

# Journal of Visualized Experiments

## VirWaTest, A Point-of-Use Method for the Detection of Viruses in Water Samples

--Manuscript Draft--

<b>Article Type:</b>	Methods Article - Author Produced Video
<b>Manuscript Number:</b>	JoVE59463R2
<b>Full Title:</b>	VirWaTest, A Point-of-Use Method for the Detection of Viruses in Water Samples
<b>Keywords:</b>	Human viruses; adenoviruses; hepatitis E virus; fecal pollution; viral concentration; viral detection; PCR; point-of-use; humanitarian; sanitation; outbreak
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<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$1200)

**TITLE:**

VirWaTest, A Point-of-Use Method for the Detection of Viruses in Water Samples

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**KEYWORDS:**

Human viruses, adenoviruses, hepatitis E virus, fecal pollution, viral concentration, viral detection, PCR, point-of-use, humanitarian, sanitation, outbreak.

**SUMMARY:**

Here we present VirWaTest, which is a simple, affordable and portable method for the concentration and detection of viruses from water samples at the point of use.

**ABSTRACT:**

Viruses excreted by humans and animals may contaminate water sources and pose a risk to human health when this water is used for drinking, food irrigation, washing, etc. The classical fecal bacteria indicator does not always check for the presence of viral pathogens so the detection of viral pathogens and viral indicators is relevant in order to adopt measures of risk mitigation, especially in humanitarian scenarios and in areas where water-borne viral outbreaks are frequent.

At present, several commercial tests allowing the quantification of fecal indicator bacteria (FIB) are available for testing at the point of use. However, such commercial tests are not available for the detection of viruses. The detection of viruses in environmental water samples requires concentrating several liters into smaller volumes. Moreover, once concentrated, the detection of viruses relies on methods such as nucleic acid extraction and molecular detection (e.g., polymerase chain reaction [PCR]-based assays) of the viral genomes.

The method described here allows the concentration of viruses from 10 L water samples, as well as the extraction of viral nucleic acids at the point of use, with simple and portable equipment. This allows the testing of water samples at the point of use for several viruses and is useful in humanitarian scenarios, as well as at any context where an equipped laboratory is not available. Alternatively, the method allows concentrating viruses present in water samples and the shipping of the concentrate to a laboratory at room temperature for further analysis.

## **INTRODUCTION:**

During the first phases of any humanitarian emergency, access to clean water supplies, sanitation, and hygiene are critical for the survival of those affected. Therefore, monitoring water quality is a priority to prevent waterborne outbreaks. It is well-known that contaminated water is frequently the origin of diseases, but it is often difficult to determine the sources of viral outbreaks such as Hepatitis E virus (HEV), even with the availability of conventional laboratory methods. The control of water quality is based on the quantification of FIB<sup>1-4</sup>. However, it has been extensively documented that there is no correlation between the absence of FIB and the presence of viral waterborne pathogens such as rotavirus (RoV), norovirus (NoV), or HEV<sup>5,6</sup>. Thus, using the water quality criteria based on FIB might result in an underestimation of risks associated with the presence of waterborne viral pathogens. The surveillance of indicator viruses, such as human adenoviruses (HAdV), or specific pathogens would be helpful in defining the exposure to viral pathogens and identifying the potential source of human infection<sup>7-10</sup> and in validating the efficacy of sanitation measures<sup>11</sup>.

Until now, the detection of viruses in these scenarios relied on skilled staff and complex logistics. VirWaTest ([virwatest.org](http://virwatest.org)) is aimed at the development of a simple, affordable, and portable method for the concentration and subsequent detection of viruses from water samples at the point of use.

The virus concentration is based on the principle of organic flocculation of 10 L water samples, by which viruses are recovered in smaller volumes<sup>12,13</sup>. The flocs are collected and added to a buffer that lyses the viruses and prevents the nucleic acids from degradation when they stored at room temperature for not more than 2 weeks.

The nucleic acid extraction method is based on the use of magnetic particles to which the nucleic acids get adsorbed. They can be transferred from one washing buffer to another and finally into the elution buffer by using a magnetic pipette to which the particles attach. Viral nucleic acid suspensions obtained can be shipped to a reference laboratory where the detection can be performed using molecular methods based on PCR. For each nucleic acid extraction, two different

quantities are tested to rule out enzymatic inhibition originated by the sample. Alternatively, with minimum equipment availability, PCR tests can be run at the point of use. The entire process is designed to be performed independently of a power supply (**Figure 1**).

