December 2012

EpiTect® ChIP OneDay Handbook

For immunoprecipitation, crosslink reversal, and DNA purification for quantitative real-time PCR analysis of chromatin immunoprecipitation

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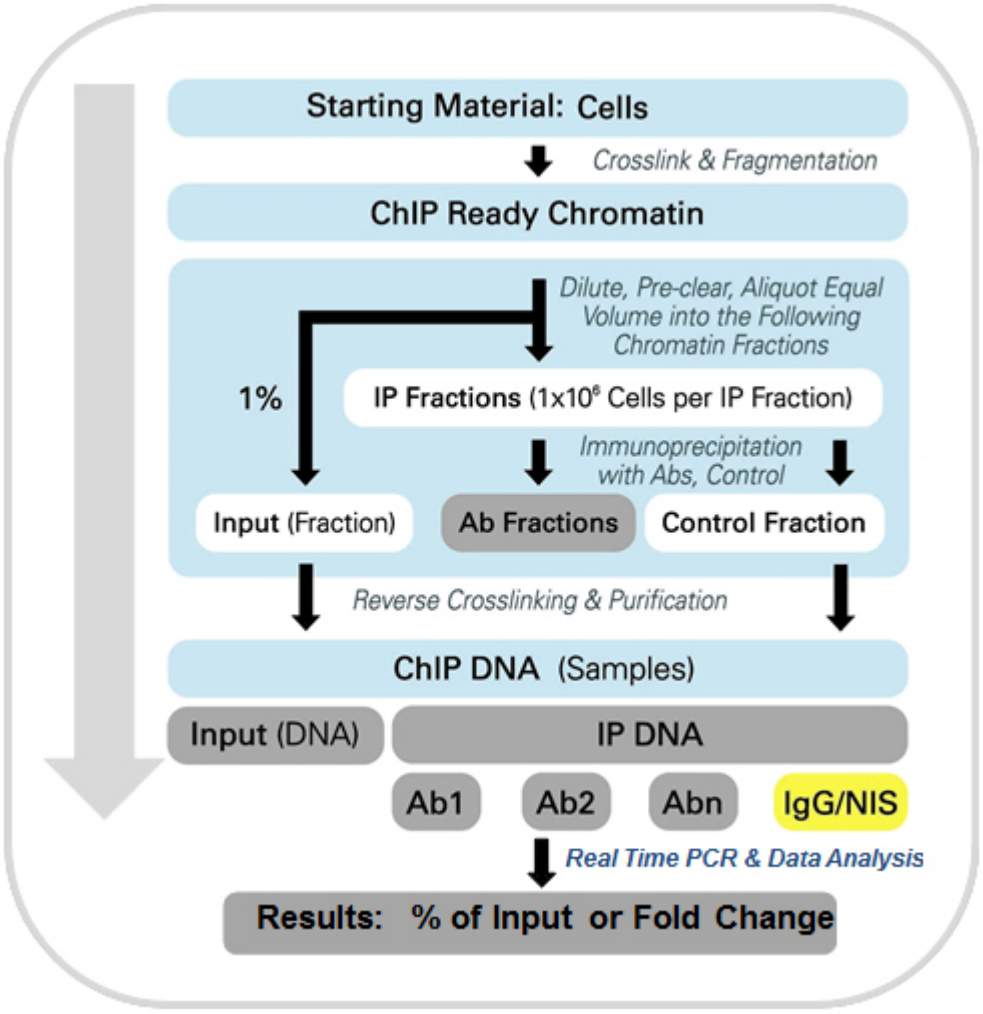
I. Background and Introduction

Chromatin Immunoprecipitation (ChIP) is rapidly becoming a very important method for understanding the mechanisms of gene regulation by transcription factors and modified histones. However, this preparative process is very tedious and time-consuming to perform, involving many steps and variables that must be optimized by each investigator in their model system. After cross-linking cells with formaldehyde, chromatin containing covalent complexes between genomic DNA and all nuclear factors is isolated and sheared by sonication into manageable sizes. Immunoprecipitation pulls down not only the target nuclear factor of interest but also any specifically bound genomic DNA sequences. Reversal of the chemical cross-linking and nucleic acid purification prepare the DNA for detection by sequencing, hybridization-based microarrays, or PCR. To help researchers, a number of commercial preparation kits are available that include all the reagents needed for this process. While some of these kits still rely on the traditional multi-day protocol to isolate ChIP DNA, others offer an innovative one-day solution. However, most kits demonstrate the success of the experiment with agarose gel-based electrophoresis rather than the gold-standard for nucleic acid detection, real-time PCR.

