



**Instructions for Use:
Bloodhound™ *ABL1* Resistance 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

| | | |
|--------------|---|--|
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|--------------|---|--|

Bloodhound™ 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 Bloodhound™ offerings are Research Use Only (RUO) assays that detect mutations in targets of interest related to ABL1 Resistance. 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

Bloodhound™ ABL1 Resistance Reagents RUO detect mutations in the *ABL1* gene; specifically in Exon 4, Exon 5, Exon 6, Exon 7 and Exon 8. The Bloodhound™ ABL1 Resistance Reagents indicate the presence of a potential mutation within a sample but does not confirm the identity of the mutation. The presence of *ABL1* mutation can be associated with diagnosis, therapeutic monitoring, and detection of minimal residual disease (MRD), therapeutic selection for response to TKIs and/or stem cell transplantation. The results from Bloodhound™ *ABL1* Resistance are qualitative.

Biological Reference of *ABL1* Mutations

The Bloodhound™ *ABL1* Reagents have been designed to PCR amplify the relevant regions in Exon 4, Exon 5, Exon 6, Exon 7 and Exon 8.

Resistance to the targeted treatment of TKI and subsequent therapeutic failure is driven by the presence of mutations in the translocated *ABL1* region.

The mutations covered in this reagent set are as follows:

| <i>ABL1 Exon 4</i> | <i>ABL1 Exons 5/6</i> | <i>ABL1 Exon 6</i> | <i>ABL1 Exon 7</i> |
|--------------------|-----------------------|--------------------|---------------------------|
| c.730A>G; p.M244V | c.895G>T; p.V299L | c.1052T>C; p.M351T | c.1187A>G; p.H396R |
| c.742C>G; p.L248V | c.895G>A; p.V299L | c.1075T>G; p.F359V | <i>ABL1 Exon 8</i> |
| c.749G>A; p.G250E | c.949T>C; p.F317L | c.1075T>A; p.F359I | c.1375G>A; p.E459K |
| c.757T>C; p.Y253H | c.949T>G; p.F317V | c.1076T>G; p.F359C | |
| c.763G>A; p.E255K | c.949T>A; p.F317I | | |
| c.764A>T; p.E255V | c.950T>G; p.F317C | | |
| | c.951C>G; p.F317L | | |
| | c.951C>A; p.F317L | | |
| | c.944C>T; p.T315I | | |
| | c.943A>G; p.T315A | | |

Principles of High Resolution Melting

Instructions for Use

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 Bloodhound™ *ABL1* Reagents Controls

The “Wild-Type Control” and “Mutant Control” sequences were constructed by synthesis and cloning for *ABL1* using the NCBI Reference Sequences:

NM_005157.6 Homo sapiens ABL proto-oncogene 1, non-receptor tyrosine kinase (ABL1), transcript variant a, mRNA

