Monitoring for epigenetic modifications at the FMR1 locus

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ABSTRACT

The vast majority of fragile X affected patients do not transcribe FMR1 due to a CGG repeat expansion in the 5'-untranslated region of the FMR1 gene. When the CGGs considerably expand, it elicits abnormal DNA methylation and histone modifications, which are responsible for FMR1 transcriptional silencing. In this chapter, we describe in detail two commonly used protocols for monitoring the epigenetic state of the FMR1 gene that bypass the difficulty in directly analyzing the CGGs. One protocol is for accurately measuring DNA methylation levels and the other is for profiling histone modifications.

Key words: FMR1, DNA methylation, histone modifications, bisulfite DNA sequencing, chromatin immune-precipitation (ChIP), PCR, qPCR

INTRODUCTION

The vast majority of fragile X syndrome (FXS) patients carry an unusual loss-of-function mutation due to a CGG tri-nucleotide microsatellite repeat expansion in the 5'-UTR of the X-linked FMR1 gene (1). When the CGGs reach the full mutation range (>200 repeats), they lead to incorrect spreading of DNA methylation from the upstream flanking region (2), coupled with a change from active to repressive histone modifications (4-9). This results in epigenetic gene silencing of FMR1 by a developmentally regulated process, eventually leading to FXS pathology. How precisely the addition of repeats results in the induction of repressive epigenetic marks remains to be elucidated.

Much effort has been invested into developing assays for recording the epigenetic status of the FMR1 gene, particularly for accurate measurement of DNA methylation levels. This is because it may serve as a valuable diagnostic tool for disease prognosis. In addition, it may provide a useful tool for investigating the yet unresolved mechanisms of FMR1 gene silencing in FXS. However, the major difficulties in monitoring the epigenetic changes that are imposed by the mutation lies in the failure to effectively PCR amplify the GC-rich repeats, particularly when they are expanded. In this chapter, we describe two commonly used methods for monitoring the epigenetic state of the 5'-end of FMR1 without the need for PCR amplification of the repeats. One method is for accurately measuring DNA methylation levels and the other is for profiling histone modifications.

Among the currently available tests for monitoring FMR1 DNA methylation (for comprehensive review see Hayward and Usdin (3)), the most sensitive and widely used assay is the one that relies on bisulfite PCR DNA sequencing in the region that is located immediately upstream to the CGGs. The technique is based on chemical pre-treatment of DNA with sodium bisulfite, which converts cytosines (Cs) into uracil by deamination, unless they are methylated. First, genomic DNA is denatured and chemically modified by sodium bisulfite, to guarantee efficient C to U conversion. Next, the 5' or 3' flanking sequence to the CGGs (a region which is typically methylated when the CGGs expand in cells of patients) is amplified by PCR using a pair of universal strand-specific primers. This permits the simultaneous amplification of methylated (unmodified) and

unmethylated (modified) molecules in a single PCR reaction. Finally, the pool of PCR products is sequenced for the identification of C to U conversion events (represented by Ts as a replacement for Cs after PCR amplification), either by single colony Sanger sequencing or locus-specific next-generation sequencing (NGS). This provides a convenient way to distinguish between methylated and unmethylated CpG sites at the single molecule level, and it permits analysis of a considerable number of CpG sites simultaneously, at single bp resolution.

For profiling of FMR1 for specific histone modifications in vivo, we use the Chromatin immune-precipitations (ChIP) procedure. ChIP is a semi-quantitative approach that determines whether a given protein/protein post-transcriptional modification binds to a specific DNA sequence in the cell. It is the method of choice for profiling histone modifications, and is commonly used for differentiating transcriptionally active (euchromatin) from inactive chromatin (heterochromatin). The technique is based on the selective enrichment of a chromatin fraction by immuno-precipitation using antibodies that specifically recognize the inspected protein. Briefly, the DNA and the proteins that are associated with chromatin are crosslinked with formaldehyde. Following cell lysis, the DNA is sheared into 250-1000bp fragments by sonication, and then selectively immuno-precipitated with an antibody that specifically interacts with the targeted protein. Next, the bound DNA fragments are purified from the immuno-precipitated chromatin fraction after crosslinking is reversed. Last, the region of interest is evaluated by qPCR for degree of enrichment by comparing amounts between antibody-immuno-precipitated (bound) and untreated control (input) DNA fractions. As ChIP relies on the immuno-precipitation reaction, it is crucial that the antibody will be highly specific and suitable for this type of experimental procedure. In addition, it is absolutely essential to include positive and negative DNA control regions in the analysis. This is to accurately assess the efficiency of the reaction by taking into account the effect of non-specific signals. One point to consider with respect to FMR1 is that the qPCR reaction, similar to the bisulfite PCR reaction, is carried out by amplification of a region that immediately flanks the CGG sequence. This is to bypass the difficulty of amplifying the CGG repetitive region. However, it should be noted that since enrichments are quantified on sheared DNA

fragments (500bp in average), they provide rough estimates at a resolution of hundreds of bps.

