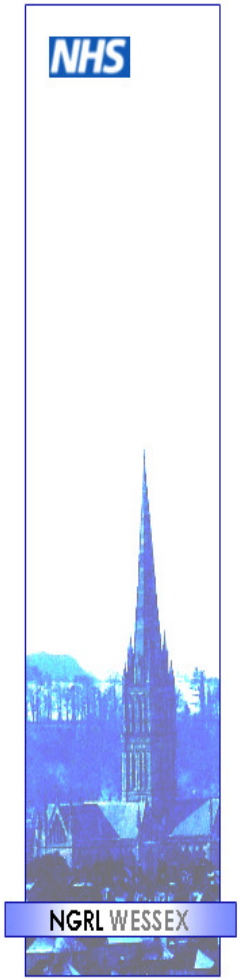
Salisbury NHS
NHS Foundation Trust



Sensitive and quantitative detection of KIT D816V in patients with systemic mastocytosis

Helen White, PhD

Senior Scientist
National Genetics Reference Lab (Wessex)

DH Department of Health

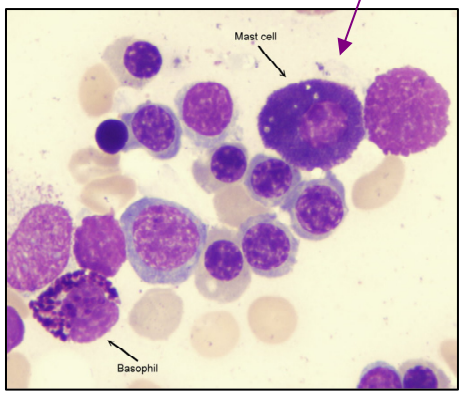
Systemic mastocytosis

- Systemic mastocytosis (SM) is a rare clonal disorder of the mast cell

- Characterised by abnormal proliferation and accumulation of mast cells

- Always involves the bone marrow and is the predominant form of mastocytosis in adults

- Patients may or may not have constitutional symptoms:
  - weight loss
  - pain
  - nausea
  - headache
  - malaise or fatigue



- Symptoms may be due to uncontrolled proliferation of mast cells or involvement of distinct organs (stomach, intestines, bone or bone marrow)

- Constitutional symptoms also can result from high levels of mast cell mediators in the blood stream

- Severity of symptoms varies from mild to life-threatening

WHO Diagnostic Criteria for Systemic Mastocytosis

If at least **1 major and 1 one minor**, or at least **3 minor criteria**, are met, the diagnosis of Systemic Mastocytosis (SM) can be established.

Major Criteria: Multifocal dense infiltrates of mast cells in bone marrow or other extracutaneous organ(s) (>15 mast cells in aggregate)

Minor Criteria:

a) Mast cells in bone marrow or other extracutaneous organ(s) show an abnormal morphology (> 25%)

b) c-kit mutation at codon 816 in extracutaneous organ(s). (Activating mutations at codon 816; in most cases, c-kit D816V)

