METAWATER

# Round Robin Test 2016

DTU Food
National Food Institute
DTU

Universitat de Barcelona
Laboratory of Virus Contaminants of Water and Food

Instituto de Ingeniería del Agua y Medio Ambiente

TECHNISCHE UNIVERSITÄT MÜNCHEN

# Round Robin Test 2016

## Content

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## b. Protocols

*Part I: Concentration methods*

**Order of samples**

**MAF: 6 – 3 – 10- 13 – 2 – 15 – 25 – 18 – 5 – 9 – 19 – 7 – 26**

**SMF: 23 – 1 – 12 – 22 – 16 – 4 – 21 – 17 – 20 – 14 – 8 – 24 – 11**

**Please note the exact date of concentration of each sample!**

### 1. Monolithic adsorption filtration (MAF)

**Equipment:**

| | |
|---|---|
| **MAF-OH (TUM)** | ☐ |
| **PTFE Bottom (TUM)** | ☐ |
| **O-Ring (TUM)** | ☐ |
| **PTFE Adaptor (TUM)** | ☐ |
| **Dispenser Tips as Housing (Carl Roth, EH 30.1)** | ☐ |
| **ph-meter and calibration solutions** | ☐ |
| **Amicon Ultra-15, PLQK Ultracel-PL Membran, 50 kDa (Merck Millipore Ltd, UFC905024)** | ☐ |

**Reagents:**

| | |
|---|---|
| **Glycine puriss p.a. (Sigma-Aldrich, 33226-250G )** | ☐ |
| **Beef Extract powder (Sigma-Aldrich, B4888)** | ☐ |
| **Sodium hypochloride (Carl-Roth, 9062.3)** | ☐ |
| **Elution Buffer (0,5 M Glycine, 3 % (w/v) Beef extract at pH 9.5)** | ☐ |

**Pathogens:**

| | |
|---|---|
| **MS2 (provided by UB) – *$10^9$ GU/ml and $10^7$ GU/ml*** | ☐ |
| **HAdV 35 (provided by UB) – *$10^9$ GU/ml and $10^7$ GU/ml*** | ☐ |
| **MNV (provided by DTU) – 4,3**$10^7$ IFU/ml ± 6*$10^5$ IFU/ml (DTU); 2,36*$10^{10}$ GU/ml (UB)*** | ☐ |
| ***E.coli* (provided by TUM)** | ☐ |
| ***E.faecalis* (provided by TUM)** | ☐ |
| ***H.pylori* (provided by UPV) – *$10^7$ CFU/ml*** | ☐ |
| ***A.castellanii* (provided by UPV) – *$10^5$ CFU/ml*** | ☐ |

The respective project partners provide protocols and primer sequences for qPCR together with the organisms.

**Wastewater samples (provided by TUM)**:

6 x 10 mL wastewater spiked with 0 copies per organism

10 x 10 mL wastewater spiked with $10^6$ copies per organism
10 x 10 mL wastewater spiked with $10^8$ copies per organism
***+ additional samples for determination of recovery***
2 x 10 mL wastewater spiked with 0 copies per organism
2 x 10 mL wastewater spiked with $10^6$ copies per organism
2 x 10 mL wastewater spiked with $10^8$ copies per organism
***+ additional samples for determination of pathogen in water matrix***
2 x 10 mL wastewater only

**Five samples** of each concentration and **three** BLANK samples are used for MAF, the other **five** as well as **three** BLANK samples for SMF.

**Two samples** of each concentration and BLANK are used for direct nucleic acid purification followed by qPCR to determine recovery rates **(will be sent on Monday, 14.03.2016 and arrive at partner institutes on 15.03.2016)**.

**Order of samples**

**MAF: 6 – 3 – 10- 13 – 2 – 15 – 25 – 18 – 5 – 9 – 19 – 7 – 26**
**Please note the exact date of concentration of each sample!**

**Sample Preparation**

All participating project partners will receive 10 mL wastewater samples spiked with adenovirus (HAdV 35), MNV strain CW3, MS2, *E.coli* (DSM 423), *E.faecalis* (OG1RF), *H.pylori* and *A.castellanii* in various concentrations.
9990 mL of mineral water (Evian) are spiked with 10 mL (= 1:1000 dilution) of wastewater as contaminated water matrix with two different concentrations.
The final concentrations investigated in the 10 L samples are: 0, $10^6$, $10^8$ copies in 10 L.
For each concentration, five samples (BLANK: three samples) will be concentrated, so that a total number of 13 samples for MAF is processed. Wastewater samples for SMF are also provided by TUM.
**Before MAF filtration all samples are adjusted to pH 3 with HCl.**

**Construction of the Monolithic Column**

To ensure the housing (1) will withstand the pressure during the filtration, the tip of the housing is cut off at a length of around 0.9 cm. To construct the monolithic column, the PTFE holder (2) is put into the housing. An O-Ring (3) and the monolithic disk (4) are put on top.

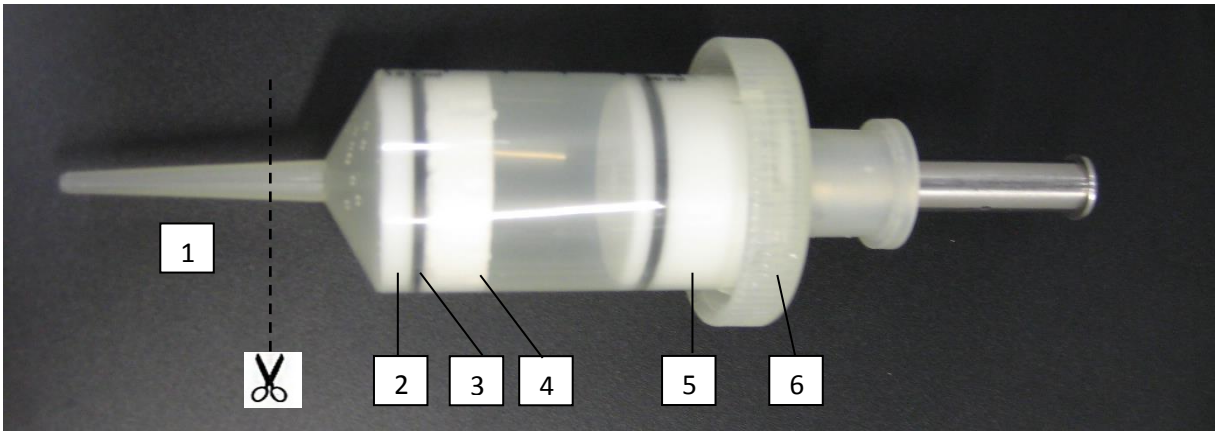


**Figure 1 BigMAF column consisting of 1: Housing, 2: PTFE holder, 3: O-Ring, 4: Monolithic disk, 5: Fitting, 6: Adapter**