There are by now plenty of commercially available antibodies for carrying out ChIP analysis for the different forms of histone modifications (particularly for the ones that are associated with the silencing of the FMR1 locus) including for histone H3 (K4me3, H3K9me2/3, and H3K27me3) and histone H4 (H4K20me3) (4-9). For each modification, appropriate positive and negative control amplicons should be selected. This is to validate the efficiency and reliability of the assay.

SUMMARY

1. EQUIPMENT AND MATERIALS

1.1. Bisulfite Reaction

1.1.1. Commercially available bisulfite reaction kit, Zymo Bisulfite Direct Kit (#D5020)

1.1.2. Eppendorf tubes 1.5ml and PCR 0.2µl tubes

1.1.3. Deionized water or TE buffer.

1.1.4. PCR thermal cycler

1.1.5. Variable speed centrifuge (benchtop or floor model, >10,000rpm)

1.2. PCR Purification, Cloning and Sequencing Analysis of Target Bisulfite DNA Fragment.

1.2.1. Fast Start Taq polymerase (Roche).

1.2.2. FMR1 gene specific converted primers

FMR1 5' (colony bisulfite):
Forward 5'- TTGAGTGTATTTTTGTAGAAATGGG-3'
Reverse 5'- CCTCTCTCTTCAAATAACCTAAAAA-3'.
Tm=55 ºC. Product size: 191bp

FMR1 3' – (colony bisulfite):
Forward 5'- GGTATTTGGTTTTAGGGTAGGTTT -3'
Reverse 5'- TTCCAACAAACCCCAAAT -3'.
Tm=55 ºC. Product size: 173bp

1.2.3. Agarose gel, ethidium bromide and electrophoresis apparatus.

1.2.4. QIAquick PCR Purification Kit (Qiagen) for purification of PCR product or QIAquick Gel Extraction Kit (Qiagen) for purification of target PCR fragment from multiple nonspecific PCR products.

1.2.5. pGEM-T Easy vector system II (Promega).

1.2.6. Competent bacteria DH5α cells

1.2.7. For bacterial culturing and positive cloning selection, bacto-tryptone (BD), yeast extract, sodium chloride, ampicillin solution, isopropyl-β-D-thiogalactoside (IPTG), X-Gal (Bio-Rad), and bacterial shaker incubator at 37 °C are required.

1.2.8. Mini prep kit

1.2.9. Colony PCR: Ready mix X5 (Biolab), SP6 and T7 primers

1.3. Bisulfite Sanger Sequencing reaction and analysis

1.3.1. BigDye 3.1V

1.3.2. PCR thermal cycler

1.3.3. Performa DTR (Dye Terminator Removal) Gel Filtration Cartridges

1.3.4. ABI No# DNA Analyzer.

1.3.5. Analysis software Finch TV and ApE-A editor

1.4. Bisulfite Deep-sequencing

1.4.1. Gene specific primers with P5 or P7 adaptors

FMR1 3' (deep-sequencing):
Forward 5'-P5- AGAGGGGTTTTTAATAGGTTTTAAGTT-3'
Reverse 5'- P7-CTTCCCTCCCTTTTCTTCTTAAT-3'.
Tm=55 ºC
P5 adaptor: Forward overhang: 5'
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]
P7 adaptor: Reverse overhang: 5'
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus- specific sequence]

1.4.2. D1000 ScreenTape kit (Agilent Technologies) and Qubit® DNA HS Assay kit (catalog #32854; Invitrogen) , (X1, Agencourt AMPure XP - Beckman Coulter

1.4.3. PrimMax Takara taq polymerase and N7XX primer (nextera barcode 1) and S5XX primer (nextera barcode 2), and 2X Primstar (ReadyMix).

1.4.4. ScreenTape kit and the Qubit® DNA HS Assay kit for quality control of prepared library and normalization processes.

1.4.5. NextSeq 500 Mid-Output Kit (150 cycles) cartridge (Illumina, San Diego, CA) and NextSeq 500 System instrument (Illumina).

1.5. Chromatin Immunoprecipitation assay materials

1.5.1. E8 supplemented medium (Gibco)

1.5.2. Vitronectin

1.5.3. Trypsin

1.5.4. 37% Formaldehyde

1.5.5. 1.25M Glycine.

1.5.6. 0.5M EDTA pH 8.0.

1.5.7. 5M NaCl

1.5.8. 1M Tris-HCl, pH 6.5

1.5.9. 10X PBS

1.5.10. Protease Inhibitor Cocktail II

1.5.11. RNase A

1.5.12. Proteinase K

1.5.13. 1M NaHCO3

1.5.14. 20% SDS

1.5.15. Antibody of interest for chromatin immunoprecipitation, anti-H3K4me2 (Upstate 07-030), anti-H3K9me3 (Abcam 8898) and anti-H3K27me (Abcam 6002).