c) Mast cells in bone marrow express CD2 and/or CD25

d) Serum total tryptase > 20 ng/mL

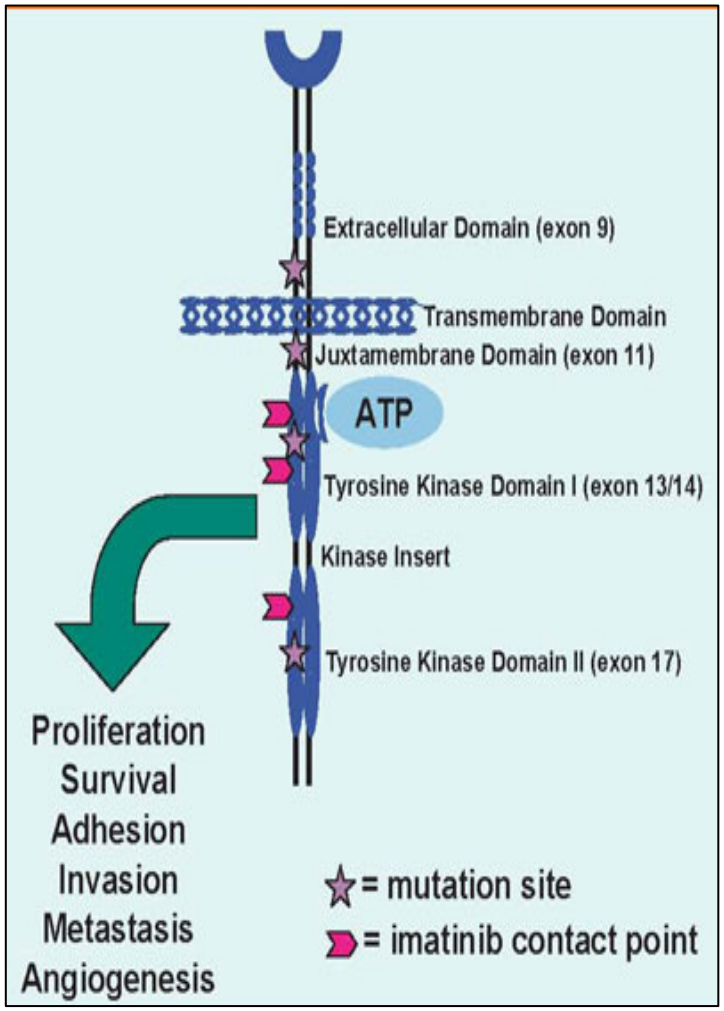
KIT D816V

- The acquired activating point mutation D816V (c. A>T 2447), found in the KIT tyrosine kinase domain present in 80 – 95% of adult patients with SM

- D816V may play a role in SM and is included in the consensus WHO SM classification criteria

- Detection of D816V is important diagnostically and also has predictive significance since the mutation confers **resistance** to the kinase inhibitor imatinib mesylate

KIT D816V



- Member of the type III transmembrane receptor protein - tyrosine kinase family

- Ligand (SCF) binding causes dimerisation leading to phophorylation and signalling to promote cell growth and proliferation

- Activating mutations e.g. D816V stabilise activated conformation leading to uncontrolled growth

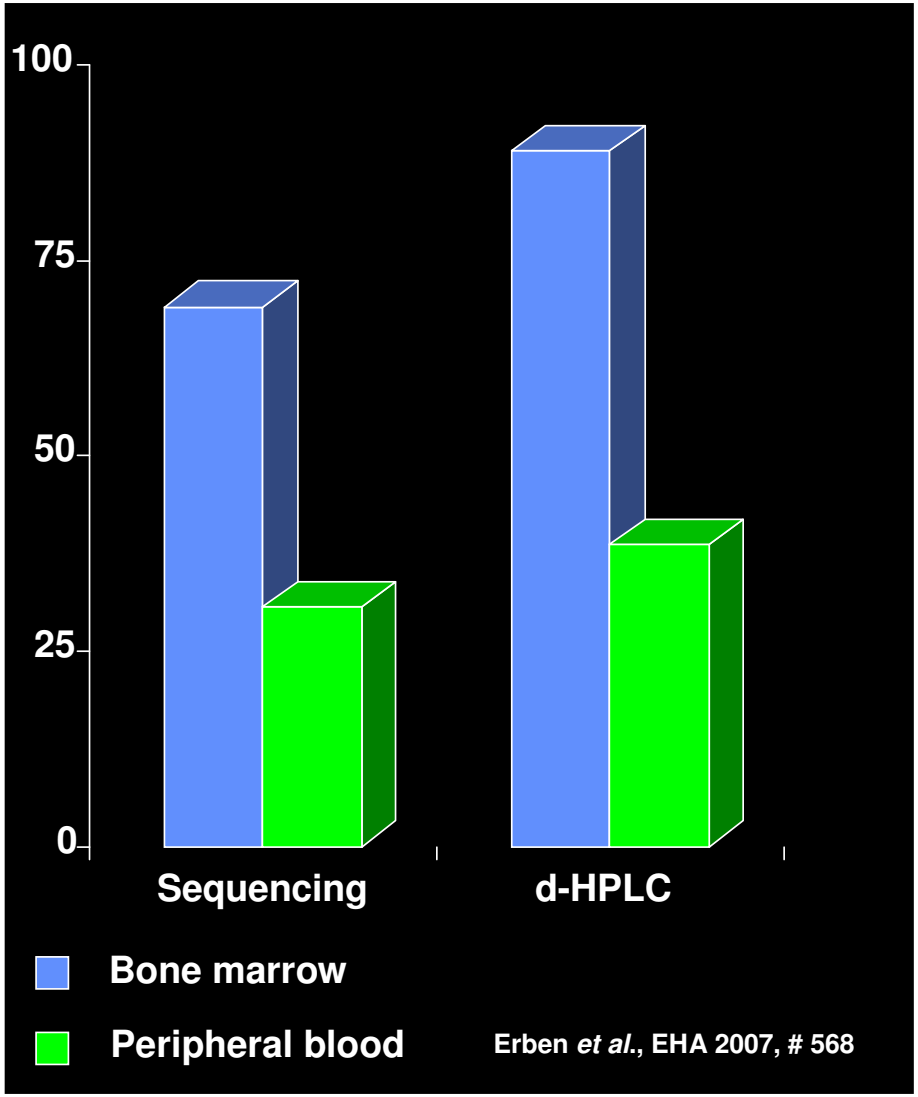
- Patients with pathogenic juxtamembrane mutations and SM patients without D816V are sensitive to imatinib mesylate

- Detection of D816V in SM patient indicates that alternative therapy should be sought

