Primerdesign™ Ltd

High risk Human Papillomavirus

Multiplex screening kit

genesig® kit

100 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Human Papillomavirus

Papillomaviruses are a diverse group of DNA viruses that infect the skin and mucous membranes of humans and a variety of animals. Although more than 100 different human papillomavirus (HPV) types have been characterized HPV6, HPV11, HPV16 and HPV18 are the most clinically relevant.

Genital or anal warts (condylomata acuminata or venereal warts) are the most easily recognised sign of genital HPV infection. Although a wide variety of HPV types can cause genital warts, types 6 and 11 accounts for about 90% of all cases. HPV types 16 and 18 are called "high-risk" types because they can cause cervical cancer, as well as anal cancer, vulvar cancer, head and neck cancers, and penile cancer. Other high-risk types include HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66 and HPV68. The high-risk types account for over 90% of cervical adenocarcinomas worldwide. HPV-induced cancers often have viral sequences integrated into the cellular DNA. Some of the HPV "early" genes, such as E6 and E7, are known to act as oncogenes that promote tumor growth and malignant transformation.

The current kit provides a single tube to screen for the presence of all high-risk HPV types listed above. The multiplex test is detected in three fluorescent channels differentiating between HPV16 and HPV18 which produce a ROX or VIC channel signal respectively and all others, which produce a signal in the FAM channel. HPV16 and HPV18 account for 70% of positive findings in clinical practice so it is helpful to know which of these are present. All other high-risk genotypes together make up the remaining 30% of clinical positives and are grouped together into the FAM channel. In this configuration, the kit gives a partial genotyping result and some additional information on which high-risk strains are present.

Specificity

The kit contains individual primers directed against each genotype detected in the kit. The primers are designed to exclude detection of 'low-risk' and other clinically irrelevant HPV genotypes.

The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis. They therefore have a very broad quantification profile.

If you require further information, or have a specific question about the detection profile of this kit then please send an email to enquiry@primerdesign.co.uk and our bioinformatics team will answer your question.

Kit contents

- **High risk HPV primer/probe mix (100 reactions BROWN)**
  ROX, FAM, VIC, and Cy5 labelled (see table below)

Target	Fluorophore
HPV16	ROX
HPV18	VIC
HPV31	FAM
HPV33	FAM
HPV35	FAM
HPV39	FAM
HPV45	FAM
HPV51	FAM
HPV52	FAM
HPV56	FAM
HPV58	FAM
HPV59	FAM
HPV66	FAM
HPV68	FAM
Endogenous control	Cy5

- **High Risk HPV positive control template (RED)**
  Positive signal for ROX, FAM and VIC channel

  As the HPV16 assay produces amplification through the ROX channel, if using a machine that recommends ROX passive reference dye please ensure ROX normalisation is switched off.

- **Lyophilised oasig™ Master Mix**
  The oasig master mix does not contain ROX, so if using a machine that requires ROX to be used a passive reference dye ensure this is turned off.

- **oasig™ resuspension buffer (BLUE)**

- **RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mix

- **Template preparation buffer (YELLOW)**
  for resuspension of positive control template

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument

Ability to detect ROX, FAM, VIC and Cy5 channels

DNA extraction kit

This kit is recommended for use with genesig Easy DNA/RNA extraction kit. However, it is designed to work well with all processes that yield high quality DNA with minimal PCR inhibitors.

Pipettors and Tips

Vortex and Centrifuge

Thin walled 1.5ml PCR reaction tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20ºC on arrival. Once the lyophilised components have been resuspended they should not be exposed to temperatures above -20ºC for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.

Primerdesign does not recommend using the kit 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. 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.

Dynamic range of test

Under optimal PCR conditions genesig kits have very high priming efficiencies of >90% and can detect between $1x10^8$ and $1x10^2$ copies of target template.

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Principles of the test

Real-time PCR

Individual primers and probes designed for each genotype have been combined into a single reaction and these can be detected through the different fluorescent channels as described in the kit contents.

The primer/probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridise to the target DNA. Fluorogenic probes are included in the same reaction mixture, which consists of a DNA probe labelled with a 5`-dye and a 3`-quencher. During qPCR 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 qPCR platforms.

Positive control

For a positive control, the kit contains a single positive control that contains templates for three of the 14 genotypes under test. One template in the positive control will give a signal through the ROX channel, another template in the positive control will give a signal through the VIC channel and the final template in the positive control will give a signal through the FAM channel. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primer/probe mix and master mix are working properly in that run. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive controls do not contaminate any other kit component, which would lead to false positive results. This can be achieved by handling these components in a post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To confirm the 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.

Endogenous control

To confirm extraction of a valid biological template, the single primer/probe mix supplied contains primers and probe designed to detect an endogenous gene. Detection of the endogenous control is through the Cy5 channel. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Resuspension 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 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/probe mix is in the base of the tube and is not spilt upon opening the tube.

2. Resuspend the high risk HPV 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	
High risk HPV primer/probe mix (**BROWN**)*	110 µl

*Please note: When the kit is not in use, it should be stored at -20ºC.

3. Resuspend the positive control template 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 foil	
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.**

4. Resuspend the lyophilised oasig 2X qPCR Master Mix in oasig resuspension buffer, according to the table below:

Component – resuspend in oasig resuspension buffer	Volume
Lyophilised oasig Master Mix	525 µl

DNA extraction

1. Complete the DNA extraction according to the manufacturer's recommended protocol.

qPCR detection protocol

1. **For each DNA sample prepare a reaction mix according to the table below:**
   Include sufficient reactions for positive and negative controls.

