FMR1 Methylation PCR: Eliminating the need for Southern Blot testing

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Associate Professor of Pathology
University of Utah School of Medicine
Medical Director, Genetics Division
ARUP Laboratories
Do you agree with this statement?

“Southern blot analysis will be replaced by faster and simpler methods such as methylation PCR.”
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Objectives and Disclosure

• To understand the diagnostic value of determining FMR1 methylation
• To review Southern blot challenges and limitations
• To learn about new approaches with PCR based assays, capable of improving the throughput and resolution of FMR1 molecular diagnostics

• Disclosures:
  – Receive commercial reagents for studies
  – Honorarium
Fragile X Syndrome

- Most common inherited form of mental retardation.
- Incidence 1:4000 males and 1:8000 females.
- Affected males have mental retardation, characteristic physical features and behavior.
- Affected females exhibit a less severe phenotype.
- Found in all populations.
Fragile X: Molecular Defect

• Tri Nucleotide Repeat (CGG) at the 5' Untranslated Region (UTR).
  – A small expansion (pre-mutation) associated with increased mRNA
    ▪ FX Ataxia, POI
  – A large expansion associated with methylation, inactivating gene expression.

<table>
<thead>
<tr>
<th>Normal</th>
<th>Premutation</th>
<th>Full Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5------&gt;44</td>
<td>45------&gt;200</td>
<td>200+</td>
</tr>
<tr>
<td>CGG</td>
<td>CGG</td>
<td>CGG</td>
</tr>
<tr>
<td>FMR-1 gene</td>
<td>FMR-1 gene</td>
<td>FMR-1 gene</td>
</tr>
</tbody>
</table>

(Chart showing normal, premutation, and full mutation with CGG repeats increasing in number)
Repeat Number Classification

- **Normal**: 5-44 repeats: Rules out diagnosis of Fragile X syndrome/carryer status.

- **Intermediate**: 45-54 repeats: Not affected but unstable, could eventually expand to a pre-mutation, then full mutation.

- **Pre-mutation**: 55-200 repeats: Carrier and at risk for expansion in next generation (females). At risk for premature ovarian insufficiency (POI) or ataxia.

- **Full mutation**: >200-230 repeats: Gene is methylated and inactive; confirms diagnosis of Fragile X syndrome.

- **Mosaic**: Both pre-mutation (un-methylated) and full mutation (methylated) present. Severity of symptoms cannot be predicted, but may be milder.
Methylation

• Variable expression of FXS
• Full mutations (>200-230 CGG repeats)
  – Mostly fully methylated
  – >230 CGG repeats without methylation
    • High functioning males (5%)
• Mosaics – may modify phenotype
  – Pre-mutation/full mutation
  – Intermediate (normal)/ full mutation
    • Contraction?
• Testing reflects status in blood
Methylation in Females

- Variable phenotype
  - <50% females with full mutations have intellectual disability
  - Other symptoms may be present
    - Avoidance personality, mood, stereotypic disorders
    - Not proven to be due to FMR1 full mutation or methylation

- X inactivation
  - Random vs skewing

- Degree of methylation not necessarily correlated with intellectual disability
Mosaicism

- Size mosaicism
- Methylation mosaicism
  - Unmethylated pre-mutation (intermediate or normal)/methylated full mutation
  - Unmethylated and methylated full mutation size range
  - Possible mechanisms
- Possible types

Methylation in Newborn Screening

• FXS screening in newborns currently not recommended
• Studies use methylation to identify only full mutations
  – Will not identify pre-mutations
  – Reduces concern for adult onset FXTAS or FXPOI
• High sensitivity/specificity in males
• Reduced sensitivity/specificity in females
• Screen males only or males and females?
Fragile X Testing

• PCR
  – Sizes normal/pre-mutation allele
  – Amplification into CGG repeat full mutation range possible
    • Preferential amplification of normal allele in females
    • Difficult to distinguish: One allele/undetected expanded allele from two normal homozygous alleles in females

• Methylation:
  – Southern blot analysis (concurrently or reflexed)
    • 80-1000+ repeats
    • Full mutations
    • Methylation
    • Sizing not accurately (± 50 CGGs)
  – mPCR
PCR Electropherogram
Chimeric PCR

20/31 CGG repeats

29/103 CGG repeats

AGGs

55 repeats

55 repeats

29/103 CGG repeats

140/800 mosaic CGG repeats
Southern Blot

Restriction Digest  
Electrophoresis

Transfer to membrane

Anneal Probe

Detect
• The FMR-1 gene region with the CGG trinucleotide repeats is flanked by Eco RI sites and a methylation sensitive enzyme site (Nru I).

