



Instructions for Use: HemeScreen[®] MPN Plated Reagents

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

Keep these Instructions for Use for future reference.

Contents

MANUFACTURER	1
HEMESCREEN® HRM PLATED REAGENTS	2
INTENDED USE	2
INDICATIONS FOR USE	2
BIOLOGICAL REFERENCE OF MPN MUTATIONS	2
PRINCIPLES OF HIGH RESOLUTION MELTING	3
TRACEABILITY OF HEMESCREEN® MPN HRM REAGENTS CONTROLS	3
HEMESCREEN® PLATED REAGENTS PREPARATION	3
OVERVIEW OF SAMPLES USING HEMESCREEN® HRM REAGENTS	3
COMPONENTS AND STORAGE CONDITIONS	5
SAFETY INFORMATION, WARNINGS, AND PRECAUTIONS	5
MATERIALS REQUIRED BUT NOT PROVIDED	5
PRIMARY SAMPLE COLLECTION, HANDLING, AND STORAGE	6
HEMESCREEN® PREPARATION AND PROCEDURE	6
1.1. Recommendations Before You Start:	6
1.2. DNA Dilutions:	6
1.3. HemeScreen® MPN Layout:	7
1.4. Preparation of Plate and Addition of DNA:	8
1.5. Amplification and Melt Curve/Dissociation on QuantStudio™ 3:	8
QUALITY CONTROL OF THE AMPLIFICATION DATA	11
2.1 Normal characteristics of HemeScreen® Amplification	11
HEMESCREEN® MPN ANALYSIS:	12
3.1. Analysis	12
3.2. Results and Further Analysis	15
TROUBLESHOOTING	17
Plate Errors:	17
Technical Errors:	17
LITERATURE REFERENCES	18
TECHNICAL SUPPORT/QUESTIONS	18

Manufacturer

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

HemeScreen® HRM Plated Reagents

The plated reagents are supplied as ready to use. This Instructions for Use is available as a download on the Precipio website, <https://www.precipiodx.com/resources/ifus>

Intended Use

For professional use only. Precipio’s HemeScreen® offerings are assays that detect 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 Reagents detect mutations relevant to Myeloproliferative Disorders (MPD) or Myelodysplastic Syndrome (MDS). The HemeScreen® HRM 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 HemeScreen® HRM Reagents will indicate the mutation status of the sample, results obtained using the HemeScreen® HRM Reagents should not be used in making clinical decisions.

Biological Reference of MPN Mutations

The HemeScreen® MPN HRM 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.

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).¹ 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.²⁻⁵ 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.¹

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	

Instructions for Use

The HemeScreen® MPN HRM Reagents are qualitative. The reagents provide a means to rapidly identify samples for relevant mutations in *MPL*, *CALR*, and *JAK2* as related to myeloproliferative neoplasms.

Principles of High Resolution Melting

High Resolution Melting (HRM) is a 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. Fragments containing a variant will have distinct melt profiles different than that of Wild-Type DNA which allows for the entire amplicon to be analyzed for mutations.

This technique does not identify an exact mutation, therefore 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.

Traceability of HemeScreen® MPN HRM Reagents Controls

The “Wild-Type Control” and “Mutant Control” sequences were constructed by synthesis and cloning for *CALR* Exon 9, *JAK2* Exons 12, 13, & 14, and *MPL* Exon 10 using the NCBI Reference Sequences:

NM_005373.3	<i>MPL</i> Exon 10
NM_004343.3	<i>CALR</i> Exon 9
NM_004972.4	<i>JAK2</i> Exons 12, 13 & 14

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.