D816V: sensitivity to TK inhibitors

Imatinib	-	Frost, 2002
INNO-406	-	Pan, 2007
Sorafenib	-	Lierman, 2007
Nilotinib (AMN107)	- + +	Verstovsek, 2006 von Bubnoff, 2005 Gleixner, 2006
Dasatinib (BMS354825)	+ + +	Shah, 2006 Schittenhelm, 2006 Gleixner, 2006
Midostaurin (PKC412)	+ + +	Growney, 2005 Gotlib, 2005 Gleixner, 2006
Rapamycin	+	Gabillot-Carré, 2006
EXEL-0862	+	Pan, 2007

Current methods and problems



Reported incidence of D816V in SM inconsistent:

- patient heterogeneity
- tissue type tested (mast cell nos. low)
- detection methods used

Methodology:

Direct sequencing (~20%)

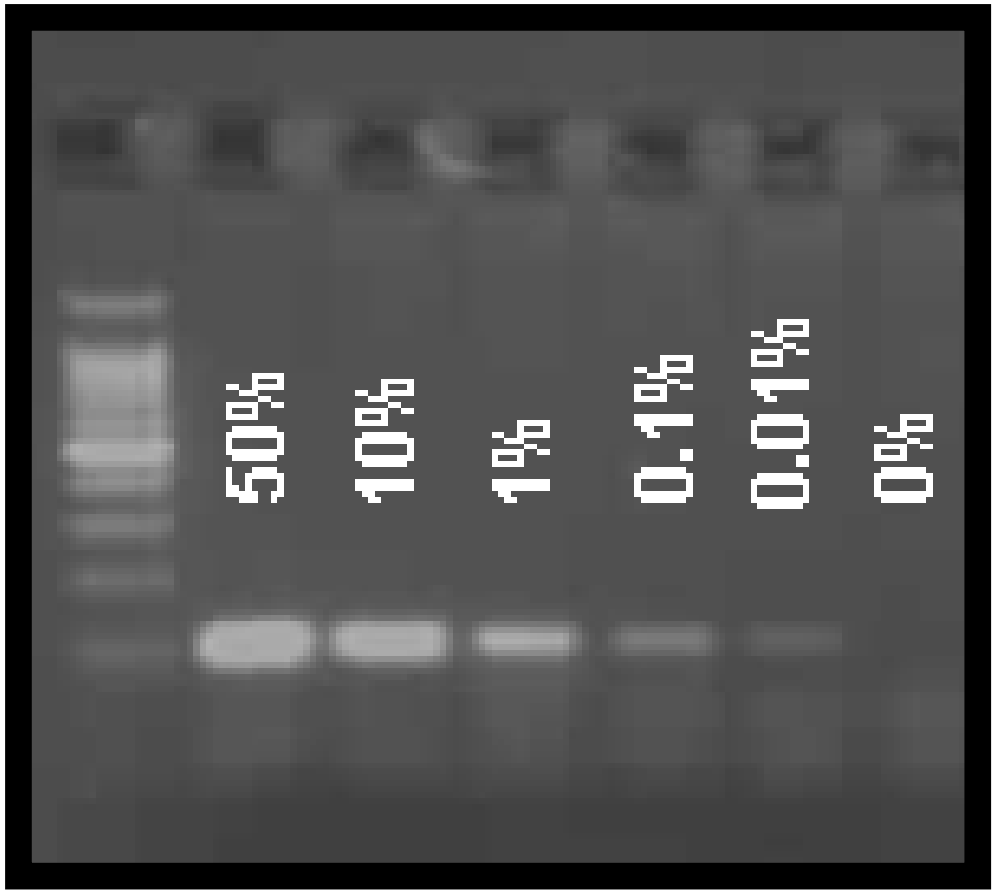
Pyrosequencing (~5-10%)

dHPLC (~5%)

ARMS (

Accurate detection of rare variant allele in background of wild type allele is difficult

Current Wessex Regional Genetics Laboratory Testing



Good lower limit of detection

Not robust

Inexpensive

Subjective interpretation at low level

Generally report as +ve or -ve

Not quantitative

Study design

Aim:

- Develop new allele specific real time PCR assay for D816V

Methodology:

- Determine the lowest limit of detection:

  serial dilutions of the HMC-1 cell line (heterozygous for D816V; 50% - 0.01%)

- Detect and quantify D816V mutation in:

  SM samples (n=20; 10 D816V positive and 10 D816V negative by ARMS)

  10 normal controls

Allele specific real time PCR assay for D816V

D816V (c. A>T 2447) : Exon 17 (97bp amplicon)

D816VmutF	GATTTTGGTCTAGCCAGCGT
D816VR	AATCCTTTGCAGGACTGTCAAG
D816Vprobe	FAM-TGTGGTTAAAGGAAACGTGAGTA-tamra

Conventional real-time PCR system

Analysed samples for total KIT using standard TaqMan assay

Re-analysed same DNA sample with allele specific assay real time assay

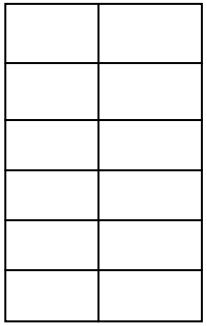
% Kit D816V = copies D816V Kit / copies total Kit

