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Standard operating procedure

for

Preparation of dsDNA and external control RNA for detection of hepatitis A virus and norovirus GI and GII

Version No.	Date	Comments
1	2019-09-22	Based on ISO 15216 part 1

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Introduction

This document describes how to generate dsDNA and EC RNA control stocks for detection of hepatitis A virus, norovirus GI and norovirus GII, as stated in ISO 15216-1:2017 annex G and H, but using the dsDNA plasmids constructed at the EURL for Foodborne Viruses. Always consider the ISO method superior to standard operating procedures published by the EURL.

Recommended equipment

Reagents and kits

TE buffer (Sigma Aldrich)
*Hin*dIII HF restriction enzyme (New England Biolabs)
Agarose (Sigma Aldrich)
TBE buffer
MinElute Gel Extraction Kit (Qiagen)
Qubit dsDNA HS kit (Thermo Fisher Scientific)
Riboprobe Combination System-T3/T7 RNA Polymerase (Promega)
RNeasy MinElute Cleanup Kit (Qiagen)
RNA UltraSense One-Step Quantitative RT-PCR System (Thermo Fisher Scientific)
Ice
Cold block

Apparatus

Microwave
DNA gel electrophoresis equipment
Gel Doc XR+ system (Bio Rad)
Qubit 3.0 (Thermo Fisher Scientific)
LightCycler 96 System (Roche)
Thermoshaker adapted to 1.5 ml tubes, capable of operating at 37 – 95 °C

Protective equipment (other than lab coat and shoes)

Latex gloves
Protective eyewear (against UV light)

General protocol

Preparation of plasmid

- Dilute the plasmid stock to 1000 ng/µl in a 1.5 ml tube using 1 x TE buffer, according to Table 1

Table 1. Dilution scheme for plasmids

Start conc. (ng/µl)	Final volume (µl)	Final conc. (ng/µl)	Volume of plasmid to add (µl)	Volume of 1 x TE buffer to add (µl)
c	100	1000	*v* = 100 000/*c*	100 - *v*

Example:

	Start conc. (ng/µl)	Final volume (µl)	Final conc. (ng/µl)	Volume of plasmid to add (µl)	Volume of 1x TE buffer to add
Noro GI	1801	100	1000	55.5	44.5
Noro GII	1955	100	1000	51.2	48.9
HAV	1332	100	1000	75.1	24.9

Plasmid linearisation

- Set a heat block adapted for 1.5 ml tubes at 37 °C
- Prepare a plasmid working solution of 100 ng/µl by adding 10 µl plasmid stock to 90 µl 1 x TE buffer
- Prepare the restriction digestion reaction in a 1.5 ml tube, according to the protocol in Table 2. Keep the restriction enzyme on a cold block

Table 2. Reagents for restriction enzyme digestion

Reagent	Final conc.	Volume to add (µl)
Restriction enzyme[1]	10 U	2
Plasmid working solution	2 µg	20
10 x CutSmart buffer	1 X	10
MQ water		68
Total		100

[1]*Hin*dIII HF (New England Biolabs)

- Incubate for 5-15 min at 37 °C
- Stop the reaction by adding 20 µl 6 x loading dye
- Store at -20 °C or proceed immediately to “Gel separation”

Gel separation

- Prepare and assemble the gel rig according to Fig. 1 (left)
- Fill the electrophoresis tank with approx. 1 l of 0.5 x TBE buffer

Fig. 1. Preparation of an agarose gel. Left: gel rig, right: a loaded agarose gel in an electrophoresis tank filled with TBE buffer

- Prepare the agarose gel solution by mixing 1 g agarose with 100 ml 1 x TBE in a glass bottle
- Microwave the glass bottle at approximately 1 min, until the agarose has dissolved completely. Do not let the solution boil
- Take out the glass bottle from the microwave (caution: hot!) and add 10 µl GelRed Nucleic Acid Gel Stain (Biotiumin) to the bottle, mix well. NOTE: make sure to wear latex gloves when you handle GelRed
- Immediately pour the agarose solution into the gel rig
- Let the gel solidify (takes approximately 20 min)
- Take out the comb from the gel rig once the gel has solidified
- Put the gel in the electrophoresis tank, as described in Fig. 1 (right)
- Prepare an un-cleaved control by mixing 2.5 µl plasmid working solution (100 ng/µl) with 2.5 µl MQ water and 1 µl 6 x LoadingDye
- Add approximately 250 ng of DNA per well by careful pipetting
    - 15 µl of the linearization reaction
    - 6 µl of un-cleaved control
- Run the gel at 100 V for 2-3 h, the negatively charged DNA will move towards the (+) pole
- Take out the gel and visualize under UV-light in the Gel Doc XR+ system (Bio Rad). NOTE! Use UV-protective eye-wear
- Linear gel bands should appear as indicated in Fig. 2
- Excise the linear gel band with a clean, sharp scalpel and place the gel band into a 1.5 ml tube. Store at -20 °C, or proceed immediately to the next step

