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Novallele Genotyping Assay User Guide

A complete set of instructions to genotype your samples using the Novallele genotyping reagents

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CANON BIOMEDICAL

Canon BioMedical is focused on empowering the biomedical research and healthcare communities through innovative technologies and solutions. Our solutions will enable clinicians and scientists to improve our health and advance science. Canon BioMedical pursues innovation in harmony with Canon's *Kyosei* philosophy — defined as "all people, regardless of race, religion, or culture, harmoniously living and working together into the future."

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Introduction

Rapid PCR with high-resolution melting (HRM) analysis enables scientists to quickly genotype samples for a wide range of genomic variations. We developed a functionally-verified chemistry that supports faster ramping rates and shortened cycling times as compared to typical PCR conditions.

This guide provides instructions to genotype samples using the Novallele genotyping reagents with a thermocycler capable of HRM. We offer two methods for you to genotype your tough targets. You may use one of our Novallele genotyping assays in conjunction with the Novallele Genotyping Mastermix as well as the corresponding Novallele Control Set. The majority of this guide is focused on this genotyping solution.

Our chemistry is ideally paired with the Novallele genotyping assays, but the Novallele Genotyping Mastermix may also be used in conjunction with the 2x Novallele Oligo Dilution Buffer to design an assay for your particular target. See "Designing your own genotyping assay," page 20, for instructions to design your own assay.

All Novallele genotyping assays, Novallele Control Sets, Novallele Genotyping Mastermix, and 2x Novallele Oligo Dilution Buffer are For Research Use Only. Not for use in diagnostic procedures.

Summary and Explanation

Within a person's twenty-three pairs of chromosomes, a majority of the three billion nucleotide bases are the same across the human race. Current estimates indicate that up to 0.1% of human genomic DNA (gDNA) may vary; meaning that any two unrelated individuals may differ at fewer than three million DNA positions. A single nucleotide polymorphism (SNP) is a variation of one nucleotide within the genome between members of the same species. SNPs are one of the most important types of genetic variations. These individual, specific variations within a gene sequence can influence a

person's disease risk and therapeutic response. Typical genetic variations also include insertions, deletions, and copy number variations (CNV).

A diverse set of genotyping technologies, including sequencing, microarrays, in situ hybridization, and PCR-based methods, are used to detect mutations. The variety of methods enables researchers to match the correct technology with not only their experimental question but also user preferences for cost, data analysis, and turnaround time.

Principle of the Procedure

The Novallele genotyping assays, Novallele Control Sets, and Novallele Genotyping Mastermix are used with a thermocycler with HRM capabilities to perform rapid PCR followed by high-resolution melting (HRM) analysis. HRM analysis is a powerful tool that detects differences in DNA sequences.

Sample type and DNA extraction

We tested gDNA extracted from cell lines and blood as well as synthetic samples including plasmids and double-stranded linear segments.

Sample preparation is critical to downstream analysis. Make sure to select a method of DNA extraction suitable for your application. Any method that yields suitable concentration, purity, and integrity of DNA is acceptable for use, but make sure to validate the method. Maintain the selected DNA extraction method as changes in salt concentration and elution buffer components can affect sample analysis. It is essential that all samples are prepared using the same method to reduce variability in the subsequent analysis.

We have achieved comparable results with the following kits during our testing:

- QIAGEN® Gentra® Puregene® Blood Kit (cat. nos. 158389, 158445, and 158467)
- QIAGEN QIAamp DNA Blood Mini Kit (cat. nos. 51106 and 51104)
- Roche MagNA Pure® Compact Nucleic Acid Isolation Kit I (cat. nos. 03730964001 and 03730972001)

Controls

We routinely use both negative and positive controls. For each thermocycler run, we recommend at least one negative no-template control (NTC) to rule out environmental contamination. To prepare the NTC reaction, substitute nuclease-free water instead of the sample.

We also recommend each thermocycler run include at least one positive control, preferably a homozygous, wild-type (WT) control; however, include a homozygous mutant (HOM) control and heterozygous mutant (HET) control in each thermocycler run if they are available. We provide controls for almost all of our assays, or you may prepare your own controls for your Novallele genotyping assay.

