Instructions for Use:
HemeScreen® CLL Plated Reagents
RUO

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

Keep these Instructions for Use for future reference.

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Manufacturer

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

HemeScreen® HRM Plated Reagents RUO

The Research Use Only (RUO) 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 Research Use Only (RUO) 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 RUO detect mutations relevant to Chronic Lymphocytic Leukemia (CLL). 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 CLL Mutations

The HemeScreen® CLL HRM 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 year 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:***	***CXCR4 Exon 2:***
c.649G>T; p.V217F	c.1866G>T; p.E622D	c.598C>T; p.Q200*
c.656C>G; p.S219C	c.1866G>C; p.E622D	c.952dup; p.T318Nfs*26
MYD88 Exon 4:	c.1873C>T; p.R625C	c.959_960del; p.V320Efs*23
c.695T>C; p.M232T	c.1874G>T; p.R625L	c.993dup; p.G332Rfs*12
c.728G>A; p.S243N	c.1984C>G; p.H662D	c.997A>T; p.K333*
MYD88 Exon 5:	c.1986C>G; p.H662Q	c.1000C>T; p.R334*
c.794T>C; p.L265P	c.1986C>A; p.H662Q	c.1005dup; p.G336Wfs*8
c.602G>A; p.R201Q	c.1996A>C; p.K666Q	c.1012_1015del; p.S338Lfs*27
c.610C>T; p.R204*	c.1996A>G; p.K666E	c.1012dup; p.S338Ffs*6
NOTCH1 Exon 34:	c.1997A>C; p.K666T	c.1013C>A; p.S338*
c.7541_7542delCT; p.P2514Rfs*4	c.1997A>G; p.K666R	c.1013C>G; p.S338*
	c.1998G>T; p.K666N	c.1014_1017del; p.S339Ffs*26
	c.1998G>C; p.K666N	c.1021del; p.S341Pfs*25
	SF3B1 Exon 15:	
	c.2098A>G; p.K700E	
	SF3B1 Exon 16:	
	c.2225G>A; p.G742D	

The HemeScreen® CLL HRM Reagents are qualitative. The reagents provide a means to rapidly identify samples for relevant mutations in *NOTCH1, SF3B1, MYD88 and CXCR4* 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® CLL HRM Reagents Controls

The "Wild-Type Control" and "Mutant Control" sequences were constructed by synthesis and cloning for *CXCR4 Exon 2, NOTCH1 Exon 34, MYD88 Exons 3, 4, & 5 and SF3B1 Exon 14, 15 & 16* using the NCBI Reference Sequences:

NM_003467.3 *CXCR4* Exon 2
NM_002468.5 *MYD88* Exon 3, 4, & 5
NM_012433.4 *SF3B1* Exon 14, 15, & 16
NM_017617.5 *NOTCH1* Exon 34

