FMR1 Methylation PCR: Eliminating the need for Southern Blot testing

Elaine Lyon, PhD, FACMG
Associate Professor of Pathology
University of Utah School of Medicine
Medical Director, Genetics Division
ARUP Laboratories

You will vote on this question after the session

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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UNIVERSITY OF UTAH SCHOOL OF MEDICINE | Department of Pathology

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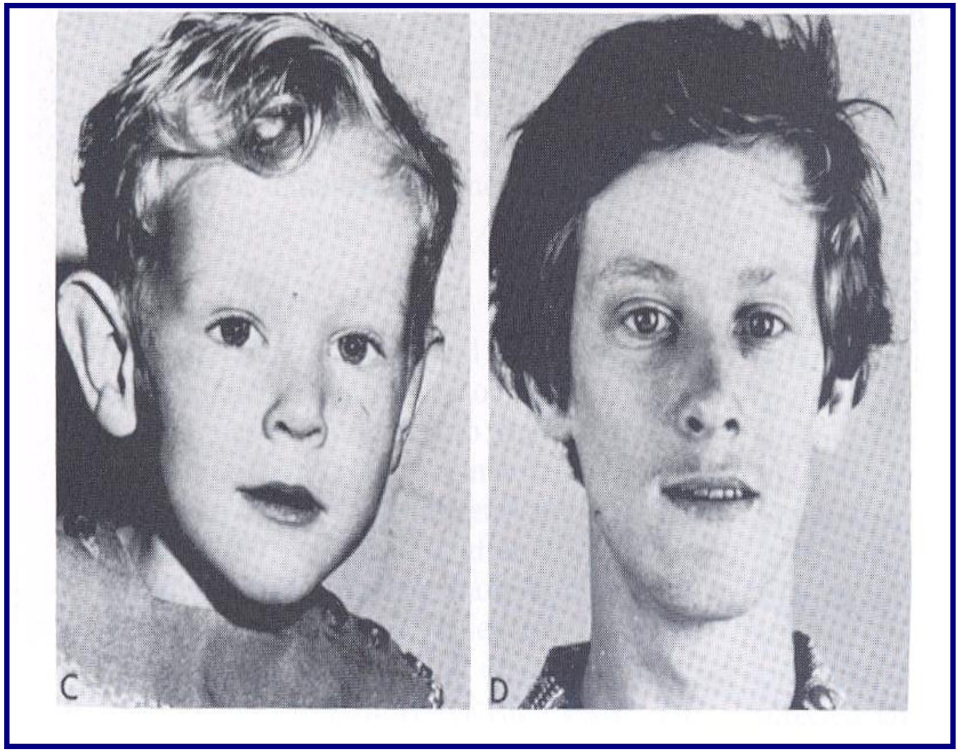
Objectives and Disclosure

- 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

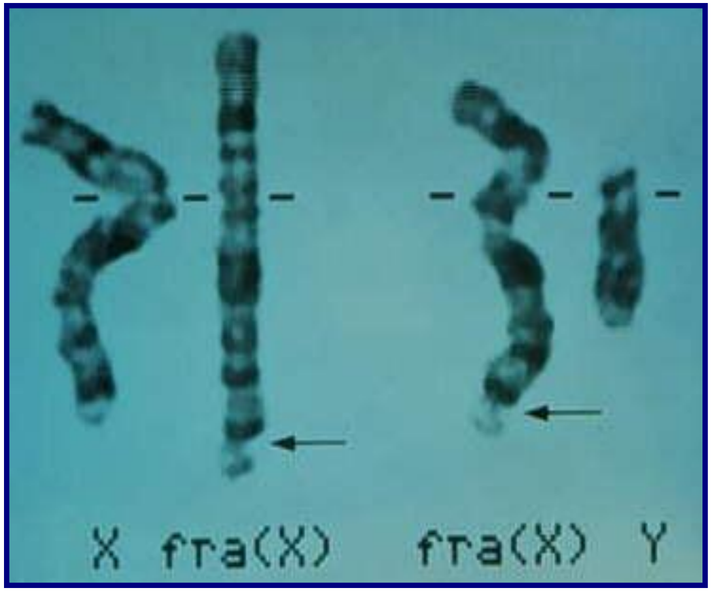
Fragile X Syndrome

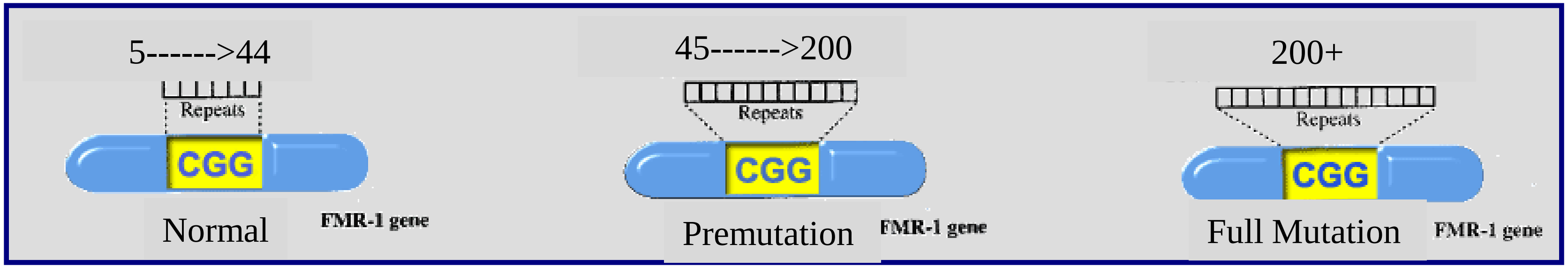
- Most common inherited form of mental retardation.
- Incidence 1:4000 males and 1:8000 females.
- Affected males have mental retardation, characteristic physical features and behavior.
- Affected females exhibit a less severe phenotype.
- Found in all populations.



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.





Repeat Number Classification

- Normal: **5-44 repeats**: Rules out diagnosis of Fragile X syndrome/carrier 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

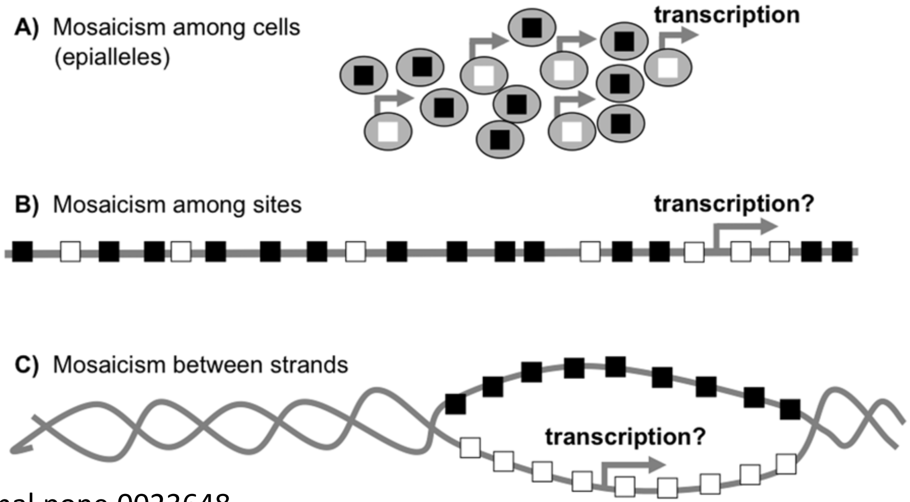
Methylation in Females

- Varible 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

Three possible types of methylation mosaicism at *FMR1:*



Stöger R, et al. (2011) PLoS ONE 6(8): e23648. doi:10.1371/journal.pone.0023648

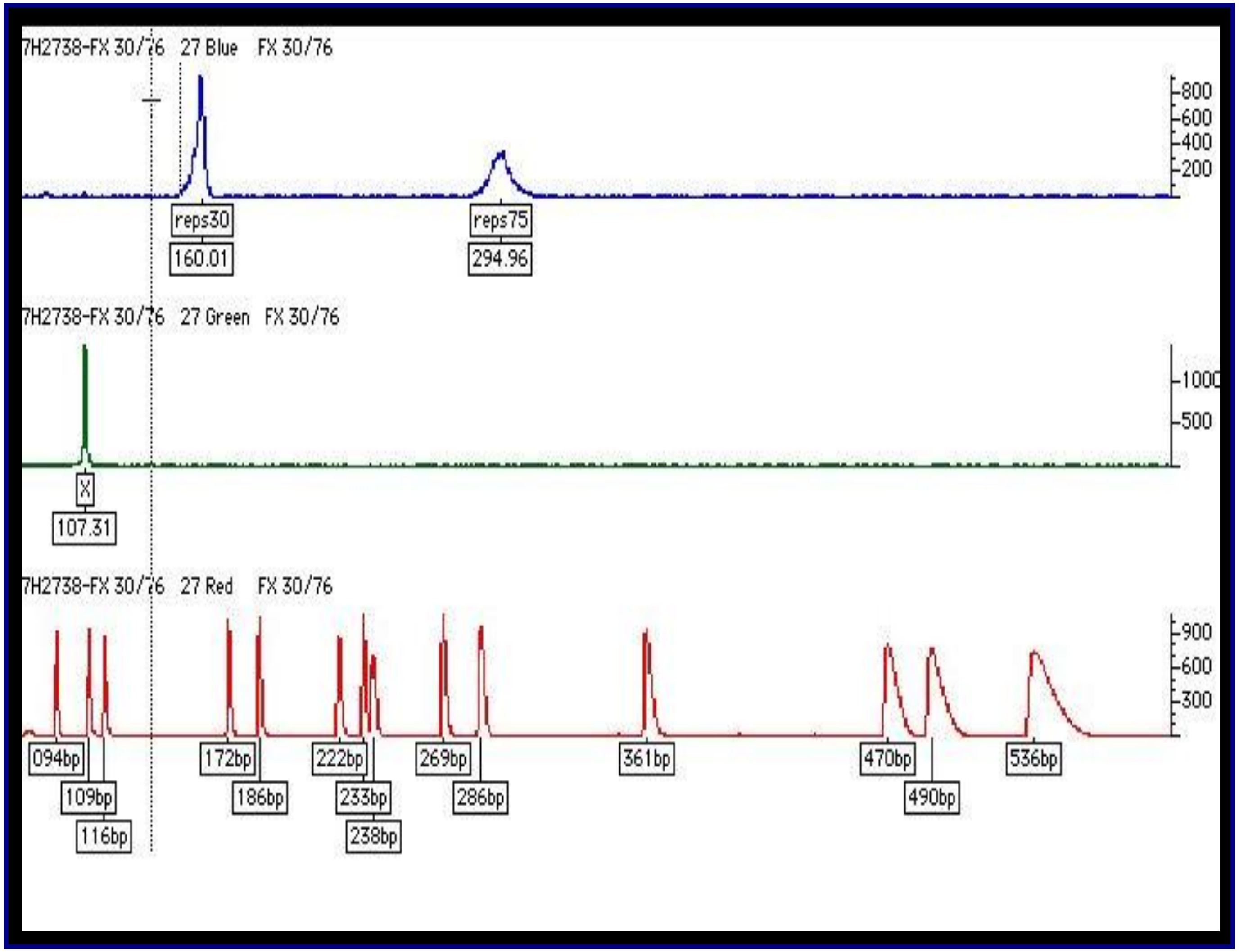
Methylation in Newborn Screening

- 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?

