

# Evaluation of High Resolution Melting Technique for Detection of JAK2-V617F Mutation in Formalin-Fixed Paraffin-Embedded Specimen from Myeloproliferative Neoplasm Cases

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## ABSTRACT

**Introduction:** Myeloproliferative neoplasm (MPN) is a group of myeloid disorders which leads to erythrocytosis, thrombocytosis and leucocytosis. MPN with BCR-ABL positive is chronic myeloid leukaemia (CML) while BCR-ABL negative MPN includes polycythaemia Vera (PV), essential thrombocytemia (ET) and primary myelofibrosis (PMF). One of the major criteria for diagnosis of BCR-ABL negative MPN is the presence of JAK2-V617F mutation which is positive in 95% of PV and around 60% of ET and MF. Beside peripheral blood specimen, formalin-fixed paraffin-embedded (FFPE) marrow specimen can be used for detection of this mutation. Unfortunately, FFPE produces low quality DNA that put a challenge for successful amplification of DNA. We aimed to evaluate the utility of High Resolution Melting (HRM) analysis for detection of JAK2-V617F mutation in FFPE specimen from MPN cases. **Materials and Methods:** This study is a descriptive cross-sectional study. Forty FFPE marrow specimens were retrieved from the years 2014-2016. Bio-Rad Precision Melt Analysis software was used for analysis of HRM data. Allele-specific PCR was done for validation of results. Positive samples were subjected to Sanger sequencing. **Results:** JAK2-V617F mutation was positive in 13 out of 40 MPN cases. Level of agreement between HRM and AS-PCR was 97.5%. **Conclusion:** HRM is a rapid and powerful diagnostic assay which is suitable for detection of JAK2-V617F mutation in FFPE marrow specimen.

**KEYWORDS:** High Resolution Melting (HRM), Allele-Specific PCR (AS-PCR), JAK2 Mutation, Myeloproliferative neoplasm (MPN), FFPE

## INTRODUCTION

Myeloproliferative neoplasm or MPN is a group of stem cell disorders in which there is an excess

proliferation of myeloid lineage with efficient maturation. MPN with BCR-ABL positive is chronic myeloid leukaemia (CML) while BCR-ABL negative MPN includes polycythaemia Vera (PV), essential thrombocytemia (ET) and primary myelofibrosis (PMF). BCR-ABL negative MPN share a common JAK2-V617F mutation. This mutation results from conversion of G (guanine) to T (thymine) at nucleotide 1849 of exon 14 of chromosome 9 in JAK2 gene and leads to substitution of valine to phenylalanine at position 617 codon.<sup>1-4</sup> JAK2-V617F mutation is present in more than 95% of PV patients and around 50-60% of ET and PMF patients.<sup>5</sup>

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The JAK2 gene is a member of tyrosine kinase family which is involved in cytokine receptor signalling. The JAK2 gene has an effect on many tissues particularly in haematopoiesis, it has an important role in signal transduction. JAK2 gene encodes cytoplasmic tyrosine kinase protein which transduces signal from cytokines and growth factor including thrombopoietin, erythropoietin, granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF). This signal transduction is important for proliferation and differentiation of the granulocytic, megakaryocytic, and erythroid lineages from pluripotent hematopoietic stem cell.<sup>6</sup>

JAK2-V617F mutation occurs in JH2 or pseudokinase domain. It is believed that the JH2 domain has negative regulatory effect on kinase domain so deletion or loss of function in JH2 domain result in hyperactivity of JH1 domain or kinase domain. Therefore, it causes consecutive activation of JAK2 signalling pathway regardless of binding of cytokines (e.g. erythropoietin) to their receptors and leads to increase production of hematopoietic cells.<sup>7</sup>

There are several methods for detection of JAK2-V617F mutation such as allele specific polymerase chain reaction (AS-PCR), Sanger sequencing, high resolution melting (HRM) analysis, restriction fragment length polymorphism PCR (RFLP-PCR). HRM analysis has its advantages compared to other methods in which it is rapid, easy to perform and does not require post amplification processing which reduces the risk of contamination.<sup>8</sup> In this study we aimed to evaluate the utility of High Resolution Melting (HRM) analysis for detection of JAK2-V617F mutation in formalin fixed paraffin embedded specimens from MPN cases.

