Evaluation of the Invader® assay platform for molecular analysis of the Factor V (G1691A) and Factor II (G20210A) mutations.

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Aim:

- To evaluate the Third Wave™ Invader® DNA assay for the detection of the FVL (G1691A) and Factor II (G20210A) mutations.
- Test 100 samples where the genotype is known for the FV and FII mutations as determined by the RFLP method.
- Compare and contrast the two methods for use in a diagnostic setting.

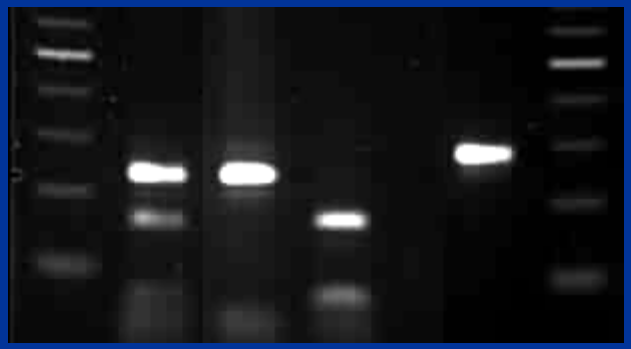
Methodology:

The RFLP method (PCR based):

Factor V Leiden:

Restriction enzyme = Mnl I

(loss of restriction site)

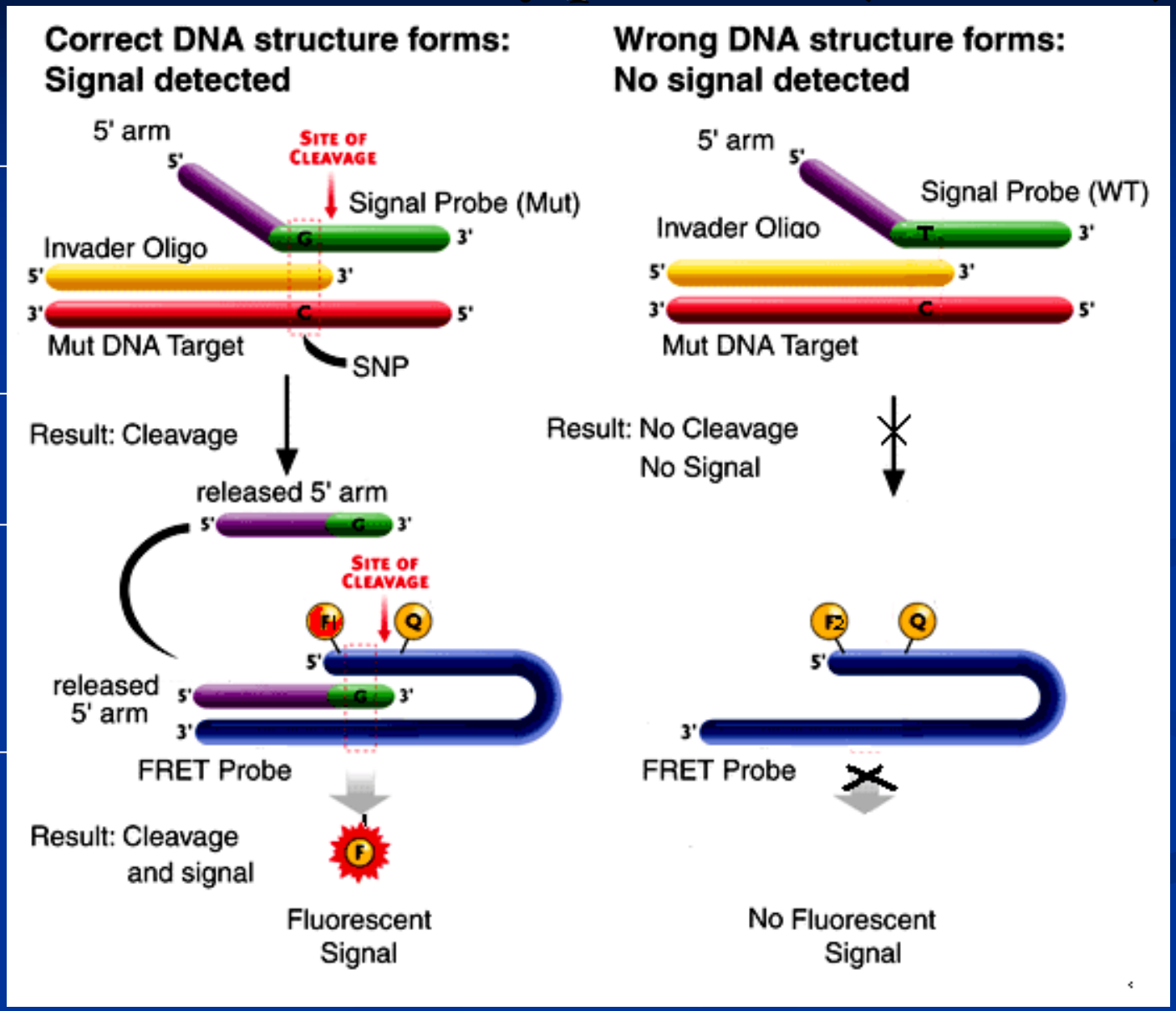


Factor II:

Restriction enzyme = Hind III

(Use of mutagenic primer and G > A creates restriction site)

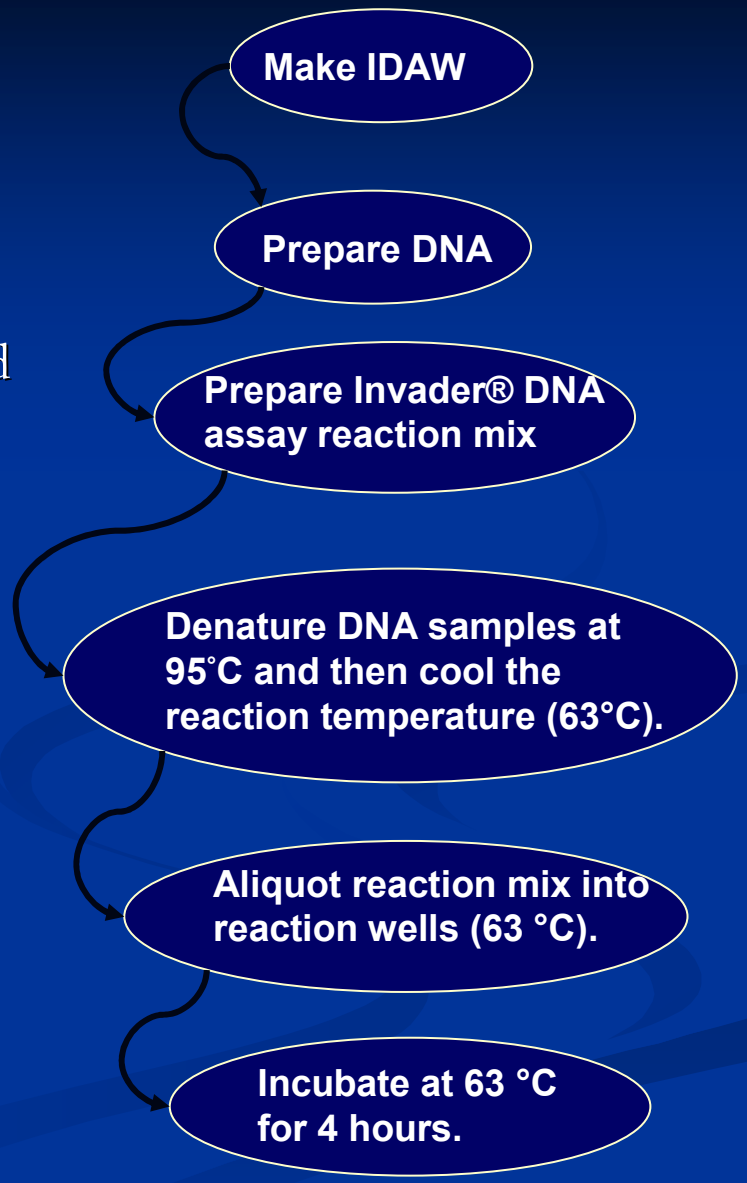
The Invader® assay platform (non-PCR) :



Invader® assay platform:

(Third Wave technologies)

- >100ng of DNA is needed
- 96 well plate format.
- biplex format F (WT) and R (Mut) signal
- Controls include a Normal, Heterozygote and Homozygote
- Fluorescence analysed using a 96 well plate reader (CytoFluor 96 well plate reader).
- An excel worksheet (IDAW) is used to calculate the net signal/ background or net fold over zero (FOZ).
- Ratio of the WT reaction to the mutant reaction.
  - Heterozygous = >0.3 to <3
  - Homozygous = <0.2
  - Normal = >5
- Total assay time = ~ 5 hours
  (hands on time =30-45min)



Results:

IDAW:

Data File Date Stamp:						Raw Data					
F Signal (Mut)	1	2	3	4	5	6	7	8	9	10	11
A	259	211									
B	684	285									
C	909	232									
D	294	217									
E	349	199									
F	318	212									
G	1508	285									
H	352	417									
R Signal (WT)	1	2	3	4	5	6	7	8	9	10	11
A	534	1678									
B	540	227									
C	245	2210									
D	253	911									
E	400	1026									
F	686	1174									
G	1374	1495									
H	1302	847									

