



PrimerDesign™ Ltd

Detection of Tamiflu resistant Swine *H1N1* Influenza Human Pandemic Strain

H275Y mutation

For general laboratory and research use only



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Introduction to Swine *H1N1* Influenza – Human Pandemic Strain

Influenza, commonly known as the flu, is an infectious disease of birds and mammals caused by an RNA virus of the family Orthomyxoviridae (the influenza viruses). In people, common symptoms of influenza are fever, sore throat, muscle pains, severe headache, coughing, and weakness and fatigue. In more serious cases, influenza causes pneumonia, which can be fatal, particularly in young children and the elderly. Although the common cold is sometimes confused with influenza, it is a much less severe disease and caused by a different virus. Similarly, gastroenteritis is sometimes called "stomach flu" or "24-hour flu", but is unrelated to influenza.

In 2009 a global outbreak of a new strain of an Influenza A virus subtype H1N1 occurred. Referred to as the "novel H1N1", and commonly called "swine flu", it is thought to be a reassortment of four known strains of influenza A virus: one endemic in (normally infecting) humans, one endemic in birds, and two endemic in pigs (swine).

The outbreak began in Mexico, with evidence that Mexico was already in the midst of an epidemic for months before the outbreak was recognized. Soon after, its government closed down most of Mexico City's public and private offices and facilities to help contain the spread. In early June 2009, as the virus spread globally, the World Health Organization (WHO) declared the outbreak to be a pandemic, but also noted that most illnesses were of "moderate severity." The virus has since spread to the Southern Hemisphere which entered its winter flu season, and to many less developed countries with limited healthcare systems. Because the virus was spreading with "unprecedented speed", and many clinics were overwhelmed testing and treating patients, WHO stopped requiring countries to report all cases, but is still monitoring unusually large outbreaks.

Western governments have used Tamiflu® (a neuramidase inhibitor anti-viral drug by Roche) extensively as a treatment strategy. Concerns have been raised that the over-use will increase the rate at which the virus develops resistance to the drug.



Principles of the test

General Principles

The PrimerDesign Tamiflu resistance genotyping kit follows the guidelines of the Centers for Disease Control and Prevention (CDC) in the United States and the World Health Organisation (WHO).

These guidelines recommend that a sample should be tested for general Influenza A first and only InfA-positive samples should then be tested for the presence of H1N1. Genotyping is only necessary for H1N1-positive samples.

Testing for Influenza A

The kit contains the M1 primer and probe set designed to detect all influenza A sequences regardless of the species of origin. Samples that test positive with the M1 primer set have an influenza A infection of unknown serotype. These positive samples should be tested with the NI primer/probe set to establish the presence of the H1N1 pandemic strain.

Testing for H1N1 — Swine Flu/Novel Flu

The NI swine flu specific primers and probe detects only the human pandemic strain and is specific to the swine H1N1 pandemic strain. Samples that test positive with the NI primer set have been exposed to swine H1N1 Influenza. Samples that test positive for M1 and negative for NI have a naturally circulating influenza A infection from an endogenous strain not related to swine flu.

Testing for Tamiflu resistant pandemic flu

Since many endemic human influenza isolates are already Tamiflu resistant, it is very important to establish that all isolates under investigation for resistance are authentic human H1N1 pandemic strain isolates of swine origin. Only samples that have tested positive for the H1N1 pandemic strain by using the NI primer/probe set should therefore be tested with the H275Y primer/probe set.

The H275Y primer and probe set is designed to genetically identify sequences that contain the mutation that leads to a Tyrosine substitution for the Histidine at position 274 which confers Tamiflu resistance. The primers and probe will detect on all swine H1N1 strains but also have some potential to detect on other influenza isolates.

A positive test result using the H275Y primer and probe multiplex indicates that the sample under investigation carries this mutation and that the isolate will be resistant to Tamiflu.

Real-time PCR detection of H1N1 influenza

Two separate primer and probe mixes are provided: M1 global influenza A and NI swine H1N1 Influenza specific. Both target sequences are detected through the **FAM** channel in separate reactions.

The primer and probe mixes provided exploit the so-called TaqMan[®] principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an



oligonucleotide labeled with a 5`-reporter dye and a downstream, 3`-quencher, During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms.