Novallele Control Set

A Novallele Control Set is available for almost all the Novallele genotyping assays, which enables you to identify the genotype of your sample by comparing the HRM analysis to the control data analysis for the WT, HET, and HOM variants.

An advantage to using a Novallele Control Set is that the controls use the same cycling conditions as the corresponding Novallele genotyping assay. A Novallele Control Set includes a WT control and a HOM control. A HET control is formulated by combining the two provided controls, although additional verification may be required to determine the correct amounts of the WT and HOM controls. Each control contains 120 µL of double-stranded DNA, which matches the target of interest, in TE buffer. Each reaction requires 3.6 µL; enough material is supplied for 30 reactions. We recommend using each control in triplicate for each PCR run.

To genotype samples, sample melt curves are compared to the Novallele Control Set melt curves.

Thermocycler

Using a thermocycler with HRM capabilities, the DNA is amplified by PCR and followed by amplicon melting. During the PCR, a fluorescent dye at saturating concentrations binds to double-stranded

DNA. The critical element is that the dye is specific and sensitive – it only fluoresces when bound to double-stranded DNA. The optical system of the HRM-enabled thermocycler captures the reduction in fluorescence intensity as double-stranded DNA denatures into single strands.

The exact fluorescence change with increasing temperature varies depending on the DNA sequence. When compared to the reference DNA, a shift in melting temperature (T_m) or a change in the melt curve shape indicates a genetic variation in the sample.

For each Novallele genotyping assay, a product specification sheet is provided that contains the assay conditions for programming your HRM-enabled thermocycler. The product specification sheets are included with each Novallele genotyping assay and may also be found at **www.canon-biomedical.com**.

We developed and verified the performance of the Novallele genotyping assays, Novallele Control Sets, and Novallale Genotyping Mastermix on the Bio-Rad® CFX Connect™, QIAGEN Rotor-Gene® Q, and Roche LightCycler® 480 instruments. Other HRM thermocyclers such as BioFire® LS32™, Thermo Fisher Scientific® QuantStudio®, and Roche LightCycler 2.0 may be compatible; however, we have only performed limited testing of the Novallele genotyping assays, Novallele Controls Sets, and Novallale Genotyping Mastermix on these instruments. For instruments other than the Bio-Rad CFX Connect, qualification of your instrument is recommended. If necessary, please contact Canon BioMedical at 1-844-CANONBIO for additional support.

PCR run

The Novallele genotyping assays in combination with the Novallele Genotyping Mastermix contain all the reagents needed to identify specific SNPs or polymorphisms, such as indels, within a given human gDNA sample. Samples containing DNA are mixed with the Novallele genotyping assay and Novallele Genotyping Mastermix in either a microcentrifuge tube or a well plate.

Each Novallele genotyping assay contains enough reagent for 160 reactions if using the recommended amount of 3.6 µL per 10 µL reaction.

Important: Novallele Genotyping Mastermix is essential for successful function of the Novallele genotyping assays. Do not replace the Novallele Genotyping Mastermix with other products.

Materials Available from Canon BioMedical

This user guide applies to multiple products that are ordered individually from Canon BioMedical as described in the table below. The Novallele genotyping assay and Novallele Control Set catalog numbers vary depending on the selected target.

Product name	Catalog number	Number of reactions	Amount
Novallele Genotyping Mastermix	40018	160	600 µL
Novallele genotyping assay	Variable	160	600 µL
Novallele Control Set	Variable	30	120 µL

Materials Required But Not Available from Canon BioMedical

Important: Make sure all instruments are calibrated according to the manufacturer's recommendations.

- 96-well or 384-well plate or applicable thermocycler reaction vessels, such as PCR tubes or capillaries
- Spectrophotometer and associated consumables such as cuvettes, etc.
- Nuclease-free water
- HRM-compatible PCR thermocycler
- Single-channel or multichannel pipettes with nuclease-free tips

- Nuclease-free microcentrifuge tubes
- Centrifuge and rotor for well plate

Warnings and Precautions

When working in a laboratory, always wear a lab coat, disposable gloves, and protective eyewear. Always follow your national and local safety policies and procedures regarding the appropriate handling and disposal of biological materials and reagents.

