Primerdesign™ Ltd

Custom Gene Of Interest (CustomGOI)

Double-Dye, Primer-only & Genotyping Assays

Handbook

CUSTOMGOI

For Research Use Only. Not for use in diagnostic procedures

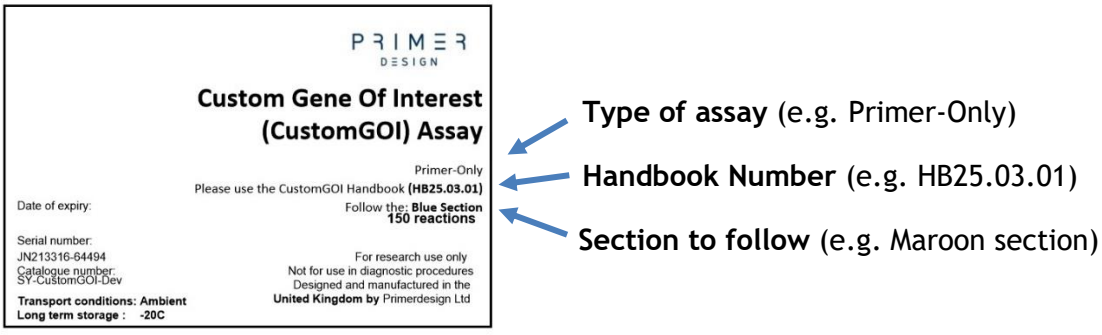
CustomGOI Handbook
CustomGOI handbook HB25.03.01
Published Date: 18 Sep 2018

Contents Page:

Where to find the correct section to follow:

Please refer to your assay format for correct handbook procedure to follow. The reference is located on front of the kits packaging material. Below is an example pouch label, the handbook number is referenced, and this number can be found on the footer of the document. The section to follow is also referenced via the colour section and type of assay (e.g. Double Dye).

Example Pouch label:



If not, or not accessible, the correct link should be supplied via the website and/or email correspondence. However, if still unclear or any other question please do not hesitate to contact us via enquiry@primerdesign.co.uk or (+44) 2380 748 830.

Introduction to CustomGOI Assays

Primer-only and/or Double Dye Assay

This assay provides reagents for the specific amplification of a target gene using real-time PCR with either Primer-only or Double-Dye (TaqMan® style) detection chemistry. When resuspended, this assay provides primers that have been tested for priming specificity, and amplification efficiency at optimal concentrations. Real-time PCR is a very sensitive technology and it is not recommended to use more or less than the specified amount of primer and probe in each reaction. However, final reaction volumes between 15 µl and 50 µl are often successful and may be tested at the user's discretion. Unfortunately, Primerdesign is not able to provide technical support for protocols other than those provided by Primerdesign.

For accurate gene expression measurements, it is essential to normalize results from your quantitative real-time PCR experiments to a fixed reference.

- Normalizing to a constitutively expressed housekeeping gene is the most common method. Primerdesign provides researchers with a range of high quality real-time PCR assays for housekeeping gene expression data.
- geNorm™ is a system for selecting optimal housekeeping genes for any biological system (e.g. cell line, tissues sample)

Both can be found at www.primerdesign.co.uk

Genotyping and SNP-Typing Assay

Each genotyping primer/probe mix contains two labelled probes homologous to the two genotypes under investigation. During qPCR amplification of the target DNA, the probes will compete to bind across the variant (mutant) region. The probe that is 100% homologous to the DNA target site will preferentially bind and give a fluorescent signal as PCR proceeds. It follows that the wild type (WT) sequence will give a strong amplification plot through one channel whilst giving a very weak signal through the alternative channel. Homozygous variant/mutant samples will give an exactly inverse result. Heterozygote samples contain both probe binding sites on each of the two alleles and therefore give an intermediate signal through both channels. The three possible genotypes can be resolved by comparing end point fluorescence. Most software platforms can perform this analysis automatically.

