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TITLE:

Genetic Variant Detection in the *CALR* gene using High Resolution Melting Analysis

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KEYWORDS:

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SUMMARY:

High resolution melting analysis (HRM) is a sensitive and rapid solution for genetic variant detection. It depends on sequence differences that result in heteroduplexes changing the shape of the melting curve. By combing HRM and agarose gel electrophoresis, different types of genetic variants such as indels can be identified.

ABSTRACT:

High resolution melting analysis (HRM) is a powerful method for genotyping and genetic variation scanning. Most HRM applications depend on saturating DNA dyes that detect sequence differences, and heteroduplexes that change the shape of the melting curve. Excellent instrument resolution and special data analysis software are needed to identify the small melting curve differences that identify a variant or genotype. Different types of genetic variants with diverse frequencies can be observed in the gene specific for patients with a specific disease, especially cancer and in the *CALR* gene in patients with Philadelphia chromosome–negative myeloproliferative neoplasms. Single nucleotide changes, insertions and/or deletions (indels) in the gene of interest can be detected by the HRM analysis. The identification of different types of

genetic variants is mostly based on the controls used in the qPCR HRM assay. However, as the product length increases, the difference between wild-type and heterozygote curves becomes smaller, and the type of genetic variant is more difficult to determine. Therefore, where indels are the prevalent genetic variant expected in the gene of interest, an additional method such as agarose gel electrophoresis can be used for the clarification of the HRM result. In some instances, an inconclusive result must be re-checked/re-diagnosed by standard Sanger sequencing. In this retrospective study, we applied the method to *JAK2* V617F-negative patients with MPN.

INTRODUCTION:

Somatic genetic variants in the calreticulin gene (*CALR*) were recognized in 2013 in patients with myeloproliferative neoplasms (MPN) such as essential thrombocythemia and primary myelofibrosis[1,2]. Since then, more than 50 genetic variants in the *CALR* gene have been discovered, inducing a +1 (−1+2) frameshift[3]. The two most frequent *CALR* genetic variants are a 52 bp deletion (NM_004343.3 (*CALR*):c.1099_1150del52, p.(Leu367Thrfs*46)), also called type 1 mutation, and a 5 bp insertion (NM_004343.3 (*CALR*):c.1154_1155insTTGTC, p.(Lys385Asnfs*47)), also called type 2 mutation. These two genetic variants represent 80% of all *CALR* genetic variants. The other ones have been classified as type 1–like or type 2–like using algorithms based on the preservation of an α helix close to wild type CALR[4]. Here, we present one of the highly sensitive and rapid methods for *CALR* genetic variant detection, the high resolution melting analysis method (HRM). This method enables the rapid detection of type 1 and type 2 genetic variants, which represent the majority of *CALR* mutations[5]. HRM was introduced in combination with real time »polymerase chain reaction« (qPCR) in 1997 as a tool to detect the mutation in factor V Leiden[6]. In comparison to Sanger sequencing that represents the golden standard technique, HRM is a more sensitive and less specific method[5]. The HRM method is a good screening method that enables a rapid analysis of a large number of samples with a great cost-benefit[5]. It is a simple PCR method performed in the presence of a fluorescent dye and does not require specific skills. Another benefit is that the procedure itself does not damage or destroy the analyzed sample that allows us to reuse the sample for electrophoresis or Sanger sequencing after the HRM procedure[7]. The only disadvantage is that it is sometimes difficult to interpret the results. Additionally, HRM does not detect the exact mutation in patients with non-type 1 or type 2 mutations[8]. In these patients, Sanger sequencing should be performed (**Figure 1**).

HRM is based on the amplification of the specific DNA region in the presence of saturating DNA fluorescent dye, which is incorporated in double-stranded DNA (dsDNA). The fluorescent dye emits light when incorporated in the dsDNA. After a progressive increase in temperature, dsDNA breaks down into single stranded DNA, which can be detected on the melting curve as a sudden decrease in fluorescence intensity. The shape of the melting curve depends on the DNA sequence that is used to detect the mutation. Melting curves of samples are compared to melting curves of known mutations or wild type CALR. Distinct melting curves represent a different mutation that is non type 1 or type 2[9].

The algorithm for the somatic genetic variant detection in the *CALR* gene by HRM, agarose gel electrophoresis and sequencing method (**Figure 1**) was used and validated in the retrospective

study published before[10].

PROTOCOL:

The study was approved by the Committee of Medical Ethics of the Republic of Slovenia. All procedures were in accordance with the Helsinki declaration.

1. Fluorescence-based quantitative real-time PCR (qPCR) and post-qPCR analysis by HRM

1.1. Resuspend primers listed in the **Table of Materials** to 100 µM with sterile, RNase and DNase free H_2O (see **Table of Materials**). Make a 10 µM working concentration primer. Primers used in the protocol were published before[2].

1.2. Quantitate granulocytes DNA by the fluorescence staining method following the manufacturer's instruction[11] (see **Table of Materials**). Prepare a 20 ng/µL DNA solution using 10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0 (see **Table of Materials**).

1.2.1. In addition to DNA samples, prepare at least three DNA controls: two positive controls with the NM_004343.3 (*CALR*): c.1099_1150del52, p.(Leu367Thrfs*46) (52 bp deletion or type 1 mutation), and NM_004343.3 (*CALR*): c.1154_1155insTTGTC, p.(Lys385Asnfs*47) (5 bp insertion or type 2 mutation) genetic variant, as well as wild-type DNA and negative control as a non-template (NTC) control.

NOTE: Before performing the HRM setup, confirm that the qPCR instrument is calibrated for HRM experiments.

1.3. Prepare qPCR HRM Master Mix according to manufacturer's protocol (see **Table of Materials**) as follows: 10 µL of 2x qPCR Master Mix with Dye, 0.2 µL of 10 µM Forward Primer, 0.2 µL of 10 µM Reverse Primer, 8.6 µL of sterile, RNase and DNase free H_2O. Use the primers from the **Table of Materials**. Run three replicates for each DNA sample and control.

1.3.1. Prepare the qPCR HRM Master Mix according to the number of samples being processed. Include excess volume of at least 10% in the calculations to provide excess volume for the loss that occurs during reagent transfers.

1.4. Mix the reaction contents by gently tapping and inverting the tube and vortexing for 2-3 s. Collect all the scattered droplets from the wall of the tube to the bottom by a brief spin.

1.5. Prepare a reaction plate appropriate for the instrument and HRM experiment (see **Table of Materials**). Transfer 19 µL of the qPCR HRM Master Mix to the appropriate wells of the 96-well optical reaction plate.

1.6. Pipet 1 µL of the negative controls, positive controls, and samples into the appropriate wells of the optical reaction plate. For the NTC, transfer 1 µL of sterile, RNase and DNase free

H_2O used for preparing the qPCR HRM Master Mix instead of DNA.

1.7. Seal the reaction plate with the optical adhesive film. Do it firmly to prevent evaporation during the run. Check that the optical adhesive film is plane across all the wells in the reaction plate to ensure correct fluorescence detection.

1.8. Spin the reaction plate at 780 x *g* at room temperature (RT) for 1 minute. Check that the liquid is at the bottom of the wells in the reaction plate. Proceed to run the assay.

NOTE: The protocol can be paused here. Store the plate at 4 °C for no more than 24 h. Allow the plate stored at 4 °C to warm to RT, and then spin the plate briefly before running it.

1.9. According to the manufacturer's instruction prepare and start the run to amplify and melt the DNA and to generate HRM fluorescence data in the qPCR instrument. In the instrument system software, assign the controls and samples to the appropriate wells.

1.10. For the *CALR* genetic variant detection, change the default instrument amplification protocol and amplify the DNA using the following thermal cycling protocol: 95 °C for 10 minutes, followed by 50 cycles of 95 °C for 10 s and 62.5 °C for 60 s.

1.11. Perform the melt curve/dissociation (HRM) stage immediately after qPCR according to the manufacturer's instructions[12] as follows: 95 °C for 10 s, 60 °C for 1 min, and then a ramp rate of 0.025 °C/s until 95 °C. Hold the temperature at 95 °C for 15 s and at 60 °C for 15 s.

