Instructions for Use:
HemeScreen® Free Flow Reagents
RUO

Please read these Instructions for Use thoroughly before you use this product.

Keep these Instructions for Use for future reference.

Contents

Manufacturer

Manufacturer		Precipio, Inc. 4 Science Park, New Haven, CT 06511 , USA Phone: 1-203-787-7888

HemeScreen® HRM Screening Reagents RUO

The Research Use Only (RUO) reagents are supplied as ready to use. This Instructions for Use is available as a download on the Precipio website, https://www.precipiodx.com/reagentrental.html.

Intended Use

***For professional use only*.** Precipio's HemeScreen® offerings are Research Use Only (RUO) assays that screen for clinically actionable somatic mutations in targets of interest related to myeloproliferative disorders and haematological cancers. The reagents are designed to be used in a clinical diagnostic laboratory by suitably trained personnel testing DNA extracted from whole blood or bone marrow using a PCR-based method and High Resolution Melt (HRM) Analysis. Samples are run in replicate analysis to confirm the presence of a mutation; however, if the exact mutation sequence is required, a sequencing method such as Sanger Sequencing is needed. In addition, if inconsistent results are observed, Sanger Sequencing or another comparable mutation detection technology can be used to confirm the exact mutation type.

Indications for Use

HemeScreen® HRM Free Flow Reagents (RUO) screen for mutations relevant to *JAK2*, *MPL*, *CALR*, *CEBPA*, *NPM1*, *FLT3*, *KIT*, *IDH1*, *IDH2*, *ASXL1*, *DNMT3A*, *RUNX1*, *SF3B1*, *WT1*, *MYD88*, *CXCR4*, *SF3B1*, and *NOTCH1*. Full list of amplicon coverage and traceability studies are found on pages 17 through 21.

The HemeScreen® HRM Screening Reagents indicate the presence of a potential mutation within a sample but does not confirm the identity of the mutation. To confirm the precise mutation detected, further analysis, such as TaqMan™ detection assays or Sanger sequencing, is required.

Although the results of analysis with this HemeScreen® HRM Screening Reagents will indicate the mutation status of the sample, results obtained using the HemeScreen® HRM Screening Reagents should not be used in making clinical decisions.

Principles of High Resolution Melting

High Resolution Melting (HRM) is a screening technique, which allows the user to determine if the PCR fragment contains a variant or not based upon the dissociation of double-stranded DNA in the presence of an intercalating dye as the temperature increases. As the temperature increases, changes in the fluorescence due to the amplicon dissociation are monitored real-time. It is expected that fragments containing a variant will have slightly different fluorescence profiles than that of Wild-Type DNA which allows for the entire amplicon to be screened for mutations.

Since this is a scanning technique and does not identify an exact mutation, any variant present in the PCR product may differentiate from the Wild-Type DNA, thus leading to a mutation positive result. HRM is a highly sensitive assay, factors such as pipetting errors, cytosine deamination and polymerase errors may give rise to these false positives.

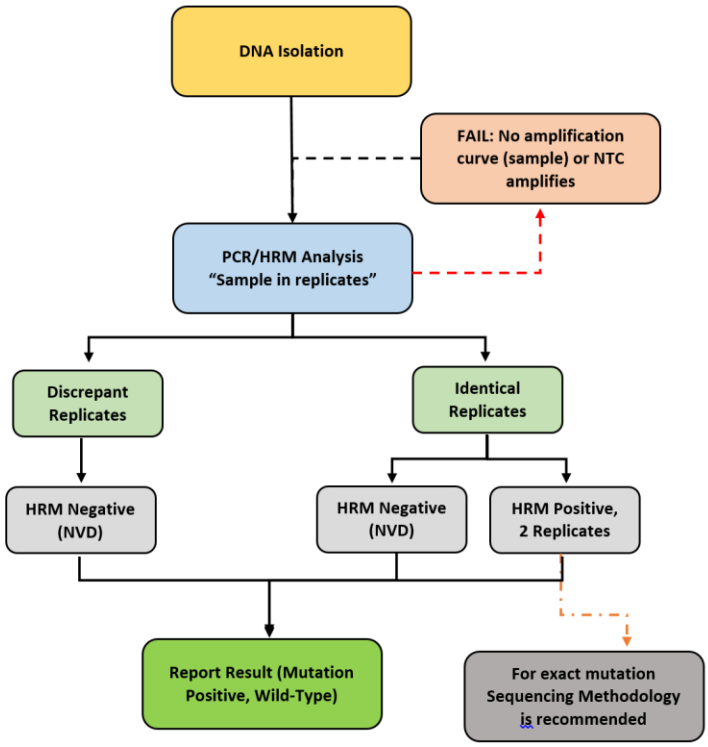
HemeScreen® Reagent Set Preparation

All reagents supplied with the HemeScreen® reagent set are ready to use. The reagents will need to be thawed and spun in a microcentrifuge before use; check details in **HemeScreen® Preparation and Procedure** section below.

Overview of Samples using HemeScreen® HRM Screening Reagents

In general, processing of samples should be carried out from start to finish as described in these Instructions for Use. If processing of a sample has to be stopped before completion of all steps, the DNA should be stored (see **Primary Sample Collection, Handling and Storage** section below). The analysis The HemeScreen® HRM Screening Reagents should only be used in the context of the workflow indicated below.

Figure 1: HemeScreen® HRM Screening Reagents Workflow



Explanation to Figure 1:

1. Isolate the DNA from peripheral blood or bone marrow using standard laboratory procedures.
2. Perform HemeScreen® MPN then review the amplification curves to ensure consistent amplification across all samples and controls
   a. If the control amplification curves are normal, proceed to HRM analysis.
      i. If an individual sample does not amplify, in either replicate, repeat HemeScreen® MPN for that sample.
      ii. If the NTC shows amplification and the CT value is <34, compare the HRM T_M to the target T_M. If the peak melt temperature is the same as the target melting temperature, the reaction is contaminated and cannot be used for further analysis. Repeat HemeScreen® MPN for the amplicon (all samples and controls) in which the NTC failed.
      iii. If the Wild-Type Control does not amplify, the PCR reaction failed. Repeat HemeScreen® MPN for the amplicon (all samples and controls) in which the Wild-Type failed.
   b. If sample amplification is acceptable, HRM analysis will result in either:
      i. A normal melt profile indicating Wild-Type, that is, HRM Negative (NVD); or
      ii. An abnormal melt profile, indicating the presence of a variant. Follow-up HRM with a sequencing methodology to verify the exact mutation or obtain the mutation allelic frequency.
      iii. If there are discrepant replicates the samples is HRM Negative (NVD). The user may repeat the sample for additional confirmation.

Components and Storage Conditions

Each HemeScreen® reagent sets contains enough reactions for 100 samples in duplicate and includes enough Mutant control for 5 reactions.

Reagent sets should be stored in the dark between -18 °C and -25 °C in a constant temperature freezer until use. Note the expiry date of each reagent set received. Do not use after the expiry date has elapsed.

Safety Information, Warnings, and Precautions

1. None of the reagents present a hazard to health in the quantities supplied. The SDS is available on our website, https://www.precipiodx.com.
2. There are no substances of animal or human origin that present a risk of infection in the supplied reagents.
3. These reagents should be used only by those persons who have been trained in the appropriate laboratory techniques.
4. Biological sample handling:
    a. All samples to be treated as hazardous and infectious, when handling whole blood or bone marrow samples always wear a suitable lab coat, disposable gloves, and protective glasses. Use a biohazard container for DNA extraction waste.
    b. Biological waste during the DNA extraction process should be disposed in accordance with national/local regulations relating to biohazard waste disposal.
    c. Liquid waste must be considered infectious and be handled and discarded according to national/local regulations relating to biohazard waste disposal.
5. Sample or Reagent Spillage
    a. Avoid contact with skin and eyes.
    b. Wear appropriate protective clothing, gloves, facemask and glasses.
    c. Absorb the material using gauze.
    d. Clean the surface with 10% (v/v) sodium hypochlorite (10% bleach) and leave the surface wet for 30 min to one hour.
    e. Wash thoroughly with water. Place the waste in suitable container and dispose of in accordance with national/local regulations relating to Bio-hazard waste disposal.
        i. If the spilt liquid contains infectious agents, clean the affected area first with laboratory detergent and water followed with 1% (v/v) sodium hypochlorite.

