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NATIONAL STANDARD OF THE
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GB 4789.42-2025

National Food Safety Standard - Food Microbiological

Examination - Examination of Norwalk Viruses

食品安全国家标准 食品微生物学检验 诺如病毒检验

Issued on: March 16, 2025

Implemented on: September 16, 2025

**Issued by: National Health Commission of the People's Republic of China;
State Administration for Market Regulation.**

Table of Contents

Foreword	3
1 Scope	4
2 Equipment and Materials	4
3 Culture Medium and Reagents.....	5
4 Examination Procedures	6
5 Operation Steps	6
6 Results and Report	12
7 Others	13
Appendix A Real-time Fluorescence RT-PCR Primers and Probes	14
Appendix B Reaction System and Parameters of Real-time Fluorescence RT-PCR...	15
Appendix C Process Control Virus Culture and Primers and Probes	17
Appendix D Preparation of External Amplification Control RNA.....	21
Appendix E Removal of RNase and Preparation of RNase-free Solutions	23

National Food Safety Standard - Food Microbiological Examination - Examination of Norwalk Viruses

1 Scope

This Standard specifies the real-time fluorescence RT-PCR examination method for Norovirus in foods.

This Standard is applicable to the detection of Norovirus in shellfish, raw vegetables, hard surface foods, soft fruits and packaged drinking water.

2 Equipment and Materials

In addition to routine sterilization and culture equipment in microbiology laboratories, other equipment and materials are as follows.

2.1 Real-time fluorescence PCR instrument.

2.2 Low-temperature centrifuge: centrifugal force $> 12,000\times g$, temperature $\leq 4\text{ }^{\circ}\text{C}$, sleeve volume suitable for 1.5 mL/50 mL centrifuge tubes.

2.3 Sterile blades or equivalent homogenizer.

2.4 Vortex mixer.

2.5 Electronic balance: with a division value of 0.1 g and 0.1 mg.

2.6 Full-temperature oscillator or equivalent device: temperature range $2\text{ }^{\circ}\text{C} \sim 60\text{ }^{\circ}\text{C}$.

2.7 Constant-temperature water bath or constant-temperature metal bath: temperature range is room temperature $\sim 100\text{ }^{\circ}\text{C}$.

2.8 Centrifuge: centrifugal force $> 5,000\times g$, sleeve volume suitable for 50 mL/15 mL centrifuge tubes.

2.9 Low-temperature refrigerator: $-80\text{ }^{\circ}\text{C}$.

2.10 Micropipette.

2.11 pH meter or precision pH test paper.

2.12 Sterile homogenizing bag: with a filter and a volume of 400 mL.

2.13 Sterile cotton swab.

2.14 Sterile shellfish peeling knife.

2.15 Rubber pad.

2.16 Sterile scissors.

2.17 Sterile tweezers.

2.18 Sterile culture dish.

2.19 RNase-free glass container, RNase-free centrifuge tube, RNase-free pipette tip, RNase-free medicine spoon, RNase-free PCR thin-walled tube: see E.1 of Appendix E.

2.20 Ultrafiltration concentration centrifuge tube (molecular weight cut-off 50 kD or 100 kD).

2.21 Sterile mixed cellulose membrane (with a pore size of 0.45 μm and a diameter of 47 mm).

3 Culture Medium and Reagents

Unless otherwise specified, all reagents used for the tests are analytically pure; the water used for the tests is RNase-free ultrapure water (see E.2.1).

3.1 G I and G II genome Norovirus primers and probes: see Appendix A.

3.2 Process control virus primers and probes: see Appendix C.

3.3 Process control virus: Mengo virus or *Escherichia coli* bacteriophage MS2, see Appendix C.

3.4 External amplification control RNA: see Appendix D.

3.5 Tris/glycine/beef extract (TGBE) buffer solution: see E.2.2.

3.6 5 \times PEG/NaCl solution: see E.2.3.

3.7 Phosphate buffer solution (PBS): see E.2.4.

3.8 Chloroform/n-butanol mixture: see E.2.5.

3.9 Proteinase K solution: see E.2.6.

3.10 75% ethanol: see E.2.7.

3.11 Trizol reagent: see E.2.8.

3.12 Hydrochloric acid (HCl) solution (6 mol/L): see E.2.9.

3.13 Sodium hydroxide (NaOH) solution (1 mol/L): see E.2.10.

3.14 Pectinase: *Aspergillus niger* pectinase, activity ≥ 5 U/mg; or *Aspergillus aculeatus* pectinase, activity $\geq 3,800$ U/mL.

3.15 Magnesium chloride.

4 Examination Procedures

See Figure 1 for the Norovirus examination procedures.

