Video Article

Efficient Chromatin Immunoprecipitation using Limiting Amounts of Biomass

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Abstract

Chromatin immunoprecipitation (ChIP) is a widely-used method for determining the interactions of different proteins with DNA in chromatin of living cells. Examples include sequence-specific DNA binding transcription factors, histones and their different modification states, enzymes such as RNA polymerases and ancillary factors, and DNA repair components. Despite its ubiquity, there is a lack of up-to-date, detailed methodologies for both bench preparation of material and for accurate analysis allowing quantitative metrics of interaction. Due to this lack of information, and also because, like any immunoprecipitation, conditions must be re-optimized for new sets of experimental conditions, the ChIP assay is susceptible to inaccurate or poorly quantitative results.

Our protocol is ultimately derived from seminal work on transcription factor:DNA interactions[1,2] , but incorporates a number of improvements to sensitivity and reproducibility for difficult-to-obtain cell types. The protocol has been used successfully[3,4] , both using qPCR to quantify DNA enrichment, or using a semi-quantitative variant of the below protocol.

This quantitative analysis of PCR-amplified material is performed computationally, and represents a limiting factor in the assay. Important controls and other considerations include the use of an isotype-matched antibody, as well as evaluation of a control region of genomic DNA, such as an intergenic region predicted not to be bound by the protein under study (or anticipated not to show changes under the experimental conditions). In addition, a standard curve of input material for every ChIP sample is used to derive absolute levels of enrichment in the experimental material. Use of standard curves helps to take into account differences between primer sets, regardless of how carefully they are designed, and also efficiency differences throughout the range of template concentrations for a single primer set. Our protocol is different from others that are available[5-8] in that we extensively cover the later, analysis phase.

Video Link

The video component of this article can be found at https://www.jove.com/video/50064/

Protocol

1. Isolation of Mouse Splenic Naïve CD4 T Cells

1. Sacrifice the mouse in a humane manner consistent with Institutional Animal Care and Use Committee (IACUC) protocols. Dissect the spleen and place it in Petri dish containing 10 ml of DMEM with 10% FBS.
2. Crush the spleen using frosted ends of two glass slides to release the splenocytes. Transfer the cell suspension in a 15 ml conical tube.
3. Collect the cells by centrifugation at 200 x g (~1,200 rpm for a clinical centrifuge with a typical rotor diameter) for 5 min at 4 °C.
4. Resuspend cells in 2 ml ACK buffer to lyse red blood cells, 1 min at room temperature (RT). Stop the reaction by adding 8 ml of DMEM with FBS.
5. Collect the cells by centrifugation at 200 x g for 5 min at 4 °C.
6. Resuspend the cells in 5 ml of DMEM containing FBS and pass through a 70 µm mesh filter (BD Falcon, Cat# 352350). Count the cells and proceed to isolation of naïve CD4 T cells using a CD4 isolation kit (Miltenyi, Cat# 130-095-248) using the manufacturer's instructions. Collect naïve CD4 T cells by centrifugation at 200 x g for 5 min at 4 °C. Resuspend the cells in 10 ml of DMEM with FBS.

2. Preparation of Chromatin

1. Add 37% formaldehyde to a final concentration of 1% to the cell suspension in DMEM and gently rock at RT (*e.g.* using a Nutator) for 15 min to cross-link DNA:protein complexes.
2. Stop crosslinking by adding 1 M glycine to a final concentration of 125 mM. Continue to rock for 5 min at RT.
3. Collect the cells by centrifugation at 200 x g for 5 min at 4 °C.

4. Wash the cells by resuspending in 5 ml of ice-cold PBS containing protease inhibitors. Wash in ice-cold PBS containing protease inhibitors 3 times total and collect the cells by centrifugation at 4 °C.
5. Resuspend the cells in 1 ml ice-cold cell lysis buffer containing protease inhibitors. Incubate on ice for 15 min. The efficiency of cell lysis can be determined by resuspending a small aliquot of cells in 0.4% trypan blue solution (Sigma, Cat# T8154) and observing with a microscope.
6. Collect the nuclei by centrifugation at 200 x g (typically ~1,200 rpm) for 5 min at 4 °C. Carefully discard the supernatant.
7. Resuspend the nuclei in 500 µl of nuclear lysis buffer containing protease inhibitors. Incubate on ice for 15 min.
8. Sonicate the cells using a Misonix Sonicator 3,000, probe size 1.6 mm: Output level 4, 15 sec burst, 4 times on ice. Each sample must be cooled on ice for 1-2 min before sonicating it again to prevent overheating of samples. Overheating can cause the reversal of cross links.
9. Centrifuge the sonicated chromatin at 16,000 x g (~13,200 rpm in a microcentrifuge) for 5 min at 4 °C.
10. Take a 20 µl aliquot of clear supernatant, add DNA loading dye and check the sonicated DNA by electrophoresis through a 2% agarose gel (ideal size of sonicated DNA for most applications is 200-500 bp).
11. Determine DNA concentration using a UV spectrophotometer. Sheared chromatin can be used immediately to set up chromatin immunoprecipitation reaction or stored at -80 °C. Typically we obtain 7.5-10 µg DNA from 2-3 X 10^6 purified CD4 T cells per mouse.