The EpiTect ChIP OneDay Kit is designed to help any biological researcher isolate and purify high-quality DNA bound to their nuclear factor of interest. The isolated DNA is ready for downstream applications including real-time PCR detection, but not for sequencing applications such as ChIP-Seq. The complete preparation kit includes all buffers and components needed for immunoprecipitation, crosslink reversal, and genomic DNA purification for detection. The general, universal kit also offers compatibility with any chosen ChIP-grade antibody. Otherwise, all you need is cross-linking agent, a sonicator, and your cell-based experiment. The protocol and reagents have been carefully optimized to take most of the guesswork out of ChIP preparation. Instructions are included for optimization of cell harvesting, sonication conditions, and real-time PCR analysis of ChIP-enriched genomic DNA that will need to be determined experimentally. The entire protocol can be completed in one day, but also includes convenient stopping points for continuing the protocol the next day. The resulting DNA is free of contaminants that confound and interfere with real-time PCR detection, in particular using the EpiTect ChIP qPCR Assays.

Overview of EpiTect ChIP OneDay Procedure



(See Page 9 for the definitions used in the procedure)

Benefits of the EpiTect ChIP OneDay Kit:

- **Speed & Ease:** Simplified protocol easily obtains ChIP DNA from cells in just 6 hours
- **Universality:** General kit compatible with any ChIP-grade antibody
- **Reliability:** High-yield and high-quality DNA for real-time PCR and highly reproducible enrichment

II. Materials Provided:

Please check the kit components immediately after you receive this package. We are not responsible for any missing items not reported within two (2) business days upon receipt.

Contents	*Quantity*	
Box 1 Store at 4 °C		
10X PBS	One 30-mL Bottle	
10X Stop Buffer	One 15-mL Bottle	
IP Lysis Buffer	One 15-mL Bottle	
IP Buffer A	One 15-mL Bottle	
Protein A Beads	One 2-mL Blue-Capped Tube	**CENTRIFUGE!**
IP Wash Buffer I	One 15-mL Bottle	
IP Wash Buffer II	One 15-mL Bottle	
IP Wash Buffer III	One 15-mL Bottle	
IP Wash Buffer IV	One 30-mL Bottle	
DNA Extraction Beads	One 2-mL Green-Capped Tube	**CENTRIFUGE!**
Elution Buffer	Three 2-mL Yellow-Capped Tubes	
Column Binding Buffer	One 15-mL Bottle	
Column Wash Buffer	One 15-mL Bottle	
DNA Spin Columns	One Bag of 12 Columns in Collection Tube	
Elution Tubes (1.7-mL)	Three Bags of 12 Tubes	
Sonication Tubes (2-mL)	One Bag of 6 Tubes	
Preclear Tubes (5-mL)	One Bag of 6 Tubes	
Box 2 Store at -20 °C		
Protease Inhibitor Cocktail	One 1.5-mL Clear-Capped Tube	
ChIP-Grade Proteinase K	One 1.5-mL Red-Capped Tube	

Storage Conditions:

- The EpiTect ChIP OneDay Kit is shipped with ice packs.
- Protease Inhibitor Cocktail (PIC) & ChIP-Grade Proteinase K in Box 2 must be stored at -20 °C.
- All other components must be stored at 4 °C in their original container to insure that no components are misplaced.
- Upon arrival, centrifuge Protein A Beads and DNA Extraction Beads at 4000 x g for 1 min at 4 ºC and return to their original position in the box.

Shelf Life:

All reagents are stable for 6 months after receipt of the kit if stored at the recommended temperature.

III. Additional Materials & Equipment Required:

Additional Materials Required:
The following components are needed for the protocol, but are not included in the kit.

