

Primerdesign™ Ltd

Detection of Prothrombin (G20210A)

snpsig™ real-time PCR
mutation detection/allelic discrimination kit

50 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Kit contents

- **Prothrombin genotyping primer/probe mix (50 reactions BROWN)**
ROX and VIC labelled
- **Wild-type positive control template (RED)**
- **Mutant positive control template (RED)**
- **RNase/DNase free water (WHITE)**
for resuspension of primer/probe mix
- **Template preparation buffer (YELLOW)**
for resuspension of positive control templates

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

oasig™ lyophilised, Precision®PLUS or Precision®FAST 2X qPCR Master Mix

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

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. 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 PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and DNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace the template DNA sample with RNase/DNase free water.

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 snpsig 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. 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 Applied Biosystems 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.

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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 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 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 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.

Thermocycling 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 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 it should be noted 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 Protocol

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

Component - Resuspend in water	Volume
Pre-PCR pack	
Prothrombin primer/probe mix (BROWN)	55 µl

- 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.

Component - Resuspend in template preparation buffer	Volume
Post-PCR heat-sealed foil	
Wild-type Positive Control Template (RED) *	500 µl
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.

qPCR detection protocol

- 1. Prepare a complete genotyping reaction mix for each primer/probe mix according to the table below:**
Include sufficient reactions for all samples, positive and negative controls.

Component	Volume
oasig, PrecisionPLUS or PrecisionFAST2X qPCR Master Mix	10 μ l
Prothrombin primer/probe mix (BROWN)	1 μ l
RNase/DNase free water (WHITE)	4 μ l
Final Volume	15 μl

- 2. Pipette 15 μ l of this mix into each well according to your qPCR experimental plate set up.**
- 3. Prepare DNA templates for each of your samples.**
- 4. Pipette 5 μ l of DNA template into each well, according to your experimental plate set up.**
To obtain a strong signal, the recommended input DNA concentration is 0.2-2ng/ μ l (1-10ng in total) with the ideal total being 5ng. For negative control wells use 5 μ l of RNase/DNase free water. The final volume in each well is 20 μ l.
- 5. 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
10ng	1:25
5ng	1:50
1ng	1:250

- 6. Pipette 5 μ l 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 oasis 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.

Protocol for oasis, PrecisionPLUS or PrecisionFAST 2X qPCR Master Mix

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x10**	Denaturation	10s	95 °C
	Extension	60s	60 °C
Cycling x35	Denaturation	10s	95 °C
	Extension (DATA COLLECTION) *	60s	68 °C

* Fluorogenic data should be collected during this step through the **ROX** and **VIC** channels

** where the sample amount is 1ng or less per well, add 5 cycles to the first stage (15 cycles)

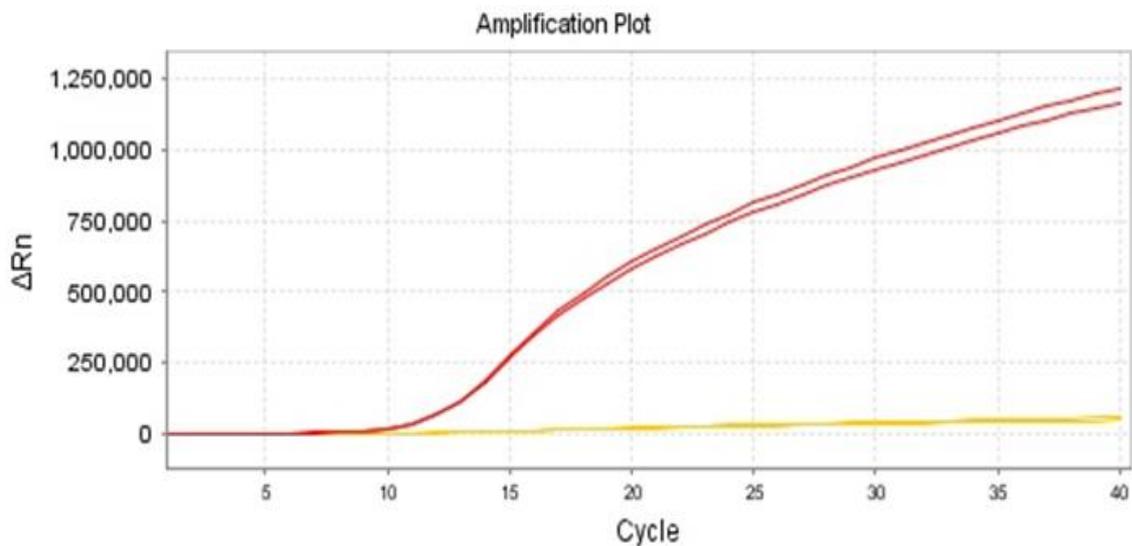
Interpretation of results

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.

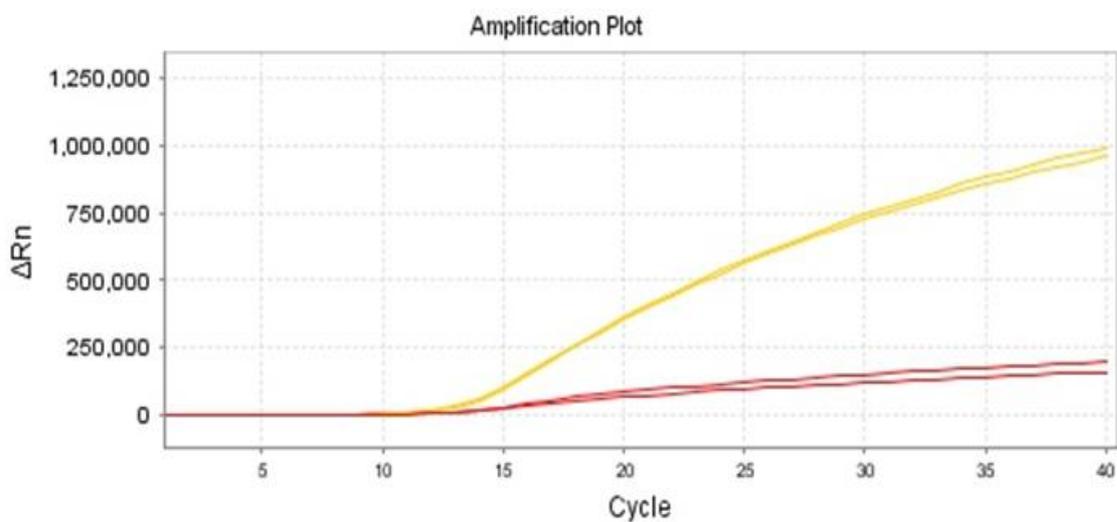
The genotype of each sample is calculated by comparing the ratio of signals between the two channels (ROX and VIC).

Sample data

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



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



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.

