Instructions for Use:
HemeScreen® AML Plated Reagents

Please read the Instructions for Use thoroughly before you use this product and keep it for future reference.

Contents

Manufacturer

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

HemeScreen® HRM Reagents

The HemeScreen 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 assays that detect 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 Reagents detect mutations relevant to Acute Myeloid Leukemia (AML). 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 DNA 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 AML Mutations

The HemeScreen® AML HRM Reagents have been designed to PCR amplify the relevant regions in *FLT3*, *KIT*, *IDH1, IDH2*, *CEBPA* and *NPM1* genes for subsequent HRM analysis.

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 detection of entire exon.
	c.395G>T; p.R132L	
	c.395G>C; p.R132P	

The HemeScreen® AML HRM Reagents do not provide quantitative results. The reagents provide a means to screen samples for relevant mutations in *FLT3*, *KIT*, *IDH1*, *IDH2, CEBPA* and *NPM1* as related to Acute Myeloid Leukemia (AML).

Principles of High Resolution Melting

High Resolution Melting (HRM) is a mutation detection technology, 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.

Traceability of HemeScreen® AML HRM Reagents Controls

The "Wild-Type Control" and "Mutant Control" sequences were constructed by synthesis and cloning for *CEBPA* Exon 1, *FLT3* Exon 14, 15, 16, 20, *IDH1* Exon 4, *IDH2* Exon 4, *KIT* Exon 9, 11, 13, 17 and *NPM1* Exon 11 using the NCBI Reference Sequences:

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

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® Reagent Set Preparation

All reagents supplied with the HemeScreen® reagent set are ready to use. The plates will need to be thawed and spun in a microcentrifuge before use; check details in **HemeScreen® Preparation and Procedure** 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). The analysis The HemeScreen® HRM Reagents should only be used in the context of the workflow indicated below.

Figure 1: HemeScreen® HRM Reagents workflow
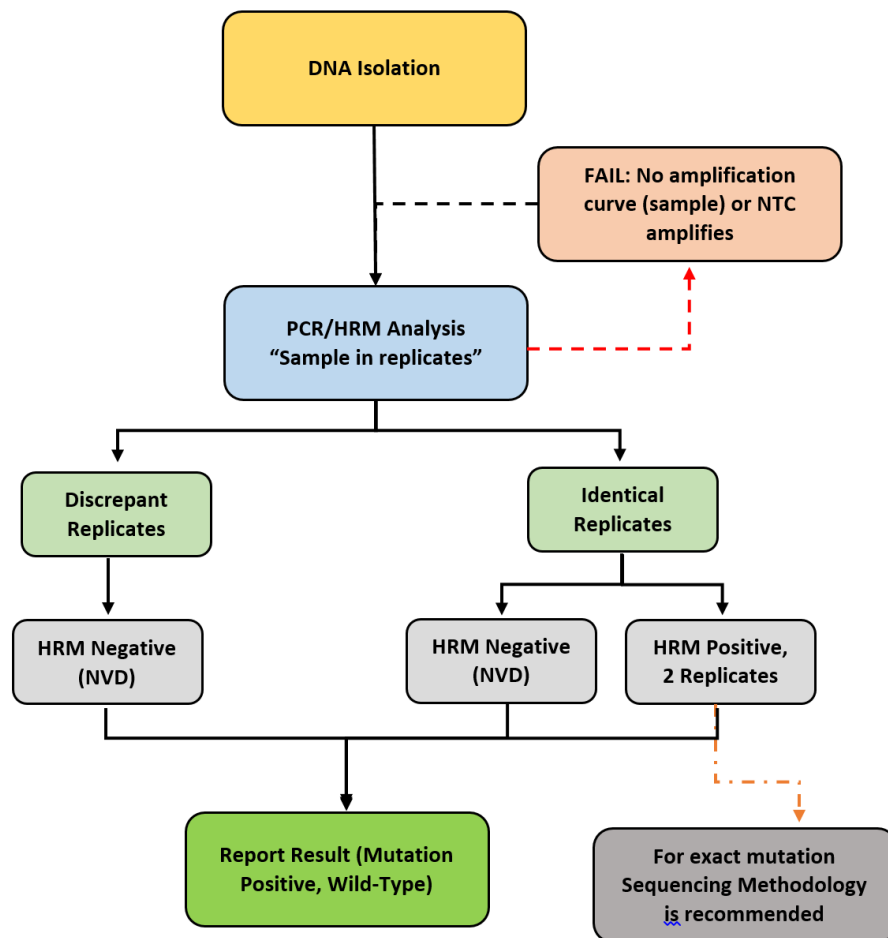
Explanation to Figure 1:

1. Isolate the DNA from peripheral blood or bone marrow using standard laboratory procedures.
2. Perform HemeScreen® AML 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® AML for that sample.
      ii. If the NTC shows amplification and the CT value is <34, compare the HRM melt temperature ™ 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® AML 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® AML 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 one of the replicates is not aligned with the positive control melt profile (melting temperature and curve peak) while the other replicate is aligned with the positive control melt profile (melting temperature and curve peak) the same is HRM negative.
      iv. If the sample melting curve is peaking after the positive control peak in more than 0.5°C then it is considered NVD.

Components and Storage Conditions

Each HemeScreen® AML plated reagent set contains plated reactions for 1 sample 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. 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™ Optical Adhesive Film (Fisher Healthcare Catalog Number **4311971**)
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/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™ field technician will create all calibration files necessary during installation. In general, calibration files are good for two years. The field technician will not complete the MeltDoctor HRM calibration plate. This plate must be ran 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.

