Primerdesign™ Ltd

R01005

genesig™PLEX Gastrointestinal Viral Real-Time PCR Multiplex Kit

Kit version: 1

Norovirus GI and GII (RNA polymerase)

Adenovirus F40 and F41 (long fibre protein)

Rotavirus A (Non-structural protein NSP5)

genesig™PLEX kit

100 tests

GENESIG

Kits by Primerdesign

Specificity of primers and probes last reviewed on:
March 2023

For general laboratory and research use only

Introduction

Norovirus Genogroups I and II

Norovirus is known to cause acute gastroenteritis. It is a small (27-38 nm), round, non-enveloped RNA virus belonging to the Caliciviridae family and is responsible for over 80% of non-bacterial outbreaks of gastroenteritis in the world. It affects individuals of all ages, with a distinct seasonal link to winter. It has a genome of 7.6 kb that is positive sense and has a single stranded linear confirmation. It encodes a major structural protein (VP1) of about 58 to 60 kDa and a minor capsid protein (VP2).

Transmission occurs predominantly through ingestion of contaminated water, food and airborne transmission, as well as contact with contaminated surfaces. The ease with which norovirus is transmitted and the low infectious dose required to establish an infection results in extensive outbreaks in numerous environments, such as hospitals, hotels and schools. There is no antiviral drug available to treat this infection and little is known about its pathogenicity. However, it has been observed that the virus can be taken up by enterocytes where translation of viral nonstructural proteins can occur; it damages and alters intestinal microvilli, leaving them blunt and broadened, thus inhibiting absorption; it causes crypt cell hyperplasia and also leads to apoptosis of enterocytes.

An incubation period of 24-48 hours is usual. Infection is characterized by the acute onset of nausea, vomiting, abdominal cramps, aching limbs, raised temperature and diarrhoea that generally last for about 48 hours. However, more severe and prolonged infection may be observed in children and the elderly. There are now ten recognised norovirus genogroups, of which three (GI, GII, and GIV) are known to affect humans. Of these three genogroups GI and GII are responsible for the majority of human infections and, since 2002 variants of the GII.4 genotype have been the most common cause of norovirus outbreaks. There have been 51 different genotypes identified within the genogroups, with a wide degree of genetic variability present even within each genotype.

Adenovirus F40 and F41

Adenoviruses are non-enveloped, icosahedral, 90–100 nm viruses with a linear, double-stranded DNA genome spanning 26–45 kb. Adenoviruses represent the largest nonenveloped viruses, because they are the maximum size able to be transported through the endosome (i.e. envelope fusion is not necessary). The virion also has a unique "spike" or fibre associated with each penton base of the capsid that aids in attachment to the host cell via the coxsackie-adenovirus receptor on the surface of the host cell. Divided into seven species (A to G), there are more than 100 subtypes of human adenovirus. In humans, the species and subtypes are associated with different conditions.

Adenovirus F40 and F41 are associated with gastrointestinal complications and is among the more frequently detected serotypes in paediatric gastroenteritis cases. Gastroenteritis

continues to be a major contributor to illness and death in low and middle-income countries and remains a prominent cause of mortality among children under the age of 5. Symptoms include diarrhoea, vomiting and fever, with diarrhoea estimated to cause 530,000 death/year worldwide in young children.

Adenoviruses are unusually stable to chemical or physical agents and adverse pH conditions, allowing for prolonged survival outside of the body. Adenoviruses can remain viable for a duration ranging from 7 days to 3 months on dry surfaces, while also demonstrating the ability to survive for several days in both tap water and sewage effluent. Adenoviruses are primarily spread via respiratory droplets, however they can also be spread by faecal routes as well.

Human Rotavirus A

Rotavirus is a genus of double-stranded RNA viruses of the Reoviridae family. Species within this genus have been named A to H, all of which cause severe diarrhoea although species A are the most common Rotavirus infection in humans. The linear RNA genome of this species contains 11 segments between 528 and 3267 nucleotides in length. The three layer capsid containing this genome has icosahedral symmetry and is around 80nm in diameter and has channels which extend inward to the core.

