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ORIGINAL RESEARCH

# Analysis of the reannealing- instead of melting-curve in the detection of JAK2 V617F mutation by HRM method

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**Background:** The Janus kinase 2 (JAK2) has an important role in the intracellular signaling in normal and neoplastic cells. JAK2 mutation, called JAK2 V617F, is frequently found in Philadelphia chromosome-negative myeloproliferative neoplasms. We aimed to assess the analytical efficiency of high-resolution melting (HRM) method using reannealing-curve analysis in comparison with routine melting-curve analysis for JAK2 V617F mutation detection.

**Method:** Twenty-three samples including one negative synthetic standard DNA, two 50% and 75% positive synthetic standard DNA samples, five wild-type samples and 15 samples positive for JAK2 V617F were examined by HRM. Melting and reannealing stages were performed, and then, raw and normalized curves were compared between the two stages.

**Results:** In melting-curve analysis, the wild-type and mutant samples had different melting temperatures (75/53°C and 75/10°C, respectively). In normalized curves corresponding to reannealing method, mutant samples were better separated from the baseline than in melting method as well as for samples with different mutant DNA burden from each other. Furthermore, wild-type samples were more homogenous in the normalized curves corresponding to reannealing than in melting method. This means that patients with a low allelic burden may be wrongly interpreted as normal in the common melting method.

**Conclusion:** We suggest the use of reannealing instead of the melting-curve analysis for the detection of sequence variations, especially for large-scale mutation and allele burden measurement in clinical settings. However, more evaluations with more sample size will better improve the benefits of reannealing-curve analysis in research and clinic.

**Keywords:** HRM, melting, reannealing

## Introduction

The Janus kinase 2 (JAK2) plays an important role in the intracellular signaling in normal and neoplastic cells.[1] A point mutation (c.1849GNT) results in a mutant protein (GTC→TTC), called JAK2 V617F in which valine is substituted by phenylalanine at position 617.[2] JAK2 V617F has been detected frequently in Philadelphia chromosome-negative myeloproliferative neoplasms, such as polycythemia vera (65–97%), essential thrombocythemia (23–57%) and primary myelofibrosis (35–57%). Accordingly, detection of this mutation has been included within the WHO diagnostic criteria for polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF).[3–6] In recent years, several methods have been developed to detect the JAK2 V617F mutation.[7,8] However, each of them has its own limitations and disadvantages, such as low sensitivity and high costs, particularly in large-scale diagnosis.[9] A more

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recently established technique, high-resolution melting (HRM) curve analysis represents a new mutation scanning technology without the need for time and cost consuming post-PCR processing.[9] HRM-curve analysis is a simple, economic and sensitive technique consisting of PCR that distinguishes DNA samples in the presence of a DNA-binding dye, based on their melt temperatures (Tm) as they are denatured from a double-stranded to a single-stranded form, by precise increasing the temperature.[10] Eva Green is a sensitive, stable and cost-effective DNA-binding dye, and therefore suitable for DNA melt curve analysis and several other PCR-based detection methods.[11] The melting pattern of a PCR product depends on its length, sequence, GC content or strand complementarity. Homozygous mutations will result in the Tm shift compared with the wild-type sample. In contrast, heterozygous mutations present different shape of the melting curve.[12,13] Recently, many researchers focused on the most efficient methods for JAK2 V617F mutation detection.[14–17] Table 1 summarizes some of the previous studies on JAK2V617F mutation detection, which focused on HRM method and compared this method with other routine molecular methods.

In this work, we aimed to describe the basis of the melting- and reannealing-curve analysis in real-time instruments and to assess the analytical efficiency of HRM method using reannealing-curve analysis in comparison with routine melting-curve analysis for JAK2 V617F mutation detection. Also, we tried to introduce a new approach to improve the sensitivity and specificity of the routine HRM method.

# Materials and methods

## Sample

A total of 23 samples including one negative synthetic standard DNA, two positive synthetic standard DNA samples (positivity of 50% and 75%), five wild-type samples (negative mutation for JAK2 V617F) and 15 mutant samples (positive for JAK2 V617F). Patients with hematocrit higher than 52% for males and 48% for females were recruited in this study. About 3 mL of venous blood were collected in EDTA for complete blood count and DNA extraction. Demographic characteristics and hematologic parameters are summarized in Table 2.