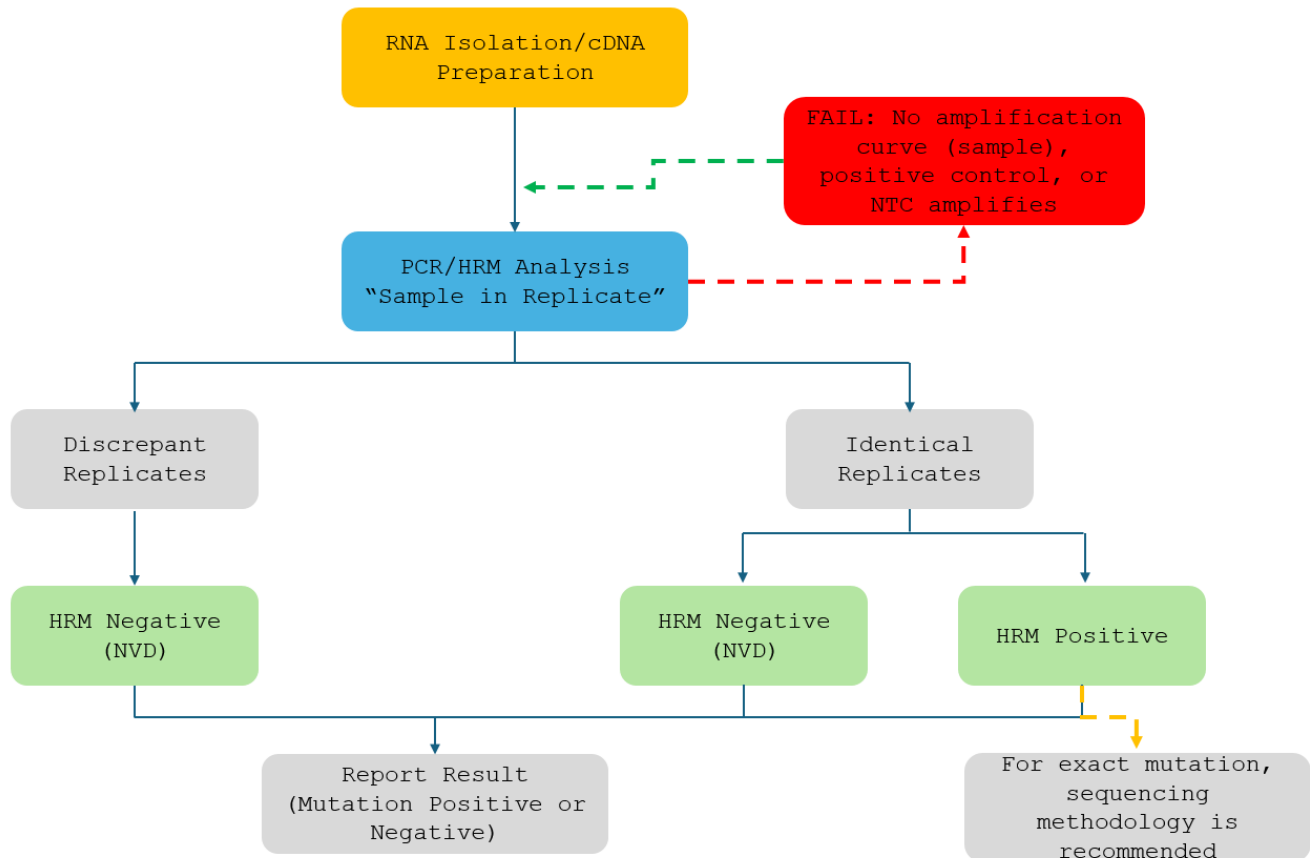
Bloodhound™ Reagents Preparation

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

Overview of Samples using Bloodhound™ 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 Bloodhound™ Reagents should only be used in the context of the workflow indicated below.

Figure 1: Bloodhound™ Reagents Workflow



Explanation to Figure 1:

1. Isolate the RNA from peripheral blood or bone marrow aspirate using standard laboratory procedures. Prepare cDNA using standard laboratory procedures. Dilute the prepared cDNA, refer to Section 1.2 for details.
2. Perform Bloodhound™ *ABL1* Resistance 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 Bloodhound™ *ABL1* for that sample.
 - ii. If the NTC shows amplification and the CT value is <346, 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 Bloodhound™ *ABL1* 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 Bloodhound™ *ABL1* 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

The Bloodhound™ ABL1 Resistance Reagents are supplied as ready to use. Bloodhound™ ABL1 Resistance Reagents can be purchased as pre-plated or free-flow. Pre-plated reactions come in 3 or 6 samples in duplicate and include a Wild-Type, Mutant, and No Template control (NTC). The free-flow reagents come as 20-samples in duplicate, see table 1 for a detailed list of set contents.

Table 1: Reagent Set Contents

| Vial | Volume |
|----------------------|---------|
| <i>ABL1</i> Exon 4 | 1020 µL |
| <i>ABL1</i> Exon 5/6 | 1020 µL |
| <i>ABL1</i> Exon 6 | 1020 µL |
| <i>ABL1</i> Exon 7 | 1020 µL |
| <i>ABL1</i> Exon 8 | 1020 µL |
| Positive Control | 75 µL |
| Wild-Type Control | 75 µL |

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

- None of the reagents present a hazard to health in the quantities supplied. The SDS is available on our website, <https://www.precipiodx.com>.
- There are no substances of animal or human origin that present a risk of infection in the supplied reagents.
- These reagents should be used only by those persons who have been trained in the appropriate laboratory techniques.
- Biological sample handling:
 - 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.
 - Biological waste during the DNA extraction process should be disposed in accordance with national/local regulations relating to biohazard waste disposal.
 - Liquid waste must be considered infectious and be handled and discarded according to national/local regulations relating to biohazard waste disposal.
- Sample or Reagent Spillage
 - Avoid contact with skin and eyes.
 - Wear appropriate protective clothing, gloves, facemask and glasses.
 - Absorb the material using gauze.
 - Clean the surface with 10% (v/v) sodium hypochlorite (10% bleach) and leave the surface wet for 30 min to one hour.
 - 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.
 - 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:

- MeltDoctor™ HRM Calibration Plate, Fast 96-well (Thermo Fisher Scientific Catalog Number 4425618)
- TE buffer (Fisher Healthcare Catalog Number AAJ75793AE)
- Nuclease free water (Fisher Healthcare Catalog Number AM9932)
- RNA Isolation Kit

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5. Reagents for reverse transcription

Consumables/Supplies:

1. MicroAmp™ Optical Adhesive Film (Fisher Healthcare Catalog Number 4360954)
2. Pipettes
3. Pipette tips
4. Vortex
5. 1.5 mL capped 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.

RNA Extraction:

NOTE: RNA degrades within 72 hours of specimen draw. It is recommended to synthesize cDNA as soon as the RNA is extracted. cDNA is more stable when stored for longer periods of time. Internal validation and optimization may be needed. While preparing cDNA reagents, store the RNA at 4 °C.

- The quality of the assay and the consistency of HRM is highly dependent of the quality of the analyte. All samples should be extracted with the same high quality RNA extraction method and protocol to avoid any variations due to reagents/buffers. The quality of RNA should be verified before use.
- No less than 20 ng/μL of initial starting RNA is required for cDNA synthesis. If yield is less than 20 ng/μL, re-extraction is necessary. Internal optimization required. Total RNA required prior to cDNA synthesis is between 0.5 and 1 μg.
- Ensure sample-to-sample uniformity.

Extracted RNA Storage if Needed:

- Extracted RNA can be stored at -20 °C for short term storage and use (up to 2 weeks) or at -80 °C for long term storage (up to 1 year).

Bloodhound™ 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 the Bloodhound™ reagents.

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 RNase Away™ solutions.