After disk insertion, the fitting (5) and adapter (6) are connected to the pump by a PTFE tube. The pump is connected to a bottle with **ultrapure water pH 3** (adjusted with HCl) to equilibrate the MAF disk. Pumping is started at a low flow rate (ca. 200 mL/min) and the adapter is hold on top of the housing until the housing is filled with water.
After the housing is filled, the adapter is pushed into the housing, forcing the water inside through the MAF disk. It is important to leave no air bubbles inside the housing. Afterwards the adapter is locked with the fitting and the flow rate is increased to the final value (up to 1 L/min) and 1 L of tap water is pumped through the column to equilibrate it. After 1 L has passed through the column the pump is stopped and connected to the sample. The column has to be filled with water all the time, and no air bubbles should be put inside to keep the pressure in the system constant. The system is now ready for the filtration

**Buffer preparation:**

**MAF Elution Buffer:**
Add beef extract powder (3% w/v) and glycine (0.5 M) to ultrapure water and stir on a magnetic stirrer until all is dissolved. Adjust the pH to 9.5 by adding HCl. Autoclave the buffer and let it cool down to room temperature before use.

**Phosphate Buffered Saline (PBS):**
145 mM NaCl, 10 mM KH2PO4, and 70 mM K2HPO4, adjusted to pH 7.6. Autoclave the buffer and let it cool down to room temperature before use.

**Filtration**

The column is fixated over a waste bag. The pump is connected to the sample, which has been adjusted to pH 3 with HCl beforehand. A flow rate of up to 1 L/min is used until all sample has passed through the column. No air should be in the column or tubings during the filtration. After filtration the column is emptied a. Pumping air through the column after filtration does not affect sample elution. The rubber tube is disconnected from the fitting and the outlet of the housing is blocked.

**Elution**

For elution, the rubber tube of the pump is disconnected from the fitting, the outlet is blocked (e.g. by a small piece of rubber tube that is blocked with a hose clamp or plug). 20 mL elution buffer (0.5 M Glycine, 3 % beef extract, pH 9,5, autoclaved) are added to the housing with a syringe and a long needle. Then, the rubber tube is reconnected to the fitting and the outlet is opened. The flow rate of the pump is set to 150 mL/min for elution. After one third of the buffer has passed through the column, the pump is switched off, the outlet of the housing is blocked again and the disk is incubated for 2 min. Afterwards the outlet is opened again and another third of the buffer is pumped through the column. The outlet is closed again and the disk is incubated for another 2 min. Then, the last third of the buffer is passed through the column.
**Note:** Monolithic disks and housing are disposable parts and **should not be used for more than one sample** in order to prevent cross contamination.

**Centrifugal Ultrafiltration**

For further concentration of samples to **1 mL**, centrifugal ultrafiltration (Amicon Ultra-15, Merck Millipore) is used according to manufacturer's instruction. (Ref 8, 9) Afterwards the samples are filled up to **5 mL** with PBS buffer and analyzed according to instructions in part II.

**Cleaning**

After each filtration the fitting, adapter and PTFE holder are cleaned in boiling water (100 °C after taking out of the fire and changing the water for every sample) for 5 min. Afterwards the parts are cooled in cool clean ultrapure water and used for the next filtration.
The rubber tube from the pump is cleaned with sodium hypochlorite (200 ppm) and afterwards ultrapure water. The tube can be dechlorinated with sodium thiosulfate (200 ppm). The disposable parts (housing and monolithic disks) are disinfected by autoclave.

**Analysis**

Samples are processed by nucleic acid extraction and qPCR as defined by the according SOP (see *part II*).

**Store concentrates at 4°C until nucleic acid extraction and further analysis!**

**1 mL of concentrate is sent to UPV for analysis of *H.pylori* and *A.castellanii* on 29.03.2016.**

**Volumes of concentrate needed for different nucleic acid extraction:**

- **500 µL** for bacteria (*E.coli* and *E.faecalis*)
- **2 x 420 µL** for viruses and bacteriophage (HAdV, MNV, MS2)
  *2 x 140 µL for HAdV extraction*
  *2 x 140 µL for MNV extraction*
  *2 x 140 µL for MS2 extraction*

## 2. Skimmed milk flocculation (SMF)

The aim of the protocol is to concentrate viruses present in 10 L of water to a final volume of **10 mL (5 mL in clean water to test higher volumes) of phosphate buffer.**

**Equipment:**

| | |
|---|---|
| **10 L plastic containers with flat bottoms (Deltalab 222805)** | ☐ |
| **High speed centrifuge (8,000xg)** | ☐ |
| **ph-meter and calibration solutions** | ☐ |
| **Sterile graduated disposable pipettes (LabClinics PN10E1)** | ☐ |
| **Sterile plastic tubes of 1.5 and 10-15 mL** | ☐ |
| **Centrifuge pots (500 mL)** | ☐ |
| **Magnetic stirrers and magnets (one per sample)** | ☐ |
| **A peristaltic pump for removing the supernatant (optional)** | ☐ |
| **Timer to switch-off the stirring after 8-10 hours** | ☐ |
| **Electric strip** | ☐ |

**Reagents (see appendix):**

| | |
|---|---|
| **Hydrochloric acid** (1N and 0,1N) | ☐ |
| **Sodium hydroxide** (4% w/v = 1 M) | ☐ |
| **Skimmed milk powder** (Difco Ref. 232100) | ☐ |
| **Phosphate buffer** (1:2 v/v of sterile $Na_2HPO_4$ 0,2M and $NaH_2PO_4$ 0,2M at pH 7.5) | ☐ |
| **Artificial sea salts** (Sigma Ref. S9883) | ☐ |

**Order of samples**

**SMF: 23 – 1 – 12 – 22 – 16 – 4 – 21 – 17 – 20 – 14 – 8 – 24 – 11**

**Please note the exact date of concentration of each sample!**

**Preparation of Pre-flocculated Skimmed Milk (PSM):**

Check the calibration of the pH-meter in the alkaline and acidic range and recalibrate if necessary. Always disinfect the pH-meter electrode with fresh hydrochloric solution (0,1 N). Prepare 100 mL of PSM 1% (w/v) for each water sample JUST BEFORE TO BE USED. In a litre of artificial seawater (33,33 g artificial sea salts into 1 L of sterile $H_2O$) add 10 g of skimmed milk powder. Stir with a magnetic stirrer and AFTER DISSOLVING adjust the pH at 3.5 (± 0.1). DO NOT AUTOCLAVE.