A quantitative PCR assay to detect HAdV, excreted by humans and found in wastewater samples in high concentrations, has been adapted to be run at the point of use. HAdV are used as human fecal viral indicators. A PCR for the quantification of MS2 bacteriophage has been also adapted since MS2 is used in VirWaTest as process control. The method can be customized for the detection of any virus of interest.

After development, the VirWaTest method has been applied by the users in two different settings in the Republic of Central Africa (RCA) and Ecuador, providing feedback on the application of the protocol in real situations.

To our knowledge, this is the first procedure that allows the concentration and detection of viruses at the point of use, independent of any power supply, large equipment, and freezing/cooling conditions. It is recommended to collect two replicates of each water sample in order to obtain robust results.

## **PROTOCOL:**

### **1. Preparation and packaging**

NOTE: The materials/equipment to be packed is listed in **Table 1**. Use gloves to handle the reagents required for the process control, the concentration reagents, the nucleic acid extraction reagents, and the nucleic acid detection reagents. Wear protective glasses to handle the reagents required for nucleic acid extraction.

#### **1.1. Process control**

1.1.1. Prepare MS2 bacteriophage stock culture (American Type Culture Collection [ATCC] 15597-B1) containing  $1 \times 10^{10}$  PFU/mL in the laboratory by following the ISO procedure 10705-1:1995 to be added as process control<sup>14</sup>.

1.1.2. Then, aliquot 10  $\mu$ L of the suspension per tube containing  $1 \times 10^8$  PFU into 10 mL tubes and let the water evaporate at 37 °C. Use one tube per water sample.

1.1.3. Additionally, prepare one tube containing 10 mL of sterile distilled water per collected sample.

#### **1.2. Concentration reagents**

1.2.1. For the preflocculated skimmed milk solution (PSM), use the following amounts to concentrate five 10 L water samples.

1.2.1.1. Prepare a plastic tube containing 5 g of skimmed milk.

1.2.1.2. Prepare a zipper plastic bag containing 16.66 g of sea salts.

1.2.1.3. Prepare a plastic bottle containing 500 mL of distilled water.

1.2.2. Prepare the reagents required for pH adjustment. Use the following amounts to adjust the pH of the PSM and of the five 10 L water samples.

1.2.2.1. Add 5 g of citric acid 1-hydrate to a 10 mL plastic tube.

CAUTION: Citric acid causes serious irritation when it comes in contact with eyes. If this occurs, rinse the eye cautiously with water for several minutes. If irritation persists, seek medical advice.

1.2.2.2. Put 2 g of sodium hydroxide into a 10 mL plastic tube.

CAUTION: Sodium hydroxide can cause severe skin burns and eye damage. If swallowed, rinse the mouth. Do not induce vomiting. If it comes in contact with the eyes, rinse cautiously with water for several minutes. Then, seek medical advice.

1.2.2.3. Put 25 mL of sterile distilled water into a plastic container.

1.2.2.4. Put 50 mL of sterile distilled water into a plastic container.

1.2.3. Prepare the sample conditioning sachet used for the flocculation, the preservative solution used to preserve the nucleic acids, and the neutralizing sachet to neutralize the discarded water and materials. Use the following amounts for each 10 L water sample to be tested.

1.2.3.1. Put 15 g of sea salts and 8 g of citric acid 1-hydrate into a zipper plastic bag for the conditioning sachet.

1.2.3.2. Add 2 mL of lysis buffer (**Table of Materials**) and 0.3 mL of nucleic acid preserving agent (**Table of Materials**) to a 5 mL tube.

CAUTION: Lysis buffer contains guanidine thiocyanate and Triton X-100. Contact with acid liberates toxic gas, and it is harmful if swallowed, inhaled, or comes in contact with the skin. If swallowed, rinse the mouth. Do not induce vomiting. If it comes in contact with the skin, wash with plenty of water. Then, seek medical advice. Nucleic acid preserving agent causes skin and eye irritation and is harmful if swallowed. If it comes in contact with the skin or the eyes, rinse with abundant water for several minutes. In any case, especially if swallowed and feeling unwell, seek medical advice.

1.2.3.3. Put 40 g of detergent powder into four zipper plastic bags.

CAUTION: Powder detergent causes eye irritation. If it comes in contact with the eyes, rinse with abundant water for several minutes. If irritation persists or if swallowed, seek medical advice.

### 1.3. Nucleic acid extraction reagents

1.3.1. Put 50  $\mu$ L of magnetic particles (**Table of Materials**) and 1 mL of ethanol 96% into a 5 mL tube for the binding buffer.