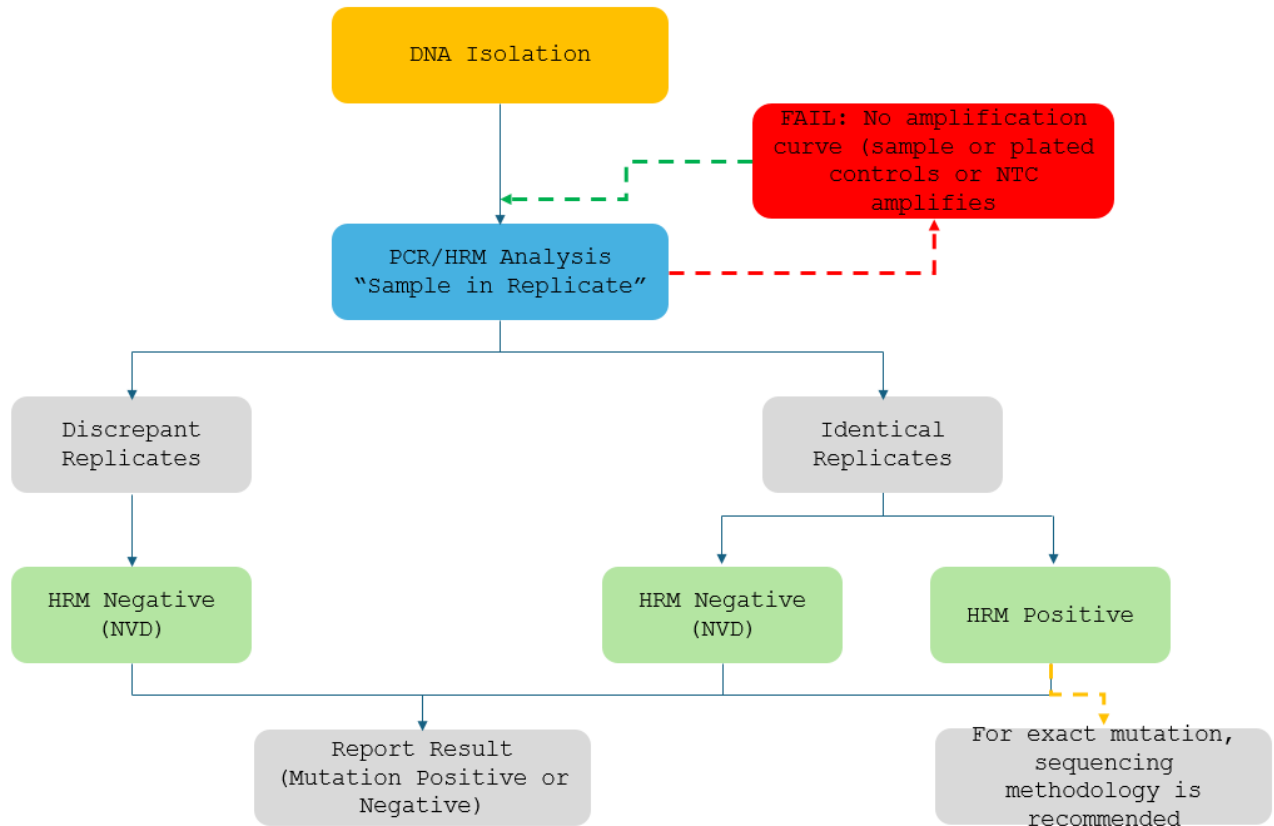
HemeScreen® Plated Reagents Preparation

All reagents supplied with the HemeScreen® plated reagents are ready to use. Each plate must be thawed and spun in a microcentrifuge before use; check details in [HemeScreen® Preparation and Procedure](#) section below.

Overview of Samples using HemeScreen® HRM 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 must be stopped before completion of all steps, the DNA should be stored (see [Primary Sample Collection, Handling, and Storage](#) section below). The HemeScreen® HRM Reagents should only be used in the context of the workflow indicated below.

Figure 1: HemeScreen® HRM Reagents Workflow



Explanation to Figure 1:

1. Isolate the DNA from peripheral blood or bone marrow aspirate using standard laboratory procedures.
2. Perform HemeScreen® MPN assay then review the amplification curves to ensure consistent amplification across all samples and controls. Refer to Section 2.1 for details.
 - 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 Plated controls do 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. Refer to Section 3.2 for details.

Components and Storage Conditions

Each HemeScreen® MPN plated reagent set contains plated reactions for 4 or 8 samples in duplicate and includes a Wild-Type, Mutant, and No Template control (NTC).

Plated reagents should be stored in the dark between $-20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ in a constant temperature freezer until use. Reagents must not be exposed to temperatures below $-22\text{ }^{\circ}\text{C}$ nor come in contact with shelving or cooling coils inside a freezer. 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 are 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 of 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 a 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)

Consumables/Supplies:

1. MicroAmp™ Optical Adhesive Film (Fisher Healthcare Catalog Number 4360954)
2. Pipettes
3. 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/ μ L) 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: New QuantStudio™ instruments are 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 a 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/ μ L in TE buffer.

OPTIONAL: Use the provided excel spreadsheet to prepare the working concentration of each sample. (See figure 2)

- 1.2.1.1. Record each sample name, DNA concentration, and associated A_{260}/A_{280} and A_{260}/A_{230} ratios.
- 1.2.1.2. Each dilution calculations will populate automatically.
 - 1.2.1.2.1. If the original sample is < 10 ng/ μ L, 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 μ L, provided enough DNA is available. The total volume may be adjusted as needed.

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

- 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.
- 1.2.5. Prior to adding the required stock of DNA to the TE buffer, vortex and spin stock DNA.
- 1.2.6. Add the required volume of each stock DNA to the tube containing TE buffer.

NOTE: Please make sure you are using different tips while adding stock DNA to 1.5 mL tubes.
- 1.2.7. Close the tubes, vortex to mix, and spin for 5 sec.
- 1.2.8. Measure all dilutions to ensure the concentration is 10 ng/ μ L. All dilutions must be between 9 and 11 ng/ μ L; Redo or

adjust dilutions if necessary, before proceeding.

1.3. HemeScreen® MPN Layout:

1.3.1. The HemeScreen® MPN Plated reagents are available in 4-Sample and 8-Sample formats.

Figure 2: HemeScreen® MPN Plate layouts for 4 samples per plate (A) and 8 samples per plate (B).

A

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		MPL_Sample 1	MPL_Sample 1	CALR_Sample 1	CALR_Sample 1	JAK12_Sample 1	JAK12_Sample 1	JAK13_Sample 1	JAK13_Sample 1	JAK14_Sample 1	JAK14_Sample 1	
C		MPL_Sample 2	MPL_Sample 2	CALR_Sample 2	CALR_Sample 2	JAK12_Sample 2	JAK12_Sample 2	JAK13_Sample 2	JAK13_Sample 2	JAK14_Sample 2	JAK14_Sample 2	
D		MPL_Sample 3	MPL_Sample 3	CALR_Sample 3	CALR_Sample 3	JAK12_Sample 3	JAK12_Sample 3	JAK13_Sample 3	JAK13_Sample 3	JAK14_Sample 3	JAK14_Sample 3	
E		MPL_Sample 4	MPL_Sample 4	CALR_Sample 4	CALR_Sample 4	JAK12_Sample 4	JAK12_Sample 4	JAK13_Sample 4	JAK13_Sample 4	JAK14_Sample 4	JAK14_Sample 4	
F			MPL_MUT		CALR_MUT		JAK12_MUT		JAK13_MUT		JAK14_MUT	
G			MPL_WT		CALR_WT		JAK12_WT		JAK13_WT		JAK14_WT	
H			MPL_NTC		CALR_NTC		JAK12_NTC		JAK13_NTC		JAK14_NTC	

Input Sample Information		DNA Extraction Info			Required Dilution Volumes	
Sample ID	ng/µL	260/280	260/230	µL Stock DNA	µL TE Buffer	
S1:	Sample 1					
S2:	Sample 2					
S3:	Sample 3					
S4:	Sample 4					

DNA Extraction Dilution Settings ?