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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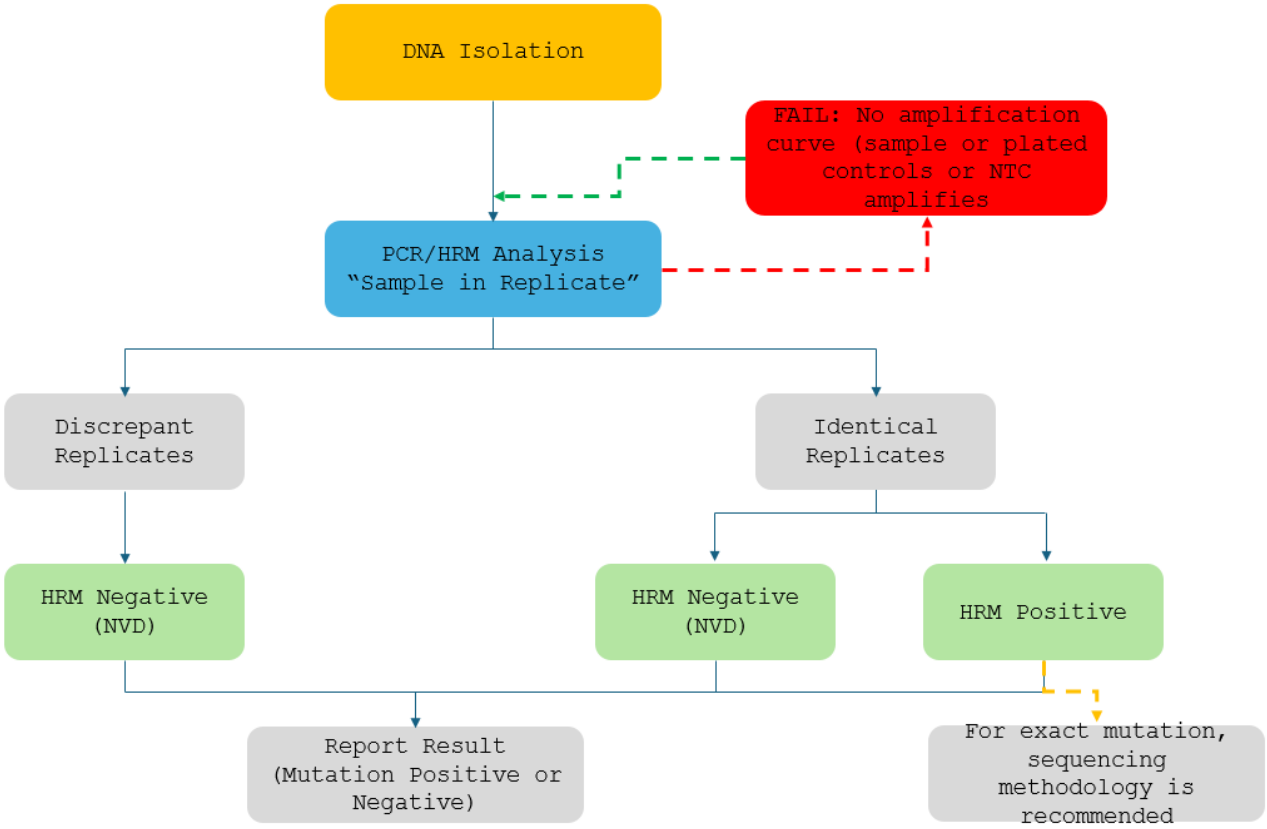
HemeScreen® Plated Reagents Preparation

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 has to be stopped before completion of all steps, the DNA should be stored (see **Primary Sample Collection, Handling, and Storage** section below for storage details). 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® CLL 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® CLL 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® CLL 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® CLL 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

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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® CLL plated reagent set contains plated reactions for 3 samples in duplicate and includes a Wild-Type, Mutant, and No Template control (NTC).

Plated reagents should be stored in the dark between -20 °C ± 2 °C in a constant temperature freezer until use. Reagents must not be exposed to temperatures below -22°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 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)

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

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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: 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/µL 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 $A_{260/280}$ 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.

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.

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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/µL. All dilutions must be between 9 and 11 ng/µL; Redo or adjust dilutions if necessary before proceeding.