CAUTION: The magnetic particles buffer contains sodium azide, which forms a toxic gas when it comes in contact with acids or heavy metal ions and is toxic if ingested or when it comes in contact with skin. If ingested or in contact with skin, rinse the mouth or the skin with abundant water and seek medical advice. Ethanol is a highly flammable liquid and vapor and causes serious eye irritation. Keep away from heat, hot surfaces, sparks, and any other ignition source. If it comes in contact with the skin or the eyes, rinse with abundant water. In case of ingestion of large amounts of water and/or if inhaled, go outside into the fresh air. In any case, seek medical advice.

1.3.2. Add 500  $\mu$ L, 600  $\mu$ L, and 200  $\mu$ L of washing buffers 1, 2, and 3, respectively (**Table of Materials**), to three 2 mL tubes, to be used as washing buffers 1, 2, and 3.

CAUTION: Washing buffers contain guanidinium thiocyanate and guanidinium chloride, which are harmful if inhaled or swallowed and irritate the skin and eyes. Contact with acids releases very toxic gas. If they come in contact with the skin or eyes, rinse with abundant water. If swallowed, rinse the mouth with abundant water. Do not induce vomiting. Always seek medical advice.

1.3.3. Add 120  $\mu$ L of elution buffer (**Table of Materials**) to a 0.5 mL tube for the elution buffer.

### 1.4. HAdV and MS2 detection reagents

NOTE: Two different nucleic acid extraction reactions may be analyzed simultaneously in each PCR assay if an 8-well thermocycler is used. For all PCR assays, prepare molecular biology water aliquoted into 1.5 mL tubes.

#### 1.4.1. HAdV detection

1.4.1.1. Prepare a mix containing 10  $\mu$ L of 22.5  $\mu$ M AdF forward primer, 10  $\mu$ L of 22.5  $\mu$ M AdR reverse primer, and 5  $\mu$ L of 11.25  $\mu$ M AdP probe (**Table 2**).

1.4.1.2. Add 2.5  $\mu$ L of the mix to tubes 1 to 8 of a tube strip. Let it dry at room temperature, close the tubes, and keep them protected from the light.

1.4.1.3. Cut the strip dividing it into two strips: tubes 1–5 and tubes 6–8.

1.4.1.4. Prepare a serial dilution of DNA suspensions containing the nucleotide sequence of the amplified HAdV region. Air-dry 1 µL of suspensions containing  $1 \times 10^5$ ,  $1 \times 10^7$ , and  $1 \times 10^9$  GC/mL into tubes 6–8.

NOTE: Keep the tubes containing the primers, probe, and the air-dried suspensions protected from light.

#### **1.4.2. MS2 detection**

1.4.2.1. Prepare a mix containing 10 µL of 25 µM pecson-2F forward primer, 10 µL of 25 µM pecson-2R reverse primer, and 2.5 µL of 25 µM PecP-2 probe (**Table 2**).

1.4.2.2. Add 2.25 µL of the mix to tubes 1 to 8 of a tube strip. Let it dry at room temperature, close the tubes, and keep them protected from the light.

1.4.2.3. Cut the strip dividing it into two strips: tubes 1–5 and tubes 6–8.

1.4.2.4. Proceed as in step 1.4.1.4 but use a standard suspension designed for MS2 instead.

### **2. Viral concentration**

NOTE: Use gloves at all times during the sample collection, reagent preparation, flocculation, floc collection, and waste disposal procedures. Wear protective glasses during the reagent preparation procedure.

#### **2.1. Sample collection**

2.1.1. Collect a 10 L water sample in a flat bottom bucket with a lid. Collect a minimum of two replicates for each sample.

NOTE: It is important to use clear buckets to visualize the pellet. If the water sample contains suspended material (e.g., sand, algae), let it sediment and, then, collect the water supernatant in a new bucket.

2.1.2. Register the collected volume, as well as the location and collection date, for each water sample.

2.1.3. Put a magnetic stirrer connected to a battery under a bucket support and place the bucket containing the water sample on its support.

NOTE: Look for a flat surface in a fresh place and keep the buckets containing the water samples from direct sunlight.

#### **2.2. Reagents preparation**

### **2.2.1. pH adjustment reagents**

2.2.1.1. Pour the citric acid powder and the sodium hydroxide pellets into the pots containing 25 and 50 mL of distilled water, respectively.

2.2.1.2. Gently shake by hand until the citric acid and the sodium hydroxide are completely dissolved.

CAUTION: Do not pour the water over the sodium hydroxide pellets. Instead, pour the sodium hydroxide pellets over the water.