3. Chromatin Immunoprecipitation (All Steps Must be Carried Out at 0-4 °C)

1. Dilute sonicated chromatin to a DNA concentration of 5-10 µg/ml (total volume= 1 ml) in ChIP dilution buffer with protease inhibitors.
2. Save 100 µl (10%) as input. Store on ice.
3. Aliquot 450 µl each into two 1.7 ml microfuge tubes labeled as isotype control (mouse or rabbit IgG) and antibody of interest. If multiple antibodies of the same isotype are used, a single isotype control will be sufficient to perform this analysis. You will need multiple isotype controls if antibodies of a different animal source or isotype are used.
4. Add 2-5 µg of specific antibody, depending on the specificity of the antibody used, or isotype control to the respective tubes.
5. Rock the tubes using a Nutator overnight at 4 °C to allow formation of chromatin-antibody complexes.
6. Add 25 µl of protein G magnetic beads (1:1 slurry of suspended beads) to the above mixture and allow to rock for at least 2 hr at 4 °C. Beads from our vendor can be used directly.
7. Place the microfuge tubes on a magnetic stand and allow the beads to collect on the magnetized side.
8. Carefully remove the solution by aspiration without disturbing the beads.
9. Add 1 ml of low salt wash solution and allow to gently rock for 5 min on a Nutator. Collect the beads using a magnetic stand and remove the wash solution. Repeat once.
10. Add 1 ml of high salt wash solution and allow to rock for 5 min on a Nutator. Collect the beads using the magnetic stand and remove the wash solution. Repeat once.
11. Add 1 ml of lithium chloride wash solution and allow to rock for 5 min on a Nutator. Collect the beads using the magnetic stand and remove the wash solution. Repeat once. The use of LiCl improves the effective removal of non-specific chromatin interactions with the beads.
12. Add 1 ml of TE solution and allow to rock for 5 min on a Nutator. Collect the beads using the magnetic stand and remove the wash solution.
13. Elute the DNA from the beads by adding 250 µl of elution buffer. Rock for 15 min at room temperature. Pipette off the eluate and save this material in a new 1.7 ml microfuge tube. Repeat once more and combine both the elutions. Discard the beads.
14. To reverse the cross-links add 5 M NaCl to a final concentration of 0.3 M and 1 µl of RNase A (20 mg/ml) to the eluted DNA. Add 400 µl of elution buffer to the inputs saved at step 3.2, to make the volume 500 µl. To the inputs add NaCl to 0.3 M, 1 µl of RNase A, 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCl pH 6.5 and 1 µl of proteinase K (20 mg/ml).
15. Incubate the tubes overnight at 65 °C in a dry heating block. To avoid evaporation of samples, seal or place a weight on the tubes to keep them from opening. A hybridization oven can also be used.
16. Let the tubes cool to RT. Add 1 ml 100% ethanol and incubate 2 hr-overnight at -80 °C to precipitate DNA.
17. Centrifuge the tubes at 16,000 x g for 15 min to pellet the DNA. Wash the DNA pellet once with 70% ethanol and air-dry the pellet. Resuspend the DNA pellet in 100 µl of autoclaved distilled water.
18. Purify DNA using QiaQuick spin columns and elute in total volume of 50 µl of elution buffer. This DNA is ready for use for PCR.