1.5.16. Protein G magnetic beads

1.5.17. DNA purification kit

1.5.18. Syber green mix

1.5.19. Primers

HOXA9 Forward 5'-CTCAGGAGCCTCGTGTCTTT-3'

Reverse 5'-GTGACCAGGTGGAGGTGTGT-3'

Tm=60 ºC, Product Size (bp) =82

CRYSTALIN Forward 5'-CCGTGGTACCAAAGCTGA-3'

Reverse 5'-AGCCGGCTGGGGTAGAAG-3'

Tm=58-62ºC, Product Size (bp) =85

APRT Forward 5'-GCCTTGACTCGCACTTTTGT-3'

Reverse 5'-TAGGCGCCATCGATTTTAAG-3'

Tm=60 ºC, Product Size (bp) =85

FMR1 promoter Forward 5’-AACTGGGATAACCGGATGCAT-3’

Reverse 5’-GGCCAGAACGCCCATTTC-3’

Tm=63 ºC, Product Size (bp) =72

1.5.20. DNase and RNase free sterile H_2O

1.5.21. Buffers

SDS Lysis Buffer

SDS	1% SDS
EDTA, pH 8	10 mM EDTA
Tris HCl, pH 8.1	50 mM Tris HCl,

Dilution Buffer 10ml

Triton X-100	1.1.%
EDTA pH=8	1.2mM
TRIS-HCl pH=8	16.7mM
NaCl	167mM
DDW	8.4ml

Low Salt Buffer 25ml

Triton X-100	1%
EDTA pH=8	2mM
TRIS-HCl pH=8	20mM
NaCl	150mM
SDS	0.1%

High Salt Buffer 25ml

Triton X-100	1%
EDTA pH=8	2mM
TRIS-HCl pH=8	20mM
NaCl	500mM
SDS	0.1%

TE buffer 25 mM

EDTA pH=8	1mM
TRIS-HCl pH=8	10mM

Elution Buffer

EDTA pH=8	1mM
TRIS-HCl pH=8	10mM
NaCl	200mM
SDS	1%

LiCl immune complex wash buffer 25ml

EDTA 0.5M	1 mM
Tris-HCl pH 8.1	10 mM
NP-40 100%	1%
LiCl 1M	250 mM
Deoxycholate	1%

1.6. Chromatin Immunoprecipitation assay materials Equipment

1.6.1. Biological and Chemical Hoods

1.6.2. CO_2 incubator

1.6.3. Vortex mixer

1.6.4. Rotating wheel/platform

1.6.5. Timer

1.6.6. Variable volume (5-1000µl) pipettes + tips

1.6.7. Centrifuge (micro and regular)

1.6.8. Variable temperature water bath

1.6.9. Sonicator Bath Vibra Cell VCX130 with 3mm microtip

1.6.10. Microfuge tubes, 1.5ml

1.6.11. Real Time PCR

1.6.12. Real Time plates

1.6.13. Filter-tip pipette tips

1.6.14. Electrophoresis equipment

2. METHODS

2.1. Measuring FMR1 methylation levels by bisulfite PCR DNA sequencing

2.1.1. Bisulfite Conversion

2.1.1.1. 2μg of genomic DNA in a total volume of 20 μl DDW is added to 130 μl of CT conversion reagent solution in a PCR tube (EZ DNA Methylation Direct Kit, Zymo). The sample is mixed and centrifuged briefly to ensure no droplets are in the cap or side of the tube.

2.1.1.2. Place the PCR tube in a thermo cycler and perform the following steps:

98°C for 8 min

64°C for 3.5 hours

4°C storage for up to 20 hours

2.1.1.3. Add 600 μl of M-Binding Buffer into a Zymo-Spin IC column and place the column into a collection tube.

2.1.1.4. Load the sample from step 2 into the column containing the M-Binding buffer. Close the cap and mix by inverting the column several times.

2.1.1.5. Centrifuge at full speed, ≥10,000xg for 30 seconds. Discard the flow-through.

2.1.1.6. Add 100μl of M-wash buffer to the column. Centrifuge at full speed for 30 seconds.

2.1.1.7. Add 200μl of M-Desulfonation Buffer to the column and let stand at room temperature (20°-30°) for 15-20 minutes. After incubation, centrifuge at full speed for 30 seconds.

2.1.1.8. Add 200μl of M-wash buffer to the column. Centrifuge at full speed for 30 seconds. Add another 200 μl of M-Wash buffer and centrifuge for 1 minute.

2.1.1.9. Place the column into a 1.5ml microcentrifuge tube. Add 10 μl of the M Elution Buffer directly to the column matrix. Wait for 1 minute and centrifuge for 30 seconds at full speed to elute the DNA.

2.1.1.10. The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long term storage, store at below -70°C **Note 1.**

2.1.2. Bisulfite PCR Amplification

Bisulfite treated DNA can be amplified with strand specific and bisulfite specific primers in a PCR reaction. Primers designed outside of a CpG region of interest will, in principle, amplify the target regardless of the methylation state of the internal sequence. Bisulfite sequencing provides an inherently more accurate assessment of the methylation state of a sample compared to PCR primers (or probes) that select for presupposed fully methylated or fully unmethylated complementary sequences, such as methylation-specific primers (MSP). Bisulfite PCR primer design is crucial for successful implementation of subsequent bisulfite sequencing analysis.