- 37 % Formaldehyde (Sigma, Catalog Number F1635)
- Silicone Cell Scraper or Rubber Policeman (VWR)
- Filter Tips
- P-200 large bore/wide-mouth pipette tip
  (VWR, Cat# 46620-642 in North America or 732-0544 in Europe)
- Wet ice / ethanol bath or wet ice bath

Equipment Required:

- Sonicator
- Rotator
- Thermomixer® (Eppendorf) or shaking water bath
- UV Spectrophotometer
- Centrifuge
- Bench-top Microcentrifuge
- Rotating Platform at 4 °C and room temperature
- Real-Time PCR Instrument

IV. Related Products

A. EpiTect ChIP Antibody Kit **Catalog Number 334481**
More information regarding the EpiTect ChIP Antibody Kit can be found at
http://www.sabiosciences.com/chipgradeantibody.php

B. EpiTect ChIP qPCR Assays **Catalog Number 334001**
More information regarding the EpiTect ChIP qPCR Assays can be found at
http://www.sabiosciences.com/chipqpcrsearch.php

C. EpiTect ChIP qPCR Arrays **Catalog Number 334211**
More information regarding the EpiTect ChIP qPCR Assays can be found at
http://www.sabiosciences.com/chipqpcrarrays.php

D. EpiTect ChIP qPCR Data Analysis
Free available at http://www.sabiosciences.com/chippcrarray_data_analysis.php

E. EpiTect ChIP Search Portal

Free available at http://www.sabiosciences.com/chipqpcrsearch.php

F. RT^2 SYBR Green qPCR Mastermixes

More information regarding the RT2 SYBR Green qPCR Mastermixes can be found at http://www.sabiosciences.com/chipqpcrmastermix.php

V. Protocol:

Definitions used in EpiTect ChIP System

ChIP Sample: Starting material for ChIP assay (cells).

NOTE: *ChIP can be performed with a single ChIP sample (detection of protein/DNA association in one condition such as histone modification), or multiple samples dependent on experimental design, such as knockout vs. wild type; treated vs. untreated; or a time course.*

ChIP Ready Chromatin: Prepared chromatin after fragmentation step ready for immunoprecipitation process**.**

IP Fraction: ChIP Ready Chromatin from each ChIP sample must be diluted, pre-cleared, and divided into equal aliquots for immunoprecipitation which are called **IP fractions**. Therefore an IP Fraction represents the fraction of diluted and pre-cleared ChIP Ready Chromatin used for the actual immunoprecipitation step with antibody of interest, control antibody, such as normal IgG or non-immune serum (NIS).

NOTE: *One ChIP sample has to be sufficient for two or more IP fractions*.

Input Fraction or Input: As an input control, 1% of **IP Fraction** from each **ChIP Sample** is set aside before the Immunoprecipitation step.

NOTE: *One ChIP sample needs only one Input control*.

Negative Control Fraction: The **IP fraction** of chromatin mixed with normal IgG or NIS that serves as negative control for ChIP-grade antibodies. Normal IgG or NIS pulls down non-specific DNA, providing a measurement of the background or noise for the ChIP assay.

Antibody Fraction: The **IP fraction** interacted with the antibody of interest to pull down its associated genomic DNA sites. This provides an estimation of the number of specific genomic sites bound or associated with the nuclear factor of interest *in vivo*.

Input DNA: Genomic DNA directly purified from **Input fraction**. It provides a measurement of the total number of interested genomic site(s) included into the **IP fraction** and serves as reference control for real time PCR performance and data normalization.

IP DNA: Genomic DNA purified from **IP fraction** after immunoprecipitation with an antibody of interest, or normal IgG/NIS.

ChIP DNA (Sample): Genomic DNA from **Input DNA** or ChIP enriched **IP DNA**.

Important Notes on ChIP Sample Preparation

The EpiTect ChIP OneDay Kit can yield reliable results with as little as two million mammalian cells per **ChIP sample**. However, the optimal amount of starting material depends on the relative abundance of the protein-associated DNA and the enrichment efficiency of the ChIP assay with the antibody of choice. Low amounts of starting material will affect the efficiency of the ChIP enrichment. The high abundance of the protein-associated DNA immunoprecipitated with highly efficient antibodies, such as anti-H3K4me3, H3K4me2 or H3ac, requires fewer cells per **ChIP sample**, but no less than 0.4 million cells. Lower amounts of starting material will yield less positive calls and will increase false negative calls.

On the contrary, lower abundance of the protein-associated DNA or lower efficient antibodies will require more cells per **ChIP sample**. A high amount of starting material will yield a greater number of positive calls though it will also increase background levels and possibly generate more false positive calls.

In general it is recommended that one million mammalian cells are required for each **IP fraction**. The actual amounts of cells required to produce high quality, quantitative results may need to be determined empirically for each experiment. Once determined, this amount should be applied uniformly for all samples within an experiment for a consistent performance.

