Development of RASSF1A assay:
universal fetal DNA marker

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Fetal specific markers

• To confirm presence of cf fetal DNA if testing result is negative

• Several candidates
  
  Control Genes (limited) – will determine that cf DNA extracted but not distinguish fetal and maternal
  
  Paternal single nucleotide polymorphisms
  
  Epigenetic markers

No one clear approach at present – many candidate markers under development
Hypermethylated RASSF1A in Maternal Plasma: A Universal Fetal DNA Marker that Improves the Reliability of Noninvasive Prenatal Diagnosis

Protocol Modifications

Chan et al. protocol (2006)

• Single enzyme digest (BstUI 60°C 16 hours)
• Probe based assay
• Beta actin promoter as digest control

Modified protocol

• Multiple enzyme digest (total digest time 4 hours)
• SYBR green assays for SRY and RASSF1A
• Melt to check amplicon specificity
• Methylation insensitive digest control on RASSF1A
• (Beta actin promoter as digest control)
Extract 2ml plasma DNA using Qiagen QIAamp circulating nucleic acid kit

70µl extraction volume

20µl

40µl digests

Methylation sensitive
- BstYI
- Bst U1
- 60°C 2 hours

- EcoRI
- HpaII
- HhaI
- 37°C 2 hours

Methylation insensitive
- Bsml
- Tsel
- 65°C 2 hours

- EcoRI
- HaeIII
- Msp1
- 37°C 2 hours

Undigested
- No enzymes
- 65°C 2 hours

- No enzymes
- 37°C 2 hours
### Digests
- Methylation sensitive
- Methylation insensitive
- Undigested

### RQ-PCR
- SRY
- RASSF1A

<table>
<thead>
<tr>
<th></th>
<th>Methylation sensitive</th>
<th>Methylation insensitive</th>
<th>Undigested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male fetus</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Female fetus</td>
<td>✗</td>
<td>✓</td>
<td>✗</td>
</tr>
<tr>
<td>No fetal DNA</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>No cell free DNA</td>
<td>✗</td>
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74% GC rich
Male fetus: SRY $+$ve and RASSF1A $+$ve

SRY: 8/9 $+$ve

RASSF1A:

Non-specific products

Undigested

Me sens

Me insens*
Female fetus: SRY -ve and RASSF1A +ve

SRY: 0/9 +ve

RASSF1A
No fetal DNA: SRY -ve and RASSF1A -ve
Future work

• NGRL (W) / RAPID validation ongoing

• Retrospective study of 66 samples - analysis near completion

  Undigested Samples (total cell free DNA): 100% had 3 replicates +ve

  Methylation sensitive (fetal DNA only)

    5  (7.5%)  3 replicates negative (no fetal DNA)
    61 (92%)  1 or more replicates +ve
    54 (83%)  2 or more replicates +ve
    48 (72%)  3 replicates +ve

  Methylation insensitive (all DNA should digest)

    61 (92%)  3 replicates negative
    5  (7.5%)  1 replicate +ve

• Prospective study of 100 samples underway - using larger volume of plasma

• Assessment of RASSF1A and beta actin digests - what combination is most useful / appropriate control

• Best practice?
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