The main route of transmission is the faecal-oral route due to contamination of food and water. The virus targets the enterocytes of the intestinal villi, enters the host cell by receptor-mediated endocytosis and replicates in the cytoplasm. After replication and construction of the new viral progeny the cell is lysed resulting in viral release as well as release of intestinal fluid which results in diarrhoea containing viral particles.

After an incubation period of around 2 days, symptoms of Rotavirus infection present with vomiting, fever and diarrhoea that can last over a week. After infection antibodies to the virus can be found in an individual, subsequent infections can occur throughout life but are mainly asymptomatic. Infection is therefore most severe in young children although new-borns are thought to have acquired immunity from trans-placental transfer of antibodies. Treatment is usually directed at alleviation of symptoms and mainly involves rehydration.

Specificity

The genesig™PLEX kit is designed for the in vitro detection of Norovirus GI and GII, Adenovirus F40 and F41, Rotavirus A.

The Adenovirus assay within this kit is predicted to detect over 95% of sequences available on the NCBI database at the time of design. For the Rotavirus A assay, 95% of the sequences from the last five years are predicted to be detected and for Norovirus GI and GII, 95% of sequences from the last three years are predicted to be detected. Due to the sequence evolution observed in the genomes of RNA viruses, a period of between three and five years is considered to be an adequate time period for analysis, depending on the number of sequences on the NCBI database.

The dynamics of genetic variation means that new sequence information may become available after the initial design. Primerdesign periodically reviews the detection profiles of our kits and when required releases new versions.

The Norovirus GII assays is predicted to cross react with Noroviruses GIX, GVI and GIV which would give signal in the FAM channel. Newly emerging evidence suggests that there will be some cross reactivity with bacteriophages found in the human gut, however the effect of this is predicted to be negligible in samples from patients suffering from gastrointestinal infections.

The Rotavirus A assay is predicted to cross react with rotavirus A infecting mammals as well as bat rotavirus and murine rotavirus, which would give signal in the VIC channel.

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

Kit contents

- **Multiplex primer/probe mix (2x 50 reactions BROWN)**
  FAM, VIC, ROX and Cy5 labelled (see table below)

Target	Fluorophore
Norovirus GI and GII	FAM
Rotavirus A	VIC
Adenovirus F40 and F41	ROX
Internal extraction control	Cy5

- **Multiplex positive control template (RED)**
- **Internal extraction control RNA (BLUE)**
- **2x Oasig Max OneStep Master Mix (GREEN)**
- **2X oasig™ Max resuspension buffer (ORANGE)**
  for resuspension of the lyophilised master mix
- **Template preparation buffer (YELLOW)**
  for resuspension of the positive control template
- **RNase/DNase free water (WHITE)**
  for resuspension of the primer/probe mix

Reagents and equipment to be supplied by the user

Real-time PCR Instrument

Extraction kit

This kit is recommended for use with exsig®Mag however, it is designed to work well with all processes that yield high quality RNA and DNA with minimal PCR inhibitors.

Pipettors and filter tips

Vortex and centrifuge1.5ml tubes

qPCR plates or 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 integrity. 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 genesig kits have very high priming efficiencies of >90% and can detect between $1X10^4$ and $1X10^2$ copies of target template.

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 genesig 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 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.

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Principles of the test

Real-time PCR

Individual primer and probes designed for each pathogen 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 and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize 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 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 qPCR platforms.

Positive control

For a positive control, the kit contains a single positive control that contains templates for the 3 targets in the test. The kit positive control will give a Norovirus GI&GII signal through the FAM channel, a Rotavirus A signal through the VIC channel and an Adenovirus F40&F41 signal through the ROX 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 primers and probes for detecting each virus are working properly in that particular 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 control does 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

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 qPCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration. Within the Multiplex primer/probe mix are primers and probes to detect the exogenous RNA using qPCR. The primers are present at PCR limiting concentrations which allows

multiplexing with the target sequence primers. Amplification of the control RNA does not interfere with detection of the target RNA/DNA even when present at low copy number. The Internal control is detected through the Cy5 channel and gives a Cq value of 28+/-3.

