BASELINE

SELECTION AND IMPROVING OF FIT-FOR-PURPOSE SAMPLING PROCEDURES FOR SPECIFIC FOODS AND RISKS

PROJECT DELIVERABLE

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Project co-ordinator

Prof. Gerardo Manfreda
Alma Mater Studiorum – Università di Bologna

Tel: +39 051 20 9 785
E-mail: gerardo.manfreda@unibo.it

Project website address www.baselineeurope.eu

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SEVENTH FRAMEWORK PROGRAMME

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SUMMARY

In this deliverable an analytical methodology based in the real-time PCR technology for the specific detection of human norovirus and/or hepatitis A in fresh produce is described. The deliverable is divided in four specific procedures: one for the nucleic acid extraction and purification from the food matrix, in this case fresh produce (i.e. lettuce), and three different procedures for the specific detection by real-time PCR assays of human noroviruses (both genogroup I and II) and hepatitis A as well as murine norovirus as virus surrogate acting as sample process control.

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Procedure 1:

Sampling and virus concentration from vegetables

The aim of this procedure is to extract and concentrate virus from 25 g of vegetables into a final volume of 500 µl of PBS buffer. This protocol is based on the protocol described in Dubois et al., 2006[1]. See Annex 1 for details of the equipments and reagents needed.

[1] Dubois E, Hennechart C, Deboosère N, Merle G, Legeay O, Burger C, Le Calvé M, Lombard B, Ferré V, Traoré O (2006) Intra-laboratory validation of a concentration method adapted for the enumeration of infectious F-specific RNA coliphage, enterovirus, and hepatitis A virus from inoculated leaves of salad vegetables. Int J Food Microbiol. 108 (2): 164-171

1. Labelling and transport of samples

All samples taken are to be labelled accordingly, including, at least the following details: Analyst, date of sampling, location and reference number to ensure traceability. All samples must be placed under refrigeration where possible (e.g. using a cool box) and taken to the laboratory as soon as possible for the next analytical steps.

2. Sampling and virus concentration from salad vegetables

A representative sample of the salad vegetable is collected into a sterile plastic bag or other appropriate container and transported to the laboratory. The sample was processed using the method of Dubois et al. (2006). Approximately 25 g sample is placed in a sterile beaker. 10 µl of the sample process control virus was pipetted onto the sample. 40 ml of Tris Glycine pH 9.5 buffer containing 1% Beef Extract (TGBE) was added to the sample. The sample was then agitated at room temperature for 20 min by rocking at 60 rpm. The liquid was decanted from the beaker through a strainer (e.g. a tea strainer) into one 50ml or two smaller centrifuge tubes, and centrifuged at 10,000 × g for 30 min at 4ºC. The supernatant(s) was decanted into a single clean tube or bottle, and the pH adjusted to 7.2. 0.25 volumes of 50% (w/v) polyethylene glycol (PEG) 8000/ 1.5 M NaCl were then added, and mixed by inversion. The suspension was then incubated with gentle rocking at 4ºC for 60 min, before centrifugation at 10,000 × g for 30 min at 4ºC. The supernatant was discarded, and the pellet compacted by centrifugation at 10,000 × g for 5 min at 4ºC before resuspension in 500 µl PBS. The suspension was then transferred to a chloroform-

resistant tube, and 500 µl chloroform:butanol (1:1) added and mixed by vortexing. The sample was allowed to stand for 5 min, and then centrifuged at 10,000 × g for 15 min at 4ºC. The aqueous phase was transferred to a clean tube and immediately used for nucleic acid extraction or stored at –20ºC. Nucleic acids were extracted using a NucliSENS® miniMAG® kit (bioMérieux) according to the manufacturer's instructions. The final elutions were performed with 150 µl elution buffer, resulting in a 300 µl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at -70ºC.

In detail:

1. Weigh 25 g of vegetable pieces and transfer to a sterile beaker.
2. Add 10µl of the sample process control virus and 40 ml of TGBE buffer to the sample.
3. Agitate at room temperature for 20 min by rocking at 60 rpm.
4. Decant the eluate from the beaker through a strainer (e.g. a tea strainer) into one 50ml or two smaller centrifuge tubes.
5. Centrifuge at 10,000 × *g* for 30 min at 4ºC.
6. Decant supernatant(s) into a single clean tube/bottle.
7. Adjust pH of the sample to 7.2 with Hydrochloric acid (1N and 0.1N).

   ATTENTION!!!!: The pH electrode should be decontaminated very carefully in freshly prepared hypochlorite (at least 1%).

8. Add 0.25 volumes of 5 × PEG/NaCl solution, mix by inversion, then incubate with gentle rocking at 4ºC for 60 min.
9. Transfer the tube(s) the centrifuge (split volume across two clean centrifuge tubes if necessary), then centrifuge at 10,000 × *g* for 30 min at 4ºC.
10. Discard the supernatant, then centrifuge at 10,000 × g for 5 min at 4ºC to compact pellet.
11. Resuspend the pellet in 500µl PBS.

    NOTE: If two tubes per sample are used, pellet from first tube in 500µl PBS should be resuspended, then transferred to the second tube where the second pellet should be resuspended in the same volume.

12. Transfer the suspension to a chloroform resistant tube, add 500 µl chloroform:butanol solution (1:1) and mix by vortexing. Allow to stand for 5 min.
13. Centrifuge at 10,000 × *g* for 15 min at 4ºC.
14. Transfer aqueous phase to a clean tube.
15. Store at -20ºC.