Final Volume: **50** µL

Final Concentration: **10** ng/µL

HemeScreen Dilution / Template Generator

- Add Plate Name
- Enter Sample IDs & DNA Extraction Info
- Click the save button to generate and save template files

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	MPL_Sample 1	MPL_Sample 1	CALR_Sample 1	CALR_Sample 1	JAK12_Sample 1	JAK12_Sample 1	JAK13_Sample 1	JAK13_Sample 1	JAK14_Sample 1	JAK14_Sample 1	MPL_MUT	MPL_WT
B	MPL_Sample 2	MPL_Sample 2	CALR_Sample 2	CALR_Sample 2	JAK12_Sample 2	JAK12_Sample 2	JAK13_Sample 2	JAK13_Sample 2	JAK14_Sample 2	JAK14_Sample 2	CALR_MUT	CALR_WT
C	MPL_Sample 3	MPL_Sample 3	CALR_Sample 3	CALR_Sample 3	JAK12_Sample 3	JAK12_Sample 3	JAK13_Sample 3	JAK13_Sample 3	JAK14_Sample 3	JAK14_Sample 3	JAK12_MUT	JAK12_WT
D	MPL_Sample 4	MPL_Sample 4	CALR_Sample 4	CALR_Sample 4	JAK12_Sample 4	JAK12_Sample 4	JAK13_Sample 4	JAK13_Sample 4	JAK14_Sample 4	JAK14_Sample 4	JAK13_MUT	JAK13_WT
E	MPL_Sample 5	MPL_Sample 5	CALR_Sample 5	CALR_Sample 5	JAK12_Sample 5	JAK12_Sample 5	JAK13_Sample 5	JAK13_Sample 5	JAK14_Sample 5	JAK14_Sample 5	JAK14_MUT	JAK14_WT
F	MPL_Sample 6	MPL_Sample 6	CALR_Sample 6	CALR_Sample 6	JAK12_Sample 6	JAK12_Sample 6	JAK13_Sample 6	JAK13_Sample 6	JAK14_Sample 6	JAK14_Sample 6	MPL_NTC	CALR_NTC
G	MPL_Sample 7	MPL_Sample 7	CALR_Sample 7	CALR_Sample 7	JAK12_Sample 7	JAK12_Sample 7	JAK13_Sample 7	JAK13_Sample 7	JAK14_Sample 7	JAK14_Sample 7	JAK12_NTC	JAK13_NTC
H	MPL_Sample 8	MPL_Sample 8	CALR_Sample 8	CALR_Sample 8	JAK12_Sample 8	JAK12_Sample 8	JAK13_Sample 8	JAK13_Sample 8	JAK14_Sample 8	JAK14_Sample 8	JAK14_NTC	

Input Sample Information		DNA Extraction Info			Required Dilution Volumes	
Sample ID	ng/µL	260/280	260/230	µL Stock DNA	µL TE Buffer	
S1:	Sample 1	103			4.9	45.1
S2:	Sample 2	62			8.1	41.9
S3:	Sample 3	71			7.0	43.0
S4:	Sample 4	99			5.1	44.9
S5:	Sample 5	153			3.3	46.7
S6:	Sample 6	37			13.5	36.5
S7:	Sample 7	44			11.4	38.6
S8:	Sample 8	13.5			37.0	13.0

DNA Extraction Dilution Settings ?

Final Volume: **50** µL

Final Concentration: **10** ng/µL

HemeScreen Dilution / Template Generator

- Add Plate Name
- Enter Sample IDs & DNA Extraction Info
- Click the save button to generate and save template files

1.3.2. HemeScreen® MPN Plate Layout Templates (xlsx) are available in the Precipio Cloud storage. The template file can facilitate plate organization and can generate QuantStudio™ plate setup files. Please make sure you pick the correct template for the correct SKU/sample number.


1.3.2.1. Enter the sample names in the “Input Sample Information” section. The 96-well plate view and the QuantStudio™ Template will automatically update.

1.3.2.2. Click to the second tab in the workbook, “QuantStudio Template.” Save As a text file.

1.4. Preparation of Plate and Addition of DNA:

- 1.4.1. Remove the plate from the freezer and thaw the plate in its packaging.
 - 1.4.1.1. Thaw the plate for a minimum of 20 min at room temperature, protected from light.
- 1.4.2. Remove the plate from the packaging. **DO NOT REMOVE** the silicone plate septa seal.
- 1.4.3. Centrifuge the plate for 2 min at 600 to 1000 x g.
 - 1.4.3.1. Confirm that the liquid in each well is at the bottom of the well and the volume is consistent well to well. If not, centrifuge the plate again for another 2 minutes.
 - 1.4.3.2. Keep the bottom of the plate clean. Fluids or other debris that contact the bottom of the plate may dirty the sample block and cause abnormal fluorescent signals.
- 1.4.4. Orient the plate so that well A1 is the top left well.
- 1.4.5. Being very careful, **SLOWLY** remove the plate septa seal from the HemeScreen® MPN Plate.
- 1.4.6. Vortex and spin down all DNA dilutions prior to use.
- 1.4.7. Add 2 µL of diluted DNA sample to each well.
 - 1.4.7.1. Pipette-mix DNA sample in each well.
 - 1.4.7.2. Controls are already added to the appropriate wells (Wild-Type, Mutant, and No Template Controls).
DO NOT ADD any DNA or water/low TE to these wells.
- 1.4.8. Place MicroAmp Optical Film over plate and seal. Ensure the plate is appropriately sealed on all edges by pressing firmly across the top of the plate and around the outside edges with a plate sealing tool.
- 1.4.9. Centrifuge the plate for 1 min to eliminate any air bubbles from the solution. Verify the optical film is adequately sealed and well volumes are consistent before placing 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 QuantStudio3 Template.edt” file from the POL folder (see [Figure 3 & 4](#)).
 - 1.5.1.1.2. This .edt file has the thermal cycler profile pre-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 unless advised by Precipio Support.
 - 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. Import the Plate layout file. Select **File -> Import Plate Setup...** Select and upload the text file created in step 1.3.2.2 (See [Figure 6](#)) to assign the sample IDs, plate layout, and target(s).
 - 1.5.1.4. Click to the “Plate” menu to confirm all sample wells have been assigned a sample name and target. If there are empty wells, confirm there is no sample name or Target assigned to the well.
 - 1.5.1.4.1. 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.
 - 1.5.1.5. On the QuantStudio Screen, press  to eject the instrument drawer.
 - 1.5.1.5.1. 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.
- 1.5.1.6. Click on **START RUN** followed by the specific instrument serial number to start the run (see [Figure 7](#)).