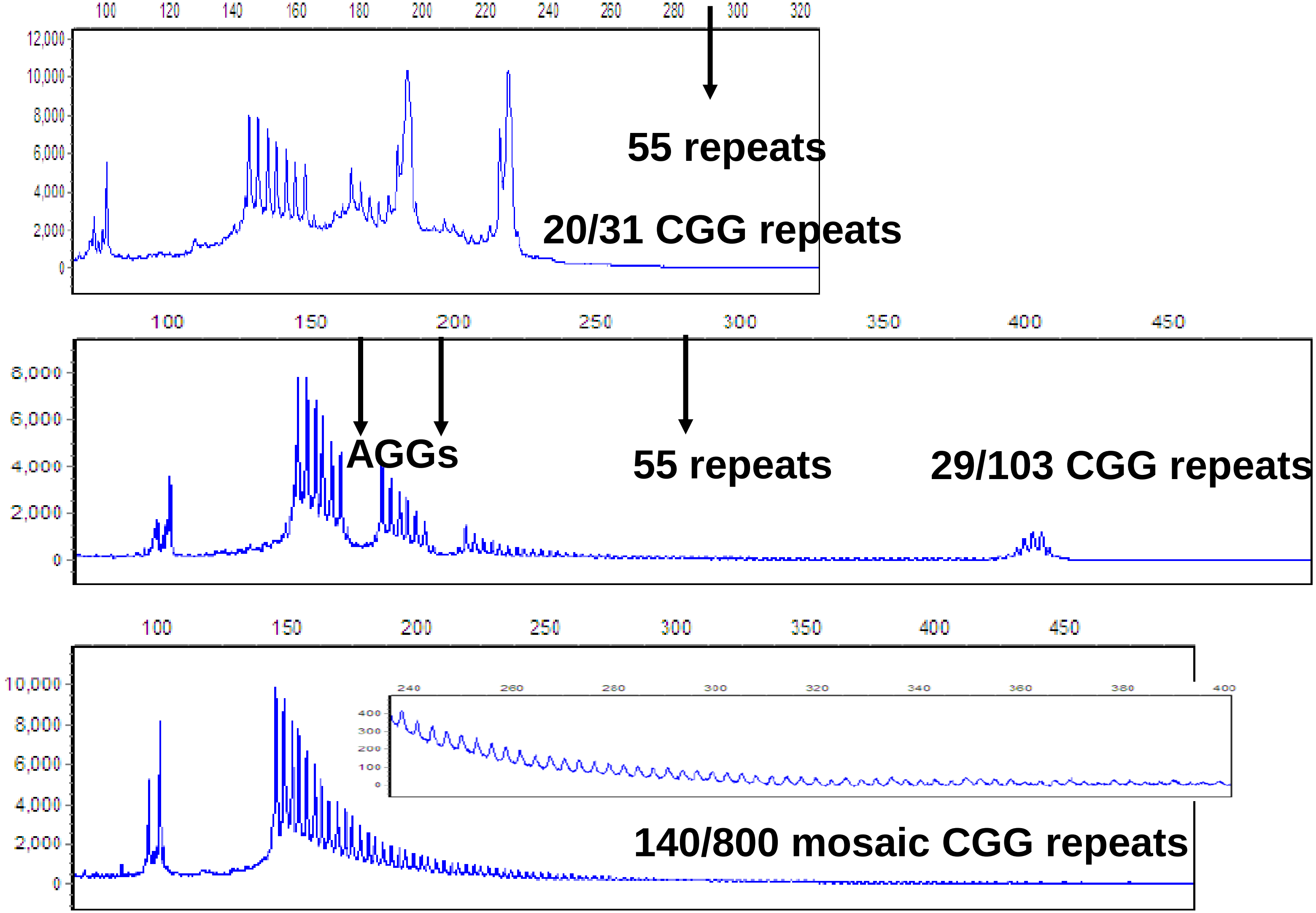
Fragile X Testing

- 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 Electropheragram



Chimeric PCR



55 repeats

20/31 CGG repeats

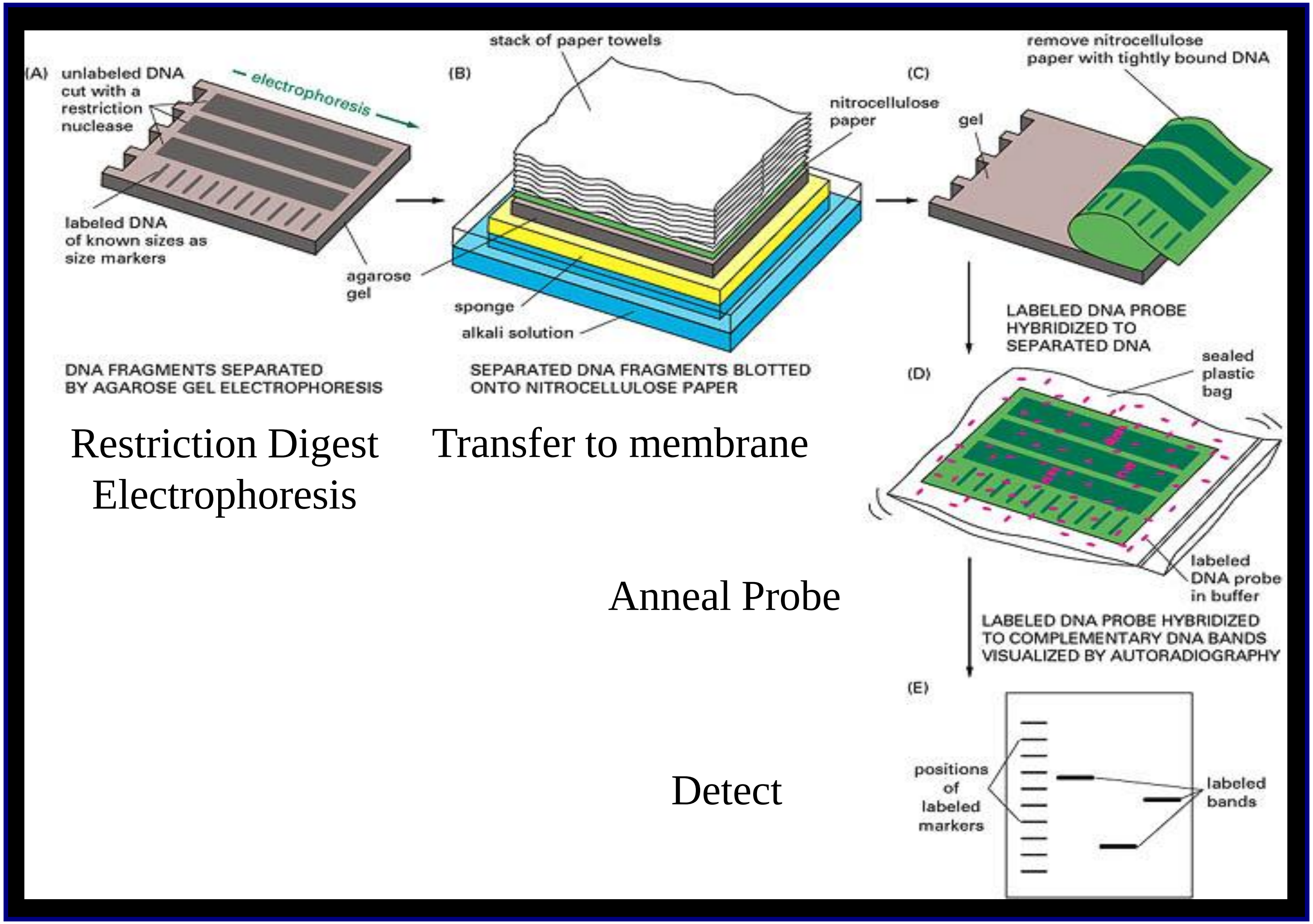
AGGs

55 repeats

29/103 CGG repeats

140/800 mosaic CGG repeats

Southern Blot



Restriction Digest
Electrophoresis

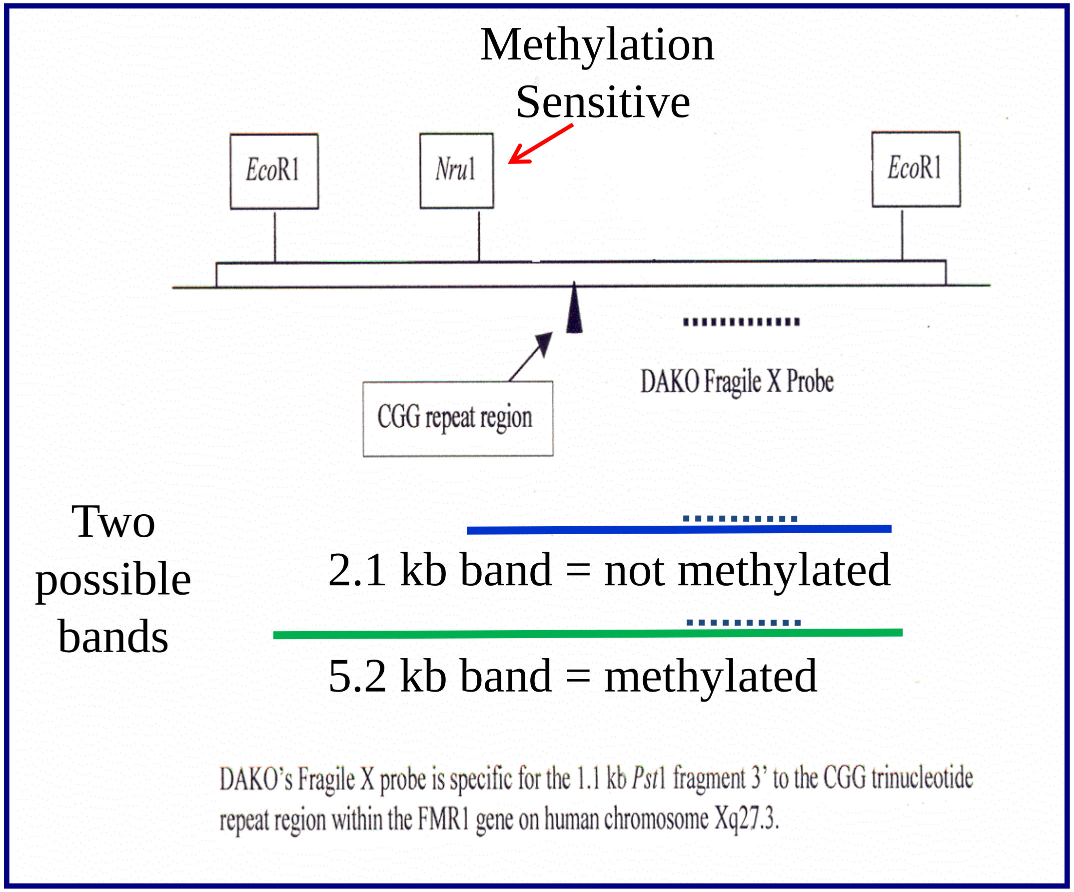
Transfer to membrane

Anneal Probe

Detect

Restriction Diagram

- The FMR-1 gene region with the CGG trinucleotide repeats is flanked by *Eco RI* sites and a methylation sensitive enzyme site (*Nru 1*).
- Full mutation has been shown to methylate the gene and prevent enzyme restriction of DNA.



Methylation Sensitive

Two possible bands

2.1 kb band = not methylated

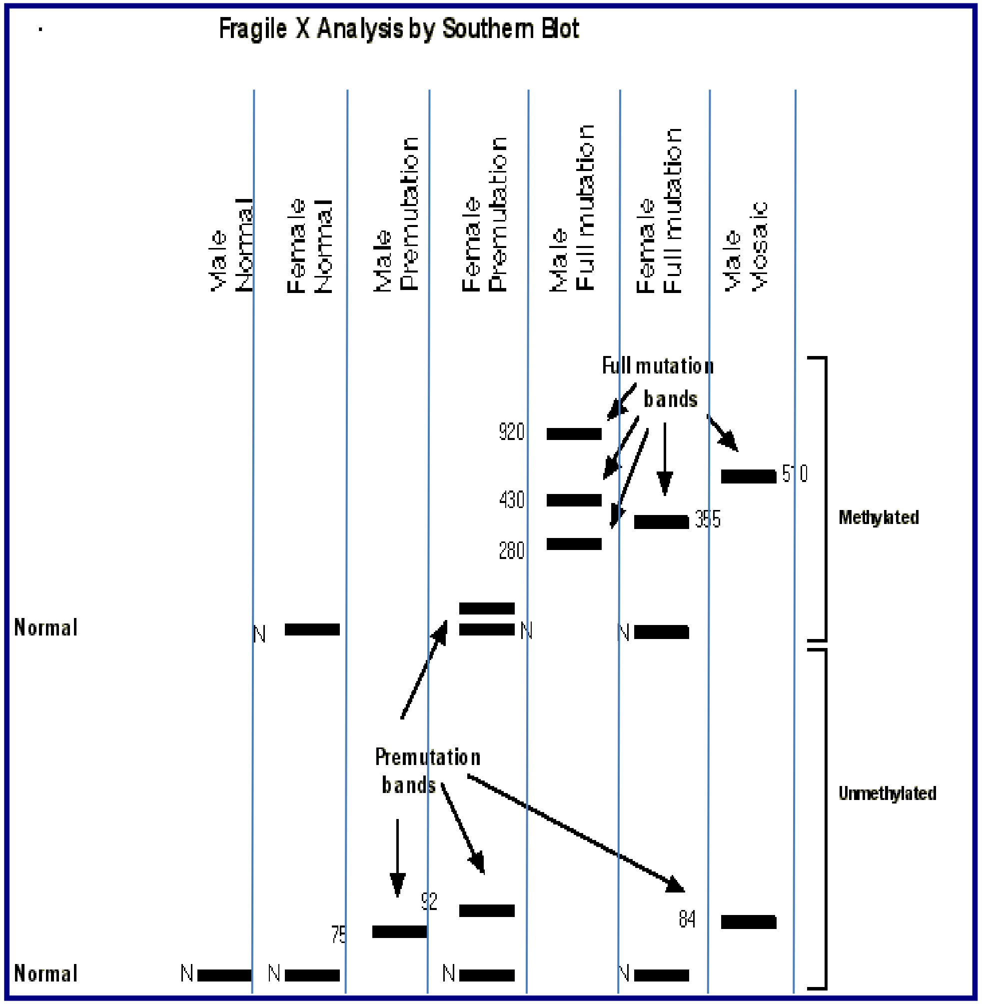
5.2 kb band = methylated

DAKO's Fragile X probe is specific for the 1.1 kb *Pst*1 fragment 3' to the CGG trinucleotide repeat region within the FMR1 gene on human chromosome Xq27.3.