Procedure 2:

Detection and quantification of Human noroviruses

The aim of this procedure is to detect and quantify Norovirus (ggI and ggII) present in food and environmental samples using quantitative real-time reverse transcriptase (QRT-)PCR. This protocol is based in the information provided by da Silva et al. [1],Svraka et al.[2], Loisy et al.[3] and Kageyama et al.[4]. See Annex 2 for details of the equipments and reagents needed. Standard curves used in QPCR are generated by using serial dilutions of known amounts of RNA IACs. Different controls are needed to guarantee the quality of the assay: a NTC (non template control) and an Internal Amplification Control –IACs- (see Annex 2 1). Quantitation will be performed using the most probable number approach. The nucleic acid extract was assayed neat, and in 10^{-1} dilution, and two replicate assays will be performed for each concentration. If both 10^{-1} replicates produced a positive signal, subsequent dilutions will be assayed until both replicates of a dilution will be negative.

Norovirus ggI reverse transcription real-time PCR

This assay is a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Svraka et al.[1], with the inclusion of an internal amplification control (IAC). The IAC and its probe are constructed as described in Diez-Valcarce et al. (in press). The reaction contains 1 × RNA Ultrasense reaction mix (Applied), 0.5 µm primer QNIF4, 0.9 µm primer NV1LCR , 0.25 µM probe NVGG1p (labelled with FAM), 50 nm IAC probe (labelled with VIC), 1 × ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and 300 copies of norovirus ggI IAC. Ten µl sample of nucleic acid extract is added, to make a final reaction volume of 20 µl. The thermocycling conditions are 15 min at 50°C, 2 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

Norovirus ggII reverse transcription real-time PCR

This assay is a one-step duplex reverse transcription real-time PCR using the primers and conditions described by da Silva et al. (2007), with the inclusion of an internal amplification control (IAC). The IAC and its probe are constructed as described in Diez-Valcarce et al. (in press). The reaction contains 1 × RNA Ultrasense reaction mix (Applied), 0.5 µm primer

QNIF2, 0.9 µm primer COG2R, 0.25 µM probe QNIFS (labelled with FAM), 50 nm IAC probe (labelled with VIC), 1 × ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and 300 copies of norovirus ggII IAC. Ten µl sample of nucleic acid extract is added, to make a final reaction volume of 20 µl. The thermocycling conditions are 15 min at 50°C, 2 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

[1] da Silva AK, Le Saux JC, Parnaudeau S, Pommepuy M, Elimelech M, Le Guyader FS. 2007. Evaluation of removal of noroviruses during wastewater treatment, using Real-Time Reverse Transcription-PCR: different behaviors of genogroups I and II. Appl Environ Microbiol. 73(24):7891-7.

[2] Svraka S, Duizer E, Vennema H, de Bruin E, van der Veer B, Dorresteijn B, Koopmans M. 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. J Clin Microbiol. 45(5):1389-94.

[3] Loisy F, Atmar RL, Guillon P, Le Cann P, Pommepuy M, Le Guyader FS. 2005. Real-time RT-PCR for norovirus screening in shellfish. J Virol Methods. 123(1):1-7.

[4] Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K. 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J Clin Microbiol. 41(4):1548-57

In detail:

1. Prepare the mix including 0.10× more reaction for inaccuracies during pipetting. Prepare mix in a clean separate area following the tables 1, 2 and 3.

Table 1. Working solutions of primers and probe.
(A starting stock solution of 100 µM for both primers and probes is assumed)

	Stock	H_2O	Final *	Molarity
Primer QNIF4	50 µl	450 µl	500 µl	10 µM
Primer NV1LCR	90 µl	410 µl	500 µl	18 µM
Primer QNIF2	50 µl	450 µl	500 µl	10 µM
Primer COG2R	90 µl	410 µl	500 µl	18 µM
Probe NVGG1p	25 µl	475 µl	500 µl	5 µM
Probe QNIFS	25 µl	475 µl	500 µl	5 µM
Probe IAC	5 µl	495 µl	500 µl	1 µM

* Distribute the final volume solution in 50 µl-aliquots

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Table 2. QRT-PCR mix (for one reaction)

Reagent	Working concentration	Final concentration	Volume (µl)
RNA Ultrasense reaction mix	5 ×	1 ×	4.00
Primer QNIF4	10 µM	500 nM	1.00
Primer NV1LCR	18 µM	900 nM	1.00
Probe NVGG1p	5 µM	250 nM	1.00
IAC probe	1 µM	50 nM	1.00
ROX reference dye	50 ×	1 ×	0.40
RNA Ultrasense enzyme mix			1.00
IAC		Around 300 copies*	0.60
Total volume of mix			10
Sample			10
Final volume			20

Table 3. QRT-PCR mix (for one reaction)

Reagent	Working concentration	Final concentration	Volume (µl)
RNA Ultrasense reaction mix	5 ×	1 ×	4.00
Primer QNIF2	10 µM	500 nM	1.00
Primer COG2R	18 µM	900 nM	1.00
Probe QNIFS	5 µM	250 nM	1.00
IAC probe	1 µM	50 nM	1.00
ROX reference dye	50 ×	1 ×	0.40
RNA Ultrasense enzyme mix			1.00
IAC		Around 300 copies*	0.60
Total volume of mix			10
Sample			10
Final volume			20

* see Appendix 2

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2. Once the mix has been prepared, aliquot 10 µl into each well including the NTC (see Appendix 1). The total volume for one reaction after addition of target will be 20 µl (10 µl mix + 10 µl sample or standard).
3. Add samples (10 µl of the ten-fold dilution of the original sample) in a separate area.
4. Add RNA standard as positive control in duplicate.
5. Add 10 µl of nuclease-free dd-water in the NTC wells
6. Close with adhesive cover and take care not to touch the cover since finger prints could interfere with the fluorescent signal register by the thermocycler.
7. Perform the QRT-PCR in an real-time PCR platform, selecting the appropriate parameters (considering the use of adhesive cover and the total volume in each well, etc):

Step description		Temperature and time	Number of cycles
Reverse transcription		50ºC for 15 min	1
Preheating		95ºC for 2 min	1
Amplification	Denaturation	95ºC for 15 s	45
	Annealing-extension	60 ºC for 1 min.	