**Conditioning of water and flocculation**

Samples must be preconditioned before flocculation. If samples present high quantity of suspended material (sand or algae) let it sediment for 15 minutes and poor carefully the water into a new container. Use one different plastic pipette for each sample.
Add a magnet into the bottle and adjust conductivity. **UB already determined conductivity of Evian water used for the round robin test. The conductivity is 314uS. 15 g sea salt should be added to reach a conductivity of 150 µS/cm$^2$.** AFTER DISSOLVING adjust the pH at 3.5 (± 0.1) by the addition of HCl 1 N. Mix the water thoroughly by vigorous stirring while adding the HCl. This step is important for

the concentration of viruses so make sure the pH has been properly adjusted. Use a new pipette in each sample. Add 100 mL of PSM 1 % for each water sample. Stir the samples for 8 hours to allow the viruses to adsorb to the flocks. Use a timer for automatically switch off the stirring and let the flocks sediment by gravity for 8 hours.

**Negative process control**

A negative process control should be included in every batch of concentrated samples. Use 10 L of Evian water and add 15 g sea salt to reach a conductivity of 150 µS/cm$^2$. Add a magnet to the bottle and adjust conductivity by addition of 10 g of artificial sea salts to each sample. AFTER DISSOLVING adjust the pH at 3.5 (± 0.1) by addition of HCl 1 N. Mix water thoroughly by vigorous stirring while adding HCl. Add 100 mL of PSM 1 % to each water sample.
Stir the samples for 8 hours to allow viruses to adsorb to the flocks. Use a timer for automatically switch off the stirring and let the flocks sediment by gravity for 8 hours.

**Collecting the flocks, centrifugation and resuspension in phosphate buffer**

Remove the supernatant using a peristaltic pump and a plastic pipette connected to a plastic tube. The supernatant can be removed also by gravity. For spiked samples the supernatant should be collected into a recipient and disinfected according with the internal procedures. In all cases, TAKE CARE to not disturb the pellet. Collect the remaining volume (approximately 500 mL) in a centrifuge bottle (e.g. Scharlab Ref. 195753501). BALANCE THE BOTTLES WITH PSM AT pH 3.5 and centrifuge at 8000 x *g*, 30 min at 4° C. As soon as the centrifuge stops, remove the centrifuge bottles. Very gently pour off and discard the supernatant. Follow appropriate measures for infectious material.
Add 7 mL of phosphate buffer to each centrifuge bottle. Once the flocks have been dissolved, measure and add phosphate buffer to reach a total volume of 10 mL. Homogenize the viral concentrate by vortexing and transfer the 5 mL to several Eppendorf.
**Store concentrates at 4°C until nucleic acid extraction and further analysis!**
**1 mL of concentrate is sent to UPV for analysis of *H.pylori* and *A.castellanii* on 29.03.2016.**

**Volumes of concentrate needed for different nucleic acid extraction:**

- **500 µL** for bacteria (*E.coli* and *E.faecalis*)
- **2 x 420 µL** for viruses and bacteriophage (HAdV, MNV, MS2)
  *2 x 140 µL for HAdV extraction*
  *2 x 140 µL for MNV extraction*
  *2 x 140 µL for MS2 extraction*

**Appendix**

*Hydrochloric Acid 1 N*

- 34.4 mL concentrated hydrochloric acid
- 400 mL deionized water

Measure 400 mL of deionized 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 expiration date.

*Sodium Hydroxide (1 M/4%)*

- 4 g sodium hydroxide
- 100 mL deionised water

Dissolve the sodium hydroxide in the deionized water in a sterile glass beaker. Once dissolved, dispense into 100 mL clean glass bottles. Label with the batch number and expiration date.

*Artificial seawater*

- 33.33 g Sea Salts (Sigma Ref. S9883)
- 1 L dechlorinated tap water

Add the Sea Salts to water and leave at room temperature overnight to dissolve if is necessary. Shake or swirl container to aid mixing.

*Preflocculated skimmed milk (PSM) 1 % (w/v)*

- 100 mL of artificial seawater.
- 1 g of skimmed milk (Difco Ref. 232100).

Dissolve using a magnetic stirrer and adjust the pH to 3.5. The flocs should be formed and are clearly visible.

*Phosphate buffer*

- 1:2 v/v of sterile Na2HPO4 0,2M and NaH2PO4 0,2M at pH 7.5.
  **Label with the batch number and expiry date.**

# *Part II: Nucleic acid preparation and analysis*

## 1. Cultivation of *E.coli* and *E.faecalis*

Preparation of cell cultures for nucleic acid analysis of bacteria.

**Equipment:**

| | |
|---|---|
| **Centrifuge** | ☐ |
| **Incubator** | ☐ |
| **Spectrophotometer** | ☐ |
| **Disposable cuvettes** | ☐ |
| **Micropipettes + filter tips** | ☐ |
| **Sterile plastic tubes of 1.5 mL** | ☐ |
| **Plastic supports** | ☐ |

**Reagents:**

| | |
|---|---|
| **NZCYM broth (Roth, X974.1)** | ☐ |
| **LB broth (luria low salt) (Sigma-Aldrich, L3397)** | ☐ |
| **Water** | ☐ |

**Cultivation protocol**

*Overnight cultures*

*E.coli* (DSM 423) is cultivated in 100 mL NZCYM broth and *E.faecalis* (OG1RF) in 100 mL LB broth (Luria low salt) overnight at 37°C. Overnight culture is centrifuged at 2100 x *g* for 10 min at 4°C. Supernatant is discarded and cell pellet is diluted in water. Concentration of bacteria solution is determined spectrophotometrically at A=670nm according to the following formula:

$$A_{670nm} = 0.03 \cong 2.3 \times 10^7 \frac{cells}{mL}$$

## 2. Nucleic acid extraction

Extraction of nucleic acids (NA) from different pathogens for qPCR analysis

### 2.1. Bacteria (QIAamp DNA Mini Kit)

For DNA purification of bacteria overnight suspension cultures or irrigation water concentrates the QIAamp DNA Mini Kit is performed according to the user's manual.