Instructions for Use

- 1.5.1.7. 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).
- 1.5.1.8. 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™.
- 1.5.1.9. PCR Product in completed plates can be stored at -20 °C up to one week.

Figure 3: Create a new experiment from template.

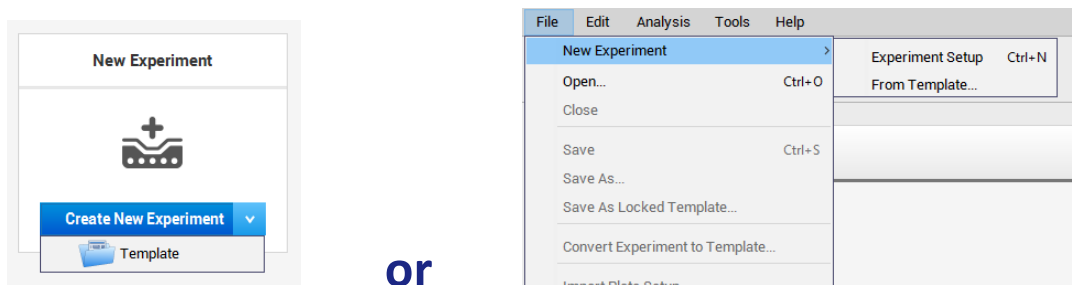
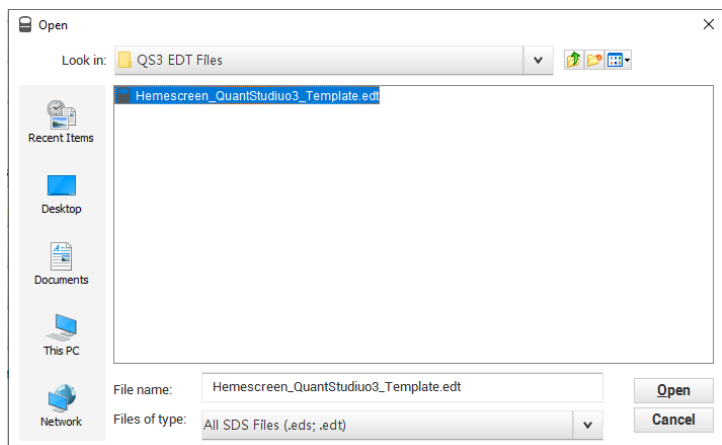


Figure 4: HemeScreen® QuantStudio™ Template.



Instructions for Use

Figure 5: HRM thermal cycler conditions.

Make sure reaction volume is 20µL (blue arrow) and data collection points are selected in step 2 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).