For more information, please consult the applicable safety data sheets (SDSs). To obtain a SDS for our products, visit **www.canon-biomedical.com** or contact us at 1-844-CANONBIO (1-844-226-6624).

Reagent Storage and Handling

When stored correctly, the materials are stable for 6 months from the date of receipt. If your products arrive damaged, please contact us at 1-844-CANONBIO (1-844-226-6624).

Novallele genotyping assays

Store Novallele genotyping assays at 2–8°C.

Novallele Genotyping MasterMix

IMPORTANT: Store the Novallele Genotyping Mastermix away from light.

Store the Novallele Genotyping Mastermix at 2–8°C and away from light as the reagent is light-sensitive.

Novallele Control Set

Store Novallele genotyping assays at –20°C. Do not freeze/thaw the product more than five times.

Procedure

We recommend testing three replicates of each sample and the following procedure is based on our recommendation.

Important points before starting

- Use sterile aerosol barrier pipette tips designed for PCR applications.
- Familiarize yourself with your HRM-enabled thermocycler before starting this protocol. Refer to the applicable thermocycler user manual and follow the manufacturer's instructions for proper operation and maintenance.
- Pipetting accuracy and precision affect the consistency of results. Make sure that all pipettes and instruments have been checked and calibrated according to the manufacturer's recommendations.
- Do not use Diethylpyrocarbonate (DEPC)-treated water. Use high-quality, nuclease-free water.
- Novallele reagents can be stored during test preparation for up to two hours at room temperature (15–25°C).

Things to do before starting

- Set up two physically separate workspaces; one for PCR setup and another for PCR and post-PCR processing operations. The physical separation will minimize amplicon contamination that may impact results and data interpretation.
- Decontaminate PCR workspaces and labware (pipettes, tube racks, etc.) with UV light and 2–10% bleach followed by wiping with 70% isopropanol.
- Thaw frozen gDNA samples thoroughly at room temperature (15–25°C) or on ice before starting.
- If using Novallele Control Set(s), thaw the controls at room temperature (15–25°C) or on ice before starting.

DNA extraction

Extract the DNA from your sample. For guidance on DNA extraction, see "Sample type and DNA extraction," page 6.

Important: Prepare all samples using the same extraction method to reduce variability in the subsequent analysis. HRM is sensitive to changes in salt and buffer components, and using the same extraction method reduces variability in the subsequent analysis.

DNA quality

After DNA extraction is complete, we recommend determining the purity and concentration of the DNA samples. DNA samples must contain fragments of at least 150bp for PCR-based analsysis.

Determine the purity of all DNA samples by UV spectrophotometric measurements or related methods. The acceptable A_{260}:A_{280} range for samples is 1.7–2.1.

The concentration, as measured by A_{260}, should be greater than or equal to 20 ng/µL. Exclude samples with a concentration of less than 20 ng/µL or concentrate the sample using ethanol precipitation. Dilute samples to 20 ng/µL using nuclease-free water or TE buffer solution (10mM Tris pH 8.0; 0.1mM EDTA).

Preparing controls

The following instructions are for preparing controls using a Novallele Control Set. You may prepare your own controls for your Novallele genotyping assay. Once prepared, treat the prepared controls as a sample in the Novallele genotyping assay of interest. Do not substitute reagents.

Recommendation: Run three replicates of each control. The instructions produce enough material for three replicates.

Preparation of the WT control

1. Pipette 11 µL of Novallele Genotyping Mastermix into a nuclease-free microcentrifuge tube.
2. Pipette 11 µL of the corresponding Novallele genotyping assay into the tube.
3. Vortex the WT control for 10–15 seconds. Centrifuge briefly.
4. Pipette 11 µL of the WT control into the tube.
5. Vortex the prepared WT control for 10–15 seconds. Centrifuge briefly.

Preparation of the HOM control

1. Pipette 11 µL of Novallele Genotyping Mastermix into a nuclease-free microcentrifuge tube.
2. Pipette 11 µL of the corresponding Novallele genotyping assay into the tube.
3. Vortex the HOM control for 10–15 seconds. Centrifuge briefly.
4. Pipette 11 µL of the HOM control into the tube.
5. Vortex the prepared HOM control for 10–15 seconds. Centrifuge briefly.

