Primerdesign

R01292

Norovirus GI/GII detection in Oysters

Kit version: 2

Target region: ORF1 gene

genesig® Easy_oys Norovirus

50 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Norovirus detection in Oysters

Norovirus is a small non-enveloped RNA virus. Transmission occurs predominantly through ingestion of contaminated water or food e.g. shellfish, but can also occur via contact with contaminated surfaces. The ease with which norovirus is transmitted and the low infectious dose required to establish an infection result in extensive outbreaks in numerous environments. Norovirus primarily affects the gastrointestinal tract, leading to acute gastroenteritis. Common symptoms include nausea, vomiting, diarrhoea, fever, muscle aches and headache. There is no antiviral drug available to treat this infection and little is known about its pathogenicity.

Oysters are a filter feeding bivalve shellfish species, highly susceptible to norovirus contamination through exposure to human sewage discharges in their habitat. Norovirus is known to accumulate in the digestive glands of oysters, with higher concentrations observed during the winter compared to summer. This is recognised as a major human health risk associated with consumption of contaminated oysters. Despite the absence of legislation at present, there is growing expectation for oyster producers to conduct viral testing using ISO 15216 for guidance. A reliable quantification method is required to estimate the norovirus genome copies per gram and the subsequent infection level to be categorized into risk groups (low-medium, medium, high, or very high). The genesig® Easy_oys Norovirus workflow developed according to ISO 15216 offers a quantitative solution for the detection of Norovirus GI and GII to control and risk manage potential outbreaks and spread.

Intended Use

The genesig® Easy_oys Norovirus kit is an RT-qPCR assay intended for quantitative detection of nucleic acid from Norovirus GI and Norovirus GII in oyster digestive tissue. This handbook serves as a comprehensive guide, providing instructions for the entire workflow, including sample processing, RNA extraction and OneStep qPCR detection. To ensure the kit's sensitive performance, reaching a limit of detection of 500 copies/g of digestive tissue, it is imperative to strictly adhere to the outlined instructions.

Specificity

This genesig® Easy kit is designed for the in vitro quantification of Norovirus GI and GII genomes using the ISO 15216 standard. Specifically, the Norovirus GI and GII assays will detect over 85% and 95% respectively of sequences available on the NCBI database at the time of last review. When reviewing this kit, sequences from the previous three-years were analysed. Due to the sequence evolution observed in the Norovirus genome over time, this is considered to be an adequate time period for analysis.

The Norovirus GI assay is predicted to cross react with Norovirus GII sequences.

The Norovirus GII assay is predicted to cross react with Noroviruses GI, GIV, GVI, GVIII and GIX.

The dynamics of genetic variation means that new sequence information may become available after the initial design. If you require further information or have a specific question about the detection profile of this kit then please send an e-mail to techsupport@primerdesign.co.uk and our team will answer your question.

Kit contents

Quantity	Component	Tube	Cap Colour
1	**genesig® Easy_oys Norovirus G1 primer/probe mix (50 reactions)** FAM labelled for Norovirus G1; VIC labelled for the Internal Control		**BROWN**
1	**genesig® Easy_oys Norovirus G2 primer/probe mix (50 reactions)** FAM labelled for Norovirus G2; VIC labelled for the Internal Control		**YELLOW**
1	**genesig® Easy_oys Norovirus G1 positive control template**		**RED**
1	**genesig® Easy_oys Norovirus G2 positive control template**		**RED**
1	**Internal extraction control RNA**		**BLUE**
2	**Lyophilised oasig® OneStep MAX 2X RT-qPCR Master Mix (50 reactions per glass vial)**		**GREEN**
2	**oasig®MAX resuspension buffer** For resuspension of the lyophilised Master Mix		**ORANGE**
1	**RNase/DNase free water** for resuspension of primer/probe mixes		**WHITE**
3	**Template preparation buffer** for resuspension of internal control template and positive control template		**YELLOW**
2	**54 genesig® q16 reaction tubes**		**N/A**