1.12. The run ends automatically. First, review and verify the amplification plot (**Figure 2**).

1.13. In the experiment menu of the instrument system software, select the amplification plot option. If no data are displayed, click the green button **Analyze**.

1.13.1. In the amplification plot tab, from the plot type drop-down menu, select the plot that displays the amplification data as the raw fluorescence readings normalized to the fluorescence from the passive reference (ΔRn) as a function of cycle number (ΔRn vs Cycle). In the plot color drop-down menu, select **Sample**.

1.14. From the graph type drop-down menu, select the linear amplification graph type. Show the baseline start and end cycle on the linear amplification graph by selecting the baseline start option. Verify that the baseline is set correctly. The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. The baseline is usually set from 3 to 15 cycles (**Figure 2**).

1.15. From the graph type drop-down menu, select the **log10 amplification graph** type. Show the threshold line on the graph by selecting the threshold option. Adjust the threshold accordingly if not set correctly. The correctly set threshold means that the threshold line crosses the exponential phase of the qPCR curves (**Figure 2**).

1.16. Verify that normal amplification curves are in all sample and positive control wells. Verify that there is no amplification in the NTC wells before cycle 40. A normal amplification plot shows an exponential increase in fluorescence that exceeds the threshold between cycles 15 and 35 (**Figure 2**). Exclude the sample wells from the analysis if there is no amplification in the well position.

NOTE: If the amplification plot looks abnormal or the NTC well indicates the amplification, identify and resolve the problem according to the manufacturer's troubleshooting guide.

1.17. Exclude the outlier with the quantification cycle (Cq) value that differs from replicates by more than 2 in the instrument system software[12]. The outliers may produce erroneous HRM results.

1.18. In the derivative melt curves, review the pre- and post-melt regions/temperature lines. The pre- and post-melt regions should be within a flat area where there are no large peaks or slopes in the fluorescent levels (**Figure 3**). If needed adjust it accordingly. Set up the start and stop of the pre- and post-melt temperature lines approximately 0.5 °C apart from each other (**Figure 3**). Restart the analysis if the parameters are adjusted by clicking on the **Analyze** button.

1.19. In the difference plot tab, in the plot settings tab, choose one of the wild-type control (homozygote) replicates as the reference DNA and restart the analysis by clicking on the **Analyze** button.

1.20. In the aligned melt curves tab, confirm that all positive controls have the correct genotype and NTC failed to amplify (**Figure 4**). From the well table, select the wells containing a positive control to highlight the corresponding melt curve in the analysis plots. Confirm that the color of the line corresponds to the correct genotype. Repeat steps for the wells containing the other positive controls and NTC.

1.21. In the aligned melt curves tab, carefully review the plot displays for the unknown samples and compare them to the plot displays for controls (**Figure 4**). From the well table, select the wells containing the unknown sample replicates, check the color of the melt curve and align them with the controls in an ordered sequence by selecting the wells containing positive controls one by one. Repeat the process for all the unknown samples.

NOTE: The unknown sample contains the variant of one of the controls if its melting curve is tightly aligned to it. Different variant groups (different colors) could be displayed indicating that the unknown sample consists of an unknown variant, not corresponding to the controls (**Figure 8**). A low level of the somatic genetic variant can be present in the patient's sample. This could influence the interpretation of the HRM result, particularly at the detection limit of the assay (**Figure 9**). In these cases, the color or even the shape of the line could closely resemble that of the wild-type genotype.

1.21.1. When the result is inconclusive, combine the HRM results with the results of the agarose gel electrophoresis and sequencing methods. Retest the sample or request and retest a new sample if needed.

1.21.2. Carefully review the data set for replicate curves and check that the alignment of each replicate within the group is tight. Exclude the replicate if it does not align tightly with the other samples in the group (outlier). Retest the sample if more outliers are present and the results are inconclusive. Be aware that the quantity and quality of the DNA sample influence the HRM results.

1.22. Analyze the result for the unknown sample in the **Difference** plot tab. Repeat the procedure described in the step 1.21 and verify that the results obtained for the unknown sample are the same.

1.23. Then, run the qPCR HRM products on the agarose gel.

NOTE: The protocol can be paused here. Store the plate at 4 °C for no more than 24 hours or at -20 °C for a longer period time but no more than 7 days. Allow the plate stored at 4 °C to warm to RT, and then spin the plate briefly before running it. Allow the plate stored at - 20 °C to thaw and warm to RT, and then spin the plate briefly before running it.

2. Agarose gel electrophoresis

2.1. Run qPCR HRM products on a 4% agarose pre-cast gel containing a fluorescent nucleic acid stain using the appropriate gel electrophoresis system (see **Table of Materials**, **Figure 5**). Run only one positive, negative, NTC and sample qPCR HRM replicate.

NOTE: Gloves should always be worn when handling gels. Any other gel electrophoresis system can fulfill this purpose if the equivalent agarose percentage, fluorescent nucleic acid stain and well format are chosen.

2.2. Run the gel electrophoresis system according to the manufacturer's protocol (see **Table of Materials**). Remove the pre-cast gel in the cassette from the package, remove the comb and insert it into the apparatus according to the manufacturer's instructions (see **Table of Materials**).

NOTE: Load the gel within 15 minutes of opening the package.

2.3. Dilute a 10 µL sample to 20 µL with sterile, RNase and DNase free H_2O. Load each well with 20 µL of diluted sample.

2.4. Dilute 3 µL of DNA size standard solution to 20 µL with sterile, RNase and DNase free H_2O (see **Table of Materials**). Load the M well with 20 µL of diluted DNA size standard solution (**Figure 6**).

2.5. Fill any empty wells with 20 µL of sterile, RNase and DNase free H_2O.

2.6. Immediately select the program according to the percentage of the gel being run and set the run time on the apparatus to 10 minutes.

2.7. Start the electrophoresis within 1 min of loading sample by pressing the **GO** button. The electrophoresis time can be extended if insufficient resolution is obtained.

NOTE: Do not exceed the maximum recommended agarose electrophoresis running time according to manufacturer's instructions.

2.8. When the electrophoresis is completed, visualize DNA in the gel using a blue light or UV transillumination. Visualizing, analyzing, and storing the electrophoresis images are mostly done by a gel imager with photo-documentation system (**Figure 5**).

2.9. Analyze and interpret the visualized qPCR HRM products gel pattern by comparing the results of the unknown sample to positive controls (**Figure 7** and **Figure 8**).

2.10. If the unknown sample HRM and gel electrophoresis results indicates that an unknown genetic variant is present, sequence the qPCR HRM product using primers described in **Table of Materials** according to the protocol described[13].

CAUTION: The agarose gels containing a fluorescent nucleic acid stain must be properly disposed of per institution regulations.

REPRESENTATIVE RESULTS:

The successfully amplified DNA region of interest with an exponential increase in fluorescence that exceeds the threshold between cycles 15 and 35 and very narrow values of the cycle of quantification (Cq) in all replicated samples and controls (**Figure 2**) is a prerequisite for the reliable identification of genetic variants by HRM analysis. This is achieved by using a precise determination of DNA with fluorescence staining and an equal amount of DNA in the qPCR HRM experiment (see step 1.2). **Figure 2** shows the successful amplification of the DNA region of interest where the Cq values of all the samples and controls are in a very narrow interval. There is no amplification in the NTC wells.

The HRM stage is performed immediately after qPCR using the protocol described in step 1.11. The active melt regions (**Figure 3**, label (c)) of the samples, the controls and the NTC are used to create their aligned melt curve plots (**Figure 4**). Therefore, the correctly set pre- and post-melt regions/temperature lines (**Figure 3A**) are important for properly visualizing and identifying genetic variants in the samples. **Figure 4A** and **Figure 4B** show the aligned melt curves and difference plots, respectively, where the identification of genetic variants is possible. The unknown samples are tightly aligned with one of the positive controls. **Figure 3B** shows incorrectly set pre- and post-melt regions/temperature lines. This results in an aligned melt curve and difference plots where correct identification of the genetic variants is more difficult (**Figure**

4C and **Figure 4D**).