Optional preparation of the HET control

Important: Use the prepared HET control immediately; do not reuse.

1. Pipette 12 µL of Novallele Genotyping Mastermix into a nuclease-free microcentrifuge tube.
2. Pipette 12 µL of the corresponding Novallele genotyping assay into the tube.
3. Vortex the WT and HOM controls for 10–15 seconds each. Centrifuge each briefly.
4. Pipette 6 µL of the WT control into the tube.
5. Pipette 6 µL of the HOM control into the tube.
6. Vortex the prepared HET control for 10–15 seconds. Centrifuge briefly.

PCR reaction setup

1. Mix the reagents by briefly inverting or vortexing the Novallele genotyping assay and Novallele Genotyping Mastermix tubes.
2. Briefly centrifuge the Novallele genotyping assay and Novallele Genotyping Mastermix tubes for 10–15 seconds.
3. Prepare a reaction for each sample according to the table below.

   Recommendations:

   - Prepare 3 replicates for each sample.
   - For each thermocycler run, include at least one NTC prepared by substituting nuclease-free water for the sample.

   Notes:

   - The calculations below include 10% more than the required amount to prevent not having enough reaction for the thermocycler run.
   - The volume of sample is based on a DNA concentration of 20 ng/µL. If using a sample with a different DNA concentration, make sure to add 66.6 ng of DNA per reaction and adjust the amount of nuclease-free water.

Table 1. Preparation of reactions.

Reagent	One reaction	Three reactions
Novallele Genotyping Mastermix	4.0 µL	11.0 µL
Novallele genotyping assay	4.0 µL	11.0 µL
Sample	4.0 µL	11.0 µL
Total	12.0 µL	33.0 µL

4. Add 10 µL of the reaction to each well of either a 96-well plate or a 384-well plate using a single-channel pipette.

   Important: Make sure to change pipette tips between each reaction to avoid cross-contamination between the wells.

5. Tightly seal the well plate as directed by the manufacturer.

6. Make sure that no bubbles are present in the wells of the plate. If bubbles are present, centrifuge the well plate at 1000 rpm for one minute for a 96-well plate or 2000 rpm for two minutes for a 384-well plate.

   Note: This is not required for QIAGEN Rotor-Gene Q or BioFire LS32 instruments.

7. Program the HRM thermocycler according to the conditions on the product specification sheet provided with the Novallele genotyping assay.

   Note: The product specification sheet for each assay is available at **www.canon-biomedical.com**.

8. Place the well plate in the thermocycler. If recommended by the manufacturer, use a compression pad with the optical film-sealed plate/disc formats.

PCR run

1. Start the PCR run.
   Important: Do not open any processed plate. Removing the strips or film releases the amplicon into the air and may cause contamination of future PCR runs.

2. After the PCR run is finished, visually inspect each well for any sign of evaporation. If evaporation is observed, note the wells as this may affect the results of data analysis.

3. Dispose of the well plate.

Data Analysis

We recommend that you analyze your data using the software for the thermocycler used to perform the PCR. Refer to the instructions provided with your thermocycler for assistance with analyzing your HRM results. Using Novallele Control Sets makes genotyping your samples easier by comparing the sample melt curves to the control melt curves.

Each Novallele genotyping assay is either a small-amplicon or a probe-based assay. Both assay types perform in the same manner, but the distinguishing peaks for genotypes are different. Small-amplicon assays generate one major melting peak at the amplicon temperature; probe-based assays generate both amplicon and probe melting peaks.

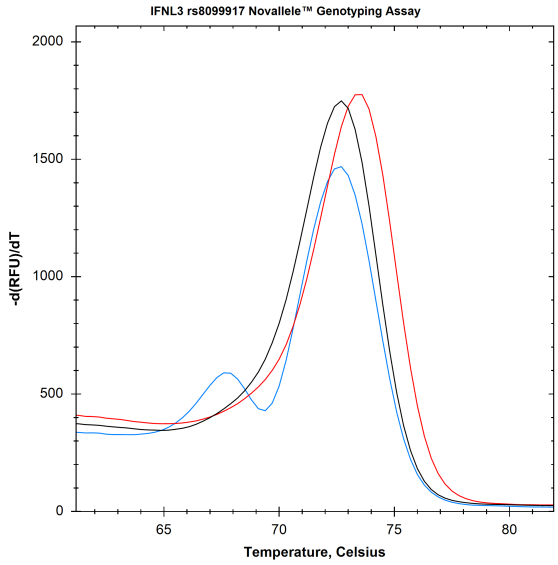
If you require assistance analyzing your results, please visit our website at **www.canon-biomedical.com**, email us at **contactus@canon-biomedical.com**, or call us at 1-844-CANONBIO (1-844-226-6624).