Materials Required but not Provided

Reagents:

1. MeltDoctor™ HRM Calibration Plate, Fast 96-well (Thermo Fisher Scientific Catalog Number 4425618)
2. TE buffer (Fisher Healthcare Catalog Number AAJ75793AE)
3. Nuclease free water (Fisher Healthcare Catalog Number AM9932)
4. DNA Extraction Kit and associated reagents

Consumables/Supplies:

1. MicroAmp™ Plates or equivalent (Fisher Healthcare Catalog Number 4483485)
2. MicroAmp™ Optical Adhesive Film (Fisher Healthcare Catalog Number 4360954)
3. Pipettes/Pipette tips
4. Vortex
5. 1.5 mL Microcentrifuge tubes
6. Microfuge capable of holding 1.5 mL tubes
7. Microfuge capable of holding 96-well plates

Instrument:

1. QuantStudio™ 3 Real Time PCR System with the 96-well, 0.1 mL block or equivalent.

NOTE: This assay has been optimized using the QuantStudio™ 3 Real-Time PCR System (96-well, 0.1 mL). Alternative platforms

may require further optimization per the platform specifications.

Primary Sample Collection, Handling and Storage

Sample type:

- This assay can be utilized with peripheral blood and bone marrow.

DNA Extraction:

- The quality of the assay and the consistency of HRM is highly dependent of the quality of the analyte. All samples and controls should be extracted with the same high quality DNA extraction method and protocol to avoid any variations due to reagents/buffers. Each extraction should have an A_{260}/A_{280} ratio of 1.8 - 2.0 and an A_{260}/A_{230} ratio of 2.0 - 2.2 and must be diluted to the same starting concentration (10 ng/µL) with the same dilution buffer. Ensure sample-to-sample uniformity. Lastly, any additional control DNA to be analyzed should be of the same quality and quantity as the sample DNA.

DNA Storage:

- Extracted DNA can be stored at 4 °C for short term storage and use (up to 4 weeks) or at -20 °C for long term storage (up to 3 years).

HemeScreen® Preparation and Procedure

NOTE: For background information and general HRM workflows, refer to the Manual, Applied Biosystems High-Resolution Melt Experiment Getting Started Guide (Publication Number MAN0014394)

NOTE: The QuantStudio™ is factory calibrated. Initial Dye calibrations expire after two years. The HRM calibration must be performed prior to the first HRM assay. The field technician will not complete the MeltDoctor™ HRM calibration plate. This plate must be run on the instrument prior to running HemeScreen®.

1.1. Recommendations Before You Start:

1.1.1. The following procedures are optional but highly recommended prior to PCR setup:

1.1.1.1. Turn on UV light inside hood or a UV crosslinker (15 min).

1.1.1.2. UV crosslink empty 1.5 mL tubes and sufficient TE buffer for DNA dilution (10 min).

1.1.1.3. Make sure all work areas are prepared for analysis of low-level mutations. This includes correct use of the PCR Workstation, dedicated pipettes, tips, 75% ETOH solution and/or DNA Away™ solutions.

1.2. DNA Dilutions:

1.2.1. Prepare the calculations to dilute all extracted sample DNA to 10 ng/µL in TE buffer.

OPTIONAL: Use the provided excel spreadsheet to prepare the working concentration of each sample.

1.2.1.1. Record each sample name, DNA concentration, and associated $A_{260/280}$ and $A_{260/230}$ ratios.

1.2.1.2. Each dilution calculations will populate automatically.

1.2.1.2.1. If the original sample is < 10 ng/µL, re-extract the sample. For best performance, a consistent DNA input is required.

1.2.1.2.2. For pipetting convenience, prepare a final dilution volume of at least 40 µL, provided enough DNA is available. The total volume may be adjusted as needed.

NOTE: It is not recommended to pipet less than 1 µL of stock DNA.

Example of the dilution calculator spreadsheet.

Sample Information Input				Calculated Dilution Volumes	
Sample ID	ng/µL	260/280	260/230	µL Stock	µL TE Buffer
Sample-1	125.4	1.8	2.1	3.19	36.81
Sample-2	45.2	1.8	2.2	8.85	31.15
Sample-3	98.5	1.9	2.0	4.06	35.94
				40.00	0.00

1.2.2. Print the sheet containing the plate layout and dilution calculation sheet.
1.2.3. Label individual 1.5 mL tubes with sample name or case number.
1.2.4. Add the required volume of TE buffer to the 1.5 mL tubes.
  1.2.4.1. Prior to adding the required volume of stock DNA to the TE buffer, ensure the sample has been adequately vortexed and spun down.
  1.2.4.2. Close the tubes, vortex, and centrifuge for 5 sec.

NOTE: Please make sure you are using different tips while adding stock DNA to 1.5 mL tubes.

1.2.5. Measure all dilutions to ensure the concentration is 10 ng/µL. All dilutions should not be more than +/- 1 µL from each other. If there are any outliers, a new dilution must be created and re-measured.

1.3. HemeScreen® Layout Setup:

1.3.1. We recommend a layout with 20 samples in duplicate and three controls. Use the layout that works best for your applications. The layout you choose will have no impact on HRM results.

Figure 2: Example HemeScreen® 20 Sample Plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	FF RXN, S1	FF RXN, S1	FF RXN, S9	FF RXN, S9	FF RXN, S17	FF RXN, S17						
B	FF RXN, S2	FF RXN, S2	FF RXN, S10	FF RXN, S10	FF RXN, S18	FF RXN, S18						
C	FF RXN, S3	FF RXN, S3	FF RXN, S11	FF RXN, S11	FF RXN, S19	FF RXN, S19						
D	FF RXN, S4	FF RXN, S4	FF RXN, S12	FF RXN, S12	FF RXN, S20	FF RXN, S20						
E	FF RXN, S5	FF RXN, S5	FF RXN, S13	FF RXN, S13								
F	FF RXN, S6	FF RXN, S6	FF RXN, S14	FF RXN, S14								
G	FF RXN, S7	FF RXN, S7	FF RXN, S15	FF RXN, S15								
H	FF RXN, S8	FF RXN, S8	FF RXN, S16	FF RXN, S16	FF RXN, MUT	FF RXN, NTC						

1.3.2. Create a plate layout containing target names and sample ID. Ensure the plate layout is saved as a .txt file. This file will be imported into the QuantStudio prior to running the HemeScreen®.

1.4. Preparation of Plate and Addition of DNA:

1.4.1. Remove the Primer Reagents with the 5% Control from freezer and thaw on ice. Thaw the reagents for a minimum of 20 min at room temperature, protected from light.
1.4.2. Reagents are **READY TO USE**.
1.4.3. Once thawed, vortex controls ~3-5 sec to mix thoroughly. Briefly centrifuge 5 sec to ensure no liquid remains on tube lids and place on ice.
1.4.4. Mix the Primer Reagents by inverting the tube several times and briefly spin it down. Keep it on ice and protect from light.
1.4.5. Aliquot 18 µL of the Primer Reagent to the relevant wells of a MicroAmp Optical 96-Well Reaction Plate as defined by your sample layout.
1.4.5.1. Add 18 µL of Primer Reagent, to designated wells for samples and controls.
1.4.5.2. To appropriate wells, add 2 µL of each DNA sample, Mutant Control (5%), or No Template Control (NTC).
1.4.5.3. Use separate pipette tips for each sample and avoid cross-contamination of the samples by splashing.
1.4.5.4. Pipette-mix to ensure sample is well mixed into reaction mixture.

NOTE: Addition of the reagent set controls last lessens the chance of contamination in test sample wells.

1.4.6. Place MicroAmp Optical Film over plate and seal.
1.4.7. Spin the plate briefly (1 min) to eliminate any air bubbles from the solution.
1.4.8. Visually inspect the plate for proper sealing. Ensure there are no air bubbles present in the plate.
1.4.9. Place the plate on the instrument.