## MATERIALS AND METHODS

### *Sample collection and DNA extraction*

A total of 40 formalin fixed paraffin embedded (FFPE) specimens were retrieved from pathology department of Tengku Ampuan Afzan Hospital (HTAA), Pahang, between 2014 and 2016 and transported to the molecular laboratory of Kulliyyah of Medicine of International Islamic University Malaysia (IIUM). All samples were diagnosed via bone marrow aspirate and trephine (BMAT) assessment as MPN. The samples included 4 cases of

PV, 4 cases of PMF, 2 cases of ET, 10 cases of unclassified MPN and 20 cases of CML. DNA were extracted from 5-8 sections of 5 micron FFPE specimens using Maxwell® RSC DNA FFPE kit (U.S) according to the manufacturer's instructions. UV/Vis SimpliNano™ spectrophotometer was used for quantification of DNA. The current study was registered under the National Medical Research Registry of Malaysia (NMRR-17-2881-38946) and was approved by Medical Research and Ethics Committee, Ministry of Health, Malaysia, and the IIUM Research Ethics Committee (IREC 2018-234).

### *High Resolution Melting (HRM) analysis*

The primers for HRM analysis is listed in Table I. Amplicon melting data of JAK2 gene were calibrated by using internal temperature control with low T<sub>m</sub> (70.25) which were complementary to each other and were blocked on their 3'-hydroxyl termini with phosphate group. HRM analysis was performed on CFX96 Bio-Rad and the result was analysed by CFX Manager V 3.1 and Precision Melt Analysis software.

All samples were tested in duplicate. PCR reactions were performed in 10 µl final volume that included 5 µl Precision Melt Supermix (Bio-Rad, Germany), 1µl primers mix (2 µM) and 4 µl DNA template (approximately 30 ng). PCR cycling protocol for JAK2 consist of an initial DNA denaturation step at 95°C for 2 min, followed by 45 cycles of denaturation step at 95°C for 10 sec, annealing step at 60°C for 30 sec and extension step at 72°C for 30 sec, and a final extension step at 72°C for 5 min. Melting program has three steps; i) a denaturation step at 95°C for 30 sec, ii) cooling down to 60°C for 1 min to facilitate the heteroduplex formation and iii) subsequent melting with continuous plate reading from 65 to 95°C with a ramp rate set to 0.2°C increment in 10 sec/step.

### *Allele specific Polymerase Chain Reaction (AS-PCR)*

The sequence of primers used in AS-PCR is listed in Table I. In this assay, one common reverse primer and two forward primers were used. First forward primer served as an internal control for mutant and wild type allele with 360-bp amplicon size while another forward primer was specific for mutant allele with 203-bp amplicon. PCR reactions were carried out in duplicate in 25 µl final volume. Each

reaction contains 12.5 µl HotStarTaq Master Mix (Qiagen, Germany), 3µl primers mix (10 µM) (1µl internal control, 1µl specific and 1 µl reverse primers), 4.5 µl dH<sub>2</sub>O and 5 µl genomic DNA (approximately 80 ng). DNA was amplified according to protocol as follow: activation of enzyme (hot star polymerase) at 95°C for 15 min, 45 cycles of denaturation step at 94°C for 30 sec, annealing step at 60°C for 30 sec and elongation step at 72°C for 1 min and an final elongation step at 72°C for 5 min. PCR products were run on 2% agarose gel with florescent dye and bands were illuminated by Enduro GDS (Labnet, U.S).

### Sanger sequencing

## STATISTICAL ANALYSIS

The level of agreement between high resolution melting analysis and allele specific PCR was shown in percentage. Microsoft Excel 2016 was used to plot the graph.