This protocol is started with four to six million adherent mammalian cells from one **ChIP sample.** This amount of cells is sufficient for three up to six **IP fractions** after carefully harvesting the cell lysate following cross-linking step. Please scale all recipes down or up according to the number of cells that you are harvesting.

A. ChIP Ready Chromatin Preparation:

This section describes the preparation of **ChIP Ready Chromatin.** It covers cell cross-linking, harvesting, lysis, and chromatin shearing based on adherent mammalian cell lines. Certain steps may need to be optimized for experiment-dependent performance. This protocol also provides several stopping points for the flexibility to optimize those steps.

Things to do before starting

- Prepare **ChIP sample**(s). For example, HeLa cells at 70-85% confluence on a 10-cm culture dish number roughly 4 to 6 million cells. Prepare one extra plate of cells to estimate cell number, if necessary.

- Warm **IP Lysis Buffer** to room temperature to prevent precipitation.

- Thaw **Protease Inhibitor Cocktail (PIC)** at room temperature. This product contains DMSO and will remain frozen below 18.4°C.

a. Cross-Linking Cells

1. **Prepare following buffers, scale up accordingly if necessary.**

   i. **1 × PBS Buffer**: Add 3.3 ml **10 × PBS** to 29.7 ml ddH_2O. Mix well and place on ice.
   ii. **FRESH Fixing Buffer (1 % Formaldehyde):** Add 0.27 ml 37 % Formaldehyde to 9.73 ml **1 × PBS Buffer**. Mix and store at room temperature in a fume hood.
   iii. **Cell Harvesting Buffer:** Add 15 µl **Protease Inhibitor Cocktail (PIC)** to 3 ml ice-cold **1 × PBS Buffer**. Mix well and place on ice.

2. Aspirate cell culture medium from the dish. Add 10 ml **FRESH Fixing Buffer** to the dish. Incubate at 37°C for 10 min.

3. Add 1.1 ml **Stop Buffer** to the dish and swirl to mix thoroughly. Incubate at room temperature for 5 min. Aspirate buffer as much as possible.

NOTE: *From this point forward, keep the samples on ice at all times.*

4. Add 9 ml ice-cold **1 × PBS** to wash the fixed cells, rocking the dish for 5 seconds. Pour off the wash buffer. Repeat once more. Aspirate as much buffer as possible.

b. Harvesting Cells

5. Add 1.5 ml ice-cold **Cell Harvesting Buffer** to the dish. Place dish on ice at an angle and scrape cells down to one edge of the dish with a silicone cell scraper or rubber policeman. Collect the cell supernatant as much as possible. Transfer all of the cell suspension to a **Sonication Tube** on ice.

6. Centrifuge the **Sonication Tube** at 800 × *g* for 10 min at 4 °C to pellet the fixed cells. Remove the supernatant. Repeat harvesting and centrifugation steps as many times as necessary to collect all of the cells. Continue with next step OR store the cell pellet at -80°C.

c. Cell Lysis

NOTE: *If samples were previous frozen and stored, thaw on ice*.

7. Add 2.2 µl **Protease Inhibitor Cocktail (PIC)** to 420 µl **IP Lysis Buffer** in a standard 1.5 ml Eppendorf tube. Mix well by pipetting.

8. Add entire volume of **IP Lysis Buffer** with **PIC** to the cell pellet. 100 µl **IP Lysis Buffer** is recommended to lyse 0.5 to 1.5 million cells.

9. Resuspend cells completely by pipetting. Incubate on ice for 10 -15 min, mixing every 5 min. Continue with next step OR store the lysate at -80°C.

d. Shearing Chromatin

NOTE: *The sonication conditions need to be optimized for different sonicators and different cell lines. It is recommended to take some trial to optimize sonication condition before beginning with sonication. Please see* **Appendix A** *for details on optimizing this step.*

10. Thaw cell lysate on ice if necessary. Place the sample tube in a wet ice / ethanol bath or wet ice bath. Make sure there is no precipitate visible in the lysate during sonication to avoid foaming.