1.2. **Procedure for Free-Flow Reagents:**

- 1.2.1. Thaw all necessary components.
- 1.2.2. Briefly vortex the assay mix and centrifuge for approximately 3-10 sec (600 to 1000 x g.) to collect the liquid in the bottom of the tube.
- 1.2.3. Plate 17 µL of each premixed primer into each reaction well in a 96-well PCR reaction plate.

NOTE: Dilute each cDNA sample, using 4 µL of cDNA to 36 µL of TE buffer to prepare enough diluted cDNA for replicate analysis of 5 amplicons (3 µL x 2 replicates x 5 amplicons = 30 µL).

- 1.2.4. Add 3 µL of the diluted cDNA product to each reaction. Mix gently by pipetting up and down.
 - 1.2.4.1. The same sample should be added to two separate wells for each assay for replicate analysis.
- 1.2.5. Add 3 µL of control into the designated reaction wells.
- 1.2.6. Add 3 µL of NTC into the designated reaction wells.
- 1.2.7. Place an optical seal over the plate and centrifuge (600 to 1000 x g., approximately 30 sec).
 - 1.2.7.1. Suspect the plate for any air bubble and well uniformity prior to placing the plate in the instrument.
- 1.2.8. Perform the run method on the instrument according to **Table 2**.

1.3. **Pre-Plated Bloodhound™ ABL1 Resistance Layout:**

- 1.3.1. The Bloodhound™ ABL1 Plated reagents are available in 3-Sample and 6-Sample formats.

Figure 2: Bloodhound™ ABL1 Plate layouts for 3 samples per plate (A) and 6 samples per plate (B).

A

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|-------------------|-------------------|---------------------|---------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| A | | | | | | | | | | | | |
| B | | ABL1 ex4_Sample 1 | ABL1 ex4_Sample 1 | ABL1 ex5/6_Sample 1 | ABL1 ex5/6_Sample 1 | ABL1 ex6_Sample 1 | ABL1 ex6_Sample 1 | ABL1 ex7_Sample 1 | ABL1 ex7_Sample 1 | ABL1 ex8_Sample 1 | ABL1 ex8_Sample 1 | ABL1 ex8_Sample 1 |
| C | | ABL1 ex4_Sample 2 | ABL1 ex4_Sample 2 | ABL1 ex5/6_Sample 2 | ABL1 ex5/6_Sample 2 | ABL1 ex6_Sample 2 | ABL1 ex6_Sample 2 | ABL1 ex7_Sample 2 | ABL1 ex7_Sample 2 | ABL1 ex8_Sample 2 | ABL1 ex8_Sample 2 | ABL1 ex8_Sample 2 |
| D | | ABL1 ex4_Sample 3 | ABL1 ex4_Sample 3 | ABL1 ex5/6_Sample 3 | ABL1 ex5/6_Sample 3 | ABL1 ex6_Sample 3 | ABL1 ex6_Sample 3 | ABL1 ex7_Sample 3 | ABL1 ex7_Sample 3 | ABL1 ex8_Sample 3 | ABL1 ex8_Sample 3 | ABL1 ex8_Sample 3 |
| E | | | ABL1 ex4_MUT | | ABL1 ex5/6_MUT | | ABL1 ex6_MUT | | ABL1 ex7_MUT | | ABL1 ex8_MUT | |
| F | | | ABL1 ex4_WT | | ABL1 ex5/6_WT | | ABL1 ex6_WT | | ABL1 ex7_WT | | ABL1 ex8_WT | |
| G | | | ABL1 ex4_NTC | | ABL1 ex5/6_NTC | | ABL1 ex6_NTC | | ABL1 ex7_NTC | | ABL1 ex8_NTC | |
| H | | | | | | | | | | | | |

B

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------------------|-------------------|---------------------|---------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|----------------|----------------|
| A | | | | | | | | | | | ABL1 ex4_MUT | ABL1 ex4_WT |
| B | ABL1 ex4_Sample 1 | ABL1 ex4_Sample 1 | ABL1 ex5/6_Sample 1 | ABL1 ex5/6_Sample 1 | ABL1 ex6_Sample 1 | ABL1 ex6_Sample 1 | ABL1 ex7_Sample 1 | ABL1 ex7_Sample 1 | ABL1 ex8_Sample 1 | ABL1 ex8_Sample 1 | ABL1 ex5/6_MUT | ABL1 ex5/6_WT |
| C | ABL1 ex4_Sample 2 | ABL1 ex4_Sample 2 | ABL1 ex5/6_Sample 2 | ABL1 ex5/6_Sample 2 | ABL1 ex6_Sample 2 | ABL1 ex6_Sample 2 | ABL1 ex7_Sample 2 | ABL1 ex7_Sample 2 | ABL1 ex8_Sample 2 | ABL1 ex8_Sample 2 | ABL1 ex6_MUT | ABL1 ex6_WT |
| D | ABL1 ex4_Sample 3 | ABL1 ex4_Sample 3 | ABL1 ex5/6_Sample 3 | ABL1 ex5/6_Sample 3 | ABL1 ex6_Sample 3 | ABL1 ex6_Sample 3 | ABL1 ex7_Sample 3 | ABL1 ex7_Sample 3 | ABL1 ex8_Sample 3 | ABL1 ex8_Sample 3 | ABL1 ex7_MUT | ABL1 ex7_WT |
| E | ABL1 ex4_Sample 4 | ABL1 ex4_Sample 4 | ABL1 ex5/6_Sample 4 | ABL1 ex5/6_Sample 4 | ABL1 ex6_Sample 4 | ABL1 ex6_Sample 4 | ABL1 ex7_Sample 4 | ABL1 ex7_Sample 4 | ABL1 ex8_Sample 4 | ABL1 ex8_Sample 4 | ABL1 ex8_MUT | ABL1 ex8_WT |
| F | ABL1 ex4_Sample 5 | ABL1 ex4_Sample 5 | ABL1 ex5/6_Sample 5 | ABL1 ex5/6_Sample 5 | ABL1 ex6_Sample 5 | ABL1 ex6_Sample 5 | ABL1 ex7_Sample 5 | ABL1 ex7_Sample 5 | ABL1 ex8_Sample 5 | ABL1 ex8_Sample 5 | ABL1 ex4_NTC | ABL1 ex5/6_NTC |
| G | ABL1 ex4_Sample 6 | ABL1 ex4_Sample 6 | ABL1 ex5/6_Sample 6 | ABL1 ex5/6_Sample 6 | ABL1 ex6_Sample 6 | ABL1 ex6_Sample 6 | ABL1 ex7_Sample 6 | ABL1 ex7_Sample 6 | ABL1 ex8_Sample 6 | ABL1 ex8_Sample 6 | ABL1 ex6_NTC | ABL1 ex7_NTC |
| H | | | | | | | | | | | ABL1 ex8_NTC | |