Genotyping by real-time PCR

The Swine H275Y primer/probe mix contains a **FAM** labeled probe homologous to the wildtype genotype and a **VIC** labeled probe homologous to the mutant Tamiflu resistant genotype. During the real-time PCR amplification of the target the probes will compete for binding. If the H275Y mutation is present the VIC labeled probe will outcompete the FAM labeled probe and give a strong amplification plots and earlier Ct values. The reverse pattern is seen if the sample contains the wild-type sequence.

Detection Positive control – MI and NI

For copy number determination and as a positive control for the PCR set up, the kit contains positive control templates. This can be used to generate a standard curve of pathogen copy number / CT value. Alternatively the positive control can be used at a single copy number for a qualitative analysis of the samples.

Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for detection of the target pathogen gene are working properly in your particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling this component in a Post PCR environment.

Genotyping Positive controls – NI Wild-type and NI H275Y Mutant genotype

For genotyping, positive controls for the Wild-type Swine NI sequence and the mutant H275Y sequence are supplied. The positive control templates provide the necessary data to enable accurate genotyping of clinical samples. This positive control is not for use in a standard curve.

Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should 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 should be ignored and the test samples repeated. Possible sources or contamination should first be explored and removed.



Internal RNA extraction control

When performing RNA extraction, it is often advantageous to have an exogenous source of RNA template that is spiked into the lysis buffer. This control RNA is then co-purified with the sample RNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration.

A separate RT primer mix and a real-time PCR primer/probe mix are supplied with this kit to detect the exogenous RNA using real-time PCR. The PCR primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control cDNA does not interfere with detection of the pathogen target cDNA even when present at low copy number. The Internal control is detected through the **VIC** channel and gives a CT value of 31+/-3.

The internal control should not be used when using the Swine H275Y genotyping primer and probe mix

Endogenous ACTB control

To confirm extraction of a valid biological template a primer and probe mix is included to detect the Actin Beta (ACTB) gene. Detection of ACTB is through the FAM channel and it is **NOT** therefore possible to perform a multiplex for ACTB and the pathogen primers. A poor ACTB signal may indicate that the sample did not contain sufficient material to produce a positive test result.

Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PrimerDesign recommend the application of 0.2 U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step (95°C for 10 minutes).



Specificity

The *PrimerDesign™ Swine H1N1 Influenza Tamiflu resistance genotyping kit* contains three primer and probe sets.

1. The M1 primer and probe set designed to detect all influenza A sequences regardless of the species of origin. Samples that test positive with the M1 primer set have an influenza A infection of unknown serotype.
2. The swine NI H1N1 Influenza specific primer/probe set detects **ONLY** the pandemic swine H1N1 Influenza. The primers and probe have 100% homology to all pandemic strains. The primers will not detect NI from any seasonal endemic human or swine NI strains.
3. The Swine H275Y primer and probe set is a duplex designed to genetically identify sequences that contain the mutation that leads to a Tyrosine substitution for the Histidine at position 275 which confers resistance to Tamiflu. The primers and probe will detect on all pandemic strains but also have some potential to detect on other influenza isolates.

It is therefore essential to confirm the isolate under investigation as a swine flu using the swine NI H1N1 Influenza specific primer/probe, prior to testing for the H275Y mutation.



Kit Contents

- MI global influenza A primer/probe mix (150 reactions **BROWN**)
- NI swine H1N1 Influenza specific primer/probe mix (150 reactions **BROWN**)
- Swine H275Y genotyping primer/probe duplex mix (150 reactions **BROWN**)
- MI positive control template (for Standard curve **RED**)
- NI positive control template (for Standard curve **RED**)
- Swine H275Y WT positive control template (**RED**)
- Swine H275Y Mutant positive control template (**RED**)
- Internal extraction control RNA (150 reactions **BLUE**)
- Internal extraction control primer/probe mix (150 reactions **BROWN**)
- Endogenous ACTB primer/probe mix (150 reactions **BROWN**)
- RT primer mix INT control, MI, NI, H275Y and ACTB (150 reactions **GREEN**)
- RNase/DNase free water

Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
Must have the capacity to read fluorescence through the FAM and VIC channel
- **Mastermix or Mastermix components**
This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x *Precision*TM Mastermix.
- **Pipettors and Tips**
- **Vortex and centrifuge**
- **Thin walled 1.5 ml PCR reaction tubes**



Kit storage and stability

This kit is stable at room temperature but should be stored at -20°C on arrival. Once the lyophilized components have been re-suspended unnecessary repeated freeze/thawing should be avoided. Under these conditions reagents are stable for six months from date of purchase.