To confirm the HRM results and to detect whether standard or next generation sequencing method is required to identify the genetic variant present in the sample, agarose gel electrophoresis is used. **Figure 7** shows the agarose gel electrophoresis of the same samples and controls that are displayed in **Figure 4**. The genetic variant in the sample can be identified by comparing the band pattern of the sample to the controls and by combining the HRM and agarose gel electrophoresis. However, the correct genetic variant identification can only be done for the samples that contain the same genetic variant as one of the controls used in the HRM assay (**Figure 7**). Samples containing rare *CALR* genetic variants differ in the HRM result and electrophoresis band pattern (**Figure 8**). In this case, the Sanger sequencing or even next generation sequencing method are used to identify the exact genetic variant.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic representation of the algorithm for the somatic genetic variant detection in the *CALR* gene by HRM, agarose gel electrophoresis and sequencing methods.

Figure 2. Amplification plot of the qPCR HRM assay for the detection of the genetic variants in the CALR gene. The amplification plot is displayed as the raw fluorescence normalized to the fluorescence from the passive reference (ΔRn) and as a function of a cycle number. The baseline is set from 3 to 15 cycles when the DNA region of interest was efficiently amplified using 20 ng of high-quality DNA in the qPCR reaction. The normal amplification plots of the DNA region of interest of all the samples are shown as green lines. Plots show an exponential increase in fluorescence that exceeds the threshold between cycles 15 and 35 in the experiment using 20 ng of high-quality DNA in qPCR reaction. The graph shows no amplification in the non-template sample wells (NTC).

Figure 3. Derivative melt curves in the qPCR HRM assay for the detection of the genetic variants in the *CALR* gene. A) An example of correctly set pre- and post-melt regions/temperature lines. The pre-melt stop temperature line must be adjacent to the start of the melt transition region. The post-melt start temperature line must be adjacent to the end of the melt transition region. The (a) label indicates the pair of lines to the left of the peaks where the pre-melt starts and stop temperatures are set. Every amplicon is double-stranded. The (b) label indicates the data peaks of the active melt region used to create the aligned melt curves plot. The (c) label indicates the pair of lines to the right of the peaks where the post-melt start and stop temperatures are set. Every amplicon is single-stranded. **B**) An example of incorrectly set pre- and post-melt regions/temperature lines. The start and stop of the pre- and post-melt temperature lines are not adjacent to the melt transition regions and are more than 0.5 °C apart from each other.

Figure 4. The aligned melt curves and difference plots in the qPCR HRM assay for the detection of the genetic variants in the *CALR* gene. A) and **B**) show the aligned melt curves and difference plots after the pre- and post-melt regions/temperature lines are correctly set. **A**) The aligned melt curves of the positive controles with 52 bp deletion (type 1 mutation), 5 bp insertion (type 2

mutation) and a wild-type are shown as orange, purple and red color, respectively. **B**) The Difference plot of the same samples as described in panel **A**. **C**) and **D**) show the aligned melt curves and difference plots after the incorrectly set pre- and post-melt regions/temperature lines for the same set of samples.

Figure 5. The gel electrophoresis system. A) The basic units of the gel electrophoresis system (see **Table of Materials**). **B**) Gel imager with photo-documentation system consisting of a CCD camera, a chamber with suitable trans/illuminating lights, and a photographic filter is shown (see **Table of Materials**).

Figure 6. Loading the gel with diluted samples and DNA size standard solution or RNase and DNase free H_2O for empty wells.

Figure 7. Analyses of the qPCR HRM products on gel electrophoresis. The gel was exposed to UV transilluminator. Image was taken by the photo-documentation system (**Figure 5B**). Wells from 1 to 3 show controls: 52 bp deletion (type 1 mutation), 5 bp insertion (type 2 mutation) and wild-type variant, respectively. The band pattern of the unknown samples in the wells 4-10 indicates them as the wild-type genetic variant. The M well represents diluted DNA size standard solution (100 to 2,000 bp).

Figure 8. HRM and gel electrophoresis analyses of the genetic variants in the *CALR* gene. A) **HRM analyses**. The aligned melt curves of the positive controls with 52 bp deletion (type 1 mutation), 5 bp insertion (type 2 mutation) and a wild-type are shown as orange, purple and red color, respectively. The unknown sample with the different genetic variant is indicated as dark blue color. **B**) Gel electrophoresis analyses. Wells from 1 to 3 show controls: 52 bp deletion (type 1 mutation), 5 bp insertion (type 2 mutation) and wild-type variant, respectively. The band pattern of the unknown sample in the lane 9 indicates the different genetic variant as controls. Other unknown samples are the wild-type variant. The M well represents the diluted DNA size standard solution (100 to 2,000 bp).

Figure 9. The limit of detection of HRM assay. A serial dilution of a sample of a 52 bp deletion (type 1 mutation) and 5 bp insertion (type 2 mutation) is presented displaying a genetic variant allele burden of approximately 50% according to Sanger sequencing analysis and the qPCR HRM assay according to the protocol described in this article. **A**) and **B**) show the aligned melt curves and difference plots for wild-type and serial dilutions of a 52 bp deletion (type 1 mutation). **C**) and **D**) show the aligned melt curves and difference plots for wild-type and serial dilutions of a 5 bp insertion (type 2 mutation). In either case, the *CALR* genetic variant could be detected in up to 1.56% dilution.

DISCUSSION:

High-resolution melting of DNA is a simple solution for genotyping and genetic variant scanning[14]. It depends on sequence differences that result in heteroduplexes that change the shape of the melting curve. Different types of genetic variants with diverse frequencies can be observed in the gene specific for a certain group of patients with cancer[1,2,15,16,10]. Deletions or insertions in the

gene of interest can be detected by the HRM analysis as we have shown in **Figure 4A**. At the implementation of the qPCR HRM assay into the routine testing, we have performed an initial evaluation study including 21 JAK2 V617F-negative ET patients with a median age of 63 years (range from 24 to 90 years). A genetic variant in the *CALR* gene was detected in 12 out of 21 JAK2 V617F-negative ET patients (proportion 0.57). A 52 bp deletion (type 1 mutation) was detected in 9 out of 21 (proportion 0.43), a 5 bp insertion (type 2 mutation) was detected in 3 out of 21 (proportion 0.143) JAK2 V617F-negative ET patients. The results are consistent with the data from the literature[1,2,17].

Reliable identification of genetic variants by HRM analysis can be achieved through the proper assay development that ensures that the experiment is optimized for HRM. Factors other than sequence can be a source of small differences in the HRM curves such as primer design, amplicon length, dye selection, choice of qPCR HRM reagent and temperature and time profiles of the qPCR HRM steps. This is the part of the very early development and optimization of the qPCR HRM assay and has already been discussed elsewhere[12,14,18]. However, reliable identification of genetic variants in the *CALR* gene with comparable sensitivity of the assays could be achieved on different HRM platforms using the same primer sequences[19]. The most critical point in the optimization process of the qPCR HRM assay was optimization of the melting and elongation temperature in the qPCR step of the qPCR HRM assay. No modification was needed for the HRM step[12]. In the routine testing, other factors influencing the HRM results becomes more important to follow.

High-quality DNA resuspended in a low-salt buffer such as TE (10 mM Tris, 1 mM EDTA or lower concentration) and the same amount of DNA in the qPCR HRM assay are important factors for successfully amplified DNA region of interests with a high signal plateau in the qPCR amplification phase (**Figure 2**). This leads to reliable identification of genetic variants by HRM analysis (**Figure 4A,4B**)[12,14]. A high-salt buffer used for the elution of DNA in the extraction protocol may subtly change the thermodynamics of the DNA melting transition. Precipitation and resuspension in low-salt buffer TE or dilution of the sample can resolve the problem of the impurities in the DNA sample. Low quality DNA can produce nonspecific PCR products or failed amplification. All this can result in a lower reproducibility and higher error rate in HRM variant detection. Additional optimization for the challenging samples such as DNA extracted from paraffin-embedded samples could be needed to obtain an optimal HRM result[15].