1.3. HemeScreen® CLL Layout:

1.3.1. The HemeScreen® CLL Plated reagents are available in 3-Sample format.

Figure 2: HemeScreen® CLL Plate layouts for 3 samples per plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	CXCR4amp1_Sample 1	CXCR4amp2_Sample 1	MYD88e3_Sample 1	MYD88e4_Sample 1	MYD88e5_Sample 1	SF3B1-E622_Sample 1	SF3B1-K666_Sample 1	SF3B1-K700_Sample 1	SF3B1-G742_Sample 1	NOTCH1_Sample 1		
B	CXCR4amp1_Sample 1	CXCR4amp2_Sample 1	MYD88e3_Sample 1	MYD88e4_Sample 1	MYD88e5_Sample 1	SF3B1-E622_Sample 1	SF3B1-K666_Sample 1	SF3B1-K700_Sample 1	SF3B1-G742_Sample 1	NOTCH1_Sample 1		
C	CXCR4amp1_Sample 2	CXCR4amp2_Sample 2	MYD88e3_Sample 2	MYD88e4_Sample 2	MYD88e5_Sample 2	SF3B1-E622_Sample 2	SF3B1-K666_Sample 2	SF3B1-K700_Sample 2	SF3B1-G742_Sample 2	NOTCH1_Sample 2		
D	CXCR4amp1_Sample 2	CXCR4amp2_Sample 2	MYD88e3_Sample 2	MYD88e4_Sample 2	MYD88e5_Sample 2	SF3B1-E622_Sample 2	SF3B1-K666_Sample 2	SF3B1-K700_Sample 2	SF3B1-G742_Sample 2	NOTCH1_Sample 2	CXCR4amp1_NTC	CXCR4amp2_NTC
E	CXCR4amp1_Sample 3	CXCR4amp2_Sample 3	MYD88e3_Sample 3	MYD88e4_Sample 3	MYD88e5_Sample 3	SF3B1-E622_Sample 3	SF3B1-K666_Sample 3	SF3B1-K700_Sample 3	SF3B1-G742_Sample 3	NOTCH1_Sample 3	MYD88e3_NTC	MYD88e4_NTC
F	CXCR4amp1_Sample 3	CXCR4amp2_Sample 3	MYD88e3_Sample 3	MYD88e4_Sample 3	MYD88e5_Sample 3	SF3B1-E622_Sample 3	SF3B1-K666_Sample 3	SF3B1-K700_Sample 3	SF3B1-G742_Sample 3	NOTCH1_Sample 3	MYD88e5_NTC	SF3B1-E622_NTC
G	CXCR4amp1_MUT	CXCR4amp2_MUT	MYD88e3_MUT	MYD88e4_MUT	MYD88e5_MUT	SF3B1-E622_MUT	SF3B1-K666_MUT	SF3B1-K700_MUT	SF3B1-G742_MUT	NOTCH1_MUT	SF3B1-K666_NTC	SF3B1-K700_NTC
H	CXCR4amp1_WT	CXCR4amp2_WT	MYD88e3_WT	MYD88e4_WT	MYD88e5_WT	SF3B1-E622_WT	SF3B1-K666_WT	SF3B1-K700_WT	SF3B1-G742_WT	NOTCH1_WT	SF3B1-G742_NTC	NOTCH1_NTC

DNA Extraction Dilution Settings
Final Volume: 100 µL
Final Concentration: 10 ng/µL

	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					

1.3.2. HemeScreen® CLL 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® CLL 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).

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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**).

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.

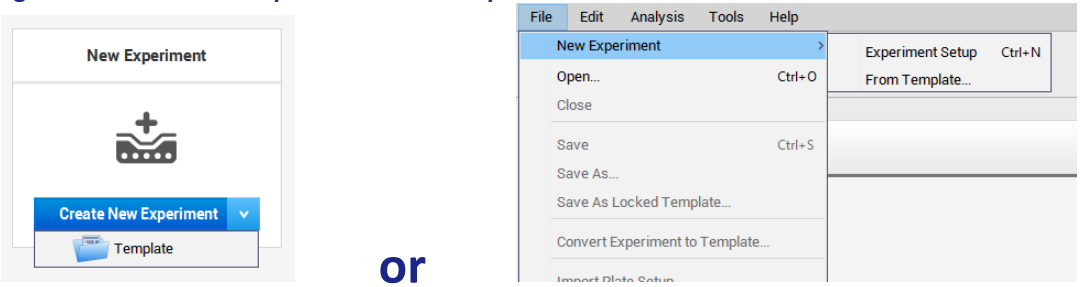

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Figure 4: HemeScreen® QuantStudio™ Template.

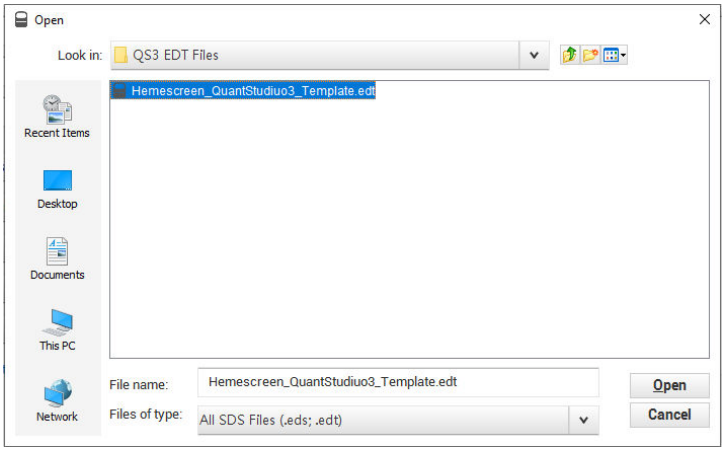

Figure 5: HRM thermal cycler conditions.

