

Sensitive and quantitative detection of KIT D816V in patients with systemic mastocytosis using allele specific real time PCR and digital PCR



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Background

- Systemic mastocytosis (SM) is a clonal disorder of the mast cell and its precursor cells
- The acquired activating point mutation D816V found in the KIT kinase domain occurs in 80 – 95% of patients with SM
- Because of its transforming ability D816V may play a role in SM and is included in the consensus WHO SM classification criteria
- Detection of D816V is important diagnostically and has predictive significance as the mutation confers resistance to the kinase inhibitor imatinib mesylate
- We have developed two methods for sensitive quantification of D816V; allele specific real time PCR (AS RQ-PCR) and digital PCR using the Fluidigm BioMark™ real-time PCR system and digital array
- This study was carried out as a secondment funded in part by the UK National Measurement Office under the Measurement for Innovators Programme

Methods

Two methods for sensitive quantification of D816V were developed; allele specific real time PCR (AS RQ-PCR) and digital PCR using the Fluidigm BioMark™ real-time PCR system and digital array. Both techniques used the same RQ-PCR design (primer sequences available on request).

AS RQ-PCR was performed using a RotorGene 6000 (QIAGEN) using 10ng DNA. Two assays were performed for each sample; one to determine total copies of KIT in the sample and one that was specific for the D816V. The mutational load of the sample was calculated using a standard curve and results were expressed as %D816V (D816V copies detected / total KIT copies detected).

Digital PCR is a method used to quantify the number of target sequences within a sample by counting amplifications from single molecules. This was performed using the Fluidigm BioMark system (figure 1a). 2.5 µl DNA (5ng/µl or 40ng/µl) was added to a PCR mix and loaded onto individual panels of the digital array (figure 1b) each of which has 756 compartments. qPCR was performed and individual reactions that contained a D816V molecule were detected (figure 1c). The number of compartments that displayed fluorescent signals (red boxes, figure 1c) versus the number of negative partitions were used to calculate the number of D816V molecules in the sample (see table in figure 2).

To determine the lowest limit of detection serial dilutions of the HMC-1 cell line (heterozygous for D816V) were analysed (50% - 0.01%). The D816V mutation was also quantified in SM samples (n=20; 11 D816V positive and 9 D816V negative as ascertained previously by 'in house' ARMS assay and 10 normal controls).

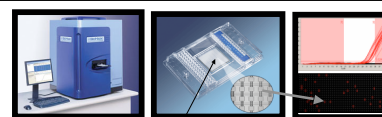
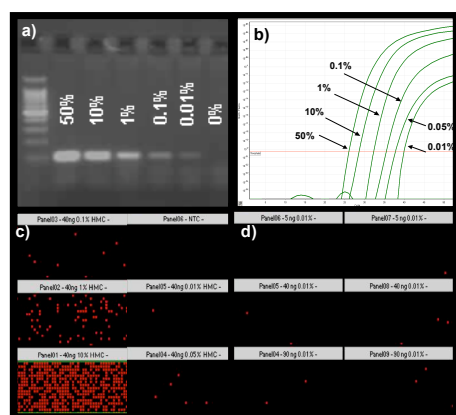


Figure 1: Fluidigm BioMark, System and Digital Array
12 panels: 765 reactions / panel

Results



		Number of positive wells expected					
DNA Stock ng/µl	Copies/µl	50% D816V	10% D816V	1% D816V	0.1% D816V	0.01% D816V	
90	2722.7	36204.5	19620.3	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

Figure 2: 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%). The lowest reliable limit of detection for D816V using a) in house ARMS, b) allele specific RQ-PCR & c) digital PCR were 0.01- 0.05%, 0.01% and 0.01% respectively. As predicted from the table, detection of 0.01% D816V using digital PCR was improved when 40ng or 90ng input DNA was used (d).

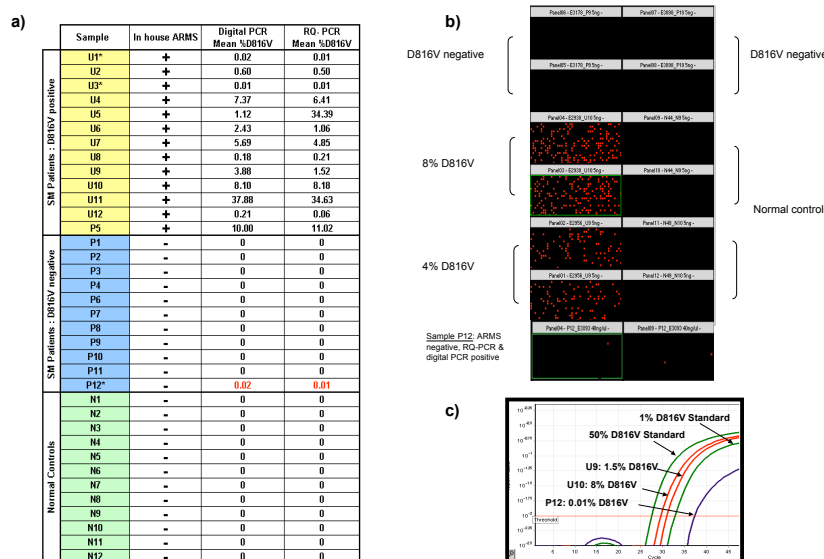


Figure 3: Results of analysis of patient samples.

a) Mean %D816V results for patient samples analysed using AS-RQPCR and digital PCR. * Digital PCR: 40ng/µl DNA sample analysed to detect <0.1% D816V.

b) Examples of digital PCR analysis. Samples U9 (8%D816V) and U10 (4% D816V) can be clearly identified as harbouring the mutation, samples P9 and P10 were negative for the mutation and the mutation was undetected in the normal controls (N9 and N10). Sample P12 (negative by in house ARMS assay) was found to be D816V positive (0.02%) by digital PCR using the 40ng/µl DNA sample.

c) Examples of samples analysed using AS-RQ PCR. Samples U9, U10 and P12 can be clearly identified as harbouring the mutation

Conclusions

- Data from both assays were highly concordant: D816V was quantified in 20 SM samples and was not detected in 10 normal controls
- To determine the lowest limit of detection serial dilutions of the HMC-1 cell line (heterozygous for D816V) were analysed (50% - 0.01%)
- The lowest limit of detection for AS RQ-PCR was 0.01 and for digital PCR was 0.01% when using 40 or 90ng/µl DNA (Figure 2)
- In a SM case that was D816V negative by 'in house' ARMS (P12) D816V was detected at 0.01 - 0.05% using both AS RQ-PCR and digital PCR
- Digital PCR has potential applications for non-invasive prenatal diagnosis and the quantification of mitochondrial and acquired mutations

Funded by:



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