8. Once the reaction is completed, store results and data as described in the user's manual of the equipment used.
9. The amount of RNA will be defined as the mean of the data obtained after correcting the dilution factor (10^{-1}).

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Procedure 3:

Detection and quantification of Hepatitis A

The aim of this procedure is to detect and quantify Hepatitis A virus present in environmental and food samples using quantitative real-time reverse transcriptase (QRT-) PCR. This protocol is based on the information provided by Costafreda et al[1]. Standard curves used in QPCR are generated by using serial dilutions of known amounts of RNA IACs. Different controls are needed to guarantee the quality of the assay: a NTC (non template control) and an Internal Amplification Control –IACs- (see Annex 3). Quantitation will be performed using the most probable number approach. The nucleic acid extract was assayed neat, and in 10^{-1} dilution, and two replicate assays will be performed for each concentration. If both 10^{-1} replicates produced a positive signal, subsequent dilutions will be assayed until both replicates of a dilution will be negative.

This assay is a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Costafreda et al. (2006), with the inclusion of an internal amplification control (IAC). The IAC and its probe are constructed as described in Diez-Valcarce et al. (in press). The reaction contained 1 × RNA Ultrasense reaction mix (Applied), 0.5 µm primer HAV68, 0.9 µm primer HAV240, 0.25 µM probe HAV150(-) (labelled with FAM), 50 nm IAC probe (labelled with VIC), 1 × ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and 300 copies of HAV IAC. Ten µl sample of nucleic acid extract are added, to make a final reaction volume of 20 µl. The thermocycling conditions are 15 min at 50°C, 2 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

[1] Costafreda, M.I., Bosch, A., Pintó, R.M. (2006). Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl Environ Microbiol. 72 (6): 3846-55

In detail:

1. Prepare the mix including 0.10× more reaction for inaccuracies during pipetting. Prepare mix in a clean separate area following the tables 4, 5 and 6.

Table 4. Working solutions of primers and probe.
(A starting stock solution of 100 µM for both primers and probes is assumed)

	Stock	H_2O	Final *	Molarity
Primer HAV68	50 µl	450 µl	500 µl	10 µM
Primer HAV240	90 µl	410 µl	500 µl	18 µM
Probe HAV150(-)	25 µl	475 µl	500 µl	5 µM
Probe IAC	5 µl	495 µl	500 µl	1 µM

* Distribute the final volume solution in 50 µl-aliquots

Table 5. QRT-PCR mix (for one reaction)

Reagent	Working concentration	Final concentration	Volume (µl)
RNA Ultrasense reaction mix	5 ×	1 ×	4.00
Primer HAV68	10 µM	500 nM	1.00
Primer HAV240	18 µM	900 nM	1.00
Probe HAV150(-)	5 µM	250 nM	1.00
IAC probe	1 µM	50 nM	1.00
ROX reference dye	50 ×	1 ×	0.40
RNA Ultrasense enzyme mix			1.00
IAC		Around 300 copies *	0.60
Total volume of mix			10
Sample			10
Final volume			20

2. Once the mix has been prepared, aliquot 10 µl into each well including the NTC (see Appendix 1). The total volume for one reaction after addition of target will be 20 µl (10 µl mix + 10 µl sample or standard).

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3. Add samples (10 µl of the ten-fold dilution of the original sample) in a separate area.
4. Add standard RNa or HAV RNA as positive control in duplicate.
5. Add 10 µl of nuclease-free dd-water in the NTC wells.
6. Close with adhesive cover and take care not to touch the cover since finger prints could interfere with the fluorescent signal register by the thermocycler.
7. Perform the QRT-PCR in an real-time PCR platform, selecting the appropriate parameters (considering the use of adhesive cover and the total volume in each well, etc):

   * see Appendix 3

   Table 6

Step description		Temperature and time	Number of cycles
Reverse transcription		50ºC for 15 min	1
Preheating		95ºC for 2 min	1
Amplification	Denaturation	95ºC for 15 s	45
	Annealing-extension	60ºC for 1 min	

8. Once the reaction is completed, store results and data as described in the user's manual of the equipment used.
9. The amount of RNA will be defined as the mean of the data obtained after correcting the dilution factor (10^{-1}).

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Procedure 4:

Detection and quantification of murine norovirus

The aim of this procedure is to detect and quantify murine norovirus used as analytical sample process control using quantitative real-time reverse transcriptase (QRT-)PCR. This protocol is based on the information provided by Baert et al[1]. Standard curves used in QPCR are generated by using serial dilutions of known amounts of RNA IACs. Different controls are needed to guarantee the quality of the assay: a NTC (non template control) and an Internal Amplification Control –IACs- (see Appendix 4). Quantitation will be performed using the most probable number approach. The nucleic acid extract was assayed neat, and in 10^{-1} dilution, and two replicate assays will be performed for each concentration. If both 10^{-1} replicates produced a positive signal, subsequent dilutions will be assayed until both replicates of a dilution will be negative.