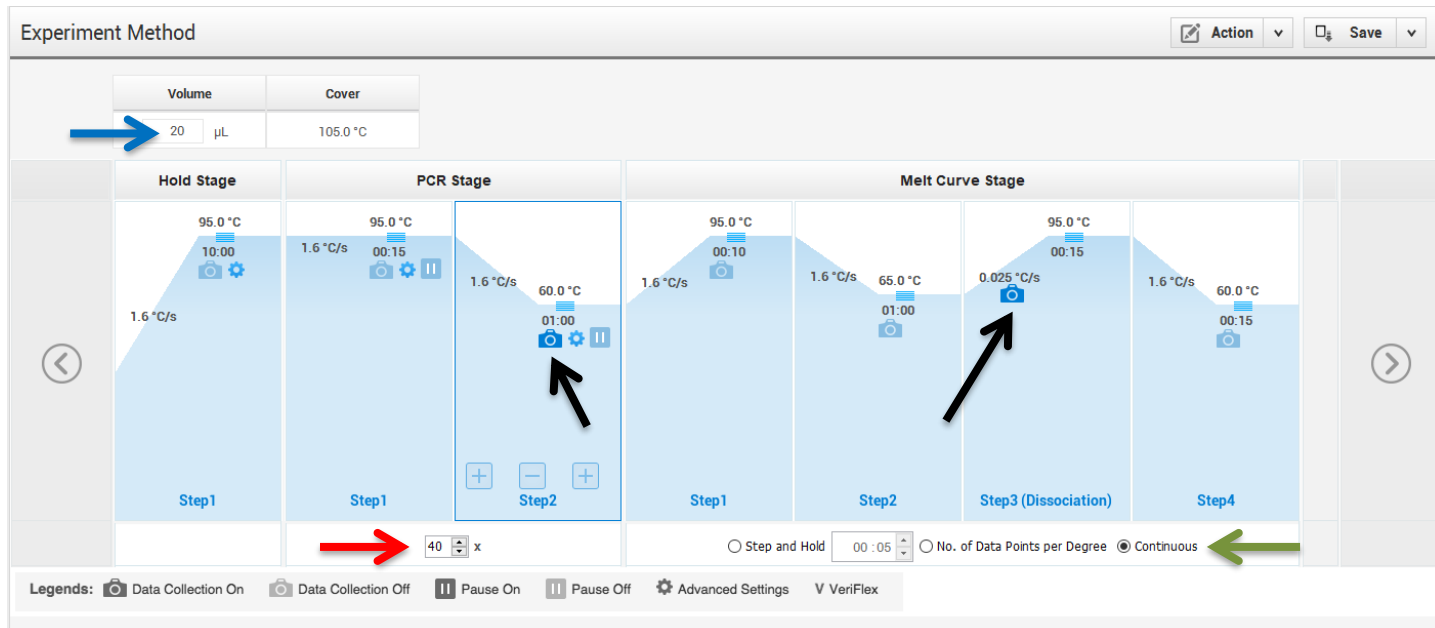


Table 1: Thermal cycling and melt curve program for the HRM reaction.

Stage	Step	Temp	Time	Ramp rate
Holding	Enzyme activation	95°C	10 min	1.6 °C/Sec
	Denature	95°C	15 sec	1.6 °C/Sec
Cycling (40 cycles)	Anneal/Extend (Data Capture on)	60°C	1 min	1.6 °C/Sec
	Denature	95°C	10 sec	1.6 °C/Sec
Melt curve/Dissociation	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.

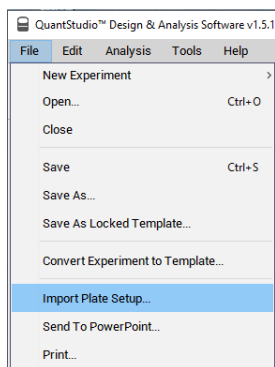


Figure 7: Starting the run.



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 and controls typically amplify between 18 - 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 NTC reactions typically have undetermined C_t values.
 - 2.1.2.2.1 In some instances, primer dimers form during the amplification and give rise to smaller, broader PCR products; however, they have undetermined or C_t values greater than 34. See [Figure 9](#).

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 T_M 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 is 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 T_M peak.
- 2.1.5 Continue to High Resolution Melt Analysis.

Figure 8: Example Amplification Plot.

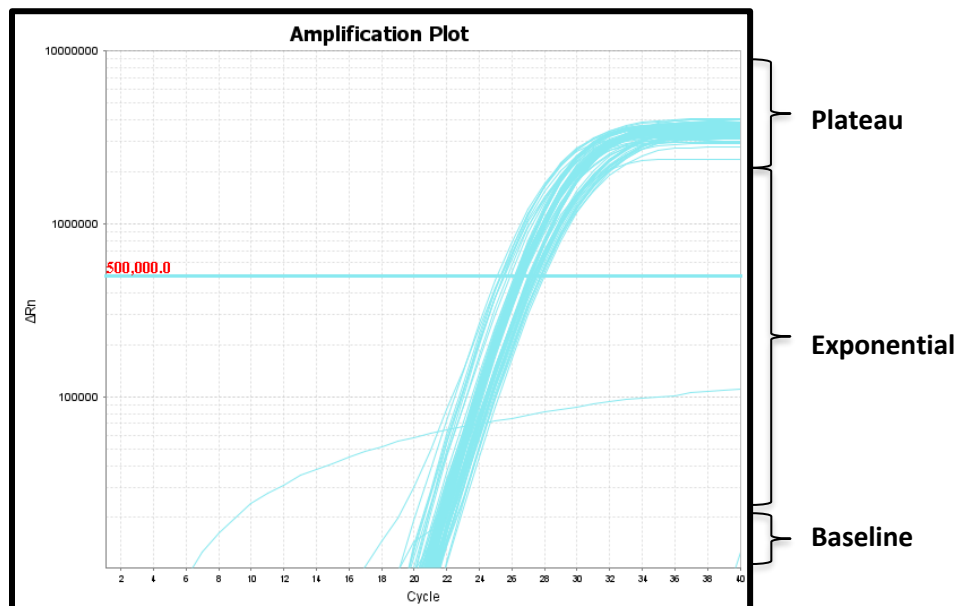
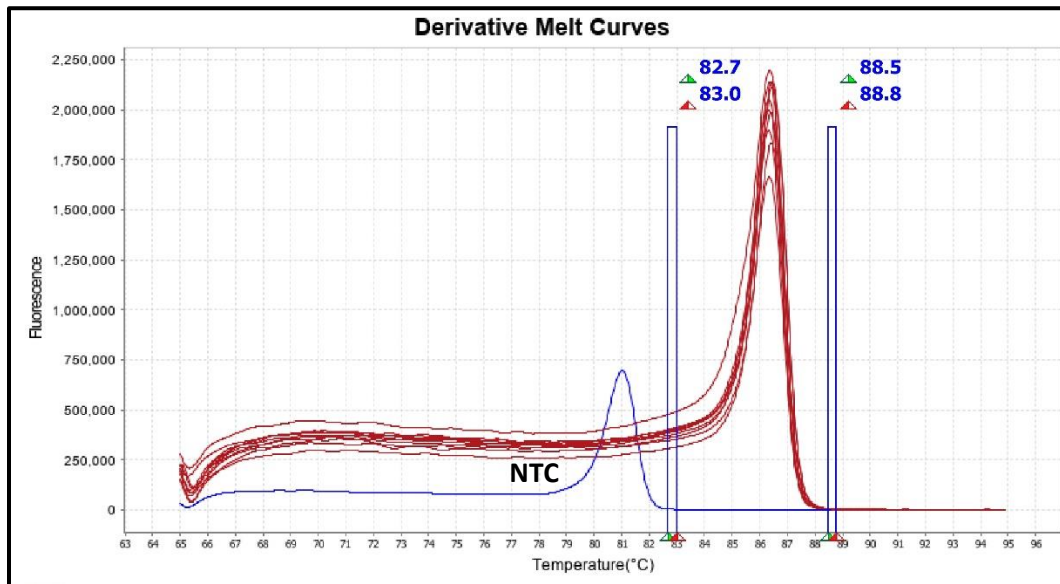


Figure 9: Example of Non-Specific Amplification in the NTC due to primer dimer formation.