1.2.1.1. Please use the provided excel spreadsheet to prepare the working concentration of each sample.

1.2.1.2. Enter each sample name, concentration, and $A_{260/280}$ and $A_{260/230}$ ratio into the Excel sheet.

1.2.1.3. The dilution calculations will populate automatically.

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

1.2.1.3.2. For pipetting convenience, prepare 130 µL provided enough DNA is available. The total volume may be adjusted to fit the extracted DNA elution amount.

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

1.2.2. Print the excel 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. Add the required volume of stock DNA and pipette mix.

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. The HemeScreen® AML plated reagents are arranged for PCR and analysis of one sample in duplicate.

Figure 2: AML Plate Layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	FLT3ex14_IDH2cdn140_Sample ID	IDH1ex4_Sample ID	FLT3ex14_IDH2cdn140_Sample ID	IDH1ex4_Sample ID	FLT3ex14_IDH2cdn140_POS	IDH1ex4_POS	FLT3ex14_IDH2cdn140_WT	IDH1ex4_WT			FLT3ex14_IDH2cdn140_NTC	IDH1ex4_NTC
B	FLT3ex15_Sample ID	CEBPAex1amp1_Sample ID	FLT3ex15_Sample ID	CEBPAex1amp1_Sample ID	FLT3ex15_POS	CEBPAex1amp1_POS	FLT3ex15_WT	CEBPAex1amp1_WT			FLT3ex15_NTC	CEBPAex1amp1_NTC
C	FLT3ex17_KITex16_Sample ID	CEBPAex1amp2_Sample ID	FLT3ex17_KITex16_Sample ID	CEBPAex1amp2_Sample ID	FLT3ex17_KITex16_POS	CEBPAex1amp2_POS	FLT3ex17_KITex16_WT	CEBPAex1amp2_WT			FLT3ex17_KITex16_NTC	CEBPAex1amp2_NTC
D	NPM1ex12_FLT3ex20_Sample ID	CEBPAex1amp3_Sample ID	NPM1ex12_FLT3ex20_Sample ID	CEBPAex1amp3_Sample ID	NPM1ex12_FLT3ex20_POS	CEBPAex1amp3_POS	NPM1ex12_FLT3ex20_WT	CEBPAex1amp3_WT			NPM1ex12_FLT3ex20_NTC	CEBPAex1amp3_NTC
E	KITex9_Sample ID	CEBPAex1amp4_Sample ID	KITex9_Sample ID	CEBPAex1amp4_Sample ID	KITex9_POS	CEBPAex1amp4_POS	KITex9_WT	CEBPAex1amp4_WT			KITex9_NTC	CEBPAex1amp4_NTC
F	KITex11_Sample ID	CEBPAex1amp5_Sample ID	KITex11_Sample ID	CEBPAex1amp5_Sample ID	KITex11_POS	CEBPAex1amp5_POS	KITex11_WT	CEBPAex1amp5_WT			KITex11_NTC	CEBPAex1amp5_NTC
G	KITex13_Sample ID	CEBPAex1amp6_Sample ID	KITex13_Sample ID	CEBPAex1amp6_Sample ID	KITex13_POS	CEBPAex1amp6_POS	KITex13_WT	CEBPAex1amp6_WT			KITex13_NTC	CEBPAex1amp6_NTC
H	IDH2cdn172_Sample ID	CEBPAex1amp7_Sample ID	IDH2cdn172_Sample ID	CEBPAex1amp7_Sample ID	IDH2cdn172_POS	CEBPAex1amp7_POS	IDH2cdn172_WT	CEBPAex1amp7_WT			IDH2cdn172_NTC	CEBPAex1amp7_NTC

↑ -------------- Add Sample -------------- ↑   -------------- Pre-plated Controls (do not add sample) --------------

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 ID	100.5			12.9	117.1

DNA Extraction Dilution Settings   **?**

DNA Extraction Dilution Settings		
Final Volume:	**130**	µL
Final Concentration:	**10**	ng/µL

HemeScreen Dilution / Template Generator
1. Add Plate Name
2. Enter Sample IDs & DNA Extraction Info
3. Click the save button to generate and save tempalte files

1.3.2. HemeScreen® AML Plate Layout Templates (.xlsx) are saved to the Precipio Storage Cloud. The template 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. Make a copy of the template file (or be sure to "Save As"). Open the copied file in Microsoft Excel.

        1.3.2.1.1. Recommended File name structure as DD_MM_YY_"Plate Barcode"

    1.3.2.2. Enter the sample names in the "Layout Maker" section." The 96-well plate view and the QuantStudio™ Template will automatically update.

    1.3.2.3. Click to the save button on the upper right hand corner.

        1.3.2.3.1. A .txt file will be automatically saved in the Templates folder located inside the POL folder.

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. The closer to room temperature the better.

    1.4.1.2. If possible protect from light.

1.4.2. Remove the plate from the packaging.

1.4.3. Centrifuge the plate for 2 minutes 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 on the bottom of the plate may dirty the sample block and cause abnormal fluorescent signals.

1.4.4. Ensure that the plate orientation is correct. Well A1 should be at the top left.

1.4.5. Being very careful, **SLOWLY** remove the plate septa from the HemeScreen® AML Plate.

1.4.6. To appropriate wells, add 2 µL of diluted DNA sample. Ensure to adequately vortex and spin down all DNA dilutions prior to use.

    1.4.6.1. Pipette-mix to ensure sample is well mixed into reaction mixture.

    1.4.6.2. 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.

1.4.7. Place MicroAmp Optical Film over plate and seal. Ensure the plate is appropriately sealed on all edges.

1.4.8. Centrifuge the plate for 1 min to eliminate any air bubbles from the solution.

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.