2.1.2.1. Primer design

2.1.2.1.1. Primers have to be ~25-30 bp in length, to ensure specificity.

2.1.2.1.2. Primer pairs should have a similar Tm, be above 50°C and not differ by more than 1-2°C

2.1.2.1.3. Primers should contain multiple (~25%) C to T bases, to ensure conversion specificity.

2.1.2.1.4. The last base at the 3' end of the primer should be a C to T to ensure amplification of converted DNA

2.1.2.1.5. CpGs should be avoided in the primer sequence to circumvent potential bias towards methylated, unmethylated or unconverted templates

2.1.2.1.6. Amplicons length should not be more than ~450 bp. **Note 2**

2.1.2.2. Bisulfite PCR reaction

2.1.2.2.1. Annealing temperature: A gradient PCR thermocycler can help to determine the appropriate annealing temperature. If there is no access to a gradient PCR thermocycler, touchdown PCR can be applied to increase the annealing sensitivity.

2.1.2.2.2. PCR reaction system: A commercially-available PCR MasterMix which mixes *Taq* DNA polymerase and dNTP with optimal salt concentration can be easily used for bisulfite PCR. If this common PCR

reaction system cannot produce a clean band, it is advisable to try a different PCR reaction system. In our laboratory, we normally use FastStart Taq polymerase from Roche to improve the bisulfite PCR results. **Note 3**

PCR mix

FMR1 F primer (2μM)	1μl
FMR1 R primer (2μM)	1μl
dNTPs (10mM)	0.5μl
FastSart Buffer containing MgCl	2.5μl
FastStar taq	0.3μl
Converted DNA	2μl
DDW	12.7 μ l

PCR cycling conditions

1. 94°C 5min
2. 95°C 30 sec
3. 55°C 30 sec
4. 72°C 30 sec
5. Go to 2 39 cycles
6. 74°C 10 min
7. 4°C ∞

2.1.2.2.3. Visualized the PCR products by agarose gel electrophoresis and analisisd by cloning and sequencing. Run 5 μl PCR products on 1.5% Agarose gel in order to verify the specificity of the primers. A single appropriately sized band indicates successful PCR amplification.

2.1.3 Bisulfite Colony Sequencing

2.1.3.1. PCR purification products. Commercially-available kits such as the RBC Kit for specific PCR fragment purification and Gel Extraction can help to isolate the target PCR product from multiple non-specific PCR bands.

2.1.3.2. PCR cloning Clone sequencing is necessary to observe the distribution of methylation patterns at the single molecule level. For this purpose we use pGEM-T Easy vector system II (Promega) which provides the T4 DNA ligase system, a pGEM-T Easy vector and competent DH5α cells as well. By using this kit, purified PCR products can be ligated to the pGEM-T Easy vector and transformed into competent DH5α cells. The DH5α cells that carry the ligated vectors can be selected on agar plates containing ampicillin/X-gal/IPTG by color change where blue colonies represent empty vector, and white colonies represent vectors inserted with target PCR products. The white colonies can then be selected and grown in LB medium+ampicillin. Plasmids containing target DNA are extracted by using a Miniprep Kit (RBC), then subjected to colony PCR and standard sequencing analysis.

2.1.3.3. Colony PCR

Mini prep products are subjected to a PCR reaction with SP6 and T7 primers

Ready mix (X5)	5µl
SP6 (2µM)	1.5µl
T7 (2µM)	1.5µl
Mini prep product	1µl
DDW	16µl

2.1.3.4. Direct PCR Sequencing Single colonies are analyzed for CpG methylation by direct sequencing (ABI 3130). After cloning and sequencing, the methylation state of individual molecules can be tabulated, in a bisulfite map, to visualize the percent of methylation.

Sequencing mix

BigDye 3.1V	1.2µl
BigDye Buffer	3.4µl
Primer (10µM) SP6/T7	0.8µl
Colony PCR product	2-3µl
DDW	11.6µl

Sequencing reaction

1.	96°C	2min
2.	96°C	10sec
3.	50°C	5sec
4.	60°C	4min
5.	Go to 2	25times
6.	10°C	∞

2.1.3.5. Purify sequencing products. Performa DTR (Dye Terminator Removal) Gel Filtration Cartridges are 800 µL spin columns assembled in a 1.5 mL microcentrifuge tube. These columns provide optimal performance for removal of unincorporated BigDye® v1.1, v3.0, and v3.1 and other dye terminators, dNTPs, salts, and other low molecular weight materials from sequencing reactions. These columns also remove DNA primers and fragments up to 20 bases, buffers, and nucleotides labeled with biotin, isotopes, and other assorted markers.

2.1.3.6. Centrifuge the Performa Gel Filtration Cartridge (BigDye® v3.1) for 3 minutes at 850 x g.

2.1.3.7. Remove the spin column and transfer to the provided 1.5 mL microcentrifuge tube.

2.1.3.8. Add the reaction sample of 10–20 µL to the center of each column.

2.1.3.9. Centrifuge for 3 minutes at 850 x g. Retain eluate.

- Up to 4 µL may be lost during sample processing.