1.5. Amplification and Melt Curve/Dissociation on QuantStudio™ 3:

1.5.1. Open the QuantStudio™ v1.5.1 software.
1.5.1.1. Create a new experiment > From Template (see **Figure 3 & 4**).
1.5.1.1.1. Select FILE, NEW EXPERIMENT, FROM TEMPLATE then browse to the "HemeScreen® QuantStudio™ 3 Template.edt" file from the POL folder (see **Figure 3 & 4**).
1.5.1.1.2. This .edt file provided by Precipio has the thermal cycler profile already programmed under Experimental Method. See **Figure 5** for a screenshot of the entire thermal cycler program.
1.5.1.1.2.1. Do not change or alter any of the parameters of the thermal cycler program.
1.5.1.1.3. See **Table 1** for the thermal cycler protocol.
1.5.1.2. Input the experiment name under the properties tab.
1.5.1.2.1. Recommended to format as DD_MM_YY_PlateBarcode_tech initials
1.5.1.2.2. Confirm the following run options:
1.5.1.2.2.1. Instrument type: QuantStudio™ 3 System
1.5.1.2.2.2. Block type: 96-Well 0.1-mL Block
1.5.1.2.2.3. Experiment type: Standard Curve
1.5.1.2.2.4. Chemistry: Other
1.5.1.2.2.5. Run mode: Standard
1.5.1.3. Click **Plate** in the **top row to open Assign Targets and Samples** tab**.**
1.5.1.4. Select **File -> Import Plate Setup…** to import the HRM Plate Layout Excel file made in earlier step. (See **Figure 6**). The samples, plate layout, and target(s) are now assigned.
1.5.1.5. Confirm all sample wells have been assigned a sample name and target. If there are empty wells, you can confirm there is no sample name or Target assigned to the well.
1.5.1.5.1. Click Advanced Setup. In the 96-well Plate Layout view, select the wells you would like to exclude from analysis and then uncheck the sample name and target from the advanced setup pane on the left side.
1.5.1.6. Touch ⏏ to eject the instrument drawer.
1.5.1.6.1. Place the plate in the drawer so well A1 of the plate is in the top left corner.

NOTE: Before clicking start run, make sure your instrument is connected to your laptop. You should see your instrument serial number in the drop-down menu.

1.5.1.7. Click on **START RUN** followed by the specific instrument serial number to start the run (see **Figure 7**).
1.5.1.8. The software will ask you to save the run. Please save the run in your designated HemeScreen® POL folder (i.e. \Egnyte\shared\HemeScreen POL**YOUR SITE**).
1.5.1.9. The run automatically starts after saving the file.

NOTE: QuantStudio™ automatically saves the run file on the instrument, if you failed to save the run on the computer you can always retrieve .eds file from the QuantStudio™.

TIP: At this stage PCR/HRM products can be stored at less than or equal to -20 °C for up to one week.

Figure 3: Create a new experiment from template.

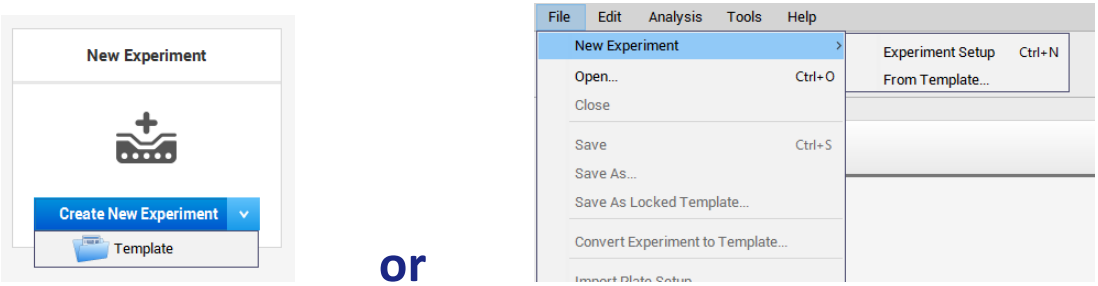


Figure 4: HemeScreen™ QuantStudio™ Template.

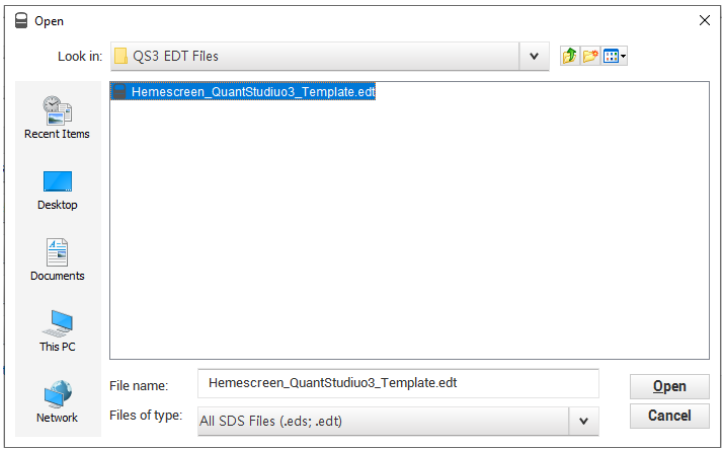


Figure 5: HRM thermal cycler conditions.

Make sure reaction volume is 20 µL (blue arrow), data collection points are selected in step 3 of PCR stage and step 3 of Melt curve stage (black arrows). Make sure PCR stage has 40 cycles (red arrow) entered and “continuous” is checked (green arrow).

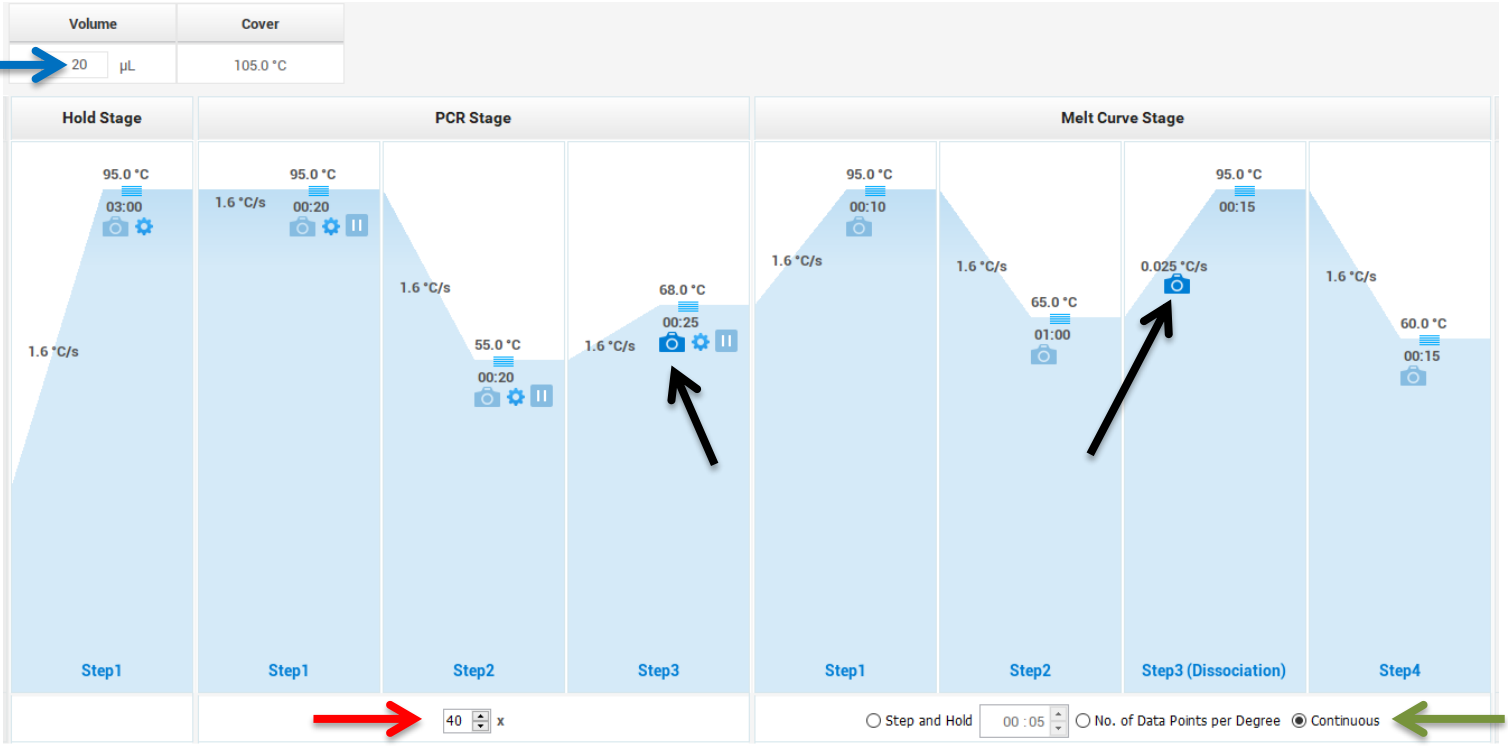


Table 1: Thermal cycling and melt curve program for the HRM reaction.