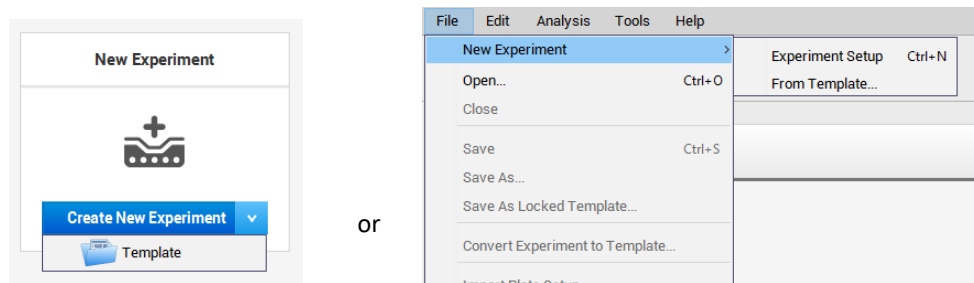**Figure 3: Create a new experiment from Template.**

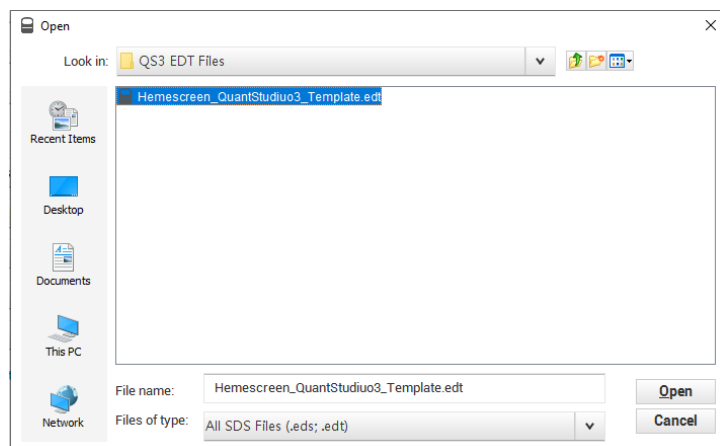**Figure 4: HemeScreen® QuantStudio™ Template.**

Figure 5: HRM thermal cycler conditions.

Make sure reaction volume is 20 µL (blue arrow), data collection points are selected in step 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).
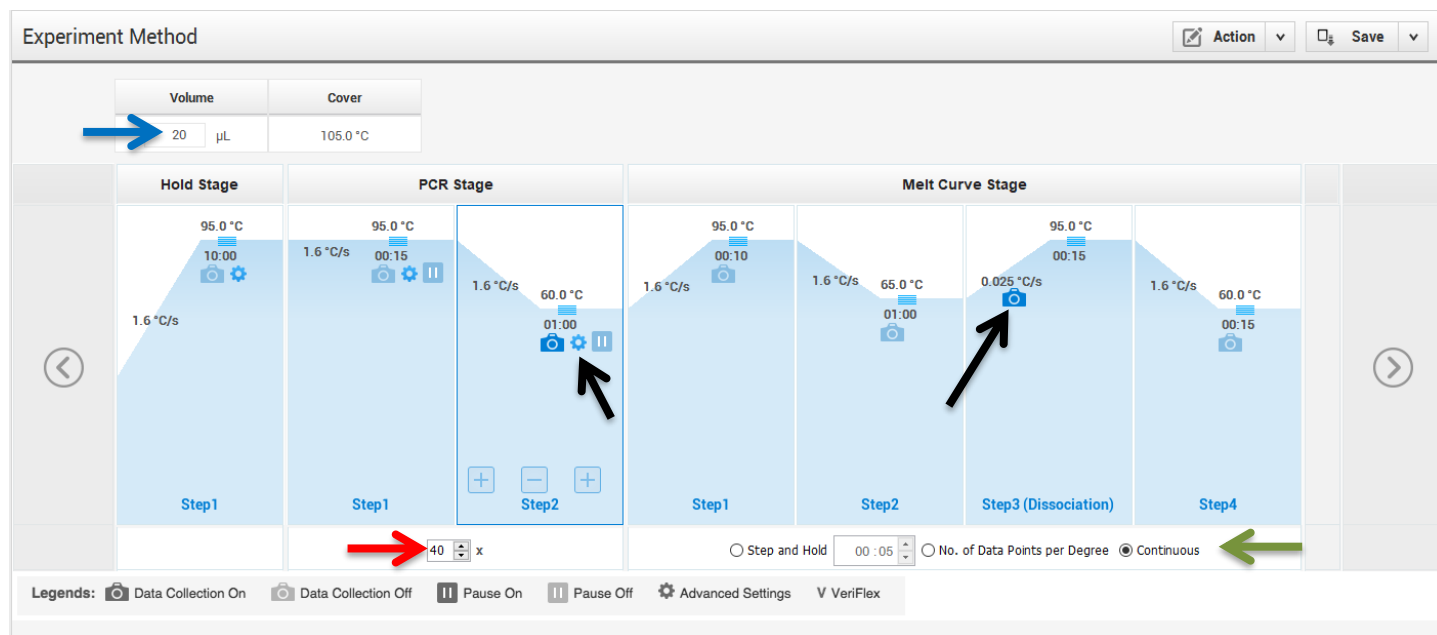
Table 1: Thermal cycling and melt curve program for the HRM reaction.

