Detection of Nonrandom X Chromosome Inactivation

This unit describes a PCR-based assay for distinguishing between the two X chromosomes in female cells and assessing the percentage of cells having each parental X chromosome active. Methylation of CpG residues in gene promoters is a major mechanism of transcriptional silencing. In mammalian female cells, hypermethylation is the way in which one X chromosome is inactivated. The X-inactivation assay described below relies on methylation sensitivity. In this unit, the highly polymorphic and therefore typically heterozygous $(CAG)_n$ region of the 5′ end of the coding region of the human androgen receptor gene (*HUMARA*), at Xq11.2, is used to distinguish and compare the methylation activity of the X chromosomes (Allen et al., 1992; Pegoraro et al., 1997).

X CHROMOSOME INACTIVATION ASSAY

In this protocol, genomic DNA samples isolated from peripheral blood, oral mucosa, or other tissues or cells are predigested with a methylation-sensitive restriction endonuclease, *Hpa*II. *Hpa*II will only cleave the DNA recognition site when the adjacent GpC island is undermethylated; hence, only the active *HUMARA* gene on the active X chromosome is digested. Following the DNA restriction digestion, the predigested samples are amplified by PCR, and only regions that are methylated, and therefore undigested, will amplify successfully. PCR products from the *Hpa*II-digested genomic DNA are then compared to a separate aliquot of the same DNA amplified without *Hpa*II digestion. Comparison of peak areas of the two *HUMARA* alleles provides data sufficient to determine X-inactivation patterns in the original cell population from which the genomic DNA was derived. Samples that show one *HUMARA* allele preferentially surviving *Hpa*II digestion are then determined to have skewed, "non-random" X inactivation (Eggan et al., 2000). Reasons for skewed X inactivation can include a clonal cell population (as in many tumors), a carrier state for X-linked cell lethal trait (Pegoraro et al., 1997; Lanasa et al., 2001), or monozygotic twinning (Nance, 1990; Lupski et al., 1991).

The X-inactivation assay comprises four steps: methylation-sensitive restriction enzyme predigestion (see Basic Protocol), followed by PCR, acrylamide gel separation, and analysis (described in the Support Protocol).

Materials

> NEBuffer 1 (New England Biolabs)
> 10 U/µl *Hpa*II restriction endonuclease
> 100 ng/µl genomic DNA sample (*APPENDIX 3B*) isolated, e.g., from peripheral blood or oral mucosa
> 100 ng/µl DNA sample that has previously shown a pattern of highly skewed X chromosome inactivation (contact authors at *ehoffman@childrens-research.org*)
>
> RNase/DNase-free microcentrifuge tubes
> Thermal cycler or water bath

Methylation-sensitive restriction pre-digestion

Two separate restriction-enzyme digestion reactions are prepared for each DNA sample—one with enzyme and one without. High-quality, high-molecular weight genomic DNA must be utilized for this assay.

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1. Prepare the following two reaction mixtures in labeled, sterile RNase/DNase-free microcentrifuge tubes, combining the reagents in the order indicated (total volume: 12.5 μl). Pipet up and down vigorously after each addition to ensure mixing.

   Reaction 1: HpaII predigestion reaction:
   5.25 μl deionized H_2O
   1.25 μl NEBuffer 1
   1.0 μl 10 U/μl *Hpa*II
   5 μl 100 ng/μl isolated DNA product (~500 ng DNA)

   Reaction 2: negative control (no HpaII):
   6.25 μl deionized H_2O
   1.25 μl NEBuffer 1
   5 μl 100 ng/μl isolated DNA product (~500 ng DNA)

2. As a positive control, prepare the above two reaction mixes using a DNA sample which has previously shown a pattern of highly skewed X-chromosome inactivation for enzyme digestion.

   Aliquots of such samples can be obtained from the authors.

3. In a thermal cycler or water bath, incubate reactions at 37°C for 120 min.

4. Finish with a 30 min incubation at 65° to deactivate the *Hpa*II.

   Further purification of cut DNA is not necessary. The digested products can be stored at 4°C for up to 48 hr, or at –20°C for extended periods of time.