Reagents and equipment to be supplied by the user

genesig® q16 (Z-genesig-q16)

exsig®Mag Nucleic Acid Extraction kit (96rxn) (R30096)

Sample Prep Solution (Z-genesigEASY- Sample prep buff)

Lyophilised Proteinase K and resuspension buffer (Z-genesigEASY-Proteinase K and Z-genesigEASY-ProK buffer)

Magnetic rack (Z-genesigEASY-MR)

Pipettes and tips

Vortex and micro centrifuge

Scalpel and shucking knife

Balance

Heat block (with optional shaking function)

1.5 ml microtube

Kit storage and stability

This kit is stable for shipping at ambient temperature but should be stored at -20°C upon arrival. Once the lyophilised components have been resuspended, they should not be exposed to temperatures above -20°C 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.

Primer Design Ltd does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

This kit was specifically optimised for oyster digestive tissue. Please ensure that the extracted nucleic acid samples are suitable in terms of purity, concentration, and RNA integrity. An internal extraction control is provided to test for non-specific PCR inhibitors.

Dynamic range of test

Under optimal PCR conditions the kit can achieve priming efficiencies between 90-110% and detect less than 100 copies of target template in the PCR reaction.

Principles of the test

Real-time PCR

A target specific primer and probe mix is provided, and this can be detected through the FAM channel.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the target cDNA. A fluorogenic probe is 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.

Positive control

The kit contains a positive control template used for copy number determination. 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 the target gene worked properly in that 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. 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 validate any positive findings 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. It is also known as a No Template Control or NTC.