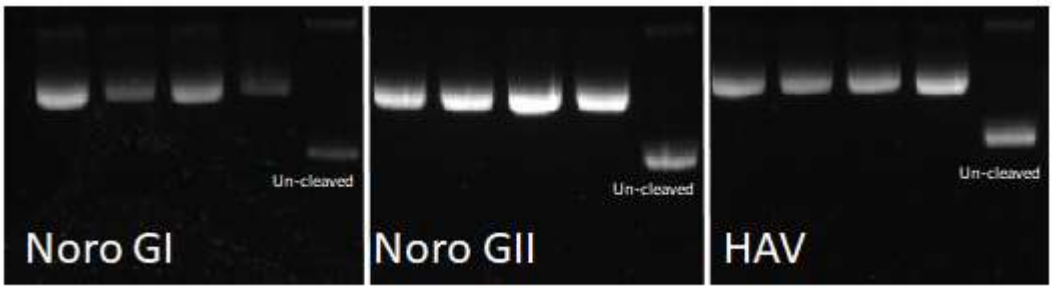

Fig. 2. Appearance of gel bands, linearised plasmid (four wells) and un-cleaved control (one well) for norovirus GI, GII and HAV

Gel purification

- Purify gel bands using MinElute Gel Extraction Kit (Qiagen), according to the manufacturer's instructions
- Store at -20 °C, or proceed immediately to the next step

Concentration measurement

- Quantify DNA with Qubit 3.0 (Thermo Fisher Scientific) using Qubit dsDNA HS kit (Thermo Fisher Scientific) according to the manufacturer's instructions
- A reaction volume of 2 µl is often appropriate
- Note the measured concentration (ng/µl)
- Store at -20 °C, or proceed immediately to the next step

In vitro transcription

- Transcribe RNA from Riboprobe® Combination System-T3/T7 RNA Polymerase (Promega).
- Prepare nucleotides by mixing equal volumes (e.g. 5 µl) of rATP, rGTP, rCTP, and rUTP each, to a total volume of 20 µl per reaction
- Mix the reagents according to the protocol in Table 3. Mix at room temperature

Table 3. Reagents for *in vitro* transcription

Reagent	Concentration	Volume to add (µl)
Transcription optimized 5x buffer		20
DTT, 100 nM		10
RNasin Ribonuclease Inhibitor	200 u	2.5
Nucleotides		20
Linearised template	Ideally 100-1000 ng	Depends on concentration
T7 polymerase	80 u	3
MQ water		Up to 100 µl
Total		100

- Pipet mix and incubate at 37 °C for 2 h
- Add 5 µL RQ1 RNase-free DNAse to the reaction, mix by pipetting
- Incubate for 15 min at 37 °C
- Store at -70 °C, or proceed immediately to the next step

Clean up of RNA

- Purify the transcripts with RNeasy® MinElute™ Cleanup Kit (Qiagen), according to the manufacturer's instructions, with the exception that the elution volume at the last step is 50 µl instead of 14 µl
- Store at -70 °C, or proceed immediately to the next step

Concentration measurement

- Quantify RNA with Qubit 3.0 (Thermo Fisher Scientific) using Qubit ssRNA HS kit (Thermo Fisher Scientific) according to the manufacturer's instructions
- A reaction volume of 2 µl is often appropriate
- Note the measured concentration (ng/µl)
- Store at -70 °C, or proceed immediately to the next step

Copy number calculation

- Calculate plasmid copy number and transcript copy number as below or by entering the measured concentration in ng/µl in the excel sheet "Copy number calculation"

Calculate the molecular weight (MV) of the plasmid/transcript by multiplying the length of the plasmid/transcript (number of nucleotides) with the relative molecular mass of an average base pair (607.4 g/mol/bp, for plasmid) or base (320.5 g/mol/b, for transcript).

Then, calculate the weight in g of each plasmid/transcript by dividing the MV with Avogadro's constant ($6.022 \cdot 10^{23}$). Multiply with 10^9 to get the weight (W) in ng.

Calculate the copy number (copies/µl) by dividing the measured concentration (in ng/µl) by W (in ng).