• If the volume loss is greater than 4 µL, this is an indication of an overly dry gel. To optimize recovery of sample, repeat centrifugation.

2.1.4. Deep-sequencing analysis.16S Library Preparation and Deep-sequencing. A D1000 ScreenTape kit (Agilent Technologies) and Qubit® DNA HSAssay kit (Invitrogen) are used for quality control of PCR amplicons, followed by bead purification (X1, Agencourt AMPure XP - Beckman Coulter), according to the manufacturer's protocol. Subsequently, the amplicons are subjected to a second PrimMax Takara PCR reaction (1st purified PCR DNA (7.5 µl), 2.5 µl N7XX primer (nextera barcode 1), 2.5 ul S5XX primer (nextera barcode 2), 12.5 µl 2X Primstar ReadyMix). The PCR program is: 98°C for 1 minute followed by 8 cycles of: 98°C for 10 seconds, 55°C for 10 seconds, 72°C for 30 seconds, then 72°C for 5 minutes and a hold at 10°C. The 2nd PCR is bead purified, followed by the use of the ScreenTape kit and the Qubit® DNA HS Assay kit for quality control and normalization of prepared libraries. Samples are pooled at 10 nM concentration, then diluted to 4 nM for denaturation according to the Illumina protocol. A multiplexed sample pool (1.5 pM including PhiX 40%) is then loaded into the NextSeq 500 Mid-Output Kit (150 cycles) cartridge (Illumina, San Diego, CA) and loaded onto a NextSeq 500 System instrument (Illumina), with 150 cycles and single-Read sequencing conditions. Data analysis: Raw sequence reads are mapped to the human genome (build GRCh38). Methylation calls are extracted after duplicate sequences had been excluded. Data visualization and analysis are performed using custom R and Java scripts. CpG methylation is calculated as the average of methylation for each CpG position, and non-CpG methylation is extracted from Bismark reports.

2.2. Monitoring for histone modifications at the FMR1 locus by Chromatin Immunoprecipitation assay

2.2.1. *In Vivo* Crosslinking

2.2.1.1. Grow the cells until they reach ~80-90% confluence (attached cells) or in suspension. For human embryonic stem cells grow under feeder free conditions on vitronectin, with the E8 supplemented medium (Gibco) on 6 well dishes we use~ 5-6 plates that are equivalent to approximately 2X10^7 cells. This will provide a chromatin preparation that can be used for up to 10 separate immunoprecipitation reactions.

2.2.1.2. De-attach the cells with trypsin-EDTA, 0.5ml per well during 5 min at 37°C.

2.2.1.3. Neutralize the trypsin by addition of full hESC growth medium containing serum.

2.2.1.4. Collect the cells into a 50ml tube, spin down the cells (5 min, 1200rpm) and discard supernatant.

2.2.1.5. Wash the cells with PBSX1, spin down the cells (5 min, 1200rpm) and remove supernatant.

2.2.1.6. Re-suspend the cells into 10 ml full medium. Count the cells and fix the concentration to 2-2.2X10^6 cell per ml with full hESC growth medium.

2.2.1.7. Add 270µl of 37% formaldehyde to 10 ml of growth media to crosslink while gently swirling the tube. **Note 4**. Final concentration of the formaldehyde is 1%. Use high quality formaldehyde. Do not use if formaldehyde is past expiration date as suggested by the manufacturer.

2.2.1.8. Incubate at room temperature for 10 min

2.2.1.9. Add 1ml of 1.25 M Glycine to the tube to quench unreacted formaldehyde. Swirl to mix and incubate at room temperature for 5 min

2.2.1.10. Centrifuge 3 min at 700 g at 4°C

2.2.1.11. Aspirate the medium by removing as much medium as possible and add 10 ml cold PBSX1.

2.2.1.12. Centrifuge 3 min at 700g at 4°C.

2.2.1.13. Remove the PBS and repeat PBS washes, step (2.2.1.12-2.2.1.13)

2.2.1.14. Add 1ml cold PBSX1 containing 2.5 µl Protease inhibitor cocktail II(X400) and transfer the cells to eppendorf tube.

2.2.1.15. Spin at 700Xg at 4°C for 5 min to pellet the cells

2.2.1.16. Discard supernatant.

2.2.1.17. Flash freeze the cells in liquid nitrogen and store in -80°C or continue to the chromatin purification step.

2.2.2. Lysis and Chromatin purification

2.2.2.1. Thaw the fixed cells pellets on ice.

2.2.2.2. Re-suspend the cells with 1 ml SDS lysis buffer containing Protease Inhibitors cocktail II. **Note 5**

2.2.2.3. Incubate for 0.5-1h on ice.

2.2.2.4. Aliquot 500 µl per microfuge tube, the cell lysate can be frozen at -80°C at this step or continue to the sonication step.

2.2.3. DNA shear by Bioruptor Sonication

2.2.3.1. If cell lysate was previously frozen, thaw on ice. Prepare: 1.5 ml tube containing the cells with the lysis buffer on fresh ice + 1.5 ml tube for equilibration + a big beaker for excess water + timer + cold water. Set the small centrifuge to 4°C.