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

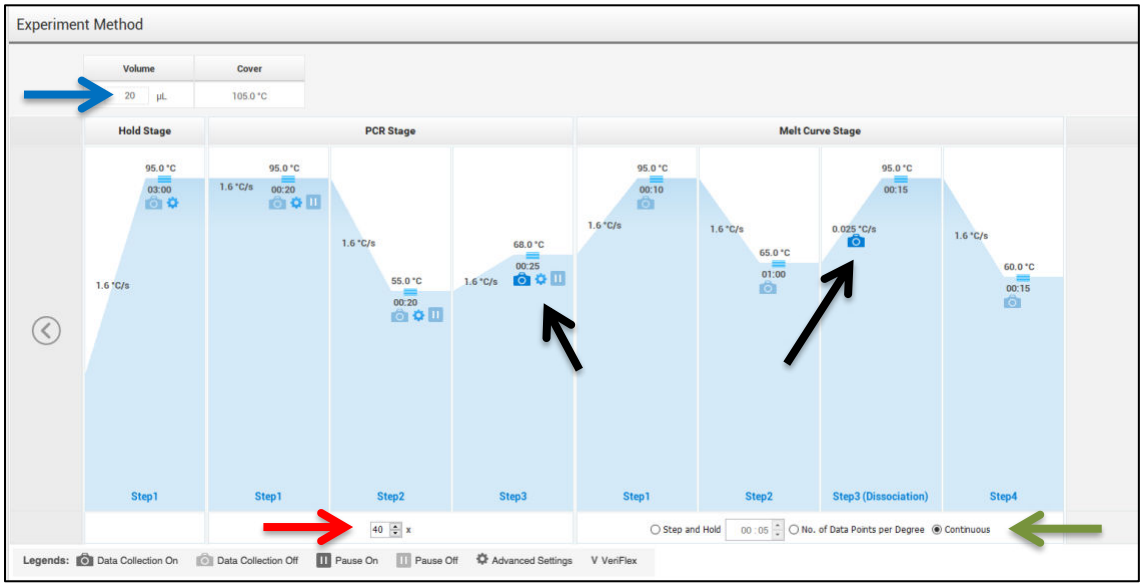

Table 1: Thermal cycling and melt curve program for the HRM reaction.

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

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Figure 6: Import Plate Layout files.

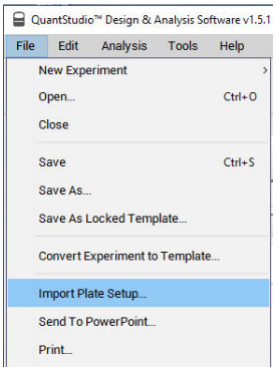

Figure 7: Starting the run.

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 amplifies 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 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 T_M peak.

2.1.5 Continue to High Resolution Melt Analysis.

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Figure 8: Example Amplification Plot.