Stage	Step	Temp	Time	Ramp rate
Holding	Enzyme activation	95°C	3 min	1.6 °C/Sec
Cycling (40 cycles)	Denature	95°C	20 sec	1.6 °C/Sec
	Primer Annealing	55°C	20 sec	1.6 °C/Sec
	Extension (Data Capture on)	68°C	25 sec	1.6 °C/Sec
Melt curve/Dissociation	Denature	95°C	10 sec	1.6 °C/Sec
	Anneal	65°C	1 min	1.6 °C/Sec
	High resolution melting (Data Capture on)	95°C	15 sec	0.025 °C/Sec
	Anneal	60°C	15 sec	1.6 °C/Sec

Figure 6: Import Plate Layout files.

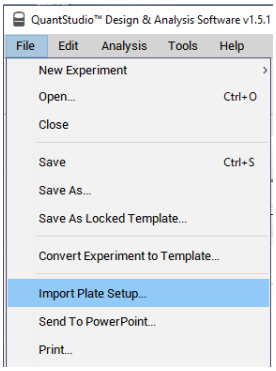
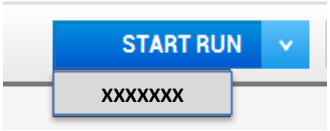


Figure 7: Starting the run.



Quality Control of the Amplification Data

NOTE: This guide is written specific to the QuantStudio™ 3 Real-Time PCR System. If you are using a similar system, please refer to that system's user guide for guidance.

2.1 Normal characteristics of HemeScreen® Amplification

2.1.1 Review the amplification data in QuantStudio™ Design & Analysis Software v1.5.1.

2.1.2 General QC metrics from the Real-Time PCR amplification are as follows; however, results may vary due to instrument to instrument variation, PCR setup, etc. See **Figure 8** for an example amplification plot.

2.1.2.1 Genomic DNA typically amplifies between 24 - 32 cycles (C_t) when 20 ng of quality DNA is added to the PCR. An exponential increase in fluorescence is expected until the reaction reaches plateau.

2.1.2.2 Plasmid control DNA typically amplifies between 18 - 28 cycles (C_t) when controls are used as directed. An exponential increase in fluorescence is expected until the reaction reaches plateau.

2.1.2.3 NTC reactions typically have undetermined C_t values.

2.1.2.3.1 In some instances, primer dimers form during the amplification and give rise to smaller, broader PCR products; however, they have higher C_t values (>34 cycles) as compared to samples and controls. See **Figure 9** for an example.

NOTE: Document which samples are outliers of the suggested QC metrics. The outliers may produce erroneous HRM results

2.1.3 In the navigation pane, click **Analysis** and then select **Melt Curve**.

2.1.4 Verify the Dissociation Curve/Melt Curve shows no unexpected Tm peaks.

2.1.4.1 Unexpected peaks may indicate contamination, primer dimers, or nonspecific amplification.

2.1.4.2 The data may appear noisy because more data is collected during a high resolution melt curve than during a standard melt curve. The extra data are required for analysis with the High Resolution Melting Software.

2.1.4.3 If the amplified sequence contains more than one variant or a more complex mutation, there may be more than one Tm peak.

2.1.5 Continue to High Resolution Melt Analysis.

Figure 8: Example Amplification Plot.

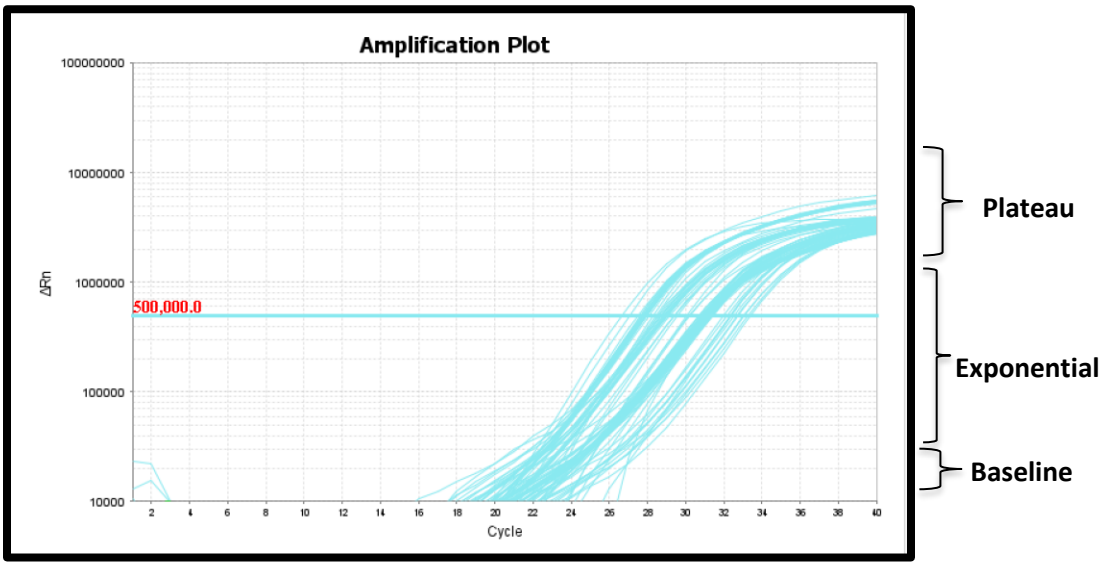
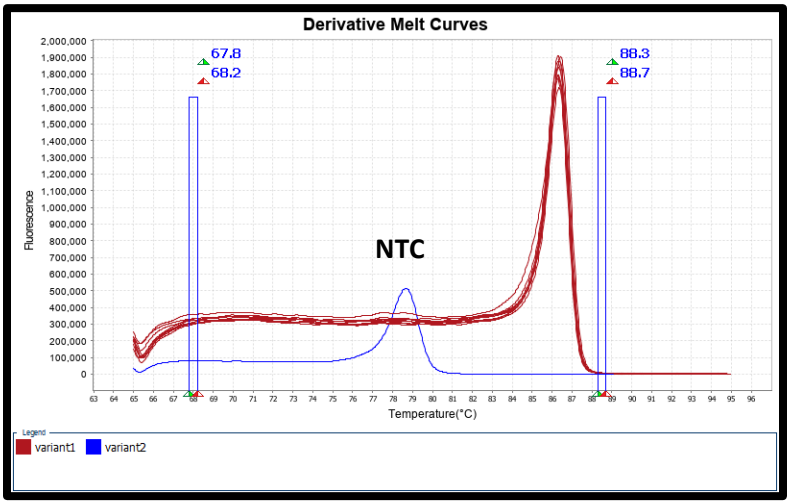


Figure 9: Example of Non-Specific Amplification in the NTC due to primer dimer formation.



*Melt temperature will vary depending on the target amplicon.

HemeScreen® Analysis:

Guidance for HRM data analysis is given in this manual; however, due to instrument-to-instrument variation, calibration variation, as well as other factors, data analysis parameters for a given data set will need to be determined by the end-user.

NOTE: For background information and general HRM Analysis, refer to the Manual, Applied Biosystems High-Resolution Melt Curve Analysis Module User Guide (Publication Number MAN0014823).