2.2.3.2. Take out the water from the sonicator. Add the cold water to the sonicator 1 cm under the line. Add ice until the water reaches the line. Insert the 1.5 ml tubes to the sonicator and close lid. Make sure it is set for high, 0.5min on-0.5min off.

2.2.3.3. Start the sonication for 7 min X 2 (the time of sonication should be optimized to reach a smear in the gel agarose with a peak at 200-300 bp following electrophoresis). **Note 6**

2.2.3.4. Centrifuge the sheered chromatin for 15 min 4°C, 14,000 rpm.

2.2.3.5. Divide the supernatant into the ChIP vials, 100µl each containing 2.2x10^6 cell equivalents which is enough for one immunoprecipitation.

2.2.3.6. You can stop at this stage and store the samples at -80°C after freezing with liquid nitrogen. Sheared crosslinked chromatin can be stored at -80°C for up to a few months.

2.2.4. Validation of sonication

2.2.4.1. Dilute the Input (use half of the Input) samples with up to 100 µl with Elution buffer and add 5 M NaCl to a final concentration of 0.2 M NaCl.

2.2.4.2. Add RNase (DNase free) and incubate for 30 min, 37°C, shaking at 1200 rpm.

2.2.4.3. Add Proteinase K and reverse crosslink for 3h, at 65 °C, shaking at 1200 rpm.

2.2.4.4. Purify using PCR/gel extraction kit and run the sonicated samples on a gel to obtain a smear which should have a peak at ~300 bp. Run 1-1.5 µg of the input in a 1.5% agarose gel, 80V for 30 min.

2.2.5. Immunoprecipitation (IP) first day

2.2.5.1. Thaw the chromatin slowly; keep it cold at all times. Remove from -20°C the Protease Inhibitor Cocktail II (PI II) and thaw at room temperature.

2.2.5.2. Prepare enough Dilution Buffer containing protease inhibitors for the number of desired immunoprecipitations and store on ice. Each IP requires the addition of 900 µl of Dilution Buffer and 4.5 µl of Protease Inhibitor Cocktail II.

2.2.5.3. Add 900 µl of Dilution Buffer containing PIC II into each tube containing 100 µl of chromatin.

2.2.5.4. Remove 10 µl of the supernatant as "Input" and save at 4^{o}C.

2.2.5.5. Add the immunoprecipitating antibody and 30 µl fully suspended protein G magnetic beads. **Note 7**.

2.2.5.6. Incubate overnight at 4^{o}C with rotation 12h (do not exceed 14 hrs).

2.2.6. Immunoprecipitation (IP) second day

2.2.6.1. Pellet Protein G magnetic beads with the magnetic separator and remove the supernatant completely.

2.2.6.2. Wash the protein G bead-antibody/chromatin complex by re-suspending the beads in 1 ml each of the cold buffers in the order listed below (all the buffers can be stored up to one year at 4°C) and incubating for 3-5 minutes on a rotating platform followed by magnetic clearance and careful removal of the supernatant fraction:

- Low Salt Immune Complex Wash Buffer, one wash.
- High Salt Immune Complex Wash Buffer, one wash.
- LiCl Immune Complex Wash Buffer, one wash.
- TE Buffer, one was

2.2.7. Elution of Protein/DNA Complexes and Reverse Crosslinks of Protein/DNA Complexes to Free DNA

2.2.7.1. Thaw Proteinase K and warm the ChIP Elution Buffer to room temperature.

2.2.7.2. Add 300 µl Elution Buffer to the beads and to the input.

2.2.7.3. Add 1 µl RNaseA and incubate 30 min at 37^{o}C, 600 rpm shaking. Add 2 µl of 20 mg/ml Proteinase K and reverse histone-DNA crosslinks by heating at 65^{o}C for 5h-O/N.

2.2.7.4. Transfer the tubes to magnetic rack and collect the supernatant to new eppendorf.

2.2.7.5. Use PCR purification kit (RBC). Elute with 50 µl pre-heated elution buffer to 60^{o}C and then another 50 µl (total of 100 µl).

2.2.8. Detection

This is the most variable step of the procedure because of the number of detection methods that can be employed and the variability of PCR primer selection. The most meaningful results will be obtained with quantitative PCR for this step and Real Time Quantitative PCR (RT-qPCR) is ideal for

implementation. Success in obtaining high-quality quantitative data is critically dependent on good primer design. In general, primers should be 20 to 30 bases long with a *T*m of 55◦ to 60◦C and optimum GC content of 50%. Amplification products should be 100 to 300 bp. Longer PCR products should be avoided, because the amplification efficiency is substantially lower, and DNA fragments that do not bind to both primers will not be amplified (this can be a significant problem since the size of DNA fragments in the samples averages ~500 bp and ranges between 100 to 1000 bp). A final primer concentration of 0.5µM works well for most primers, but in some instances, improved product specificity may be obtained by lowering the final primer concentration 5 to 10-fold. Immunoprecipitated chromatin is analyzed using real-time PCR, looking for enrichments at the FMR1 promoter region, 300bp upstream to the transcription start site (TSS). HoxA9 and APRT are used as positive and negative controls, respectively, for both H3K9me3 and H3K27me3; and APRT and Crystalin are used as positive and negative controls for H3K4me2, respectively. Enrichments (ΔΔCt) are normalized to positive controls, and FMR1 and negative controls are presented in graphs. The results can be presented also as relative enrichment with respect to the negative control. The positive control shows that the immunoprecipititation succeeded and the negative control gives the baseline noise of the assay. The data represents average values of three to five independent ChIP experiments. Error bars represent standard error (paired t test, *p < 0.05, **p < 0.01, ***p < 0.001). **Note 8**

