Validation of two new PCR technologies for diagnostic testing of the Fragile X CGG repeat as well as *FMR1* methylation status

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Introduction

- Almost all cases of Fragile X syndrome are caused by an expansion of a CGG repeat in the 5' UTR of the FMR1 gene to >200 repeats.
- For FRAX diagnostics we are validating two new PCR technologies from Asuragen Inc. USA:
  1. a gene-specific *FMR1* PCR and a CGG repeat primed PCR
  2. a prototype PCR method that was developed to detect the *FMR1* methylation status, which is currently only detected by Southern blot analysis.

Methods

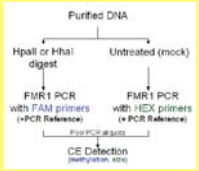
Gene-specific *FMR1* PCR and CGG repeat primed PCR
Samples: 75 (25 prenatal and 50 postnatal) including full expanded alleles (FM), premutations (PM), intermediates, homozygous normal females and normal males were tested.

PCR's were performed on 20-40 ng DNA according the supplied protocol from Asuragen Inc. USA.

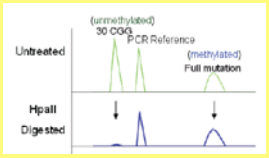
Gene specific PCR contains two primers outside the CGG repeat in FMR1, while the repeat primed PCR contains 3 primers: two outside the CGG repeat and one inside the CGG repeat.

Data were analyzed using Genemarker software and results compared to previous obtained genotype calls and Southern blot analysis.

Prototype *FMR1* methylation PCR
Samples: 45 (19 prenatal and 26 postnatal) including alleles in all ranges were tested.
80 ng of DNA is used and treated as indicated below:



In case of a methylated allele, this allele will not be digested by the methylation sensitive enzyme. Unmethylated alleles will be digested. By running untreated and digested DNA by CE, the methylation status can be determined of different alleles.

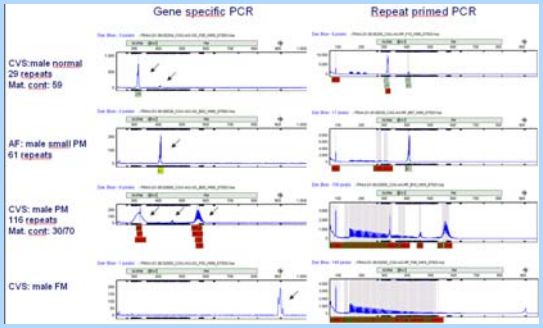


Calculating the percent of methylation:
(Peak height of allele (FAM)/ Peak height reference peak (FAM))/ (Peak height of allele (HEX)/ Peak height reference peak (HEX)) * 100% .

All results were compared to previous Southern blot data.

Results

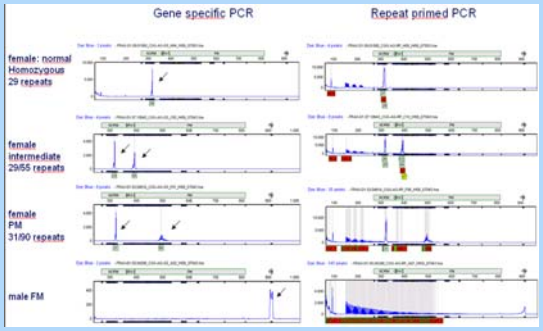
Gene-specific *FMR1* PCR (GS-PCR) and CGG repeat primed PCR (RP-PCR)



- 25 prenatal samples were tested:
  - all samples succeeded in GS and RP-PCR
  - all results were concordant with previous results
  - repeats >200 can be detected
  - maternal contamination is detected

Samples tested

Allele	N	
Normal	12	6-50 repeats
Intermediate	0	50-58 repeats
PM	4	59-200 repeats
FM	9	> 200 repeats



- 50 postnatal samples were tested:
  - all samples succeeded in GS-PCR
  - 3 samples failed in RP-PCR
  - all results were concordant with previous results
  - repeats >200 can be detected

Samples tested

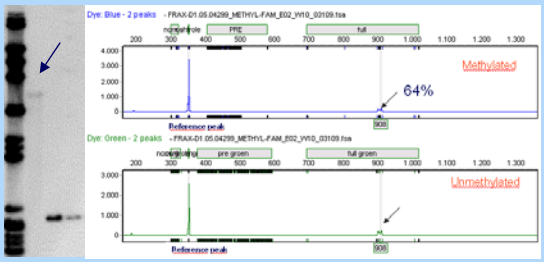
Allele	N	
Normal	33	6-50 repeats
Intermediate	3	50-58 repeats
PM	8	59-200 repeats
FM	6	> 200 repeats

Prototype *FMR1* methylation PCR

Samples tested

Prenatal samples			Postnatal samples	
Allele	N		Allele	N
Normal	6	6-50 repeats	Normal	8
Intermediate	0	50-58 repeats	Intermediate	3
PM	4	59-200 repeats	PM	9
FM	9	> 200 repeats	FM	6

- Overall high concordance between Southern blot analysis and FMR1 methylation PCR
- There is generally a lower than expected percent methylation observed for different samples
- Postnatal sample concordance was greater than prenatal sample concordance with Southern blot
- Two prenatal samples with no result on Southern blotting could be resolved using the methylation PCR
- Two postnatal samples gave no result for unmethylated run



Southern blot data versus *FMR1* methylation PCR of a postnatal sample.

On Southern blot the full mutation appears to be almost completely methylated, while in general the *FMR1* methylation PCR shows lower methylation percentages. We hypothesize that this discrepancy might be caused by using different restriction enzymes in both methods. For the Southern blot, DNA is digested using HindIII/EagI while for the *FMR1* methylation PCR HpaII is used. Furthermore, it could also be due to a difference in resolution.

Conclusions & Discussion

2 new *FMR1* PCR kits (GS-PCR and RP PCR)
- 75 samples (25 prenatal: 50 postnatal) tested
- All results were concordant with previous genotype calls
- The advantage of these kits: resolves zygosity status
- Disadvantage of these kits: no gender specific peaks

Prototype *FMR1* methylation PCR
- 45 samples (19 prenatal; 26 postnatal) tested
- Overall high concordance with previous Southern blot data
- Generally a lower than expected percent methylation observed
- Advantage of this kit: could possibly replace Southern blotting
- More samples need to be screened to determine the reproducibility and robustness of this kit

References

Asuragen Inc. (http://www.asuragen.com/)

For questions or remarks, please email E.M.J.Boon@lumc.nl

Data will also be presented at the Eurogentest Satellite meeting: Sunday June 13 Time 10.15-12.30