- Dilute to appropriate concentrations (e.g. 10^6 copies/µl) in 1 x TE-buffer (Sigma-Aldrich)
- Store at -70 °C until use, or for a maximum of six months

DNA contamination check

- Prepare target specific RT-qPCR mastermixes according to Table 4 with target specific primers and probe (Table 5), using RNA UltraSense One-Step Quantitative RT-PCR System (Thermo Fisher Scientific). Master mix templates are provided in the excel sheet "RT-qPCR mastermix"
- Prepare two aliquots of master mix, one labelled + RT (14 reactions) and one - RT (4 reactions)

Table 4. RT-qPCR mix

Reagent	Stock conc. (µM)	Volume (µl)	Final conc. (nM)
RNA Ultrasense 5X Reaction Mix		5	
RNA Ultrasense Enzyme Mix		1.25	
Forward primer	20	0.625	500
Reverse primer	20	1.125	900
Probe	10	0.625	250
MQ water		11.375	
Template		5	
Total		25	

Table 5. Primers and probes

Target	Type	Name	Sequence (5'-3')
Hepatitis A virus	F	HAV 68	TCA CCG CCG TTT GCC TAG
	R	HAV 240	GGA GAG CCC TGG AAG AAA G
	P	HAV 150	[FAM]-CCT GAA CCT GCA GGA ATT AA-[MGB][BHQ1]
Norovirus GI	F	QNIF4	CGC TGG ATG CGN TTC CAT
	R	NV1LCR	CCT TAG ACG CCA TCA TCA TTT AC
	P	TM9	[FAM]-TGG ACA GGA GAT CGC- [MGB][BHQ1]
Norovirus GII	F	QNIF2	ATG TTC AGR TGG ATG AGR TTC TCW GA
	R	COG2R	TCG ACG CCA TCT TCA TTC ACA
	P	QNIFS	[FAM]-AGC ACG TGG GAG G GC GAT CG-[BHQ1]

FAM = 6-carboxyfluorescein, MGB = minor groove binder, BHQ1 = black hole quencher 1, NFQ = non-fluorescent quencher

- Inactivate the - RT aliquot by heating at 95 °C for 5 min
- Mix 20 µl master mix with 5 µl sample. Include a serial dilution of linearised DNA as a calibration curve. A suggested plate layout is shown in Fig 3

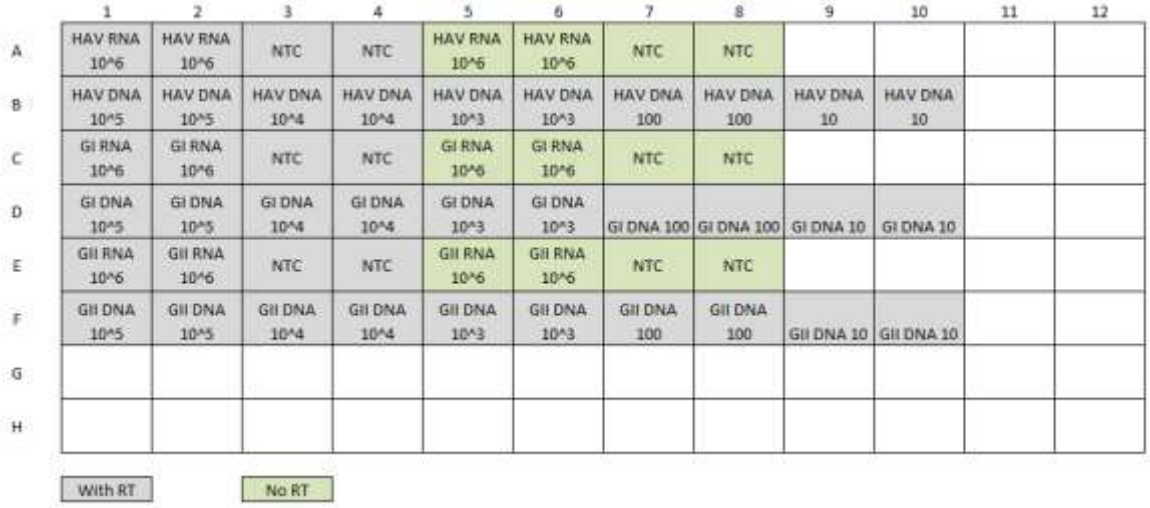

Fig. 3 Example of plate layout. Concentrations indicate copies/µl

- Run the RT-qPCR according to the protocol in Table 5

Table 5. RT-qPCR protocol

Step	Temp (°C)	Time
RT	55	1 h
Preheating	95	5 min
45 cycles of		
Denaturation	95	15 s
Annealing-Extension	60	1 min
	65	1 min

- Evaluate results by comparing the concentrations of RNA transcript obtained by + RT and - RT wells. Calculate the percentage of DNA in the transcript by dividing the concentration obtained in the - RT wells by the concentration obtained in the corresponding + RT wells, and multiplying with 100