4. Preparation of the PCR Reaction (All Steps Must be Carried Out on Ice)

1. Collect all the DNA samples from ChIP and corresponding input samples. Also, collect all the PCR reagents and primers for targeted region as well as a control region. Use a "hotstart" Taq DNA polymerase. We will use a SYBR Green-based assay to quantify DNA amplification in this procedure, but qPCR mastermix kits can be used if necessary.
2. Make a serial dilution of input DNA to generate a standard curve in the qPCR analysis: for example 10%, 1%, 0.1% and 0.01% in elution buffer (from the QiaQuick spin columns in step 3.18). The concentration range and increment of this standard curve template set can vary based on the expected levels of ChIP enrichment, etc. Dispense 10 µl of input DNA samples in respective wells of a 96-well plate (Genemate, cat# T-3182-1), in duplicate.
3. Dispense 10 ml ChIP DNA from isotype control as well as specific antibody in respective wells, in triplicate.
4. Make a "master mix" containing either target primers or control primers (0.1 µM final concentration of each primer) and dispense 10 µl into respective wells. For example, in the template below dispense master mix-containing targeted primers into wells marked green and dispense master mix with control primers into wells marked red.
5. Cover the PCR plate with optical plastic sealing film and centrifuge at 500 x g in a clinical centrifuge (~1,200 rpm) for 1 min at RT to collect everything at the bottom of the well.
6. Start the PCR reaction. We will use the LightCycler 480 II (Roche) operating LightCycler 480SW 1.5 software to run the qPCR in this example.

Pre-incubation: 1 cycle: 95 °C/5 min.

Amplification: 40-45 cycles: 94 °C/5 sec, 60 °C/5 sec, 72 °C/10 sec (annealing temperature should be 2-3 °C less than the melting temperature of the primers).

Melting curve: 1 cycle.

Cooling: 1 cycle.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10% Input	10% Input	Isotype	Specific								
B	1% Input	1% Input	Isotype	Specific								
C	0.1% Input	0.1% Input	Isotype	Specific								
D	0.01% Input	0.01% Input										
E	10% Input	10% Input	Isotype	Specific								
F	1% Input	1% Input	Isotype	Specific								
G	0.1% Input	0.1% Input	Isotype	Specific								
H	0.01% Input	0.01% Input										

Green: Use targeted region primers.

Red: Use control region primers.

5. Analysis

1. Program the qPCR software for quantification of absolute quantity of DNA. Importantly, the use of a standard curve to interpolate DNA amounts in the unknown specific and isotype samples allows the evaluation and matching of the quality and efficiency of all primer sets over the range of concentrations found in the samples. Furthermore, it also provides a more reliable method to convert the Cq (C_t or C_p) values to final relative fold enrichment results than using ΔΔCq and related methods.
2. Go to the sample editor and mark the wells containing input samples of various dilutions (10%, 1%, 0.1% and 0.01%) with their standard curve values. Also label the wells with unknown ChIP samples, exactly as the PCR plate is laid out. In equivalent software used in other qPCR machines, designate subsets corresponding to reactions for each primer pair, standard curve value and experimental and isotype control sample accordingly.
3. In the LightCycler software, go to the subset editor and mark the subsets including the wells with input samples as well as the unknown ChIP samples. The unknown samples will be quantified using the standard curve generated from the known concentrations of input samples for the analysis. In equivalent software used in other qPCR machines, designate subsets corresponding to all the reactions for each primer pairs.
4. After the PCR amplification is complete, perform the analysis with "Abs Quant/2nd Derivative Max" and select the subset for analysis and press OK. In equivalent software used in other qPCR machines, convert Cq value to the DNA amount in each subset.
5. Press "Calculate" which will generate the standard curve using the known concentrations of input samples and will display the absolute quantity of DNA present in the unknown samples.
6. If the quality of sheared DNA is good, and if the primers bind specifically to the targeted region, PCR efficiency should be close to 2.
7. Perform a melting curve analysis with "Tm Calling" for the same subset, if available. A single peak indicates that only one specific product was amplified. Amplification of specific DNA can also be tested by electrophoresing the PCR product along with DNA ladder through an agarose gel.
8. Export data as a tab-delimited text file, which can be opened in Microsoft Excel for further analysis.
9. Using Microsoft Excel, divide the DNA amount for the specific antibody with the isotype control for targeted primers. Repeat this step for the control region primers. If multiple antibodies of the same isotype are used for different immunoprecipitations, normalize each of these with the values from the same isotype control. Moreover, if multiple targeted primer pairs are used, the DNA quantities from a single control primer pair set should be used for normalization of each target (**Figures 1** and **2**). The output values represent the specific antibody immunoprecipitation fold enrichment at each location relative to non-specific antibody background immunoprecipitation.
10. Divide the fold enrichment (to isotype control) for targeted primers with the fold enrichment (to isotype control) for control region primers to obtain enrichment relative to a control unbound region (**Figure 1**). Importantly, note that because of the generation of standard curves with total input DNA for each sample, each of the above immunoprecipitation values interpolated from the standard curves are already normalized to, and expressed as, the fraction of total input by the machine software.
11. If the stringency of the buffers used for immunoprecipitation or washing is too high, it is possible that robust amplification from samples immunoprecipitated with specific antibodies will be observed, while amplification from isotype control immunoprecipitated samples will not be observed. In such a scenario, it is better to subtract the amount of Isotype control ChIP from specific antibody ChIP for both the targeted region and the unbound region. Relative enrichment can be calculated by dividing the difference for targeted region by difference for unbound region (**Figure 3**).