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 non specific 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 PrimerDesign pathogen detection kits have very high priming efficiencies of $>95\%$ and can detect between 1×10^8 and 1×10^2 copies of target template.



Bench side Protocol —InfA and/or H1N1 detection

To minimise 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 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. Reconstitute the kit components 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	Volume
Pre-PCR box	
NI H1N1 Influenza specific primer/probe mix (150 reactions BROWN)	165 µl
MI global influenza A primer/probe mix (150 reactions BROWN)	165 µl
Swine H275Y duplex Primer/Probe mix (BROWN)	165 µl
Endogenous ACTB primer/probe mix (BROWN)	165 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
RT primer mix INT control/Pathogen/ACTB (GREEN)	165 µl
Pre-PCR heat sealed envelope	
Internal extraction control RNA (BLUE)	600 µl
Post-PCR heat sealed envelope	
NI Positive Control Template (RED) *	500 µl
MI Positive Control Template (RED) *	500 µl
Swine H275Y WT Positive Control Template (RED) *	500 µl
Swine H275Y Mutant Positive Control Template (RED) *	500 µl

* 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.

RNA extraction

The internal extraction control RNA can be added either to the RNA lysis/extraction buffer or to the RNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control RNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal strength.

1. Add 4µl of the Internal extraction control RNA (**BLUE**) to each sample in RNA lysis/extraction buffer

2. Complete RNA extraction according to the manufactures protocols



Reverse Transcription

The protocols below are for PrimerDesign *qScript* Reverse Transcription kit. If you are using an alternative RT kit then follow the manufacturer's instructions.

Annealing Step

1. For each RNA sample add the following reagents to a thin walled 0.2 ml PCR tube or equivalent.

Component	1 reaction
RNA template (2ng-2µg)	X µl
RT primer mix (GREEN)	1 µl
RNAse/DNAse free water (WHITE)	X µl
Final volume	10 µl

2. Apply a lid to each sample and then heat to 65°C for 5 minutes.

This can be achieved using a thermostatically controlled “hot block” or heated water bath. Do not heat the RNA to a higher temperature as this can lead to RNA degradation.

3. Immediately cool the tubes in an ice water bath.

For optimal results, samples are transferred directly from 65°C to the ice. Do not allow the samples to cool prior to cooling on ice.



Extension step

1. Make up a mix according to the protocol below;

You will need 10µl for each RT reaction.

Component	1 reaction
qScript 10X Buffer (WHITE)	2µl
dNTP mix 10mM of each (ORANGE)	1µl
DTT 100mM (BLACK)	2µl
RNAse/DNAse free water(WHITE)	4µl
qScript enzyme (WHITE)	1µl
Final volume	10 µl

2. Add 10 µl of this mix to each of the samples on ice.

3. Apply a lid to each sample, mix tubes by briefly vortexing and incubate at 55°C for 20 minutes.

This can be achieved using a thermostatically controlled “hot-block” or heated water bath

4. Heat inactivate the reaction by incubation at 75°C for 15 minutes.

5. Store cDNA samples at -20°C until use.



Real-time PCR detection of Influenza A and/or Swine flu H1N1

1. Prepare a reaction mix according to the tables below:

Include sufficient reactions for the standard curve wells (6 samples in duplicate) and also the negative control.

MI global influenza A or NI Swine H1N1 Pathogen detection mix

Component	l reaction
2X Precision™ Mastermix	10 µl
MI or NI primer/probe mix (BROWN)	1 µl
Internal extraction control primer/probe mix (BROWN)	1 µl
RNAse/DNAse Free water	3 µl
Final volume	15 µl

Endogenous ACTB detection mix (Optional)

Component	l reaction
2 X Precision™ Mastermix	10 µl
Endogenous ACTB primer/probe mix (BROWN)	1 µl
RNAse/DNAse Free water	4 µl
Final volume	15 µl

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

3. Prepare sample cDNA templates for each of your samples (suggested concentration 5ng/µl) in RNAse/DNAse free water.

If the concentration of RNA that was used to make the cDNA is not known, then dilute your RT reaction mix 1:5 (10µl of sample DNA and 40µl of water)

4. Pipette 5µl of diluted cDNA template into each well, according to your experimental plate set up.

For negative control wells use 5µl of RNAse/DNAse free water. The final volume in each well is 20µl



5. Preparation of standard curve dilution series

- 1) Pipette 900µl of RNase/DNAse free water into 5 tubes and label 2-6
- 2) Pipette 100µl of Positive Control Template (**RED**) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 100µl from tube 2 into tube 3
- 5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (RED)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

6. Pipette 5µl of standard template into each well, according to your experimental plate set up.

The final volume in each well is 20µl.