Small-amplicon assays

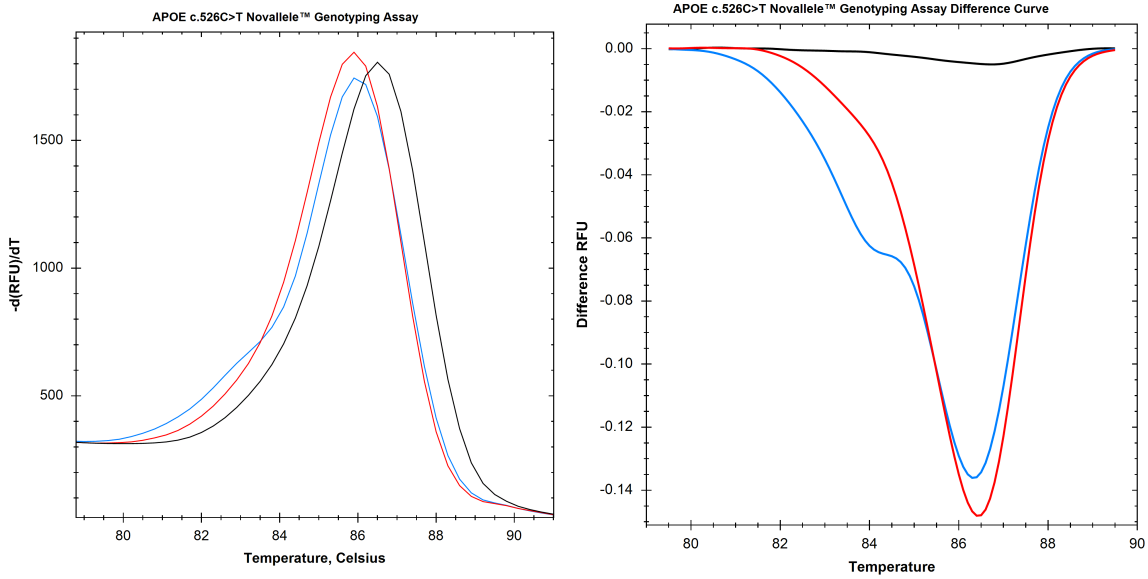
Using the first derivative (-df/dT) of the melt curves, small-amplicon assays are analyzed using the amplicon peaks. The HOM variant is detected by the change in T_m, and the HET variant is detected by a change in shape. Depending on the thermocycler HRM performance, the definition of a HET sample in a small-amplicon assay may not be very clear. In these cases, consider analyzing the data using a difference plot method if the instrument software has this capability.

The two examples below display the WT in black, the HET variant in blue, and the HOM variant in red. The first example has a very distinguished HET melt peak; the second example has a HET melt peak that is not strongly distinguished from the other peak. A difference plot was used to distinguish the HET variant.

Example of a small-amplicon melt curve with a highly distinguished HET variant.



Example of a small-amplicon melt curve with a HET variant that is not strongly distinguished.