- 1.3.2. Bloodhound™ ABL1 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. Procedure for Pre-plated Reagents

- 1.4.1. Remove the cDNA from the freezer and allow to thaw.
 - 1.4.2. Remove the plate from the freezer and thaw the plate in its packaging.
 - 1.4.2.1. Thaw the plate for a minimum of 20 min at room temperature, protected from light.
 - 1.4.3. Remove the plate from the packaging. **DO NOT REMOVE** the silicone plate septa seal.
 - 1.4.4. Centrifuge the plate for 2 min at 600 to 1000 x g.
 - 1.4.4.1. 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.
 - 1.4.4.2. 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.
 - 1.4.5. Orient the plate so that well A1 is the top left well.
 - 1.4.6. Being very careful, **SLOWLY** remove the plate septa seal from the Bloodhound™ ABL1 Plate.
 - 1.4.7. Vortex and spin down all cDNA dilutions prior to use.
 - 1.4.8. Dilute each cDNA sample, using 4 µL of cDNA to 36 µL of TE buffer.
 - 1.4.9. Add 3 µL of diluted cDNA sample to each well.
 - 1.4.9.1. Pipette-mix cDNA sample in each well.
 - 1.4.9.2. 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.
- 1.4.10. 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.
 - 1.4.11. 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 “Bloodhound MPN 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.

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- 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 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.

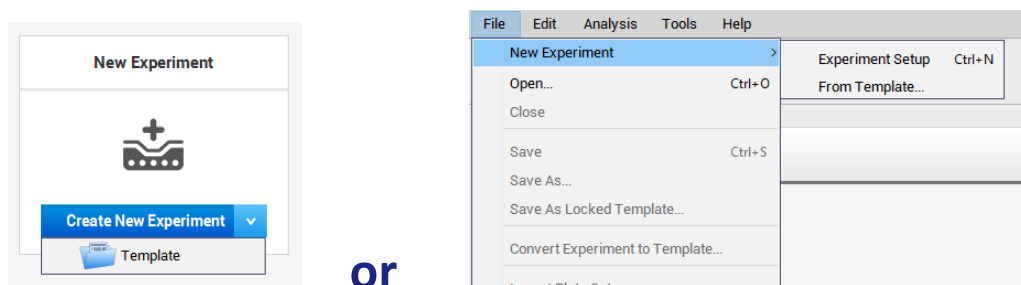
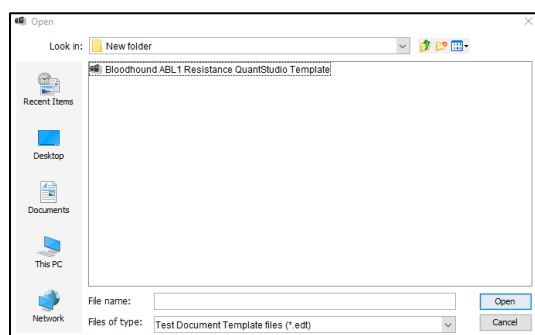


Figure 4: Bloodhound™ QuantStudio™ Template.



Instructions for Use

Figure 5: HRM thermal cycler conditions.

Make sure reaction volume is 20 μL (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).