Amplification Protocol

Amplification conditions using PrimerDesign 2X Precision™ MasterMix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37°C
	Enzyme Activation (if required)***	10 mins	95°C
50 cycles	Denaturation	10s	95°C
	DATA COLLECTION*	60s	60°C

* Fluorogenic data for the NI gene is through the FAM and the INT control is detected in the VIC channels

** Required if your mastermix includes UNG to prevent PCR carryover contamination

*** Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.



Interpretation of Results

Pathogen	Internal control	Negative control	Positive control	Interpretation
+ve	+ve	-ve	+ve	+ve
+ve	-ve	-ve	+ve	+ve
-ve	+ve	-ve	+ve	-ve
-ve	-ve	-ve	-ve	Exp Fail
+ve	+ve	+ve	+ve	Exp Fail

Internal PCR control

When used according to the above protocols, assuming a 100% extraction efficiency, that 1:4 of extracted RNA is used in the RT reaction and that 1:10 of the RT reaction is used in an individual well, then a CT value of 31 is expected. However this can vary significantly depending on the extraction efficiency, the quantity of RNA added to the RT and PCR reaction and the individual machine settings. CT values of 31 ± 3 are within the normal range. When amplifying a pathogen sample with high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous ACTB control

The signal obtained from the ACTB primer and probe set will vary according to the amount of biological material present in a given sample.



Bench side Protocol — Real-time PCR genotyping of NI positive samples

Samples that give a positive result with the NI Swine H1N1 specific primers and probe can be screened for Tamiflu resistance provided that the Ct value obtained was between 15 and 28. Samples giving a Ct value less than 15 should be diluted accordingly. Samples giving a Ct value greater than 28 contain insufficient biological material for genotyping.

1. Prepare a reaction mix according to the tables below:

Include sufficient reactions for the standard curve wells (6 samples in duplicate) and also the negative control.

Swine H275Y genotyping mix

Component	l reaction
2X Precision™ Mastermix	10 µl
Swine H275Y primer/probe mix (BROWN)	1 µl
RNAse/DNAse Free water	4 µl
Final volume	15 µl

n.b. The Internal extraction control primers and probe cannot be used in the genotyping test

- Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.**
- Prepare sample cDNA templates for each of your samples (suggested concentration 5ng/µl) in RNAse/DNAse free water.**
- Pipette 5µl of diluted cDNA template into each well, according to your experimental plate set up.**
For negative control wells use 5µl of RNAse/DNAse free water. The final volume in each well is 20µl
- Preparation of positive control wells for WT and H275Y.**
For positive control wells use 5µl of WT positive control template in duplicate And 5µl of H275Y positive control template in duplicate in separate wells.



Amplification Protocol

Amplification conditions using PrimerDesign 2X Precision™ MasterMix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37°C
	Enzyme Activation (if required)***	10 mins	95°C
50 cycles	Denaturation	10s	95°C
	DATA COLLECTION*	60s	60°C

* Fluorogenic data for the WT genotype is through the FAM channel and the H275Y mutation is detected in the VIC channel

** Required if your mastermix includes UNG to prevent PCR carryover contamination

*** Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.

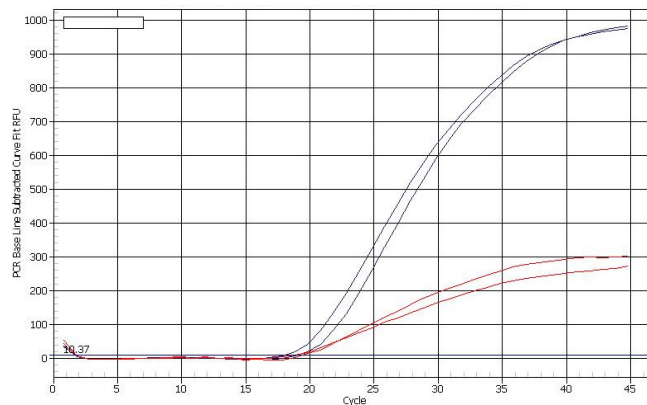


Interpretation of Results

Genotyping is based on the ratio of signal between the two Channels (FAM and VIC). The two positive controls demonstrate the expected levels of FAM and VIC fluorescence for both wild type and H275Y sequences. Wild type sequence will have strong amplification plots with high end point fluorescence in the FAM channel; they will have weak amplification plots with later CT values in the VIC channel (Fig.1a). The reverse pattern is seen for H275Y sequences (Fig.1b).