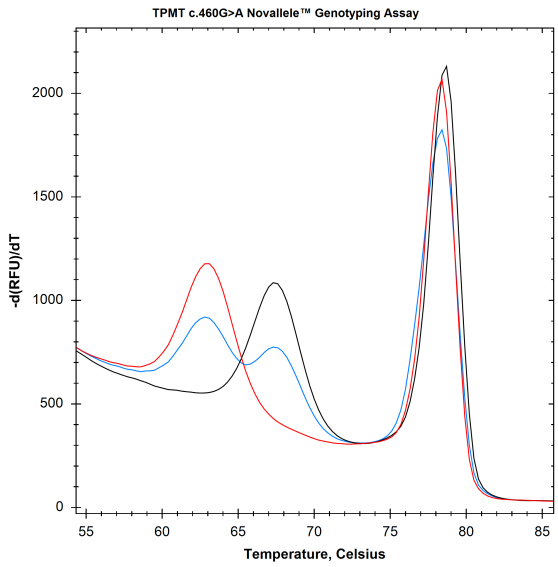


Probe-based assays

Probe-based assays use an unlabeled probe By using the first derivative (-df/dT) of the melt curves, genotyping of probe-based assays is done by analyzing the probe region.. In addition to the primary amplicon peak, the probe-based assays have an unlabeled-probe peak at a lower temperature that is used to differentiate genotypes. The WT and HOM variant peak positions, as compared to each other, depend on the nature of the mutation. The HOM variant can have either a lower or higher T_m as compared to the WT.

The example below displays the WT in black, the HET variant in blue, and the HOM variant in red.

Example of a probe-based melt curve:



Designing your own genotyping assay

The Novallele Genotyping Mastermix is a laboratory-optimized, fast PCR chemistry formulation that supports HRM. You can use the Novallele Genotyping Mastermix in conjunction with the 2x Novallele Oligo Dilution Buffer to design an assay for your particular target. You must use the Novallele Genotyping Mastermix and 2x Novallele Oligo Dilution Buffer together – do not replace the 2x Novallele Oligo Dilution Buffer with other reagents.

Designing DIY assay mixtures

Use the one of the tables below to prepare your assay mixture based on whether your assay is a short-amplicon or an unlabeled-probe assay. The assay is prepared by diluting the primers and probes in the 2x Novallele Oligo Dilution Buffer, which contains all of the necessary chemistry for use with the Novallele Genotyping Mastermix.

The preparation of the unlabeled-probe assay is based on a 1:5 ratio of limited to excess primer; you may need to adjust this ratio based on your assay design.

Table 2. Preparation of a short-amplicon assay mixture.

Reagent	Concentration	Volume
2x Novallele Oligo Dilution Buffer	2x	300 µL
Forward primer	100 µM	18 µL
Reverse primer	100 µM	18 µL
Nuclease-free water	N/A	264 µL
Final volume	N/A	600 µL

Table 3. Preparation of an unlabeled -probe assay mixture.

Reagent	Concentration	Volume
2x Novallele Oligo Dilution Buffer	2x	300.0 µL
Limited primer	100 µM	3.6 µL
Excess primer	100 µM	18.0 µL
Unlabeled	100 µM	18.0 µL
Nuclease-free water	N/A	260.4 µL
Final volume	N/A	600.0 µL

Follow the Novallele Genotyping Assay PCR reaction set up as described in the "Procedure" section, page 12, of this user guide and substitute the DIY Assay mixture for the Novallele Genotyping Assay in the protocol. While the conversion of a DIY Assay for use with the Novallele Genotyping Mastermix is a simple procedure, the user will need to determine optimal cycling parameters.

Troubleshooting

Hopefully you do not have any problems with your Novallele products, but this section provides helpful hints from the scientists that developed the Novallele products. Additional information and content can also be found at **www.canon-biomedical.com**. If you require assistance beyond these resources, please email us at **contactus@canon-biomedical.com** or call us at 1-844-CANONBIO (1-844-226-6624).

How do I analyze my data?

Please refer to the user manual of your thermocycler instrument for instructions on analyzing data. Generally both small-amplicon and probe-based assays are analyzed using the first derivative of the melt curve, known as the derivative plot. Depending on the thermocycler HRM performance, the

definition of a HET sample in a small-amplicon assay may not be very clear. In these cases, consider analyzing the data using a difference plot method if the instrument software has this capability.

Examples of genotyping data analysis are available for every Novallele genotyping assay at **www.canon-biomedical.com**.

There is an additional shoulder or feature observed in the melt curve for one of my samples. What does this mean?

Extra features on the melt curve can originate from many sources. Additional melt curve features may result from the magnitude of smoothing used for data analysis but could also indicate an additional sequence variation within the region being analyzed.

The extra feature may also be contamination or an artifact caused by an instrument error or particulate contamination that disrupted the imaging process.