11. Insert the probe (1/16" microtip) to a 2 - 3 mm depth into the lysate. Shear lysate using optimized conditions.

    For example, lysate can be sheared under the following settings using Sonicator 3000 (MISONIX, Part # S3000) with a 1/16" microtip:

    i. Power: 0.5 W; Time: 2 seconds ON / 15 seconds OFF; Total Time: 16 seconds (8 times per round); 3 rounds.
    ii. Mix the lysate by pipeting up-and-down after every round and avoid precipitation before the next round.

NOTE: *Make sure there is no precipitate visible in the lysate before centrifugation. The white precipitate can cause chromatin loss.*

12. Centrifuge the lysate to pellet the debris at 14,000 × *g* for 10 min at 4 °C.

13. CAREFULLY transfer the supernatant to a new **Elution Tube** without disturbing the debris pellet. Continue with next step OR snap freeze the **ChIP Ready Chromatin** at -80°C.

B. Protein/DNA Immunoprecipitation (IP):

This section describes the immunoprecipitation procedure with a ChIP-grade antibody of choice. This protocol requires a 100 µl aliquot of **ChIP Ready Chromatin** (10-100 µg chromatin or 0.2-2 million cell equivalents) for each **IP Fraction.**

Things to do before starting next step

- Make sure enough ChIP desired antibodies are available (sold separately, check our

website for detail information: http://www.sabiosciences.com/chipgradeantibody.php). It is necessary to include at least two **IP Fractions** for each ChIP assay, one for the antibody of interest, one for normal IgG/NIS as negative control. A third one as a positive control, for example, using anti-RNA Polymerase II, or anti-Acetylated Histone H3, is highly recommended to ensure reliability of ChIP assay.

- Thaw **Protease Inhibitor Cocktail (PIC)** at room temperature.

- Thaw aliquots of ChIP Ready Chromatin on ice if samples were frozen.

e. Pre-Clear

14. For each **IP Fraction**, Transfer 900 µl **IP Buffer A** containing 7.5 µl **PIC** into 1.7-ml **Elution Tube.** Add 100 µl aliquot of **ChIP Ready Chromatin** into the Tube. Mix well by pipetting and place on ice.

    For multiple immunoprecipitations, scale up the **IP Buffer A** as needed. **5-mL Pre-Clear tubes** are provided in the Kit for more than two **IP Fractions** pre-clearing to minimize handling and to ensure consistency.

15. Add 50 µl **Protein A Beads** for each **IP Fraction.** Cap the tube tightly and incubate for 50 min at 4 ºC with rotation.

    To insure a homogeneous suspension, invert the **Protein A Beads** tube several times, and pipette up-and-down 5 times using a P-200 large bore/wide-mouth pipette tip immediately before each volume withdrawal.

16. Centrifuge the samples at 4000 × *g* for 1 min at 4 ºC.

17. Place the tube on the ice for 1 min to let the Protein A Beads settle completely.

18. Transfer **Pre-Cleared Chromatin** (supernatant) to **NEW** ice-cold **5-mL Tube**. Avoid disturbing the bead pellet.

f. IP Fractions

19. Remove 10 µl of the supernatant (1 % of one 1 ml IP fraction) to another 1.7-mL **Elution Tube** as "**Input Fraction**" and store at 4 ºC until ready for **DNA Isolation & DNA Purification**.

NOTE: *Multiple IP fractions from the same one **ChIP Sample** will share one **Input Fraction.***

20. Transfer 1 ml aliquot of the supernatant into each labeled 1.7-mL **Elution Tubes**.

21. Add the appropriate antibody into each supernatant fraction ("**IP Fraction"**):

For antibody from EpiTect ChIP antibody Kit:

i. **Negative Control IP Fraction**: Add 2-4 µg Control IgG.
ii. **Positive Control IP Fraction**: Add 2-4 µg anti-RNA Polymerase II antibody.
iii. **Antibody IP Fraction**: Add 2-4 µg antibody of interest, e.g., 2 µg Anti-H3ac, or 4 µg Anti-Human p53.

For user-provided antibody, the appropriate amount of antibody needs to be empirically determined.

22. Incubate **IP Fractions** on rotator at 4 ºC for 2 hours or overnight if necessary.

For user-provided antibody, follow the antibody manufacturer's recommendations.

NOTE: *The antibody incubation time depends on the antibody, the physical number of targets, and other experiment-specific variables.*

g. Immunoprecipitation

23. Add 60 µl **Protein A Beads** to each **IP fraction**. Incubate on a rotator at 4 ºC for one hour.

To insure a homogeneous suspension, invert the **Protein A Beads** tube several times, and pipette up-and-down 5 times using a P-200 large bore/wide-mouth pipette tip immediately before each volume withdrawal.