ΔΔCt calculation

$$\text{Average } C_t^{\text{(INPUT)}} - \text{Average } C_t^{\text{(IP)}} = \Delta C_t$$

Subtract ΔC_t for the experimental primer pair from ΔC_t of the negative control $\Delta C_t^{\text{exp}} - \Delta C_t^{\text{control}} = \Delta\Delta C_t$

NOTES

1. **Completeness of bisulfite conversion**

   After bisulfite conversion PCR analysis is often the first indicator to evaluate the success of the bisulfite conversion. For a pilot experiment, select gDNA of known purity and quantity, not exceeding 300 ng for a 150 uL bisulfite conversion reaction. The most common sources of poor bisulfite conversion are from insufficient denaturation due to excess gDNA concentration or poor sample purity and possible renaturation of the freshly denatured gDNA. Testing XX normal cells for FMR1 methylation can serve as a 50/50 methylated/non-methylated control for bisulfite conversion efficiency.

2. **Primer design**

   Primers designed outside of a CpG region of interest will, in principle, amplify the target regardless of the methylation state of the internal sequence. Bisulfite sequencing provides an inherently more accurate assessment of the methylation state of a sample compared to PCR primers (or probes) that select for presupposed fully methylated or fully unmethylated complementary sequences, such as Methyl specific primers.

   Methyl Primer Express® Software is a free online primer design tool (geared specifically to facilitate methylation studies) which assists primer design for both methylated and unmethylated bisulfite modified DNA. Users simply cut and paste in the selected genomic sequence, the software then performs an *in-silico* bisulfite conversion (C's are converted to T's), and aids in the selection of primers. Methyl Primer Express software is available for free download at: (http://marketing.appliedbiosystems.com/mk/get/GAAS_CLINICAL_METHYLATED?_A=77005&_D=50613&_V=0#)

3. **PCR bias which is a variable for each sample and each analysis.**

   Bisulfite PCR amplification can be performed as a regular PCR reaction. However, PCR conditions for amplifying bisulfite-treated material should be carefully optimized since the bisulfite treatment reduces the specificity of DNA double strands. It is recommended to use 1-4μl of eluted DNA for each PCR, however, up to 10μl can be used if necessary. The elution volume can be ≥10μl depending on the

requirements of the experiment but small elution volumes will yield more concentrated DNA.

4. **Cross-linking**

   This treatment crosslinks the proteins to the DNA ensuring co-precipitation of the DNA with the protein of interest.

   The extent of formaldehyde cross-linking is an important variable that in principle may be modified by changing the duration of crosslinking, the concentration of formaldehyde, or the temperature at which the cross-linking is performed. For some applications where protein cross-linking is particularly efficient (as for histones) it might be useful to decrease the cross-linking time or formaldehyde concentration. In particular, histone tails have a number of lysine residues that are likely to be modified by formaldehyde, and such modified lysines may interfere with the binding of antibodies against specific peptides corresponding to modified histones (e.g., by acetylation, phosphorylation, methylation).

5. **Cell lysis**

   Although complete lysis of all cells is not absolutely necessary (and may be difficult to achieve), it is very important that lysis be as efficient as possible. Efficient lysis is important to obtain a reproducible degree of cell breakage among a group of samples to reliably compare results. Significant differences in cell lysis efficiency will result in immunoprecipitations with different ratios of antibody to chromatin, which will possibly alter immunoprecipitation efficiency.

6. **Sonication**

   Shearing DNA to a small size (~500 bp average) by sonication is the critical factor in achieving resolution between a DNA sequence where a particular protein is bound and a nearby (*cis*-) DNA sequence that does not bind that protein. In addition, fragmentation of the chromatin is essential for its solubilization from the ruptured cells. The ability to fragment and solubilize the chromatin depends on the extent of chromatin cross-linking. In general, more cross-linking results in larger fragment size and lower solubility, resulting in lower yield (Orlando et al., 1997). Because of the importance of this variable, the DNA size should be assessed to confirm that the

desired degree of fragmentation has been achieved, and it should be reassessed if fixation conditions are change.