• Full mutation has been shown to methylate the gene and prevent enzyme restriction of DNA.
Southern Schematic

- Normal females show
  - one methylated allele (5.2 kb)
  - one un-methylated allele (2.1 kb)

- Normal males
  - one un-methylated allele (2.1 kb)
Southern Example

- Size standards
- Full mutation, Male
- Pre-mutation, Female
- Normal, Female
Simplify Methylation Analysis

- Methylation PCR (mPCR)
  - Sodium Bisulfate Methylation Modification
  - Restriction digest

- Approaches
  - MLPA
  - Real-time PCR
  - Mass Spectrometry
  - Capillary Electrophoresis
MLPA

Nygren AOH et al. JMD 2008; 10 (6):496-501
MLPA Results

- **Male patient groups:**
  - **Straight arrows,** methylation-specific *FMR1* probes;
  - **curved arrows,** methylation-specific *FMR2* probes;
  - **arrowheads,** digestion control probes.
Real-Time PCR

• TaqMan
  – Methylated
  – Unmethylated

• Melt curves

Real-Time PCR Results

- Inexpensive
- Sensitive/specific for males
- 82% sensitive for females (PPV: 97%) for genotype
- Unable to predict phenotype (intellectual disability)

Melt Analysis

Real-time PCR – Melt Analysis

Reagents provided by Celera
‘Melting Peaks’

Normal Females

Titration % methylation

Reagents provided by Celera
Site Specific Analysis

- Hairpin bisulfite modification
- MALDI TOF
- Compare to levels of mRNA or protein

Stöger R et al. PLOS One 2011 e23648
Godler et al. Human Molecular Genetics 2010; doi:10.1093/hmg/ddq037 1-15
mPCR and Capillary Electrophoresis

- Results visually similar to Southern blots
- Methylation status of each CGG-repeat subpopulation
- Restriction digest followed by PCR
- Digestion controls with each sample
- Reference range (categories)
  - <20% unmethylated
  - 20-80% partially methylated
  - >80% fully methylated
mPCR Workflow Overview (CE)

8 µL of Purified gDNA (~20 ng/µL) + 2 µL of Control DNA

4 µL 4 µL

Control Enzyme (FAM) Digestion Enzyme (HEX)

1 µL 1 µL

PCR FAM Primers PCR HEX Primers

2 µL of Pooled PCR Amplicons, 11 µL HiDi+ 2 µL of ROX 1000 Size Std
Denature at 95°C – 5 min, cool at 4°C – 2 min.

ABI 3130 XL DATA Analysis

From http://www.asuragen.com/Diagnostics/US/Products/Methylation_PCR_FragileX/
Pilot Sample Study Summary

• 25 Pilot clinical samples were prepared and run at ARUP and sent to Asuragen.

• The Pilot Sample results were in high agreement for size and methylation status.
  – 23/25 concordant with 2 technical issues
  – 1 sample had no Hex reference peak
  – 1 sample had a technical issue, 1 sample (FX#35) with an under call on % methylation
  – Both samples were resolved at Asuragen
Normal Female

- mPCR: Normal allele, 29/30
- Note: Blue signal saturated (as expected for alleles in the normal range).
- Need 5 sec injection data for accurate determination of methylation status for NOR alleles.
Male, Pre-mutation, Unmethylated

Digestion controls

Size standards
- Full mutation male
- Pre-mutation female
- Normal female

Male, Pre-mutation, Unmethylated
Female, Full Mutation, Fully Methylated

Size standards
Full mutation male
Pre-mutation female
Normal female
Male, Full mutation, Mosaic

