Tamiflu resistance detection kit

H1N1 (H275Y)

genesig® real-time PCR mutation detection/allelic discrimination kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

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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/mutant region. The probe that is 100% homologous to the DNA binding site will preferentially bind and give a fluorescent signal as qPCR 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 will give an intermediate signal 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 kit 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 should 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 viral RNA added to each test. The optimum sample RNA 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 should be included every time the kit is used. In this instance, 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 of contamination should first be explored and removed.

Thermo-cycling parameters

The optimum cycling parameters for Primerdesign genotyping kits 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 RNA (<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 it should be noted that the reported cycle threshold (Cq) values produced by this program will be lower than usual and that this is expected.

Master Mix compatibility

PrecisionPLUS OneStep Master Mix and oasig OneStep 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 OneStep Master Mix or oasig OneStep Master Mix is used.

Kit contents

- **H1N1 (H275Y) genotyping primer/probe mix (150 reactions, BROWN)**
- **Wild type (WT) positive control template (RED)**
- **Mutant (SNP) positive control template (RED)**
- **RNase/DNase free water (WHITE)**
  for resuspension of genotyping primer/probe mix
- **Template preparation buffer (YELLOW)**
  for resuspension of positive control templates

The contents above are required for genotyping.

The kit will also contain the items listed below. These are for H1N1 detection.

- **Swine-M1 primer/probe mix (150 reactions, BROWN)**
- **Swine-N1 primer/probe mix (150 reactions, BROWN)**
- **Swine M1/N1 RT primer mix (150 reactions, GREEN)**
- **Human endogenous control (150 reactions, BROWN)**
- **2 x internal control primer/probe mix (150 reactions, BROWN)**
- **2 x internal control RNA templates (BLUE)**
- **Swine-M1 positive control template (RED)**
- **Swine-N1 positive control template (RED)**

Instructions for use of the H1N1 kit can be found in the H1N1 kit handbook at www.genesig.com.

Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
  Must be able to read fluorescence through the VIC and ROX channels.

- **qPCR Master Mix**
  This kit is designed to work well with Primerdesign PrecisionPLUS OneStep 2X RT-qPCR Master Mix or oasig OneStep 2X RT-qPCR Master Mix. Primerdesign can only guarantee accurate genotyping results when PrecisionPLUS OneStep or oasig OneStep Master Mix is used.

- **Pipettors and Tips**

- **Vortex and centrifuge**

- **Thin walled 0.2 ml PCR reaction tubes**

Kit storage

This kit is stable at room temperature but should be stored at -20ºC on arrival. Primerdesign does not recommend using the kit after the expiry date stated on the pack. Once the lyophilised components have been resuspended, unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.

Suitable sample material

All kinds of sample material suited for qPCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration and RNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace the test sample with RNase/DNase free water.

Licensing agreement and limitations of use

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

Primerdesign satisfaction guarantee

Primerdesign takes pride in the quality of all our 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 quality assurance system, all Primerdesign products are monitored to ensure the highest levels of performance and reliability.

Notices and disclaimers

During the warranty period Primerdesign genesig 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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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.

Bench-side protocol

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. The positive control template is a significant contamination risk and should therefore be pipetted after negative control and sample wells.

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 genotyping primer/probe mix in the RNase/DNase water supplied, according to the table below:**
To ensure complete resuspension, vortex the tube thoroughly.

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

3. **Resuspend the positive control templates in the template resuspension buffer supplied, according to the table below:**
To ensure complete resuspension, vortex the tube thoroughly.

Component – resuspend in template preparation buffer	Volume
Post-PCR heat sealed capsules	
WT positive control template (**RED**)*	500µl
Mutant positive control template (**RED**)*	500µl

*** 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. **Prepare a complete genotyping reaction mix, according to table below:**
Prepare sufficient reactions for all samples. Include 2 extra reactions for the wild type and variant positive controls and 1 extra sample as a 'no template control'.

Component	1 reaction	e.g. 50 reactions
oasig OneStep or PrecisionPLUS OneStep Master Mix	10µl	500µl
Genotyping primer/probe mix (**BROWN**)	1µl	50µl
RNase/DNase free water (**WHITE**)	4µl	200µl
Final volume	**15µl**	**750µl**

Please note, if using oasig OneStep Master Mix the ROX passive reference dye supplied as part of the kit will not be required.