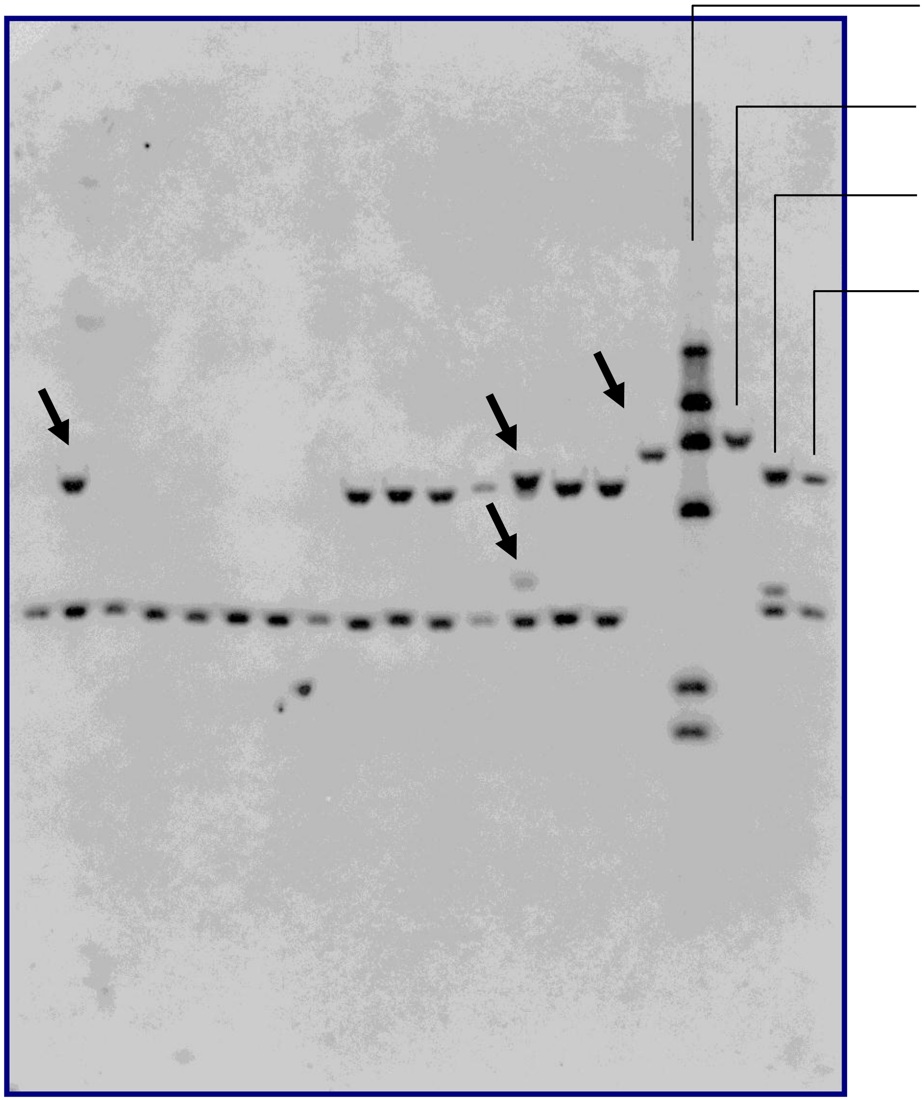
Southern Schematic

- 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

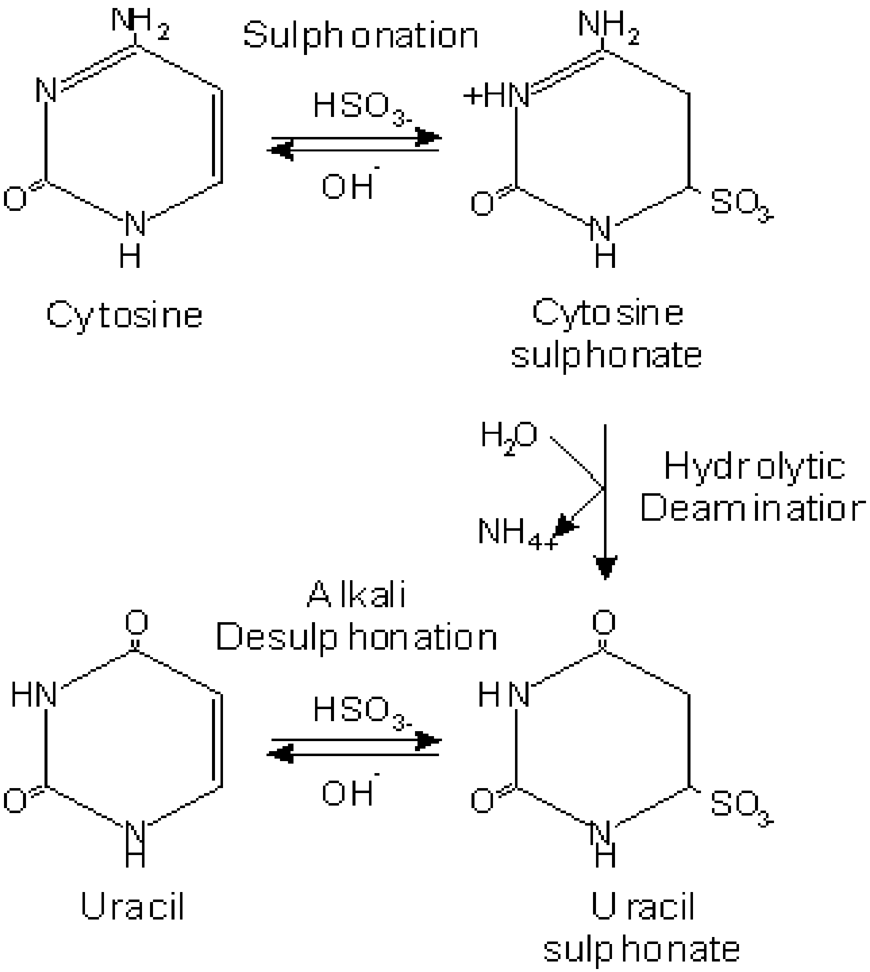
Full mutation , Male

Pre-mutation , Female

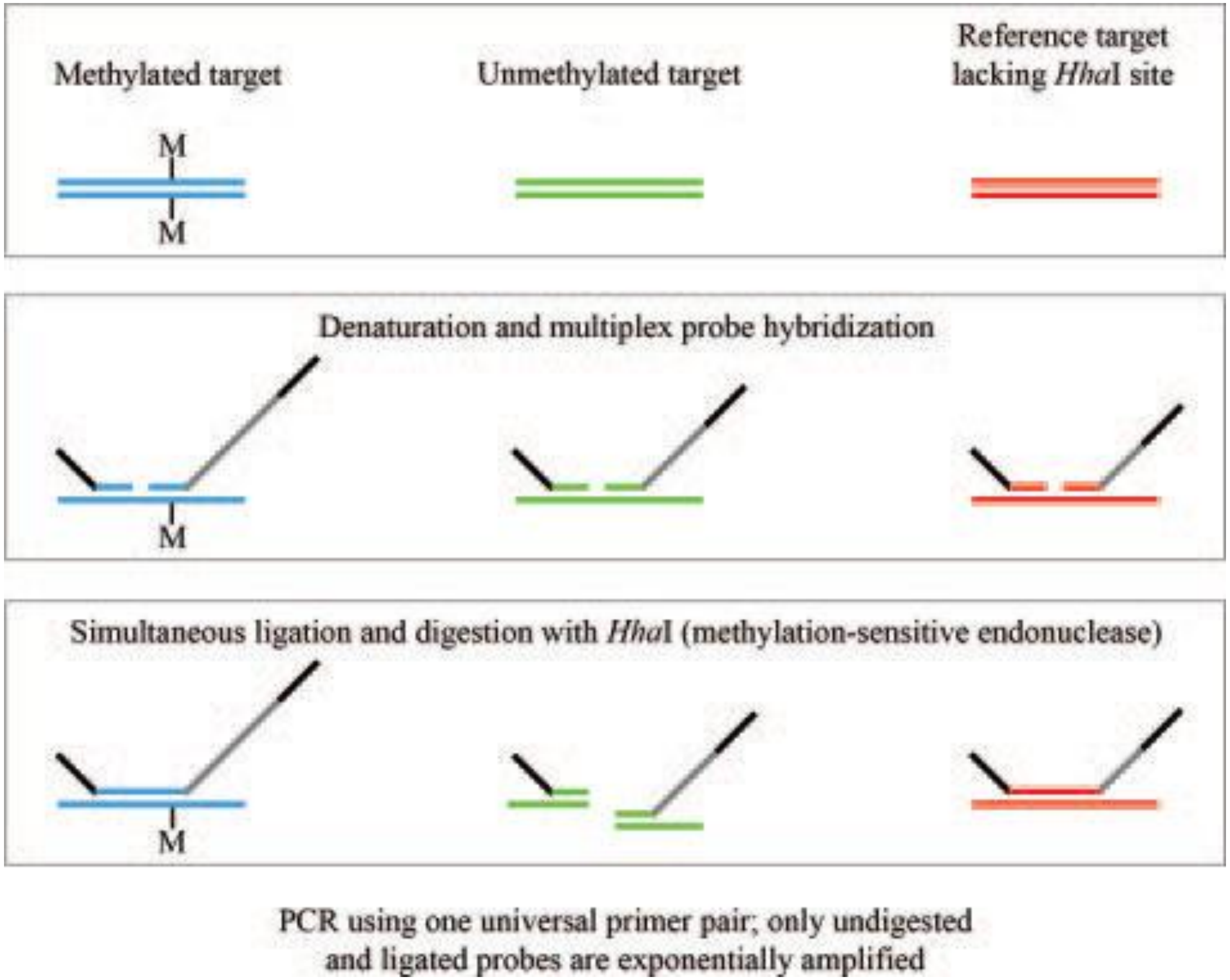
Normal , Female

Simplify Methylation Analysis

- Methylation PCR (mPCR)
  - Sodium Bisulfate Methylation Modification
  - Restriction digest
- Approaches
  - MLPA
  - Real-time PCR
  - Mass Spectrometry
  - Capillary Electrophoresis



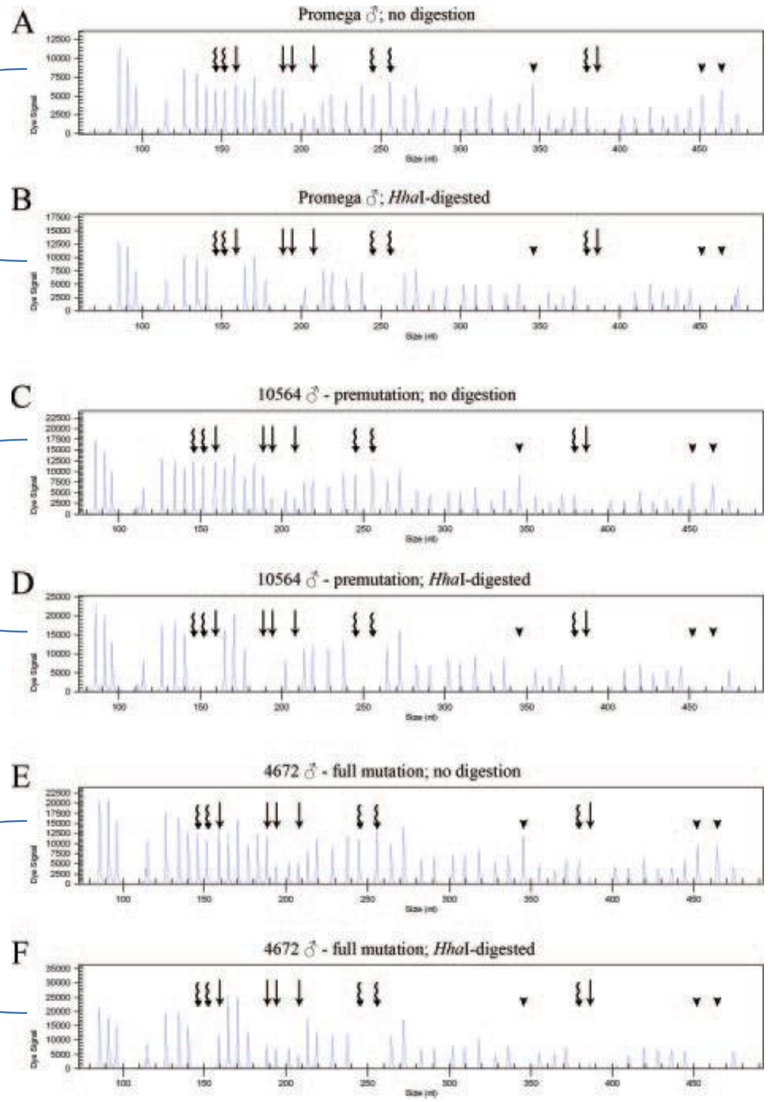
MLPA



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.