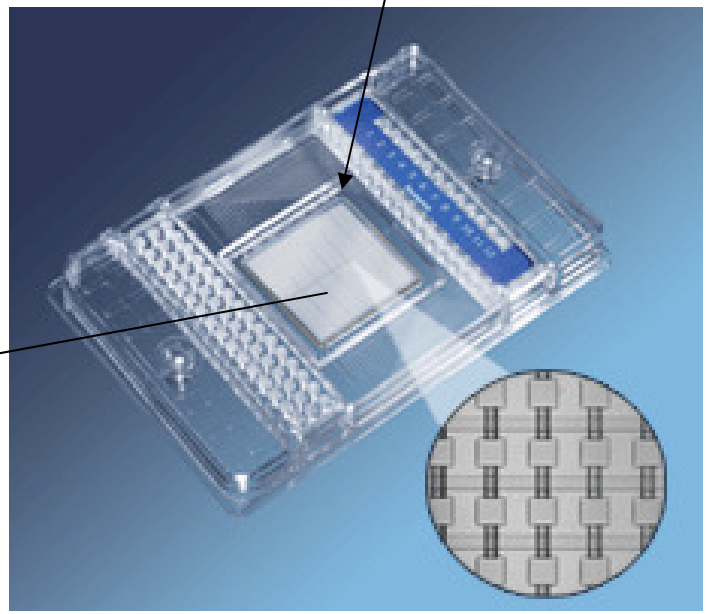
	Total Kit		D816V		
	Ct	Copies	Ct	Copies	%D816V
50% D816V	23.89	6,526.00	27.33	3,716.37	56.947
10% D816V	23.65	7,627.80	30.04	717.406	9.405
1% D816V	23.85	6,699.80	33.66	79.998	1.194
0.1% D816V	23.62	7,780.40	37.64	7.156	0.092
0.05% D816V	23.62	7,754.70	40.79	1.055	0.014
0.01% D816V	23.61	7,814.40	41.8	0.572	0.007
0% D816V	23.62	7,772.20	-	-	-

Fluidigm BioMark™ real-time PCR system and digital array

Digital PCR is a method used to quantify the number of target sequences within a sample by counting amplifications from single molecules