HemeScreen® MPN Analysis:

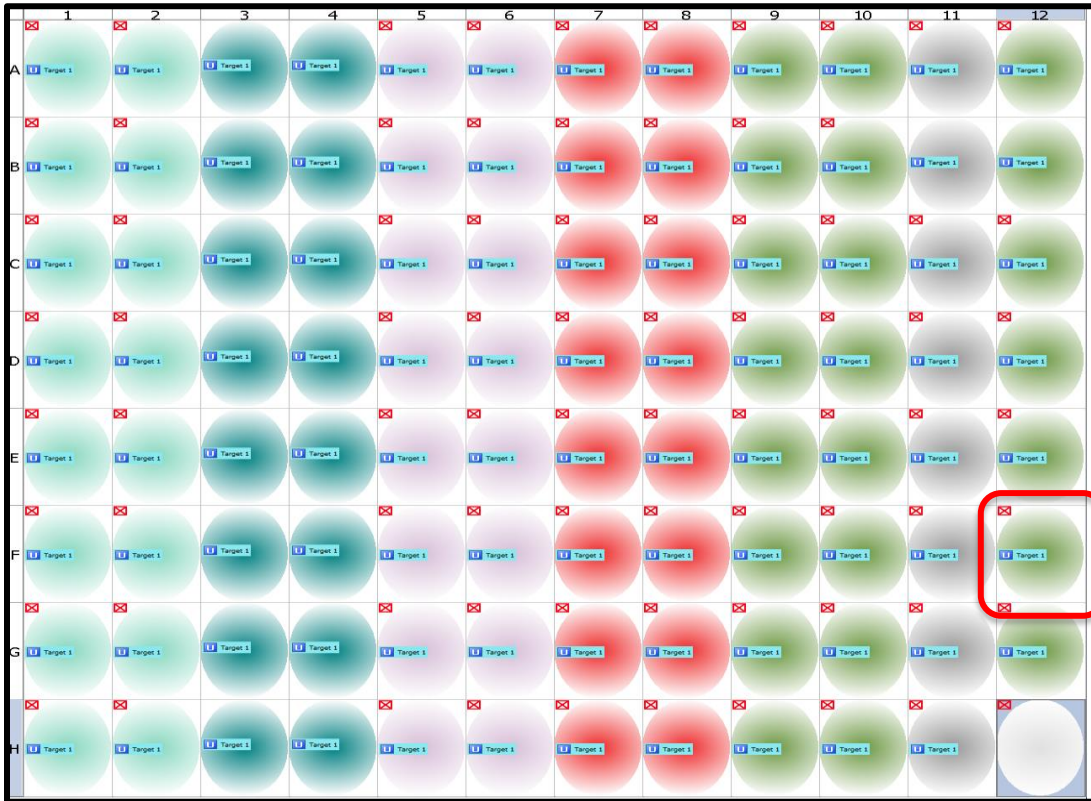
Guidance for HRM data analysis is given in this manual; however, due to instrument-to-instrument variation, calibration variation, as well as other factors, data analysis parameters for a given data set will need to be determined by the end-user.

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

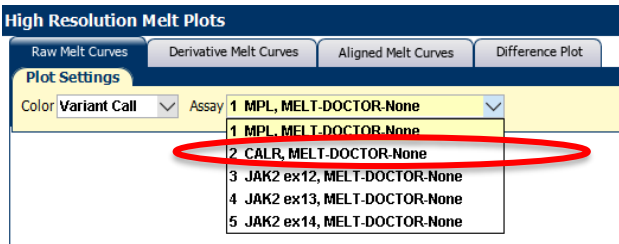
- 3.1.1. Review the melt curves using High Resolution Melt Software v3.2.
- 3.1.2. Open High Resolution Melt Software v3.2. Click **Open** on the main window to open the saved *.eds run file saved in step 1.5.1.8.
- 3.1.3. HemeScreen® MPN contains 5 targets of interest. Each target of interest must be analyzed one at a time (i.e., *CALR* only).
 - 3.1.3.1. Confirm NTC pass and omit from analysis. 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](#)).
- 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. Select the assay name (e.g. *CALR*) in the Assay dropdown menu ([Figure 11](#)). The data for the selected assay will appear in the High Resolution Melt Plots tabs. For example, if "*CALR*" is selected on an 8 sample plate layout, data from columns 3 and 4 and B11 and B12.
 - 3.1.4.3. Review the data. If there are any samples with poor amplification, omit from analysis. Note that samples with variations give rise to different possible peak shapes.

Figure 10: Example plate layout with omitted NTC reactions for CALR. The well outlined in red is the CALR NTC reaction.



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

Figure 11: Assay dropdown menu.