Reagents and Equipment to Be Supplied by User

- **Real-Time PCR instrument**
- **Master Mix or Master Mix components**
  - This assay is designed to work well with all commercially available Master Mixes. However, we recommend the use of Primerdesign **2x PrecisionPLUS® or Oasig™ Master Mixes**. Also, offer PrecisionFAST®, for superior fast performance.
    - We only guarantee quality of results with these Master Mixes
- **Pipettors and Tips**
- **Vortex and centrifuge**
- **Sample template**
  - If using RNA, the quality of cDNA will directly affect the quality of data generated using this kit. Primerdesign recommends the use of the Primerdesign Precision nanoScript™ 2 Reverse Transcription kit to generate cDNA from RNA. This is because of its known quality to convert RNA to cDNA.

Assay storage and stability

This assay is stable at room temperature but must be stored at -20ºC on arrival. Once the Lyophilised components have been resuspended they must not be exposed to temperatures above -20°C for longer than 30 minutes at a time and unnecessary repeated freeze/thawing must be avoided. The assay is stable for six months from the date of resuspension under these circumstances. If a standard curve dilution series is prepared this can be stored frozen for an extended period. If you see any degradation in this serial dilution a fresh standard curve can be prepared from the positive control.
Primerdesign does not recommend using the assay after the expiry date stated on the pack.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity (An internal PCR control is supplied to test for nonspecific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template RNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions CustomGOI detection assays have very high priming efficiencies of >90%.

Licensing Agreement and Limitations of use

PCR is covered by several patents owned by Hoffman-Roche Inc and Hoffman-LaRoche, Ltd. Purchase of Primerdesign assays does not include or provide license with respect to any patents owned by Hoffman-La Roche or others.

Primerdesign Satisfaction Guarantee

At Primerdesign takes pride in the quality of all of its products. Should this product fail to perform satisfactorily when used according to the protocols in this manual, Primerdesign will replace the item free of charge.

Quality Control

As part of our ISO9001 and ISO13485 certified quality assurance systems, all Primerdesign products are verified to ensure the highest levels of performance and reliability.

Notices and disclaimers

This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period Primerdesign CustomGOI detection assays allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

Trademarks

Primerdesign™ is a trademark of Primerdesign Ltd.

The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG. BI, ABI PRISM® GeneAmp® and MicroAmp® are registered trademarks of the Applera Genomics (Applied Biosystems Corporation). BIOMEK® is a registered trademark of Beckman Instruments, Inc.; iCycler™ is a registered trademark of Bio-Rad Laboratories, Rotor-Gene is a trademark of Corbett Research. LightCycler™ is a registered trademark of the Idaho Technology Inc. GeneAmp®, TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc., The purchase of the Primerdesign ™ reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc

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CustomGOI Assay

Double-Dye Assay

150 tests

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Assay contents

N.B. *Once resuspended the assays must remain at* ***-20ºC*** *until ready to use.*

- **Target specific primer and probe mix (BROWN Lid)**

- **RNase/DNase free water (WHITE Lid)**

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Resuspension and PCR-Setup protocol

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- To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment.
- Ideally this would be a designated PCR lab or PCR cabinet.
- Filter tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening.**
   - This will ensure Lyophilised primer and probe mix is in the **base of the tube** and is not spilt upon opening the tube.

2. **Resuspend the primer/probe mixes in the RNase/DNase free water supplied, according to the table below:**
   - To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes, and vortex again before use.

Component - resuspend in water	Volume
Pre-PCR pack	
Target primer/probe mix (BROWN Lid)	**165 µl**

   - ***If using Oasig™ Master mix**, please resuspend using the supplied Master Mix resuspension buffer, by adding 525 µL directly to the vial.*
   - *Invert the master mix, 10 times to ensure complete resuspension*

3. **When using Primerdesign 2x PrecisionPLUS® or Oasig™ qPCR Master Mix, make up a mix containing all qPCR reagents according to the protocol below.**
   To ensure complete resuspension, vortex each tube thoroughly.