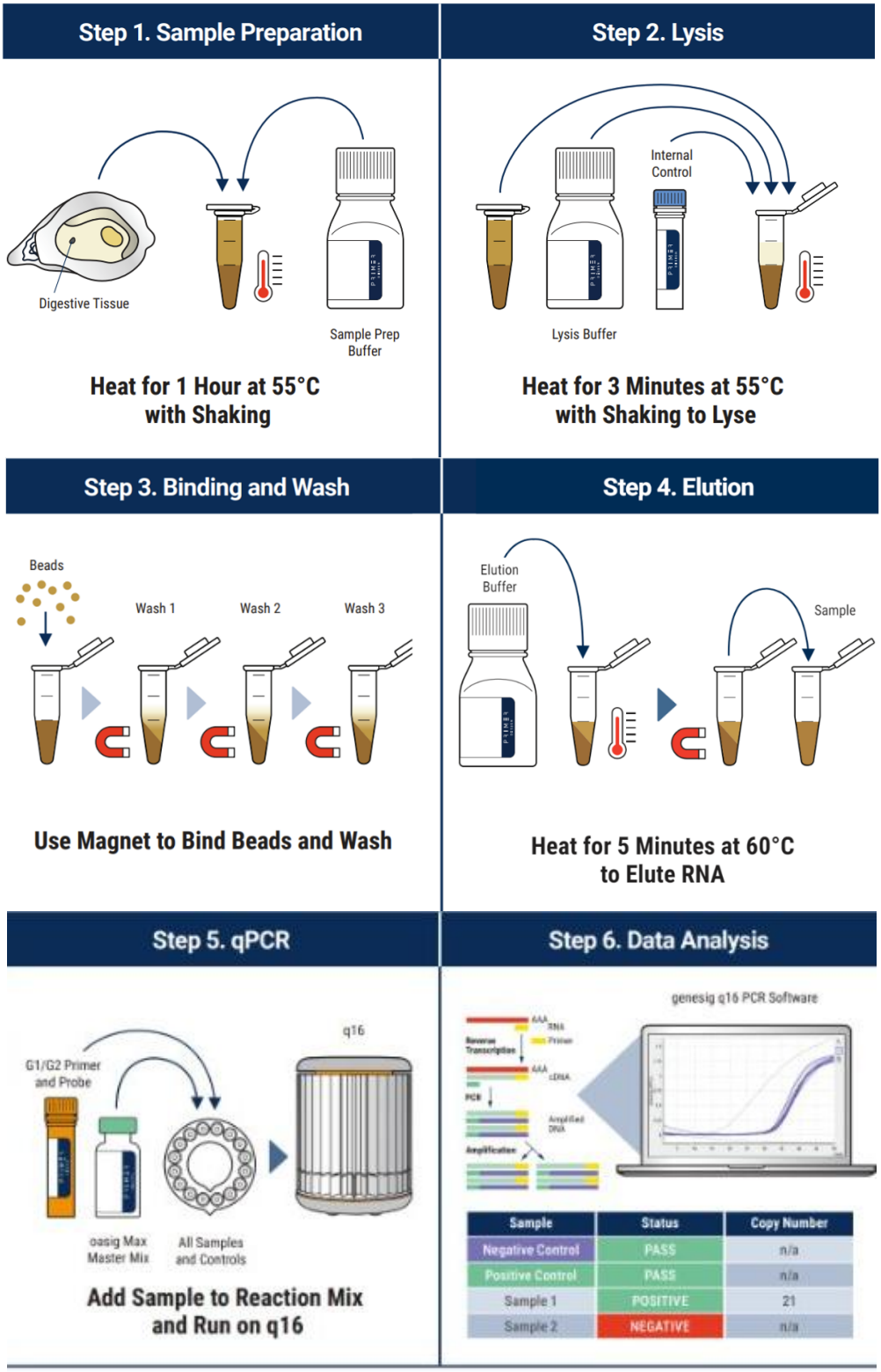
Internal RNA extraction control

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.

Amplification of the control RNA does not interfere with detection of the target RNA even when present at low copy number. The Internal control is detected through the VIC channel and gives a Cq value ≤ 31 depending on the level of sample dilution.

Oyster Extraction Workflow

A summary of the Oyster Extraction Workflow can be found in the table below:



Resuspension protocol

To minimize the risk of contamination with foreign RNA/DNA, we recommend that all pipetting is 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.

Please note: Each tube should be pulse-spun in a centrifuge before opening, if possible. This will ensure lyophilised primer/probe mix or template is in the base of the tube and is not lost upon opening the tube.

Proteinase K

1. **Resuspend the No.1 Proteinase K using the No.1a Proteinase K buffer, according to the table below:**

Component - resuspend in No.1a Proteinase K buffer	Volume
No.1 Proteinase K	500 µl

2. **It is recommended to dispense proteinase K into 100 µl single use aliquots, which should be stored at -20°C.**
   Vortex before dispensing to ensure complete resuspension.

Internal RNA Control

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

Component - resuspend in template preparation buffer	Volume
Internal extraction control RNA (**BLUE**)	1000 µl

2. **It is recommended to dispense the internal control into 25 µl single use aliquots, which should be stored at -20°C.**
   Vortex before dispensing to ensure complete resuspension.

Positive Control

1. **Resuspend each positive control template 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
genesig®Easy_oys Norovirus G1 Positive Control Template (**RED**) *	800 µl
genesig®Easy_oys Norovirus G2 Positive Control Template (**RED**) *	800 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

2. **It is recommended to dispense the positive control into 100 µl aliquots, which should be stored at -20°C.**
   Vortex before dispensing to ensure complete resuspension. Do not exceed five freeze-thaw cycles.

Reaction Mix

It is recommended to resuspend the full kit and to prepare single use aliquots in PCR tubes for subsequent use. The reaction mix should be stored at -20°C. The procedure below makes 50 reactions.

1. **Resuspend the primer/probe mixes in the RNase/DNase free water supplied, according to the table below:**
   To ensure complete resuspension, allow the primer/probe mix to rehydrate for 10 minutes at room temperature. Vortex each tube thoroughly. Failure to mix well can produce poor kit performance.

Component - resuspend in water	Volume
genesig® Easy_oys Norovirus G1 primer/probe mix (**BROWN**)	110 µl
genesig® Easy_oys Norovirus G2 primer/probe mix (**YELLOW**)	110 µl

2. **Resuspend the lyophilised oasig® OneStep MAX 2X RT-qPCR Master Mix in oasig® MAX resuspension buffer, according to the table below:**
   Swirl gently to mix and incubate at room temperature for two minutes or until the solution is clear.