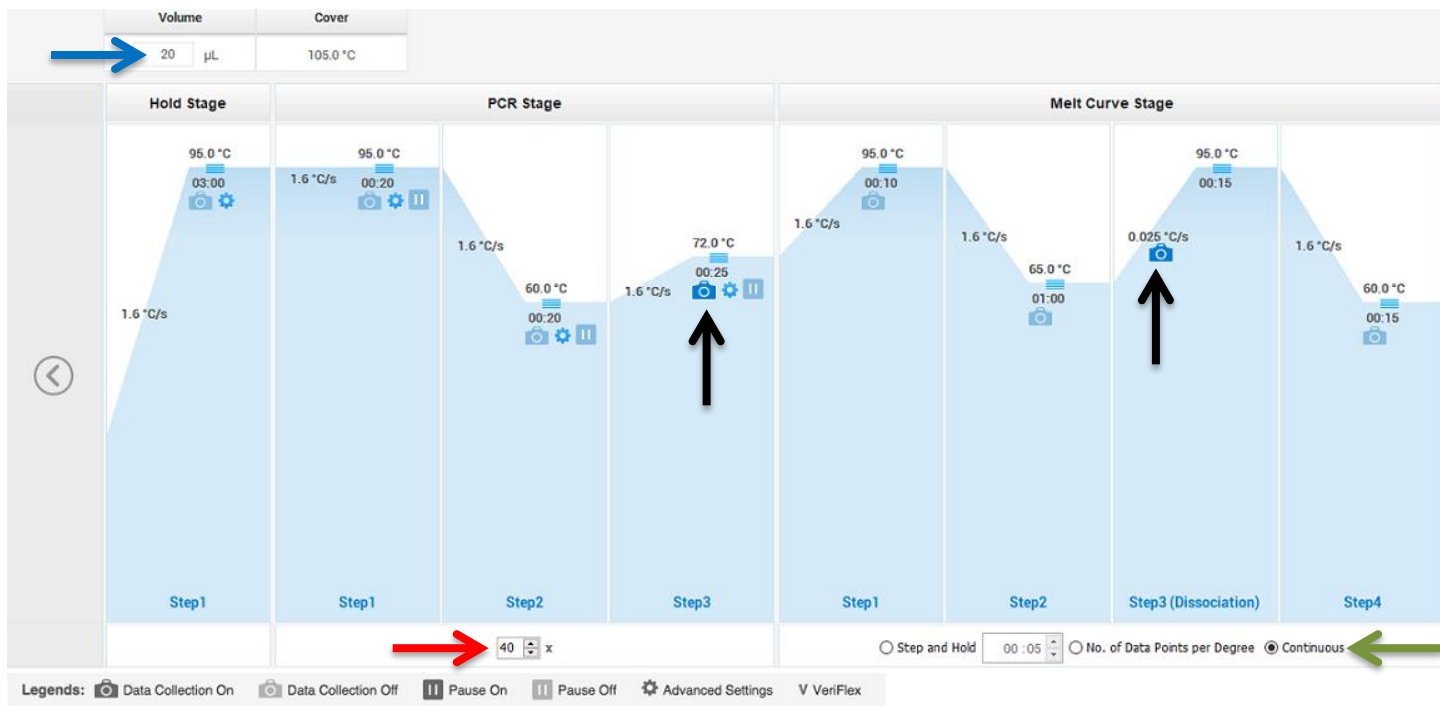


Table 2: Thermal cycling and melt curve program for the HRM reaction.

| Stage | Step | Temp | Time | Ramp rate |
|-------------------------|---|-----------------------|--------|-------------------------------------|
| Holding | Denature | 95 $^{\circ}\text{C}$ | 3 min | 1.6 $^{\circ}\text{C}/\text{Sec}$ |
| | Denature | 95 $^{\circ}\text{C}$ | 20 sec | 1.6 $^{\circ}\text{C}/\text{Sec}$ |
| Cycling (40 cycles) | Anneal | 60 $^{\circ}\text{C}$ | 20 sec | 1.6 $^{\circ}\text{C}/\text{Sec}$ |
| | Extend (Data Capture on) | 72 $^{\circ}\text{C}$ | 25 sec | 1.6 $^{\circ}\text{C}/\text{Sec}$ |
| | Denature | 95 $^{\circ}\text{C}$ | 10 sec | 1.6 $^{\circ}\text{C}/\text{Sec}$ |
| Melt curve/Dissociation | Anneal | 65 $^{\circ}\text{C}$ | 1 min | 1.6 $^{\circ}\text{C}/\text{Sec}$ |
| | High resolution melting (Data Capture on) | 95 $^{\circ}\text{C}$ | 15 sec | 0.025 $^{\circ}\text{C}/\text{Sec}$ |
| | Final | 60 $^{\circ}\text{C}$ | 15 sec | 1.6 $^{\circ}\text{C}/\text{Sec}$ |

Figure 6: Import Plate Layout files.

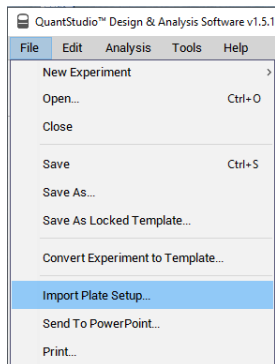
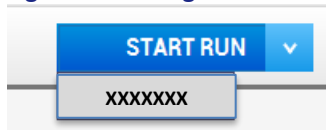


Figure 7: Starting the run.



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 Bloodhound™ 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 cDNA and controls typically amplify between 22 - 32 cycles (C_t). 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](#).
- 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.

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

Figure 8: Example Amplification Plot.