Component	Per Reaction
Resuspended primer/probe mix (BROWN Lid)	**1 µl**
Primerdesign 2x PrecisionPLUS® Or Oasig™ Master Mix	**10 µl**
RNAse/DNAse free water (WHITE Lid)	**4 µl**
Final Volume	**15 µl**

4. **Pipette 15 µl of this mix into each well according to your real-time PCR experimental plate set up**

5. **Prepare Sample for each of your reaction**
   - If the concentration of cDNA is not known, then dilute your RT reactions 1:10 (10 µl of RT and 90 µl of water), suggested concentration 5ng/µl.

6. **Pipette 5 µl of each sample into its specific reaction well, according to your experimental plate set up.**
   - The final volume in each well is 20 µl

7. **Include the following negative control wells.**
   - Include wells where the sample is replaced with RNase/DNase free water. Any amplification in this sample is indicative of template cross contamination between wells, or contamination of one or more reagents.
   - If running cDNA samples, include wells where the equivalent concentration of RNA is added minus the reverse transcription step. Amplification of these wells may indicate genomic DNA contamination of your RNA sample. A DNase treatment step is highly recommended during RNA extraction to prevent this occurring.

qPCR amplification protocol

Amplification conditions using Oasig™ or PrecisionPLUS® 2X qPCR Master Mix.

	Step	Time	Temp
Cycling x40**	Enzyme activation	2 min	95 °C
	Denaturation	10 s	95 °C
	Annealing/Extension *DATA COLLECTION* *	60 s	60 °C

Amplification conditions using Primerdesign 2X PrecisionFast® qPCR Master Mix

	Step	Time	Temp
Cycling x40**	Enzyme activation	2 min	95 °C
	Denaturation	5 s	95 °C
	Annealing/Extension *DATA COLLECTION* *	20 s	60 °C

*Fluorogenic data must be acquired during this step through the FAM channel (or alternative channel if specified).
**For low copy number targets, giving late detection, a further 10 cycles may be needed to generate the complete amplification plot.

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CustomGOI Assay

Primer-Only (SYBR®green) Assay

150 tests

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Assay contents

N.B. Once resuspended the Assays must remain at -20ºC until ready to use.

- **Target specific primer mix (Blue Lid)**

- **RNase/DNase free water (WHITE Lid)**

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1. **Pulse-spin each tube in a centrifuge before opening.**
   - This will ensure Lyophilised primer mix is in the **base of the tube** and is not spilt upon opening the tube.

2. **Resuspend the primer mixes in the RNase/DNase free water supplied, according to the table below:**
   - To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes, and vortex again before use.

Component - resuspend in water	Volume
Pre-PCR pack	
Target primer mix (Blue Lid)	**165 µl**

3. **When using Primerdesign 2x PrecisionPLUS® or PrecisionFAST® qPCR Master Mix, make up a mix containing all qPCR reagents according to the protocol below.**
   To ensure complete resuspension, vortex each tube thoroughly.

Component	Per Reaction
Resuspended primer mix (Blue Lid)*	**1 µl**
Primerdesign 2x Precision®PLUS Master Mix (containing SYBRgreen®)	**10 µl**
RNase/DNase free water (WHITE Lid)	**4 µl**
Final Volume	**15 µl**

*working concentration of primers = 300 nM in a 20 µl reaction

4. **Pipette 15 µl of this mix into each well according to your real-time PCR experimental plate set up**

5. **Prepare Sample for each of your reaction**
   - If the concentration of cDNA is not known, then dilute your RT reactions 1:10 (10 µl of RT and 90 µl of water), suggested concentration 5ng/µl.