3.1. Analysis

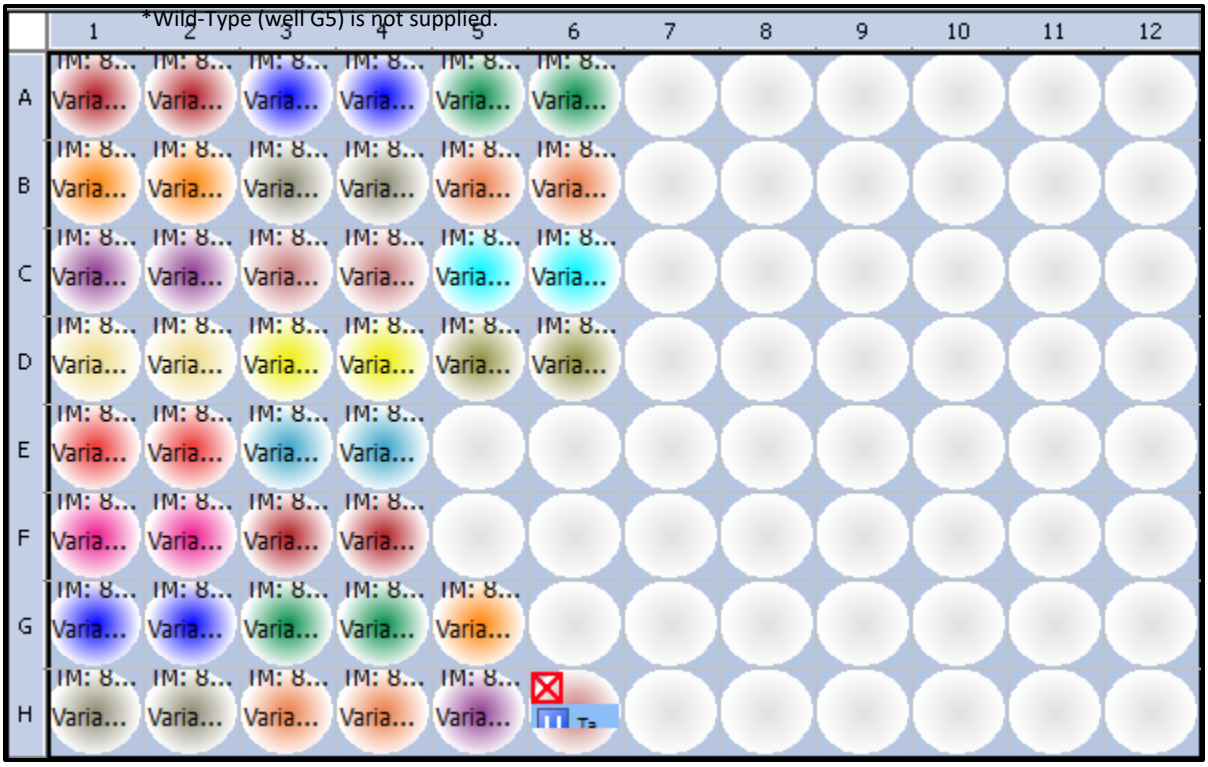
3.1.1. Review the melt curves using High Resolution Melt Software v3.2.

3.1.2. Open High Resolution Melt Software v3.2. Click **Open** on the main window to open the saved *.eds run file saved in step 1.5.1.8.

3.1.3. The example plate layout below includes five targets.. Each target of interest must be analyzed one at a time (i.e., *CALR* only).

3.1.3.1. To omit samples or NTCs from analysis, select specific samples in the **Plate Layout** tab, and then right-click and choose **Omit**. Omitted samples are indicated by "☒" in the **Plate Layout** Tab. (See **Figure 10**).

Figure 10: Example plate layout with omitted NTC reaction.



NOTE: Omit any outliers and NTCs from further analysis (unless NTCs exhibited possible contamination in previous steps).

3.1.4. To analyze each target:

3.1.4.1. Click the **Derivative Melt Curves** tab in the High Resolution Melt Plots pane.

3.1.4.2. Omit any wells that do not contain the target of interest. Wells can be omitted by selecting, right clicking and selecting "omit". Select any wells that remain, ensure there is a WT, MUT and NTC selected for the target of interest.

3.1.4.3. Select Analyze on the upper right hand corner.

3.1.4.4. Review the data. If there are any outliers (samples with poor amplification), omit from analysis at this point. Note that samples with variations give rise to different possible peak shapes.

3.1.4.5. Review and adjust the pre-melt and post-melt regions to optimize your separation and variant calls. For most targets, set the pre-melt and post-melt regions as close as possible to the derivative curve peak (see **Figure 11**).

3.1.4.5.1. If no data appears in the plots, click the green Analyze button and double check that the wells you are analyzing are selected.

3.1.4.5.2. The pre-melt and the post-melt Start and Stop temperature lines should be approximately 0.2 - 0.5

°C apart from each other.

3.1.4.6. Click the **Analyze** button on the top right of the main window.

3.1.4.7. Navigate to the **Difference Plot** tab to review each sample curve.

3.1.4.7.1. Select the reference control (Wild-Type) from the Reference dropdown list (see **Figure 12**). The Wild-Type control well location is determined by the plate layout.

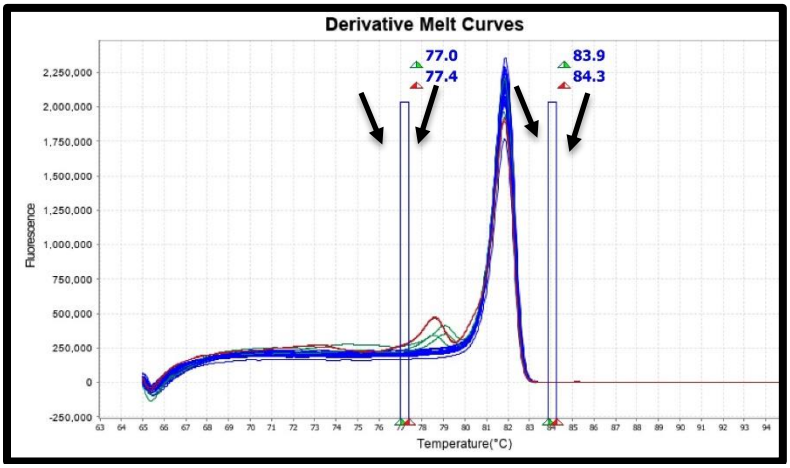
3.1.4.7.2. **Figure 13** shows the Difference Plot that corresponds with **Figure 11**.

3.1.4.8. In some cases, the automated calls may require further review.

3.1.4.8.1. Visually inspect both the Difference Plot and Derivative Melt Curves to verify that no samples exhibit unique melt profiles that may indicate possible variants.

3.1.4.8.2. A manual call can be assigned to these samples by highlighting the curve in either the Difference or Derivative Melt Curve plots, right-clicking and selecting Manual Call.

Figure 11: Example of Derivative Melt Curve.



*Melt temperature will vary depending on the target amplicon.

Figure 12: Reference Drop Down Menu.

