Standard capsid integrity assay for shellfish, leafy greens and berry samples

Heat and RNase treatment

1. A risk assessment should be performed ensuring safe handling and decontamination procedures.

2. For shellfish extraction prepare a 1 M Tris-HCl buffer solution pH8.8 (1 litre)
    a. Dissolve 121.14 g Tris (Fisher #10785341) in 800 ml dH_2O.
    b. Adjust pH to 8.8 with the appropriate volume of concentrated HCl in a fume cupboard with stirring (wear gloves and safety glasses and note precautions for handling HCl). Adjust final volume to 1 litre with deionized water.
    c. Autoclave, aliquot and store at 4ºC.

3. For shellfish samples re-suspend 500 mg of defrosted chopped digestive tissues in 500 µl ice cold 1 M Tris pH 8.8.

4. Ensure the sample is adequately dispersed by vortexing for 30 seconds. Incubate the sample for 10 minutes on ice, followed by micro centrifuging at 13000 RPM for 5 minutes. Place the sample on ice until required.

5. For berries and leafy greens follow the sample preparation method 8.2.4 described in ISO/TS 15216 and use 4x100 µl of plant extract in step 7.

6. [Optional spiking. If a GI or GII stool isolate is spiked into the shellfish sample, prepare a 10% stool solution in ice cold Dulbecco's PBS pH 7.2 (without magnesium or calcium) (Sigma cat no. D8537) and add 12 µl of the 10% stool to the combined sample preparation prior to step].

7. Prepare 4 appropriately labelled thin-walled PCR tubes for each sample. Place tubes on ice, then add 100 µl of shellfish sample supernatant or 100 µl of plant extract to each of the 4 tubes per sample.

8. For each food sample, retain two subsamples on ice, and transfer two subsamples to a thermal cycler and subject to heat treatment at 80°C for 2 minutes using the max/standard ramp rates with cooling to 4°C at the end of the heating period. Transfer heat-treated subsamples to ice.

9. Prepare RNase ONE stock by mixing 100 µl of 10X reaction buffer with 10 µl (100 units) of RNase ONE, (this volume sufficient for 10 treatments i.e. 5 food samples), keep on ice.

10. Add 11 µl RNase ONE stock to the two heat-treated subsamples per food sample, mix by pipetting gently, then transfer again to a thermal cycler. Subject to a temperature of 37°C for 15 minutes using the max/standard ramp rates with cooling to 4°C at the end of the heating period, and then keep on ice.

11. Add 11 µl ice cold RNase 10X reaction buffer to both non heat-treated subsamples per food sample.

12. Add 29 µl ice cold 1 M Tris pH 8.8 to each tube to increase the total volume of the reaction mix to 140 µl. Proceed immediately to RNA extraction.

RNA Extraction

RNA extraction uses the QIAamp Viral RNA Mini Kit (250) Cat. No./ID: 52906.

1. Prepare four appropriately labelled 1.5 ml microfuge tubes for each food sample.

2. Add 560 µL of Buffer AVL with added carrier RNA (prepared following the manufacturer's instructions) to each tube.

3. Add the total 140 µL volume of each subsample reaction mix to its corresponding tube. Mix thoroughly by gentle inversion. Incubate the tubes at room temperature for 10 minutes.

4. Add 560 µL of 100% Ethanol to each tube and mix thoroughly by gentle inversion

5. Add 630 µL of each reaction-ethanol mix to a Mini column (in a 2ml collection tube) close the cap and centrifuge for 1 minute at 6000 x g.

6. Discard the filtrate. Add the remaining reaction-ethanol mix (630 µL) to each corresponding Mini column, replace cap and centrifuge again for 1 minute at 6000 x g.

7. Discard the filtrate and add 500 µL of Buffer AW1 (wash buffer 1) to each Mini column, replace cap and centrifuge again for 1 minute at 6000 x g.

8. Discard the filtrate and add 500 µL of Buffer AW2 (wash buffer 2) to each Mini column, replace cap and centrifuge again for 3 minutes at full speed.

9. Discard the filtrate and centrifuge again, for 1 minute at full speed to remove residual buffer from each column.

10. Transfer each Mini column into a new, appropriately-labelled 1.5ml microcentrifuge tube Discard the 2ml collection tubes and filtrate.

11. Carefully add 60 µL of Buffer AVE to the centre of each Mini column, incubate at room temperature for 1 minute then centrifuge for 1 minute at 6000 x g.

12. Discard the Mini columns and store the tubes with the purified RNA at 4°C for up to 8 hrs or -80°C for longer periods.

RT-qPCR and calculation of results

1. Using qRT-PCR conditions and reagents as described in ISO/TS 15216, assay 3 x 5 µL of each subsample RNA for each food sample for norovirus GI and GII alongside 3 x 5 µL water as negative control, and a dsDNA dilution series. For each food sample and each genogroup include all reactions for untreated and heat/RNase treated subsamples on the same qRT-PCR plate.

