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

O. N. Wood, D. J. Bunyan, J. F. Harvey, NCP Cross

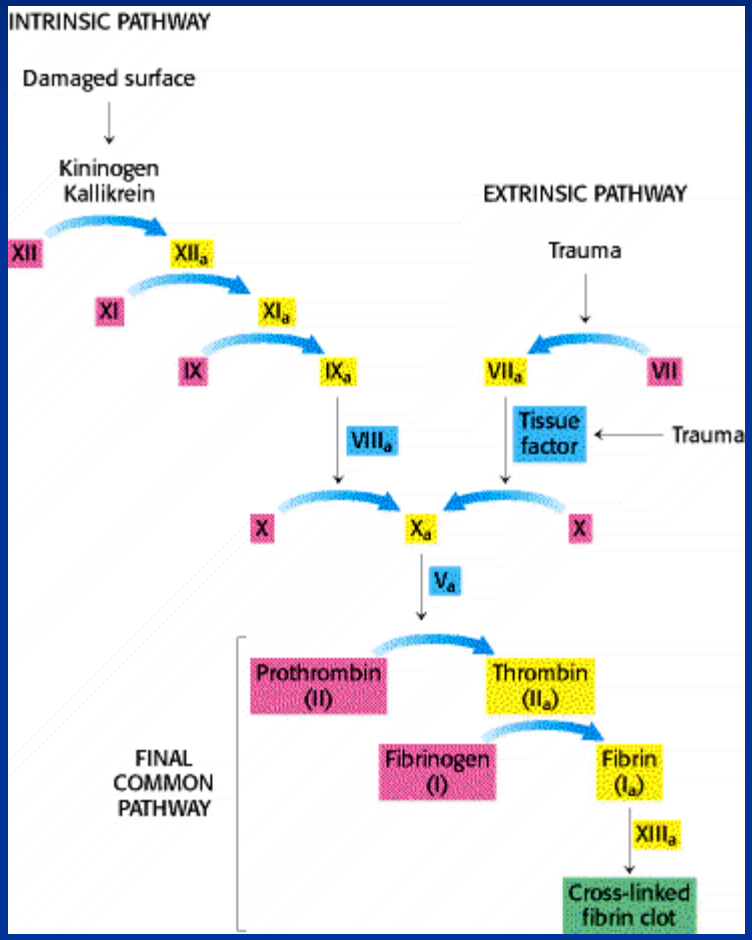
Wessex Regional Genetics Laboratory

and

National Genetics Reference Laboratory.

Introduction:

Blood clotting cascade:



- Activated Factor V (FVa) is involved in the conversion of prothrombin to thrombin.
- Thrombin then acts on fibrinogen to form fibrin.
- Fibrin forms the main constituent of the blood clot.

Thrombophilia:

- Pathological activation of the clotting cascade leading to venous thromoboembolism (VTE).
- Incidence = 1/ 1000.

Clinical manifestations:

- Deep vein thrombosis (DVT) of the lower limbs and pulmonary embolism.

Genetic, acquired and mixed risk factors for thrombosis:

Inherited	acquired	Mixed
Anti-thrombin deficiency	Age	Hyperhomocysteinemia
Protein C deficiency	Immobilisation	Increased Factor VII levels
Factor V Leiden	Surgery	Increased Factor XI levels
Factor II G20210A	Malignancy	Increased factor IX levels
	Pregnancy	Increased Fibrinogen levels
	Oral contraceptives	
	HRT	

Genetic thrombophilia:

- Individuals with genetic thrombophilia show an increased RISK of recurrent thrombosis with an earlier onset (<45 yrs).

Factor V (Proaccelerin):

- Factor V Leiden mutation (Arg506Glu) affects an activated protein C (APC) cleavage site and causes APC resistance.
- **Pathology** = lowered turnover of FVa and an increased conversion of prothrombin to thrombin.

– Heterozygous = 5% of general population and 18% of thrombophilia patients. Increased risk of thrombosis = 2 to 7-fold higher

– Homozygous mutations carriers have increased risk of 40-80 fold higher.

Cont:

Factor II (prothrombin):

- The prothrombin mutation G20210A locates to the 3'-untranslated region and causes hyperthrombinemia.
  - It is present in 2% of the population and 7% of individuals with thrombosis.
  - FII (G20210A) is linked to a 2 to 5-fold increase in risk. of thrombosis, homozygosity of the mutation is associated with a further increase in risk of thrombosis.