Another cause may be that the DNA quality is not optimal. Analyze the sample to confirm the A_{260}:A_{280} ratio is within the range of 1.7-2.1.

We recommend repeating the assay on the sample, if possible.

I am using the Novallele Control Set, and my threshold cycle (C_t)/crossing point-PCR-cycle (C_p) is not the same for my controls and gDNA samples. Is this okay?

Due to the nature of our manufacturing process, the copy number of the WT and HOM controls is not always identical, which causes a difference in C_t/C_p between the controls; however, this difference will not affect your genotyping call.

I am using the Novallele Control Set, and my controls have different C_t/C_p values. Is this okay?

Slight concentration variations due to the nature of our manufacturing process, and, therefore, the C_t/C_p values could differ for each control; however, this difference will not affect your genotyping call.

I am using the Novallele Control Set, and there are small T_m differences between the gDNA sample and controls. Is this okay?

Small T_m differences are likely between gDNA and controls. The T_m may differ, but the difference between the melting temperatures should remain the same between the WT and HOM variants.

I am using a Novallele Control Set, and the two peaks of my HET control appear uneven or asymmetrical. I followed the procedure to prepare the HET control. What can I do?

When using a probe-based Novallele genotyping assay, equal or almost equal peaks for the HET control are expected in the probe region of the derivative (-dF/dT) plot. One peak corresponds to the WT control, and the other peak corresponds to the HOM control. If the peaks are highly asymmetrical or one peak is almost non-existent, the HET control may contain unequal amounts of the WT control and HOM control — despite the fact that the two controls were mixed in equal volumes during preparation.

If one of the HET control peaks is one-third the height of the other peak, perform the following experiment to identify the correct ratio of the WT control to the HOM control.

Generate HET material with various WT: HOM ratios such as 1:2, 1:3, and 1:5 such that the excess material corresponds to the lower HET control peak. Test all of the HET material simultaneously to find the correct ration to use as the control for future experiments.

Due to the nature of gDNA, the HET control peaks will not always be equal. Find example control graphs for all of our assays at **www.canon-biomedical.com**. Use these graphs as reference for your experiments.

Contact Information

We strive to deliver the highest quality technical support. Our support team is composed of experienced scientists with extensive academic and industry laboratory expertise in genotyping and

PCR technologies. If you have any questions about our products or HRM, please do not hesitate to contact us.

For technical assistance or more information, please visit our website at **www.canon-biomedical.com**, email us at **contactus@canon-biomedical.com**, or call us at 1-844-CANONBIO (1-844-226-6624).

Ordering Information

Product	Description	Catalog number
Novallele genotyping assays	Genetic variation-specific PCR assay	Variable
Novallele Control Set	WT and HOM controls for a Novallele genotyping assay	Variable
Novallele Genotyping Mastermix	Rapid PCR chemistry formulation for High Resolution Melt analysis	40018

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The purchase of this product grants the purchaser rights under certain Roche patents to use it solely for investigational research or providing human in vitro diagnostic services. No general patent or other license of any kind other than this specific right of use from purchase is granted hereby.

Canon BioMedical warrants that each Novallele genotyping assay and Novallele Genotyping Mastermix, each a "Novallele Product," sold to the Customer will substantially perform in accordance with the written specifications therefor set forth in the applicable package insert or user manual originally included by Canon BioMedical with each Novallele Product delivered to the Customer until the instructed "use by" date set forth on the Novallele Product packaging. Canon BioMedical's sole liability for, and the Customer's sole remedy, under this limited warranty will be for Canon BioMedical to replace the nonconforming Novallele Product with a conforming Novallele Product, provided that in each case Canon BioMedical is notified and returned by the Customer during the warranty period of the nonconforming Novallele Product as instructed by an authorized Canon BioMedical employee. The warranty period on any replacement Novallele Product will be the period from the shipment date until the replacement Novallele Product "use by" date.

A copy of the Canon BioMedical standard terms and conditions applicable to the Novallele genotyping assays and Novallele Genotyping Mastermix can be found on www.canon-biomedical.com. If you have questions about product specifications or performance, please call Canon BioMedical Support at 1-844-CANONBIO or visit www.canon-biomedical.com.

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We are here to assist.

Please email or call to speak with our technical support staff about HRM or to place an order.

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