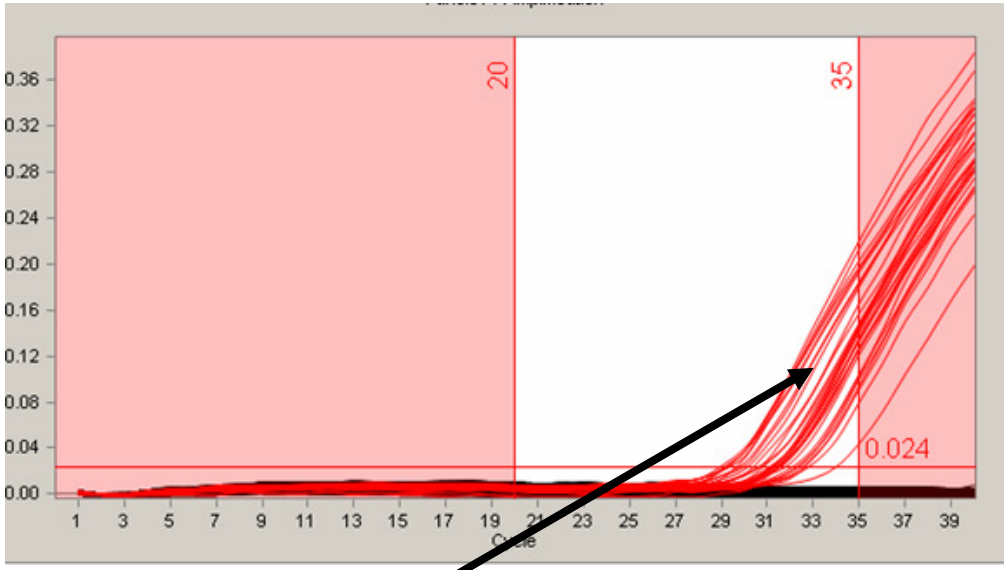
12 panels: 765 reactions / panel

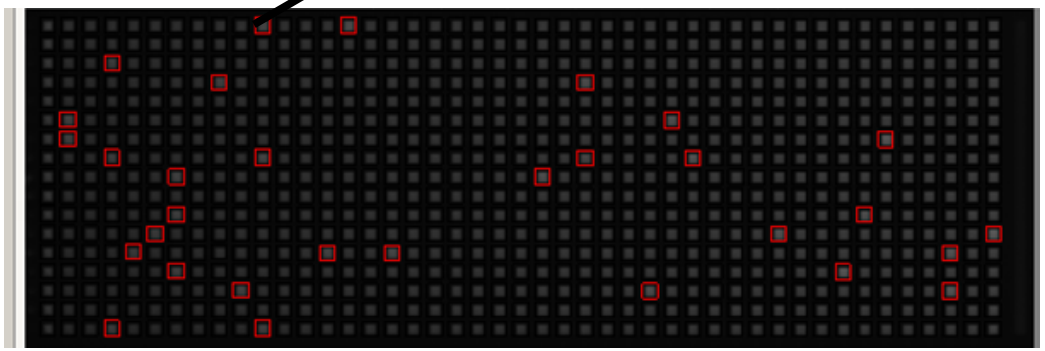




2.5 µl DNA (5ng/µl or 40ng/µl) was added to a PCR mix and loaded onto individual panels of the digital array. qPCR was performed and individual reactions that contained a D816V molecule are detected.

765 reaction wells





The number of wells that display fluorescent signals versus the number of negative wells are used to calculate the number of D816V molecules in the sample

5ng / ul DNA = 2178 total copies / panel

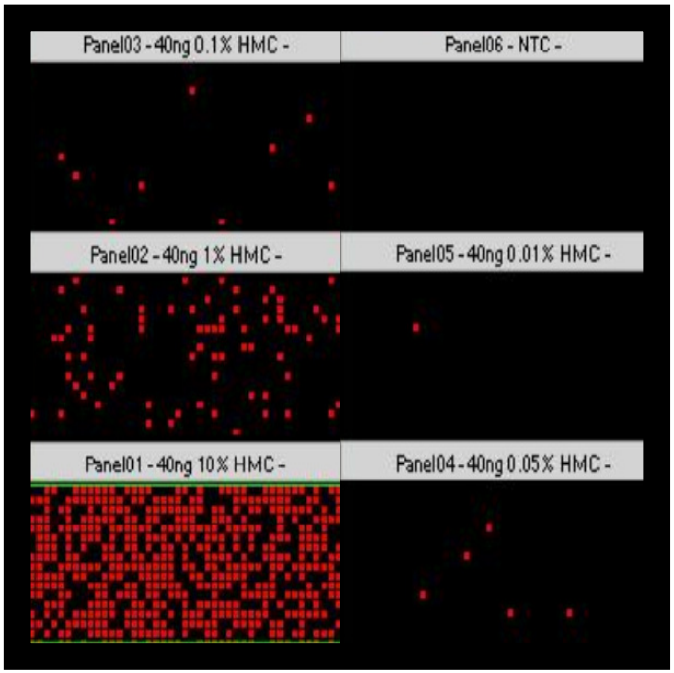
Lowest limit of detection

To determine the limit of detection of D816V for each technique, serial dilutions of the HMC-1 cell line (heterozygous for D816V) were analysed (50% - 0.01%)

			Number of positive wells expected				
DNA Stock ng / µl	Copies / µl*	Copies on panel	50% D816V	10% D816V	1% D816V	0.1% D816V	0.01% D816V
90	27272.7	39204.5	19602.3	3920.5	392.0	39.2	**3.9**
40	12121.2	17424.2	8712.1	1742.4	174.2	17.4	**1.7**
5	1515.2	2178.0	1089.0	217.8	21.8	2.2	**0.2**

			Number of positive wells expected				
DNA Stock ng / µl	**Copies / µl***	**Copies on panel**	**50% D816V**	**10% D816V**	**1% D816V**	**0.1% D816V**	**0.01% D816V**
90	27272.7	39204.5	19602.3	3920.5	392.0	39.2	**3.9**
40	12121.2	17424.2	8712.1	1742.4	174.2	17.4	**1.7**
5	1515.2	2178.0	1089.0	217.8	21.8	2.2	**0.2**