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Baert et al. (2008)[1], with the inclusion of an internal amplification control (IAC). The IAC and its probe are constructed as described in Diez-Valcarce et al. (in press). The reaction contained 1 × RNA Ultrasense reaction mix (Applied), 0.2 µm each primer, 0.2 µM probe MGB-ORF1/ORF2 (labelled with FAM), 50 nm IAC probe (labelled with VIC), 1 × ROX reference dye (Invitrogen), 1 µl RNA Ultrasense enzyme mix (Invitrogen) and 600 copies of murine norovirus IAC. Ten µl sample of nucleic acid extract was added, to make a final reaction volume of 20 µl. The thermocycling conditions were 15 min at 50°C, 2 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

[1] Baert, L., Wobus, C.E., Van Coillie, E., Thackray, L.B., Debevere,J., and Uyttendaele, M. (2008) Detection of Murine Norovirus 1 by Using Plaque Assay, Transfection Assay, and Real-Time Reverse Transcription-PCR before and after Heat Exposure. Appl. Environm. Microbiol. 74: 543-546

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In detail:

1. Prepare the mix including 0.10× more reaction for inaccuracies during pipetting. Prepare mix in a clean separate area following the tables 7, 8 and 9.

Table 7. Working solutions of primers and probe.
(A starting stock solution of 100 µM for both primers and probes is assumed)

	Stock	H_2O	Final *	Molarity
Primer Fw-ORF1/ORF2	20 µl	480 µl	500 µl	4 µM
Primer Rv-ORF1/ORF2	20 µl	480 µl	500 µl	4 µM
Probe MGB-ORF1/ORF2	20 µl	480 µl	500 µl	4 µM
Probe IAC	5 µl	495 µl	500 µl	1 µM

* Distribute the final volume solution in 50 µl-aliquots

Table 8. QRT-PCR mix (for one reaction)

Reagent	Working concentration	Final concentration	Volume (µl)
RNA Ultrasense reaction mix	5 ×	1 ×	4.00
Primer Fw-ORF1/ORF2	4 µM	200 nM	1.00
Primer Rv-ORF1/ORF2	4 µM	200 nM	1.00
Probe MGB-ORF1/ORF2	4 µM	200 nM	1.00
IAC probe	1 µM	50 nM	1.00
ROX reference dye	50 ×	1 ×	0.40
RNA Ultrasense enzyme mix			1.00
IAC		Approx 300 copies*	0.60
Total volume of mix			10
Sample			10
Final volume			20

2. Once the mix has been prepared aliquot 10 µl into each well including the NTC (see Appendix 1). The total volume for one reaction after addition of target will be 20 µl (10 µl mix + 10 µl sample or standard).

3. Add samples (10 µl of the ten-fold dilution of the original sample) in a separate area.
4. Add standard MnoV RNA in duplicate as positive control.
5. Add 10 µl of nuclease-free dd-water in the NTC wells.
6. Close with adhesive cover and take care not to touch the cover since finger prints could interfere with the fluorescent signal register by the thermocycler.
7. Perform the QRT-PCR in an real-time PCR platform, selecting the appropriate parameters (considering the use of adhesive cover and the total volume in each well, etc):

[*] See Appendix 4

Table 9

Step description		Temperature and time	Number of cycles
Reverse transcription		50ºC for 15 min	1
Preheating		95ºC for 2 min	1
Amplification	Denaturation	95ºC for 15 s	40
	Annealing-extension	60ºC for 1 minute	

8. Once the reaction is completed, store results and data as described in the user's manual of the equipment used.
9. The amount of RNA will be defined as the mean of the data obtained after correcting the dilution factor (10^{-1}).

ANNEXES

Annex 1:

EQUIPMENT AND REAGENTS FOR SAMPLING AND VIRUS CONCENTRATION FROM VEGETABLES

EQUIPMENT

- Sterile graduated disposable pipettes (10 ml; 5 ml and 1 ml).
- Micropipette tips of a range of sizes, 1000 µl, 200 µl and 20 µl
- Pipette filler.
- Vortex mixer.
- Thermo-shaker operating at 60ºC and 1400 rpm or equivalent.
- Rocking platform or equivalent for use at room temperature and 4ºC at 60 rpm.
- Aspirator or equivalent apparatus for removing supernatant.
- Heating block capable of operating at 99°C or equivalent.
- Refrigerated centrifuge(s) and rotor(s) capable of running at 10,000 × *g* with capacity for 20 ml tubes and for narrow gauge chloroform resistant tubes.
- Centrifuge and microcentrifuge tubes/bottles of a range of sizes, 1.5 ml, 15 ml, 50 ml. 1.5-ml tubes with screw caps are necessary. The exact selection of tubes required will depend on the centrifuges and rotors available in each laboratory.
- Bench centrifuge and rotor capable of running at 1,500 × *g* with capacity for 15 / 50 ml tubes.
- Centrifuge.
- pH Meter with temperature electrode or a thermometer.
- Refrigerator.
- Measuring cylinder (500 ml).
- Volumetric flasks (100 and 1000 ml)

REAGENTS

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

- Polyethylene Glycol (PEG)
- TGBE buffer

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- NaCl (SV)
- Phosphate Buffered Saline (PBS)
- Potassium chloride (if required for production of PBS buffer)
- Na_2HPO_4 (if required for production of PBS buffer)
- KH_2PO_4 (if required for production of PBS buffer)
- Tris base
- Glycine
- Beef Extract (3% w/v) in glycine buffer (0.05M)
- Beef extract powder
- Pectinase
- Chloroform
- Butanol
- Sodium hydroxide (4% w/v [= 1N])
- Hydrochloric acid (1N and 0.1N) (for adjusting pH)
- Double distilled water.