Size standards

Full mutation male
Pre-mutation female
Normal female

250 repeats
## Comparison between mPCR and Southern

<table>
<thead>
<tr>
<th>Background Information</th>
<th>ARUP - mPCR, Methylation Comparison</th>
<th>ARUP - SB Results</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample ID</td>
<td>Sex</td>
<td>ARUP - mPCR</td>
<td>ARUP - SB Results</td>
</tr>
<tr>
<td>FX#1</td>
<td>F</td>
<td>Fully methylated FM</td>
<td>Fully methylated FM-size mosaic</td>
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<tr>
<td>FX#3</td>
<td>F</td>
<td>Fully methylated FM</td>
<td>Fully methylated FM</td>
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<td>M</td>
<td>Normal</td>
<td>N/A</td>
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<td>FX#8</td>
<td>M</td>
<td>Unmethylated PM</td>
<td>Premutation unmethylated PM</td>
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<tr>
<td>FX#11</td>
<td>M</td>
<td>Mostly methylated, maybe some indication of partial</td>
<td>Methylated - may have low level unmethylated mosaic</td>
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<td>FX#15</td>
<td>F</td>
<td>Partial Methylation (Female PM)</td>
<td>methylated/unmethylated PM</td>
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<tr>
<td>FX#17</td>
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<td>Partial Methylation (Female PM)</td>
<td>methylated/unmethylated PM</td>
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<td>FX#18</td>
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<td>FX#21</td>
<td>F</td>
<td>Partial Methylation (Female PM)</td>
<td>methylated/unmethylated PM</td>
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<tr>
<td>FX#26</td>
<td>F</td>
<td>Fully methylated FM</td>
<td>Fully methylated FM</td>
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<tr>
<td>FX#28</td>
<td>M</td>
<td>Unmethylated PM</td>
<td>Premutation unmethylated PM</td>
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<tr>
<td>FX#29</td>
<td>M</td>
<td>Fully methylated FM</td>
<td>Fully methylated FM-size mosaic</td>
</tr>
<tr>
<td>FX#33</td>
<td>F</td>
<td>Partial Methylation (Female PM)</td>
<td>Skewed, premutation mostly unmethylated PM</td>
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</table>
Comparison between mPCR and Southern

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Sex</th>
<th>ARUP - mPCR</th>
<th>ARUP - SB Results</th>
<th>Agreement</th>
</tr>
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<tbody>
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<td>FX#34</td>
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<td>Normal</td>
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<td>Possible mosaic with partial methylation in FM allele, likely undercalled as a technical error</td>
<td>Fully methylated FM-size mosaic</td>
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<tr>
<td>FX#37</td>
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<td>Mostly methylated PM with unmethylated FM</td>
<td>Premutation unmethylated/size mosaic</td>
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<td>FX#38</td>
<td>F</td>
<td>Fully methylated FM</td>
<td>Fully methylated FM</td>
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<td>FX#39</td>
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<td>Partial Methylation (Female PM)</td>
<td>Premutation</td>
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<td>FX#48</td>
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<td>Fully methylated FM (maybe some indication of partial)</td>
<td>Methylated FM - may have low level unmethylated</td>
<td>Yes</td>
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<td>FX#54</td>
<td>M</td>
<td>Fully methylated FM</td>
<td>Fully methylated FM</td>
<td>Yes</td>
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<td>FX#55</td>
<td>M</td>
<td>No Reference Peak in HEX (technical)</td>
<td>Fully methylated</td>
<td>Yes</td>
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<td>FX#60</td>
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<td>Partial Methylation (Female PM)</td>
<td>Possibly skewed premutation mostly unmethylated PM</td>
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<td>FX#165</td>
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<tr>
<td>Neg-Size-Std</td>
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# Summary Report

## AmpliDex® FMR1 mPCR Summary Sheet

**Job ID:** mPCR-4-05-2012  
**Operator:** Jane  
**Data Processed:** 07/21/2012  
**Samples:** 24  
**Source File:** mPCR-4-05-2012_AmpliReport.XLS