Stage	Step	Temp	Time	Ramp rate
Holding	Enzyme activation	95 °C	10 min	1.6 °C/Sec
Cycling (40 cycles)	Denature	95 °C	15 sec	1.6 °C/Sec
	Anneal/extend	60 °C	1 min	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	95 °C	15 sec	0.025 °C/Sec
	Anneal	60 °C	15 sec	1.6 °C/Sec

Figure 6: Import Plate Layout files.
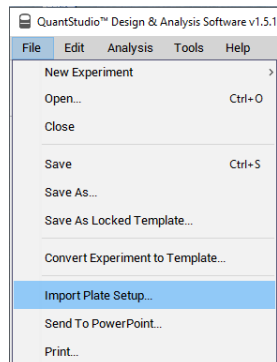
Figure 7: Starting the run.
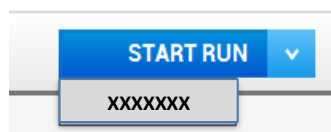
Quality Control of the Amplification Data

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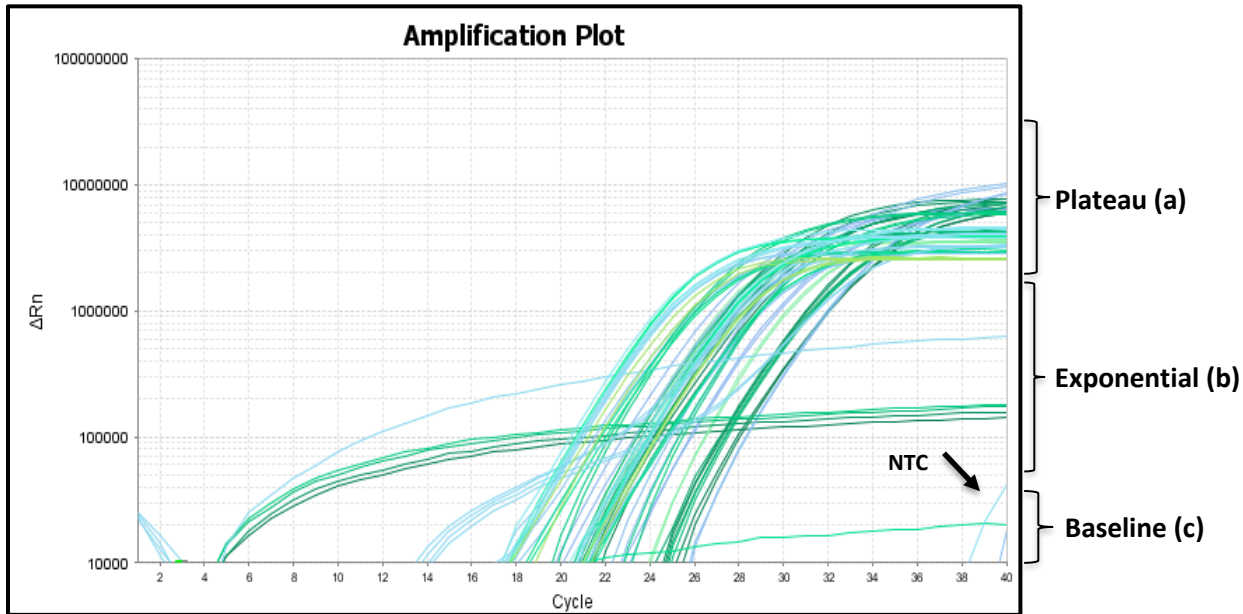
Figure 9: Example of Non-Specific Amplification in the NTC due to primer dimer formation.
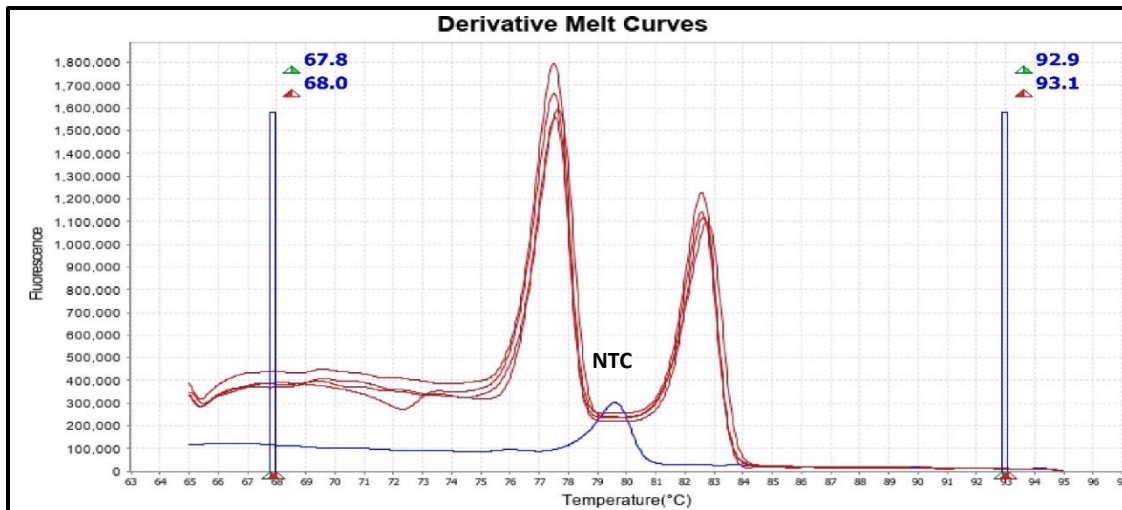
HemeScreen® AML Analysis:

NOTE: Three of the 16 reactions in the AML Panel contain two amplicons. For these duplex reactions, each amplicon will require separate analysis. Examples are shown below. The first amplicon mentioned in the **Target** column has the lowest melt temperature in the respective traces. The indicated Derivative Peak Temperatures are approximate and may be slightly different depending on your instrument.

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).

Table 2: Reactions that contain multiple targets.