PREPARATION OF THE REAGENTS

Hydrochloric Acid (1N)

34.4 ml concentrated hydrochloric acid

400 ml deionised water

Measure 400 ml of deionised water in a measuring cylinder and then pour into a clean 500 ml glass bottle. Using a 10 ml disposable pipette add 34.4 ml of concentrated hydrochloric acid. Label with the batch number and the expiry date.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
Stock	Room temperature	1 year
In-use	Room temperature	1 year

Hydrochloric Acid (0.1N)

10 ml 1N hydrochloric acid

90 ml deionised water

In a 100 ml volumetric flask add approximately 50 ml of deionised water. With a 10 ml disposable pipette add 10 ml of 1N hydrochloric acid and bring to the volume with deionised water. Label with the batch number and the expiry date. For storage and expiry dates see above.

pH Buffers

pH buffers are supplied ready to use and a small volume should be aliquoted to a plastic universal when needed for use. After use, the aliquoted buffer and universal should be discarded.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
In-use	Room temperature	Day of use

Phosphate Buffered Saline (PBS)

10 Dulbecco's PBS tablets in 1 litre deionised water

or 2 Invitrogen or 2 Gibco PBS tablets in 1 litre deionised water

Using a magnetic stirrer, dissolve the PBS tablets in the deionised water. Once dissolved, aseptically dispense 10 ml volumes into sterile universals. If commercial PBS tablets are unavailable, please make in-house as set out below.

In-house preparation:

Add 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and dissolve in approximately 800 ml molecular grade water in a beaker. Mix by stirring until the solids are dissolved. Adjust the pH to 7.3 and bring to 1000 ml in a volumetric flask with molecular grade water.

Sterilise all solutions according to local procedures e.g. autoclave at 121°C for 15 minutes. Check the sterility, of each batch made by plating out 100 µl onto Nutrient Agar plates and record on the QC sheet. Label with the volume, batch number and the expiry date.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
In-house Stock	Room temperature	4 months
In-use	4°C (± 3°C)	4 months
In-media	4°C (± 3°C)	1 week

5× PEG/NaCl solution (50% (w/v) PEG 8000, 1.5M NaCl)

Add 500g PEG 8000, 87 g NaCl and 450 ml molecular grade water to a beaker. Mix with gentle shaking/stirring until the solids are dissolved and then adjust the volume to 1000 ml in a volumetric flask.

	Storage	Expiry
Stock	Room temperature	4 months
In-use	Room temperature	4 months

Chloroform:Butanol

Add together equal volumes of chloroform and butanol. Shake to mix.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
Stock	Room temperature	1 year
In-use	4°C (± 3°C)	4 months

Tris Glycine 1% Beef Extract (TGBE) Buffer

Add 12.1 g Tris base, 3.8 g glycine, 10 g beef extract powder and 800ml molecular grade water to a bottle. Mix with stirring until the solids are dissolved. Adjust the pH to 9.5 and bring to 1000 ml in a volumetric flask.

	Storage	Expiry
Supplier's Stock	Room temperature	Supplier's use-by date
Stock	Room temperature	4 months
In-use	2 – 8 ºC	4 months

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Annex 2:

EQUIPMENT AND REAGENTS FOR DETECTION OF HUMAN NOROVIRUSES

EQUIPMENT

- Real-time PCR platform
- Micropipettes of a range of sizes, 1000µl, 200µl and 20µl
- Gloves
- Vortex mixer
- Microcentrifuge
- Refrigerator
- Freezer

REAGENTS AND PLASTICWARE

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

- RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, Catalog Number - 11732-927).
- Tris-EDTA pH 8 (Ambion, Applied Biosystems).
- Nuclease-free water.
- 96-well optical reaction plates (Applied Biosystems. Cat. No. 4306737 for 20 units or 43426659 for 500 units) or similar form other companies.
- Optical adhesive covers (Applied Biosystems. Cat. No. 4311971 for 100 units or 4360954 for 25 units) or similar form other companies.
- Optical adhesive cover starter kit (Cat. No 4313663) or similar form other companies.
- Optical caps and its corresponding installing tool (Cat. No 4323032 and 4330015) or similar form other companies.
- Micropipette tips of a range of sizes, 1000 µl, 200 µl and 20 µl.

OLIGONUCLEOTIDES

NOROVIRUS GGI

- Forward primer: QNIF4 (5’- CGC TGG ATG CGN TTC CAT -3’)
- Reverse primer: NV1LCR (5’- CCT TAG ACG CCA TCA TCA TTT AC -3’)
- Norovirus GG I Probe: NVGG1p (5’-FAM- TGG ACA GGA GAY CGC RAT CT-BHQ1-3’)

NOROVIRUS GGII

- Forward primer: QNIF2 (5’- ATG TTC AGR TGG ATG AGR TTC TCW GA -3’)
- Reverse primer: COG2R (5’- TCG ACG CCA TCT TCA TTC ACA -3’)

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- Norovirus GG I Probe: QNIFS (5'- FAM- AGC ACG TGG GAG GGC GAT CG -BHQ1-3')
- IAC MGB TaqMan probe: IACP (5'-VIC- CCA TAC ACA TAG GTC AGG –MGB- NFQ- 3' at a final concentration of 0.100 µM.

* Black hole quencher or non-fluorescent quenchers are strongly recommended instead of TAMRA

Controls

Non-template control (NTC): The assay must include a NTC to prove mix does not produce fluorescence. This control comprises 10 µl of QRT-PCR mix and 10 µl of nuclease-free dd-water.