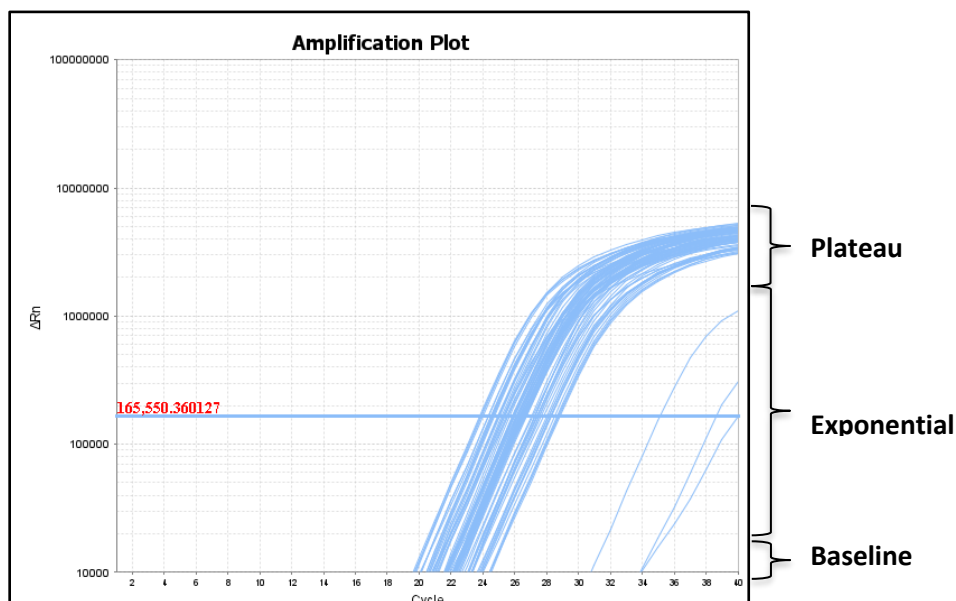
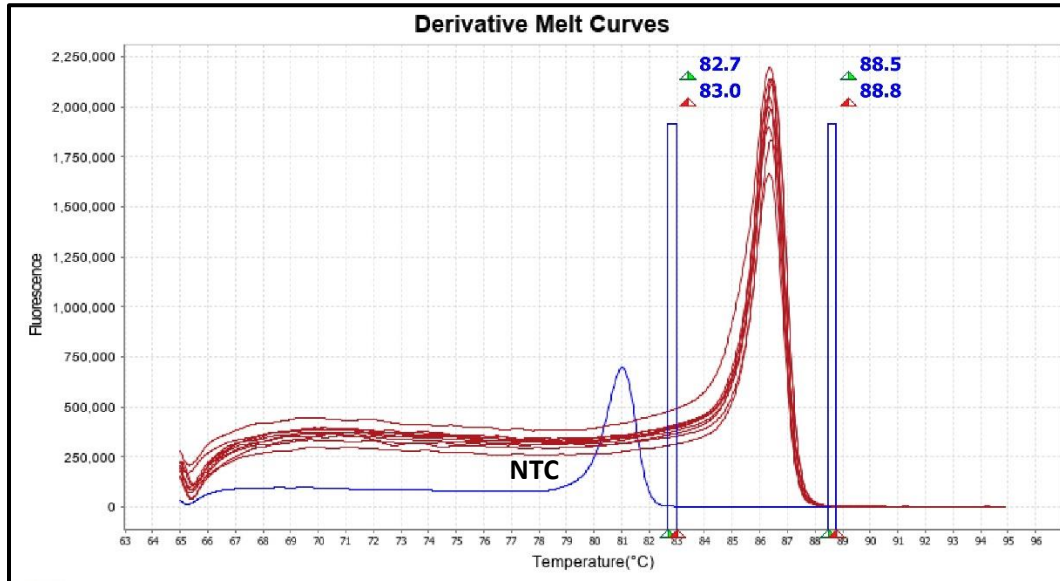


Figure 9: Example of Non-Specific Amplification in the NTC due to primer dimer formation.



Bloodhound™ ABL1 Resistance Analysis:

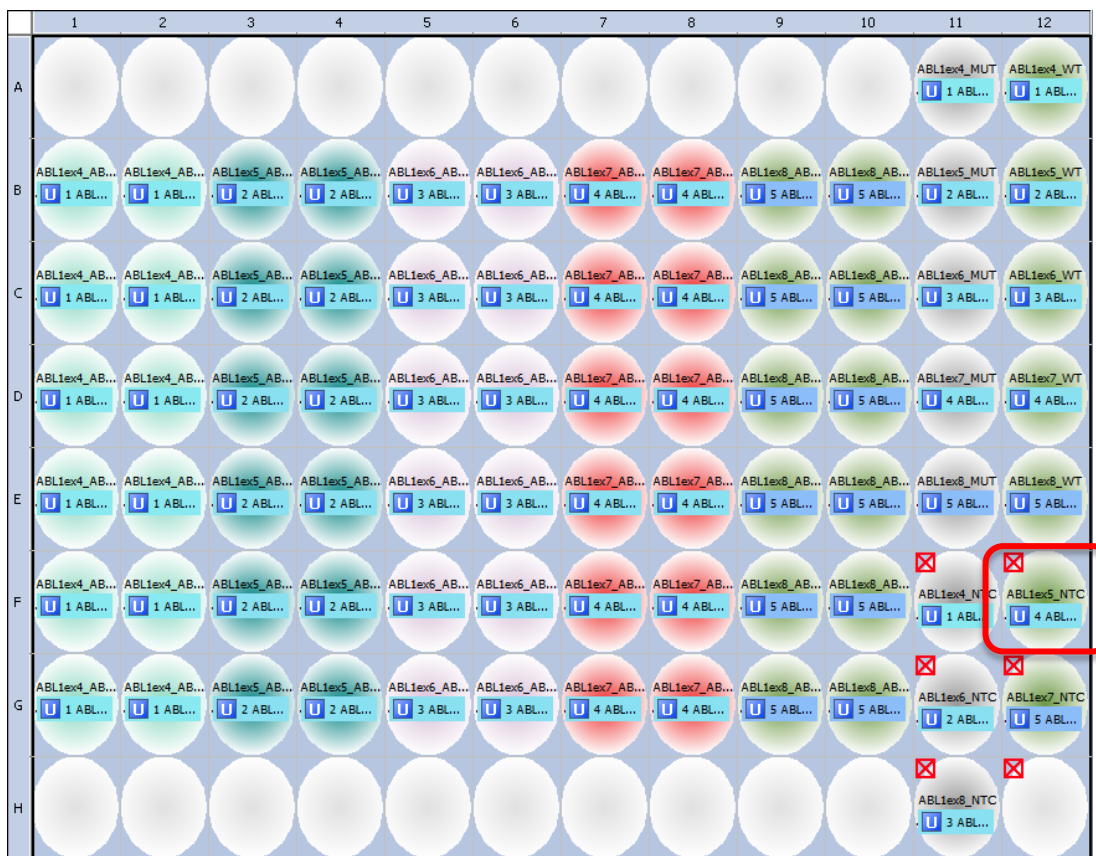
Guidance for HRM data analysis is given in this manual; however, due to instrument-to-instrument variation, calibration variation, as well as other factors, data analysis parameters for a given data set will need to be determined by the end-user.