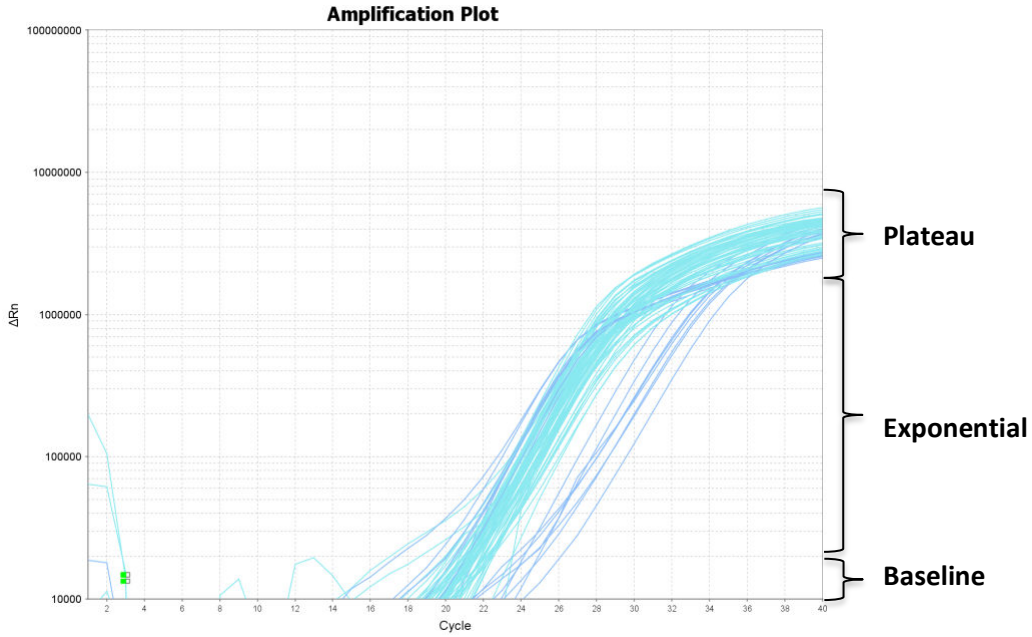
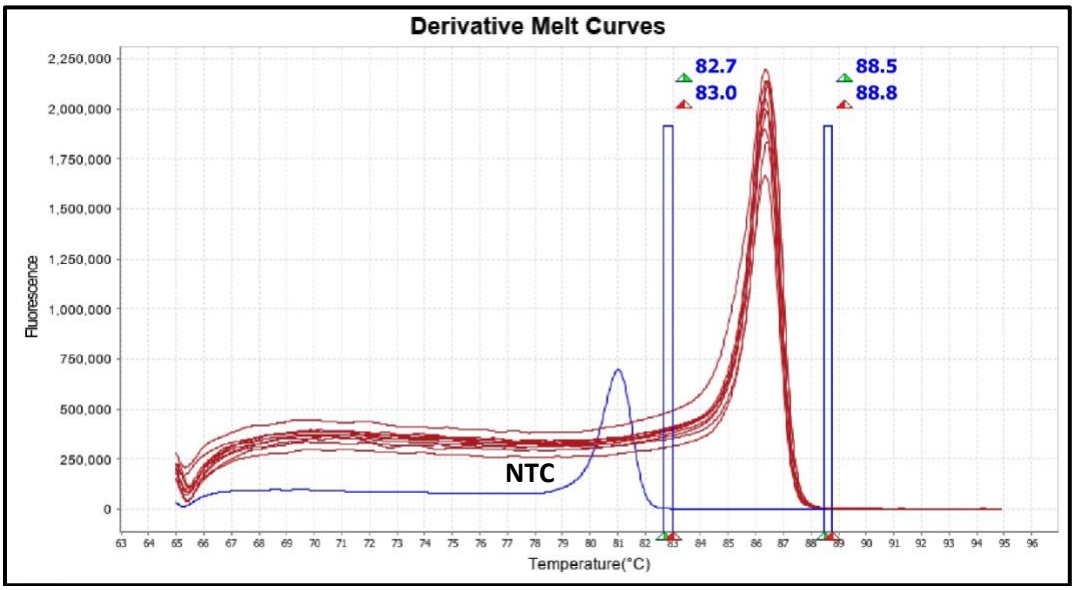


Figure 9: Example of Non-Specific Amplification in the NTC due to primer dimer formation.



HemeScreen® CLL 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.

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3.1.3. HemeScreen® CLL contains 10 targets of interest. Each target of interest must be analyzed one at a time (i.e., *NOTCH1* 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**).