### **2.2.2. Preflocculated skimmed milk**

2.2.2.1. Place a stand-up bag on a magnetic stirrer and pour 500 mL of distilled water in it. Drop a stirring magnet inside the bag and turn on the magnetic stirrer.

2.2.2.2. Pour the contents of a sea salt sachet and of a skimmed milk tube into the stand-up bag and stir for 5 min at medium speed to dissolve them.

2.2.2.3. Add 3 mL of citric acid to the PSM solution using a Pasteur pipette to make sure the pH lies between 3.4 and 3.6. Measure the pH of the PSM solution using pH indicator strips. Add smaller amounts of citric acid and sodium hydroxide as the pH gets closer to 3.5.

2.2.2.4. Turn off the magnetic stirrer and make sure the flocs are visible. Use a flashlight to help to visualize the flocs.

### **2.3. Flocculation**

2.3.1. Drop a stirring magnet into the water, set the magnetic stirrer at maximum speed, and turn it on. Make sure the stirring magnet starts spinning.

2.3.2. Add 10 mL of distilled water to a process control tube. Invert the tube a few times to rehydrate the viral stock and pour the solution into the water.

2.3.3. Pour the contents of one sample conditioning sachet into the water and stir for 5 min.

2.3.4. Measure the pH of the water sample using pH indicator strips. Add a few drops of citric acid or sodium hydroxide to the water sample to make sure the pH lies between 3.4 and 3.6. Add smaller amounts of citric acid and sodium hydroxide as the pH gets closer to 3.5.

2.3.5. Add 100 mL of the PSM solution using a 100 mL container.

2.3.6. Seal the bucket with the lid, set the magnetic stirrer at minimum speed, and leave the



water stirring for 8 h.

2.3.7. Turn off the magnetic stirrer and let the water still for at least 5 h to allow the flocs to settle.

## **2.4. Floc collection**

2.4.1. Build a siphoning system composed of a pipette controller, two pipettes, and a plastic tube, to aspirate the water supernatant for discharging the water on the flocs.

2.4.1.1. Attach a 10 mL tape-end pipette to the pipette controller. Then, attach the tip of the pipette to the plastic tube.

2.4.1.2. Remove the filter of a 10 mL open-tip pipette using tweezers and attach the pipette to the plastic tube.

2.4.2. Place an empty bucket on the floor.

2.4.3. Immerse the tip of the open-tip pipette in the water. Make sure to keep it close to the water surface to avoid disturbing the flocs. Aspirate the water supernatant until it reaches the tape-end pipette.

2.4.4. Pinch the plastic tube by the end attached to the tape-end pipette and detach it from the pipette.

2.4.5. Place the tube in an empty bucket. Release the pressure on the tube to let the water flow into the empty bucket.

2.4.6. Pinch the plastic tube to stop the water flow when the water level is about to reach the stirring magnet and move the pipette away from the bucket.

2.4.7. Shake the bucket to resuspend the flocs and pour them into a 500 mL stand-up plastic bag. Allow the flocs to settle for 1 h more.

2.4.8. Carefully aspirate the water supernatant using a 50 mL pipette and a manual pipette controller, avoiding disturbing the flocs.

2.4.9. Aspirate the flocs using a 100 mL pipette and transfer them to a 50 mL graduated centrifuge tube. Note down the final volume of sample concentrate and transfer 1 mL of it to a 5 mL tube containing the preservative solution, using a Pasteur pipette.

NOTE: It is important that the centrifuge tube is graduated so the final volume of the concentrated sample can be measured as accurately as possible and registered.

2.4.10. Perform the nucleic acid extraction in the field. Alternatively, store the preservative solution containing the viruses at 20–30 °C for up to 15 days or ship them to a reference laboratory for further analysis.

## **2.5. Waste disposal and material reuse**

2.5.1. Add the contents of a neutralizing agent sachet to the bucket containing the discarded water. Mix the water, and then, leave it still for 30 min.

2.5.2. Dispose of the treated water as general waste.

2.5.3. Place the pipettes and the plastic tube inside the bucket. Fill the bucket with water and pour in the contents of one neutralizing agent sachet.

2.5.4. Wash them for at least 30 min to sterilize them and rinse them with abundant clean water to remove any remaining neutralizing agent.

## **3. Nucleic acid extraction**

NOTE: Use gloves and always wear protective glasses during the nucleic acid extraction procedure.

### **3.1. Magnetic pipette operation**

3.1.1. Get familiarized with the operation of the magnetic pipette before performing the extraction.

### **3.2. Nucleic acid extraction**

3.2.1. Arrange the tubes containing the reagents required for the extraction in a rack, from left to right: binding buffer, washing buffers, and elution buffer. Set the appropriate number of tubes according to the number of samples or replicates being analyzed.