5 Operation Steps

5.1 Sample Processing

The samples to be tested shall generally be transported in an environment below 4 °C. The laboratory shall detect the samples as soon as possible after receiving them. If the samples cannot be detected within 24 hours, they shall be stored in a -80 °C refrigerator, taken out before the test, and placed at room temperature, until the samples are completely thawed. Cross-contamination shall be prevented during sample processing and PCR detection. Each sample can be set up for 2 ~ 3 parallel processing. During sample processing, pay attention to replacing test consumables, such as scissors, tweezers and pipette tips to prevent cross-contamination between samples.

5.2 Virus Extraction

5.2.1 Soft fruits and raw vegetables

5.2.1.1 Cut 25 g of soft fruits (a general term for soft and juicy fleshy fruits developed from the ovary, such as strawberries and grapes) or raw vegetables (for example, lettuce) into small pieces of about 2.5 cm × 2.5 cm × 2.5 cm under sterile conditions (if the fruit or vegetable is smaller than this volume, it can be left uncut).

5.2.1.2 Transfer the sample pieces to a sterile homogenizing bag (with a filter and a volume of 400 mL), add 10 μL of process control virus, and then, add 40 mL of TGBE buffer solution (for soft fruit samples, it is necessary to add 30 U *Aspergillus niger* pectinase, or 1,140 U *Aspergillus aculeatus* pectinase).

5.2.1.3 Use a full-temperature oscillator, at room temperature and 60 times/min, oscillate for 20 minutes. For acidic soft fruits (soft fruits with a pH of less than 7.0 after the ash from the incineration of soft fruits is dissolved in water, such as strawberries, waxberries and raspberries), during the oscillation process, it is necessary to determine the pH every 10 minutes. When the pH is lower than 9.0, use 1 mol/L NaOH to adjust the pH to 9.5. Each time the pH is adjusted, extend the oscillation time by 10 minutes.

5.2.1.4 Transfer the extracting solution to a 50 mL centrifuge tube. If the volume is relatively large, use 2 centrifuge tubes. 10,000×g, 4 °C, centrifuge for 30 min. Take the supernatant to a sterile test tube or conical flask and use 6 mol/L HCl to adjust the pH to 7.0.

5.2.1.5 Add 5 × PEG/NaCl solution that is 0.25 times the volume of the extracting solution to the extracting solution, so that the final content of PEG/NaCl in the extracting solution is 100 g/L PEG, 0.3 mol/L NaCl. At room temperature, shake it well for 1 min, then, at 4 °C and 60 times/min, oscillate it for 60 min or let it stand at 4 °C overnight. 10,000×g, 4 °C, centrifuge for 30 min and discard the supernatant. 10,000×g, 4 °C, centrifuge for 5 min to compact the precipitate and discard the supernatant.

5.2.1.6 Add 500 μL of PBS to re-suspend the precipitate. If the sample is raw vegetables, directly transfer the re-suspension to an RNase-free centrifuge tube, determine and record the volume of the re-suspension for subsequent RNA extraction. If the sample is soft fruit, transfer the re-suspension to a chloroform-resistant sterile centrifuge tube, add 500 μL of chloroform/n-butanol mixture, conduct vortex to mix it, let it stand at room temperature for 5 min, 10,000×g, 4 °C, centrifuge for 15 min, transfer the supernatant to an RNase-free centrifuge tube, determine and record the volume of the supernatant for subsequent RNA extraction.

5.2.2 Hard surface foods

5.2.2.1 Use PBS to moisten a sterile cotton swab and vigorously wipe the food surface (such as carrots, melons, nuts, 50 cm² ≤ wiping area ≤ 100 cm²), and record the wiping area. Add 10 μL of process control virus to the cotton swab. If the food surface is too rough, the cotton swab may be damaged. Multiple cotton swabs may be used, but only one of the cotton swabs needs to be added with the process control virus.

5.2.4.7 Use 6 mol/L hydrochloric acid to adjust the pH of the extracting solution to 7.0 and transfer all the extracting solution to the ultrafiltration concentration centrifuge tube.

5.2.4.8 At 5,000×g and room temperature, centrifuge for 15 min. Subsequently, transfer the extracting solution in the concentration tube to an RNase-free centrifuge tube, and determine and record the volume of the extracting solution for subsequent RNA extraction.

5.3 Viral RNA Extraction and Purification

NOTE: viral RNA can be manually extracted and purified or using an equivalent commercial viral RNA extraction kit. After viral RNA extraction is completed, RNase inhibitors may be added to extend the RNA storage time. During the operation, disposable rubber or latex gloves shall be worn and frequently replaced. The extracted RNA can be immediately tested or stored at 4 °C for no more than 8 hours or stored in a -80 °C refrigerator for no more than 30 days.