## RESULTS

### High Resolution Melting analysis results

In this current study, we are able to distinguish heterozygous mutants (G/T) (guanine/thymine) from the wild type (G/G) (guanine/guanine) allele. Wild type allele is shown in horizontal base line. Figure 1 shows JAK2-V617F mutation which can be

Table I: Primers for detection of JAK2-V617F mutation

Assay	Primer sequences in 5'- 3' orientation		Amplicon size
HRM	Forward	AAGCAGCAAGTATGATGAGC	112 bp
	Reverse	AGAAAGGCATTAGAAAGCCTGTA	
	Forward (internal temperature calibrator)	ATCGTGATTTCATAGTTATCTAAGTAGTTGGCATTAA-	50bp
	Reverse (internal temperature calibrator)	TAATTCATTT GCGGTCAGTCGGCCTAGCGGTAGCCAGCTGCGGCAC- TGCCTGACGCTCAG	
AS-PCR	Forward (internal control)	ATCTATAGTCATGCTGAAAGTAGGAGAAAG	364 bp
	Forward (specific)	AGCATTGGTTTTAAATTATGGAGTATATT	203 bp
	Reverse (common)	CTGAATAGTCCTACAGTGTTTTTCAGTTTCA	

Positive samples were subjected to Sanger sequencing (Figure 4). In order to amplify DNA for sequencing, we used the same primers that were used in HRM assay. The total volume of PCR products were run on 2% agarose gel that contained florescent dye and the band with 112-bp product were excised. MinElute gel extraction kit (Qiagen, Germany) was used for purification of DNA. 10 µl of purified DNA was sent to the First Base Laboratories Sdn, Bhd (Selangor, Malaysia) for sequencing.

### Limit of detection

To find analytical sensitivity of HRM assay, wild type DNA was diluted with mutated DNA (approximately 60% mutant) in 1:2 ratio to get different concentration of mutant samples (around 30%, 15%, 7.5%, 3.75%, 1.875 % and 0.94%). HRM assay were performed and the melting curves were normalized to get temperature-shifted difference curve using precision melt analysis software.

differentiate from wild type by using difference plot curve and adjusting temperature. JAK2-V617F mutation was present in 12 cases (30%) of 40 MPN patients including CML.

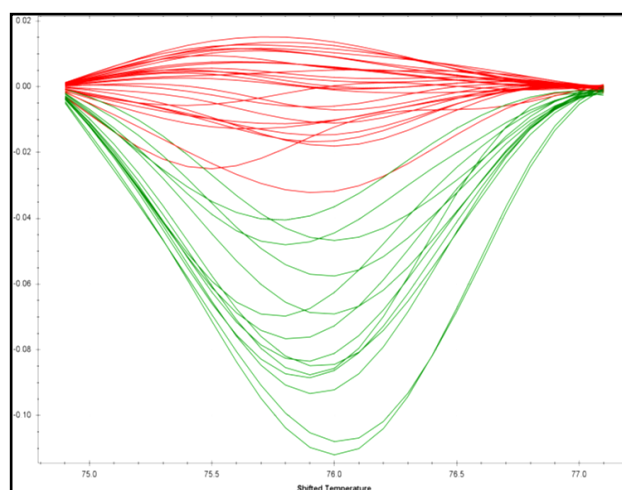
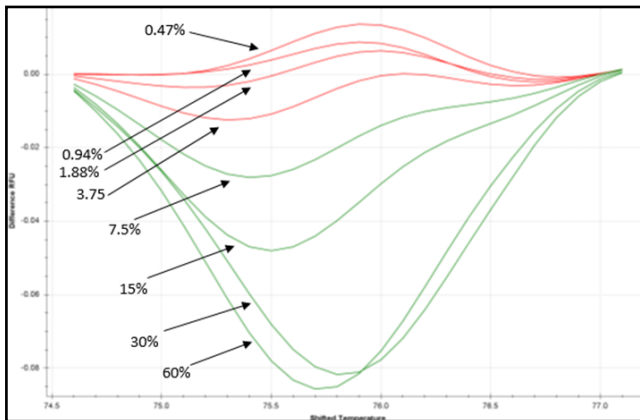


Figure 1: The temperature shifted difference curve of JAK2-V617F. The red curves indicate wild type allele whilst the green curves represent mutant allele.