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

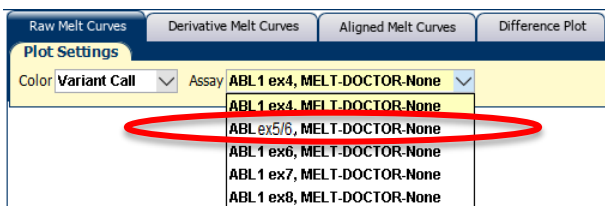
- 3.1.1. Review the melt curves using High Resolution Melt Software v3.2.
- 3.1.2. Open High Resolution Melt Software v3.2. Click **Open** on the main window to open the saved *.eds run file saved in step 1.5.1.8.
- 3.1.3. Bloodhound™ ABL1 Resistance contains 5 targets of interest. Each target of interest must be analyzed one at a time (i.e., ABL1 exon 5/6 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. ABL1 exon 5/6) 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 "ABL1 exon 5/6" is selected on a 3 sample plate layout, data from columns 4 and 5.
 - 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 *ABL1* exon 5/6. The well outlined in red is the *ABL1* exon 5/6 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.

- 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.

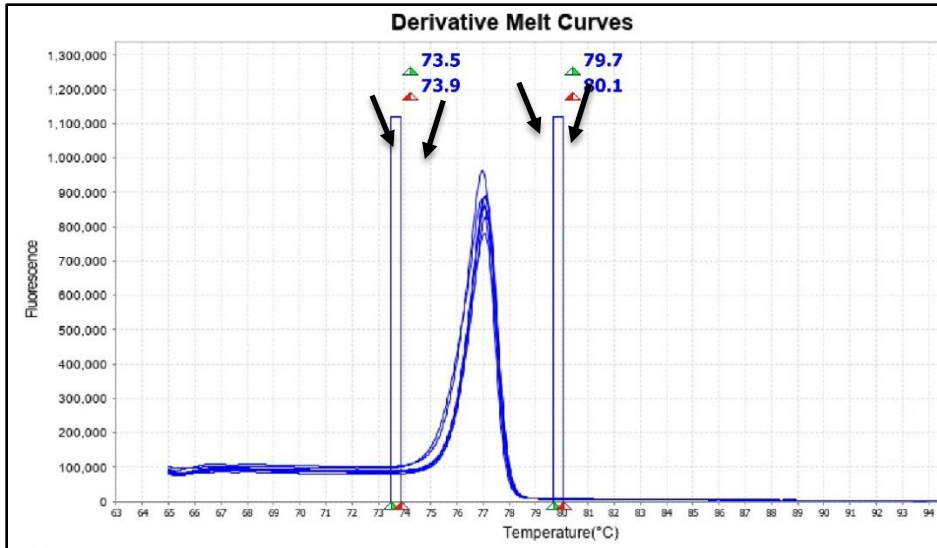


Figure 13: Reference Dropdown Menu.