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<th>ID</th>
<th>Sample File</th>
<th>Allele Ranges Detected</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
<th>Peak 4</th>
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<th>Peak 6</th>
<th>Peak 7</th>
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<tr>
<td>FX#101_A01_001.fsa</td>
<td>90% 0.99</td>
<td>✓</td>
<td>49</td>
<td>9%</td>
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<td></td>
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<tr>
<td>FX#109_A03_001.fsa</td>
<td>90% 1.33</td>
<td>✓</td>
<td>30</td>
<td>33%</td>
<td>47</td>
<td>90%</td>
<td></td>
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<tr>
<td>FX#110_A03_003.fsa</td>
<td>92% 2.38</td>
<td>✓</td>
<td>51</td>
<td>52%</td>
<td>95</td>
<td>100%</td>
<td>111</td>
<td>100%</td>
<td>116</td>
<td>7%</td>
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<tr>
<td>FX#114_B01_003.fsa</td>
<td>93% 1.05</td>
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<td>29</td>
<td>41%</td>
<td>46</td>
<td>34%</td>
<td></td>
<td></td>
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<tr>
<td>FX#119_D05_006.fsa</td>
<td>95% 0.84</td>
<td>✓</td>
<td>30</td>
<td>44%</td>
<td></td>
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<tr>
<td>FX#120_D03_007.fsa</td>
<td>96% 0.8</td>
<td>✓</td>
<td>32</td>
<td>46%</td>
<td>61</td>
<td>100%</td>
<td>&gt;200</td>
<td>15%</td>
<td>&gt;200</td>
<td>85%</td>
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<tr>
<td>FX#122_C01_006.fsa</td>
<td>96% 0.98</td>
<td>✓</td>
<td>23</td>
<td>36%</td>
<td>33</td>
<td>61%</td>
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<td>65</td>
<td>2%</td>
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<td>96% 0.78</td>
<td>✓</td>
<td>30</td>
<td>11%</td>
<td>&gt;200</td>
<td>100%</td>
<td>&gt;200</td>
<td>100%</td>
<td>&gt;200</td>
<td>93%</td>
</tr>
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<td>FX#130_F03_011.fsa</td>
<td>91% 1.19</td>
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<td>30</td>
<td>4%</td>
<td>&gt;200</td>
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<td>100%</td>
<td>&gt;200</td>
<td>93%</td>
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<tr>
<td>FX#131_E01_009.fsa</td>
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<td>5%</td>
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<td>FX#132_F01_011.fsa</td>
<td>94% 1.08</td>
<td>✓</td>
<td>30</td>
<td>40%</td>
<td>&gt;200</td>
<td>73%</td>
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<td>72%</td>
<td>&gt;200</td>
<td>85%</td>
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<td>50%</td>
<td>85</td>
<td>90%</td>
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<tr>
<td>FX#140_H03_015.fsa</td>
<td>92% 0.98</td>
<td>✓</td>
<td>23</td>
<td>25%</td>
<td>47</td>
<td>100%</td>
<td></td>
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<tr>
<td>FX#141_G01_013.fsa</td>
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<td>✓</td>
<td>23</td>
<td>12%</td>
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<td>FX#149_A04_002.fsa</td>
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<td>23</td>
<td>66%</td>
<td>86</td>
<td>22%</td>
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</table>
ARUP Study: mPCR/CE

- 200 clinical samples submitted for FXS analysis and enriched for pre-mutations/full mutations
  - 88 males, 112 females
  - 36 normal, 36 intermediate, 65 pre-mutation, 63 full mutations
- Test set ARUP/Asuragen comparison
  - 25 samples: tested at Asuragen/ARUP
    - Reproducibility: 2X (ARUP)
- Accuracy – 175 additional samples
  - 90 analyzed to date
    - Switching from GeneMapper to GeneMarker
  - Southern analysis available for pre-mutations/full mutations
- Reproducibility
  - 2X for all full mutations
  - In progress: precision studies with full/pre/intermediate and normal alleles
Male Full Mutation

Fully Methylated

>200 CGG

98%, 100%

99%

Confidential, not intended for distribution
Female, Fully Methylated Size Mosaic

20

>200 >200

~70% Me

100% 100%, 100%

Full male
Pre-mutation female Normal female
Skewed X Inactivation

Signal height in the linear range

5 s injection data; mPCR at Asuragen

PM mostly unmethylated
NOR mostly methylated
FAM
HEX

38 CGG
76
78

81% Me
29% Me
3% Me

212.59 1.35
D10. C 3HP
155 909

344.75 NOR
2938

455.6115 PM
3802

3500xL_FXS_Methyl/hai

Pre-mutation female
Normal female
Full mutation male
Size standards
Male, High Repeats

Size standards
Full mutation male
Pre-mutation female
Normal female

Unmethylated PM smear

76%
9%
5%
Male, Full Mutation

Size standards
Full mutation male
Pre-mutation female
Normal female
Next Steps

• Continue reproducibility study
  – between run, within run

• Confirm reference range

• Evaluate skewed X inactivation
  – Normal allele, pre-mutation alleles

• Side-by-side with clinical samples
Conclusions

• mPCR for FX
  – Several methods available
    • Overall methylation status
      – MLPA
      – Real-time PCR
    • Specific subpopulations
      – CE (validation nearly complete)
  – Standardize methylation percentages
    • Improve understanding of methylation patterns to clinical severity
  – Reduce/replace Southern analysis
Thanks to

• ARUP
  – Mohamed Jama, MS
  – Serene Gibson
  – Alison Millson, MT(ASCP)
  – Ping Yu, MS
  – Cindy Meadows, BS, MB(ASCP)CM
  – Samuel Egbert

• Asuragen
  – Stela Filipovic-Sadic, MS
  – Adrian Gonzales, BS
  – Andrew Hadd, PhD
  – Gary J. Latham, PhD

• Celera
  – Aaron Hamilton, PhD