Large differences in the DNA amount used in the qPCR HRM assay can impact the resulting melting temperature (Tm) and consequently lead to inconclusive results. Therefore, the precise determination of DNA concentration and dilution of all DNA samples are mandatory[20]. Ultraviolet (UV) absorption and fluorescence staining methods accurately determine the concentrations of high-purity DNA solutions[21]. The UV absorbance method could be used for evaluating the purity of the DNA solutions by performing ratio absorbance measurements at A260/A280 and A260/230. However, the fluorescent staining method is more sensitive to the degradation of DNA than the UV absorption method and can more accurately determine DNA concentration[21]. Therefore, it is more appropriate for the standardization of quantification and dilution of all DNA samples in the HRM analysis[20,21].

The indels are the main genetic variants in the *CALR* gene observed in MPN patients[1]. As the product length changes and increases, the difference between wild-type and heterozygote curves becomes smaller, and the variant detection becomes harder[7]. Therefore, an additional method for the clarification of such genetic variant should be used.

A good example is agarose gel electrophoresis. This is a simple and effective method to separate DNA fragments of different sizes[22,23]. DNA molecules are separated by size within the agarose gel so that the traveled distance is inversely proportional to the logarithm of its molecular weight[24]. **Figure 4A, Figure 4B** and **Figure 7** show examples where the two most common types of *CALR* genetic variant, 52 bp deletion and 5 bp insertion, are identified by combining qPCR HRM and agarose gel electrophoresis results. However, when the HRM result and agarose gel electrophoresis band pattern indicate a different genetic variant (including single nucleotide change) as in the controls (**Figure 8**), Sanger sequencing is still needed to identify the exact genetic variant[10].

A low level of somatic genetic variant in the *CALR* gene can be present in the patient's sample. making an interpretation of the qPCR HRM, agarose gel electrophoresis and Sanger sequencing results more demanding, particularly at the detection limit of the assay (**Figure 9**). Any melting curve shape line differing from the wild type one indicates the genetic variant to be present in the sample. The sensitivity of the qPCR HRM assay is lower than 5% (**Figure 9** and reference[19]) and is more sensitive than the Sanger sequencing method, whose sensitivity was reported to be 15-20%[19]. In these cases, the next generation deep sequencing method that detect larger indels could be applied and confirm the HRM result. In conclusion, the HRM analysis is a powerful method for genotyping and genetic variation scanning of somatic genetic variants in the *CALR* gene. The identification of different types of genetic variant is mostly based on the controls used in the qPCR HRM assay. More reliable results are obtained by combining the HRM results with results from agarose gel electrophoresis. In case of inconclusive results, standard Sanger sequencing or even more sensitive next generation sequencing method can be used to properly identify the genetic variant.

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The authors have no conflicts of interest to disclose.

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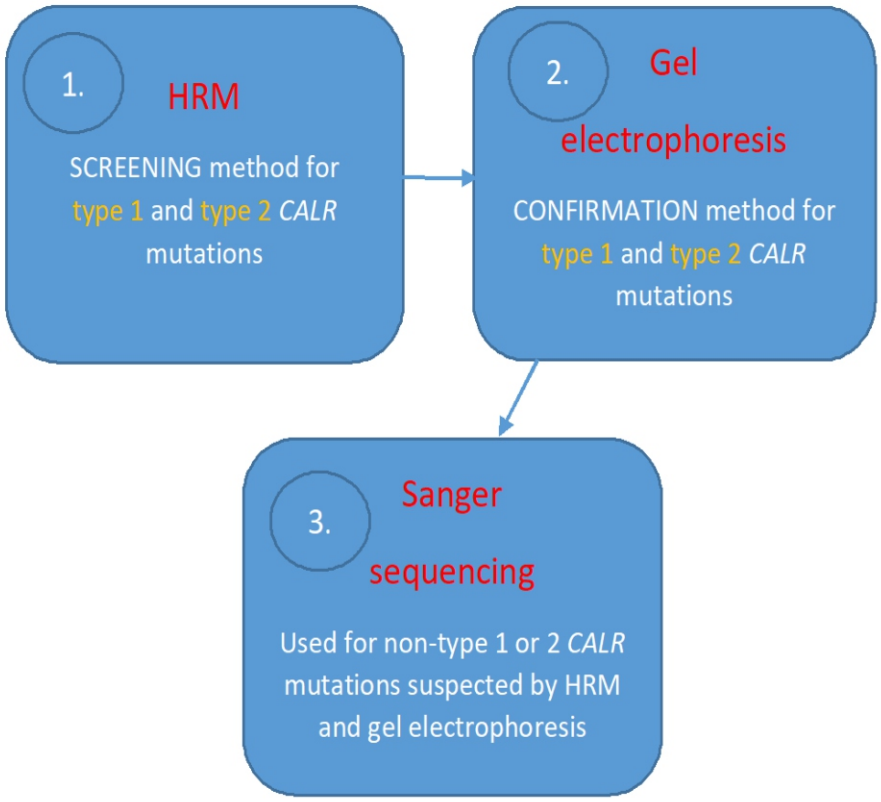
Figure



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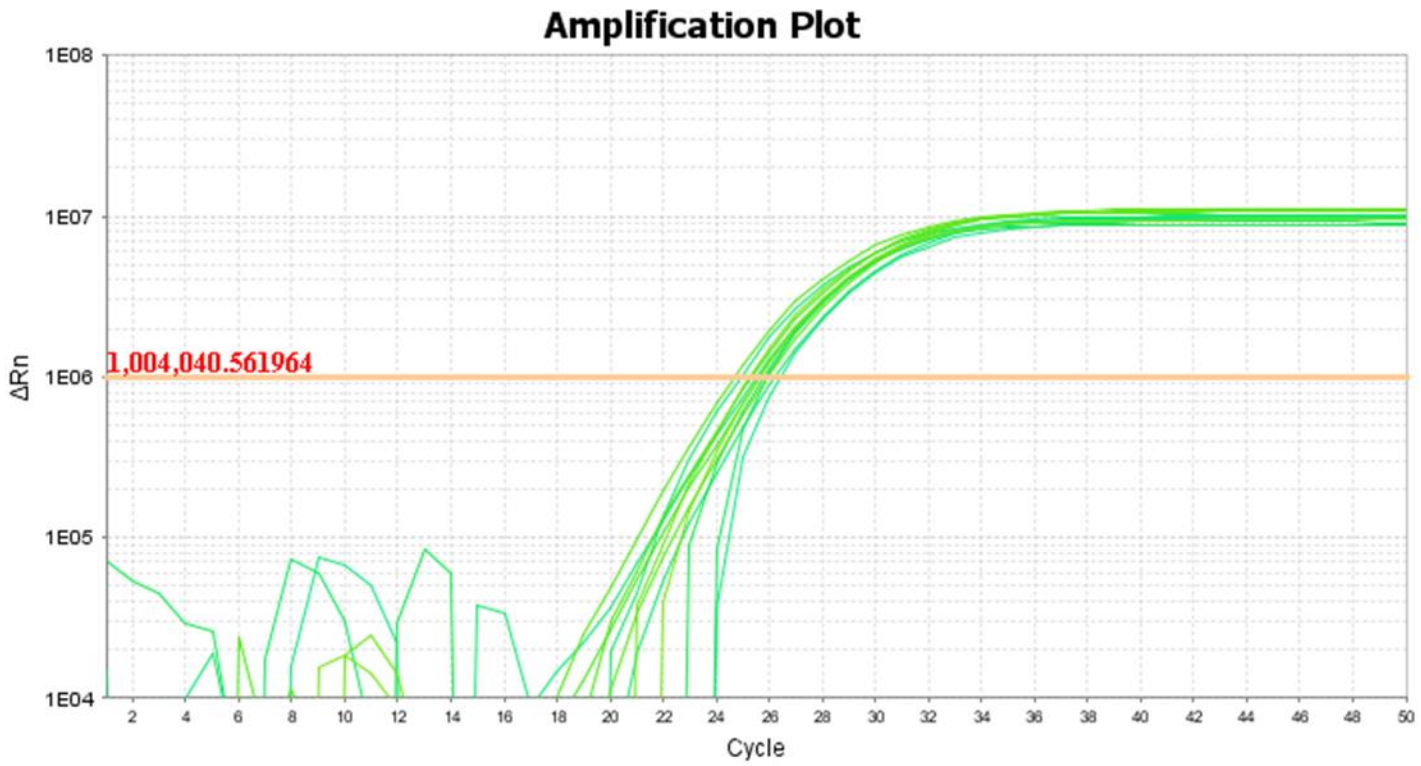