6. **Pipette 5 µl of each sample into its specific reaction well, according to your experimental plate set up.**
   - The final volume in each well is 20 µl

7. **Include the following negative control wells.**
   - Include wells where the sample is replaced with RNAse/DNAse free water. Any amplification in this sample is indicative of template cross contamination between wells, or contamination of one or more reagents.
   - If running cDNA samples, include wells where the equivalent concentration of RNA is added minus the reverse transcription step. Amplification of these wells may indicate genomic DNA contamination of your RNA sample. A DNAse treatment step is highly recommended during RNA extraction to prevent this occurring.

qPCR amplification protocol

Amplification conditions using PrecisionPLUS® 2X qPCR Master Mix (premixed with SYBRgreen®).

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x40***	Denaturation	10 s	95 °C
	Annealing/Extension *DATA COLLECTION* *	60 s	60 °C
Melt Curve	Melt Curve Analysis**		

Amplification conditions using Primerdesign 2 x PrecisionFAST® qPCR Master mix (premixed with SYBRgreen®).

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x40***	Denaturation	5 s	95 °C
	Annealing/Extension *DATA COLLECTION* *	20 s	60 °C
Melt Curve	Melt Curve Analysis**		

*Fluorogenic data must be acquired during this step through the SYBR channel.

**A post PCR run melt curve can be used to prove the specificity of the primers. See the manufacturer's instructions for your hardware platform

***For low copy number targets, giving late detection, a further 10 cycles may be needed to generate the complete amplification plot.

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CustomGOI Assay

Custom Genotyping and SNP-Typing Assay

50 tests

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Assay contents

N.B. Once resuspended the Assays must remain at -20ºC until ready to use.

- **Custom genotyping primer/probe mix (BROWN Lid)**

- **Wild-type positive control template (RED Lid)**

- **Mutant positive control template (RED Lid)**

- **Template preparation buffer (YELLOW Lid)**

- **RNase/DNase free water (WHITE Lid)**

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Principles of the test

Genotyping by real-time PCR using hydrolysis probes

Each genotyping primer/probe mix contains two labelled probes homologous to the two genotypes under investigation. During qPCR amplification of the target DNA the probes will compete for binding across the variant region. The probe that is 100% homologous to the DNA binding site will preferentially bind and give a fluorescent signal as PCR proceeds. It follows that the wild-type sequence will give a strong amplification plot through one channel whilst giving a very weak signal through the alternative channel. Homozygous variant samples will give an exactly inverse result. Heterozygote samples contain both probe binding sites on each of the two alleles and therefore give an intermediate single through both channels. The three possible genotypes can be resolved by comparing end point fluorescence. Most hardware platforms can perform this analysis automatically.

Positive controls

The Assay contains positive control templates for each of the two genotypes. These can be run as parallel samples to give control signals for each genotype. In order to provide good positive control data that is directly relevant to the samples under test, the control DNA must be used at a similar copy number to the sample DNA. This protocol contains guidelines for varied dilution of the positive control templates depending on the level of genomic DNA added to each sample. The optimum sample DNA level is 5ng. Positive control templates are a potential contamination risk to subsequent tests so must be handled carefully.

Negative control

To confirm absence of contamination, a negative control reaction is recommended to be included every time the assay is used. In this instance, the RNase/DNase free water must be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results is recommended to be ignored and the test samples repeated. Possible sources of contamination is recommended to be first explored and removed.

Thermocycling parameters

The optimum cycling parameters for Primerdesign genotyping assays is a two-step cycling procedure. The first set of cycles are designed for optimal PCR amplification. Where the test samples contain very low levels of input gDNA (<1ng), additional first stage cycles can be added. The second set of cycles are optimal for probe discrimination and therefore cycle at a higher temperature. Fluorescent data is only collected during the second set of amplification cycles. These parameters can be programmed into most machines but note that the reported Cycle threshold values produced by this program will be lower than usual and that this is expected.

Master mix compatibility

PrecisionPLUS® Master Mix, PrecisionFAST® Master Mix and oasig Master Mix contain the enzyme, nucleotides, buffers and salts at precisely the correct concentration for this application. The annealing temperatures of the primer and probe have been carefully calibrated and any change in the reaction buffer can significantly alter the performance of the assay. For this reason, Primerdesign can only guarantee accurate genotyping results when PrecisionPLUS®, PrecisionFAST® or oasig™ Master Mix is used.