**Equipment:**

| | |
|---|---|
| **Centrifuge** | ☐ |
| **Water bath or rocking platform set to 56 °C and 70 °C** | ☐ |
| **Micropipettes + filter tips** | ☐ |
| **DNA LoBind tubes (Eppendorf, Cat. No. 0030108051)** | ☐ |

**Reagents:**

| | |
|---|---|
| **QIAamp® DNA Mini kit (Qiagen, Cat. No. 51304)** | ☐ |
| **Ethanol (96-100%)** | ☐ |

**Extraction protocol**

All centrifugation steps are carried out at room temperature (15–25 °C)

1. Pipet 500 µl of prepared dilution of dilution series into a 1.5 ml microcentrifuge tube, and centrifuge for 5 min at 5000 x *g* (7500 rpm).

2. Add 180 µl Buffer ATL to the pellet

3. Add 20 µl proteinase K, mix by vortexing, and incubate at 56 °C until the pellet is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

4. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

5. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70 °C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

6. Add 200 µl ethanol (96–100 %) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

7. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20000 x *g*; 14,000 rpm) for 3 min.

10. Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp Mini spin column in a clean 1.5 ml DNALoBind tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 100 µl Buffer AE or distilled water. Incubate at room temperature for 5 min, and then centrifuge at 6000 x *g* (8000 rpm) for
    1 min.

12. Repeat step 11.

## 2.2. Viruses (QIAamp Viral RNA Mini Kit)

For viral DNA and RNA purification the QIAamp Viral RNA Mini Kit is performed according to the user's manual. The total volume of viral concentrate used is **140 µl**. **Extractions for MNV, MS2 and HAdV are done two times to get the needed volume necessary for qPCR analysis.**

**Equipment:**

| | |
|---|---|
| **Centrifuge** | ☐ |
| **QIACUBE + tubes+ adaptors** (optional) | ☐ |
| **Micropipettes + filter tips** | ☐ |
| **Sterile graduated disposable micropipettes** | ☐ |
| **Sterile plastic tubes of 1.5 mL** | ☐ |
| **Plastic supports** | ☐ |

**Reagents (see appendix):**

| | |
|---|---|
| **QIAamp Viral RNA Mini Kit** (**Qiagen, Cat. No. 52906)** | ☐ |
| **Ethanol (96-100%)** | ☐ |

**Extraction protocol**

1. Pipet 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.
2. Add 140 µl of viral concentrate to the 560 µl of Buffer AVL-Carrier. Mix the tube 15 seconds.
3. Incubate at room temperature (15-25 °C) for 10 min.
4. Spin the tubes to remove drops from the inside of the lid.
5. Add 560 µl of ethanol (96-100 %) to the sample. Mix by vortexing during 15 seconds. Spin the tubes to remove drops from the inside of the lid.
6. Carefully apply 630µl of the solution from the step 5 to a QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Centrifuge at 6000xg for 1 min. Place the QIAamp spin column into a clean 2mL collection tube and discard the tube containing the filtrate.
7. Repeat the step 6 until all sample has passed through the spin column.
8. Add 500 µl of buffer AW1. Centrifuge at 6000 x *g* for 1 min. Keep the spin column, and discard the collection tube and the filtrate.
9. Add 500 µl of buffer AW2. Centrifuge at 20000 x *g* for 3 min. Keep the spin column and discard the collection tube and the filtrate.
10. Repeat step number 9 but centrifuge for 1 min.

11. Place the QIAamp Mini Spin Column into a clean 1.5mL centrifuge tube (collection tube). Open the column and add 60 µl of AVE. Incubate the Spin Column for 2 min and centrifuge at 6000 x *g* for 1 min.

12. Store the eluted NA at -80 °C for further molecular analysis.

## 3. qPCR

### 3.1. Standard preparation

Standard curves for qPCR analysis are based on culture or synthetic DNA fragments (gBlocks® Gene Fragments) standards.

#### 3.1.1. *E.coli* and *E.faecalis*

According to the determined concentration of bacterial culture suspension (see *part* II, 1.) a dilution series from $10^8$ to $10^1$ CFU/mL in a final volume of 500 µl is prepared. Bacterial DNA of each dilution is extracted as described in 2.1.1.
Serial dilutions are analysed in triplicates by qPCR and standard curve is generated.

#### 3.1.2. HAdV, MS2 and MNV (gBlocks® Gene Fragments)

1. Create an account at www.idtdna.com
2. Go to products and services
3. Select gBlocks® Gene Fragments. The price depends on the size of the DNA sequence
4. Select Order
5. Insert name of sequence and length of synthetic DNA you want to synthesize (sequences below)
6. When stock arrives follow instruction for resuspension
7. Centrifuge tube for 3-5 sec at a minimum of 3000 x *g* to ensure the material is in the bottom of the tube
8. Add TE buffer to reach a final concentration of 10 ng/µl. The amount of DNA in ng is written in the sheet attached to the product

| Properties | |
|---|---|
| Length: | 346 |
| Amount Delivered: | 500ng |
| GC Content: | 58.09% |
| Molecular Weight: | 213696.6 |
| fmoles/ng: | 4.68 |
| µg/$OD_{260}$: | 50 |

1. Vortex briefly
2. Incubate at 50 °C for 20 minutes
3. Briefly vortex and centrifuge
4. The resuspended synthetic DNA has to be stored at -20 °C
5. Quantify the gBlocks® Gene Fragment to know the exact amount of DNA per µl