Representative Results

The Chromatin Immunoprecipitation (ChIP) protocol presented here controls for differences, if any, in the amount of DNA used in PCR through use of a primer pair that amplifies an unbound region of genome, thus serving as a "loading control". In the example shown in **Figure 3**, we have

used the coding region of the mouse *Actb* gene as a region unbound to our protein of interest and the transcription factor NFAT binding site on mouse *Il2* promoter as target region. Alternatively, a region several kilobases upstream or downstream of the targeted region known to have no binding of the protein of interest can be chosen for this purpose. When designing a primer pair for the targeted region, the ideal size of PCR product is approximately 100-200 bp. It is also important to computationally verify that these primers do not bind anywhere else in the genome.

Relative enrichment of a protein bound to different regions of the genome can be compared if the same set of isotype control and specific antibodies are chosen. Enrichment of various proteins on the same target region will depend on the specificity and affinity of the antibody used as well as the amount of the specific protein bound to DNA. For example, relative enrichment in the case of ChIPs performed for histones will typically return a higher enrichment value when compared to a transcription factor that may bind to DNA in response to various cellular signals. If the specificity of the antibody is good, we typically observe a 2-10 fold enrichment over the unbound control region (**Figures 3** and **4**).

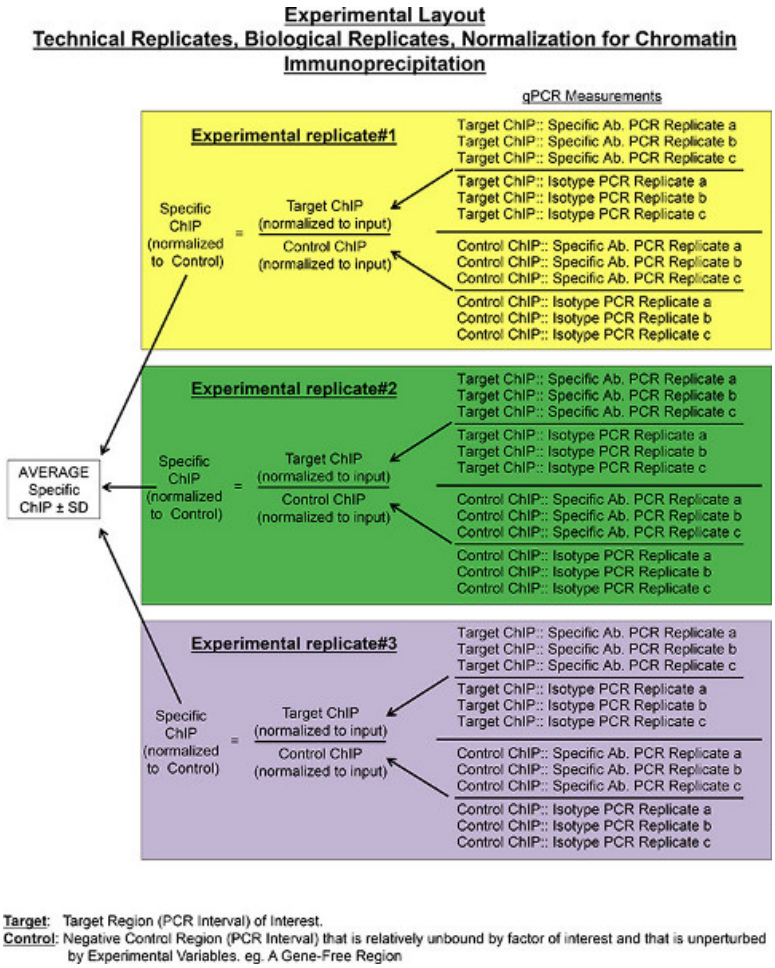

Figure 1. Experimental layout describing calculations to obtain relative enrichment of ChIP for specific antibody at the target site. Quantitative PCR is used to obtain DNA amounts in ChIP samples immunoprecipitated using a specific antibody and its isotype control. Quantitative PCR is performed using primers spanning target sequence and unbound control region of genome. For each ChIP sample, PCR is performed in triplicate, indicated as technical replicates a, b and c, and relative enrichment is calculated for each replicate as explained in the protocol. Calculations for average technical output values are performed. Variance can also be analyzed at this step, and ideally should be low. The technical error calculations however are not included in the final formula for calculation of error between biological replicates. Rather, the measured biological variation itself also inherently contains the technical variation. Three experimental replicates (in boxes colored yellow, green and purple) are used per ChIP experiment to obtain an average ChIP signal and its standard deviation (SD). Click here to view larger figure.

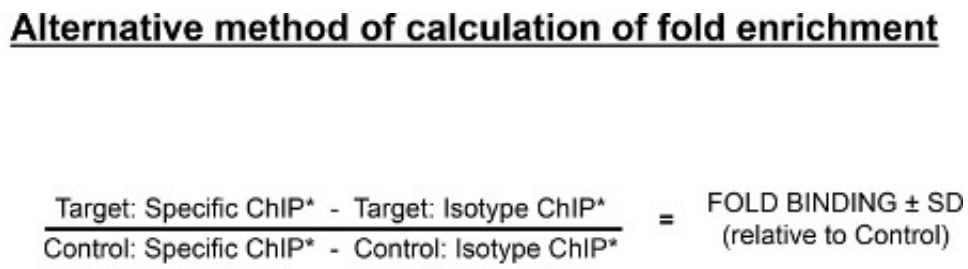

Figure 2. Alternative method of calculation of ChIP enrichment. In cases where the amount of DNA immunoprecipitated from the isotype antibody is extremely low and qPCR amplication is poor, this amount of DNA can be subtracted from the amount of DNA immunoprecipitated using a specific antibody to obtain the fold enrichment for every technical replicate. This method of calculation must be used in all three experimental replicates to obtain an average ChIP signal and its standard deviation (SD), as described in **Figure 1**.