5. Proceed with PCR amplification, dye labeling, and gel electrophoresis of the restriction fragments (see Support Protocol).

PCR AMPLIFICATION AND LABELING OF DIGESTED AND UNDIGESTED DNA TEMPLATES

In this protocol, PCR amplification is performed on the separate PCR reactions for the predigested and undigested DNA templates generated in the Basic Protocol (reactions 1 and 2 above). A key issue is the accurate quantitation of peak area of the *HUMARA* alleles after amplification, and the method used for PCR and primer synthesis depends on the detection technique employed. Possible detection methods include radioactive nucleotide precursor incorporation in the PCR reaction or primer end labeling with [γ-^{32}P]ATP, followed by electrophoresis on sequencing gels, and quantitation via phosphor imager or other methods. The authors and others have found that the most accurate method is the use of primers tagged covalently with either fluorescent molecules or infrared-absorbing dyes, followed by detection of fluorescent signals using an appropriate automated sequencer. PCR primers labeled with infrared or fluorescent dyes are typically synthesized with a modified 5′ base containing the fluor or dye on a spacer arm. Only one of the two PCR primers need to be labeled. Also, depending on the strength of the dye and coupling efficiency, one must empirically identify the correct dilution of labeled primer with unlabeled primer, and/or the correct PCR cycle number, so that the amount of labeled PCR product is in the linear range of the detection system used. Here, an example is provided of the forward primer synthesized with the LI-COR IR labeled dye (1 pmol/μl), with 20 cycles of PCR, and detection of products on an IR sequencer, here a LI-COR 4200S DNA Analyzer. Other labeling and detection methods can be substituted as mentioned below.

Materials

Red Taq DNA polymerase and 10× PCR reaction buffer (Sigma)

1.25 mM dNTP mix (1.25 mM each dNTP; see recipe)

1 pmol/μl labeled Met-F forward primer (see recipe for primers)

5 pmol/μl unlabeled Met-R1 reverse primer (see recipe for primers)

50 ng/μl *Hpa*II-predigested and positive and negative-control DNA (see Basic Protocol)

100-bp DNA ladder (Life Technologies)

IR2 stop solution/loading dye (LI-COR)

96-well PCR plates (e.g., Perkin-Elmer)

Thermal cycler

LI-COR 4200S DNA Analyzer

Additional reagents and equipment for PCR (*UNIT 2.5; CPMB UNIT 15.1*)

Perform PCR amplification

1. Prepare the following master mix for each reaction, for single-day use (note that there are two reactions per sample, and also positive controls; see Basic Protocol):

   > 6.0 μl deionized H_2O
   > 1.25 μl 10× PCR reaction buffer
   > 1.0 μl 1.25 mM dNTP mix
   > 1.0 μl 1 pmol/μl labeled Met-F forward primer
   > 0.25 μl 5 pmol/μl unlabeled Met-R1 reverse primer
   > 1.0 μl 1 U/μl Red Taq DNA polymerase

   The total volume is 10.5 μl total/reaction.

2. Place a 10.5-μl aliquot of the master mix for each sample into the appropriate well of a prelabeled PCR plate.

3. Add 2 μl of 50 ng/μl *Hpa*II-digested and positive and negative control DNA samples prepared in the Basic Protocol to the correspondingly labeled wells of the PCR plate containing the master mix.