6. Insert the data in the provided excel sheet

| **Concentrations** | | | | | |
|---|---|---|---|---|---|
| qubit measure | 7,29 | ng/ul | | | |
| molecular weight | 213696,6 | | | | |
| lenght in base pairs | 273 | | | | |
| **Standard calculation** | | | | | |
| | | | | | |
| lenght | 273 | **pb** | | | |
| plasmid concentration | 7,29 | **ng/µl** | | | |
| Molecular weight | 213696,60 | **u.m.a** | | | |
| concentration genomic copies /gr | 2,82E+18 | **cg/gr** | | | |
| ng for 1E+11 genomic copies | 35,49 | **ng** | | | |
| volume that has 1E+11 genomic copies | 4,87 | **µl** | | | |
| volumen of TE buffer | 995,13 | **ul** | | | |
| | | | | | |
| | | | | | |
| stock concentration / ml | 1,00E+11 | 1,00E+10 | 1,00E+09 | 1,00E+08 | 1,00E+07 |
| | | | | | |
| | | | | | |
| genomic copies in 10 ul of reaction | 1,00E+09 | 1,00E+08 | 1,00E+07 | 1,00E+06 | 1,00E+05 |
| genomic copies in 5 ul of reaction | 5,00E+08 | 5,00E+07 | 5,00E+06 | 5,00E+05 | 5,00E+04 |

10. The dilution of $10^{11}$ genomic copies is diluted in TE buffer to a final V=1ml

11. Serially dilute the DNA in order to obtain dilutions per 10 or 5 µl

12. Test each dilution by triplicate in a qPCR reaction. Make sure signal (fluorescence, Ct values<40) appear after $5x10^0$ copies/5µl for RNA or $1x10^0$ copies/10µl for DNA triplicates. If the signal appears at lower dilutions, the standard curve is not well constructed

**Sequences of gBlocks® Gene Fragments for qPCR of HAdV, bacteriophage MS2 and *A.castellanii***

**HAdV**

ATGATGCCGCAATGGTCTTACATGCACATCGCCGGGCAGGACGCCTCGGAGTATCTGAGCCCGGGCACACACA
CACACCTGGTGCAATTTGCCCGCGCCACCGATACGTACTTCAGCCTGGGGAACAAGTTCAGAAATCCCGCTGC
GATTCGTGCCAGTCGACCGCGAGGACACCGCTTATTCTTACAAAGTGCGCTTTACGCTGGCCGTGGGCGACAA
CCGGGTGTTGGACATGGCCAGCACCTACTTTGACATCCGCGGCGTGCTGGATCG

**MS2**

CGTCGTAAGGTGCCTACAAGCGAAGTGGGTCATCGTGGGGTCGCCCGTACGAGGAGAAAGCCGGTTTCGGCT
TCTCCCTCGACGCACGCTCCTGCTACAGCCTCTTCCCTGTAAGCCAAAACTTGACTTACATCGAAGTGCCGCAG
AACGTTGCGAACCGGGCGTCGACCGAAGTCCTGCAAAAGGTCACCCAGGGTAATTTTAACCTTGGTGTTGCTT

TAGCAGAGGCCAGGTCGACAGCCTCACAACTCGCGACGCAAACCATTGCGCTCGTGAAGGCGTACACTGCCGC
TCGTCGCGGTAATTGGCGCCAGGCGCTCCGCTACCTTGCCCTAAACGAACTGTC

**MNV**

ACCAGTTTGGGTGGTACGGTCGTCTTGATCGTGCCAGCATCGACCGCCAGCTCCTCTGGACTAAAGGACCTAC
CCACCAGAACCCCTTTGAGACTCTCCCTGGACATGCTCAGAGACCCTCCCAACTAATGGCCCTGCTCGGTGAGG
C

## 3.2. Primer and probe sequences

| Pathogen | Ref | Name | Sequence (5'➔3') | Gene | Working solutions* | Final PCR conc |
|---|---|---|---|---|---|---|
| | | AdF | CWTACATGCACATCKCSGG | | 22.5 µl | 0.9 µM |
| **HAdV** | 1, 2 | AdR | CRCGGGCRAAYTGCACCAG | Hexon protein | 22.5 µl | 0.9 µM |
| (DNA) | | AdP1 | 6-FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ1 | (69 bp) | 11.25 µl | 0.225 µM |
| | | pecson-2F | AAGGTGCCTACAAGCGAAGT | | 25 µl | 1 µM |
| **MS2** | 3 | pecson-2R | TTCGTTTAGGGCAAGGTAGC | Mat protein | 25 µl | 1 µM |
| (RNA) | | PecP-2 | 6-FAM-ATCGTGGGGTCGCCCGTACG-BHQ1 | (335 bp) | 25 µl | 0.25 µM |
| | | E.coli-FWD | CAATTTTCGTGTCCCCTTCG | | 22.5 µl | 0.5 µM |
| ***E.coli*** | 4 | E.coli-REV | CATCACCCGAAGATGAGTTTT | ITS | 22.5 µl | 0.9 µM |
| (DNA) | | E.coli-probe | 6-FAM-TTGCTGGTTTGTGAGTGAAAGTCGCC-BHQ-1 | (148 bp) | 11.25 µl | 0.25 µM |
| | | E.faec-FWD | TGTGGCAACAGGGATCAAGA | | 22.5 µl | 0.5 µM |
| ***E.faecalis*** | 5 | E.faec-REV | CATAACCAGCATTTTCAGCGAT | groES | 22.5 µl | 0.9 µM |
| (DNA) | | E.faec-probe | 6-FAM-TCGTTCGTGCATTAGAAGA-BHQ1 | (77 bp) | 11.25 µl | 0.25 µM |
| | | G54763F | TGATCGTGCCAGCATCGA | | 12,5 µl | 500 nM |
| **MNV** | 6, 7 | G54863R | GTTGGGAGGGTCTCTGAGCAT | polyprotein | 22,5 µl | 900 nM |
| (RNA) | | G54808P | 6-FAM-CTACCCACCAGAACCCCTTTGAGACTC-BHQ1 | (101 bp) | 25 µl | 250 nM |

*Volume of starting stock solution for qPCR mix of primer or probe to prepare **100 µl** of working solution (adjust to 100µl with DNA-RNA free water). A starting stock solution of **100 µM** for both primer and probe is assumed.