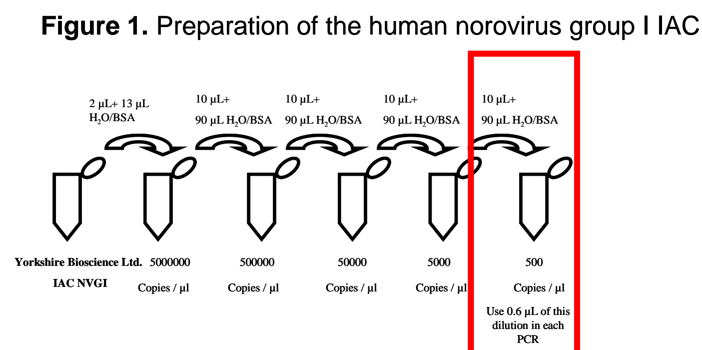
Internal amplification control (IAC): The IAC must be added to every well, to verify that the reaction has worked and has not failed, e.g. through inhibition or instrument failure or operator error.

Contamination: Standard precautions should be applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules. Decontaminate the micropipettes after each use. Use UV or cleaning products which can be obtained from your local micropipette suppliers.

PREPARATION OF THE HUMAN NOROVIRUS GROUP I IAC

The vial labelled **IAC-NVGI** contains **the human norovirus group I RNA IAC.** To include in each PCR the optimum number of IAC copies, dilutions of the original IAC should be made as follows (Each dilution was made in nucleases free water with 0.1 mg/mL of BSA):

Figure 1. Preparation of the human norovirus group I IAC



Therefore, and based on the quantification done by real-time PCR of the a series of ten-fold dilutions, approximately **300 copies** of the IAC should be added in each PCR, which has been demonstrated to be the amount of IAC that has less influence in the limit of detection of the human norovirus group I system using the commercial IAC that can be provided by Yorkshire. Results of the different assays made to chose the most suitable amount of IAC copies are shown in table **10** (all the experiments were performed by ITACyL).

Table 10.

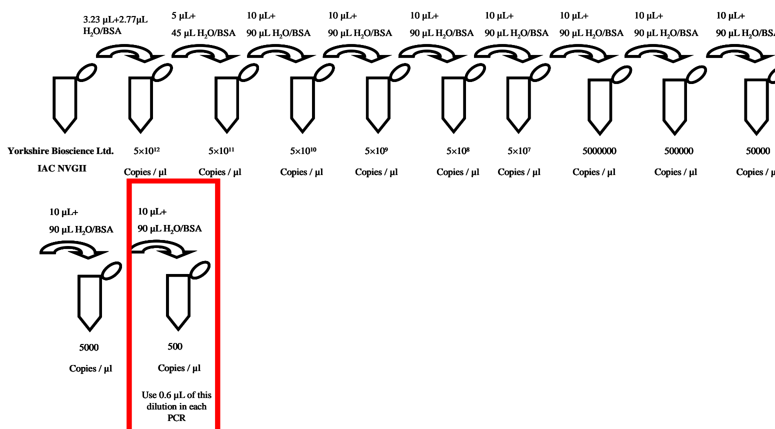
IAC																	
No IAC		3000 IAC				300 IAC				30 IAC				3 IAC			
hNVGIP	hNVGIP	hNVGIP	hNVGIP	IACP	IACP	hNVGIP	hNVGIP	IACP	IACP	hNVGIP	hNVGIP	IACP	IACP	hNVGIP	hNVGIP	IACP	IACP
26.65 0.68	5/5*	29.69 0.34	5/5	23.99 0.15	5/5	29.78 0.21	5/5	28.38 0.10	5/5	29.77 0.16	5/5	32.80 0.14	5/5	30.45 1.24	3/3	40.21 2.85	3/3
30.91 0.90	5/5	34.41 0.29	5/5	24.31 0.13	5/5	33.49 0.64	4/5	28.03 0.35	4/5	33.30 0.46	5/5	31.51 2.22	5/5	33.82 0.35	5/5	37.30 0.57	5/5
34.32 3.31	5/5	40.91 0.70	5/5	24.23 0.08	5/5	37.79 1.28	5/5	28.25 0.21	5/5	38.29 0.38	5/5	32.90 0.32	5/5	38.14 0.92	5/5	39.61 1.55	4/5

* positive reactions out of total reactions (i.e. five positive signals out of 5 replicates)

PREPARATION OF THE HUMAN NOROVIRUS GROUP II IAC

The vial labelled **IAC-NVGII** contains **the human norovirus group II RNA IAC.** To include in each PCR the optimum number of IAC copies, dilutions of the original IAC should be made as follows (Each dilution was made in nucleases free water with 0.1 mg/mL of BSA).

Figure 2. Preparation of the human norovirus group II IAC



Therefore, and based on the quantification done by real-time PCR of the a series of ten-fold dilutions, approximately **300 copies** of the IAC should be added in each PCR, which has been demonstrated to be the amount of IAC that has less influence in the limit of detection of the human norovirus group II system using the commercial IAC can be provided by Yorkshire Bioscience Ltd. Results of the different assays made to chose the most suitable amount of IAC copies are shown in table **11** (all the experiments were performed by ITACyL).

Table 11.

IAC																	
No IAC		3000 IAC				300 IAC				30 IAC				3 IAC			
hNVGIIP	hNVGIIP	hNVGIIP	hNVGIIP	IACP	IACP	hNVGIIP	hNVGIIP	IACP	IACP	hNVGIIP	hNVGIIP	IACP	IACP	hNVGIIP	hNVGIIP	IACP	IACP
21.15 0.41	5/5*	21.35 0.34	5/5	25.18 0.50	5/5	21.0 0.23	5/5	25.27 0.29	5/5	21.20 0.30	5/5	25.18 0.42	5/5	21.09 0.19	5/5	25.40 0.38	5/5
23.49 0.20	5/5	23.76 0.13	5/5	28.48 0.38 0.39	5/5	23.93 0.13	5/5	28.38 0.27	5/5	24.02 0.11	5/5	28.44 0.48	5/5	24.09 0.21	5/5	28.44 0.45	5/5
27.41 0.39	5/5	27.60 0.24	5/5	32.77 0.42	5/5	27.54 0.09	5/5	32.65 0.03	5/5	27.54 0.34	5/5	32.93 0.79	5/5	27.30 0.62	4/5	32.92 0.83	4/5