- 3.1.4.4. Adjust the pre-melt and post-melt regions (blue lines surrounding the derivative peak) to define the area melt region. This helps to focus in and optimize your separation and variant calling. For most targets, set the pre-melt and post-melt regions as close as possible to the base of the derivative curve peak (see [Figure 12](#)).
 - 3.1.4.4.1. If no data appears in the plots, click the green Analyze button and double check that the wells you are analyzing are selected.
 - 3.1.4.4.2. The pre-melt and the post-melt Start and Stop temperature lines should be approximately 0.2 - 0.5°C apart from each other.
- 3.1.4.5. Click the **Analyze** button on the top right of the main window.
- 3.1.4.6. Navigate to the Difference Plot tab to analyze each sample curve.
 - 3.1.4.6.1. Select the reference control (Wild-Type) from the Reference dropdown list (see [Figure 13](#)).
 - 3.1.4.6.2. [Figure 14](#) shows the Difference Plot that corresponds with [Figure 12](#).
- 3.1.4.7. Review variant calls. Curves that group within the normal control are normal or No Variant Detected (NVD). Curves that group with the positive control are abnormal or mutant detected.

Instructions for Use

- 3.1.4.7.1. Visually inspect both the Difference Plot and Derivative Melt Curves to verify that no samples exhibit unique melt profiles that may indicate possible variants.
- 3.1.4.7.2. 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 12: Example of Derivative Melt Curve.

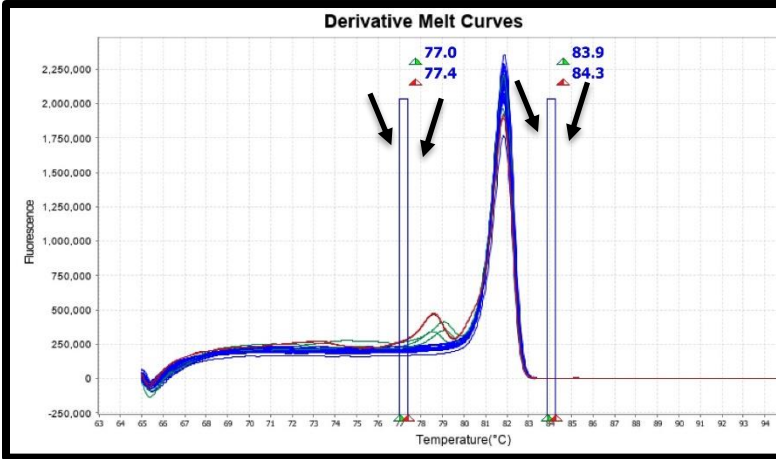


Figure 13: Reference Dropdown Menu.

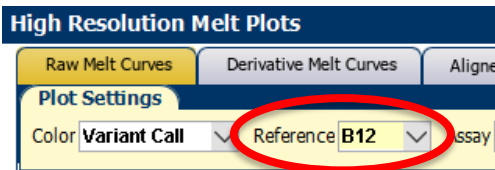
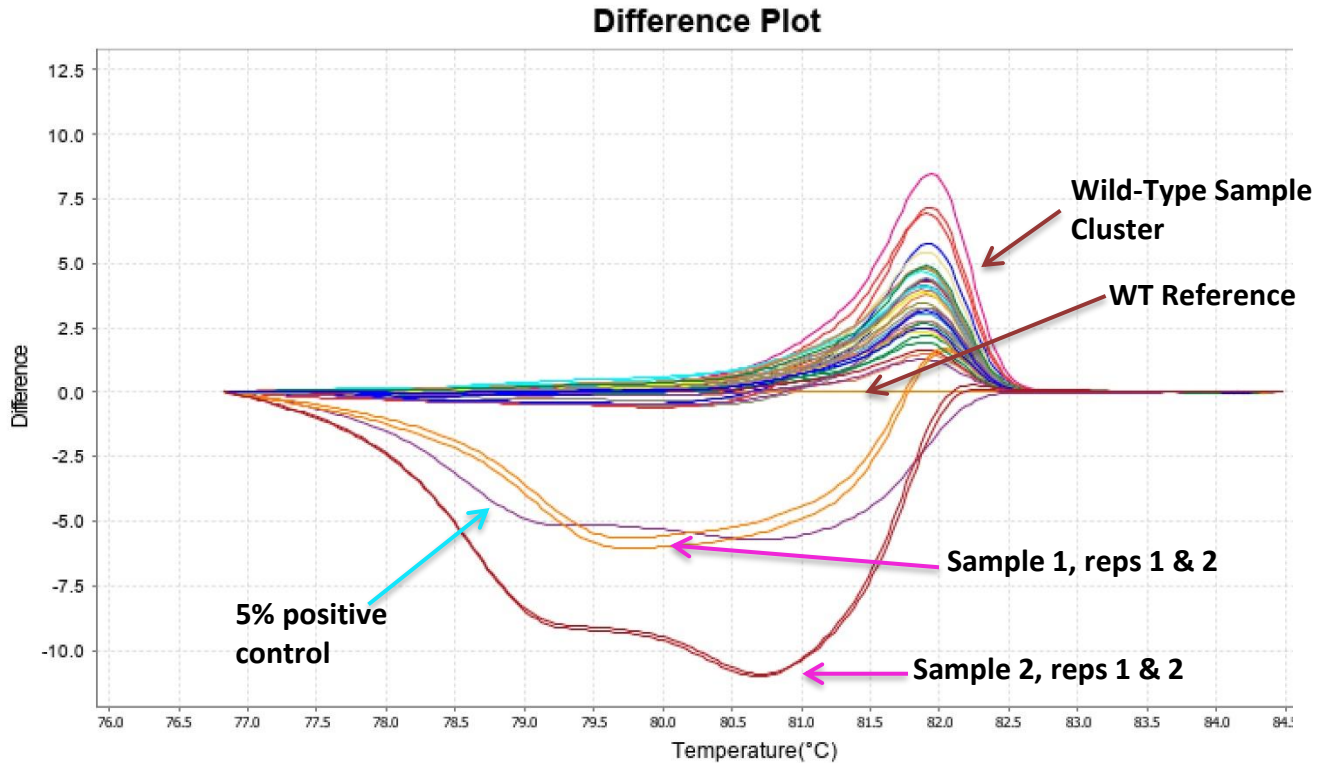


Figure 14: Example CALR Exon 9 Difference Plot.