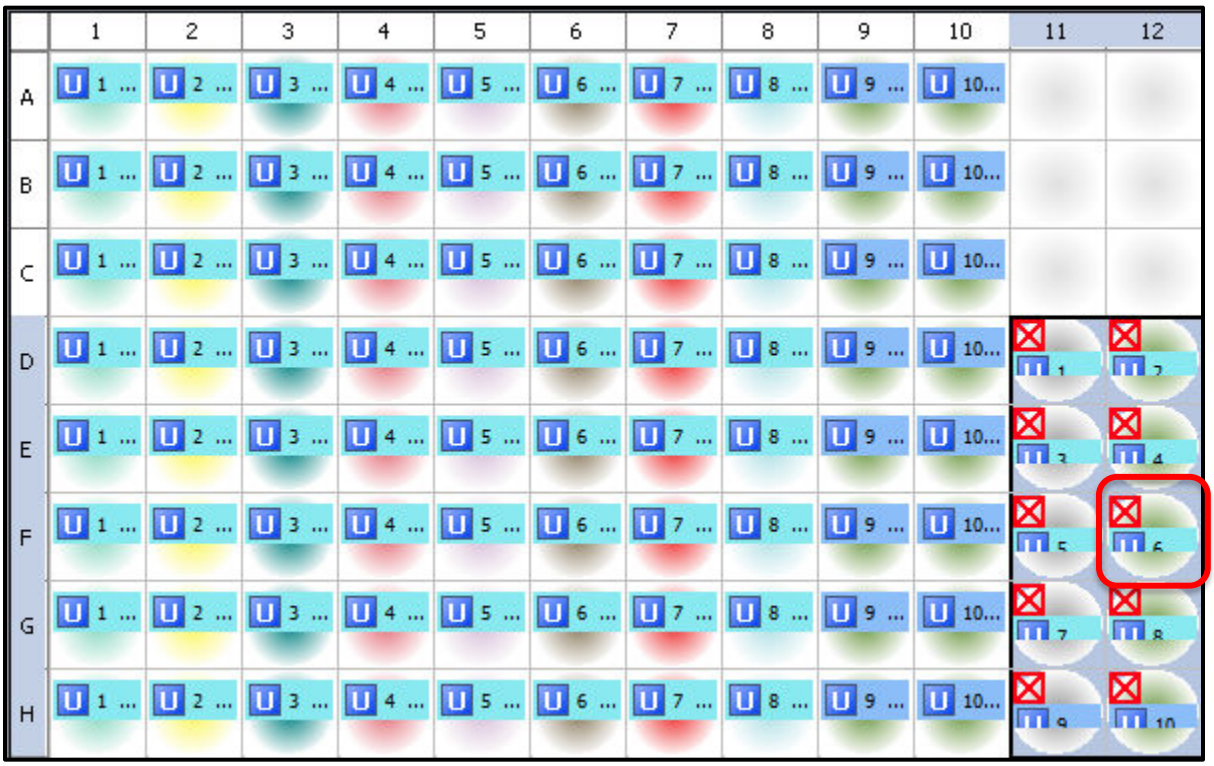
3.1.4. To analyze each target:

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. *SF3B1 E622*) 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 "*SF3B1* E622" is selected from the plate layout, data from columns 6 and F12.

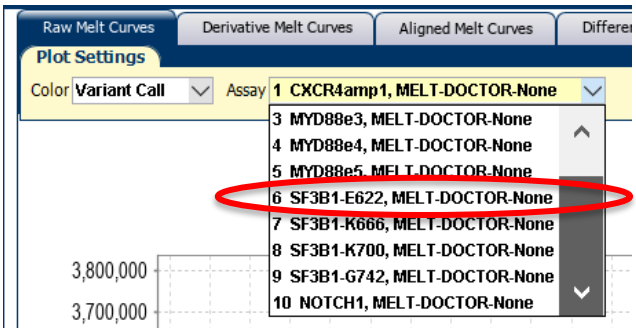
3.1.4.3. Review the data. If there are any samples with poor amplification, omit from analysis. Note that samples with variations give rise to different possible peak shapes.

Figure 10: Example plate layout with omitted NTC reactions. The well outlined in red is the NOTCH1 NTC reaction.



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

Figure 11: Assay dropdown menu.



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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.

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.