## 3.3. Parameters

**Equipment:**

**qPCR platform**
**Centrifuge**
**96 well qPCR reaction plate corresponding to qPCR platform**

| qPCR Platform | 96 well plate | adhesive cover foil | adhesive cover starter kit |
|---|---|---|---|
| ABI PRISM®HID 7700 SDS | Applied Biosystems, 4306737 | Applied Biosystems, 4311971 | Applied Biosystems, 4313663 |
| LightCycler® 480 System | Roche, 4729692001 | Roche, 4729692001 | - |

**Optical caps + corresponding installing tool** *(optional)* ☐
**(Applied Biosystems, Cat. No. 4323032 and 4330015)** ☐
**DNA LoBind tubes (Eppendorf, Cat. No. 0030108051)** ☐
**Micropipettes + filter tips** ☐

**Reagents:**

*For qPCR* (HAdV, *E.coli*, *E.faecalis*)*:*
**TaqMan Environmental PCR Master Mix (Applied Biosystems, 4396838)** ☐

*For qRT-PCR* (*MNV, MS2*)*:*
**RNA Ultrasense™ One-Step Quantitative RT-PCR System (Invitrogen, 11732-927)** ☐
**All solutions for qPCRs should be prepared in DNA LoBind tubes.**

**Master mix preparation**

Prepare master mix freshly in a clean separated area on ice.
**To avoid pipetting inaccuracies it is recommended to prepare 0.10x additional master mix solution.**

*Master mix for:*

HAdV, MNV, MS2, *E.coli*, *E.faecalis*

| qPCR Master Mix | DNA | RNA |
|---|---|---|
| Environmental Mix 2x | 12.5 µl | ------ |
| Ultrasense Mix 5x | ------ | 5 µl |
| Primer Forward | 1 µl | 1 µl |
| Primer Reverse | 1 µl | 1 µl |
| Probe | 0.5 µl | 0.25 µl |
| ROX (dil 1/10) | ------ | 0.5 µl |
| Enzyme Mix | ------ | 1.25 µl |
| $H_2O$ | ------ | 11 µl |
| TOTAL VOLUME OF MIX | 15 µl | 20 µl |
| Sample | 10 µl | 5 µl |

**LightCycler480**

| qPCR Master Mix | DNA | RNA |
|---|---|---|
| Environmental Mix 2x | 12.5 µl | ------ |
| Ultrasense Mix 5x | ------ | 5 µl |
| Primer Forward | 1 µl | 1 µl |
| Primer Reverse | 1 µl | 1 µl |
| Probe | 0.5 µl | 0.25 µl |
| BSA (20x) | ------ | 1.25 µl |
| Enzyme Mix | ------ | 1.25 µl |
| $H_2O$ | ------ | 10.25 µl |
| TOTAL VOLUME OF MIX | 15 µl | 20 µl |
| Sample | 10 µl | 5 µl |

For qPCR of DNA virus add 15 µl of master mix, for qRT-PCR of RNA virus add 20 µl of master mix to a 96 well qPCR reaction plate kept on ice.

**Analysis of target**

Add 10 µl of extracted DNA or 5 µl of extracted RNA template to each well. Cover wells and spin plate to ensure that no liquid is attached to the sides of the wells.

*Standard curves used for qPCR and qRT-PCR are generated by using serial dilutions of:*

*Virus and protozoa:* known amounts of a synthetic DNA construct (gBlocks® Gene Fragments, *part II*, 3.1.2.) containing the target region of the PCR.

*Bacteria:* known concentration of extracted bacteria overnight culture (e.g. $10^8$ to $10^1$ CFU/mL) (also see *part II*, 3.1.)

**Controls**

*Negative process control (NPC):*
Add 10 µl of extracted DNA or 5 µl of extracted RNA of negative process control extraction to each well. If positive results are obtained, samples belonging to corresponding concentration batch should not be used for calculations.

*Non-template control (NTC):*
**Two wells per plate set-up** containing master mix and 10 µl of molecular grade water for DNA and 5µl for RNA. NTC proves that master mix itself does not show any fluorescent signal. An amplification signal (distinguishable from background fluorescence) indicates cross-contamination or nonspecific amplification.

*Negative extraction control:*
**Two wells per concentration/extraction set-up** containing master mix and 5 µL (RNA) or 10 µl (DNA) extracts from the negative extraction control (a sample processed during the NA extraction part, where water has been added instead of pathogen).

*Positive NA extraction control:*
**Two wells per concentration/extraction set-up** containing master mix and 5 µL (RNA) or 10 µl (DNA) of extracts from the positive extraction control (a sample processed during the NA extraction part, where pathogen has been added ideally in the same amount as was spiked in the test samples).

*Standard curve:*
To determine the recovered pathogen, a standard curve of pathogen RT-qPCR or qPCR is generated by amplifying **duplets** of 10-fold dilutions of the positive NA extraction control of the pathogen.

*Enzymatic inhibition control:* (suggested)
It is advisable to evaluate potential enzymatic inhibition due to inhibitors present in each of the different environmental matrices evaluated.

*For DNA:*

1 µl of reference suspension containing $10^5$GC/10 µl in 10 µl of water

1 µl of reference suspension containing $10^5$GC/10 µl in 10 µl of sample

1 µl of reference suspension containing $10^5$GC/10 µl in 10 µl of sample diluted 1:10

*For RNA:*

1 µl of reference suspension containing $10^5$GC/10 µl in 5 µl of water

1 µl of reference suspension containing $10^5$GC/10 µl in 5 µl of sample

1 µl of reference suspension containing $10^5$GC/10 µl in 5 µl of sample diluted 1:10

*Process control (MS2):*

Extract 140 µl of the stock suspension of MS2 phage used to spike all processed samples. A quantification of MS2 per sample will be performed to estimate virus recovery/extraction efficiency. If negative results are obtained or concentration efficiency is lower than <0.1% results obtained for this sample should be discarded.

**Standard solution (dsDNA)**

In a separate area add $10^1$, $10^2$, $10^3$, $10^4$ and $10^5$ GC/10 µl per reaction for DNA or $5x10^1$, $5x10^2$, $5x10^3$, $5x10^4$ and $5x10^5$ GC/5 µl for RNA of the dsDNA control (gBlocks® Gene Fragments, *part II*, 3.1.2.) previously diluted in a suitable buffer (e.g. TE buffer). Use a micropipette exclusively used for this purpose. Add the reference solution in duplicates or triplicates.