Component	Volume
oasig Master Mix	10 µl
High Risk HPV primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final volume	**15 µl**

2. **Pipette 15µl of these mixes into each well according to your qPCR experimental plate set up.**

3. **Pipette 5µl of DNA template into each well according to your experimental plate set up.**
   For negative control (NTC) wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl.

4. **Pipette 5µl of positive control template into the positive control wells.**

qPCR amplification protocol

Amplification conditions using oasig 2X qPCR Master Mix

	Step	Time	Temp
X 50 cycles	Enzyme activation	2 mins	95°C
	Denaturation	10 secs	95°C
	DATA COLLECTION*	60 secs	60°C

* Fluorogenic data should be collected during this step through the ROX, FAM, VIC and Cy5 channels.

If using a machine that recommends ROX to be used a passive reference dye ensure this is switched off.

Interpretation of results

Positive control

The positive control well should give an amplification plot through the ROX, FAM and VIC channel. The positive control signals indicate that the kit is working correctly. The number of copies of template packaged in the positive control varies for each channel and therefore the Cq value will be different. The expected Cq values are listed below:

Channel	Expected Cq value
ROX	21-25
VIC	17-21
FAM	24-29

If a negative result is obtained in either the ROX, VIC or FAM channel, then the test results are invalid and must be repeated

No template control (NTC)

The NTC should give a flat line (flat amplification plots) through all channels. Signals in the NTC indicate cross contamination during plate set up.

Sample data

Each sample should give a signal through the Cy5 channel, however, the signal obtained may vary according to the amount of biological material present. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample. If the endogenous control fails to amplify the test has failed because neither the sample quality nor quantity was high enough. In these situations, try increasing the concentration of the DNA sample that is added to the reaction, or checking the DNA/RNA extraction protocol for any user errors during preparation and repeat the DNA extraction. Please note, poor extractions can be caused by overloading the DNA extraction with too much starting material.

Presence of HPV is detected in the channels indicated in the kit contents section. A positive signal in the ROX channel indicates a positive test for HPV16. A positive signal in the VIC channel indicates a positive test for HPV18, whereas a positive signal in the FAM channel indicates positive tests for HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66 or HPV68. Mixed infections are relatively common having reported frequencies up to 25% depending on the clinical setting. That means that samples may give positive signals through multiple channels such as FAM and VIC e.g. a sample containing HPV18 (VIC channel) and HPV68 (FAM channel). Mixed infections can also lead to unusually shaped curves where more than one amplification take place in the same channel e.g. a sample where both HPV58 and HPV66 are present and amplify and produce fluorescence in the FAM channel.

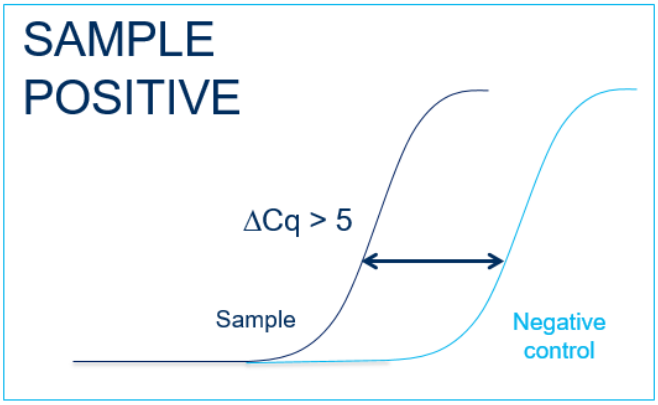
Summary of data interpretation

Target (ROX/FAM/VIC)	Endogenous control (Cy5)	Positive Control	Negative Control	Interpretation
ROX +	**+ / -**	**+**	**-**	**HPV16 POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**HPV18 POSITIVE RESULT**
FAM +	**+ / -**	**+**	**-**	**OTHER HIGH-RISK TYPE POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

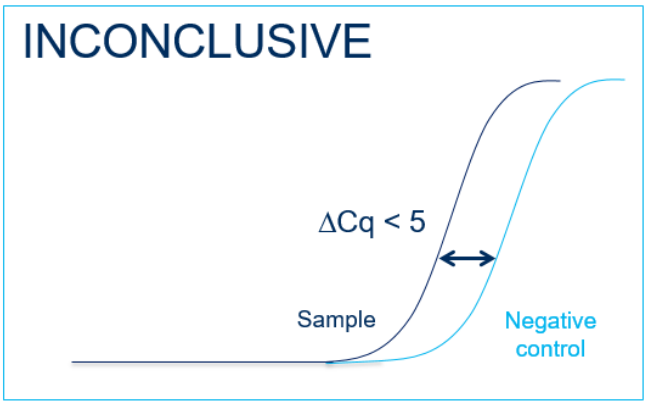
+ / -	**+ / -**	**+**	**≤35**	**EXPERIMENT FAILED** Due to test contamination
+ / -	**+ / -**	**+**	**>35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

Positive control template (RED) is expected to amplify between Cq 17 and 29. Please note the specific Cq ranges for each channel listed on page 13. Failure to satisfy these quality control criteria is a strong indication that the experiment has been compromised.

* Where the test sample is positive and the negative control is positive with a Cq >35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.