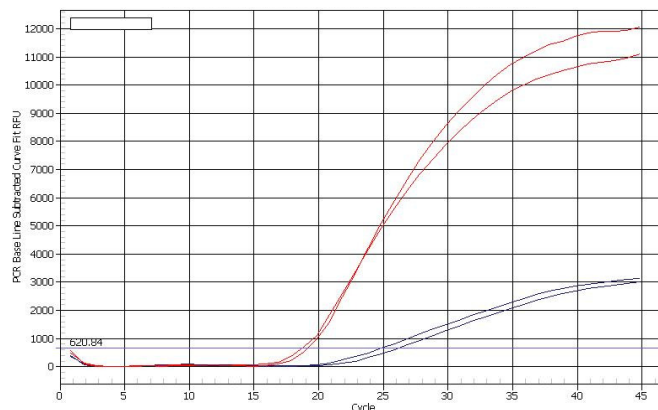
This data can be best be visualized by using a cluster analysis, plotting the FAM end point fluorescence on one axis and VIC channel data on the other (Fig.2). Many hardware platforms will perform this analysis automatically. Follow the manufactures instructions for your individual software.

Fig.1a



Example FAM channel analysis: Wild Type positive control data showing a strong signal (Blue). A much weaker signal (lower end point fluorescence and later Ct values) is present on the H275Y mutant template (red).

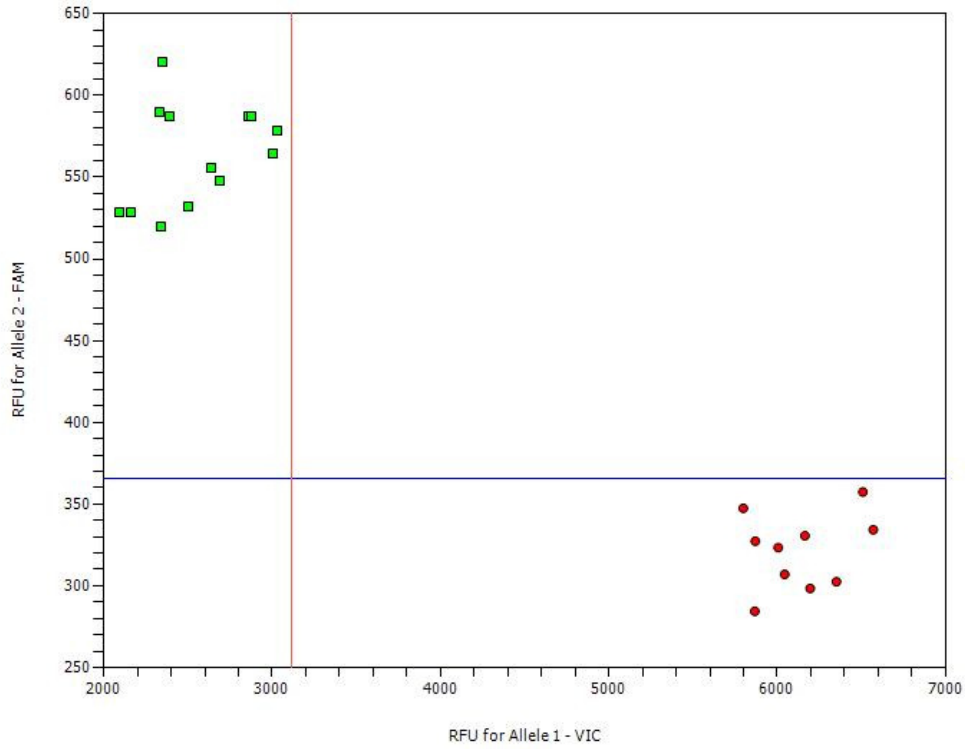
Fig.1b



Example VIC channel analysis: Example H275Y mutant positive control data showing a strong signal (red). A much weaker signal (lower end point fluorescence and later Ct values) is present on the Wild Type template (blue).



Fig.2



Cluster analysis data showing FAM fluorescence vs. VIC fluorescence. Each genotype is shown as a cluster of points and is clearly distinguishable. Green = wild-type sample. Red = mutant H275Y homozygote.



Notices and disclaimers

During the warranty period PrimerDesign pathogen detection kits 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.

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