## Ethical considerations

This study was approved by ethical committee of Kerman University of Medical Sciences. The ethics approval code is IR.KMU.REC.1395.812. This code obtained before the study starts and the patients provided written informed consent conducted in accordance with the Declaration of Helsinki.

## DNA extraction

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and was kept at 4°C before use. DNA purity was determined by calculating the ratio of absorbance at 260 and 280 nm (A260/A280) using Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, Thermo Scientific, Wilmington, DE, USA).

**Table 1** Some of the previous studies on JAK2V617F mutation detection

| Reference | Sample size | Mutation type | Detection methods | Findings |
|---|---|---|---|---|
| Cao et al[14] | 58 PV samples | JAK2V617F | PCR-RFLP, ARMS-PCR, sequencing | Sequencing was gold standard method and other methods was also appropriate to detect mutation |
| Carillo et al[15] | 28 PV samples | JAK2V617F | Nested HRM, sequencing | Modified HRM was more effective than routine HRM for detection of JAK2V617F mutation |
| Lin et al[16] | 24 Myeloproliferative disorder patients | JAK2V617F | HRM, AS-PCR | Compared the limit of detection (LOD) between the different methods for JAK2V617F mutation detection and showed the (LOD) for HRM is 6%. |
| Matsumoto et al[17] | 38 PV samples | JAK2 and Calreticulin | HRM, sequencing | HRM is a sensitive method for scanning the JAK2 and Calreticulin genes |
| Wang et al[18] | 4 Osteogenesis imperfect patients | COL1A1 | HRM, sequencing | They found two new mutations in COL1A1 gene and introduced HRM analysis for gene scanning as an attractive option for medical laboratories |

**Table 2** Demographic characteristics and hematologic parameters

| Variable | Mutant Jak2 (JAK2V617F) | Wild-type Jak2 |
|---|---|---|
| **Sample size** | 15 | 5 |
| **Male (%)** | 13 (65%) | 4 |
| **Female (%)** | 7 (35%) | 1 |
| **Age** | | |
| Mean | 67.2 | 58.3 |
| SD | 1.7 | 2.5 |
| **Hemoglobin (g/L)** | | |
| Mean | 193 | 142 |
| SD | 21.2 | 3.1 |
| Minimum | 240 | 152 |
| Maximum | 181 | 131 |
| **Hematocrit (%)** | | |
| Mean | 59.3 | 42.6 |
| SD | 5.1 | 8.9 |
| Minimum | 69.7 | 46 |
| Maximum | 54.4 | 39 |
| **Platelet ($10^9$/L)** | | |
| Mean | 470 | 240 |
| SD | 252 | 122 |
| Minimum | 885 | 220 |
| Maximum | 362 | 410 |
| **WBC ($10^9$/L)** | | |
| Mean | 10.1 | 7.4 |
| SD | 3.2 | 2.1 |
| Minimum | 6.4 | 4.3 |
| Maximum | 14.2 | 9.2 |

## HRM-curve analysis

The specific primers for using in the amplification stage of HRM were designed using Vector NTI version11 as follows:

JAK2, F: 5′-TTGAAGCAGCAAGTATGATG-3′;
JAK2, R: 5′-CTTACTCTCGTCTCCACAG-3′.

The target region was 86 bp in length and located in the 1783–1869 nucleotide position. These primers were synthesized by Eurofins MWG Operon (Ebensburg, Germany) (Figure 1).

Concentration of DNA samples was set as 100 pmol/μL to make minimum interference in melting analysis and better interpretations and comparison of the results.

HRM method was conducted by Rotor-Gene Q real-time thermal cycler and results were confirmed by sequencing and TaqMan Probe methods. The HRM reaction mix without DNA template was used as non-template control. To check the integrity and overall quality, PCR products were run on 2% agarose gel and visualized by staining with ethidium bromide. The final reaction volume of 20 μL consisted of 10 μL of Master Mix (typit HRM; Qiagen), 1.4 μl primer pair and 2 μL DNA in 6.6 μL distilled water. The thermal cycling conditions consisted of initial melting stage at 95°C for 5 mins followed by 35 cycles of 5 s at 94°C, annealing at 60°C for 10 s and then a final extension stage of 5 mins at 72°C.