Target(s)	Sample Wells	Mutant Control	WT Control	Derivative Peak Temperature 1	Derivative Peak Temperature 2
FLT3 Exon 14 and *IDH2* Exon 4 Codon 140	A1, A3	A5	A7	*FLT3* Exon 14 = 77 °C	*IDH2* Exon 4 = 81.5 °C
KIT Exon 17 and *FLT3* Exon 16	C1, C3	C5	C7	*KIT* Exon 17 = 77 °C	*FLT3* Exon 16 = 82 °C
NPM1 Exon 12 and *FLT3* Exon 20	D1, D3	D5	D7	*NPM1 Exon 12* = 73.5 °C	*FLT3* Exon 20 = 79 °C

*Derivative Peak Temperatures are approximate and may differ instrument to instrument.

3.1. Analysis

3.1.1. Open High Resolution Melt Software v3.2.

3.1.2. Click **Open** on the main window and select the result file (*.eds) saved in step 1.5.1.8.

3.1.3. HemeScreen® AML contains 19 targets of interest in 16 reactions. Each target of interest must be analyzed one at a time (i.e., *FLT3 Exon 14* 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 reactions.
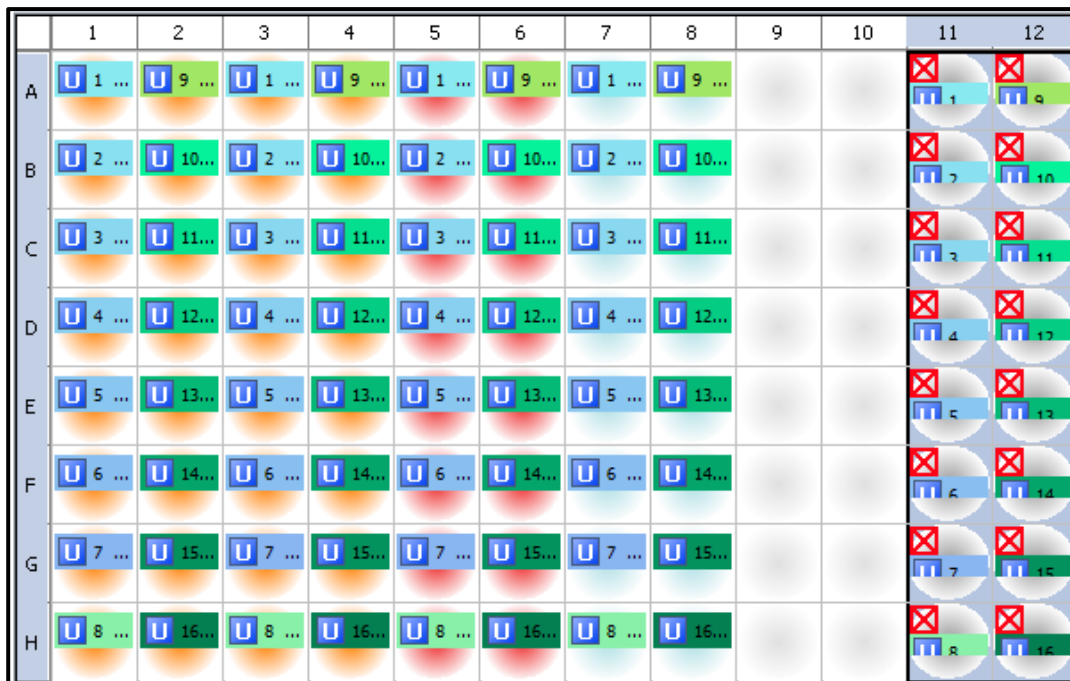
**NOTE:** Omit any outliers and NTCs from further analysis (unless NTCs exhibited possible contamination in previous steps).

3.1.4. **To analyze wells with a single amplicon:**

    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. *KIT* Exon 9) 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 "*KIT* Exon 9" is selected, data from wells E1, E3, E5, and E7 will be displayed (if NTC is omitted).

Figure 11: Assay dropdown menu.
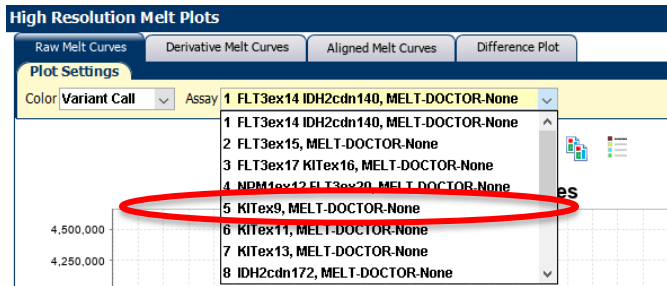
    3.1.4.3. Review and adjust the pre-melt and post-melt regions to optimize your separation and variant calls. For most targets, set the pre-melt and post-melt regions as close as possible to the derivative curve peak (see **Figure 12**).

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

        3.1.4.3.2. The pre-melt and the post-melt Start and Stop temperature lines should be approximately 0.2 - 0.5 °C apart from each other.

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

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

        3.1.4.5.1. Select the reference control (Wild-Type) from the Reference dropdown list (see **Figure 13**).

        3.1.4.5.2. **Figure 14** shows the Difference Plot that corresponds with **Figure** 12**12**.

Figure 12: Example of Derivative Melt Curves for a single amplicon reaction.