Difference Plot shows a dark orange Wild-Type Control reference line at Difference = 0. Samples that cluster with the Wild-Type reference are considered NVD. The purple curve is the 5% Positive Control. The red and orange curves, Sample 1 and Sample 2, more closely match the profile of the positive control (purple). These are called positive since both replicates differentiate from the Wild-Type group and both replicates match the positive control silhouette,



3.2. Results and Further Analysis

3.2.1. If both replicate curves are NVD, the case is classified as NVD.

3.2.2. If both replicate curves match the positive profile, the case is classified as variant detected.

3.2.3. If the replicate curves differ from each other, further analysis might be required.

3.2.3.1. For example, in [Figure 15](#), one replicate curve dips below the WT reference, and the other replicate peaks above the WT reference. The dips and peaks differentiate at the same temperature, and variation between these replicates is due to slight variations in the melt and the selected reference curve. A dip vs a peak does not indicate an abnormal result. In this case, the sample will be deemed as NVD. Neither replicate is grouping 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 16](#)). The black line in [Figure 15](#) and [Figure 16](#) provides a visual ruler showing all specimens behaving in a similar manner that is different than the MUT control.

Figure 15: Example *CALR* Exon 9 Discrepant Replicate curves.

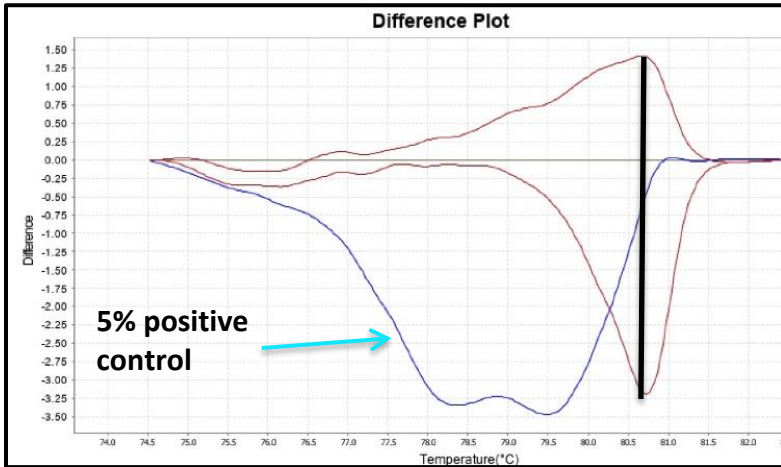
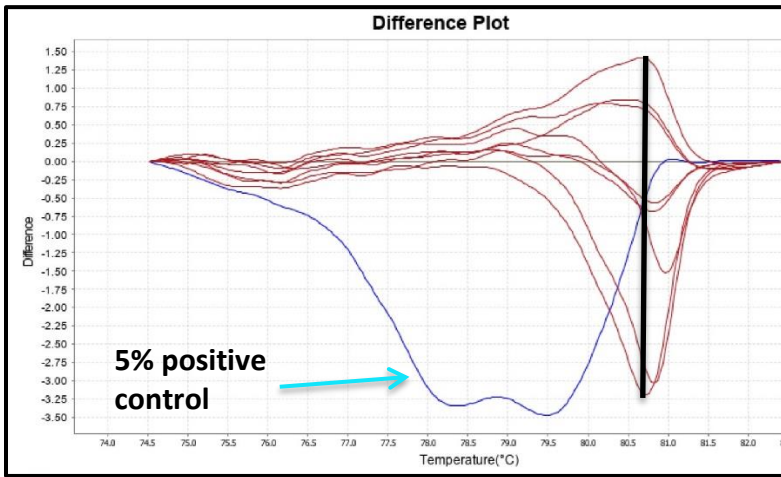


Figure 16: Example *CALR* Exon 9 Discrepant Replicate curves with WT Group.



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	<i>In vitro</i> diagnostic medical device	Sterilized using aseptic processing techniques	Keep away from light
			
Use By:	Catalog number	Manufacturer	Batch Code
			
European Conformity	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.

Literature References

1. Brown, V., S. Borinstein, D. Friedman. 2018. JAK2. *My Cancer Genome*
<https://www.mycancergenome.org/content/disease/myeloproliferative-neoplasms/jak2/?tab=0> (Copyright 2010-2017 MY CANCER GENOME)
2. Xia, D. and Hasserjian, R. P., Molecular testing for JAK2, MPL, and CALR in myeloproliferative neoplasms.
PMID: 27727468; DOI: 10.1002/ajh.24578
3. I. Panovska-Stavridis, A. Eftimov, M. Ivanovski, A. Pivkova-Veljanovska, L. Cevreska, S. Hermouet, et al. Essential thrombocythemia associated with germline JAK2 G571S variant and somatic CALR type 1 mutation.
PMID: 27009537; DOI: 10.1016/j.clml.2016.02.039
4. Marchioli R., Finazzi G., Specchia G., Cacciola R., Cavazzina R., Cilloni D. Cardiovascular events and intensity of treatment in polycythemia vera
PMID: 23216616; DOI: 10.1056/NEJMoa1208500
5. Plo, Isabelle, and Caroline Marty. "Not just another kinase mutation!"
PMID: 31877214; DOI: 10.1182/blood.2019003650

Technical Support/Questions

Technical Support Telephone: +1.203.787.7888 ext. 509

TechSupport@precipiodx.com

www.precipiodx.com

Precipio, Inc.
4 Science Park
New Haven, CT 06511, USA