on the fixation conditions used to prepare the FFPE samples, the type of tissue you are working with or due to the differences between normal and tumor tissues. It may be necessary to combine multiple tubes of the same sample together and use a tissue tearor for 40 seconds to help disrupt the cells.
	It is recommended to perform dounce homogenization following use of a tissue homogenizer. Use a dounce homogenizer with a small clearance pestle (Active Motif Catalog Nos. 40401 & 40415). If the material is difficult to work with, start with a loose pestle to break up the cells and then switch to the tight-fitting pestle..
Low solubility of chromatin following sonication	Solubility can sometimes be increased by heating the samples prior to the single tube sonication (C.11). Samples can be heated for 2 hours at 65°C or for 2 hours at 80°C. The concern with the heat treatment is it increases the likelihood that there will be a reversal of cross-links on the chromatin which will decrease the efficiency of ChIP.
	Sonication samples were emulsified. Avoid emulsification by turning up the power of the sonicator gradually. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample for 4 minutes at 8,000 rpm in a 4°C microcentrifuge to remove trapped air.
Low chromatin yield	Poor quality FFPE samples. Depending on the formalin fixation and storage conditions, FFPE samples may experience degradation and loss of antigenicity over time. These samples may not be suitable for chromatin extraction and downstream ChIP analysis. Consider using alternative sample material.
	Consider using additional tissue material and repeating the chromatin preparation. Depending on the size and quality of the sample it may be difficult to obtain sufficient chromatin from 5 - 10 slides/sections.
	Additional processing of the insoluble pellet may be necessary to obtain additional chromatin. Follow the recommendations in Section F to dilute the insoluble pellet. Repeat the sonication with an additional 20-40 pulses. Evaluate the secondary sonication by qPCR.

Section K. Related Products

ChIP-IT® Kits	Format	Catalog No.
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® High Sensitivity	16 rxns	53040
ChIP-IT® qPCR Analysis Kit	10 rxns	53029
ChIP-IT® ChIP-Seq	10 libraries	53041
ChIP-IT® FFPE	16 rxns	53045
ChIP-IT® FFPE Chromatin Preparation Kit	5 rxns	53030
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™ Probe Sonicator	110 V	53051
EpiShear™ Cooled Sonication Platform, 1.5 ml	1 platform	53080
ChIP-IT® Protein G Magnetic Beads	25 rxns	53014
Protein G Agarose Columns	30 rxns	53039
Siliconized Tubes, 1.7 ml	25 tubes	53036
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

Modified Histones Array	Format	Catalog No.
MODified™ Histone Peptide Array	1 array	13001

Histone Modification FP Binding Assay	Format	Catalog No.
HiLite™ Histone H3 Methyl-Lys9 / Lys27 FP Binding Assay	1 kit	57001

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

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