Normal control

Pre-mutation

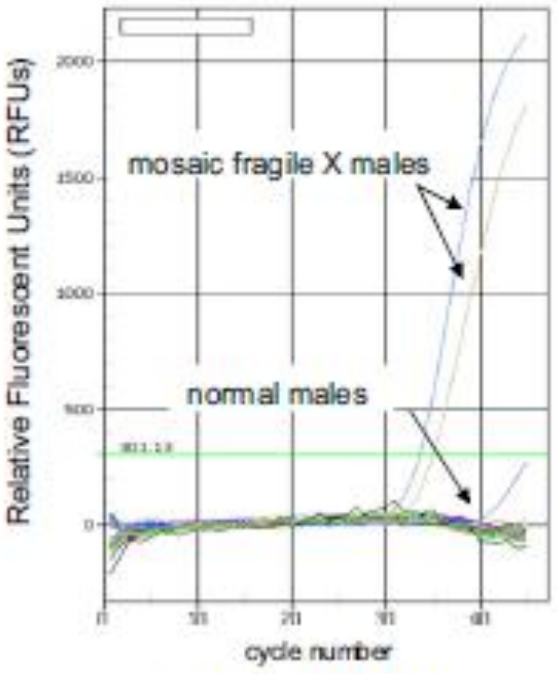
Full mutation

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

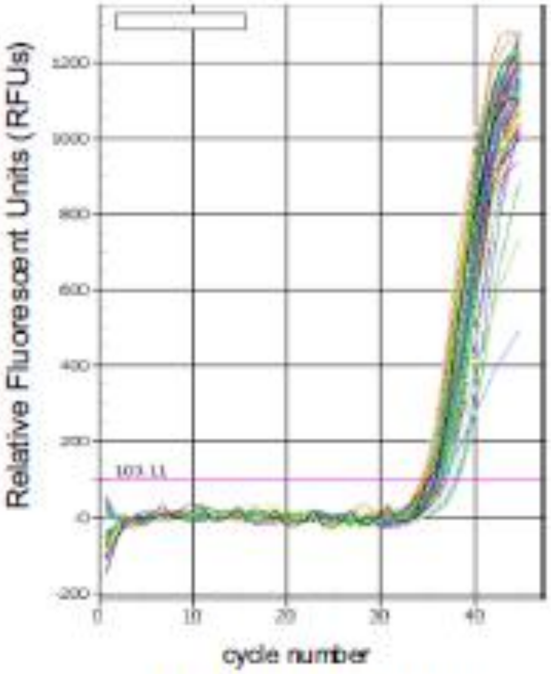
Real-Time PCR

- TaqMan
  - Methylated
  - Unmethylated

- Melt curves



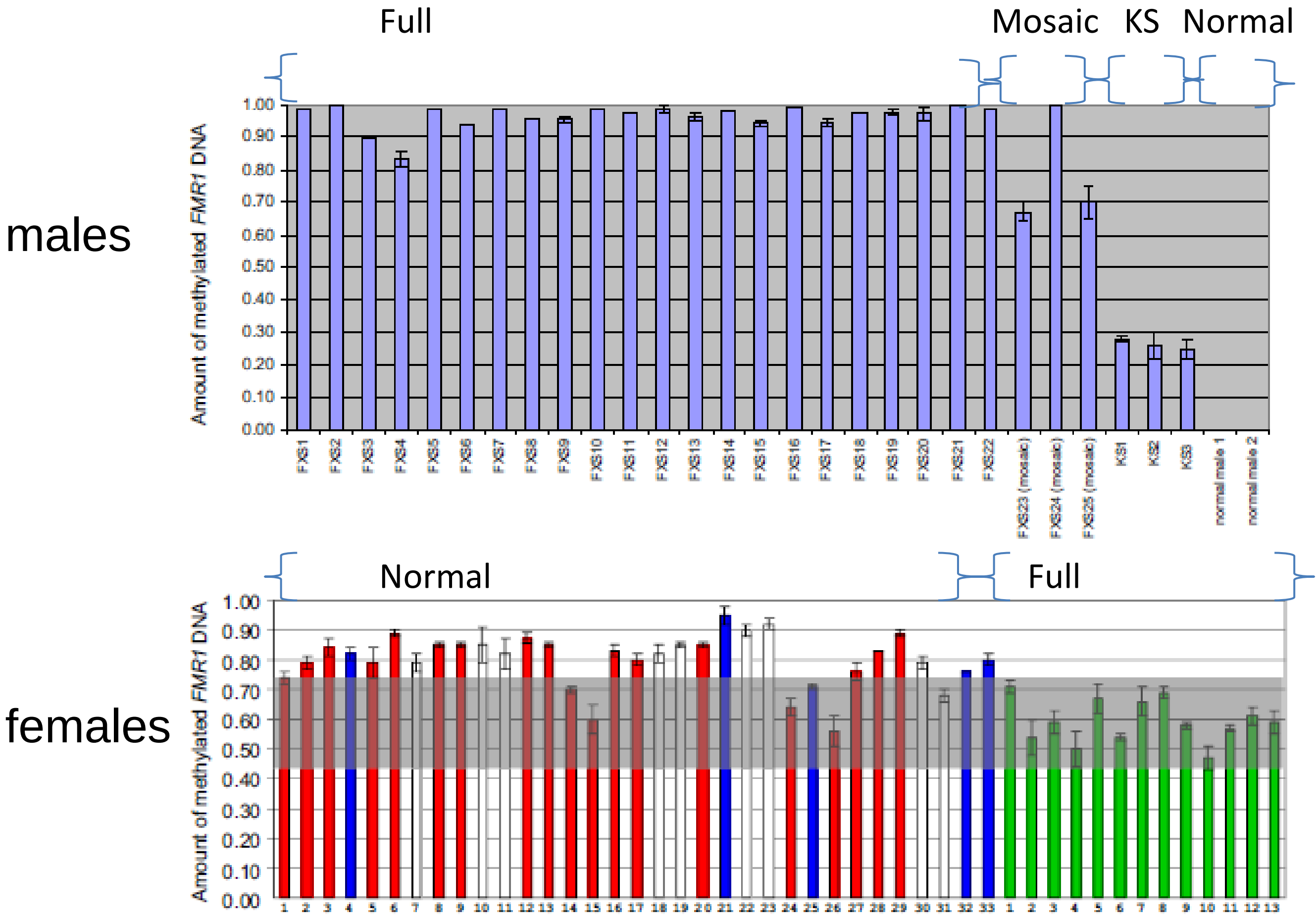
Methylated *FMR1* DNA



Unmethylated *FMR1* DNA

Coffee B et al. AJHG 2009; 85:503-514.

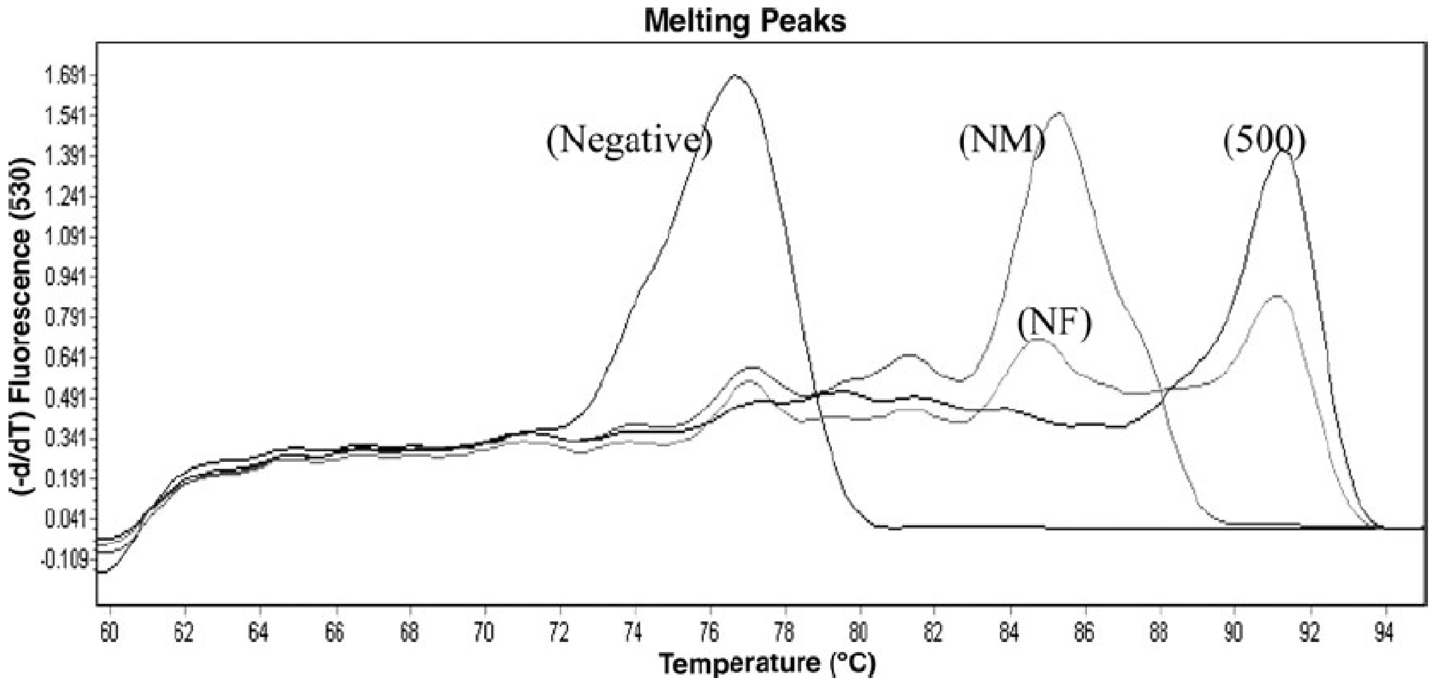
Real-Time PCR Results



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

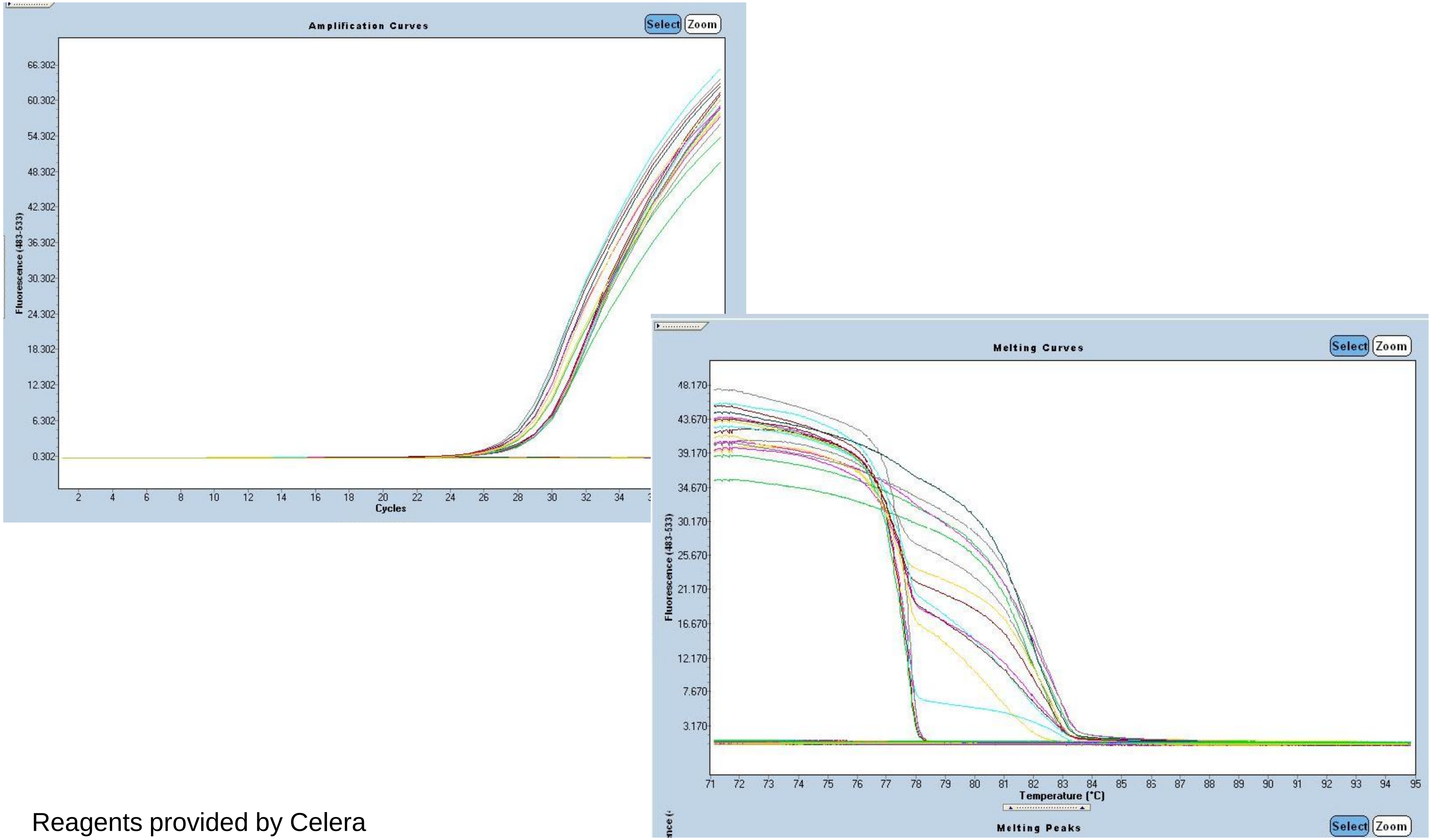
Coffee B et al. AJHG 2009; 85:503-514.