24. Centrifuge the **IP fractions** at 4000 × *g* for 1 min at 4 ºC.

25. Carefully place the tubes on ice for 1 min to let the **Protein A Beads** settle completely.

26. Remove and discard the supernatant from each **IP Fraction**.

To avoid disturbing the bead pellet, use a P-1000 pipette tip to withdraw 900 µl, following by P-200 pipette tip to withdraw the remaining volume.

27. Wash the bead pellet containing Protein A Beads-antibody/chromatin complex by resuspending the bead pellet in 1 ml each of the cold **Wash Buffers** as listed below and incubating for 4 min on a rotating platform. Spin down briefly at 4000 × *g* for 1 min and carefully remove supernatant.

i. **IP Wash buffer I**, one wash

ii. **IP Wash buffer II**, one wash

iii. **IP Wash buffer III**, one wash

iv. **IP Wash buffer IV**, two washes.

NOTE: *To reduce background, remove as much supernatant as possible in the last wash. Continue with next step OR store at 4°C overnight.*

C. DNA Isolation & Purification:

This section describes crosslink reversal and the purification of DNA from the **IP Fractions** with sufficiently high quality and yield for real-time PCR.

Things to do before starting next step

Set one Thermomixer or water bath or oven at 45 °C and another at 95 °C.

h. DNA Isolation

28. Add 30 µl **Elution Buffer** and 2 µl **ChIP-Grade Proteinase K** directly to the each **IP Fraction** including the previously saved and stored **Input Fraction from step19**.

29. Incubate on Thermomixer shaking at 500 rpm or water bath or oven at 45 °C for 30 min.

30. Add 100 µl **DNA Extraction Beads** directly to each **IP Fraction** sample. Cap the tubes. Vortex for 10 seconds.

    To insure a homogeneous suspension, invert beads tube several times, and pipette up-and-down 5 times using a P-200 large bore/wide-mouth pipette tip immediately before each volume withdrawal.

31. Incubate on Thermomixer shaking at 500 rpm or water bath or oven 95 °C for 10 min.

32. Centrifuge the samples at 14,000 × *g* for 1 min at room temperature.

33. Transfer 100 µl of each supernatant to a **NEW** labeled **Elution Tube**, **<u>without</u>** disturbing the pellet.

34. Add another 100 µl **Elution Buffer** to each sample. Vortex for 20 seconds. Centrifuge at 14,000 × *g* for 1 min at room temperature.

35. Transfer 100 µl of each supernatant and pool the supernatants in the new tubes for a final 200 µl volume supernatant for each sample. Continue with next step OR store the supernatants at -20°C or 4 °C.

i. DNA Purification

Things to do before starting next step

- Add 7.2 ml ethanol to the **Column Wash Buffer** bottle, and mix well before use.

- Thaw supernatants, if necessary.

36. Add 400 µl **Column Binding Buffer** to each 200 µl **IP Fraction** sample. Mix well by pipetting. Immediately transfer the entire supernatant to its own **DNA Spin Column** (in 2-mL **Collection Tube**) without wetting the rim, close the lid, and centrifuge at 11,000 × *g* for 1 min at room temperature.

37. Remove **DNA Spin Column** from **Collection Tube**. Discard flow-through material. Place **DNA Spin Column** back into the same **Collection Tube**.

38. Carefully open the **DNA Spin column** and add 600 µl **Column Wash Buffer**, containing ethanol, to each column, without wetting the rim, close the lid, and centrifuge at 11,000 × *g* for 1 min at room temperature.

39. Remove **DNA Spin Column** from **Collection Tube**. Discard flow-through material. Place **DNA Spin Column** back into the same **Collection Tube**. Centrifuge at 11,000 × *g* for 2 min at room temperature to remove any residual washing buffer.

NOTE: *Insure that the tip of the DNA Spin Column DOES NOT come into contact with the flow-through material while removing it from the centrifuge and its Collection Tube.*

40. Place the **DNA Spin Column** into a clean **Elution Tube,** and discard the **Collection Tube**. Add 100 µl **Elution Buffer** to the center of the column membrane. Close the lid and incubate at room temperature for 1 min.