Optimization of DNA Sonication

Optimal conditions required for shearing crosslinked DNA to 200-1000 base pairs in length depend on the cell type, cell concentration per lysis buffer and the sonicator equipment, including the power settings and number of pulses. Following the rotocol below, determine the optimal conditions required.

a. Generate at least 4 different microfuge tubes containing a variety of cell equivalent concentrations in the range of 5 x 106 per ml to 5 x 107 per ml. Each microfuge tube should contain approximately 300-400µl of cell lysate.

b. Be sure to keep the samples on ice at all times during the sonication processes, sonication generates heat which will denature the chromatin.

c. Remove 1 x 10^5 cell equivalents from each condition prior to sonication for analysis of unsheared DNA.

d. For each cell concentration, sonicate each tube for a different number of 10 sec pulses depending on the number of tubes. For example, sonicate the first tube for 1 x 10 seconds, the second tube for 2 x10 seconds, the third tube for 3 x 10 seconds and the fourth tube for 4 x 10 seconds.

e. Repeat for all cell concentrations.

f. Remove 5µl of the sonicated chromatin from each condition to a fresh tube.

g. To all 5µl samples (unsheared and sheared), add 90µl ddH_2O and 4µl 5M NaCl.

h. Incubate at least 4-5 hours to overnight at 65°C to reverse the DNA – protein crosslinks.

i. Add 1µl of RNase A and incubate for 30 minutes at 37°C.

j. Add 2µl 0.5M EDTA, 4µl 1M Tris-HCl and 1µl Proteinase K and incubate at 45°C for 1-2 hours.

k. Load 10µl (1 x 10^4 cell equivalents) and 20µl (2 x 10^4 cell equivalents) on a 1-2% agarose gel with a 100bp DNA marker.

• Loading different amounts helps to avoid under- or over-loading.

l. Observe which of the shearing conditions gives a smear of DNA in the range of 200bp-1000bp.

m. Repeat optimization of the shearing conditions if the results indicate that the fragmented DNA is not in the desired size range. Once optimal conditions have been determined, it is advised that the user does not alter the cell concentration or volume of lysate per microfuge tube for subsequent chromatin immunoprecipitation experiments.

7. **Immunoprecipitation**

The success of this procedure relies on the use of an antibody that will specifically and tightly bind its target protein in the buffer and wash conditions used. In addition, the antibody should be present in excess with respect to its target protein so that differences in the amount of protein-DNA complexes of interest will be accurately measured.When quantitative PCR will be performed in real time using SYBR Green, high-quality primer pairs should result in ~1.9-fold amplification/cycle. Such amplification efficiency can be determined from quantitative analysis of raw fluorescence data for each cycle. Amplification efficiencies <1.8 are likely to cause problems, particularly if detection of the PCR product requires 30 cycles or more. Specificity information may also be obtained by running dissociation curves on reactions following the conclusion of the PCR run.

8. **Quantitation**

When quantitative PCR is performed in real time using SYBR Green, the linear range is directly visualized and the quality of the reactions can be directly assessed. For reactions involving a given primer, the curves should be superimposable with respect to shape, and they should differ only in the number of cycles needed to reach the threshold (*Ct*). Amplification efficiencies should be ~1.9-fold/cycle. If the curves have different shapes and/or amplification efficiencies are <1.8, the reactions are not equivalent and accurate quantitation is impossible.

Controls

The control, which is usually meaningful, is to examine a given pair of input and immunoprecipitated samples for multiple genomic regions. Control genomic regions should all give the same background level of apparent immunoprecipitation efficiency. Fragments bound by the protein of interest will give higher apparent immunoprecipitation efficiencies, and the difference can be expressed as fold

enrichment over the background level. In the best cases, enrichments can be >100-fold, but even a factor of two can be meaningful if the experiment is repeated enough times and the experimentally determined error is sufficiently low. The advantage of this approach is that identical samples are used to directly determine relative protein association to different genomic regions. Furthermore, differences in fold enrichment for different genomic regions represent relative quantitative measurements of protein association in vivo.

Data interpretation

In most experiments, it is presumed that the protein of interest associates specifically with certain genomic regions and associates only nonspecifically with other genomic regions. In general, it is very difficult to distinguish true nonspecific association from experimental background of the cross-linking procedure. In this regard, immunoprecipitations with the antibody of interest generally give 2- to 3-fold higher immunoprecipitation efficiencies than immunoprecipitations with control (or no) antibodies, but it is unclear whether this effect is physiologically meaningful or an experimental artifact. Moreover, for a given pair of INPUT and IP samples, the fold enrichment of a given genomic region over the background is directly related to the level of protein association in vivo. It is useful to define “relative protein occupancies” for different regions by subtracting the background from the observed immunoprecipitation efficiencies. For some experiments, particularly those involving histone modifications, it is inappropriate to analyze the data in terms of occupancy units and specific versus nonspecific binding sites. Histones associate with essentially all genomic regions, and the level of a particular chromatin modification typically occurs in a continuum. Thus, it is very difficult to determine whether a given region is devoid of a particular modification, although information in this regard can be obtained in control immunoprecipitations using an off-target antibody. For these reasons, quantitative analysis of the relative level of a given histone modification is best presented using simple immunoprecipitation efficiencies. Again, to account for sample-to sample variations, a specific genomic region should be given an arbitrarily defined value, which is used to determine the relative levels of all other genomic regions.

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