Melt Analysis



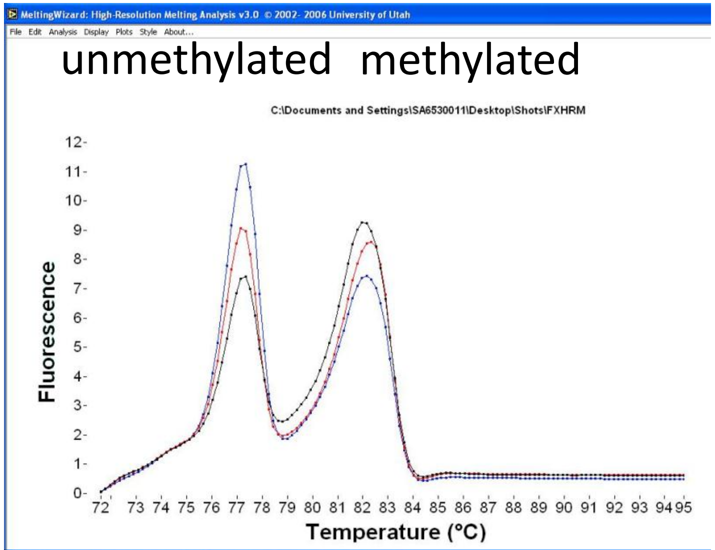
Elias MH et al. Genet Test Molec Bio 2011; 15(6):387-393.

Real-time PCR – Melt Analysis

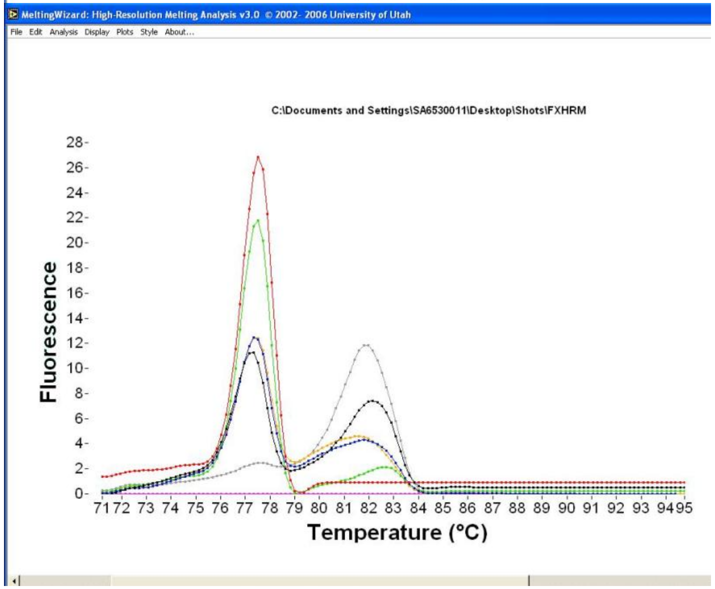


Reagents provided by Celera

‘Melting Peaks’



Normal Females

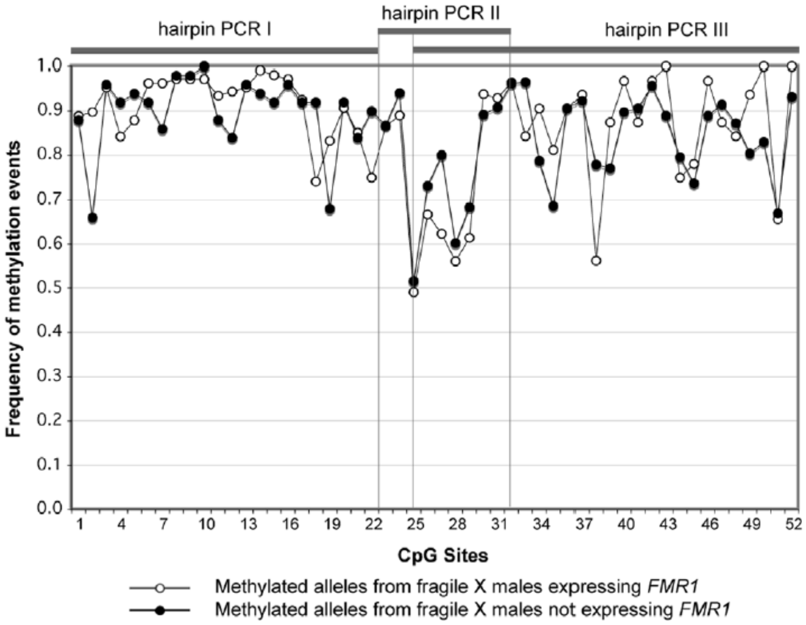


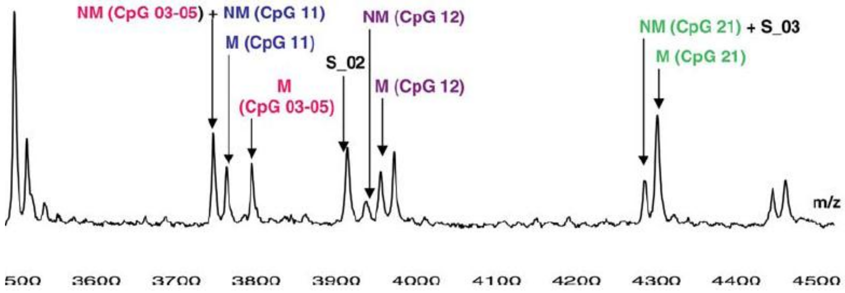
Titration % methylation

Reagents provided by Celera

Site Specific Analysis

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





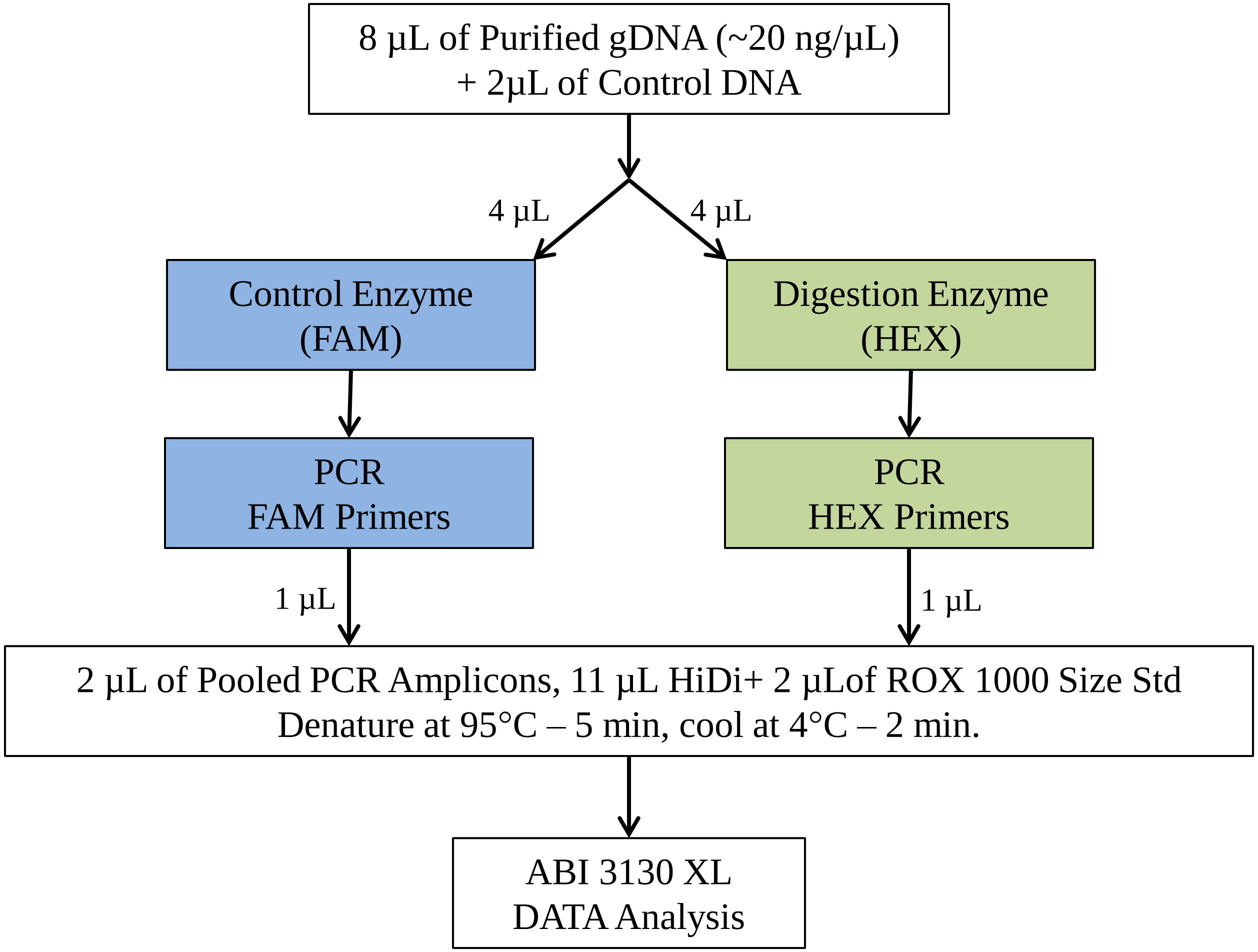
Stöger R et al. PLOS One 2011 e23648

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)
+ 2µL of Control DNA

4 µL

4 µL

Control Enzyme
(FAM)

Digestion Enzyme
(HEX)

PCR
FAM Primers

PCR
HEX Primers

1 µL

1 µL

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

ABI 3130 XL
DATA Analysis

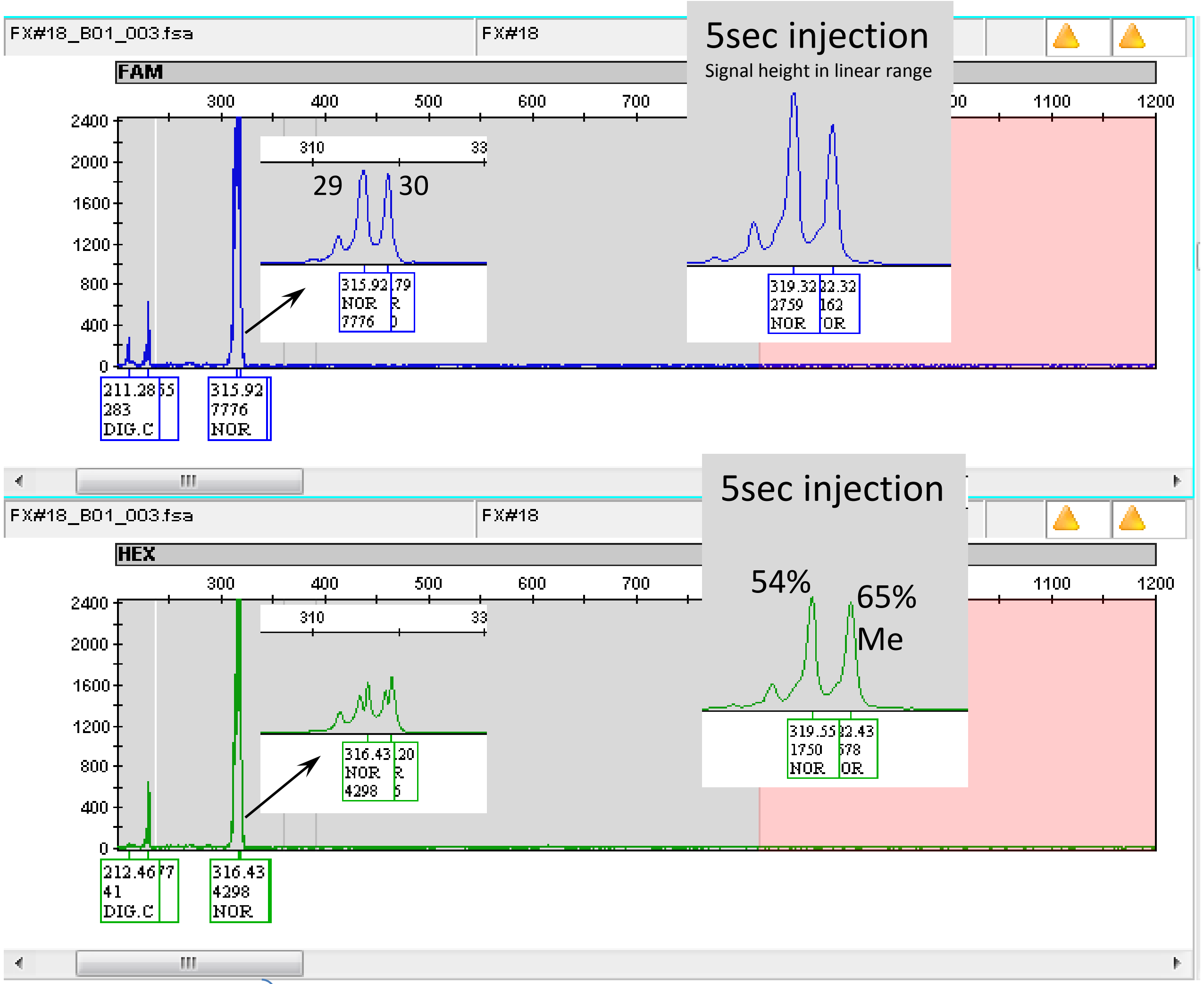
From http://www.asuragen.com/Diagnostics/US/Products/Methylation_PCR_FragileX/