41. Centrifuge at 11,000 × *g* for 1 min at room temperature.

42. Repeat the elution step with another 100 µl of **Elution Buffer** for a final elution volume of 200 µl for each **ChIP DNA Sample**. The purified **ChIP DNA Samples** can be stored at -20°C or 4 °C.

D. ChIP DNA Detection & Data Analysis

Purified **ChIP DNA** can then be analyzed by multiple down stream application. This section describes the quantitative detection of ChIP DNA by SYBR green real-time PCR. Please refer to the **EpiTect ChIP qPCR Assay** Handbook for details.

j. Real time PCR Reaction

43. Design ChIP qPCR Assays for quantitative analysis of ChIP DNA. For each **ChIP Sample,** suggest to perform the following ChIP qPCR Assays.

Recommended ChIP qPCR Assays for the ChIP DNA from ONE ChIP Sample

ChIP qPCR Assay	Primer Targeted Genomic Site	ChIP DNA from ONE ChIP Sample							ChIP qPCR Assays
		Input	Negative Control IP (IgG/NIS)	Positive Control IP (Anti-poly 2)	Antibody #1 IP	Antibody #2 IP	……	Antibody #N IP	
	Positive Control	√	√	√	×	×	×	×	3
	Negative Control	√	√	√	√	√	√	√	N+3
	Site of Interest #1	√	√	×	√	√	√	√	N+2
	Site of Interest #2	√	√	×	√	√	√	√	N+2
	……	√	√	×	√	√	√	√	N+2
	Site of Interest #N	√	√	×	√	√	√	√	N+2

NOTE: *ChIP qPCR Positive and Negative Control Assays are included in the EpiTect ChIP Antibody Kit (visit: http://www.sabiosciences.com/chipgradeantibody.php), also sold separately. To find primers of your interest, please use our "EpiTect ChIP Search Portal" available at http://www.sabiosciences.com/chipqpcrsearch.php.*

44. Use 1/100th (2 µl) of the purified **ChIP DNA** in each real-time PCR in a total volume of 25 µl, or 1/250th (0.8 µl) the DNA in a total volume of 10 µl with a RT^2 SYBR Green PCR Master Mix matched with your instrument.

Recommended Preparation of Sample Cocktails for Real-Time PCR

Component	Amount (µl)		
RT2 SYBR Green qPCR Master Mixes	12.5	5	
ddH_2O	10.5	3.8	
EpiTect ChIP qPCR Assay Primers	1	0.4	Final primer concentration is 0.4 µM
ChIP DNA	2	0.8	
Total Volume	**25**	**10**	

45. Seal the wells/tubes after all reaction wells/tubes have been dispensed, and centrifuge briefly to remove air bubbles before placing into your real-time PCR instrument. Perform Real-Time PCR according to the program as listed below.

Cycles	Duration	Temperature
1	10 minutes	95 °C
40	15 seconds	95 °C
	1 minute	60 °C
1	1 minutes	95 °C
	30 seconds	55 °C
	30 seconds	95 °C

NOTE: *If using your own primers or PCR reagent, the appropriate PCR condition needs to be optimized.*

k. Real time PCR Data Analysis

46. Set Threshold Fluorescence Value to 0.2 and export all C_t value data and labels to an Excel spreadsheet. Calculate raw C_t values into percentage of input and fold difference using free Excel-based **EpiTecChIP qPCR Data Analysis Template** (Download from: http://www.sabiosciences.com/chippcrarray_data_analysis.php).

How to Calculate the ChIP Enrichment?

Our **Excel-based ChIP qPCR Data Analysis Template** provides a guideline and template for performing calculations either as a **"% Input**" or **"Fold Change**" for characterizing individual experimental samples for their loci specific enrichment, or as a "**Differential Occupancy** (**Fold Change**)" when comparing multiple experimental samples.

i. Normalize each IP DNA fractions' C_t value to the Input DNA fraction C_t value for the same qPCR Assay (ΔC_t) to account for chromatin sample preparation differences.

$$\Delta C_{t\ [\text{normalized IP}]} = (C_{t\ [\text{IP}]} - (C_{t\ [\text{Input}]} - \text{Log2 (Input Dilution Factor)}))$$

Where, Input Dilution Factor = (fraction of the input chromatin saved)-1
Average normalized IP C_t values for replicate samples if applied

ii. Calculate the "**% Input**" for each **IP fraction** (linear conversion of the normalized IP ΔC_t).