Resuspension and PCR-Setup protocol

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- To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment.
- Ideally this would be a designated PCR lab or PCR cabinet.
- Filter tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening.**
   - This will ensure Lyophilised primer and probe mix is in the **base of the tube** and is not spilt upon opening the tube.

2. **Resuspend the primer/probe mixes in the RNase/DNase free water supplied, according to the table below:**
   - To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes, and vortex again before use.

Component - resuspend in water	Volume
Pre-PCR pack	
Target primer/probe mix (BROWN Lid)	**55 µl**

   - ***If using oasig™ Master mix**, please resuspend using the supplied Master Mix resuspension buffer, by adding 525 µL directly to the vial. Do not add rox.*
   - *Invert the master mix, 10 times to ensure complete resuspension*

3. **Resuspend the positive control templates in the template preparation buffer supplied, according to the table below.**
   - To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes, and vortex again before use.

Component - resuspend in Template Preparation Buffer	Volume
Post-PCR Heat Sealed Foil	
Wild-type Positive Control Template (RED Lid) *	**500 µl**
Mutant Positive Control Template (RED Lid) *	**500 µl**

#PRIMERDESIGNTOPTIP

- * This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

4. **When using Primerdesign 2x PrecisionPLUS®, Oasig™ or PrecisionFAST® qPCR Master Mix, make up a mix containing all qPCR reagents according to the protocol below.**
   To ensure complete resuspension, vortex each tube thoroughly.

Component	Per Reaction
Resuspended primer/probe mix (BROWN Lid)	**1 µl**
Primerdesign 2x Precision®PLUS Or Oasig Master Mix™	**10 µl**
RNase/DNase free water (WHITE Lid)	**4 µl**
Final Volume	**15 µl**

5. **Pipette 15 µl of this mix into each well according to your real-time PCR experimental plate set up**

6. **Prepare DNA Samples for each of your reactions**

7. **Pipette 5 µl of each sample into its specific reaction well, according to your experimental plate set up.**
   - The final volume in each well is 20 µl
   - To obtain a strong signal, the recommended input DNA concentration is 0.2-2 ng/µl (1-10 ng in total) with the ideal total being 5 ng. For negative control wells use 5 µl of RNase/DNase free water.

8. **Dilute positive control DNA in template preparation buffer**
   - It is important that the input copy number for the positive control DNA is matched to the amount of sample DNA being added to each reaction. The positive control DNA must be diluted according to the table below:

Sample Input DNA	Positive Control Dilution Factor
10 ng	**1 : 25**
5 ng	**1 : 50**
1 ng	**1 : 250**

9. **Pipette 5 µl of each positive control DNA according to your experimental plate set up.**

qPCR amplification protocol

For machines that can be programmed to include 2 cycling stages the following protocol is recommended for optimum resolution between genotypes.

If using oasig™ Master Mix, DO NOT add ROX to the master mix as a passive reference. If using a machine that uses ROX as a passive reference, then the passive reference must be turned off or set to “none” indicating no passive reference.

Amplification conditions using Oasig™, PrecisionPLUS® or PrecisionFAST® 2X qPCR Master Mix.