Component - resuspend in oasig® MAX resuspension buffer	Volume
Lyophilised oasig® OneStep MAX 2X RT-qPCR Master Mix (**GREEN**)	525 µl

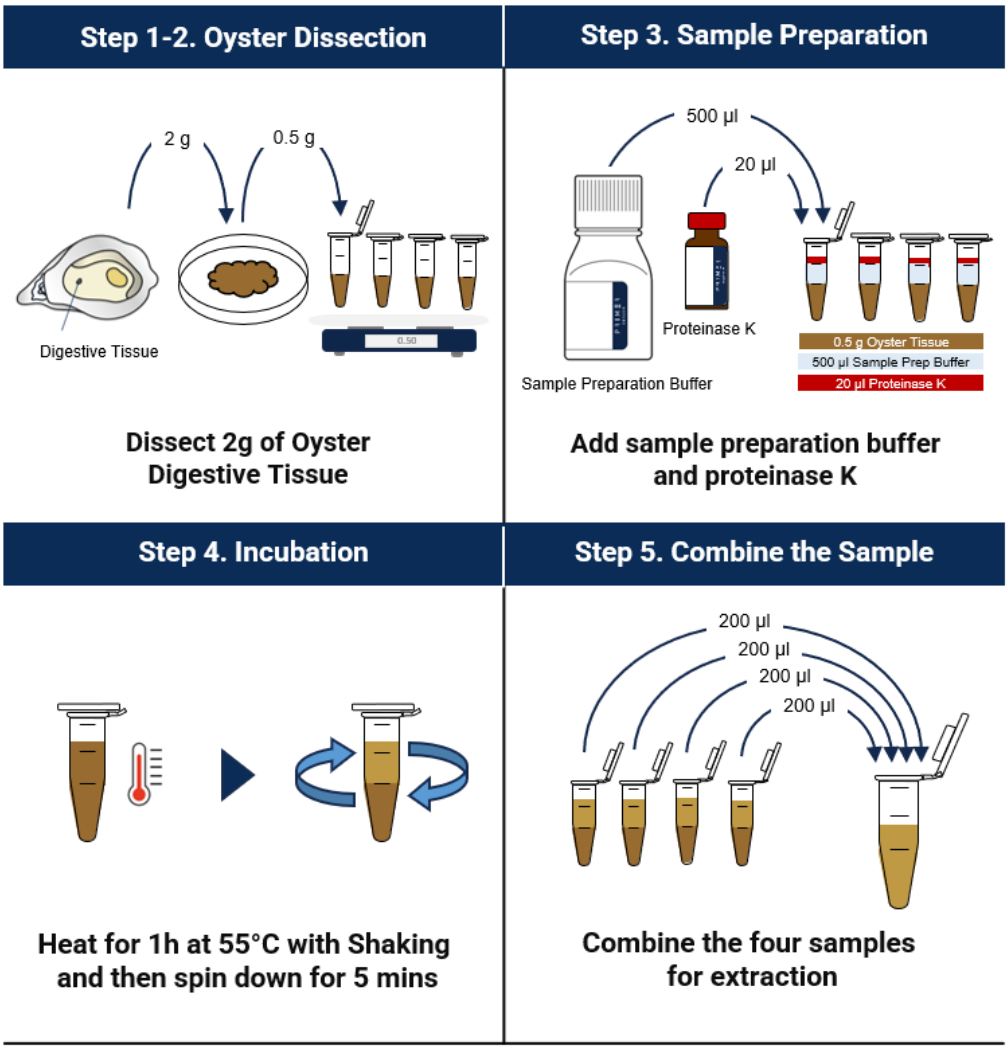
3. **Combine the primer/probe mix and oasig® OneStep MAX 2X RT-qPCR Master Mix to prepare a reaction mix according to the table below:**
   Reaction mix should be prepared separately for Norovirus G1 and Norovirus G2. The table includes volumes per PCR tube. Use the total volumes to prepare the reaction mix.