After that, HRM analysis was continued in the two stages: first, melting stage by precise increasing the temperature and second, reannealing stage by precise decreasing the temperature. Melting stage consisted of temperature increase from 60°C to 94°C with a ramp rate of 0.02%. This procedure starts with double-strand DNA (dsDNA) molecules with 100% fluorescence emission due to the binding interactions between Eva Green dye and dsDNA molecules. By the temperature increase, the dsDNA began melting at which all ssDNA molecules dissociate gradually and fluorescence starts to reduce. Tm is the temperature at which one-half of dsDNA is separated to ssDNA. On the other hand, fluorescence emission is about 50%. In contrast, reannealing stage starts with a gradual temperature decrease from 94°C to 60°C with a ramp rate of 0.02% that all of ssDNA molecules associate together gradually to form ssDNA, and therefore, fluorescent emission increases to 100%. In both methods, there are some variations between samples because of the different DNA copy number and fluorescence emission status (depending on the pre-HRM PCR reaction). Therefore, the normalized region should be used to overcome this defect. The first normalized region for the reannealing stage is considered as 0% fluorescent emission and the second normalized region is considered as 100%. Conversely, the first and second normalized regions for melting stage are considered as 100% and 0% fluorescent emission, respectively. These regions are the same for all samples and melting analysis is performed to find the Tm or 50% fluorescent emission. The amplification plot for each sample was generated by the Rotor-Gene Q- Pure Detection software linked to the instrument. The generated data were subsequently imported to the HRM software for melting-curve analysis. The fluorescence change of each sample was plotted against temperature and normalized to produce the aligned melt curves. Finally, the results of melting and reannealing stages were compared.

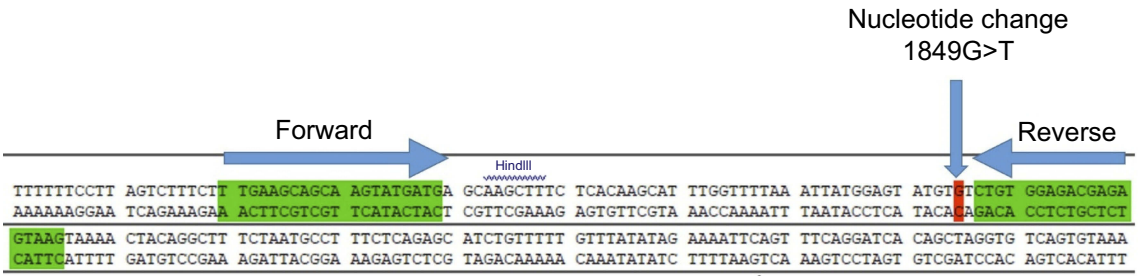


**Figure 1** Schematic view of amplification target for HRM method and JAK2 V617F (C.1849G>T) point mutation.

## Results

Quality and concentration of DNA samples were also quickly estimated by agarose gel electrophoresis (Figure 2).

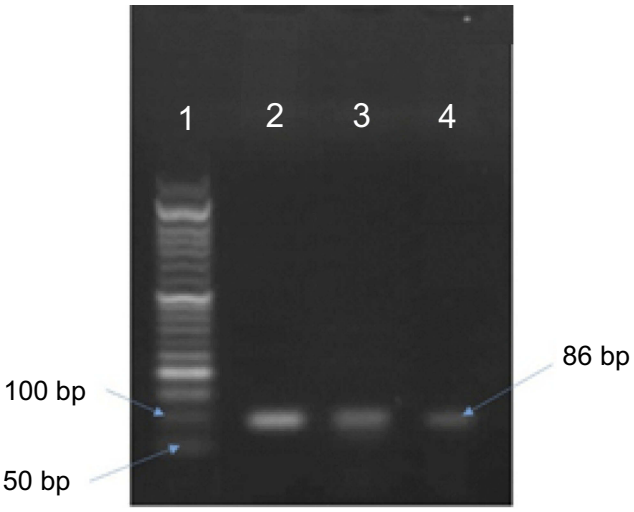


**Figure 2** Electrophoresis of PCR products on 1% agarose gel. Amplified product is 86 bp. 1: Size marker (50 bp); 2, 3: samples, 4: positive control.