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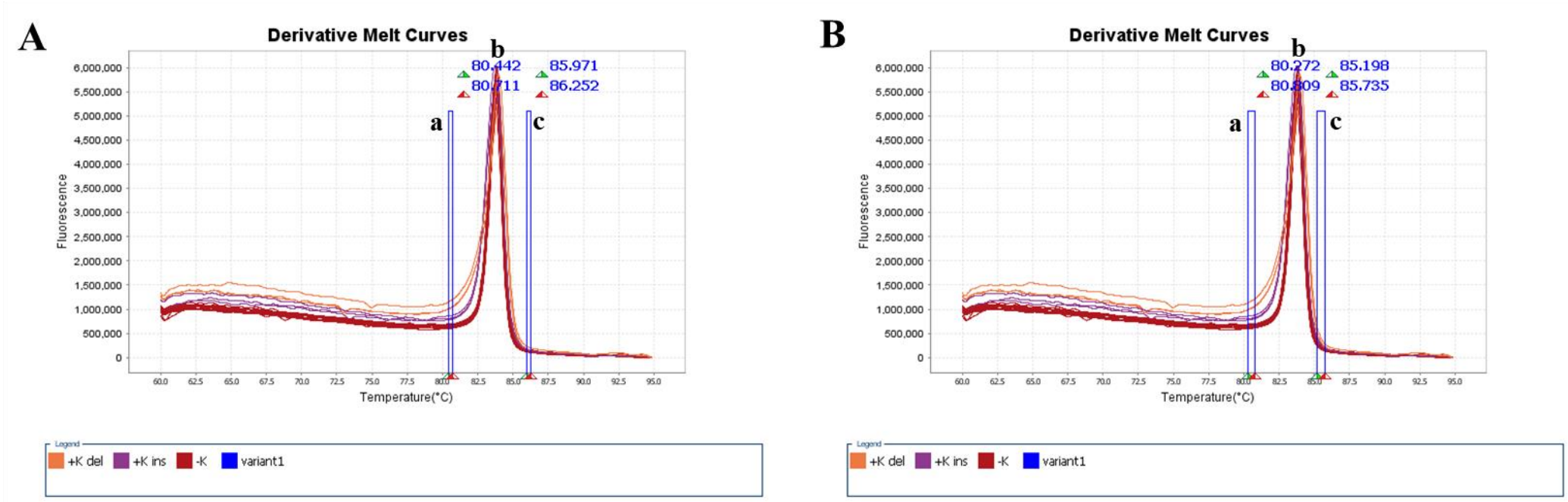

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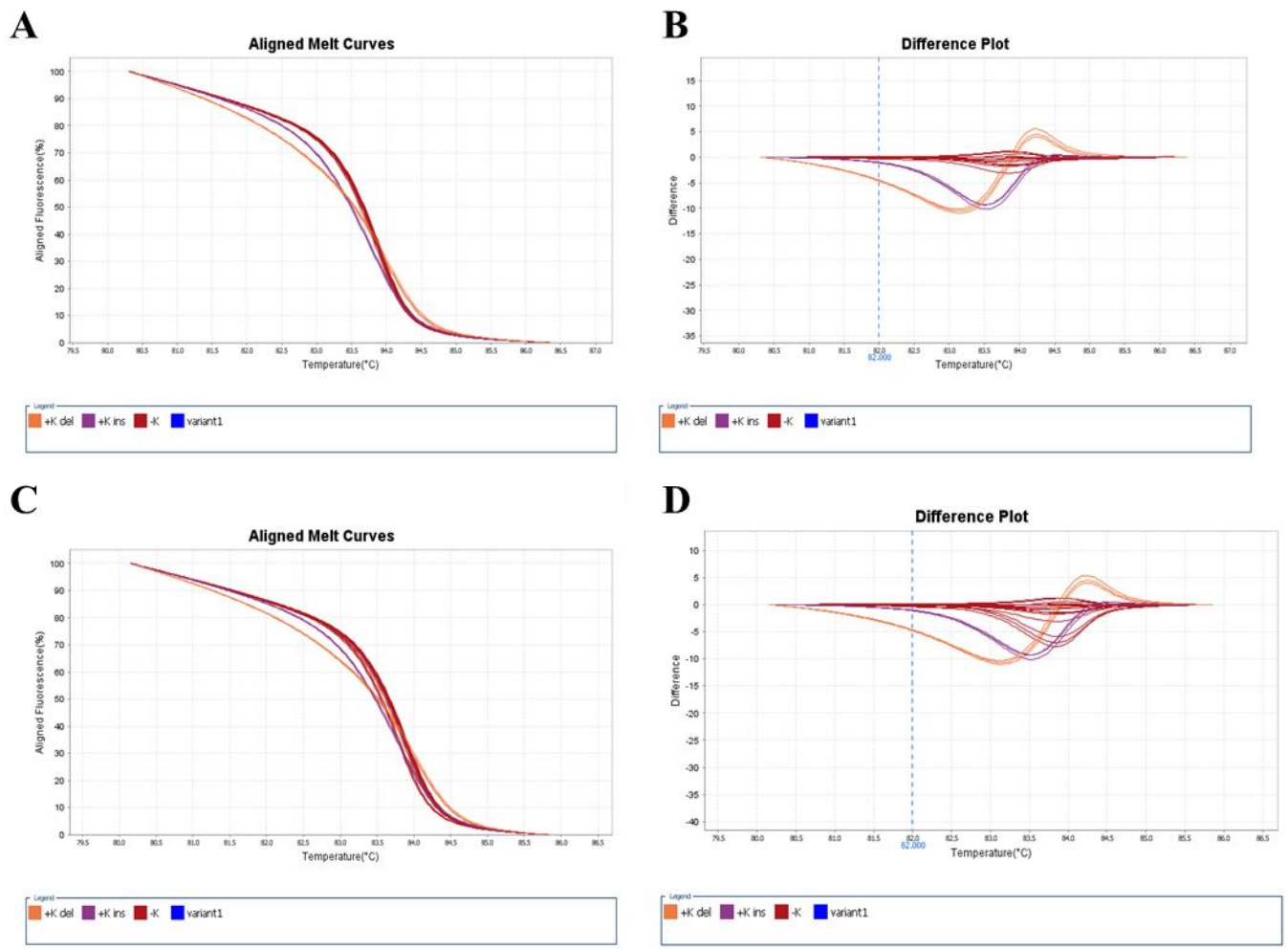