Resuspension protocol

To minimise the risk of contamination with foreign RNA, 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 lost 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	
Multiplex primer/probe mix (**BROWN**) (volume each tube)	55µl

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

Component – resuspend in template preparation buffer	Volume
Pre-PCR heat-sealed foil	
Internal extraction control RNA (**BLUE**)	500µl
Post-PCR heat-sealed foil	
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. **Resuspend the lyophilised OneStep Master Mix in oasig™ Max resuspension buffer, according to the table below:**

Component – resuspend in oasig resuspension buffer	Volume
Lyophilised Oasig Max OneStep Master Mix (**GREEN**)	525µl

RNA extraction

The internal extraction control RNA can be added to either lysis/extraction buffer or to the 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.

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

2. **Complete RNA extraction according to the manufacturer's recommended protocols.**

OneStep RT-qPCR detection protocol

For optimum performance and sensitivity
All pipetting steps and experimental plate set up should be performed on ice. After the plate is prepared, proceed immediately to the OneStep amplification protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artifacts that reduce the sensitivity of detection.

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

Component	Volume
Oasig Max OneStep Master Mix (**GREEN**)	10µl
Multiplex primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (**WHITE**)	4µl
Final volume	**15µl**

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

3. **Pipette 5µl of RNA sample 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.

4. **Pipette 5µl of positive control template into each well according to your plate set up.**
   The positive control contains templates for Norovirus, Adenovirus and Rotavirus. The final volume in each well is 20µl.

OneStep RT-qPCR amplification protocol

Amplification conditions using lyophilised OneStep Master Mix

	Step	Time	Temp
	Reverse transcription	10 mins	55°C
	Enzyme activation	2 mins	95°C
Cycling x 50	Denaturation	10 secs	95°C
	DATA COLLECTION*	60 secs	60°C

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

Interpretation of results

Positive control

The positive control well should give an amplification plot through the FAM channel (Norovirus), the VIC channel (Rotavirus) and the ROX channel (Adenovirus). There is no internal control template within the positive control so the Cy5 channel should give no signal (flat amplification plot). The positive control signals indicate that the kit is working correctly to detect each virus.

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.

Internal RNA extraction control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of RNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a target sample with a 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.

Sample data

Presence of the viruses are detected in the channels indicated in the kit contents section. Positive signals indicate positive tests for those viruses. It may be possible for samples to contain multiple viruses, therefore positive results in the FAM, VIC and ROX channels may be present.

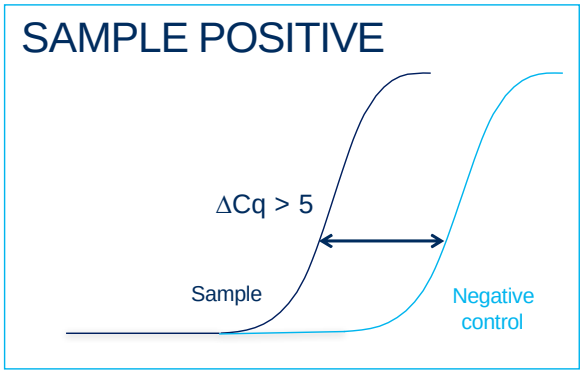
Summary of data interpretation

Target (FAM/VIC/ROX)	Internal extraction control (Cy5)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**NOROVIRUS POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**ROTAVIRUS POSITIVE RESULT**
ROX +	**+ / -**	**+**	**-**	**ADENOVIRUS POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

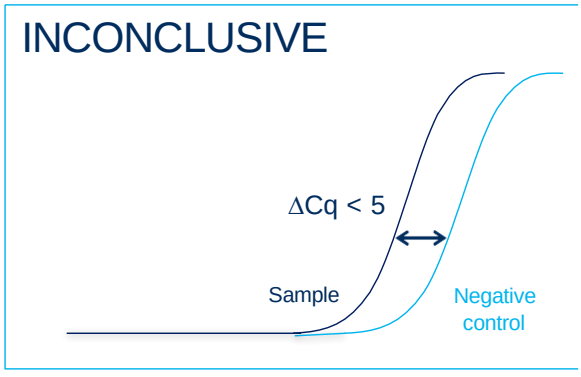
+ / -	**+ / -**	**+**	**≤35**	**EXPERIMENT FAILED** Due to test contamination
+ / -	**+ / -**	**+**	**>35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

Positive control template (RED) is expected to amplify between Cq 22.6 and 29.6. Failure to satisfy this quality control criterion 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.