40ng



0.1%

16

NTC

1%

98

0.01%

2

10%

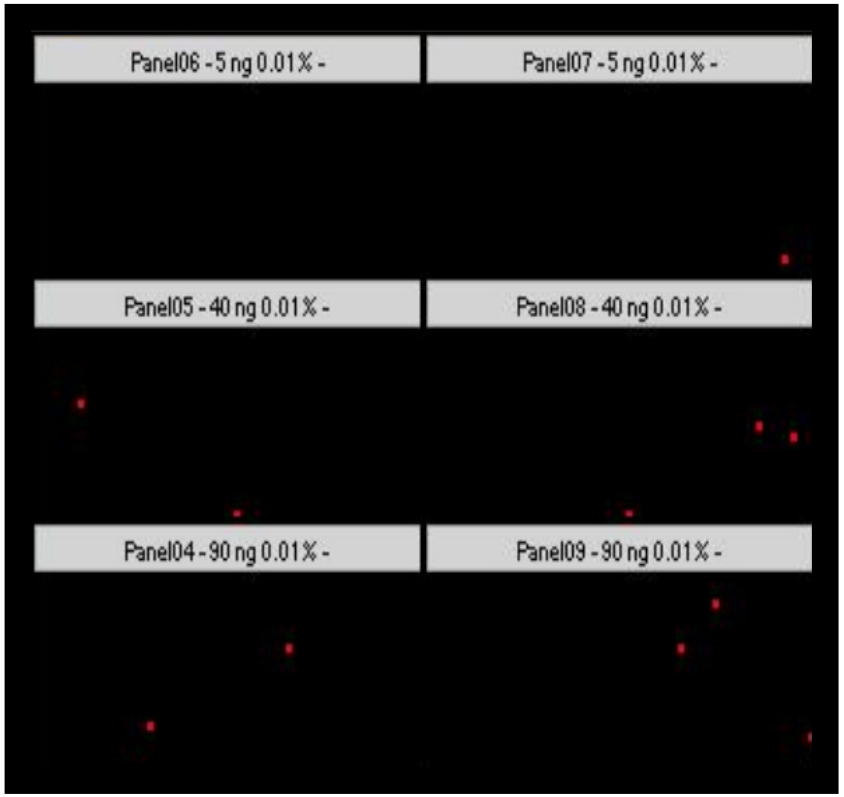
864

0.05%

8

			Number of positive wells expected				
DNA Stock ng / µl	Copies / µl*	Copies on panel	50% D816V	10% D816V	1% D816V	0.1% D816V	0.01% D816V
90	27272.7	39204.5	19602.3	3920.5	392.0	39.2	**3.9**
40	12121.2	17424.2	8712.1	1742.4	174.2	17.4	**1.7**
5	1515.2	2178.0	1089.0	217.8	21.8	2.2	**0.2**

0.01%



5ng

0.5

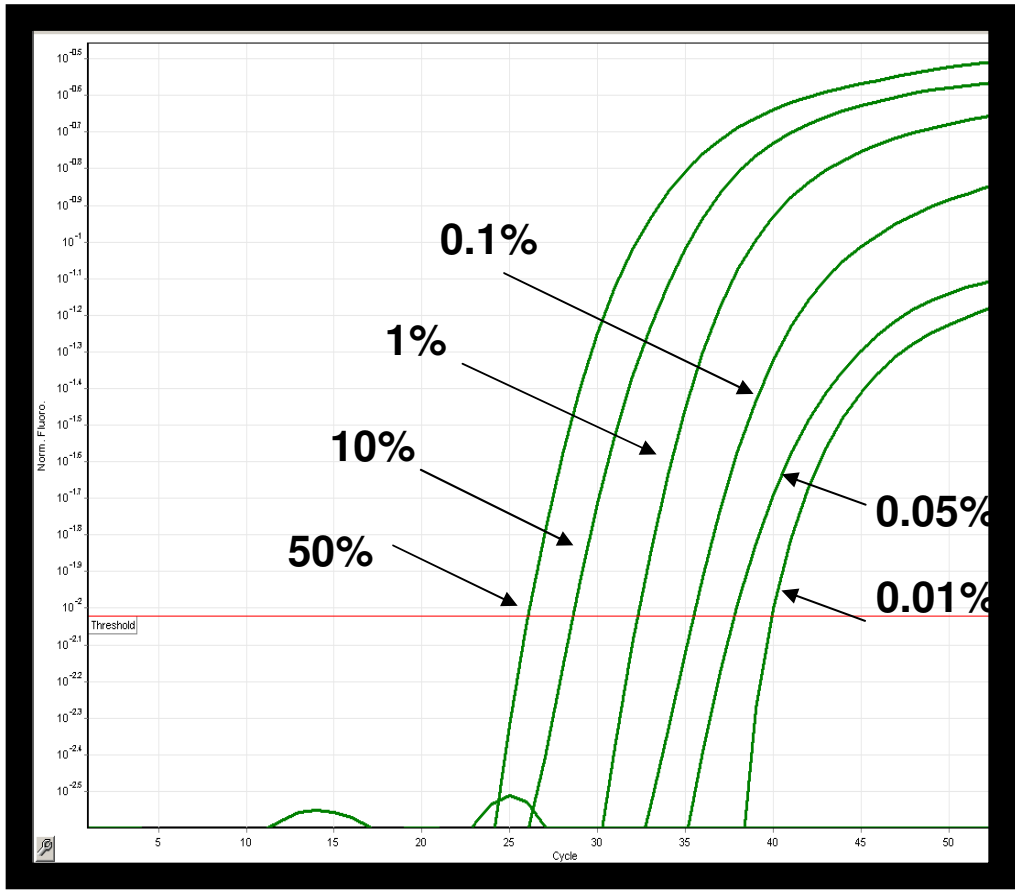
40ng

3

90ng

2.5

Conventional real-time PCR system



Lowest limit of detection 0.01%

Analysis of patient DNA samples

Samples tested:

SM samples (n=20) previously tested by WRGL ARMS assay:

11 D816V positive (n=11)
9 D816V negative (n=9)

Normal Controls (n=10)

DNA samples quantified using Nanodrop spectorphotometer

Diluted to 5ng/µl (to detect 10% - 0.1%)

Assayed using conventional real time PCR assay and digital PCR assay (in duplicate)

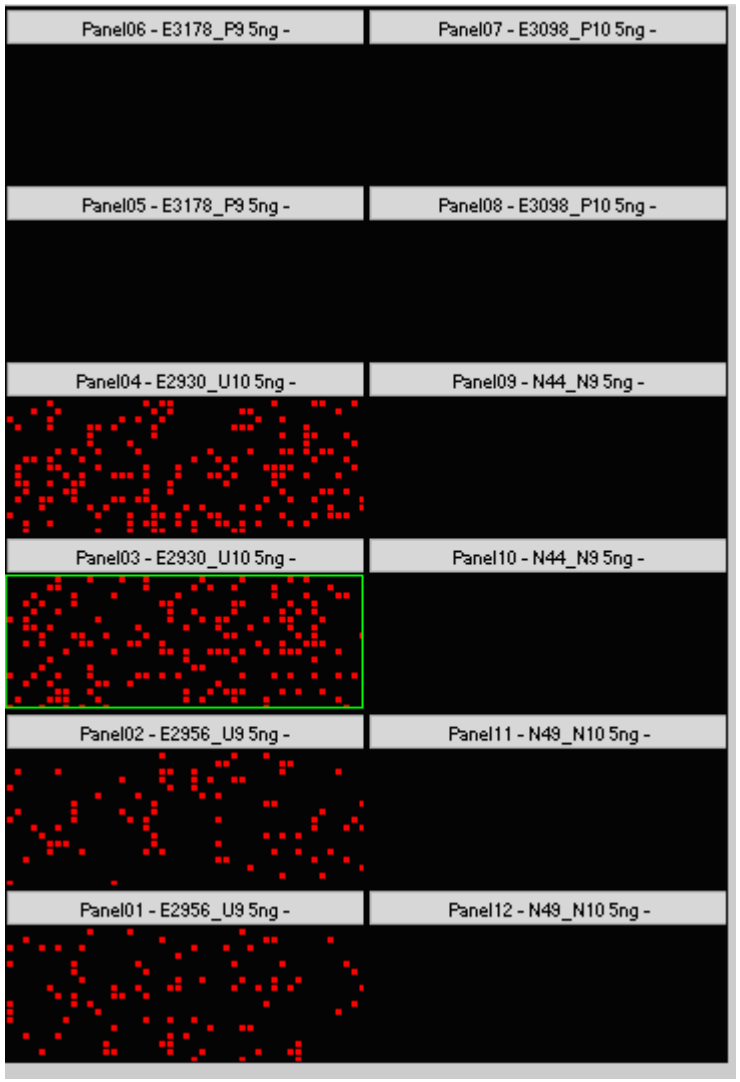
%D816V calculated

Example of Digital Array with Patient samples

D816V negative SM patient DNA

8% D816V

4% D816V

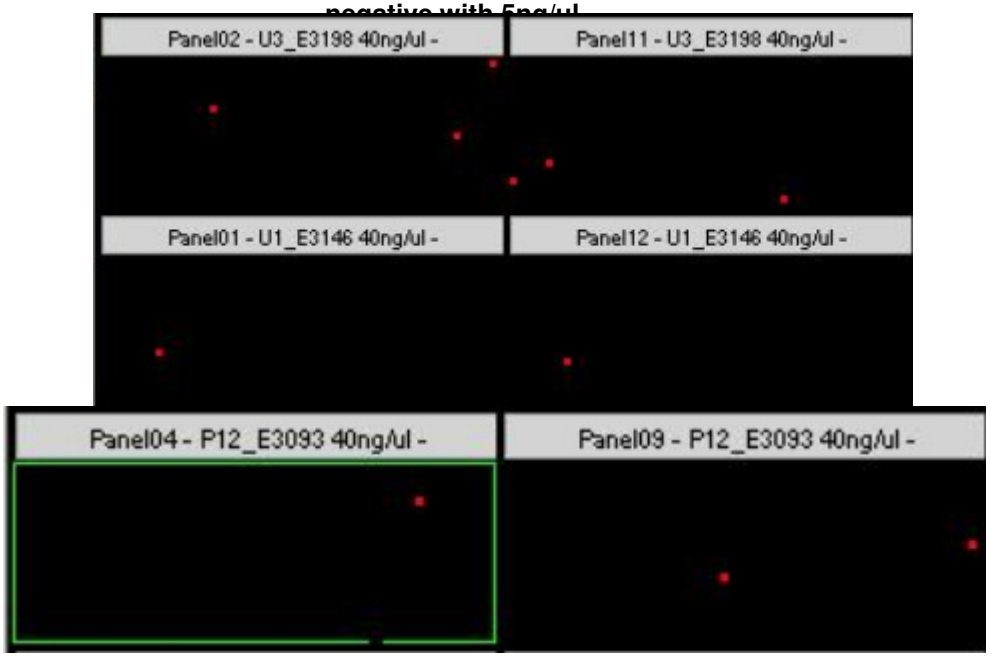


D816V negative SM patient DNA

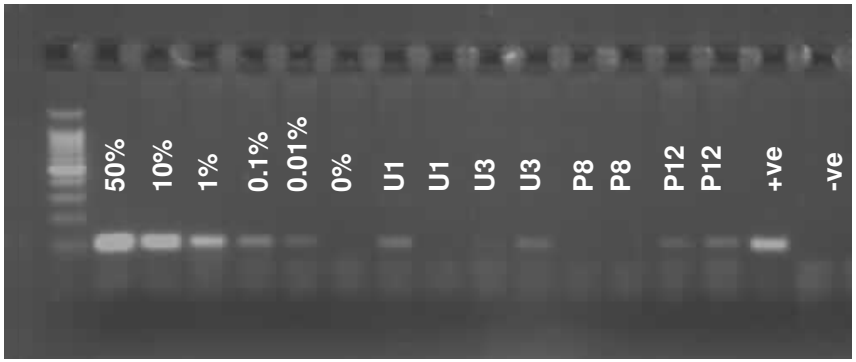
Normal controls

	Sample	In house ARMS	Digital PCR Mean %D816V	RQ- PCR Mean %D816V
SM Patients : D816V positive	U1*	+	0.02	0.01
	U2	+	0.60	0.50
	U3*	+	0.01	0.01
	U4	+	7.37	6.41
	U5	+	1.12	34.39
	U6	+	2.43	1.06
	U7	+	5.69	4.85
	U8	+	0.18	0.21
	U9	+	3.88	1.52
	U10	+	8.10	8.18
	U11	+	37.88	34.63
	U12	+	0.21	0.06
	P5	+	10.00	11.02
SM Patients : D816V negative	P1	-	0	0
	P2	-	0	0
	P3	-	0	0
	P4	-	0	0
	P6	-	0	0
	P7	-	0	0
	P8	-	0	0
	P9	-	0	0
	P10	-	0	0
	P11	-	0	0
	P12*	-	0.02	0.01
Normal Controls	N1	-	0	0
	N2	-	0	0
	N3	-	0	0
	N4	-	0	0
	N5	-	0	0
	N6	-	0	0
	N7	-	0	0
	N8	-	0	0
	N9	-	0	0
	N10	-	0	0
	N11	-	0	0
	N12	-	0	0