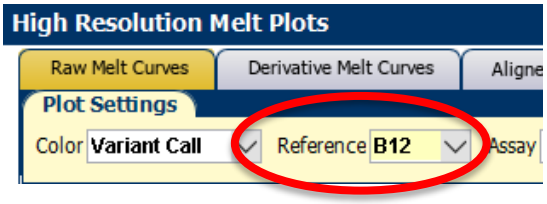
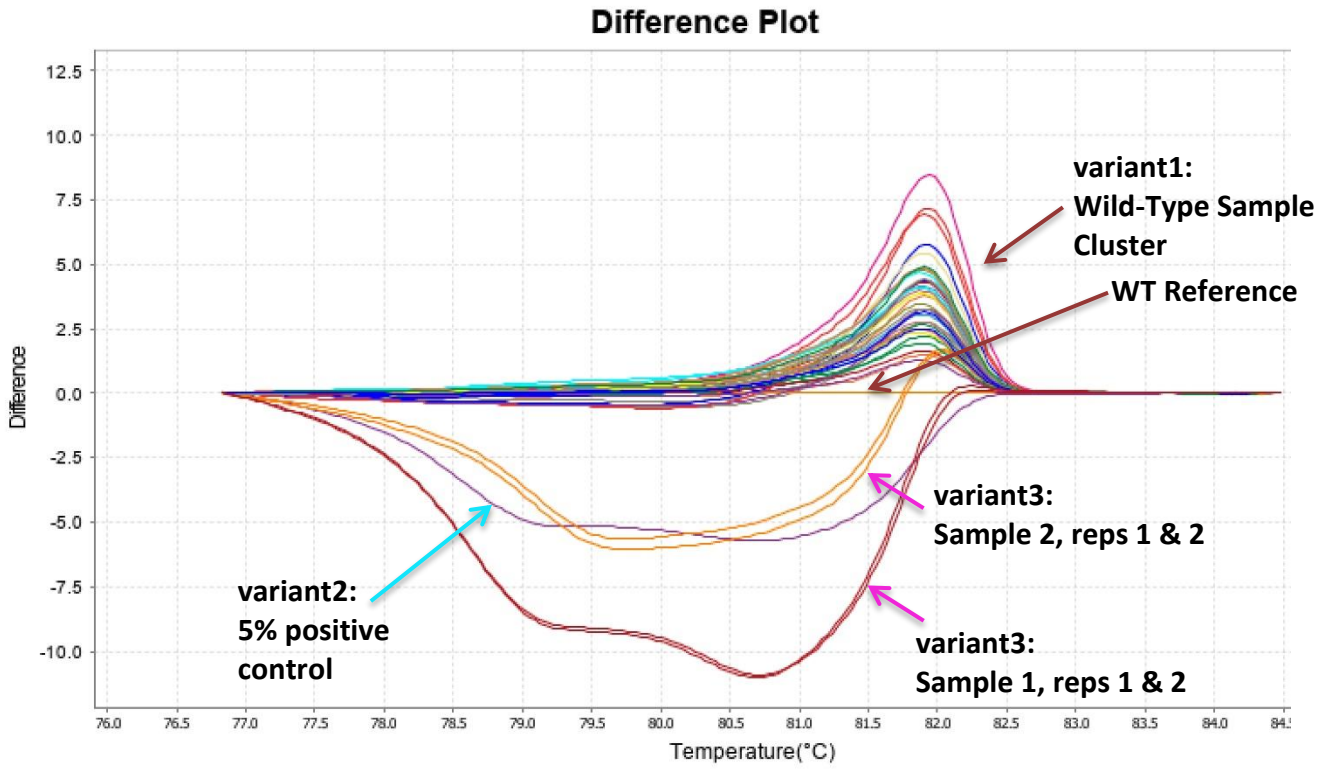


Figure 13: Example *CALR* Exon 9 Difference Plot.

The Difference Plot shows a Wild-Type reference (dark orange) is the flat line at Difference = 0. The cluster of samples will screen NVD. The samples in duplicate (red lines and orange lines) screen positive, since the melt temperature aligns with that of the 5% control (light purple line) and whose difference in fluorescence between the sample curve and the reference curve is greater than that of the 5% control.



*Melt temperature will vary depending on the target amplicon.

3.2. Confirmatory Procedure

3.2.1. Once a specimen has been identified as variant detected (see **Figure 13** above), the sample can be reported out, or confirmed with a secondary method as desired.

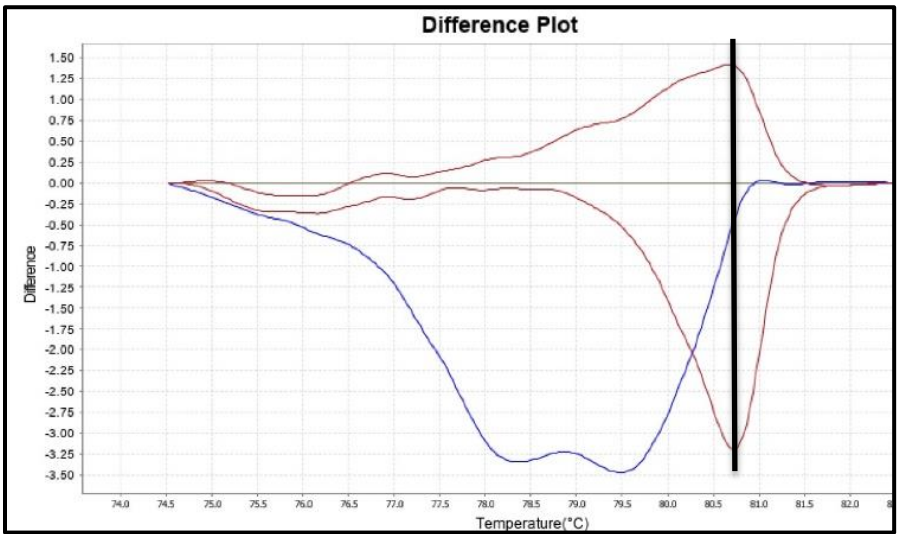
3.2.2. If both replicates are NVD, the case is classified as NVD.

3.2.3. If both replicates are detected, the case is classified as variant detected.

3.2.4. If the replicates are behaving differently, further analysis might be required.

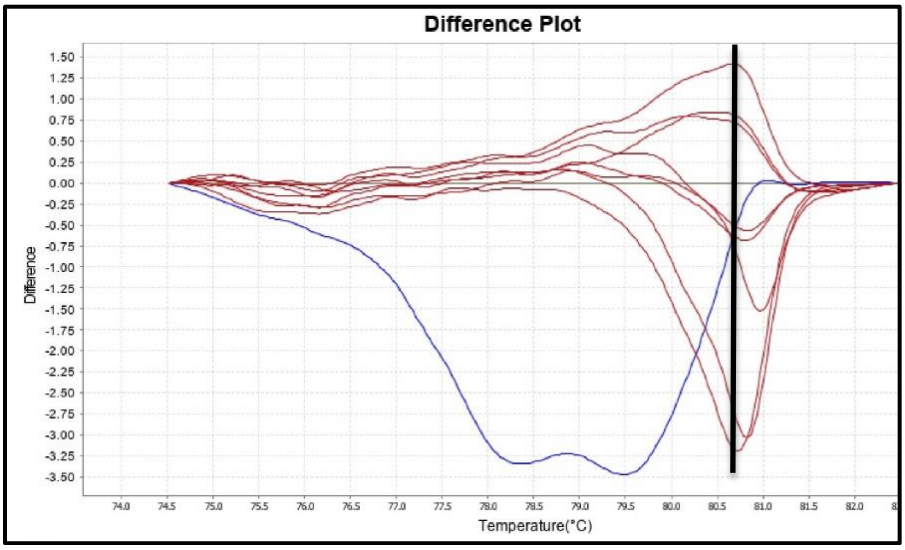
3.2.5. For example Figure 14. In this example, one replicate is dipping below the WT baseline, and the other replicate is peaking above the WT baseline. In this case, the sample will be deemed as NVD. This answer is due to the overall behavior of the replicates. Neither replicate is dipping below the WT baseline with tracking with the MUT control. In addition, even though one replicate is dipping below the WT baseline, it is still grouping with the NVD samples (see Figure 15). The black line seen in Figure 14 and Figure 15 provides a visual ruler showing all NVD specimens behaving in a similar manner that is different than the MUT control.

Figure 14: Example *CALR* Exon 9 Discrepant Replicates.



*Melt temperature will vary depending on the target amplicon.

Figure 15: Example *CALR* Exon 9 Discrepant Replicates with WT Group.



*Melt temperature will vary depending on the target amplicon.

Troubleshooting

Plate Errors:

Plate sealing errors	Make sure the plate is sealed completely. Failure to properly seal each well may lead to evaporation or contaminate the sample block which will affect the analysis.
Air bubbles	Remove any air bubbles as possible by flicking or tapping on the wells before running the plate. Centrifuge the plate for 1 min or longer prior to each run.
Dilution errors	If the dilution measures greater or less than 10ng/µL, repeat the dilution. If the problem persists, re-measure the stock solution to ensure accuracy of the dilution numbers.

Technical Errors:

Cross contamination	To avoid cross contamination, set up HRM reactions in designated pre-PCR areas. Good laboratory practices, especially cleaning the bench and pipettes after reaction setup, are also required to avoid contamination.
NTC contamination	NTC amplification at C_t values < 34 or the Derivative Melt Curve Peaks align at the same melt temp with the Wild-Type or Mutation controls indicate contamination. Repeat the PCR run for samples and controls for that amplicon.