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

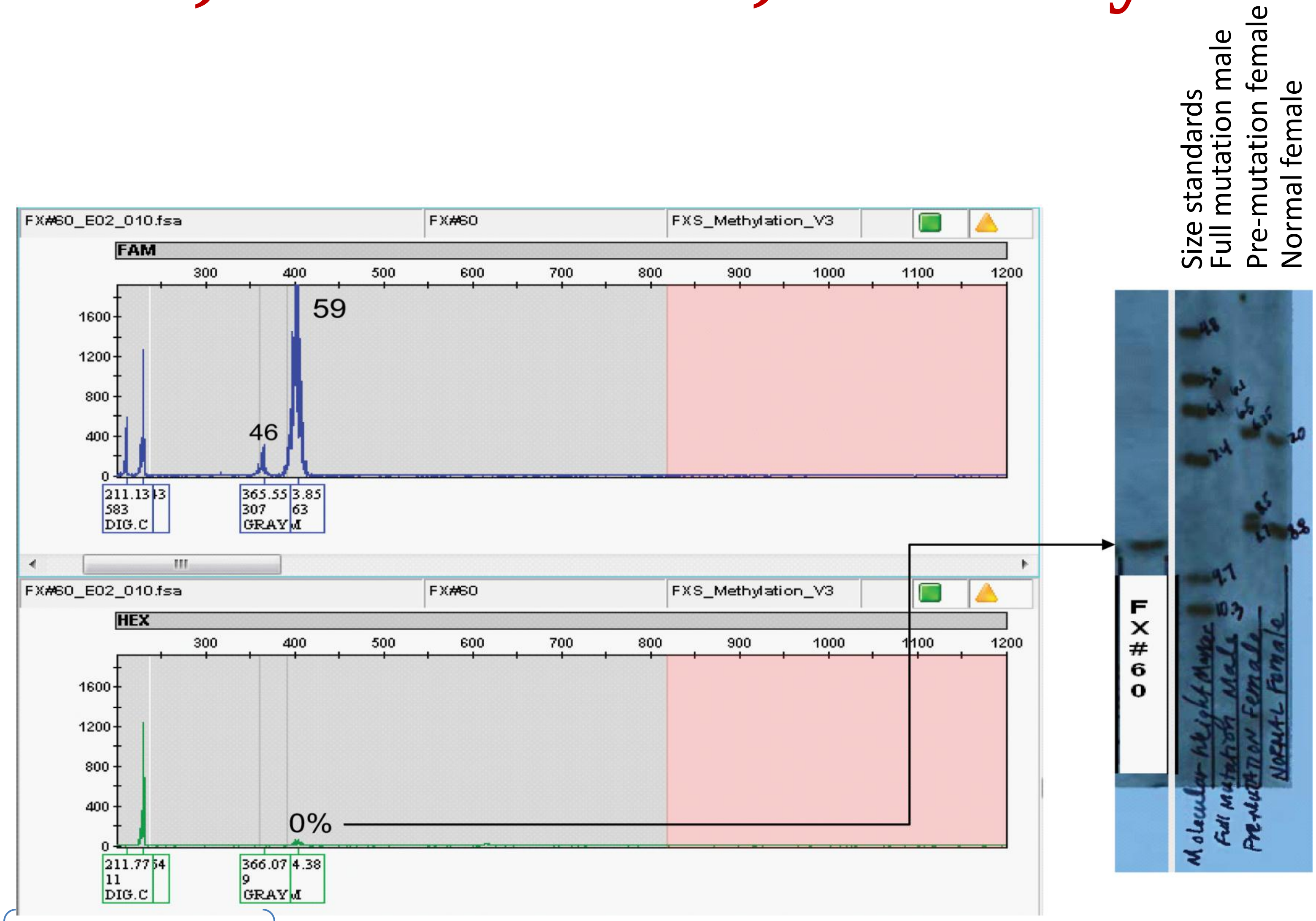
Normal Female



- 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.*

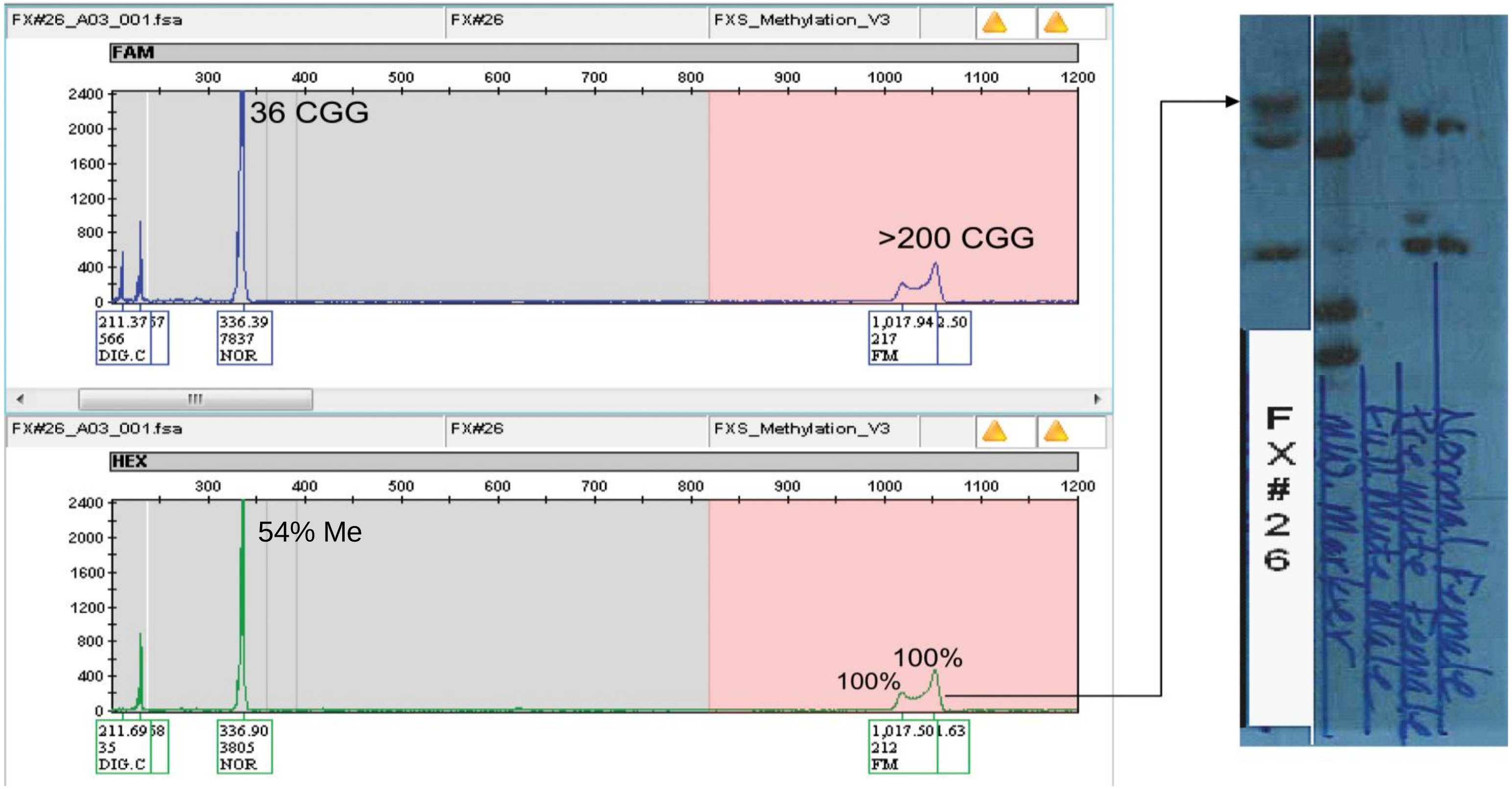
Digestion control

Male, Pre-mutation, Unmethylated

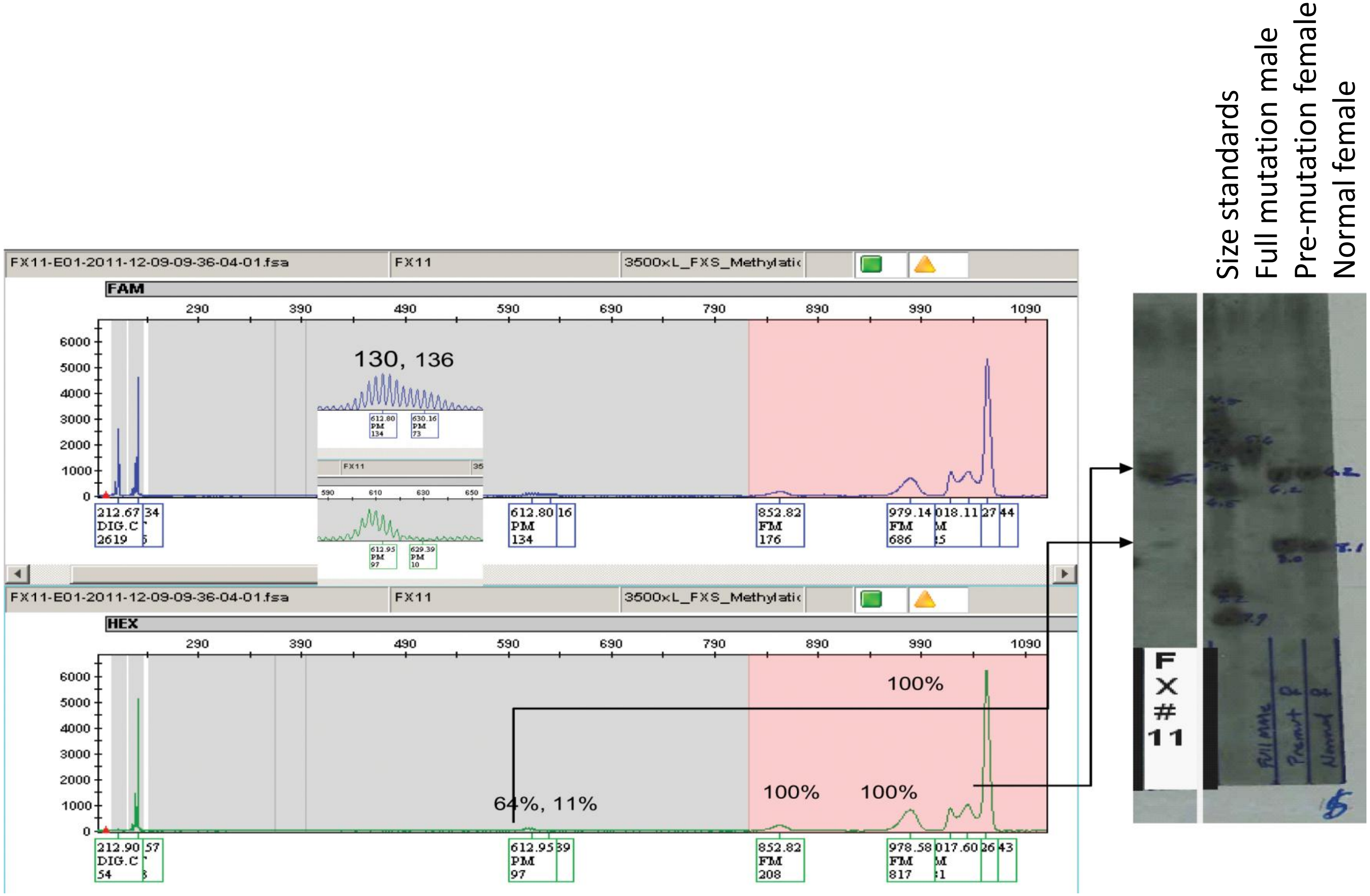


Digestion controls

Female, Full Mutation, Fully Methylated



Male, Full mutation, Mosaic



250 repeats

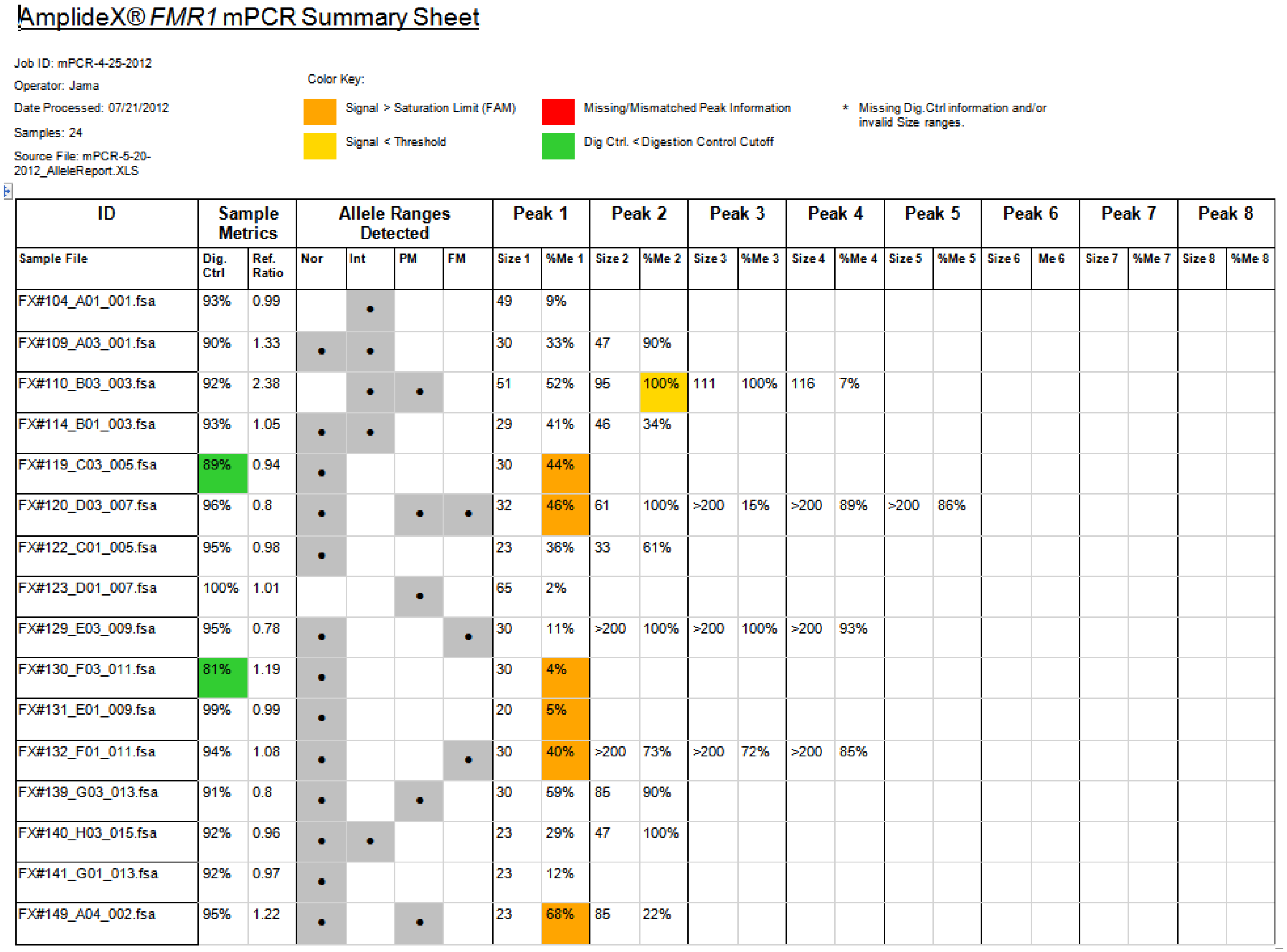
Comparison between mPCR and Southern

Background Information		ARUP - mPCR, Methylation Comparison		Agreement
Sample ID	Sex	ARUP - mPCR	ARUP - SB Results	
FX#1	F	Fully methylated FM	Fully methylated FM-size mosaic	Yes
FX#3	F	Fully methylated FM	Fully methylated FM	Yes
FX#5	M	Normal	N/A	Yes
FX#8	M	Unmethylated PM	Premutation unmethylated PM	Yes
FX#11	M	Mostly methylated, maybe some indication of partial	Methylated - may have low level unmethylated mosaic	Yes
FX#15	F	Partial Methylation (Female PM)	methylated/ unmethylated PM	Yes
FX#17	F	Partial Methylation (Female PM)	methylated/ unmethylated PM	Yes
FX#18	F	Normal	N/A	Yes
FX#21	F	Partial Methylation (Female PM)	methylated/ unmethylated PM	Yes
FX#26	F	Fully methylated FM	Fully methylated FM	Yes
FX#28	M	Unmethylated PM	Premutation unmethylated PM	Yes
FX#29	M	Fully methylated FM	Fully methylated FM-size mosaic	Yes
FX#33	F	Partial Methylation (Female PM)	Skewed, premutation mostly unmethylated PM	Yes

Comparison between mPCR and Southern

Background Information		ARUP - mPCR, Methylation Comparison		Agreement
Sample ID	Sex	ARUP - mPCR	ARUP - SB Results	
FX#34	F	Normal	N/A	Yes
FX#35	M	Possible mosaic with partial methylation in FM allele, likely undercalled as a technical error	Fully methylated FM-size mosaic	Yes
FX#37	M	Mostly methylated PM with unmethylated FM	Premutation unmethylated/size mosaic	Yes
FX#38	F	Fully methylated FM	Fully methylated FM	Yes
FX#39	F	Partial Methylation (Female PM)	Premutation	Yes
FX#48	F	Fully methylated FM (maybe some indication of partial)	Methylated FM - may have low level unmethylated	Yes
FX#54	M	Fully methylated FM	Fully methylated FM	Yes