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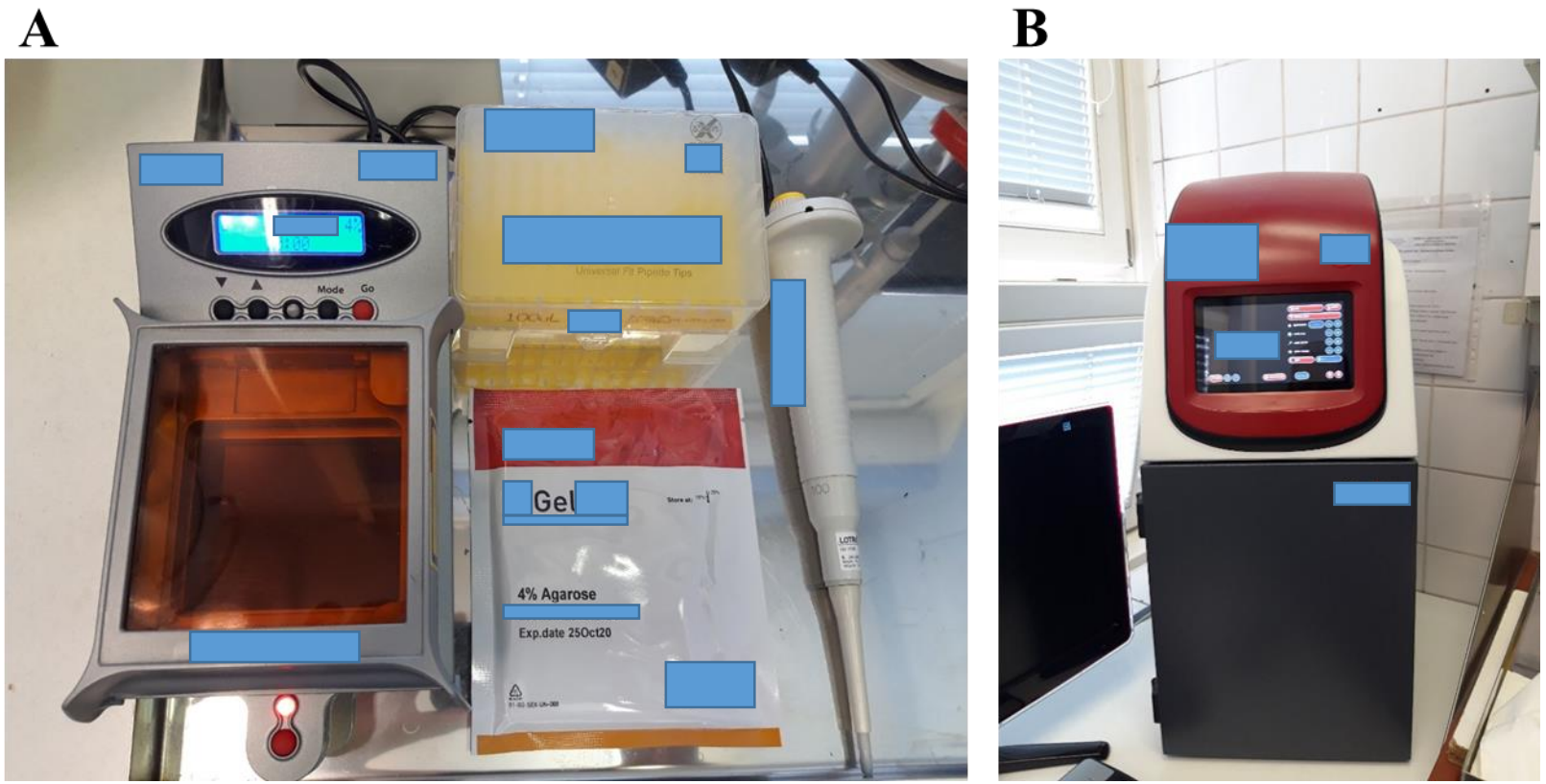

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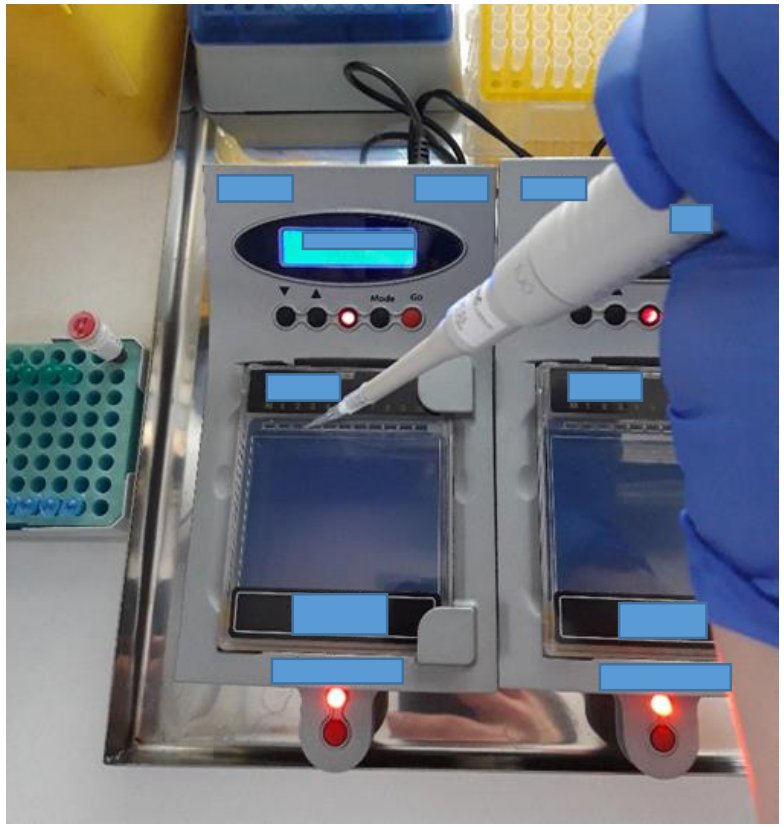

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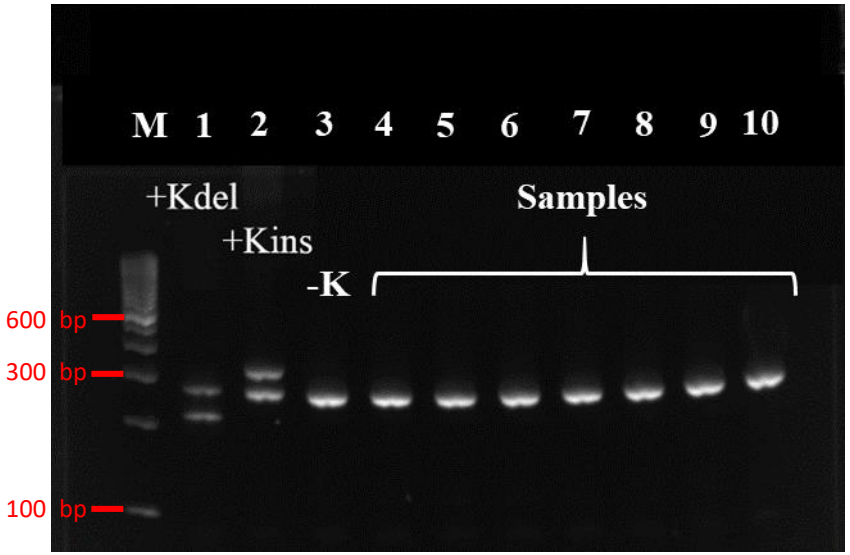