Explanation of Symbols and Warnings

			
Caution, consult accompanying documents	*In vitro* diagnostic medical device	Sterilized using aseptic processing techniques	Keep away from light
			
Use By:	Catalog number	Manufacturer	Batch Code
			
Research Use Only	Consult instructions for use	Temperature Limitation	

Each manufactured lot of HemeScreen® is performance-tested on positive control cell lines to ensure product performance

Each user must make an independent judgment on whether this product is suitable for use in applications conducted in their laboratory. Precipio does not guarantee the successful outcome of any testing based solely on the use of HemeScreen®. Precipio's contribution to these procedures is simply at the step of providing reagents for these procedures.

Biological Reference of MPN Mutations

The HemeScreen® MPN HRM Screening Reagents have been designed to PCR amplify the relevant regions in *MPL*, *CALR*, *JAK2* exon 12, *JAK2* exon 13 and *JAK2* exon 14 genes for subsequent HRM analysis. The HemeScreen® MPN HRM Reagents do not provide quantitative results.

Background of the genes chosen for this panel:

Myeloproliferative neoplasms (MPNs) arise from issues stemming from the bone marrow which lead to abnormally high numbers of certain blood cell types in the blood. Classic MPNs include disorders such as polycythemia vera (PV; Red blood cells), essential thrombocythemia (ET; Platelets), and primary myelofibrosis (PMF; Fibers and blasts). Specific variants in the *JAK2*, *CALR*, and *MPL* genes (see above) are useful biomarkers for these diseases as they can play a role in either disease diagnosis or provide information regarding disease prognosis. These essentially mutually exclusive variants occur in a relatively high frequency, as 98% of sample with PV and 50-65% of samples with ET or PMF exhibit mutations in *JAK2*. Variants in *CALR* and *MPL* are observed in approximately 20-25% or 5-7% of both ET and PMF samples respectively, with only 10-15% of these samples exhibiting triple-negative morphology.

The mutations covered in this reagent set are as follows:

MPL Exon 10:	***CALR Exon 9:***
c.1544G>T; p.W515L	c.1099_1150del; p.L367fs*46
c.1543_1544TG>AA; p.W515K	c.1154_1155insTTGTC; p.K385fs*47
JAK2 Exon 12:	***JAK2 Exon 14:***
c.1611_1616delTCACAA; p.F537_K539delinsL	c.1849G>T; p.V617F
c.1624_1629delAATGAA; p. N542_E543del	
c.1615_1616delAAinsTT; p.K539L	
JAK2 Exon 13:	
c.1711G>A; p.G571S	
c.314G>T; p.G105V	

Biological Reference of AML Mutations

The HemeScreen® AML HRM Screening Reagents have been designed to PCR amplify the relevant regions in *FLT3*, *KIT*, *IDH1, IDH2*, *CEBPA* and *NPM1* genes for subsequent HRM analysis. The HemeScreen® AML HRM Reagents do not provide quantitative results.

Background of the genes chosen for this panel:

- *FLT3* (fms-like tyrosine kinase) mutation is an unfavorable prognostic marker. *FLT3*-ITD is the most common mutation. *FLT3*-TKD mutations (seen in <5% of cases) when combined with *NPM1* mutation has a greater overall prognosis. *FLT3* inhibitors are often paired with chemotherapy for treatment.
- *KIT* mutation expression is found in approximately 80% of cases. *KIT* mutation has a poor prognosis and clinical outcome. *RUNX1* cytogenetic mutation is commonly associated with KIT mutation. Treatments include chemotherapy as well as inhibitors such as Dasatinib and Radotinib.
- *IDH1* (isocitrate dehydrogenase 1) mutation is generally associated with decreased complete remission. *IDH1* mutation is often paired with *NPM1* mutation and normal cytogenetics. *IDH1* mutation has poor prognosis, especially when paired with *FLT3*. When *IDH1* is paired with cytogenetic abnormalities such as PML/RARA, the overall prognosis worsens. *IDH1* mutation alone has a more favorable outcome.
- *IDH2* (isocitrate dehydrogenase 2) mutation is generally paired with normal cytogenetics and does not affect overall prognosis. *IDH2* mutations are often not associated with other prognostic AML mutations, such as *FLT3*, *CEBPA* and *NPM1*; however, they can be associated with *IDH1* mutation.
- *CEBPA* (CCAAT/enhancer binding protein α) mutations can be seen in 15% - 19% of AML samples. *CEBPA* mutations have a favorable prognosis, when no *FLT3* mutation is present; regardless of cytogenetic abnormalities.

- *NPM1* (Nucleophosmin 1) mutations are most common (~50% of cases), and are usually seen in conjunction with other AML-associated mutations. *NPM1* mutations have been suggested as a monitoring tool for MRD due to its stable nature during the course of disease. *NPM1* mutation has a favorable prognosis when it is the only abnormality.

The mutations covered in this reagent set are as follows:

***KIT* Exon 9:**	***KIT* Exon 17:**	***IDH2* Exon 4:**
c.1504_1509dup; p.A502_Y503dup	c.2446G>C; p.D816H	c.418C>G; p.R140G
***KIT* Exon 11:**	c.2446G>T; p.D816Y	c.418C>T; p.R140W
c.1669_1674del; p.W557_K558del	c.2446_2447GA>AT; p.D816I	c.419G>A; p.R140Q
c.1669_1683del; p.W557_E561del	c.2447A>T; p.D816V	c.419G>T; p.R140L
c.1669T>C; p.W557R	c.2458G>T; p.D820Y	c.515G>T; p.R172M
c.1669T>G; p.W557G	c.2459A>G; p.D820G	c.514A>T; p.R172W
c.1669T>A; p.W557R	c.2464A>T; p.N822Y	c.515G>A; p.R172K
c.1676T>G; p.V559G	c.2466T>G; p.N822K	c.516G>T; p.R172S
c.1676T>A; p.V559D	c.2466T>A; p.N822K	c.516G>C; p.R172S
c.1676T>C; p.V559A	c.2467T>G; p.Y823D	***NPM1* Exon 11:**
c.1679T>A; p.V560D	c.2474T>C; p.V825A	c.860_863dup; p.W288Cfs*12
c.1727T>C; p.L576P	***IDH1* Exon 4:**	***FLT3* Exon 14:**
***KIT* Exon 13:**	c.299G>A; p.R100Q	Internal Tandem Duplications
c.1924A>G; p.K642E	c.298C>T; p.R100*	***FLT3* Exon 15:**
c.1961T>C; p.V654A	c.313G>C; p.G105R	Internal Tandem Duplications
Full exon coverage	c.314G>T; p.G105V	***FLT3* Exon 16:**
	c.314G>A; p.G105D	Internal Tandem Duplications
	c.394C>T; p.R132C	***FLT3* Exon 20:**
	c.394C>G; p.R132G	Mutations in codons 835 and 836
	c.394C>A; p.R132S	***CEBPA* Exon 1:**
	c.395G>A; p.R132H	Mutation screening of entire exon.
	c.395G>T; p.R132L	
	c.395G>C; p.R132P	

Biological Reference of Cytopenia Mutations

The HemeScreen® Cytopenia HRM Screening Reagents have been designed to PCR amplify the relevant regions in *WT1*, *ASXL1*, *RUNX1*, *DNMT3A*, and *SF3B1* genes for subsequent HRM analysis. The HemeScreen® Cytopenia HRM Reagents do not provide quantitative results.

Background of the genes chosen for this panel:

- *WT*1 (Wilms' Tumor 1) can lead to higher relapse rate and poor prognosis in patients with acute myeloid leukemia (AML). *WT*1 mutations may be observed in 6-15% of AML cases, often with patients who have *FLT*3-ITD and/or *CEBPA* mutations. Induction chemotherapy resistance has been associated with a *WT*1 mutation. *WT*1 mutations can be used as a tool in determining MRD.
- *ASXL*1 (additional sex combs like 1) mutations can be seen in myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), chronic myelomonocytic leukemia (CMML), refractory cytopenia, persistent cytopenia with early detection of MDS and AML. When present, the mutation is associated with poor prognosis and more aggressive disease. *ASXL*1 mutation occurs in CMML often (~45% of cases), and is rarely seen in PV. *ASXL*1 is often associated with *RUNX*1 cytogenetic abnormalities in both MDS and AML, as well as *CEBPA* in AML. Poor prognosis and aggressive disease is often paired with *ASXL*1 mutation.
- *RUNX1* (runt-related transcription factor 1) mutation is commonly seen in chemotherapy-related MDS, but is also seen in MDS, AML, as well as AML after MDS. Recently *RUNX*1 mutation has been discovered in CML patients. *RUNX*1 mutation is also associated with abnormal cytogenetics, including monosomy 7, trisomy 21 and trisomy 13. *RUNX*1 and monosomy 7 can lead to rapid progression of AML when the patient is being treated for MDS. CEBPA and *NPM*1 are not commonly associated with mutated *RUNX*1. Whereas, *FLT*3 mutation is more commonly associated with *RUNX*1 mutation, approximately 30% in *FLT3*-ITD

cases and 11% in *FLT*3-TKD cases. *RUNX*1 mutation has a poor prognosis regardless of cytogenetic abnormalities and should be considered in the diagnosis of AML.