Component	Volume (PCR well)	Total Volume
oasig® OneStep MAX 2X RT-qPCR Master Mix (**GREEN**)	10 µl	500 µl
Norovirus G1 (**BROWN**) or Norovirus G2 (**YELLOW**) primer/probe mix	2 µl	100 µl
Final Volume	**12 µl**	**600 µl**

4. **Pipette 12 µl into each PCR tube to use directly or store at -20°C before use.**
   Vortex before dispensing to ensure complete resuspension.

Sample Processing and RNA Extraction Protocol

Sample Preparation



1. **Collect and shuck a minimum of ten oysters.**

2. **Dissect the oyster digestive tissue and use a blade to homogenise the tissue.**
   Avoid dissecting white tissue, if possible, as this will introduce more PCR inhibitors.

3. **Collect a total of 2 g of digestive oyster tissue and combine with sample preparation solution and proteinase K according to the table below:**
   A total of 2 g of tissue should be divided into four 1.5 ml tubes for extraction. Vortex thoroughly to ensure complete resuspension. If required, flick the tube to remove tissue from the bottom of the tube. Refer to resuspension protocol for preparation of proteinase K.

Component	Volume
Oyster digestive tissue	0.5 g
Sample Prep Solution	500 µl
No.1 Proteinase K	20 µl

4. **Incubate each tube for one hour at 55°C using a heat block with periodic shaking.**
   If a heat block with optional shaking is not available, it is recommended to vortex every ten minutes.

5. **Centrifuge each tube for five minutes. Once settled, transfer and combine 200 µl from each tube into a new 1.5 ml tube.**
   Avoid pipetting the top fatty layer and any of the tissue settled at the bottom of the tube. Tilt the tube to make it easier to pipette the solution. The final volume should be 800 µl. A volume of 200 µl from this sample be used for extraction.

Lysis

1. **Combine the sample with the exsig® Mag lysis buffer and the internal control in a new 1.5 ml tube, according to the table below:**
   Refer to the resuspension protocol for preparation of the internal control. The internal control must be added last. Two extractions are recommended for each sample.

Component	Volume
Oyster Sample	200 µl
exsig® Mag lysis buffer	200 µl
Internal extraction control RNA (**BLUE**)	10 µl

2. **Incubate for three minutes at 55°C using a heat block with constant shaking.**
   Vortex before incubation to ensure complete resuspension. If a heat block with optional shaking is not available, it is recommended to vortex every minute.

3. **Allow the sample(s) to cool to room temperature for approximately two minutes.**

Binding and Wash

1. **Add 320 µl of exsig® Mag Binding Buffer and 40 µl of the exsig® Mag Particle Suspension to each sample.**
   Ensure that the beads in the exsig® Mag Particle Suspension are homogenous before pipetting.

2. **Vortex and incubate at room temperature for five minutes with constant shaking.**

3. **Pulse centrifuge and place onto the magnetic rack for two minutes.**

4. **Remove and discard the supernatant.**
   Avoid removing any of the magnetic beads. If required, leave some of the supernatant.

5. **Add 400 µl of exsig® Mag wash buffer 1.**

6. **Remove from magnetic rack and vortex to mix.**

7. **Incubate for one minute at room temperature with constant shaking.**

8. **Pulse centrifuge and place onto the magnetic rack for two minutes.**
   After centrifuging, check that the magnetic beads are not attached to the tube lid.

9. **Remove and discard the supernatant.**

10. **Repeat steps 5-9 for exsig® Mag wash buffer 2.**

11. **Repeat steps 5-9 for exsig® Mag wash buffer 3.**
    All of the supernatant must be removed to avoid dilution of the sample and to minimise inhibition.

Elution

1. **Add 50 µl of exsig® Mag Elution Buffer.**

2. **Incubate for five minutes at 60°C using a heat block with constant shaking.**
   Vortex before incubation to ensure complete resuspension.