5.3.1 Virus lysis

Add all the viral extracting solution to the centrifuge tube, add an equal volume of Trizol reagent, mix it well, conduct vigorous vortex oscillation for 1 min (about 1,800 r/min), leave at room temperature for 5 min, add 0.2 times the volume of chloroform, conduct vortex mixing for 30 s (not too vigorously to avoid the formation of an emulsion layer, or invert by hand to mix), at 12,000×g, centrifuge for 5 min, transfer the upper-layer aqueous phase to a new RNase-free centrifuge tube to avoid sucking out the middle layer.

5.3.2 Viral RNA extraction

Add an equal volume of isopropanol to the centrifuge tube, invert and mix, leave at room temperature for 5 min, at 12,000×g and room temperature, centrifuge for 5 min, discard the supernatant, invert it on absorbent paper, and wipe the liquid dry (different samples shall be wiped dry with different independent absorbent papers) to prevent cross-contamination.

5.3.3 Viral RNA purification

5.3.3.1 Add 1 mL of 75% ethanol, invert and wash the RNA precipitate, at 12,000×g and 4 °C, centrifuge for 10 min, and carefully discard the supernatant.

5.3.3.2 Repeat adding 1 mL of 75% ethanol, invert and wash the RNA precipitate, at 12,000×g and 4 °C, centrifuge for 10 min, and carefully discard the supernatant. Then, invert the centrifuge tube on absorbent paper and wipe the liquid dry (different samples shall be wiped dry with different independent absorbent papers) to prevent cross-contamination. Or use a micropipette to dry it, use one pipette tip for each sample, and do not touch the precipitate with the pipette tip. Dry it at room temperature for 3 min. Do not excessively dry it to prevent RNA from being insoluble in water.

5.3.3.3 Add 50 µL of RNase-free ultrapure water, gently mix it, dissolve the RNA precipitated at the bottom of the centrifuge tube, at 2,000×g and room temperature, centrifuge for 5 s and

store on ice for later use.

5.4 Quality Control

5.4.1 Blank control

Use RNase-free ultrapure water as blank control (A reaction well).

5.4.2 Negative control

Extract RNA from the same type of food sample, in which, norovirus is not detected, as negative control (B reaction well).

5.4.3 Positive control

Use external amplification control RNA as positive control (J reaction well).

5.4.4 Process control virus

The extraction efficiency of process control virus in the sample represents the extraction efficiency of norovirus in the sample, which serves as process control of virus extraction.

5.4.4.1 Extract viral RNA from 10 μL of process control virus stock solution (approximately 10^{10} PFU/mL) in accordance with the steps in 5.3 and purify it.

5.4.4.2 Perform 10-fold gradient dilution of the extracted and purified process control virus RNA (D ~ G reaction wells), add primers and probes of process control virus, and use the reaction system and parameters in Appendix B to determine the Ct values of undiluted and gradient diluted process control virus RNA.

5.4.4.3 With the lg value of the undiluted and gradient diluted process control virus RNA concentration as the X axis and the corresponding Ct value as the Y axis to draw a standard curve; it is required that the standard curve $r^2 \geq 0.98$. Set the undiluted process control virus RNA concentration to 1, and the process control virus RNA concentration after 10-fold gradient dilution respectively to 10^{-1} , 10^{-2} and 10^{-3} , etc.

5.4.4.4 Add process control virus primers and probes to the food sample RNA containing process control virus (C reaction well), use the reaction system and parameters in Appendix B, determine the Ct value, substitute it into the standard curve, and calculate the process control virus RNA concentration after virus extraction and other steps.

5.4.4.5 Calculate the extraction efficiency. Extraction efficiency = process control virus RNA concentration after virus extraction and other steps $\times 100\%$, that is, (C reaction well) Ct value corresponding concentration $\times 100\%$.

5.4.5 External amplification control

By adding external amplification control RNA, the amplification inhibition index is calculated

as amplification control.

5.4.5.1 Respectively add the external amplification control RNA (10^5 copies/ μL ~ 10^6 copies/ μL) to the RNA of the food sample containing the process control virus (H reaction well), the 10-fold diluted RNA of the food sample containing the process control virus (I reaction well), and the RNase-free ultrapure water (J reaction well), and meanwhile, add the primers and probes of the G I or G II genome. Use the reaction system and parameters in Appendix B to perform real-time fluorescence RT-PCR reaction and determine the Ct value.