  Note: If the DNA concentration exceeds 0.1 %, perform DNAse treatment on the stock transcript according to the protocol below, and test by using RT-qPCR with and without RT again

  For a 100 µl sample, add 5 µL RQ1 RNase-free DNAse from Riboprobe® Combination System-T3/T7 RNA Polymerase (Promega), mix by pipetting and incubate for 15 min at 37 °C. Adjust volumes if necessary (*i.e*. 2.5 µl DNAse is added to a 50 µl sample)

- Store RNA transcripts with a DNA concentration of <0.1 % at -70 °C

Appendix 1. Information about the plasmids used at the EURL for Foodborne Viruses

Plasmids were purchased from Eurofins Genomics, with the following insert sequences:

Hepatitis A virus

Insert sequence

TAATACGACTCACTATAGGTGGAAGTCCATGGTGAGGGGACTTGATACCTCACCGCCGTTTGCCTAGGCTATAGGCTAAAT
TTTCCCTTTCGGATCCCCCTTTCCTATTCCCTTTGTTTTGCTTGTAAATATTGATTTGTAAATATTGATTCCTGCAGGTTCAGG
GTTCTTAAATCTGTTTCTCTATAAGAACACTCATTTCACGCTTTCTGTCTTCTTTCTTCCAGGGCTCTCCCCTTGCCCTAGGCT
CTGGCCGTTGCGCCCG**AAGCTT**

Size: 2721 bp
Transcript size: 249 b
Position: base 54-299 in NC001489.1
Sense: plus
T7 ->
GGATCC: extra bases to get a *Bam*HI restriction site
G: mismatch within the probe region, but according to the ISO-standard
AAGCTT: *Hind*III restriction site
Vector: pEXA2, Eurofins

PCR-system:

HAV68: TCACCGCCGTTTGCCTAG
HAV150: CCTGAACCTGCAGGAATTAA, reverse complement: TTAATTCCTGCAGGTTCAGG
HAV240: GGAGAGCCCTGGAAGAAAG reverse complement: CTTTCTTCCAGGGCTCTCC

Norovirus GI

Insert sequence

TAATACGACTCACTATAGGGATTTCCAGCAAGGTCATACATGAAATCAAGACTGGTGGATTGGAAATGTATGTCCCAGGAT
GGCAGGCCATGTTCCGCTGGATGCGCTTCCATGACCTCGGATTGTGGACAGGAGATCGCGATCTTCTGCGGATCCGAATTC
GTAAATGATGATGGCGTCTAAGGACGCTACATCAAGCGTGGATGGCGCTAGTGGCGCTGGTCAGTTGGTACCGGAGGTT
AATGCTTCTGACCCTCTTGC**AAGCTT**

Size: 2717 bp
Transcript size: 245 b
Position: 5215-5452 in M87661.2
Sense: plus
T7 ->
GGAT: extra bases to get a *Bam*HI-site
AAGCTT: *Hind*III restriction site
Vector: pEXA2, Eurofins

PCR-system:

QNIF4: CGCTGGATGCGNTTCCAT

TM9: TGGACAGGAGATCGC
NV1LCR: CCTTAGACGCCATCATCATTTAC (reverse complement: GTAAATGATGATGGCGTCTAAGG)

Norovirus GII

Insert sequence

TAATACGACTCACTATAGGACAGCAAAATTAGCAAGCTAGTCATTGCAGAACTGAAGGAAGGTGGCATGGATTTTTACGT
GCCCAGACAAGAGCCAATGTTCAGATGGATGAGATTCTCAGATCTGAGCACGTGGGAGGGCGATCGCAATCTGGCTCGG
ATCCAGCTTTGTGAATGAAGATGGCGTCGAATGACGCCAACCCATCTGATGGGTCCGCAGCCAACCTCGTCCCAGAGGTC
AATAATGAGGTTATGGCTCTGGAGCCCGTT**AAGCTT**

Size: 2725 bp
Transcript size: 243 b
Position: 4935-5180 in Lordsdale (X86557.1)
Sense: plus
T7 ->
GGAT: extra bases to get a *Bam*HI-site
AAGCTT: *Hind*III restriction site
Vector: pEXA2, Eurofins

PCR-system:

QNIF2: ATGTTCAGRTGGATGAGRTTCTCWGA
QNIFS: AGCACGTGGGAGGGCGATCG
COG2R: TCG ACG CCA TCT TCA TTC ACA (reverse complement: TGTGAATGAAGATGGCGTCGA)

Vector map

https://www.eurofinsgenomics.eu/media/892663/pex-a2_map_seq_v12.pdf

Size: 2450 bp