2. Record Cq values for each PCR reaction.

3. Calculate ΔCq for each shellfish sample and genogroup as follows:-

   ΔCq = average Cq (heat treated, RNase treated subsamples) – average Cq (untreated subsamples)

   NOTE: For any sample and genogroup where one or more PCR reactions from the untreated subsamples is negative, or gives a quantity of <1 copy/µL by reference to the standard curve generated from the dsDNA dilution series, the ΔCq shall be recorded as “not quantifiable” and shall not be used for subsequent calculations. For the heat/RNase treated subsamples, for any PCR reaction that is negative, or that gives a quantity of <1 copy/µL by reference to the standard curve, a censored value equivalent to 1 copy/µL shall be used to calculate the average Cq.

   For each sample carry out an unpaired one-tailed t-test to compare the 6 Cq values obtained for untreated samples with the 6 Cq values (including censored values) obtained for heat/RNase treated subsamples. If the obtained p value is >0.05 then the ΔCq is not significantly different from zero.

   Calculate upper and lower 95% confidence limits for the ΔCq using the method for computing a confidence interval on the difference between means http://onlinestatbook.com/2/estimation/difference_means.html and using the appropriate t-statistic for a one-tailed test.

   NOTE: where the calculated ΔCq is negative (average Cq [heat treated, RNase treated subsamples] < average Cq [untreated subsamples]), the ΔCq shall be set to a censored value of 0.00. In such cases the t-test is not applicable while upper and lower 95% confidence limits for the ΔCq shall be calculated by adding and subtracting the calculated error parameters to/from the censored value of 0.00.

4. Calculate percentage of exposed virus RT-qPCR target in each sample using the following formula:-
   Percentage exposed virus RT-qPCR target = $(1 - 10^{(\Delta Cq/m)}) \times 100\%$

where m is the slope of a standard curve obtained by plotting log10 copies/µL vs. Cq value for the dsDNA dilution series.

Calculate upper and lower confidence limits for the percentage of exposed RT-qPCR target in each sample by repeating the calculation using the upper and lower confidence limits for ΔCq determined at stage 3.

NOTE: where the lower limit for the ΔCq is negative, the calculated percentage using the above formula will also be negative; in these cases percentages for the lower limit shall be recorded as 0%.

Expression of Results

1. For each sample and genogroup where no censored Cq values have been used for calculations, and where the ΔCq is greater than and significantly different from zero, express the result in terms of ΔCq and percentage exposed virus genome as:-

   ΔCq = a

   Exposed RT-qPCR target = x% (y – z%)

   where a is the calculated ΔCq, x is the calculated percentage exposed RT-qPCR target, and y and z are the lower and upper confidence limits for the percentage exposed RT-qPCR target.

2. For each sample and genogroup where no censored Cq values have been used for calculations, and where the ΔCq is greater than but **NOT** significantly different from zero, express the result in terms of ΔCq and percentage exposed RT-qPCR target as:-

   ΔCq = a (not significantly greater than zero)

   Exposed RT-qPCR target = x% (0 – z%) (not significantly greater than zero)

   where a is the calculated ΔCq, x is the calculated percentage exposed RT-qPCR target, and z is the upper confidence limit for the exposed RT-qPCR target (note: where ΔCq is not significantly different from 0, lower limit for percentage exposed RT-qPCR target will be 0%).

3. For each sample and genogroup where no censored Cq values have been used for calculations, and where ΔCq calculated from the raw data is either **negative** (and therefore set at a censored value of 0.00), or exactly 0.00, express the result in terms of ΔCq and percentage exposed RT-qPCR target as:-

   ΔCq = 0.00

Exposed RT-qPCR target = 0% (0 – z%) (not significantly greater than zero)

where z is the upper confidence limit for the exposed RT-qPCR target.

4. For each sample and genogroup where censored Cq values for the heat/RNase treated subsamples have been used for calculations, and where the ΔCq is greater than and significantly different from zero, express the result in terms of ΔCq and percentage exposed RT-qPCR target as:-

   ΔCq ≥ a

   Exposed RT-qPCR target ≥ y%

   where a is the calculated ΔCq, and y is the lower confidence limit for the exposed RT-qPCR target

5. For each sample and genogroup where censored Cq values for the heat/RNase treated subsamples have been used for calculations, and where the ΔCq is **NOT** significantly different from zero, express the result in terms of ΔCq and percentage exposed RT-qPCR target as:-

   ΔCq not quantifiable

   Exposed RT-qPCR target not quantifiable

6. For each sample and genogroup where one or more PCR reactions from the untreated subsamples is negative, or gives a quantity of <1 copy/µL by reference to the standard curve generated from the dsDNA dilution series, express the result in terms of ΔCq and percentage exposed RT-qPCR target as:-

   ΔCq not quantifiable

   Exposed RT-qPCR target not quantifiable