* positive reactions out of total reactions (five positive signals out of 5 replicates)

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Annex 3:

EQUIPMENT AND REAGENTS FOR DETECTION OF HEPATITIS A VIRUS

EQUIPMENT

- Real-time PCR platform
- Micropipettes of a range of sizes, 1000µl, 200µl and 20µl
- Gloves
- Vortex mixer
- Microcentrifuge
- Refrigerator
- Freezer

REAGENTS AND PLASTICWARE

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

- RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, Catalog Number - 11732-927).
- Tris-EDTA pH 8 (Ambion, Applied Biosystems).
- Nuclease-free water.
- 96-well optical reaction plates (Applied Biosystems. Cat. No. 4306737 for 20 units or 43426659 for 500 units) or similar form other companies.
- Optical adhesive covers (Applied Biosystems. Cat. No. 4311971 for 100 units or 4360954 for 25 units) or similar form other companies.
- Optical adhesive cover starter kit (Cat. No 4313663) or similar form other companies.
- Optical caps and its corresponding installing tool (Cat. No 4323032 and 4330015) or similar form other companies.
- Micropipette tips of a range of sizes, 1000µl, 200µl and 20µl.

OLIGONUCLEOTIDES

- Forward primer: HAV68 (5'- TCA CCG CCG TTT GCC -3')
- Reverse primer: HAV240 (5'- GGA GAG CCC TGG AAG AAA G -3')
- HAV Probe (Taqman MGB probe): HAV150(-) (5'-FAM- CCT GAA CCT GCA GGA ATT AA –MGB-NFQ-3')
- IAC MGB TaqMan probe: IACP (5'-VIC- CCA TAC ACA TAG GTC AGG –MGB- NFQ- 3' at a final concentration of 0.100 µM.

[*] Black hole quencher or non-fluorescent quenchers are strongly recommended instead of TAMRA

MGB: Minor groove binder

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Controls

Non-template control (NTC): The assay must include a NTC to prove mix does not produce fluorescence. This control comprises 10 µl of QRT-PCR mix and 10 µl of nuclease-free dd-water.

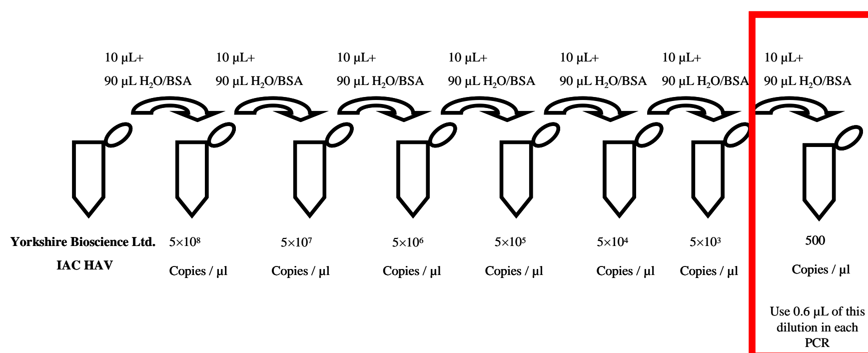
Internal amplification control (IAC): The IAC must be added to every well, to verify that the reaction has worked and has not failed, e.g. through inhibition or instrument failure or operator error.

Contamination: Standard precautions should be applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules. Decontaminate the micropipettes after each use. Use UV or cleaning products which can be obtained from your local micropipette suppliers.

PREPARATION OF THE HEPATITIS A VIRUS IAC

The vial labelled IAC-HAV contains the hepatitis A RNA IAC. To include in each PCR the optimum number of IAC copies, dilutions of the original IAC should be made as follows (Each dilution was made in nucleases free water with 0.1 mg/mL of BSA):

Figure 3. Preparation of the Hepatitis A Virus IAC



Therefore, and based on the quantification done by real-time PCR of the a series of ten-fold dilutions, approximately **300 copies** of the IAC should be added in each PCR, which has been demonstrated to be the amount of IAC that has less influence in the limit of detection of the hepatitis A virus system using the commercial IAC can be provided by Yorkshire Bioscience Ltd. Results of the different assays made to chose the most suitable amount of IAC copies are shown in table 12 (all the experiments were performed by ITACyL).

Table 12.

								IAC									
No IAC		3000 IAC				300 IAC				30 IAC				3 IAC			
HAVP	HAVP	HAVP	HAVP	IACP	IACP	HAVP	HAVP	IACP	IACP	HAVP	HAVP	IACP	IACP	HAVP	HAVP	IACP	IACP
19.20 0.43	5/5*	19.29 0.30	5/5	23.91 0.22	5/5	20.42 0.43	5/5	31.66 0.48	5/5	20.51 0.61	5/5	36.92	1/5	20.19 1.08	5/5		0/5
22.23 0.34	5/5	22.22 0.45	5/5	24.80 0.14	5/5	22.57 0.78	5/5	30.13 0.57	5/5	22.96 0.15	5/5	39.78 0.38	5/5	23.11 0.24	5/5		0/5
25.48 1.35	5/5	25.24 0.77	5/5	24.89 0.12	5/5	26.28 0.61	5/5	30.29 0.31	5/5	26.89 0.29	5/5	38.45 0.85	5/5	26.64 0.26	5/5		0/5

* positive reactions out of total reactions (five positive signals out of 5 replicates)