Example of a qPCR 96 well template:

plate 1

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1(DNA) 5(RNA) | 1(DNA) 5(RNA) | 1e+1(DNA) 5e+1(RNA) | 1e+1(DNA) 5e+1(RNA) | 1e+2(DNA) 5e+2(RNA) | 1e+2(DNA) 5e+2(RNA) | 1e+3(DNA) 5e+3(RNA) | 1e+3(DNA) 5e+3(RNA) | 1e+4(DNA) 5e+4(RNA) | 1e+4(DNA) 5e+4(RNA) | 1e+5(DNA) 5e+5(RNA) | 1e+5(DNA) 5e+5(RNA) |
| B | 1e+6(DNA) 5e+6(RNA) | 1e+6(DNA) 5e+6(RNA) | NTC | NTC | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | SMF 1 | SMF 1 | SMF 1/10 | SMF 1/10 | SMF 2 | SMF 2 | SMF 2/10 | SMF 2/10 | SMF 3 | SMF 3 | SMF 3/10 | SMF 3/10 |
| F | SMF 4 | SMF 4 | SMF 4/10 | SMF 4/10 | SMF 5 | SMF 5 | SMF 5/10 | SMF 5/10 | MAF 1 | MAF 1 | MAF 1/10 | MAF 1/10 |
| G | MAF 2 | MAF 2 | MAF 2/10 | MAF 2/10 | MAF 3 | MAF 3 | MAF 3/10 | MAF 3/10 | MAF 4 | MAF 4 | MAF 4/10 | MAF 4/10 |
| H | MAF 5 | MAF 5 | MAF 5/10 | MAF 5/10 | Inoculum High | Inoculum High | Inoculum High/10 | Inoculum High/11 | CNE | CNE | CNE/10 | CNE/10 |

| Plate 2 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 1(DNA) 5(RNA) | 1(DNA) 5(RNA) | 1e+1(DNA) 5e+1(RNA) | 1e+1(DNA) 5e+1(RNA) | 1e+2(DNA) 5e+2(RNA) | 1e+2(DNA) 5e+2(RNA) | 1e+3(DNA) 5e+3(RNA) | 1e+3(DNA) 5e+3(RNA) | 1e+4(DNA) 5e+4(RNA) | 1e+4(DNA) 5e+4(RNA) | 1e+5(DNA) 5e+5(RNA) | 1e+5(DNA) 5e+5(RNA) |
| B | 1e+6(DNA) 5e+6(RNA) | 1e+6(DNA) 5e+6(RNA) | NTC | NTC | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | SMF 6 | SMF 6 | SMF 6/10 | SMF 6/10 | SMF 7 | SMF 7 | SMF 7/10 | SMF 7/10 | SMF 8 | SMF 8 | SMF 8/10 | SMF 8/10 |
| F | SMF 9 | SMF 9 | SMF 9/10 | SMF 9/10 | SMF 10 | SMF 10 | SMF 10/10 | SMF 10/10 | MAF 6 | MAF 6 | MAF 6/10 | MAF 6/10 |
| G | MAF 7 | MAF 7 | MAF 7/10 | MAF 7/10 | MAF 8 | MAF 8 | MAF 8/10 | MAF 8/10 | MAF 9 | MAF 9 | MAF 9/10 | MAF 9/10 |
| H | MAF 10 | MAF 10 | MAF 10/10 | MAF 10/10 | Inoculum Low | Inoculum Low | Inoculum Low/10 | Inoculum Low/11 | CNE | CNE | CNE/10 | CNE/10 |

## qPCR program

qPCR or RT-qPCR are performed on an adequate system selecting the appropriate parameters. Different qPCR systems can be used to run qPCR or RT-qPCR respectively. The table below displays the institutes and systems on which qPCRs or qRT-PCRs for certain pathogen were established.

| pathogen | established qPCR system | institute |
|---|---|---|
| HAdV<br>MS2 | ABI PRISM®HID 7700 SDS (Applied Biosystems) & Mx3000P (Stratagene) | UB |
| MNV | StepOne Plus (Applied Biosystems) | DTU |
| *E.coli*<br>*E.faecalis* | LightCycler480 (Roche) | TUM |

*qPCR parameters (TaqMan assay)*

| Pathogen | Cycles | Temperature [°C] | Time [min] |
|---|---|---|---|
| HAdV | 1 | 95 | 10:00 |
| | 40 | 95 | 00:15 |
| | | 60 | 01:00 |
| MS2 | *following the RT-qPCR (60 min at 55 °C)* | | |
| | 1 | 95 | 05:00 |
| | 40 | 95 | 00:15 |
| | | 60 | 01:00 |
| | | 65 | 01:00 |
| MNV | *following the RT-qPCR (60 min at 55 °C)* | | |
| | 1 | 95 | 05:00 |
| | 45 | 95 | 00:15 |
| | | 60 | 01:00 |
| | | 65 | 01:00 |

| Pathogen | Cycles | Temperature [°C] | Time [min] |
|---|---|---|---|
| *E.coli* | 1 | 95 | 10:00 |
| | 45 | 95 | 00:15 |
| | | 60 | 01:00 |
| *E.faecalis* | 1 | 95 | 10:00 |
| | 45 | 95 | 00:15 |
| | | 60 | 01:00 |

## c. References

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## d. Experimental overview

**Provided pathogens**

DTU → MNV
UB → HAdV, MS2 → TUM
UPV → *A.castellanii*, *H.pylori*

**Sample conditions:**
- Viral stocks of **10 mL** with **$10^9$particles/mL**
- *A.castellanii* and *H.pylori* stocks of **10 mL** with **$10^9$cells/mL**

**Spiking of 500 mL waste water per concentration with all pathogens**

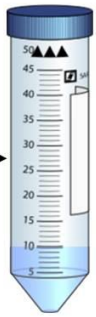
MNV
HAdV
MS2
*A.castellanii*
*H.pylori*
*E.coli* (provided by TUM)
*E.faecalis* (provided by TUM)

Different concentrations of pathogens [cells/10 L] or [particles/10 L]: **$10^8$, $10^6$, $10^0$**

**For 1x$10^8$cells or particles/10 L:**
465 mL waste water
+ 5 mL MNV [$10^9$particles/mL]
+ 5 mL HAdV [$10^9$particles/mL]
+ 5 mL MS2 [$10^9$particles/mL]
+ 5 mL *A.Castellanii* [$10^9$cells/mL]
+ 5 mL *H.pylori* [$10^9$cells/mL]
+ 5 mL *E.coli* [$10^9$cells/mL]
+ 5 mL *E.faecalis* [$10^9$cells/mL]