- *DNMT3A* (DNA methyltransferase 3A) mutation can be seen in AML and MDS. Patients who have MDS with *DNMT3A* mutation have an increased chance of disease transformation to AML. *DNMT3A* mutation has poor prognosis for AML patients, and can be used to monitor treatment. *DNMT3A* mutation is often seen with *IDH*2 and SF3B1 mutations.
- SF3B1 (splicing factor 3b subunit 1) mutation identifies a condition characterized by ring sideroblasts (RS), ineffective erythropoiesis, and indolent clinical course. A large body of evidence supports recognition of SF3B1-mutant MDS as a distinct nosologic entity.

The mutations covered in this reagent set are as follows:

***WT1* Exon 7:**	***RUNX1* Exon 4:**	***SF3B1* Exon 14:**
c.1110dup; p.V371Cfs*14	c.167T>C; p.L56S	c.1866G>T; p.E622D
c.1142C>A; p.S381*	c.319C>T; p.R107C	c.1866G>C; p.E622D
***WT1* Exon 9:**	***RUNX1* Exon 5:**	c.1873C>T; p.R625C
c.1384C>T; p.R462W	c.422C>A; p.S141*	c.1874G>T; p.R625L
c.1385G>A; p.R462Q	c.485G>A; p.R162K	c.1984C>G; p.H662D
c.1385G>C; p.R462P	c.496C>T; p.R166*	c.1986C>G; p.H662Q
c.1390G>A; p.D464N	***RUNX1* Exon 6:**	c.1986C>A; p.H662Q
***ASXL1* Exon 13 codon 591:**	c.592G>A; p.D198N	c.1996A>C; p.K666Q
c.1772dup; p.Y591*	c.602G>A; p.R201Q	c.1996A>G; p.K666E
***ASXL1* Exon 13 codon 635**:	c.610C>T; p.R204*	c.1997A>C; p.K666T
c.1900_1922del; p.E635Rfs*15	***RUNX1* Exon 8:**	c.1997A>G; p.K666R
c.1934dup; p.G646Wfs*12	c.958C>T; p.R320*	c.1998G>T; p.K666N
***ASXL1* Exon 13 codon 693:**	***DNMT3A* exon 23:**	c.1998G>C; p.K666N
c.2077C>T; p.R693*	c.2645G>A; p.R882H	***SF3B1* Exon 15:**
***ASXL1* Exon 13 codon 808:**	c.2644C>T; p.R882C	c.2098A>G; p.K700E
c.2423del; p.P808Lfs*10	c.2644C>A; p.R882S	***SF3B1* Exon 16:**
***ASXL1* Exon 13 codon 1102:**	c.2645G>C; p.R882P	c.2225G>A; p.G742D
c.3306G>T; p.E1102D	c.2644C>G; p.R882G	
	c.2645G>T; p.R882L	

Biological Reference of CLL Mutations

The HemeScreen® CLL HRM Screening Reagents have been designed to PCR amplify the relevant regions in *NOTCH1*, *SF3B1*, *MYD88* and *CXCR4* genes for subsequent HRM analysis.

Background of the genes chosen for this panel:

- *CXCR4* receptor protein activates signaling pathways for proliferation, cell growth and differentiation. *CXCR4* overexpression is associated with more aggressive disease. Studies show an increase in migratory potential in those with high levels of *CXCR4*.
- *MYD88* codes for a protein involved in signaling within immune cells. MYD88 mutation is often related with IgVH-mutation and has shown no significant impact on overall survival. *MYD88* mutation is exclusive of *NOTCH1* and *SF3B1*. *MYD88* mutation appears less frequently when CD38 is greater than or equal to 30%.
- *NOTCH1* (Notch Homolog 1) is considered an oncogene and a tumor suppressor due to the diverse function. Poor prognosis, shorter treatment-free survival and <10 yr overall survival is commonly seen in *NOTCH1* mutated CLL. *NOTCH1* mutation is seen with unmutated IgVH, deletion 13q14, deletion 17p and trisomy 12. Individuals with *NOTCH1* mutated CLL have a higher incidence of Richter's syndrome. *NOTCH1* mutation is often correlated with CD38 being greater than or equal to 30%.
- *SF3B1* (Splicing Factor 3b Subunit 1) is involved in DNA repair; mutation of this gene causes dysregulation of the maintenance of DNA. *SF3B1* mutation is commonly associated with unmutated IgVH, fludarabine-resistance, and concurrent *TP53* mutation. This mutation is associated with poor outcome, <10 yr survival. Studies have sown SF3B1 mutation are later events in diseases

progression.

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The mutations covered in this reagent set are as follows:

MYD88 Exon 3:	***SF3B1* Exon 14:**
c.649G>T; p.V217F	c.1866G>T; p.E622D
c.656C>G; p.5219C	c.1866G>C; p.E622D
MYD88 Exon 4:	c.1873C>T; p.R625C
c.695T>C; p.M232T	c.1874G>T; p.R625L
c.728G>A; p.S243N	c.1984C>G; p.H662D
MYD88 Exon 5:	c.1986C>G; p.H662Q
c.794T>C; p.L265P	c.1986C>A; p.H662Q
c.602G>A; p.R201Q	c.1996A>C; p.K666Q
c.610C>T; p.R204*	c.1996A>G; p.K666E
NOTCH1 Exon 34:	c.1997A>C; p.K666T
c.7541_7542delCT; p.P2514Rfs*4	c.1997A>G; p.K666R
CXCR4 Exon 2:	c.1998G>T; p.K666N
c.598C>T; p.Q200*	c.1998G>C; p.K666N
c.952dup; p.T318Nfs*26	***SF3B1* Exon 15:**
c.959_960del; p.V320Efs*23	c.2098A>G; p.K700E
c.993dup; p.G332Rfs*12	***SF3B1* Exon 16:**
c.997A>T; p.K333*	c.2225G>A; p.G742D
c.1000C>T; p.R334*	
c.1005dup; p.G336Wfs*8	
c.1012_1015del; p.S338Lfs*27	
c.1012dup; p.S338Ffs*6	
c.1013C>A; p.S338*	
c.1013C>G; p.S338*	
c.1014_1017del; p.S339Ffs*26	
c.1021del; p.S341Pfs*25	

Traceability of HemeScreen® HRM Reagent Controls

The “Wild-Type Control” and “Mutant Control” sequences were constructed by synthesis and cloning using the NCBI Reference Sequences:

NC_000001.11 MPL Exon 10
NC_000019.10 CALR Exon 9
NC_000009.12 JAK2 Exons 12, 13 & 14
NC_000019.10 CEBPA Exon 1
NC_000013.11 FLT3 Exons 14, 15, 16, & 20
NC_000002.12 IDH1 Exon 4
NC_000015.10 IDH2 Exon 4
NC_000004.12 KIT Exons 9, 11, 13, & 17
NC_000005.10 NPM1 Exon 11
NC_000020.11 ASXL1
NC_000002.12 DNMT3A
NC_000021.9 RUNX1
NC_000002.12 SF3B1
NC_000011.10 WT1

NC_000009.12 NOTCH1 Exon 34
NC_000002.12 CXCR4 Exon 2
NC_000003.12 MYD88 Exon 3, 4, & 5

A restriction enzyme site was added 5' and 3' of each cloning region in order to linearize the plasmid which reduces the possibility of contamination. DNA sequence was confirmed using Sanger sequencing with the primers supplied in this reagent set.

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