Co-inheritance of FVL and FII mutations:

- Compound heterozygosity for FVL (G1691A) and the Factor II (G20210A) mutations occurs in 1/1000.
  - This is associated with a 20-fold increase in risk of thrombosis.

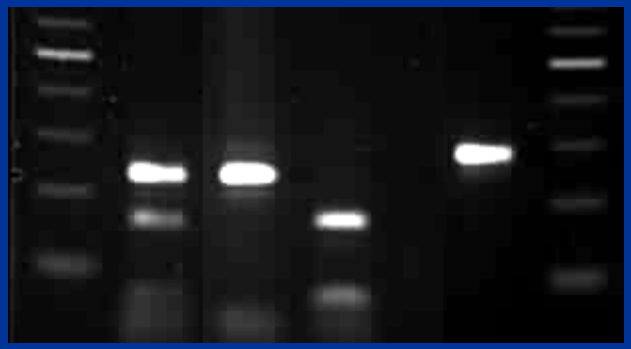
Methodology:

The RFLP method (PCR based):

Factor V Leiden:

Restriction enzyme = Mnl I

(loss of restriction site)

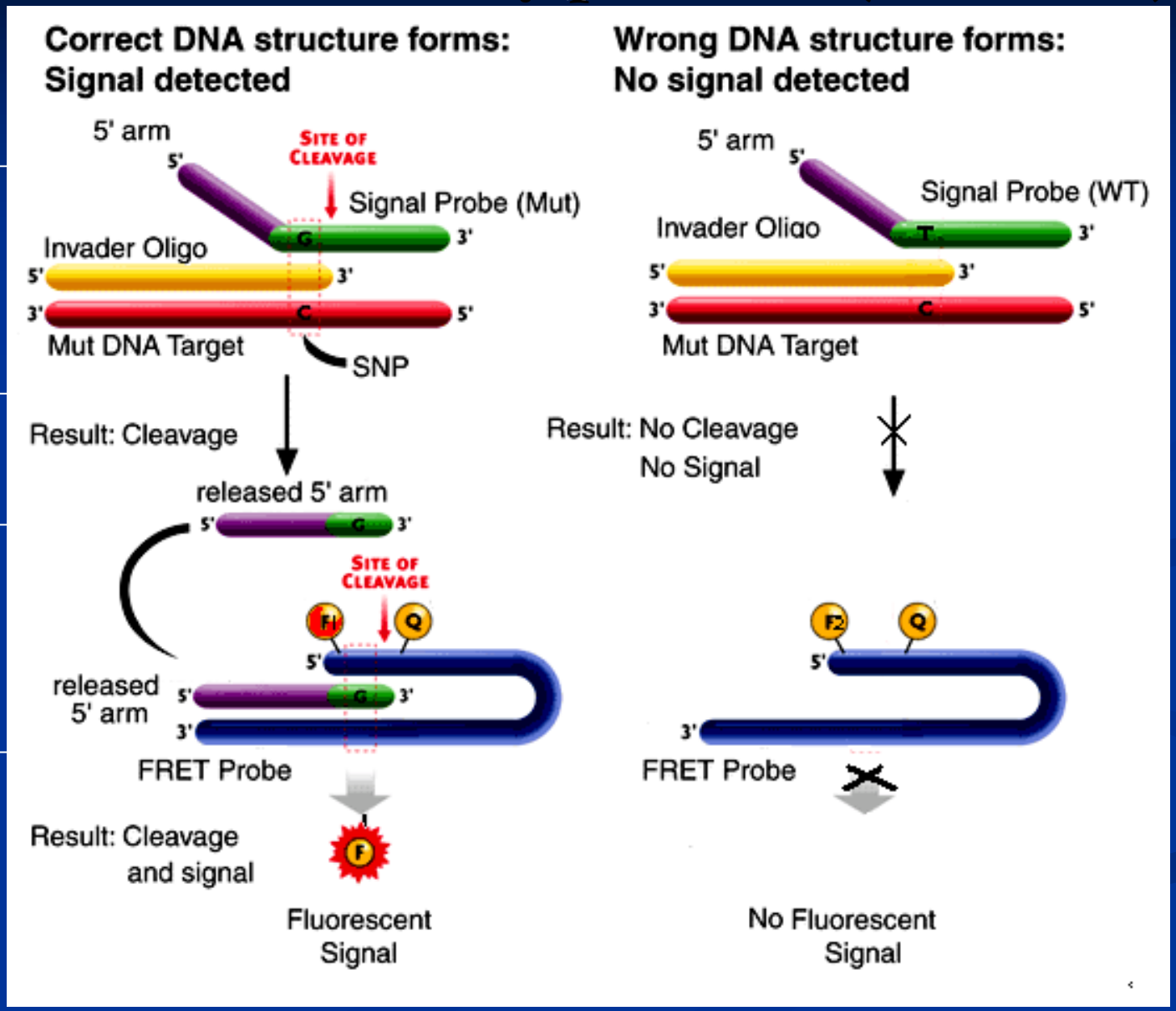


Factor II:

Restriction enzyme = Hind III

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

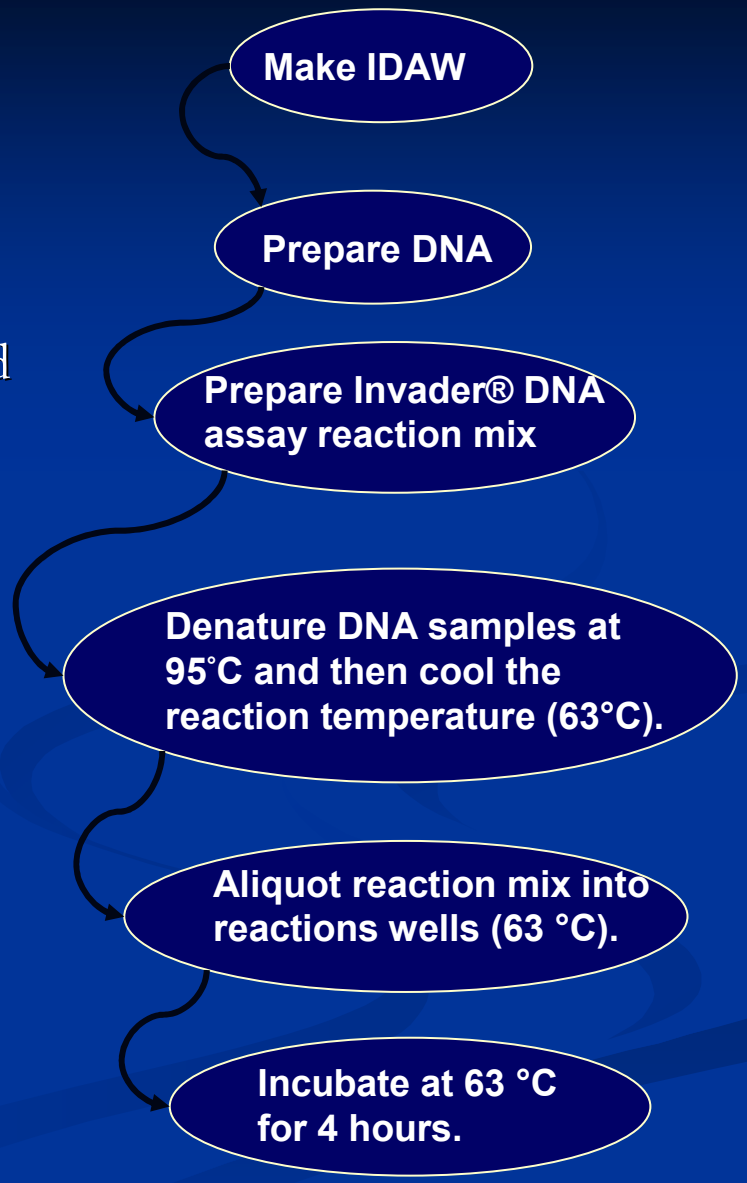
The Invader® assay platform (non-PCR) :



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)



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.

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	X
% Concordance	100%	100%
% Repeated	5.5%	3.6%
% Fail	3.6%	2.7%

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

Other applications of the Invader assay platform:

- Cystic fibrosis testing
- Conexin 26
- MTHFR (methylenetetrahydrofolate reductase)
- ApoE
- Rett syndrome
- Prenatal chromosomal analysis

Conclusion:

- The invader offers cheap rapid detection of SNP's
- Highly reproducible results with 100% concordance to existing methods.
- It is non-PCR based.
- Wide applications to other areas of molecular diagnostics.
- Highly dependant on template concentration as it affects the reaction dynamics and overall signal strength.
- More expensive than the existing RFLP method but is less labour intensive and is more rapid with results in ~ 5 hours.

Acknowledgements:

Wessex Regional Genetics Laboratory:

- Dr John Harvey
- Dr Dave Robinson

The National Genetics Reference Laboratory:

- Dr DJ Bunyan
- Dr Helen White

Third Wave Technologies:

- Dr Erin Dorn