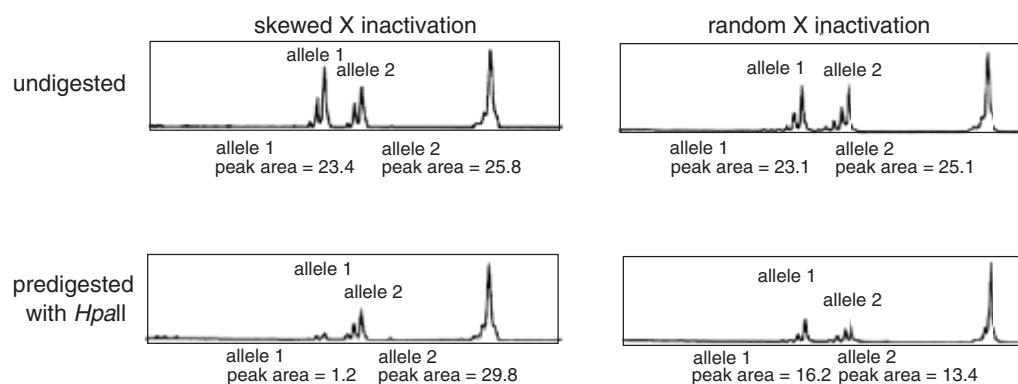4. Place the PCR plate in a thermocycler and perform PCR under the following conditions:

1 cycle:	3 min	94°C	(denaturation)
19 cycles:	30 sec	94°C	(denaturation)
	30 sec	60°C	(annealing)
	45 sec	72°C	(extension)
Final step:	indefinitely	4°C	(hold)

   Recommended PCR product storage is at –20°C, not to exceed 1 week.

Separate PCR products by electrophoresis

5. Thaw the PCR products if they have been in storage and add 3 μl of IR2 stop solution/loading dye to each of the 12.5-μl samples, pipetting vigorously up and down to mix.

6. Denature at 93°C for 3 min in the thermal cycler, and immediately place on ice.

   Labeled primers are light-sensitive and should be kept covered or in the dark whenever possible.

7. Run cut PCR products besides uncut products on the LI-COR 4200S DNA Analyzer with molecular weight markers on the outside lanes. Follow operations manual for the instrument to load and electrophorese the samples on an acrylamide denaturing gel.

   Products are ~250 to 300 bp.

Figure 9.7.1 Example of results showing extremely skewed pattern of X inactivation and random X inactivation. The upper automated sequencer traces correspond to the undigested, PCR-amplified *HUMARA* locus and show two alleles of the androgen receptor corresponding to the normal (paternal), and, in the case of the skewed example, mutant (maternal) X. The lower traces show the two samples after predigestion with the methylation-sensitive restriction endonuclease *Hpa*II and PCR amplification of *HUMARA*. The X chromosome that is active is undermethylated, and, once digested with the restriction enzyme, cannot be PCR-amplified and thus disappears from the lower trace. In the lower right trace, because both alleles exist in the methylated and unmethylated states, both alleles appear equally decreased after enzymatic predigestion, indicating a random pattern of X inactivation.

Perform X-inactivation dosage analysis

8. Manually assign lanes from the completed image files. Quantitate the relative activation/inactivation ratios of androgen-receptor alleles for each sample by first identifying the two peaks corresponding to the two X chromosome alleles in the undigested sample, then normalizing the peak densities to compensate for any unequal amplification of alleles. Next, multiply this normalization factor by the digested sample peak ratio.

This quantitation process is illustrated in Figure 9.7.1, which includes the equation and a sample calculation.

REAGENTS AND SOLUTIONS

*Use double-distilled sterile water (e.g., Sigma) in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2D**; for suppliers, see **SUPPLIERS APPENDIX**.*

dNTP mix, 1.25 mM (1.25 mM each dNTP)

Purchase 25 mM dATP, dCTP, dGTP, and dTTP from Perkin-Elmer. Microcentrifuge each nucleotide 30 sec at maximum speed. Mix the individual nucleotides in the following proportions:
125 µl 25 mM dATP
125 µl 25 mM dCTP
125 µl 25 mM dGTP
125 µl 25 mM dTTP

Add 500 µl double-distilled water for a total volume of 1000 µl.

The concentration of each nucleotide in the final dNTP mix is 1.25 mM. To avoid freeze/thawing the entire stock, it is recommended that the 1000 µl be divided into ten 100-µl aliquots.

Primers

Labeled forward primer: Met-F: 5′-TCC AGA ATC TGT TCC AGA GCG TGC-3′ labeled with LI-COR IRDye 700 (available from LI-COR).