### Analytical sensitivity of HRM for detection of JAK2-V617F mutation

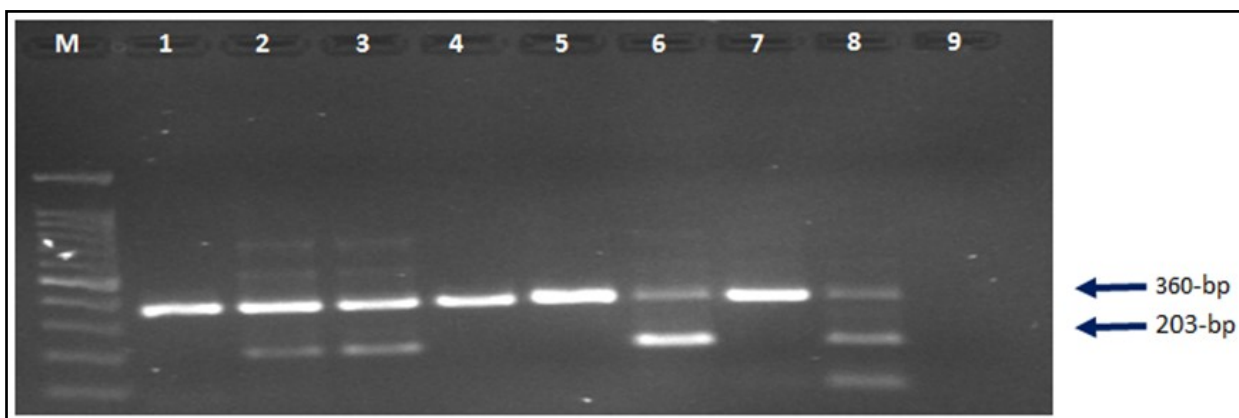
We found that the lowest concentration in which JAK2-V617F mutation could be detected is 7.5%, which was shown in Figure 2.



**Figure 2:** HRM analysis of mixture of wild type and mutant allele at various percentage of JAK2 mutant. The HRM analysis identified mutation plot from 60% mutant down to 7.5% mutant.

### Allele specific PCR results

The JAK2-V617F mutation was successfully detected by allele specific PCR. The presence of one band with 360-bp amplicon represent homozygous wild type and the presence of two bands with 360-bp and 203-bp indicates heterozygous mutant for JAK2 exon 14 mutation (Figure 3). JAK2-V617F mutation was present in 13 (32.5%) of 40 MPN patients including CML. In addition, positive samples were subjected to Sanger sequencing (Figure 4) for confirmation of the results.



**Figure 3** Agarose gel electrophoresis of 7 samples. (Lane M: 100-bp DNA marker, Lanes 1, 4, 5 and 7: wild type, Lanes 2, 3, 6 heterozygous Mutant, Lane 8: positive control, Lane 9: NTC) M: DNA Marker; NTC: No Template Control

### Comparison of HRM and AS-PCR

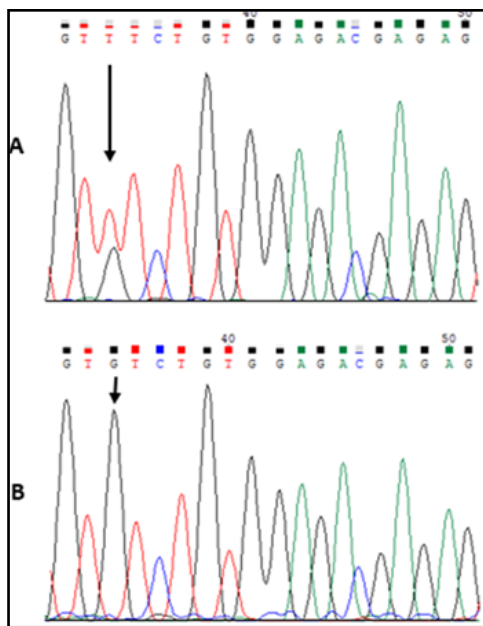
Of the 40 samples analysed, 27 (67.5%) samples showed wild types by both HRM and AS-PCR assays. Analysis of HRM revealed that 12 (30%) samples were mutant for the JAK2-V617F mutation while analysis using AS-PCR demonstrated that 13 (32.5%) samples carried the mutation. Based on these results, level of agreement between HRM and AS-PCR was 97.5% (39/40). Only one discrepancy was found between two assays, which was an ET case that showed negative result in HRM and positive result in AS-PCR.