5.4.5.2 Calculate the amplification inhibition index. Amplification inhibition index = Ct value (RNA of food sample containing process control virus + external amplification control RNA) – Ct value (RNase-free ultrapure water + external amplification control RNA), that is, amplification inhibition index = Ct value (H reaction well) – Ct value (J reaction well). If the inhibition index is ≥ 2.00 , the amplification inhibition index of RNA of the 10-fold diluted food sample needs to be compared, that is, amplification inhibition index = Ct value (I reaction well) – Ct value (J reaction well).

5.5 Real-time Fluorescence RT-PCR

The real-time fluorescence RT-PCR reaction system and reaction parameters are detailed in Appendix B. The amount of each reagent added to the reaction system can be appropriately adjusted in accordance with the specific situation or different total reaction volumes. Commercial kits can be used, and the operation can be carried out in accordance with the instructions of the kit. The reaction wells can also be added or adjusted to achieve independent detection of Norovirus G I and G II genome in one reaction. First, prepare the fluorescent RT-PCR reaction pre-mix. In accordance with the order and sample volume of Table B.1 in Appendix B, successively mix the RT-PCR buffer solution, MgSO_4 , dNTPs, reverse transcriptase, DNA polymerase and RNase-free ultrapure water to prepare the fluorescent RT-PCR reaction pre-mix. Respectively add the reaction pre-mix to different reaction wells (15.88 $\mu\text{L}/\text{well}$), and then, add the following substances to each reaction well to respectively detect the Norovirus of G I or G II genome and process control virus, and calculate the amplification inhibition index.

A reaction well: blank control, add primers and probes of 5.0 μL of RNase-free ultrapure water + 4.12 μL of G I or G II genome Norovirus;

B reaction well: negative control, add primers and probes of 5.0 μL of RNA of the negative control sample + 4.12 μL of G I or G II genome Norovirus;

C reaction well: add primers and probes of 5.0 μL of RNA of the food sample containing process control virus + 4.12 μL of process control virus;

D reaction well: add primers and probes of 5.0 μL of RNA of process control virus + 4.12 μL of process control virus;

E reaction well: add primers and probes of 5.0 μL of RNA of 10-fold diluted process control virus + 4.12 μL of process control virus;

F reaction well: add primers and probes of 5.0 μL of RNA of 100-fold diluted process control virus + 4.12 μL of process control virus;

G reaction well: add primers and probes of 5.0 μL of RNA of 1,000-fold diluted process control virus + 4.12 μL of process control virus;

H reaction well: amplification control 1, add primers and probes of 5.0 μL of RNA of the food sample containing process control virus + 1.0 μL of external amplification control RNA + 4.12 μL of G I or G II genome Norovirus;

I reaction well: amplification control 2, add primers and probes of 5.0 μL of 10-fold diluted RNA of the food sample containing process control virus + 1.0 μL of external amplification control RNA + 4.12 μL of G I or G II genome Norovirus;

J reaction well: amplification control 3/positive control, add primers and probes of 5.0 μL of RNase-free ultrapure water + 1.0 μL of external amplification control RNA + 4.12 μL of G I or G II genome Norovirus;

K reaction well: sample 1, add primers and probes of 5.0 μL of RNA of the food sample containing process control virus + 4.12 μL of G I or G II genome Norovirus;

L reaction well: sample 2, add primers and probes of 5.0 μL of 10-fold diluted RNA of the food sample containing process control virus + 4.12 μL of G I or G II genome Norovirus.

6 Results and Report

6.1 Determination of Examination Validity

6.1.1 The examination results are valid only if the following quality control requirements are satisfied: blank control negative (A reaction well), negative control negative (B reaction well), positive control positive (J reaction well).

6.1.2 Process control (C ~ G reaction wells) must satisfy the following requirements: process control virus extraction efficiency $\geq 1\%$; if the extraction efficiency is $< 1\%$, re-detection is required; if the extraction efficiency is $< 1\%$ and the Norovirus detection result is positive, the detection result is determined to be valid.

6.1.3 Amplification control (H ~ J reaction wells) must satisfy the following requirements: amplification inhibition index < 2.00 ; if the amplification inhibition index is ≥ 2.00 , the amplification inhibition index of RNA of the 10-fold diluted food sample needs to be compared; if the amplification inhibition index of RNA of the 10-fold diluted food sample is < 2.00 , then, the amplification is valid, and the Ct value of RNA of the 10-fold diluted food sample needs to be used as the detection result; if the amplification inhibition index of RNA of the 10-fold diluted food sample is ≥ 2.00 , the amplification is invalid and re-detection is required; if the amplification inhibition index of RNA of the 10-fold diluted food sample is ≥ 2.00 , the Norovirus detection result is positive and the detection result is determined to be valid.

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