## HRM analysis by melting and reannealing methods

According to the results of melting-curve analysis, the wild-type and mutant DNA samples had different melting temperatures (75/53°C and 75/10°C, respectively) (Figure 3). First and second normalization regions were defined in the raw curves (Figures 4 and 5). Then, normalized curves were obtained for both melting and reannealing methods in which the difference in melting temperatures was apparent (Figures 6A and 7A). For the better discrimination between wild-type and mutant samples, one wild-type sample was defined as baseline (normalized minus G). As shown in Figures 6B and 7B, mutant sample was completely separated from the baseline. In this context, samples with more mutant DNA burden were in more distance from the baseline (50% and 75% mutant samples compared to the baseline). Interestingly, in normalized minus G curves corresponding to reannealing method, mutant samples were better separated from the baseline than in melting method as well as for samples with different mutant DNA burden from each other (for example, 50% from 75% mutant samples). Furthermore, wild-type

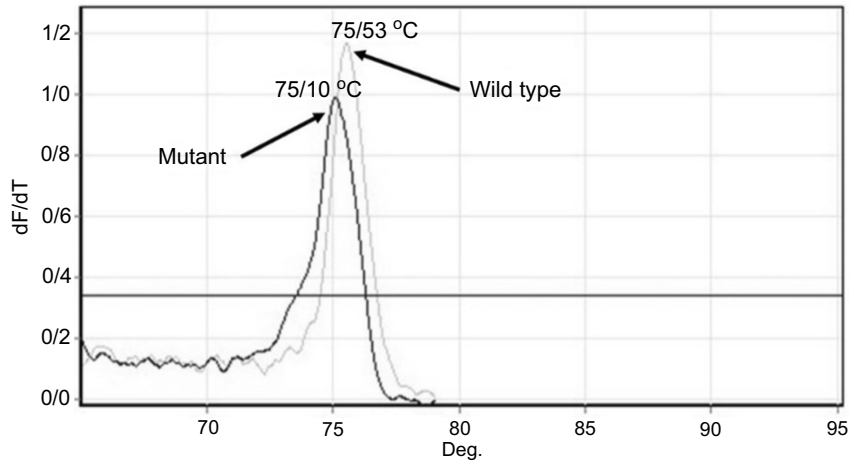


**Figure 3** Melting curve of the wild-type and mutant DNA samples.

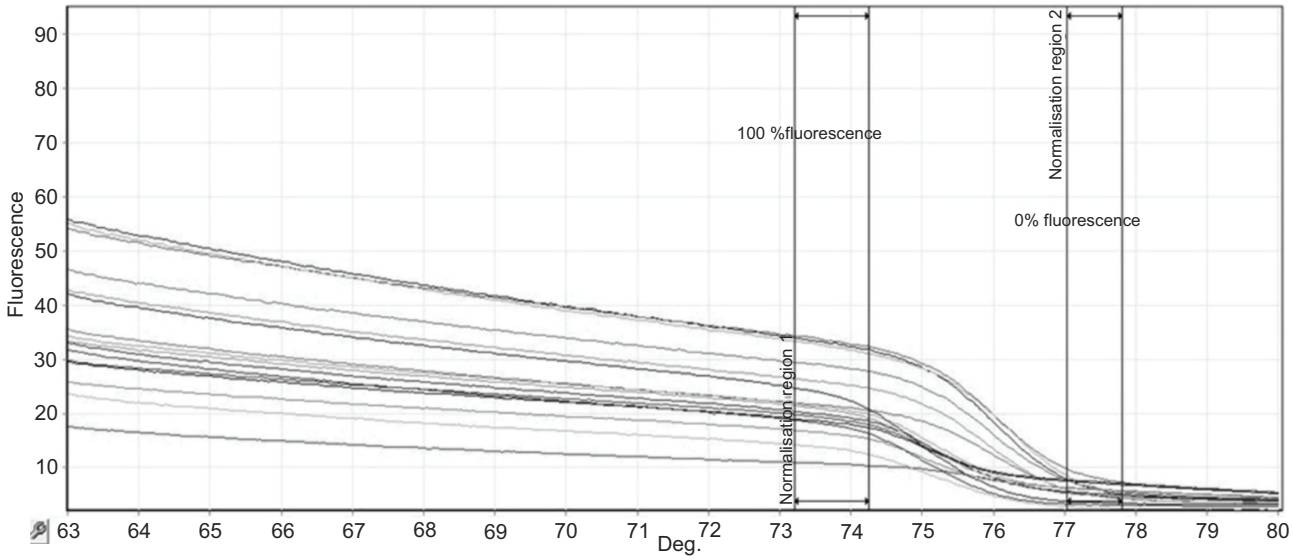