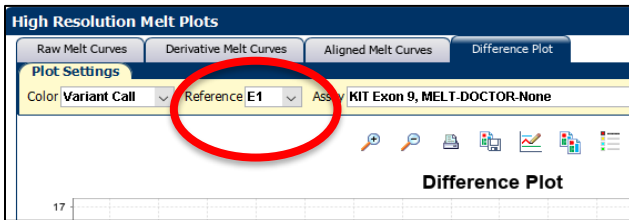Single target reactions will have one peak. Pre-Melt and Post-Melt Regions are assigned.
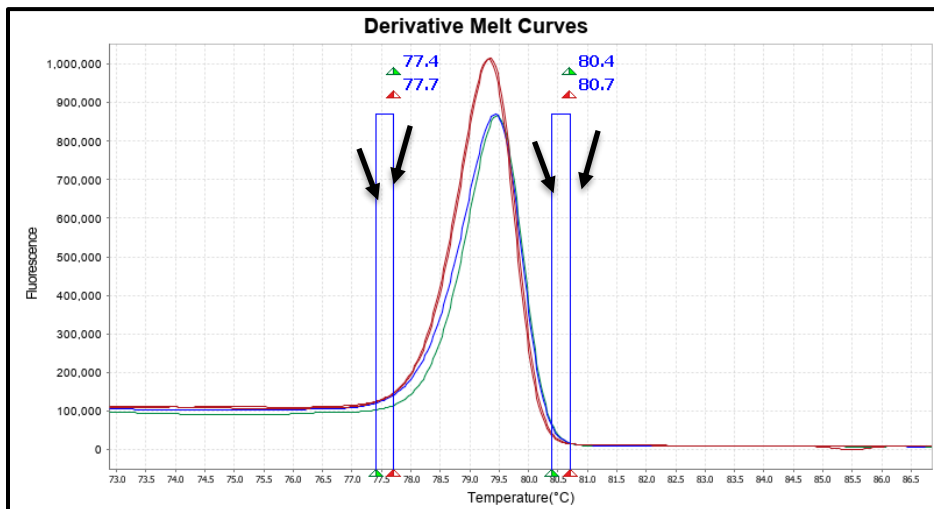
Figure 13: Reference Drop Down Menu.

Figure 14: Example *KIT Exon 9* Difference Plot.

The Difference Plot shows a Wild-Type reference (green) is the flat green line at Difference = 0 along with the 5% mutation positive control (blue) that has a peak Difference at 79.2 °C and both sample replicates (red) that have been differentiated into distinct groups with a peak Difference at 79.5 °C. In this case the sample replicates are screened NVD. The replicate curves do not align with and exceed the positive reference. They also do not peak at an earlier temperature than the positive control peak.
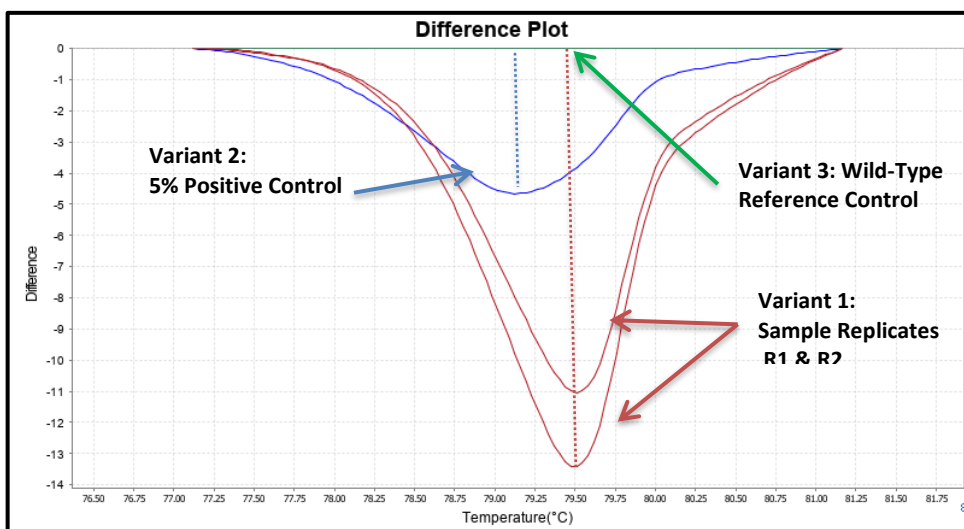
3.1.5. **To analyze wells with multiple amplicons:**
   3.1.5.1. Click the **Derivative Melt Curves** tab in the High Resolution Melt Plots pane.
   3.1.5.2. Select the assay name (e.g. *NPM1 Exon 12* and *FLT3* Exon 20) in the Assay dropdown menu (**Figure 13**).
   3.1.5.3. Manually set the pre-melt and post-melt regions to span a single peak.
      3.1.5.3.1. Set the pre-melt and post-melt regions as close as possible to the amplicon peak (see **Figure 15** and **Figure 16**Figure 1). The melt regions (indicated by the black arrows) should be approximately 0.2 - 0.5 °C apart from each other.
   3.1.5.4. Click **Analyze** on the top right of the main window to update the results.
   3.1.5.5. Navigate to the **Difference Plot** tab to review the software's automatic calls.
      3.1.5.5.1. Select the reference control (Wild-Type) from the Reference dropdown list (see **Figure 13**).
      3.1.5.5.2. **Figure 17** is the Difference Plot that corresponds with **Figure 16**
   3.1.5.6. In some cases, the automated calls may require further review.
      3.1.5.6.1. Visually inspect both the Difference Plot and Derivative Melt Curves to verify that no samples exhibit unique melt profiles that may indicate possible variants.
      3.1.5.6.2. 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 15: Derivative Melt Curve for a multiplex reaction with two amplicons.