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

OR, calculate Assay Site IP Fold Enrichment above the sample specific background (linear conversion of the first $\Delta\Delta C_t$).

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

iii. Normalize the specific enrichment

$$\%\ Input_{[Normalized]} = \%\ Input_{[Antibody]} - \%\ Input_{[IgG/NIS]}$$

iv. Determine the difference between the treated sample (S2) and the control sample (S1)

$$\textbf{Fold Change} = \%\ Input_{[Normalized]}\ (S2) / \%\ Input_{[Normalized]}\ (S1)$$

VI. Troubleshooting & FAQ

For FAQs, please visit our website: http://www.sabiosciences.com/support_faq.php?target=chipqpcr

If you have any questions, please contact a Technical Support Representative by phone at 1-888-503-3187 or 301-682-9200 or by email at support@sabiosciences.net.

Appendix A: Chromatin Shearing Optimization

Chromatin shearing conditions need to be optimized for your ChIP samples (cell line of interest) BEFORE performing chromatin immunoprecipitation. Mock samples for optimizing shearing conditions may be prepared using untreated cells and following protocol steps for **Cell Cross-linking**, **Harvesting**, and **Lysis** in this manual, scaling up as needed. The Sonicator 3000 (MISONIX, Part# S3000) with 1/16" microtip is used for chromatin sonication in this protocol.

A. Chromatin Shearing

1. Prepare approximately 420 µl cell lysate samples by following the protocol. Transfer 10 µl of Unsheared Control DNA to a 1.7-mL microcentrifuge tube, labeled ***S0***.

2. Place the tube containing the remaining cell lysate sample on an ice / ethanol bath rack, or a rack with wet ice.

3. Insert the probe (1/16" microtip) to a 2 - 3 mm depth into the cell lysate.

4. Shear chromatin with following settings:
   Power: 0.5 W; Time: 2 seconds ON / 15 seconds OFF; 16 seconds total time (or eight times per round)

5. After the round, mix the cell lysate by pipetting up and down. Transfer 10 µl of the sheared chromatin to a 1.7-mL microcentrifuge tube, labeled ***S1***.

6. Repeat the sonication round twice saving 10 µl of the sheared chromatin each time into 1.7-mL microcentrifuge tubes labeled ***S2*** and ***S3***, respectively.

NOTE: *If sample starts to bubble:*
Stop the sonication immediately!
Centrifuge the sample at 6000 × g for 5 min at 4 °C to eliminate bubbles.
Continue the sonication with previous settings.

If bubbling continues, adjust the settings to the following:
Power: 2.5 W; Time: 10 seconds ON / 30 seconds OFF; Total Time: 1 to 3 min (6 to 18 times per round)
For example, use a 3-min setting (and 18 times per round) if foaming happens the first time

B. Agarose Gel Electrophoretic Analysis:

7. Prepare a 1.2 % agarose gel.

8. Add 2 µl **ChIP-Grade Proteinase K** and 8 µl ddH_2O to the tubes ***S0***, ***S1***, ***S2***, and ***S3***.

9. Vortex the tubes, and centrifuge briefly to collect material at the bottom of their tubes.

10. Incubate at room temperature for 10 min.

11. Add 50 µl of **DNA Extraction Beads** to each tube. Vortex for about 10 seconds.

12. Incubate on thermomixer shaking at 500 rpm at 95°C for 10 min, or incubate on other incubator alternatively.

13. Centrifuge the tubes at 14,000 × *g* at room temperature for 1 min.

14. Transfer 20 µl of each supernatant to a new tube.

15. Add 4 µl 6 × DNA Loading Dye and mix well.

16. Load samples into separate wells of the gel and proceed to gel analysis.

The appropriate sonication conditions should yield sheared chromatin DNA running as a smear with an average size between 500-1500 bp. The smear can be extended through to 3 kb on the agarose gel, but no larger than 10 kb.

Ordering Information

Product	Contents	Cat. no.
EpiTect ChIP OneDay Kit (12)	Buffers, Protein A Beads, DNA extraction beads, DNA Spin Columns, tubes, Protease Inhibitor Cocktail, ChIP-Grade Proteinase K	334471

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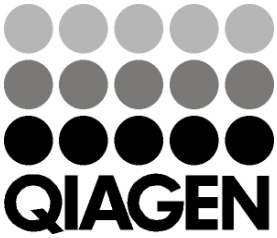

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