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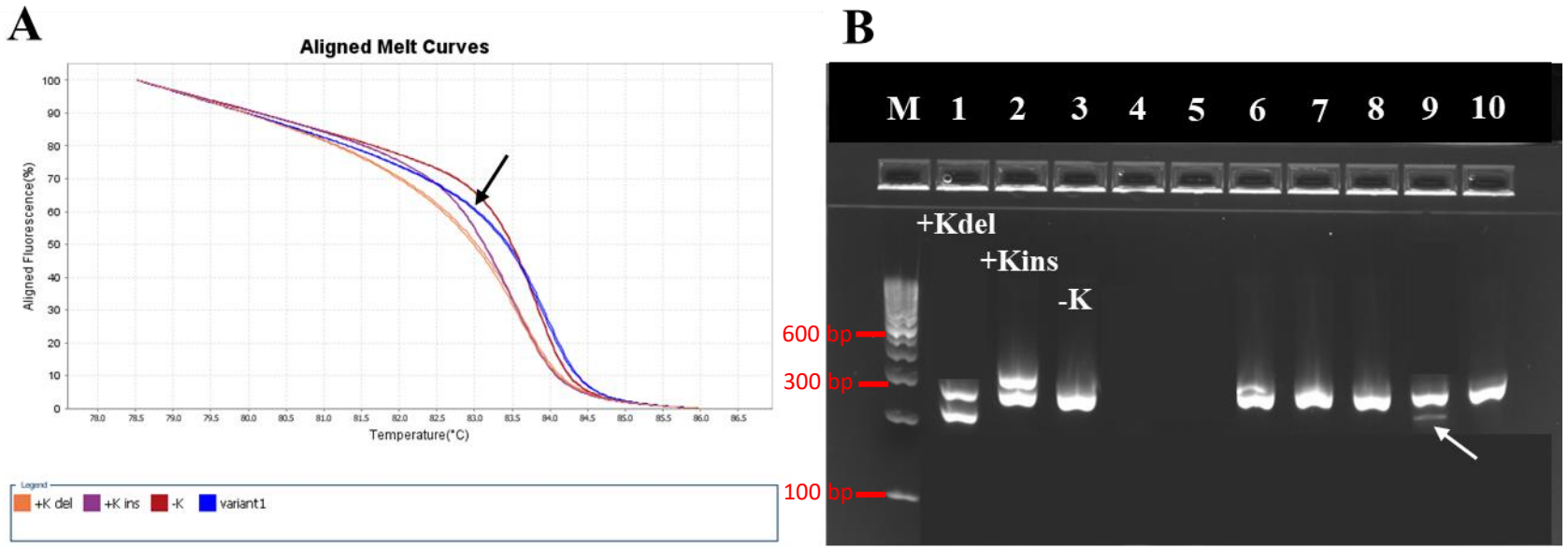

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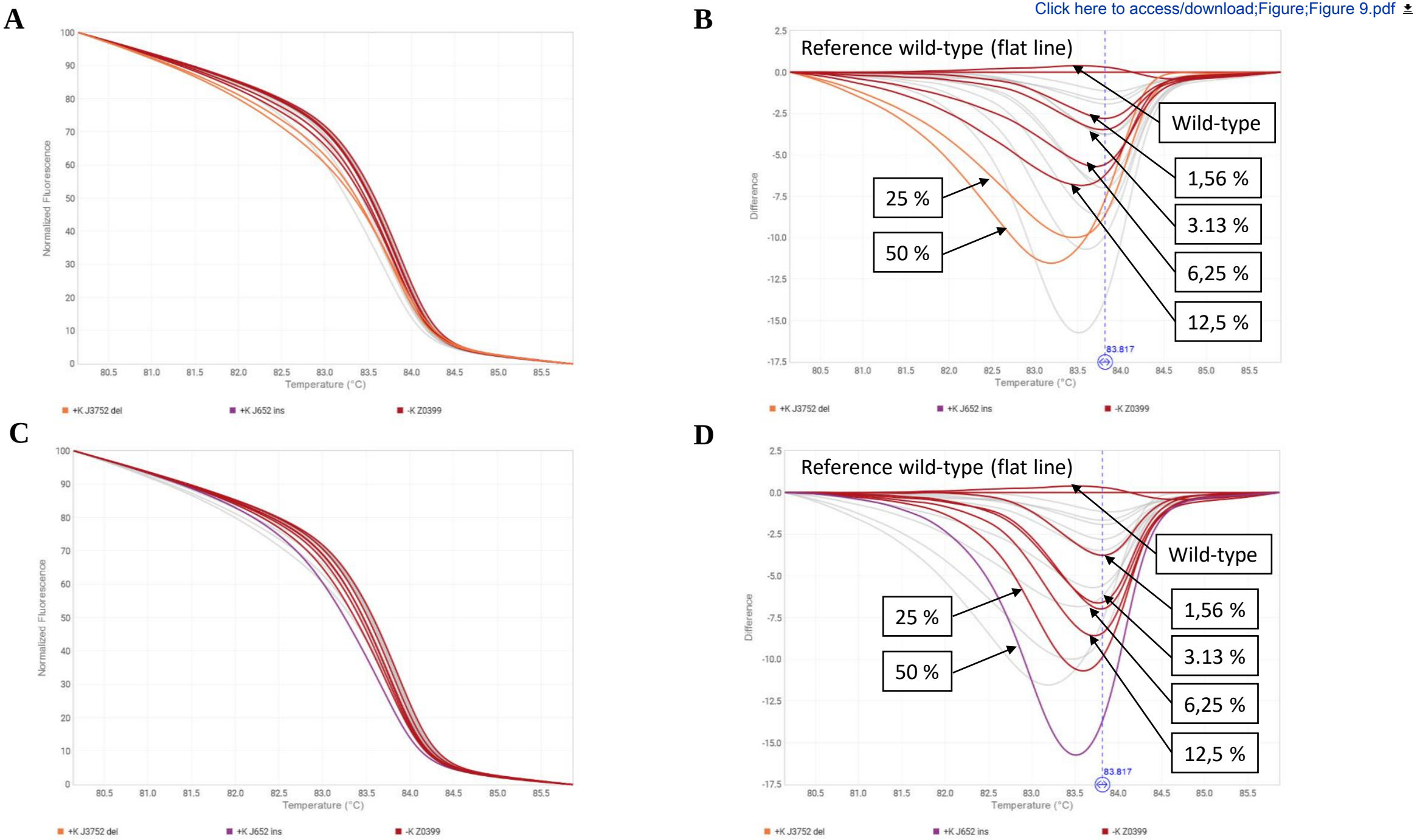

Name	Company
E-Gel EX 4% Agarose	Invitrogen, Thermo Fischer Scientific
Fuorometer 3.0 QUBIT	Invitrogen, Thermo Fischer Scientific
Invitrogen E-Gel iBase and E-Gel Safe Imager Combo Kit	Invitrogen, Thermo Fischer Scientific
MeltDoctor HRM MasterMix 2X	Applied Biosystem, Thermo Fischer Scientific
MicroAmp Fast 96-well Reaction Plate (0.1 mL)	Applied Biosystems, Thermo Fischer Scientific
MicroAmp Optical adhesive film	Applied Biosystems, Thermo Fischer Scientific
NuGenius	Syngene
Primer CALRex9 Forward	Eurofins Genomics
Primer CALRex9 Reverse	Eurofins Genomics
QIAamp DNA Mini Kit	QIAGEN
Qubit Assay Tubes	Invitrogen, Thermo Fischer Scientific
QUBIT dsDNA HS assay	Invitrogen, Thermo Fischer Scientific
Trackit 100bp DNA Ladder	Invitrogen, Thermo Fischer Scientific
ViiA7 Real-Time PCR System	Applied Biosystems, Thermo Fischer Scientific
Water nuclease free	VWR, Life Science

Catalog Number	Comments
G401004	
Q33216	
G6465EU	
4415440	Components: AmpliTaqGold 360 DNA Polymerase, MeltDoctor trade HRM dye, dNTP blend including dUTP, Magnesium salts and other buffer components, precisely formulated to obtain optimal HRM results
4346907	
4311971	
NG-1045	Gel documentation systems
	Sequence: 5'GGCAAGGCCCTGAGGTGT'3 (High-Purity, Salt-Free)
	Sequence: 5'GGCCTCAGTCCAGCCCTG'3 (High-Purity, Salt-Free)
51306	DNA isolation kit with the buffer for DNA dilution.
Q32856	
Q32854	
10488058	Ladder consists of 13 individual fragments with the reference bands at 2000, 1500, and 600 bp.
4453534	
436912C	RNase, DNase and Protease free water

Dear Dr. Nguyen,

The following concerns have been raised regarding our manuscript. We have tried to address all of them and have improved our manuscript accordingly. These are our point by point answers:

Editorial comments:

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 1.12: Provide an example figure showing the desired plot.

• **Protocol Numbering:** Add a one-line space between each protocol step.

– *has been included in the manuscript.*

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
2) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
3) Notes cannot be filmed and should be excluded from highlighting.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

• **Figures:**

-We added an additional figure according to the comment of the reviewer #1, Figure 1. Schematic representation of the algorithm for the somatic genetic variant detection in the CALR gene by HRM, agarose gel electrophoresis and sequencing methods. The number of the other figures changed accordingly.

1) Fig 6, fig 7b: provide molecular weight markers.