Unlabeled reverse primer: Met-R1: 5′-GCT GTG AAG GTT GCT GTT CCT CAT-3′ (available from Life Technologies).

COMMENTARY

Background Information

Mammals ensure dosage compensation of X-linked genes by transcriptionally silencing one X chromosome in female somatic cells, a process known as X-inactivation. X-inactivation most often occurs randomly, during the 64- to 100-cell stage of embryonic development. The result is a mosaic of cells expressing one or the other X chromosome, usually with equal numbers of cells expressing each of the two X chromosomes. Nonrandom X inactivation, or "skewing," occurs when one X chromosome is expressed in a higher percentage of cells than the other. Skewing can be attributed to stochastic factors or to cloning (tumors), or can be familial (X-linked cell lethal).

The process and means of maintaining X chromosome inactivation has interested investigators since the 1960s. The inactive X chromosome was first identified by its late replication patterns and heterochromatic nature (Willard, 2001).

Critical Parameters and Troubleshooting

If the *HUMARA* site is uninformative due to homozygosity, the polymorphic $(CAG)_n$ region flanking the fragile X gene locus (*FRAXA*), at Xq27.3, can be substituted (see *UNIT 9.5*, Basic Protocol 1, for PCR conditions). The exception to this protocol is that one primer must be synthesized with infrared dyes (LI-COR).

Also, keep in mind that when making a PCR master mix, both the digested and undigested reactions are amplified for each subject DNA sample, so the number of PCR reactions is double the sample DNA number.

It is important to stress that X-chromosome inactivation analysis performed on cell lines may show a skewed pattern of X-chromosome because of the clonal nature of the cell lines. Samples tested from subjects with certain types of leukemia will also show skewing in peripheral blood cells due to clonal expansion of a single cell with one X chromosome active.

It is also conceivable that a single subject will show different patterns of X chromosome inactivation in different tissues; however, in practice this is generally not observed. An exception to this is seen in X-linked dominant conditions, where cells with the mutant X active in certain tissues show a growth disadvantage. Other examples include subjects with X-linked severe combined immunodeficiency (*SCIDX1*), who show skewing in blood but not other tissues (Puck et al., 1992), and subjects with incontinentia pigmenti, who show significant skewing in peripheral blood leukocytes against the mutant X, with the associated skin lesions activating only the mutant X (Parrish et al., 1996). Also the concordance of skewing

between tissues is lost with age (Sharp et al., 2000).

It is important to be familiar with the quantitative analysis hardware and software. An overabundance of labeled PCR product can saturate the photomultiplier tubes (PMTs) used by many fluorescence and light detection systems. This saturation is indicated by a flattened apex of the quantification curve. Reducing the concentration of DNA and/or the number of amplification cycles will remedy this problem. Due to the visual nature of the software, discrepancies between calculated results and visual results are readily apparent, making positive controls absolutely necessary to determine if analysis is accurate.

Anticipated Results

The rate of skewing can vary depending on the population in question. Among the general population, researchers have found 4% to 7% of women less than 25 years of age to be highly skewed (10:1), and that the rate of skewing increases with age to 16% to >20% in woman over the age of 60 (Busque et al., 1996; Sharp, et al., 2000). Lanasa et al. (2001) found a group of women with recurrent abortions to have a 14% rate of skewing. Manifesting carriers of X-linked conditions such as DMD should theoretically all be skewed (Pegoraro et al, 1994).

Time Considerations

An experiment with 25 DNA samples including controls is a day-long project, but materials can be stored between steps for scheduling flexibility.

The digestion will take 2.5 hr in the thermal cycler or water bath after about 30 min of sample and reaction preparation. The PCR reaction also takes ~30 min to prepare and load the sample plate, then <1 hr in the thermal cycler or water baths. The acrylamide gel will polymerize in 45 min to 1 hr after ~20 min of plate/reagent preparation. The samples will then take ~30 min to load. The gel will need to run for ~2.5 hr and analysis can take 30 min to 1 hr. Again, storage of samples can occur post-digestion or post-PCR for experiments not completed in 1 day.

Literature Cited

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