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x10**	Denaturation	10 s	95 °C
	Annealing/Extension	60 s	60 °C
Cycling x35	Denaturation	10 s	95 °C
	Annealing/Extension *DATA COLLECTION* *	60 s	66 or 68 °C ***

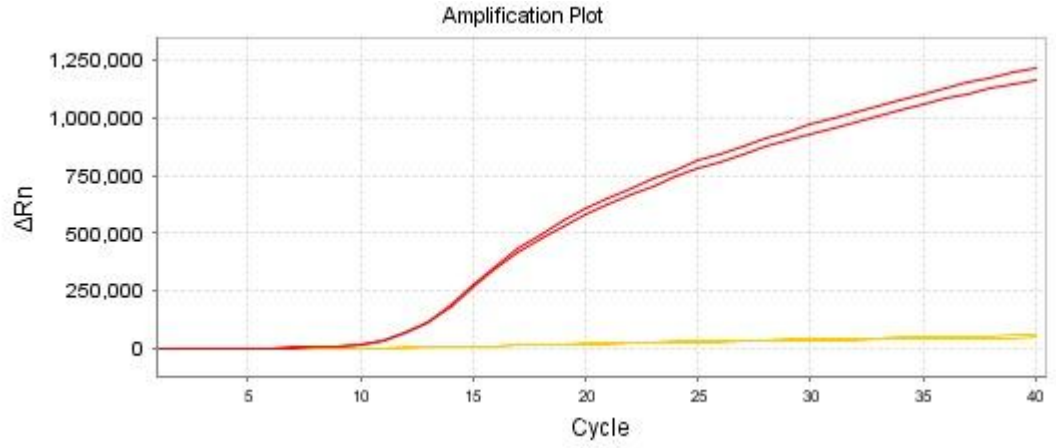
* Fluorogenic data must be acquired during this step through the ROX / VIC channel.
** Where the sample amount is 1ng or less per well, add 5 cycles to the first stage (15 cycles)
*** Please refer to your datasheet provided for correct annealing temperature usage for your assay

Interpretation of results

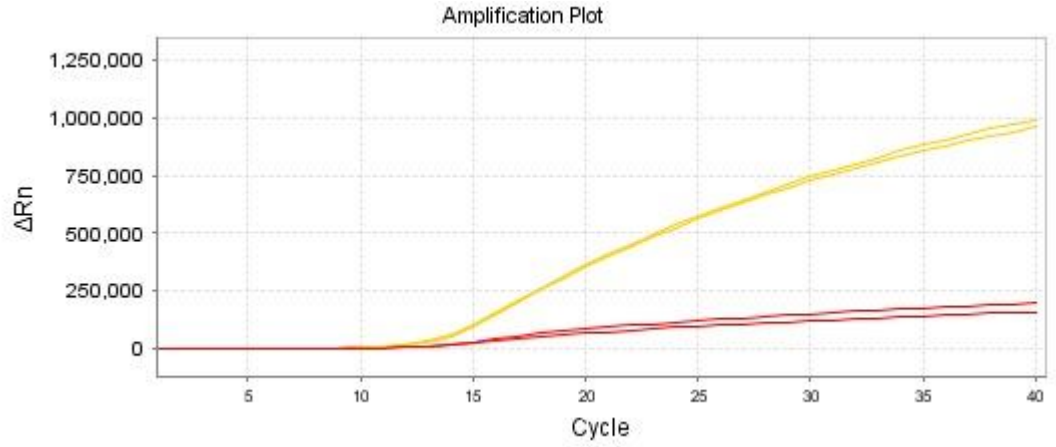
The wild type probe is labelled to read through the ROX channel whilst the mutant probe is labelled to read through the VIC channel. On wild-type sequences the ROX channel will give a strong amplification plot and the VIC channel none or very low detection. The signals are reversed on mutant samples. Heterozygote samples will give an intermediate signal through both ROX and VIC channels.

Sample data

Wild type sample (**Mutant signal**, **WT signal**)



Mutant DNA sample (**WT signal**, **Mutant signal**)



The genotype of each sample is calculated by comparing the ratio of signals between the two channels (ROX and VIC).

The raw data above can be best visualized by using a cluster analysis; plotting the end point fluorescence data from the ROX channel on one axis and the end point fluorescence data from the VIC channel on the other axis. Most qPCR software platforms will perform this analysis automatically so follow the manufacturer's instructions for your software. The data is quickly resolved into clusters corresponding to the wild type, heterozygous and

homozygous variant samples.