- *Molecular weight marker was added to the fig 7 (before fig 6) and fig 8b (before fig 7b).*

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are E-gel EX, biotix, utip, Invitrogen,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding

language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

- *Have been changed, blur out*

2) Fig 4,5: blur out product names.

- Have been changed, blur out (Fig 5,6)

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors demonstrated that HRM and agarose gel electrophoresis are effective methods to identify types of CALR mutation.

Major Concerns:

1. The abstract was not correlated with the manuscript since it did not provide the information about methods and results of the manuscript. – *the abstract consists of the description of the method and we added our clinical results*
2. The authors should describe more information about material and methods.
- Population (MPN patients, which type?) and sample size calculation – *we have included the necessary patient population information in the text*
- Ethical consideration for analysis of specimen – *it was addressed in the text*
- Overview of method such as performed HRM, gel electrophoresis, and Sanger sequencing concurrently or in sequential? – *we have included a figure showing our step-wise approach to CALR mutation detection with HRM used as a screening method, gel electrophoresis as a confirmation method and Sanger sequencing in determining non type 1 or type 2 mutations*
- Describe about statistical analysis – *does not apply to our manuscript*

Minor Concerns:

1. The conclusion that "HRM is powerful method for genotyping and genetic variation scanning" should be specific for CALR mutation according to this study.

Reviewer #2:

Manuscript Summary:

Pajič et al describe the use of high resolution melt curve analysis to detect somatic mutations in the CALR gene. They discuss the use of the ViiA7 Real-Time PCR System and associated software to analyse a sample number of samples and controls for type 1 and type 2 CALR mutations.

Major Concerns:

1) The manuscript is very specific to the type of machine and reagents used. Although it demonstrates that CALR mutations can be detected using HRM the protocol presented is not directly transferable to other HRM platforms. The most difficult aspect of HRM is the analysis and applying the correct software parameters and this will vary greatly for each amplicon analysed and each HRM platform used.

- It is also true for many other molecular genetic tests. However, the most important part in the successful implementation of the PCR (qPCR)-based test on different platforms are the primer sequences used in the PCR (qPCR) protocols. Some of the examples are standardization of the PCR primers and protocols in A Europe Against Cancer Program (1,2,3). Such a standardization effort has not been performed for the genetic variant detection in the CALR gene, yet. Therefore, we have searched for the most suitable primer sequences for the our HRM assay and found them in the principal study, where the genetic variants in the CALR gene in patients with Philadelphia chromosome–negative myeloproliferative neoplasms were found (4). We and other (5) demonstrated that the published primer sequences (4) are suitable for the HRM analysis with comparable sensitivity on different HRM platforms.

Nevertheless, the presented protocol covers the most critical steps in the evaluation of the HRM results that can be transferable to other HRM platforms.

We have discussed the subject in the discussion section.

References:

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*2. Gabert, J., Beillard, E., van der Velden, V. et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia – A Europe Against Cancer Program. Leukemia **17,** 2318–2357 (2003). https://doi.org/10.1038/sj.leu.2403135;*

3. van Dongen, J., Langerak, A., Brüggemann, M. et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 17, 2257–2317 (2003). https://doi.org/10.1038/sj.leu.2403202)

*4. Klampfl, T. et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. N. Engl. J. Med. **369**, 2379–2390 (2013).*

5. Bilbao-Sieyro, C. et al. High Resolution Melting Analysis: A Rapid and Accurate Method to Detect CALR Mutations. PLoS One 9, e103511 (2014).

2) No information is provided regarding the level of detection of somatic mutation. This is a very important information to ascertain (or at least discuss) as it impacts greatly on the follow up confirmation of cases where the melt curve is shifted from the normal controls and also determines the lower limit of detection of the technique for this particular amplicon. It would have been useful to see a dilution series of the two positive controls to see the effect of mutational load on the ability of the technique to detect the mutations a variety of levels. In many cases HRM will detect mutations at <5% and so there needs to be some discussion of how to handle these cases as Sanger sequencing will generally only confirm mutations present at c.20%. For cases with low mutational burden a technique such as NGS may be needed to confirm the mutations.
- we have included our data for the level of detection of somatic mutation in the CALR gene (Figure 9) and discuss it in the Representative results and Discussion sections.

3) More data from patient samples could be included to add data concerning the sensitivity and specificity of the technique – *we have included data on CALR mutation detection from our center in the discussion*

4) The section on gel electrophoresis should be removed or significantly shortened. This is a standard laboratory technique and does not need to be discussed. It does not require expensive equipment to carry out.
-we significantly shortened the agarose gel electrophoresis section. The agarose gel electrophoresis is the step that we always perform in routine practice and therefore cannot be removed completely.

Minor Concerns:
Suggest that the title is changed to 'Genetic Variant Detection in the CALR gene using High Resolution Melting Analysis' – *the title has been changed*

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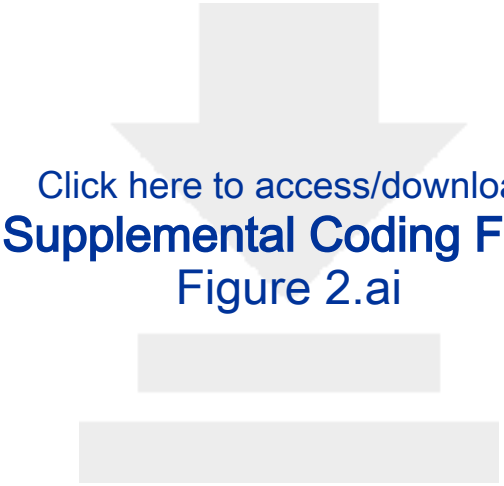

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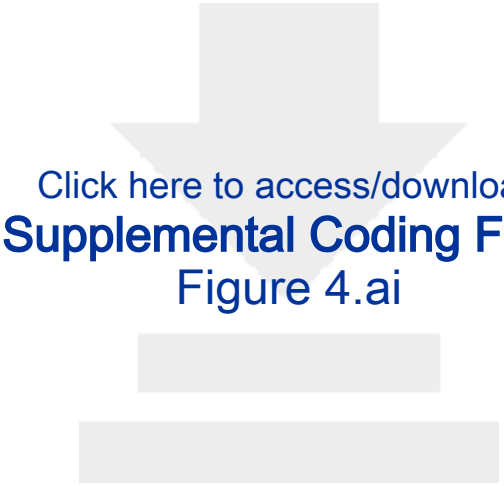

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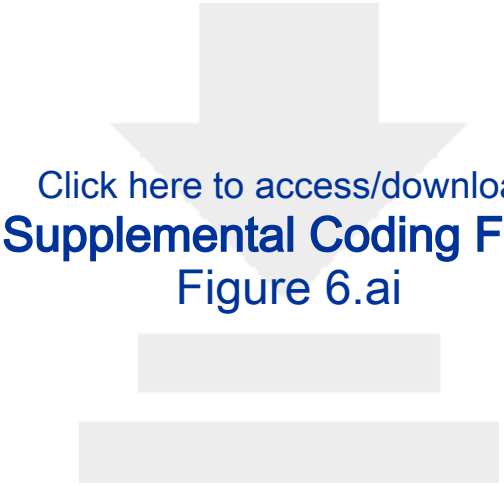

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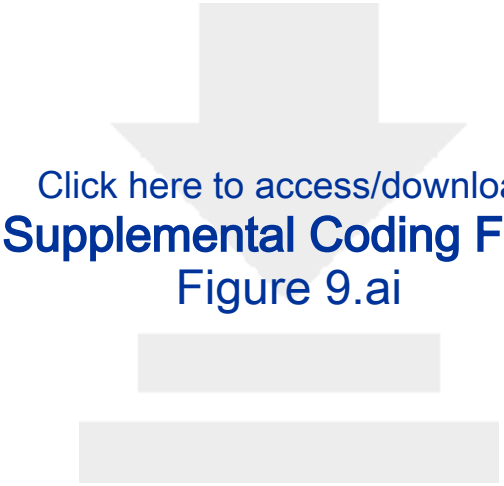

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