			Lot Numbers:			FVL (1691) Control 1 (WT)
Operator:	Date:	DNA Reaction Buffer 1 (B)	0	FVL (1691) FRET R Cassette (R)	0	FVL (1691) Control 2 (Het)
oly2	30/01/2004	FVL (1691) Invader Oligo (I)	0	FVL (1691) FRET F Cassette (F)	0	FVL (1691) Control 3 (Mut)
		FVL (1691) Primary Probes (P)	0	Cleavase X Enzyme 40 ng/µl (E)	0	Control 4 (No Target Blank)

Invader Data Analysis - FVL (G1691A) Biplex Assay — Version 040202

Sample	Invader Genotype	F Signal	R Signal	F Signal FOZ	R Signal FOZ	Net F Signal FOZ	Net R Signal FOZ	RATIO	Data	Action
FVL (1691) Control 1	WT	259	534	0.88	2.11	0.04	1.11	27.767	VALID	NONE
FVL (1691) Control 2	HET	684	540	2.33	2.13	1.33	1.13	0.855	VALID	NONE
FVL (1691) Control 3	MUT	909	245	3.09	0.97	2.09	0.04	0.019	VALID	NONE
Control 4		294	253						VALID	NONE
66(4ul)	EQ1	349	400	1.19	1.58	0.19	0.58	3.106	INVALID	REPEAT SAMPLE
6(4ul)	WT	318	686	1.08	2.71	0.08	1.71	20.965	VALID	NONE
9	HET	1508	1374	5.13	5.43	4.13	4.43	1.073	VALID	NONE
10	WT	352	1302	1.20	5.15	0.20	4.15	21.017	VALID	NONE
11	WT	211	1678	0.72	6.63	0.04	5.63	140.810	VALID	NONE
12	-	285	227	0.97	0.90	0.04	0.04	1.000	INVALID	REPEAT SAMPLE
13	WT	232	2210	0.79	8.74	0.04	7.74	193.379	VALID	NONE
19	WT	217	911	0.74	3.60	0.04	2.60	65.020	VALID	NONE
15	WT	199	1026	0.68	4.06	0.04	3.06	76.383	VALID	NONE
16	WT	212	1174	0.72	4.64	0.04	3.64	91.008	VALID	NONE
17	WT	285	1495	0.97	5.91	0.04	4.91	122.727	VALID	NONE
18	WT	417	847	1.42	3.35	0.42	2.35	5.612	VALID	NONE

Factor V and Factor II Invader results:

	Factor V	Factor II
Number of samples	110	110
Normal	71	98
Heterozygous	33	9
Homozygous	2	0
Fails	4 (3.6%)	3 (2.7%)
% Repeated	5.5%	3.6%
% Concordance	100%	100%

Discussion:

- The genotypes obtained from the Invader® assay showed 100% concordance to the RFLP method showing that it is suitable for use in diagnostic molecular genetics.

Repeated samples: Factor V 5.5% and Factor II 3.6%.

Possible reasons:

- Low signal as a result of low DNA concentration meaning the patient sample doesn't exceed the background fluorescence seen in the no target blank control.
  - when repeated with more DNA the correct genotype was obtained.

Failed samples: Factor V 3.6% and Factor II 2.7%.

- The samples that could not be genotyped were due to a low DNA concentration.

- 50 /110 samples tested were from Southampton Human genetics unit (total volume 10-15µl).
- All failed samples were from this source and could not be quantified or genotyped due to sample being depleted.

Comparison of Invader and RFLP:

	RFLP	Invader platform
Total time	7-25 hours	4-5 hours
Hands on time	2-3 hours	<45 mins
Number of steps	8	4
	1: Prepare sample 2: Make master mix 3: Make digest mix 4: Add digest mix to samples 5: Restriction digest 6: Pour gel 7: Load gel 8: Image gel	1: Prepare sample 2: Make master mix 3: Add master mix to plate 4: Read plate
Analysis	Gel (subjectivity)	Excel spreadsheet (IDAW)
Visualisation	EtBr (mutagenic)	FRET (fluorescence)

Cost per test:

Invader assay platform = £ 8 per result

RFLP = Approx. £ 2-3

However, the Invader assay requires the use of a flourescence plate reader which costs around £9000

Other applications of the Invader assay platform:

- Cystic fibrosis testing
- Connexin 26
- MTHFR (methylenetetrahydrofolate reductase)
- ApoE
- Hexosaminidase A (Tay Sachs)

Conclusion:

- The invader offers cheap, rapid detection of SNP's
- Highly reproducible results with 100% concordance to existing methods.
- It is non-PCR based.
- Can test up to 92 samples at once
- Wide applications to other areas of molecular diagnostics.
- Highly dependant on template concentration as it affects the reaction dynamics and overall signal strength. However, 120/120 tests on DNA from our lab worked first time.
- More expensive than the existing RFLP method but is less labour intensive and is more rapid with results in ~ 5 hours.