**For 1x$10^6$cells or particles/10 L:**
465 mL waste water
+ 5 mL MNV [$10^7$particles/mL]
+ 5 mL HAdV [$10^7$particles/mL]
+ 5 mL MS2 [$10^7$particles/mL]
+ 5 mL *A.Castellanii* [$10^7$cells/mL]
+ 5 mL *H.pylori* [$10^7$cells/mL]
+ 5 mL *E.coli* [$10^7$cells/mL]
+ 5 mL *E.faecalis* [$10^7$cells/mL]

**10 mL aliquots of each concentration sent to partner institutes**
(will be sent at 4°C;
store at **4°C** for **max. 1 week**)



**Partner institute get:**
*for MAF:*
**6**x $10^8$ aliquots (1x extra for evaluation of inoculated pathogens)
**6**x $10^6$ aliquots (1x extra for evaluation of inoculated pathogens)
3x $10^0$ aliquots
*for SMF:*
**6**x $10^8$ aliquots (1x extra for evaluation of inoculated pathogens)
**6**x $10^6$ aliquots (1x extra for evaluation of inoculated pathogens)
3x $10^0$ aliquots

**Spiking of 10 L evian water**


**# samples of 10 L spiked Evian water:**
13 samples for MAF concentration
(5x $10^8$, 5x $10^6$, 3x $10^0$)
13 samples for SMF concentration
(5x $10^8$, 5x $10^6$, 3x $10^0$)

2x $10^8$ as well as 2x $10^6$ aliquots should be left over for evaluation of inoculated pathogens

---

**10 L spiked evian water**


13 samples
13 samples

**Concentration of pathogens at each partner institute**

**MAF + CeUF** *(MAF SOP TUM)*

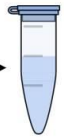
$V_{sample}$ = 10 L → (MAF) 20 mL → (centrifugal ultrafiltration) 1 mL

**SMF** *(SMF SOP UB)*

**Extraction of nucleic acids**

UPV
Instituto de Ingeniería del Agua y Medio Ambiente

*A.castellanii*
*H.pylori*
sent to



concentration to $V_{final}$ = 1 mL

MNV
HAdV
MS2
*E.coli*
*E.faecalis*

| method | # concentrated samples | | |
|---|---|---|---|
| | $10^8$ | $10^6$ | $10^0$ (blank) |
| MAF | 5 | 5 | 3 |
| SMF | 5 | 5 | 3 |

UB and TUM (DTU aswell?!) send concentrated samples of ***H.pylori*** and ***A.castellanii*** to UPV for DNA extraction and qPCR analysis

**Harmonized SOPs for NA extraction**
*Kits:*
- Bacteria/protozoa:
  QIAamp DNA Mini Kit
  (*H.pylori*:
  GeneJet™ genomic DNA purification kit)
- Virus:
  QIAamp viral RNA Mini Kit

**Extracted nucleic acids**

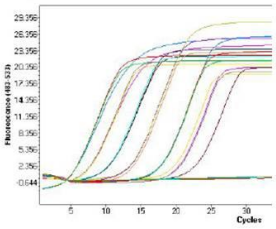
**Extracted nucleic acids of:**

- 13 samples for MAF concentration ($5x\ 10^8$, $5x\ 10^6$, $3x\ 10^0$)
- 13 samples for SMF concentration ($5x\ 10^8$, $5x\ 10^6$, $3x\ 10^0$)
- $2x\ 10^8$ aliquots (not spiked in Evian)
- $2x\ 10^6$ aliquots (not spiked in Evian)

**TaqMan-based qPCR analysis**

**qRT-PCR of MS2 and MNV** (SOP UB/DTU; based on RNA Ultrasense™ One-Step Quantitative RT-PCR System)

**qPCR of**
HAdV (SOP UB)
*A.Castellanii* (SOP UPV)
*H.Pylori* (SOP UPV) (SYBR Green)
*E.coli* (SOP TUM)
*E.faecalis* (SOP TUM)



**Harmonized SOPs for qPCR of certain pathogen**
SOPs should contain:

- primer and probe sequences
- qPCR parameters
- preparation of standard

UPV performs qPCRs of ***H.pylori*** and ***A.castellanii*** for concentrated samples of UB and TUM (aswell of DTU?!)

**Comparison of results between partner institutes**

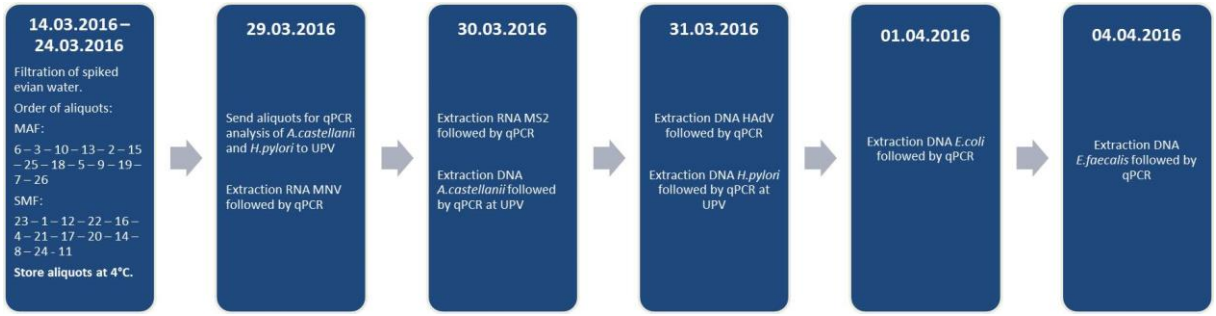
**vs.**

**MAF vs. SMF for certain pathogen:**

- recovery rates
- reproducibility

**Agreement of final concentration method used in project**

## e. Planned schedule (last update: 11.03.2016)




## f. Timetable sample concentration

**Note the date of sample concentration and send back to TUM**

| Extraction method | sample no. | date | | Extraction method | sample no. | date |
|---|---|---|---|---|---|---|
| MAF | 6 | | | SMF | 23 | |
| | 3 | | | | 1 | |
| | 10 | | | | 12 | |
| | 13 | | | | 22 | |
| | 2 | | | | 16 | |
| | 15 | | | | 4 | |
| | 25 | | | | 21 | |
| | 18 | | | | 17 | |
| | 5 | | | | 20 | |
| | 9 | | | | 14 | |
| | 19 | | | | 8 | |
| | 7 | | | | 24 | |
| | 26 | | | | 11 | |