Development of a targeted Next-Generation Sequencing (NGS) assay for acquired mutations in myeloproliferative neoplasms (MPN)

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Background

MPN are a group of rare, heterogeneous bone marrow disorders characterised by the uncontrolled production of one or more myeloid cell lineages[1]. Polycythemia vera, primary myelofibrosis and essential thrombocythemia are subtypes of MPN associated with acquired mutations commonly identified in *JAK2*, *CALR* and *MPL*[2]. Our current testing protocol involves sequential mutation testing using multiple laboratory techniques (see Figure 1).

Aims

•Design a targeted NGS assay to capture affected regions down to >1% mutation burden[3,4]
•Replace current testing protocol with single assay to improve testing efficiency and cost, plus manage increasing demand
•Implement assay into routine diagnostic use

Figure 1: Current testing pathway for MPN

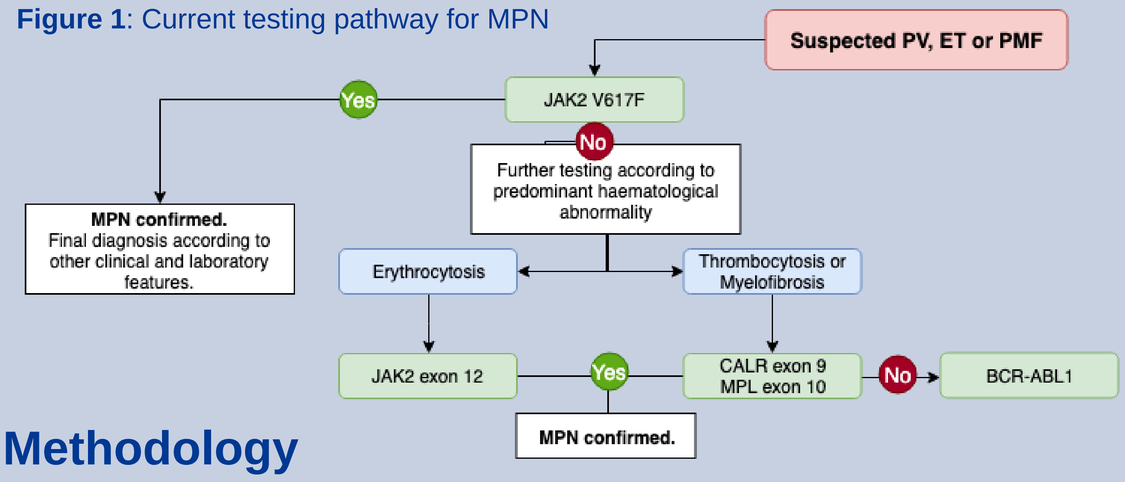
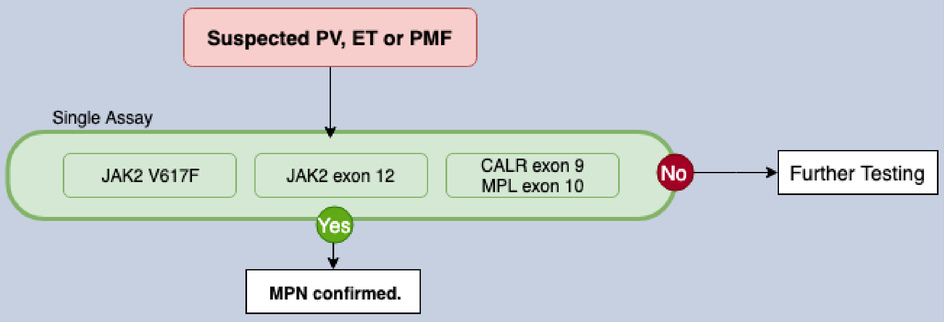


Figure 2: Proposed molecular genetics testing pathway for MPN



Methodology

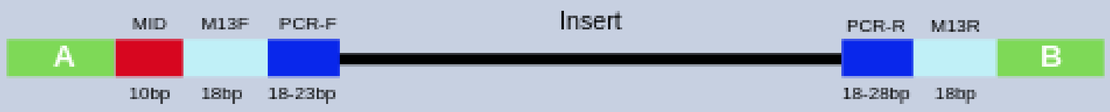
Concept

•Designed PCR primers using Genetics Ark and Primer3 to capture the minimum regions shown in Table 1.
•Four target regions multiplexed using Qiagen PCR master mix. PCR products visualised using agarose gel electrophoresis (Figure 4)
•Multiplex PCR incorporated sequence specific primers containing M13 tags for the 4 regions, different pairs of index primers (to allow pooling of up to 96 samples), and Illumina sequencing adaptors (Figure 3)
•NGS performed using MiSeq 300 cycles PE v2 kit
•Further optimisation focused on an iterative process of adjusting primer concentration to achieve even coverage of each amplicon in NGS data