Reactions that contain two targets will have two peaks. Each of these peaks must be analyzed one at a time by manually setting the melting regions to surround a single peak and then pressing Analyze.
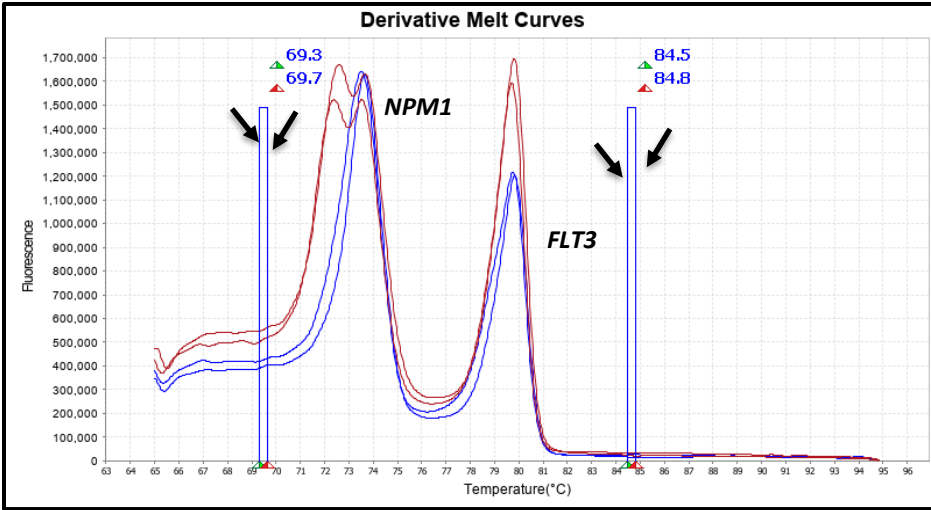

Figure 16: Derivative Melt Curve for the *NPM1 Exon 12* and *FLT3* Exon 20 multiplex reaction.

The pre-melt and post-melt regions are set specifically for *NPM1* analysis. Notice the double peaks in the sample curves for the *NPM1* amplicon.
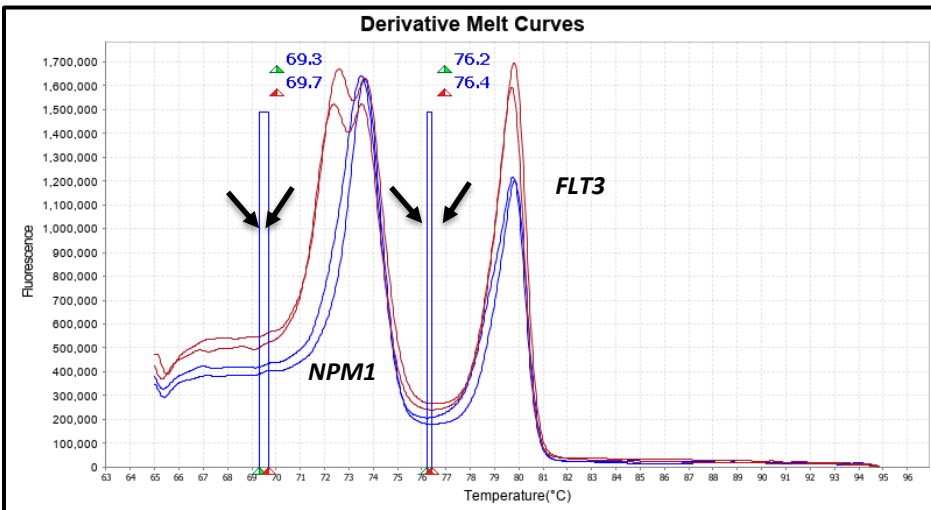
Figure 17: Difference Plot results for *NPM1*

The Difference Plot shows a Wild-Type reference is the flat blue line at Difference = 0 along with the 5% mutation positive control (blue) that has a peak Difference at 73.4 and both sample replicates (red) that have been differentiated into distinct groups with a peak Difference at 72.9 °C. The sample replicates in this example screen positive since the melt data curve aligns with/peaks at a lower temperature and surpasses the 5% positive control (blue).
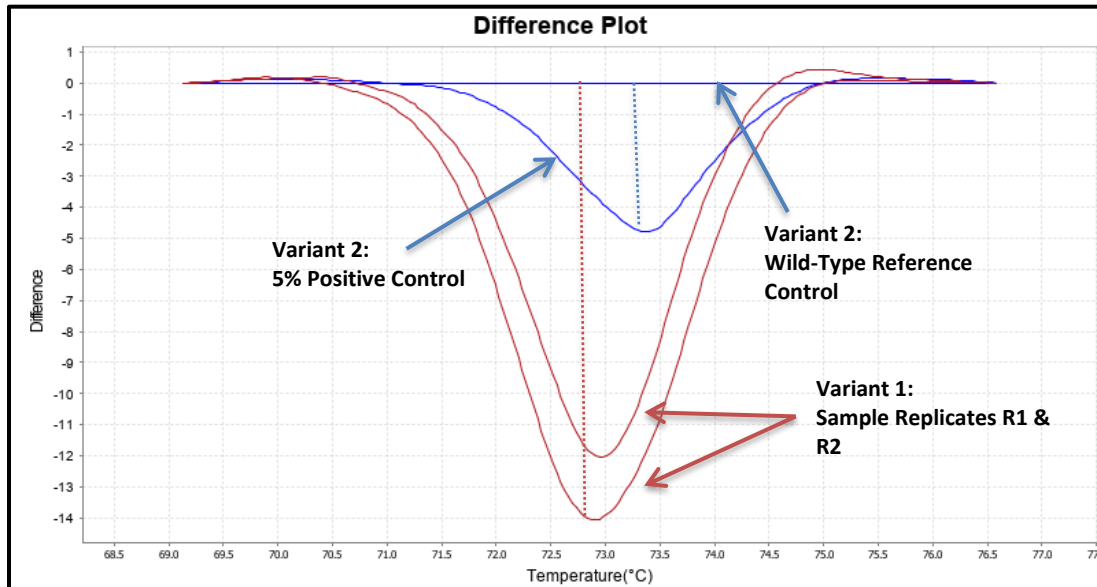
3.1.5.7. Next, repeat the process to analyze the second target. In this example the second target is *FLT3* Exon 20.
    3.1.5.7.1. Navigate to the Derivative Melt Curve tab and manually set the pre-melt and post-melt curves to span the second peak as seen in **Figure 18**.
    3.1.5.7.2. Click Analyze on the top right of the main window to update the results.
    3.1.5.7.3. Click on the **Difference Plot** tab to view the results.
    3.1.5.7.4. Select the reference control (Wild-Type) from the Reference dropdown list and click Analyze to view the results.
    3.1.5.7.5. See **Figure 19** for the **Difference Plot** results using the parameters shown in **Figure 18**.
    3.1.5.7.6. The Wild-Type Control is the flat blue line at Difference = 0 in the **Difference Plot**.