Using 40ng/ul positive by digital PCR -



V low level positive with ARMS on retesting



Summary

D816V mutation was quantified in 20 SM samples and 10 normal controls

Data from conventional real time PCR and digital PCR assays were highly concordant and the mutation was not identified in the normal controls

To determine the lowest limit of detection serial dilutions of the HMC-1 cell line (heterozygous for D816V) were also analysed (50% - 0.01%)

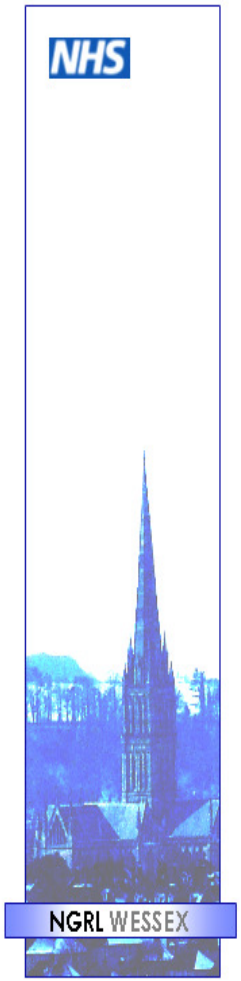
D816V was detected at c. 0.05% by AS RQ-PCR and digital PCR in one SM case that was D816V negative by ARMS

Digital PCR can detect mutation at 0.01% if using 40 or 90ng/ul DNA sample

Digital PCR could be useful for NIPD of fetal sex and paternally inherited mutations

Important to determine input DNA concentrations for digital PCR required to detect very low (or high) level mutations - dynamic range limited when compared to conventional real time PCR

Acknowledgements



NGRL (Wessex)

Mrs Vicky Hall
Prof Nick Cross
Mrs Kathy Waghorn

LGC

Dr Dan Scott
Dr Carole Foy
Miss Becky Sanders

This study was carried out as a secondment funded in part by the UK National Measurement Office under the Measurement for Innovators Programme