Table 1: The four regions of interest with chromosome coordinates showing the mutation Hotspots captured by the assay (from hg19 genome build)

Gene	Region	Recurrent mutation(s)	Chromosome coordinates
JAK2	exon 14	V617F, C618R	chr9:5073769-5073776
JAK2	exon 12	across exon	chr9:5069925-5070052
MPL	exon 10	c.1097_1254	chr19:13054569-13054727
CALR	exon 9	S505N, W515W/K/L/A	chr1:43814978-43815010

Figure 3: Construct structure of amplicons. PCR-F and PCR-R represent the forward and reverse region specific primers which include M13F and M13R tags. Multiplex indices (MID) used to identify samples and Illumina sequencing adaptors (A + B) for binding onto flow cell.



Samples

•Tested DNA samples from 12 anonymised MPN patients with genetic variants in 1 of the 4 regions (results in Table 2)
•Screened further samples to analyse a breadth of mutation genotypes and sensitivities to determine the limit of detection (results in Table 3)

Bioinformatics pipeline

•Alignment, quality control (QC) checks and variant calling
•Use of bioinformatic tools such as Vardict for SNV calling and Amplicon Indel Hunter for larger indels

Results

1. Visualised successful amplification and sequencing of regions of interest on Integrative Genomics Viewer (IGV) and obtained even and high depth of coverage (Figure 5)
2. Detection of known genetic variants in 12 positive control samples with 100% concordance (Table 2)
3. Determination of limit of detection in a breadth of mutation types (Table 3)

Figure 4: Electrophoresis gel showing amplification of the four amplicons in a single reaction

Table 2: List of 12 positive MPN control samples where variants were detected

Patient	Variant	Result
1, 2, 3	**JAK2 exon 14:** p.V617F	Detected
	JAK2 exon 12:	
4	p.K539L	Detected
5	6bp deletion	Detected
6	6bp deletion	Detected
	MPL exon 10:	
7	p.W515R	Detected
8	p.S505N	Detected
9	p.W515L	Detected
	CALR exon 9:	
10	52bp deletion	Detected
11	c.1112delA	Detected
12	5bp insertion	Detected

Figure 5: Image taken from IGV showing sequence reads for normal control sample for *MPL* exon 10

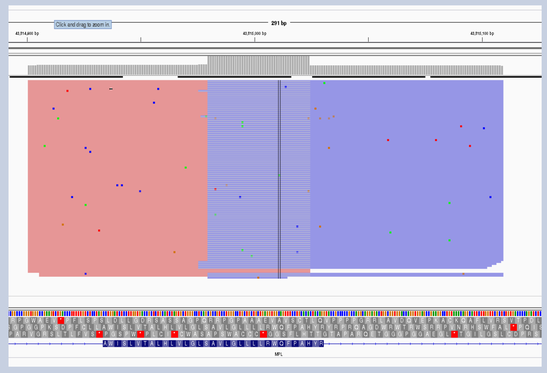


Table 3: Variants called using bioinformatics pipeline to percentage of mutation level, green and red shows variants detected and not detected respectively. *using aiHunter

Limit of Detection (%)	0.5	1	2	5	10	Neat
JAK2 exon 14 V617F						
JAK2 exon 12 K539L						
JAK2 exon 12 6bp deletion						
MPL exon 10 W515L						
CALR exon 9 52bp deletion*						
CALR exon 9 5bp insertion						

Next Steps

- Further work is currently being undertaken to test whether assay is capable of detecting a wide range of differently sized insertions and deletions especially in JAK2 exon 12.
- Exploring approach involving longer sequencing reads (up to 300bp) in order to improve variant calling algorithms.
- Complete validation and gain accreditation of this targeted NGS assay to aid in the accurate diagnosis of MPN with reduced turnaround times.

References

1. Mead, A.J., Mullally, A. (2017). 'Myeloproliferative neoplasm stem cells', *Blood,* 129(12), pp.1607-1616.
2. Nangalia, J., Grinfield, J. and Green A.R. (2016). 'Pathogenesis of myeloproliferative disorders', *The Annual Review of Pathology: Mechanisms of Disease,* 11, pp.101-26.
3. Vannucchi, A.M., Barbui, T., Cervantes, F., et al. (2015). 'Philadelphia chromosome-negative chronic myeloproliferative neoplasms: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow up', *Annals of Oncology,* 26(5), pp.85-99.
4. Bench A., White HE., Foroni L et al. (2013) Molecular diagnosis of the myeloproliferative neoplasms: UK guidelines for the detection of JAK2 V617F and other relevant mutations, Br. J. Haematol. 160: 25-34.