FX#55	M	No Reference Peak in HEX (technical)	Fully methylated	Yes
FX#60	M	Unmethylated PM	Premutation unmethylated PM	Yes
FX#74	F	Partial Methylation (Female PM)	Possibly skewed pre-mutation mostly unmethylated PM	Yes
FX#85	M	Unmethylated	Premutation unmethylated	Yes
FX#165	F	Fully methylated FM	Fully methylated FM - size mosaic	Yes
ARUP-NTC				
Neg		ND		
Neg-Size-Std		ND		

Summary Report



AmplideX® *FMR1* mPCR Summary Sheet

Job ID: mPCR-4-25-2012

Operator: Jama

Date Processed: 07/21/2012

Samples: 24

Source File: mPCR-5-20-2012_AlleleReport.XLS

Color Key:

🟧	Signal > Saturation Limit (FAM)	🟥	Missing/Mismatched Peak Information
🟨	Signal < Threshold	🟩	Dig Ctrl. < Digestion Control Cutoff

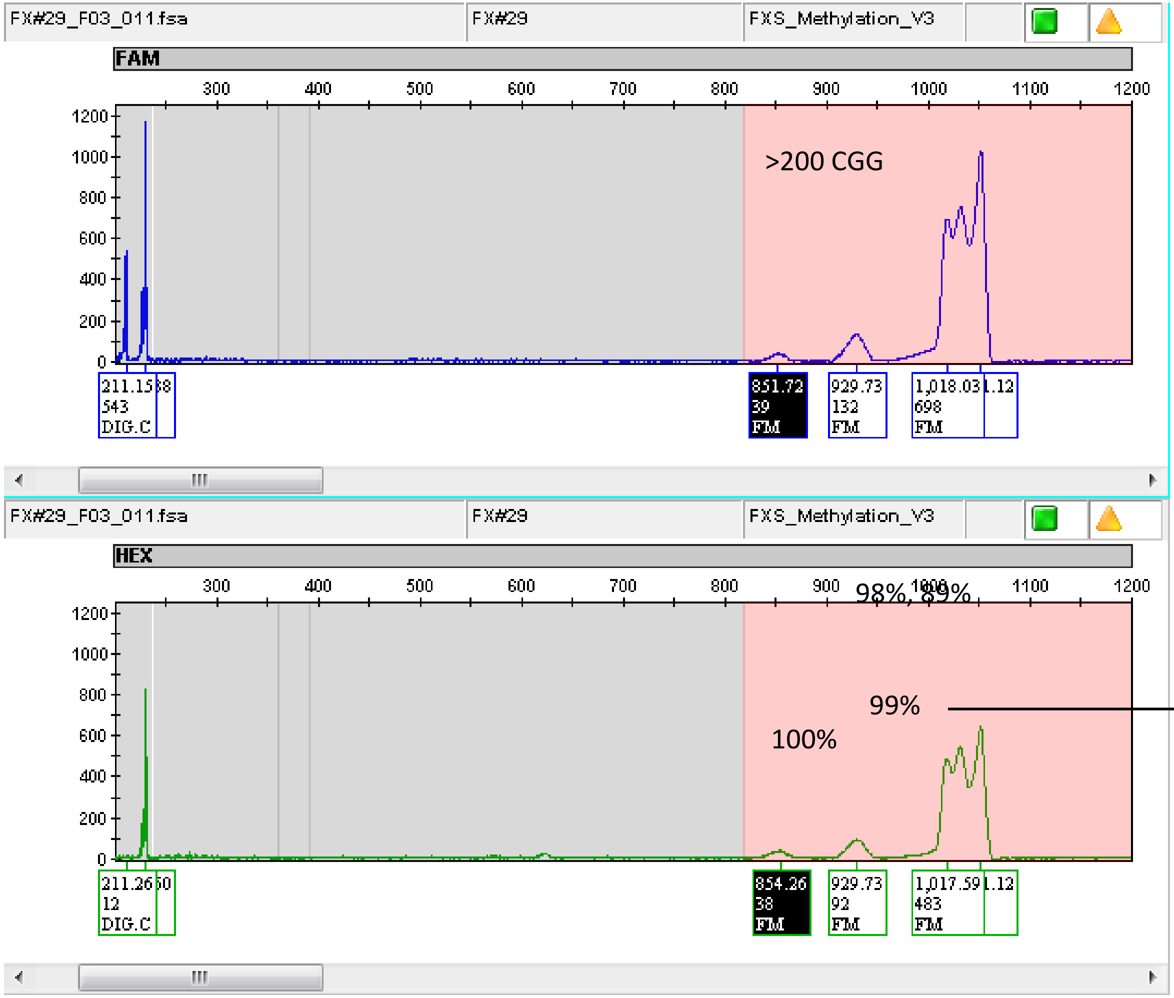
* Missing Dig.Ctrl information and/or invalid Size ranges.

ID	Sample Metrics		Allele Ranges Detected				Peak 1		Peak 2		Peak 3		Peak 4		Peak 5		Peak 6		Peak 7		Peak 8	
Sample File	**Dig. Ctrl**	**Ref. Ratio**	**Nor**	**Int**	**PM**	**FM**	**Size 1**	**%Me 1**	**Size 2**	**%Me 2**	**Size 3**	**%Me 3**	**Size 4**	**%Me 4**	**Size 5**	**%Me 5**	**Size 6**	**Me 6**	**Size 7**	**%Me 7**	**Size 8**	**%Me 8**
FX#104_A01_001.fsa	93%	0.99		●			49	9%														
FX#109_A03_001.fsa	90%	1.33	●	●			30	33%	47	90%												
FX#110_B03_003.fsa	92%	2.38		●	●		51	52%	95	100%	111	100%	116	7%								
FX#114_B01_003.fsa	93%	1.05	●	●			29	41%	46	34%												
FX#119_C03_005.fsa	89%	0.94	●				30	44%														
FX#120_D03_007.fsa	96%	0.8	●		●	●	32	46%	61	100%	>200	15%	>200	89%	>200	86%						
FX#122_C01_005.fsa	95%	0.98	●				23	36%	33	61%												
FX#123_D01_007.fsa	100%	1.01			●		65	2%														
FX#129_E03_009.fsa	95%	0.78	●			●	30	11%	>200	100%	>200	100%	>200	93%								
FX#130_F03_011.fsa	81%	1.19	●				30	4%														
FX#131_E01_009.fsa	99%	0.99	●				20	5%														
FX#132_F01_011.fsa	94%	1.08	●			●	30	40%	>200	73%	>200	72%	>200	85%								
FX#139_G03_013.fsa	91%	0.8	●		●		30	59%	85	90%												
FX#140_H03_015.fsa	92%	0.96	●	●			23	29%	47	100%												
FX#141_G01_013.fsa	92%	0.97	●				23	12%														
FX#149_A04_002.fsa	95%	1.22	●		●		23	68%	85	22%												