### DISCUSSION AND CONCLUSION

According to the World Health Organization (WHO) (2016) revision for myeloid malignancy classification, one of major criteria for diagnosis of MPN beside bone marrow morphology is the presence of mutation.<sup>9</sup> JAK2-V617F mutation is the most frequent mutation in BCR-ABL negative MPN. This mutation was identified in approximately 95% of patients with PV and around 60% in PMF and ET.<sup>10</sup> There are many methods for detection of this mutation such as Sanger sequencing, restriction fragment length polymorphism (RFLP-PCR), allele specific PCR (AS-PCR) and high resolution melting (HRM) analysis.<sup>8</sup>

HRM is a technique for detection of known and unknown variations in DNA. It was first described by Gundry et al. in 2002 and later Wittwer et al. in 2003 used LCGreen dye instead of labelled primer to increase sensitivity of HRM assay.<sup>11,12</sup> HRM is based

on dissociation of DNA by gradually increasing the temperature after PCR and monitoring its melting temperature which depends on DNA sequence, GC content and length.<sup>13</sup> HRM has its advantages compared to the conventional methods such as AS-PCR. Since HRM is performed in closed tube and does not require post PCR processing (e.g. gel electrophoresis), therefore, it is faster, easy to perform and less likely to become contaminated. However, it is less sensitive compared to AS-PCR (sensitivity of 6% in HRM compared to 0.01% in AS-PCR) and its sensitivity decreases by increasing amplicons size more than 300bp.<sup>14,15</sup>



**Figure 4:** Sequencing chromatograms of wild type and mutant allele. A is heterozygous mutant (1849 G>T) while B is wild type (G). Arrow indicates position of the mutation.

In this study, a total of 40 formalin fixed paraffin embedded (FFPE) marrow specimens of MPN patients were retrieved from Tengku Ampuan Afzan Hospital (HTAA) between 2014 and 2016. We found that by using HRM assay we were able to detect JAK2 mutation in around 7.5% of heterozygous mutant sample. Our result is consistent with previous study by Lin et al. who reported the sensitivity of 6% by using HRM assay.<sup>16</sup>

Our data revealed that the results of HRM assay corresponded well with the results of AS-PCR with the concordance rate of 97.5%, which was comparable with the results of Er et al. who reported the concordance rate of 100% with ARMS assay.<sup>17</sup> We found only one discrepancy (ET patient) between these two assays, which was negative in

HRM and positive in AS-PCR assay. This discrepancy could be due to the different reaction condition

used in the assays. In this study, around 30 ng of gDNA was used in HRM assay while around 80 ng of gDNA was used in AS-PCR assay. Therefore, differences in concentration of DNA template would likely result in discrepancy in these assays.

In conclusion, our results demonstrated that HRM analysis is a rapid and powerful diagnostic assay for detection of JAK2-V617F mutation in FFPE specimens in MPN patients.

**Table II:** Comparison of HRM analysis and AS-PCR results for detection of JAK2-V617F mutation

HRM*	AS-PCR# (N= 40)		Level of agreement
	Wild	Mutant	
Wild	27	1	
Mutant	0	12	39/40 (97.5%)
Total	27	13	

\*high resolution melting; #allele specific polymerase chain reaction

#### CONFLICT OF INTEREST

None.

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