5. **Dispense 15µl of the reaction mix according to your plate layout.**

6. **Dispense 5µl of extracted RNA sample according to your plate set up.**
   To obtain a strong signal, the recommended input RNA concentration is 0.2 – 2 ng/µl (1-10ng in total) with the ideal total being 5ng. Substitute sample RNA with RNase/DNase free water as a negative control.

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

Sample input RNA	+ve Control Dilution factor
10ng	1:25
5ng	1:50
1ng	1:250

8. **Dispense 5µl of each positive control DNA template according to your 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 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.

Recommended protocol when using PrecisionPLUS OneStep or OneStep oasig 2X RT-qPCR Master Mix

	Step	Time	Temp
	Reverse transcription	10 mins	55°C
	Enzyme activation	2 mins	95°C
X 10 cycles*	Denaturation	10s	95°C
	Extension	60s	60°C
X 35 cycles	Denaturation	10s	95°C
	Extension **(DATA COLLECTION)** **	60s	66°C

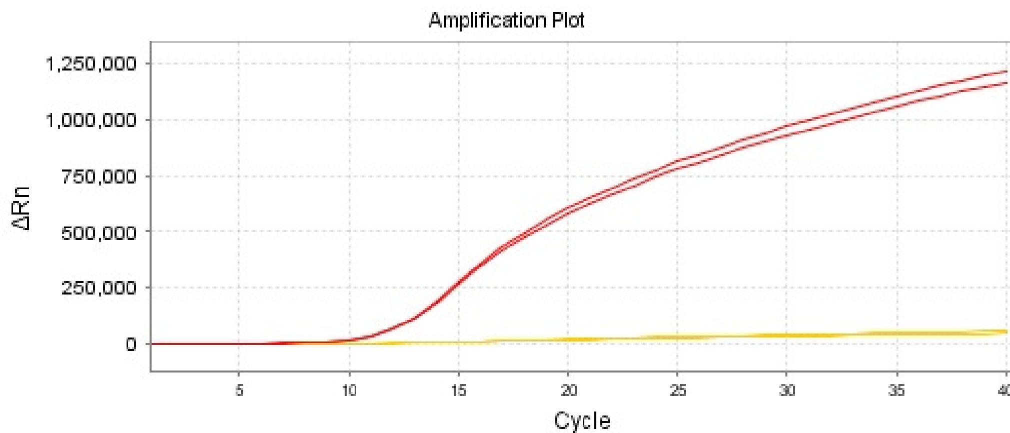
* Where the sample amount is 1ng or less per well add 5 cycles to the first stage (15 cycles)
** Fluorogenic data should be collected through the ROX and VIC channels.

Interpretation of results

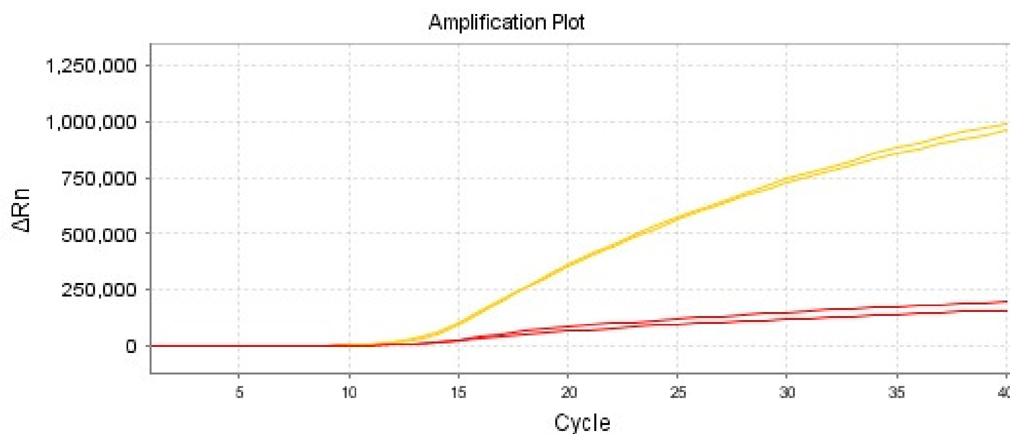
The wild type probe is labelled to read through the ROX channel whilst the mutant probe is labelled to read through the VIC channel. On wild type sequences the ROX channel will give a strong amplification plot and the VIC channel none or very low detection. The signals are reversed on mutant samples.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

Sample data

Wild type sample (**WT signal, Mutant 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 best be visualised 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 are quickly resolved into clusters corresponding to the wild type, heterozygote and homozygous variant samples.