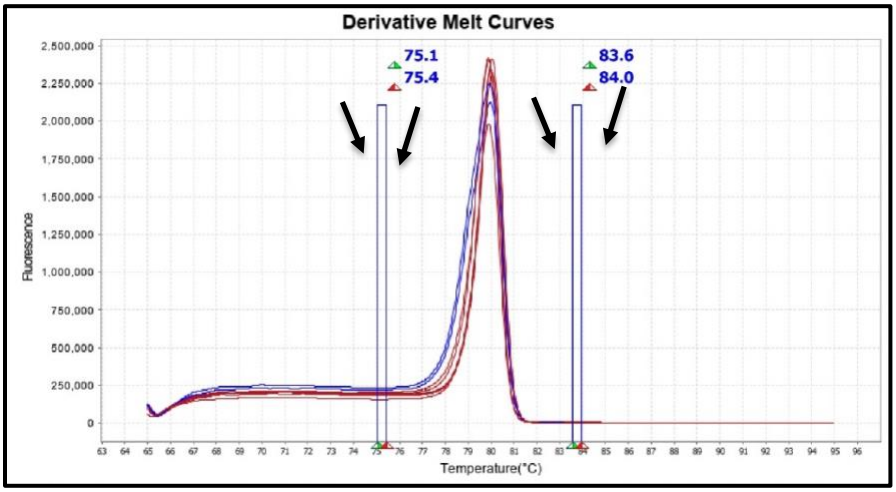

Figure 13: Reference Dropdown Menu.

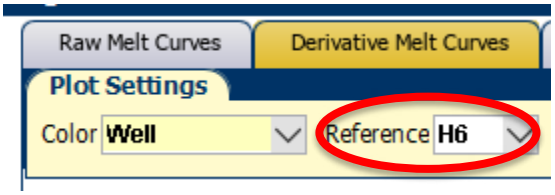

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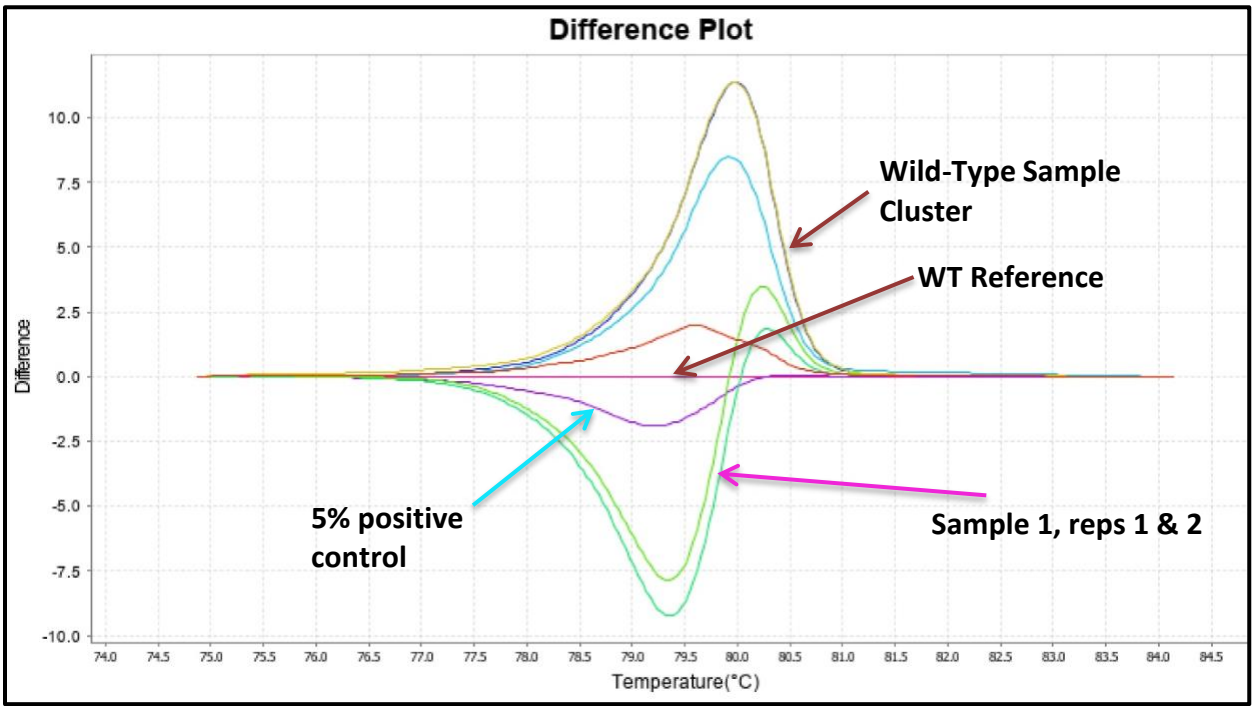
Figure 14: Example *SF3B1* E622 Difference Plot.