**Figure 4** Raw melt curves with normalized region.

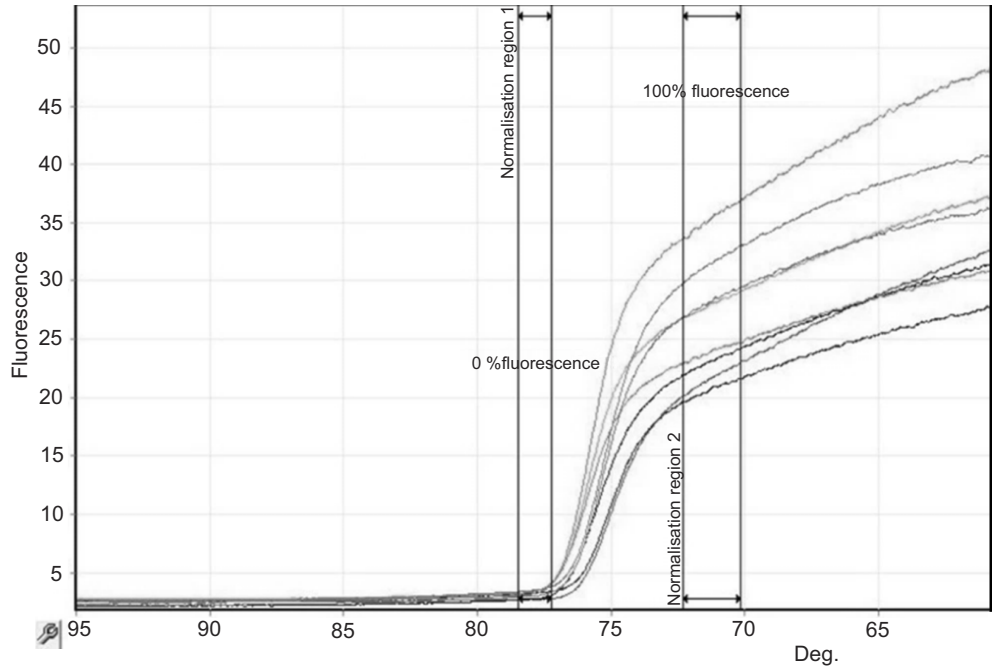


**Figure 5** Raw reannealing curves with normalized region.

samples were more homogenous in the normalized minus G curves corresponding to reannealing than in melting method. This means that patients with a low allelic burden may be wrongly interpreted as normal in melting method.

## Discussion

HRM analysis is an efficient and sensitive PCR-based approach for determining the gene mutation with capability to differentiate heterozygous and homozygous mutations. Furthermore, this method provides the possibility of allele burden measurement in clinical evaluations.[19,20] Previous studies using the dilution series of wild-type and mutant DNA samples have shown an analytical sensitivity of about 5% for HRM method in the detection of mutations.[19] In a study by Chien-Yu Lin et al, the limit of detection for HRM method was 6%, and in comparison, with AS-PCR it was 2.5%; this finding shows that the HRM method is a sensitive method, can also be performed in closed tube, and is cost-effective. Due to the

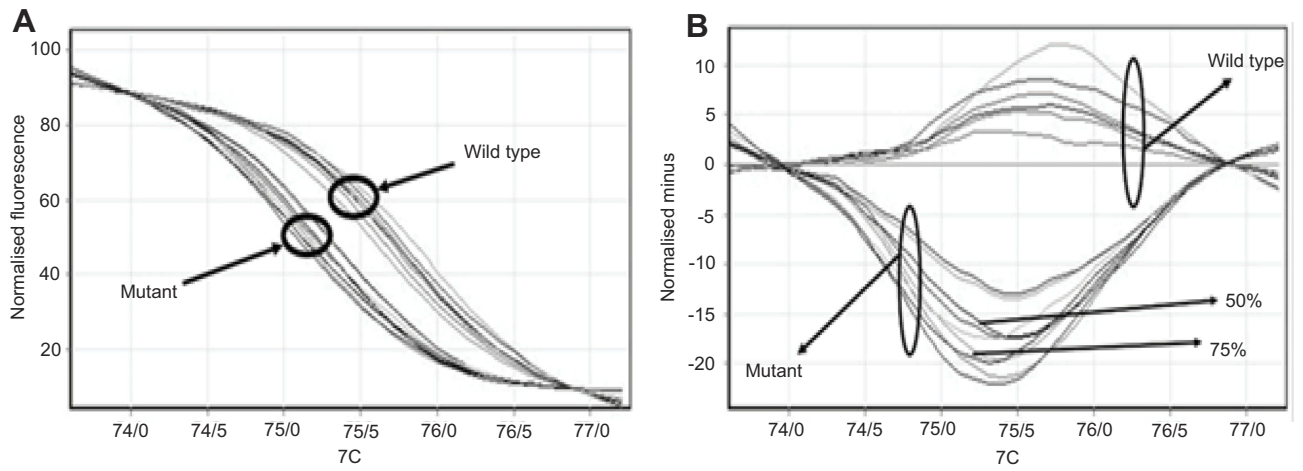


**Figure 6** (**A**) Normalized curves for melting method; (**B**) normalized minus G curves for melting method: wild type, mutant and, 50% and 75% synthetic positive samples are shifted from the baseline.