3. **Pulse centrifuge and place onto the magnetic rack for three minutes.**

4. **Transfer the eluate to a new 1.5 ml tube.**
   Avoid transferring any of the magnetic beads. This eluate will be used as your sample for PCR.

RT-qPCR detection Protocol

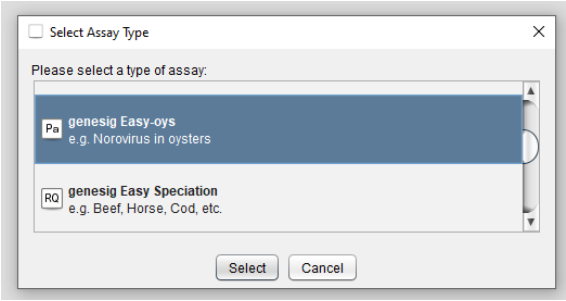
1. **Prepare the reaction mix according to the resuspension protocol or defrost reaction mix PCR tubes.**
   Each tube should contain 12 µl of reaction mix for Norovirus G1 or Norovirus G2. The number of tubes required should include one positive control and one negative control for each assay. A minimum of two replicates per sample are required for each assay.

2. **Pipette 8 µl of RNase/DNase free water (WHITE) into the NTC wells.**

3. **Pipette 8 µl of sample into each well, according to your experimental plate set up.**
   Pipette tips should always be changed between samples.

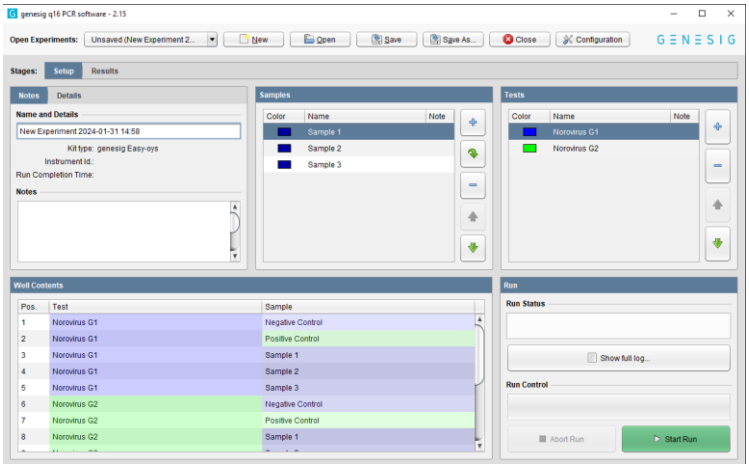
4. **Pipette 8 µl of the positive control (RED) into the corresponding PCT wells.**
   Refer to the resuspension protocol for the preparation of the Norovirus G1_v3.0 Positive Control Template or the Norovirus G2_v2.0 Positive Control Template.
   The positive control MUST be added last due to the high contamination risk.

5. **Open the genesig® q16 software and select the genesig® Easy_oys module.**



6. **Centrifuge the PCR tubes and load into the q16 instrument.**
   Ensure all the solution is at the bottom of the PCR tube. If required, use empty tubes to balance.

7. **Add a file name and label the samples.**
   If testing for both Norovirus G1 and G2, add and label a “test” for each assay.



8. **Start the run.**

Interpretation of results

Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
+	+ / -	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
-	+	+	-	**NO DETECTION OF TARGET**
+ / -	+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
-	-	+	-	**SAMPLE PREPARATION FAILED**
+ / -	+ / -	-	+ / -	**EXPERIMENT FAILED**

For positive results, the number of copies in the PCR well will be reported by the software.

A genesig®Easy_oys Norovirus calculator is available upon request. Please contact technical support techsupport@primerdesign.co.uk.

Positive Control

The positive control template is expected to amplify between Cq 17 – 19 in the FAM channel. If the positive control is outside this Cq range, do not report the copy number, as the sample is only valid as a qualitative result. The positive control criteria must pass to be used for quantification.

Internal PCR Control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency and the presence of PCR inhibitors. Cq values of ≤ 31 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 result.

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