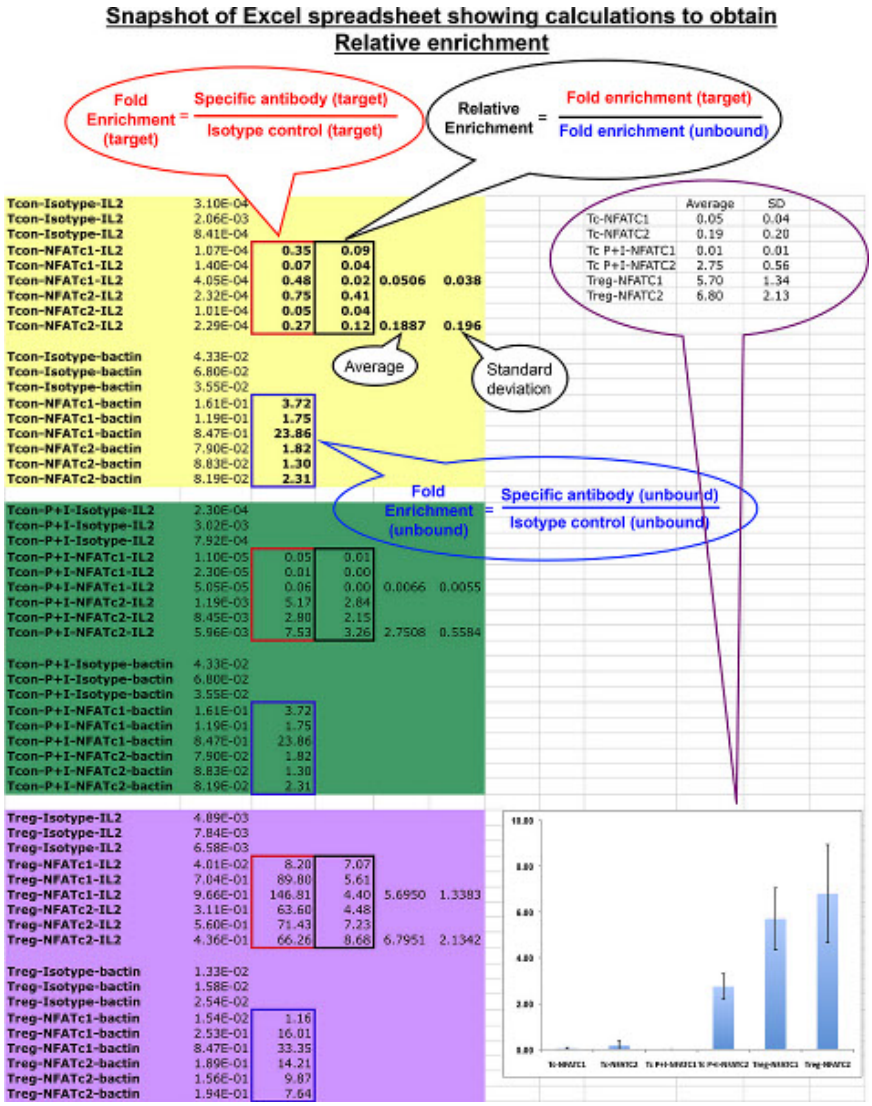

Figure 3. Snapshot of Microsoft Excel spreadsheet showing calculations to obtain relative enrichment. Data from three experimental repeats (in boxes colored yellow, green and purple) with three technical replicates each was used to calculate fold enrichment. Use of the calculation protocol described in **Figure 1** is depicted in this spreadsheet. Click here to view larger figure.

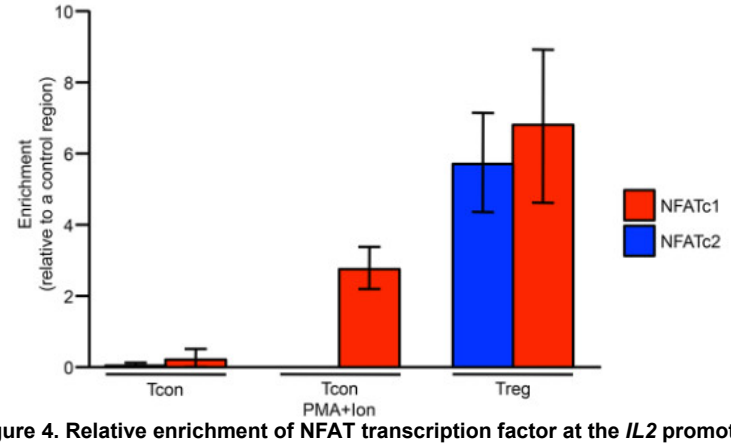

Figure 4. Relative enrichment of NFAT transcription factor at the *IL2* promoter. Conventional CD4 T cells (Tcon) and regulatory CD4 T cells (Tregs) were isolated from Foxp3EGFP mice. A small fraction of Tcon cells was stimulated with PMA and Ionomycin for 30 min. ChIP was performed as explained in the experimental protocol. Data presented here is from three experimental repeats with three technical replicates each. Relative enrichment of transcription factors NFATc1 and NFATc2 on the mouse *IL2* promoter in these cell types is depicted as fold enrichment over an unbound region[3].

Discussion

The protocol above provides a robust method of accurately quantifying DNA enrichment from primary lymphocytes using ChIP. One major reason for robustness in this protocol is the inclusion of biological replicates. The above protocol uses three replicates, the enrichment for which is calculated independently. The outputs are then averaged to provide a degree of enrichment and standard deviations calculated to provide a measure of variability. For each sample, three technical replicates are also performed to eliminate variation in sample handling, PCR amplification, etc. The amount of variation in the technical repeats is typically much less than that observed between different biological samples. A second reason for robustness is the inclusion of multiple technical controls, including isotype-matched control antibodies, the use of a standard input DNA curve, and the use of a control amplicon corresponding to a non-specific genomic region.

Limitations of the above method are similar to those encountered generally using ChIP. The method suffers from somewhat poor spatial resolution, the limiting factor being the shearing of DNA fragments by sonication. Therefore, the resolving power of the method is only approximately 500 bp of DNA. Experiments that require higher resolution can make use of micrococcal nuclease digestion[8] , or exonuclease digestion of recovered fragments (ChIP-exo)[9,10] . In addition, ChIP preparations described above coupled to high-throughput sequencing of recovered fragments (ChIPseq, ChIP-seq or ChIP-SEQ)[11,12] in place of qPCR, produces a sampling of differently sheared enriched fragments, the intersection of which may be taken as a *bona fide* site of interaction. Nevertheless, the method presented here provides a robust way of carefully and accurately quantifying DNA association with transcription factors, nucleosome components, modified histones and other proteins at specific sites of interest.

Disclosures

No conflict of interest declared.

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