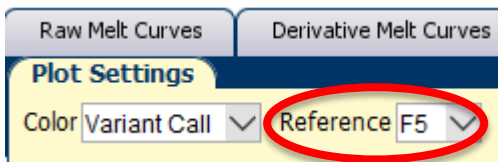
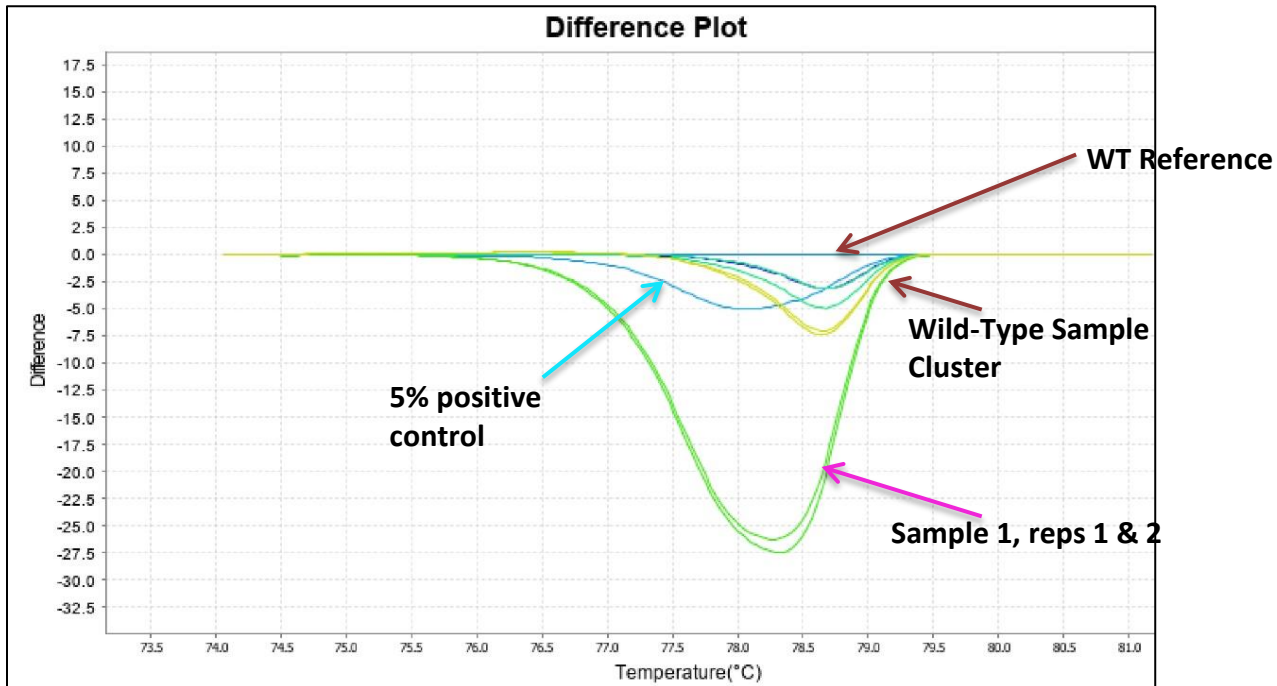


Figure 14: Example *ABL1* exon 5/6 Difference Plot.

Difference Plot shows a dark yellow Wild-Type Control reference line at Difference = 0. Samples that cluster with the Wild-Type reference are considered NVD. The light blue curve is the 5% Positive Control. The lime green curves, Sample 1, more closely match the profile of the positive control (light blue). 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 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 Discrepant Replicate curves.

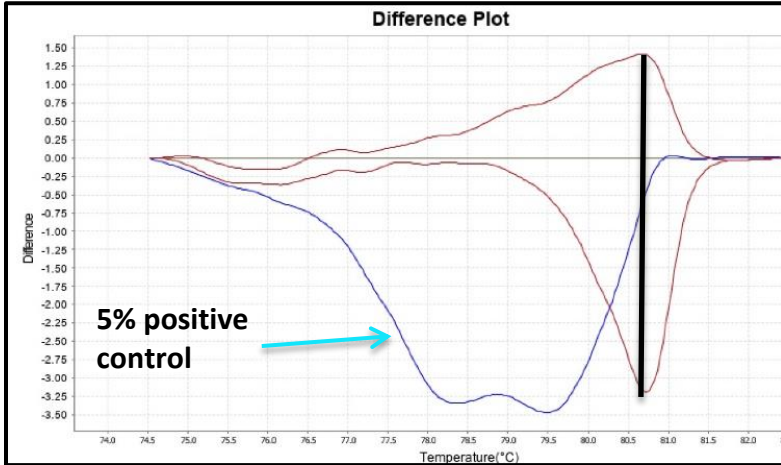
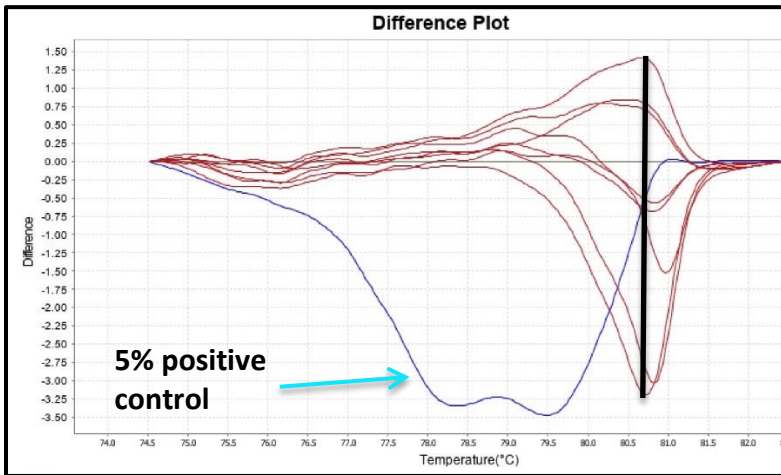


Figure 16: Example Discrepant Replicate curves with WT Group.



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. |

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 |
|  |  |  | |
| Research Use Only | Consult instructions for use | Temperature Limitation | |

Each manufactured lot of Bloodhound™ 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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