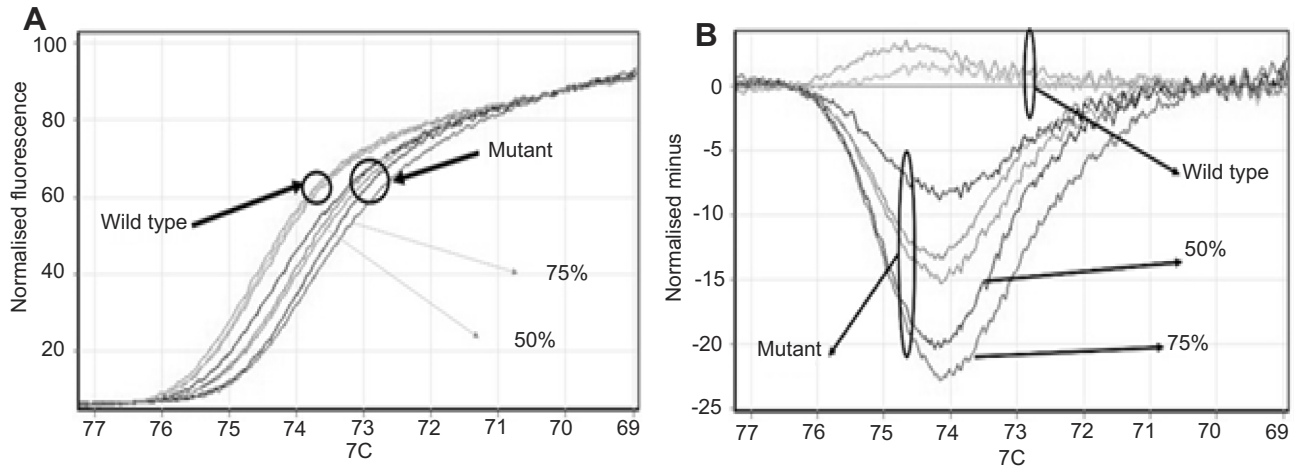


**Figure 7** (**A**) Normalized curves for reannealing method; (**B**) normalized minus G curves for melting method: wild type, mutant and, 50% and 75% synthetic positive samples are shifted from the baseline and better separated from each other than in melting method.

sensitivity of this method (with 6% limit of detection),[16] it can be useful in following up the patients' response to the treatment and determining MRD for them. So the analysis of HRM is essential for detection. Here for the first time, we compared the reannealing- and melting-curve analysis methods following the HRM analysis with the aim to introduce the more efficient one for JAK2 V617F mutation detection and better differentiation of the samples with different sequence alterations. To the best of our knowledge, there is no previous study that focused on the reannealing step following the HRM-curve analysis. Therefore, there was no previous report for discussion and result comparison. In a study by Serge Carillo et al,[15] nested PCR before the common HRM analysis was introduced as Nested HRM which improved the efficacy of the HRM-curve analysis. They reported that Nested HRM had higher sensitivity than common HRM analysis in clinical applications in comparison with other currently used methods. According to the results, samples with mutation allele burden were better differentiated in reannealing than melting step, as well as better separation of the wild type from the mutant samples. Since the length of the PCR products is similar, the Tm shift in curves is directly related to the sequence alterations. Therefore, the Tm shift in the sample with 70% mutant allele burden was more than in sample with 30% mutant allele burden. Complete separation of the mutant from wild-type samples is an essential step in mutation detection and allele burden measurement. In conclusion, we suggest the use of reannealing instead of the melting-curve analysis for the detection of sequence variations, especially for large-scale mutation and allele burden measurement in clinical settings. However,

more evaluations with more sample size will better improve the benefits of reannealing-curve analysis in research and clinics.

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## Disclosure

The authors declare that they have no conflict of interests in this work.

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