Figure 18: Derivative Melt Curve for the *NPM1* Exon 12 and *FLT3* Exon 20 multiplex.

Multiplex showing the pre-melt and post-melt Start and Stop temperature settings for *FLT3* analysis.
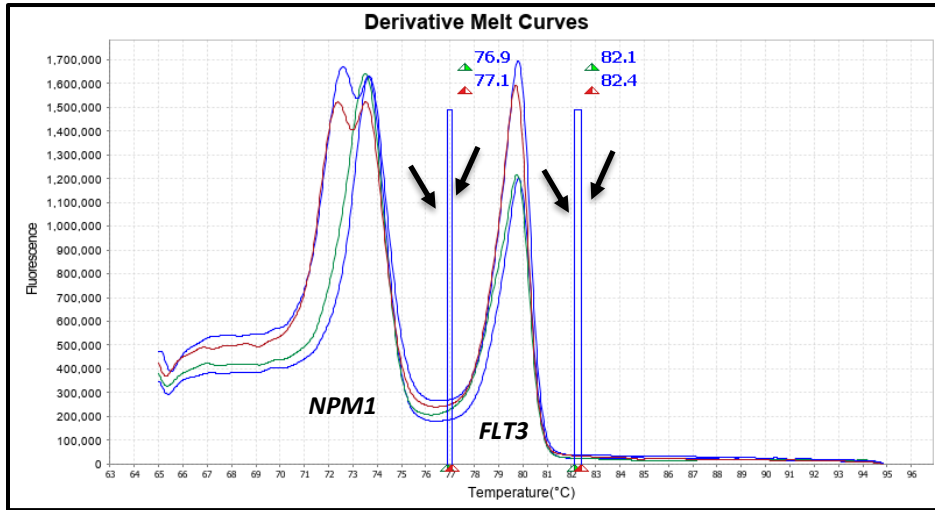

Figure 19: Difference Plot results for *FLT3* Exon 20.

The two sample curves should behave similarly and, in this case, do give similar patterns peaking at 79.9 °C. Even though the range in the difference of the two replicates is present (red and blue peaks), the two traces, though, do not resemble the 5% mutant control. The sample replicates in this example screen WT since the peak for the sample replicates at 79.8 °C is not similar to the known positive sample (green) which has a peak at 79.2 °C.
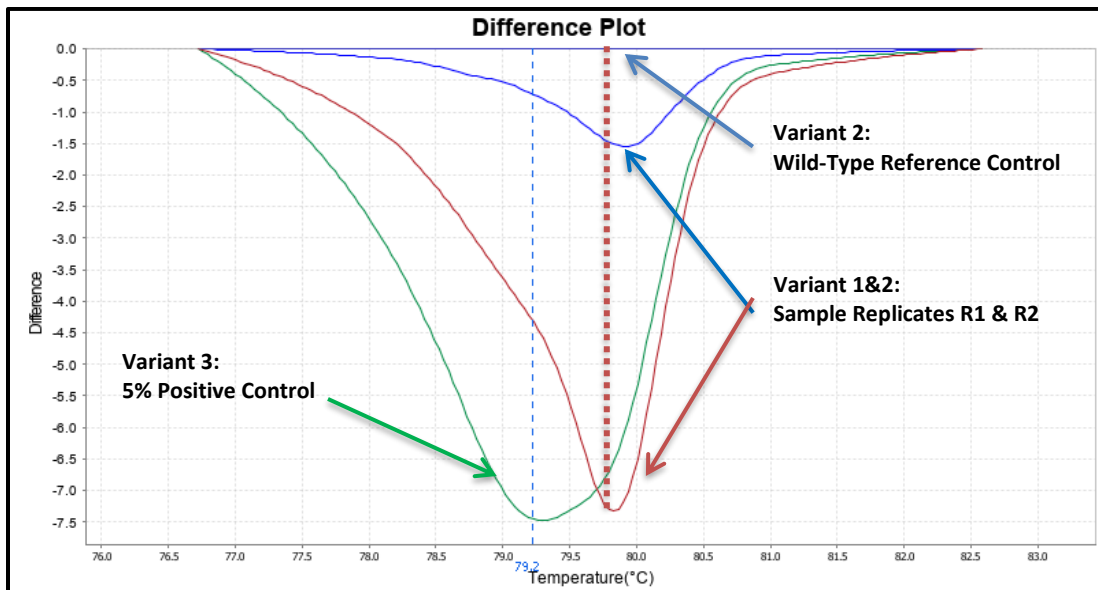
3.2. **Confirmatory Procedure**

    3.2.1. Once a specimen has been identified as variant detected (see **Figure 17** 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 positive, the case is classified as variant detected.

    3.2.4. If one of the replicates is not aligned with the positive control melt profile (melting temperature and curve peak) while the other replicate is aligned with the positive control melt profile (melting temperature and curve peak) the same is HRM negative.

    3.2.5. If the sample melting curve is peaking after the positive control peak in more than 0.5°C then it is considered NVD.

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 minute 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	**IVD** In vitro diagnostic medical device	STERILE A Sterilized using aseptic processing techniques	Keep away from light
Use By:	REF Catalog number	Manufacturer	LOT Batch Code
CE 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 In Vitro Diagnostic 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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