Annex 4:

EQUIPMENT AND REAGENTS FOR DETECTION OF MURINE NOROVIRUS

EQUIPMENT

- Real-time PCR platform
- Micropipettes of a range of sizes, 1000µl, 200µl and 20µl
- Gloves
- Vortex mixer
- Microcentrifuge
- Refrigerator
- Freezer

REAGENTS AND PLASTICWARE

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

- RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, Catalog Number - 11732-927).
- Tris-EDTA pH 8 (Ambion, Applied Biosystems).
- Nuclease-free water.
- 96-well optical reaction plates (Applied Biosystems. Cat. No. 4306737 for 20 units or 43426659 for 500 units) or similar form other companies.
- Optical adhesive covers (Applied Biosystems. Cat. No. 4311971 for 100 units or 4360954 for 25 units) or similar form other companies.
- Optical adhesive cover starter kit (Cat. No 4313663) or similar form other companies.
- Optical caps and its corresponding installing tool (Cat. No 4323032 and 4330015) or similar form other companies.
- Micropipette tips of a range of sizes, 1000µl, 200µl and 20µl.

OLIGONUCLEOTIDES

- Forward primer: Fw-ORF1/ORF2 (5'- CAC GCC ACC GAT CTG TTC TG-3')
- Reverse primer: Rv-ORF1/ORF2 (5'- GCG CTG CGC CAT CAC TC-3')
- mNoV Probe (Taqman MGB probe): MGB-ORF1/ORF2 (5'-FAM- CGC TTT GGA ACA ATG –MGB– NFQ -3')
- IAC MGB TaqMan probe: IACP (5'-VIC- CCA TAC ACA TAG GTC AGG –MGB-NFQ- 3' at a final concentration of 0.100 µM.

* Black hole quencher or non-fluorescent quenchers are strongly recommended instead of TAMRA

MGB: Minor groove binder

Controls

Non-template control (NTC): The assay must include a NTC to prove mix does not produce fluorescence. This control comprises 10 µl of QRT-PCR mix and 10 µl of nuclease-free dd-water.

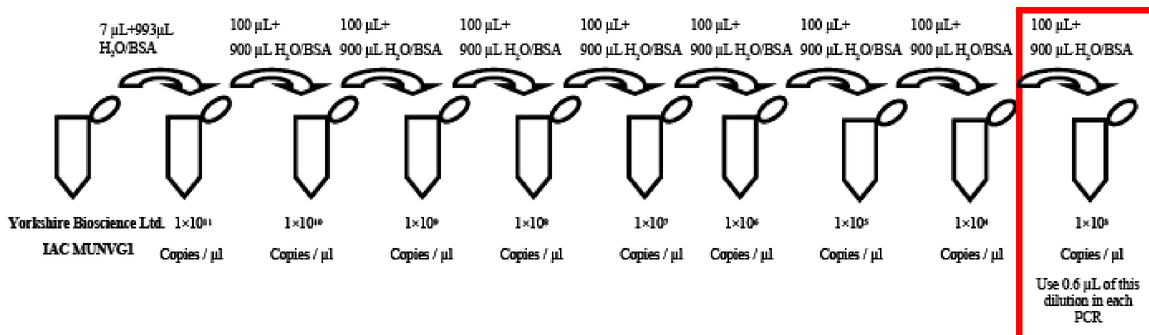
Internal amplification control (IAC): The IAC must be added to every well, to verify that the reaction has worked and has not failed, e.g. through inhibition or instrument failure or operator error.

Contamination: Standard precautions should be applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules. Decontaminate the micropipettes after each use. Use UV or cleaning products which can be obtained from your local micropipette suppliers.

PREPARATION OF THE MURINE NOROVIRUS IAC

The vial labeled IAC-MUNVG1 contains the murine norovirus RNA IAC. To include in each PCR the optimum number of IAC copies, dilutions of the original IAC should be made as follows (Each dilution was made in nucleases free water with 0.1 mg/mL of BSA):

Figure 4: Preparation of the murine norovirus IAC



Therefore, and based on the quantification done by spectrophotometry, fluorometry and ten-fold dilutions real-time PCR, approximately 600 copies of the IAC are added in each PCR, which has been demonstrated to be the amount of IAC that has less influence in the limit of detection of the murine norovirus system. Results of the different assays made to chose the most suitable amount of IAC copies are shown in table 13 (all the experiments were performed by ITACyL).

Table 13.

MNV		IAC																	
		No IAC		6000 IAC				600 IAC				60 IAC				6 IAC			
		MNVP	MNVP	MNVP	MNVP	IACP	IACP	MNVP	MNVP	IACP	IACP	MNVP	MNVP	IACP	IACP	MNVP	MNVP	IACP	IACP
100	C_T	32.20	5/5*	34.95	5/5	32.69	5/5	35.64	5/5	36.46	5/5	35.97	5/5	39.95	5/5	34.97	4/5	41.62	1/5
	SD	0.20		0.36		0.12		0.48		0.31		0.46		1.71		0.18			
10	C_T	35.21	5/5	38.99	4/5	32.84	5/5	38.74	5/5	36.07	5/5	39.41	5/5	38.75	2/5	38.42	5/5	40.67	1/5
	SD	0.16		0.91		0.10		0.56		0.44		1.08		0.49		0.83			
1	C_T	38.03	4/5	42.19	3/5	32.69	5/5	41.07	3/5	36.01	5/5	40.70	2/5	39.59	5/5	39.36	2/5	40.71	3/5
	SD	0.53		1.93		0.07		0.62		0.66		0.03		1.42		0.47		1.11	

* positive reactions out of total reactions (five positive signals out of 5 replicates)

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