Difference Plot shows a dark pink 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 green and teal curves, Sample 1, 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 compared to the selected reference curve and do not indicate a mutant signal. A dip vs a peak does not indicate an abnormal result. In this case, the sample is NVD. Neither replicate is grouping with the MUT control. In addition, **Figure 16** shows the same sample replicates in context with other samples and even though one replicate is dipping below the WT baseline, it is still grouping with other NVD samples (The black line in **Figure 15** and **Figure 16** provides a visual ruler indicating all specimens behaving in a similar manner that is different than the MUT control.

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Figure 15: Example Discrepant Replicate curves.

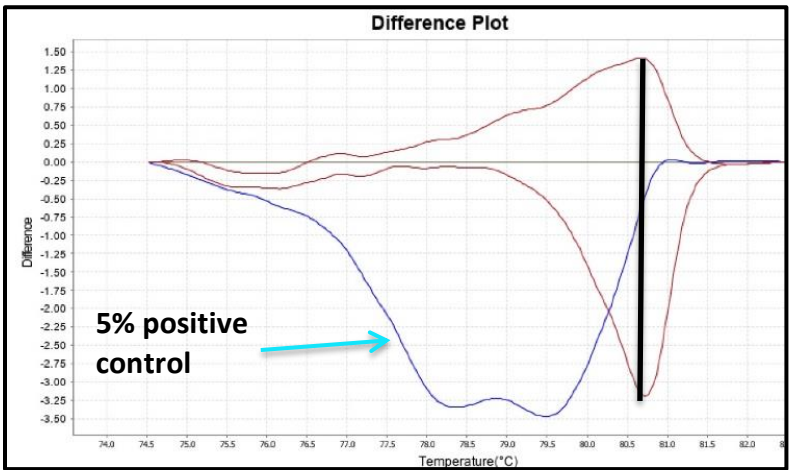
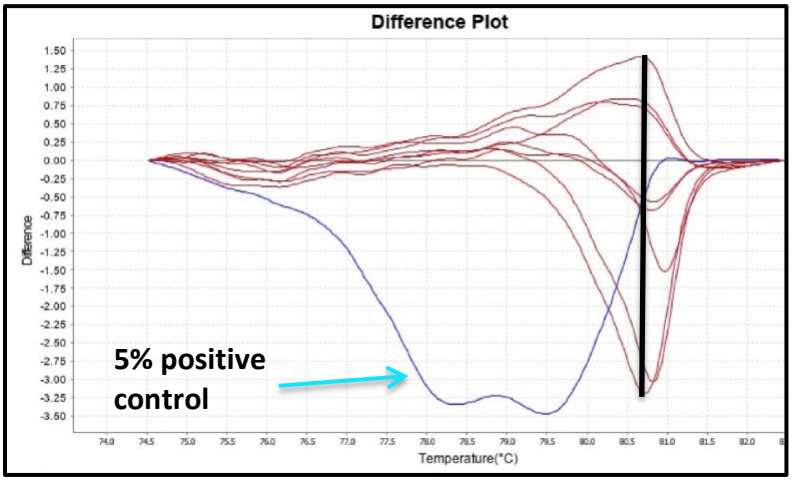


Figure 16: Example Discrepant Replicate curves with WT Group.



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Troubleshooting

Pre Analytical:

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.
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.
Dilution trouble	A sample that is too diluted (<10 ng/µl of DNA) the sample may not amplify as expected. Repeat dilution is recommended.
Extraction	Degraded DNA, low DNA quality or sample quality will impact the amplification of the sample. Repeat extraction may be necessary.
Machine Setup	Make sure the correct protocol is selected on the instrument prior to running the plate. Ensure the plate has been placed into the machine using the correct orientation with A1 in the upper left hand corner of the block prior to closing the instrument.

Analytical:

Amplification Curves	Each reaction has internal controls. If there is no amplification of the internal controls, repeat DNA extraction and DNA dilution for this sample.

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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.

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   PMID: 23568491; DOI: 10.1182/blood-2013-02-427641

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