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

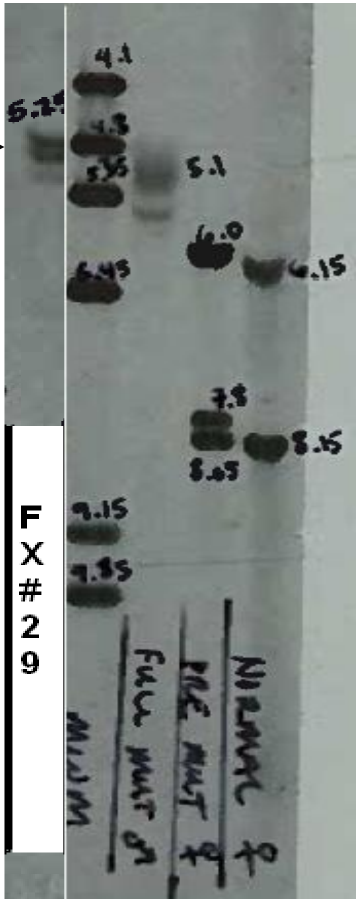


>200 CGG

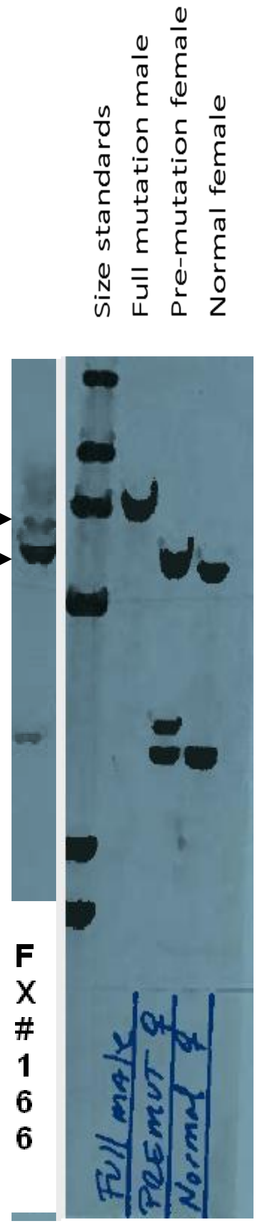
98%, 89%

100%

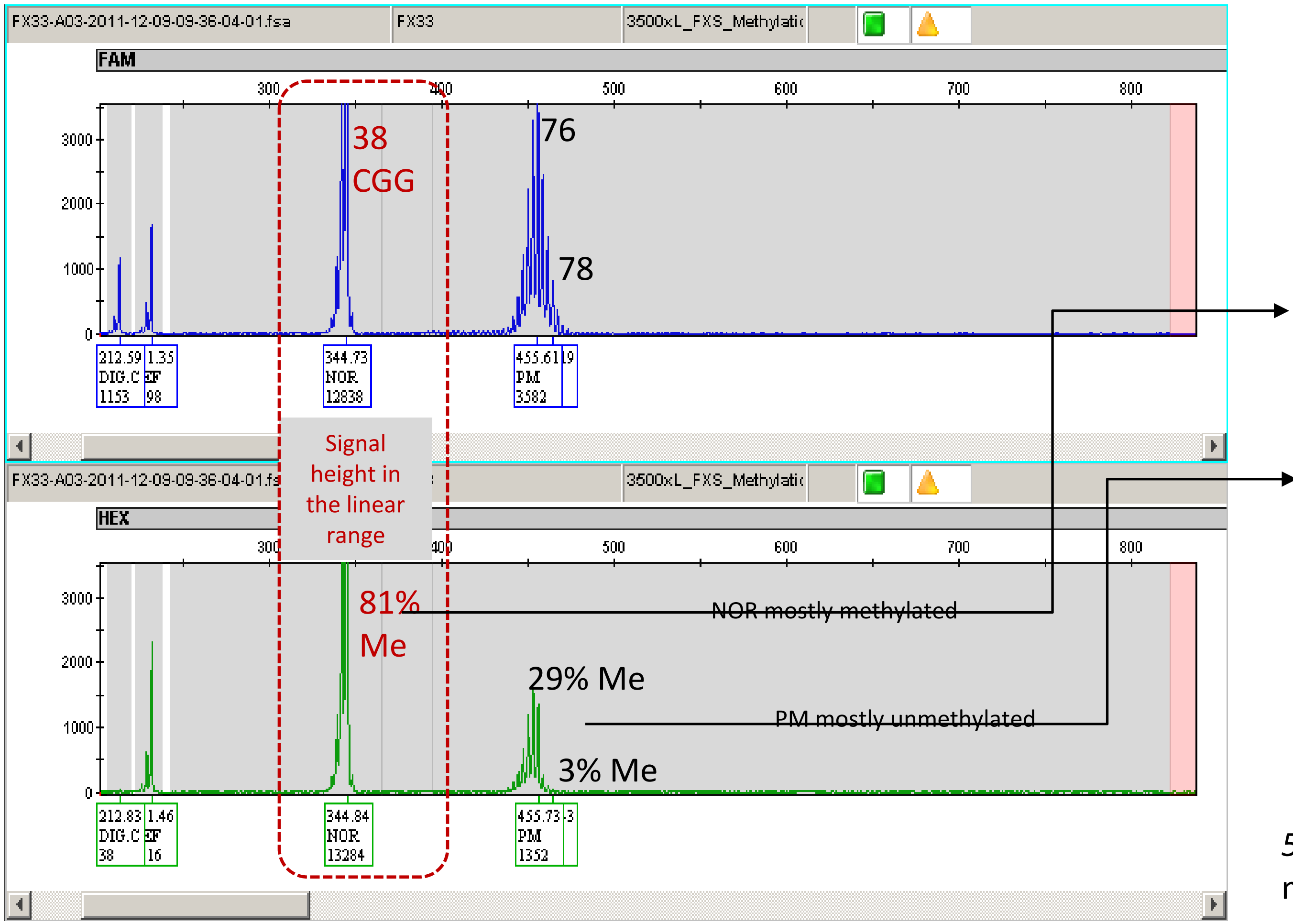
99%



Female, Fully Methylated Size Mosaic



Skewed X Inactivation



38 CGG

76

78

Signal height in the linear range

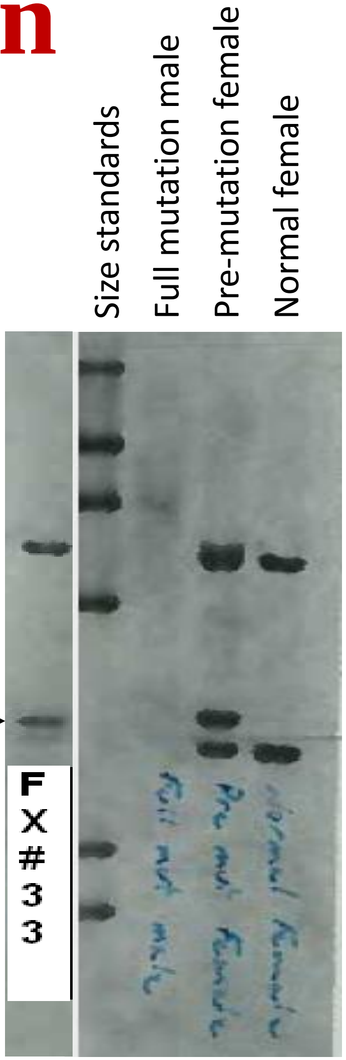
81% Me

NOR mostly methylated

29% Me

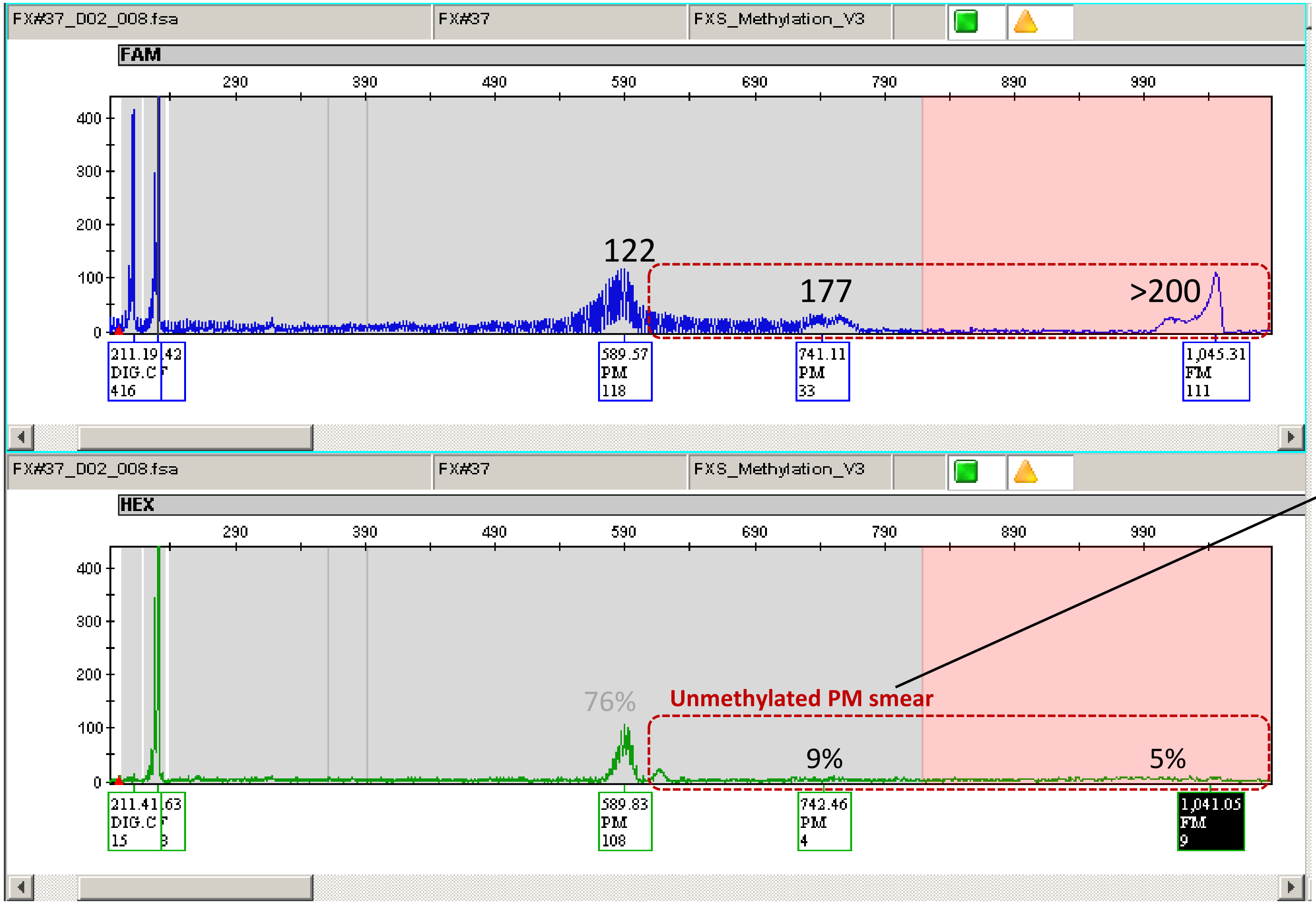
PM mostly unmethylated

3% Me

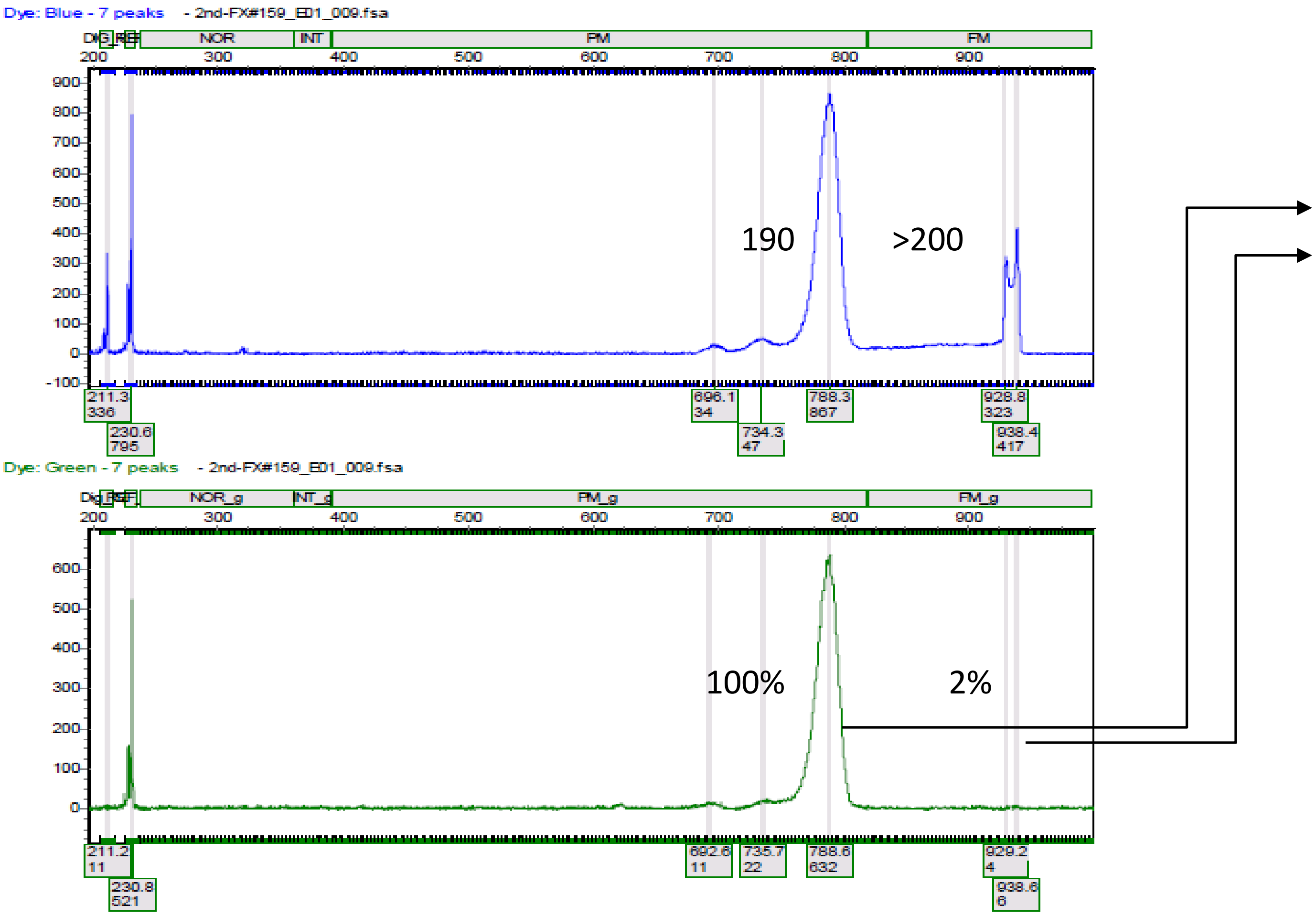


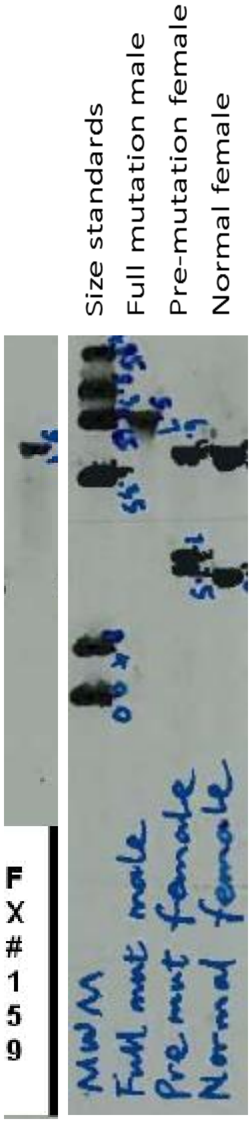
5 s injection data;
mPCR at Asuragen

Male, High Repeats



Male, Full Mutation





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

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