3.2.2. Transfer 1 mL of sample concentrate to the tube containing the binding buffer, using a disposable Pasteur pipette. Invert the tube 8x to 10x to homogenize the solution.

3.2.3. Incubate the solution at room temperature for 10 min while continuously mixing, either using a solar-powered orbital or manually.

3.2.4. Collect the magnetic particles using the magnetic pipette and a clean tip, which can be reused for the three washing buffers.

3.2.5. Release the magnetic particles into the tube containing 500 µL of washing buffer 1. Wash them by gently shaking the solution with the tip for 30 s and collect them.

3.2.6. Transfer the magnetic particles to the tube containing 600 µL of washing buffer 2. Wash them by gently shaking the solution with the tip for 30 s and collect them.

3.2.7. Transfer the magnetic particles to the tube containing 200 µL of washing buffer 3. Wash them by gently shaking the solution with the tip for 30 s and collect them.

3.2.8. Release the magnetic particles into the tube containing the elution buffer and discard the tip.

3.2.9. Incubate the solution at room temperature for 5 min while continuously mixing, using the solar-powered orbital.

NOTE: Mixing the magnetic particles in the elution buffer is a crucial step. In case of lacking an orbital, continuously mix by hand during the incubation time.

3.2.10. Replace the tip on the magnetic pipette by a new one and collect the magnetic particles from the elution buffer. Make sure to completely remove the magnetic particles from the elution buffer since they can interfere with molecular detection methods.

3.2.11. Discard the tip with the particles attached to it. Proceed with the PCR analysis, ship the elution buffer to a reference laboratory, or store the elution buffer in at 4 °C for further analysis.

#### **4. Viral detection with a battery-operated eight-tube real-time thermocycler**

NOTE: Use gloves and always wear protective glasses during the nucleic acid detection procedure. Replace the gloves for new ones when performing a new PCR experiment to avoid cross-contamination.

##### **4.1. HAdV quantitative PCR**

4.1.1. Add 14 µL of nuclease-free water to tubes 2, 4, and 5.

4.1.2. Add 4 µL of PCR mix to tubes 1–5.

4.1.3. Add 14 µL of nucleic acid extraction from sample A to tube 1 and 14 µL from sample B to tube 3.

4.1.4. Add 2 µL of extracted nucleic acids from sample A to tube 2 and 2 µL from sample B to tube 4. Close the five-tube strip.

4.1.5. Add 14 µL of nuclease-free water to tubes 6, 7, and 8.

4.1.6. Add 4 µL of this mix to tubes 6, 7, and 8, and close them.

441  
442 4.1.7. Put both strips in the thermocycler and select the appropriate run (**Table 3**).

443  
444 4.1.8. Discard the water tube and keep the extracted nucleic acid in the freezer, if possible, or, if  
445 not, in a fresh place protected from light in case a second test needs to be performed.

446  
447 4.1.9. Clean the micropipettes, the rack, the working material, and all material properly with a  
448 nucleic acid cleaner, and discard the plastic bag containing the used tip, gloves, and tubes.

449  
450 CAUTION: Nucleic acid remover is a very flammable liquid and steam. Do not breathe in the spray  
451 mist and avoid any contact of the liquid with the eyes or the skin. Otherwise, immediately rinse  
452 with abundant water and seek medical advice.

453  
454 4.1.10. Collect the results and analyze the obtained data.

## 455 **4.2. MS2 quantitative PCR**

456  
457  
458 4.2.1. Add 14 µL of nuclease-free water to tubes 2, 4, and 5.

459  
460 4.2.2. Add 4 µL of PCR mix and 0.5 µL of reverse transcriptase enzyme to tubes 1–5.

461  
462 4.2.3. Add 14 µL of nucleic acid extraction from sample A to tube 1 and 14 µL from sample B to  
463 tube 3.

464  
465 4.2.4. Add 1 µL of extracted nucleic acids from sample A to tube 2 and 1 µL from sample B to tube  
466 4. Close the five-tube strip.

467  
468 4.2.5. Add 15.5 µL of nuclease-free water to tubes 5, 6, 7, and 8, and close them.

469  
470 4.2.6. Add 4 µL of PCR mix and 0.5 µL of reverse transcriptase enzyme to tubes 6, 7, and 8.

471  
472 4.2.7. Put both strips in the thermocycler and select the appropriate run (**Table 3**).

473  
474 4.2.8. Proceed as described in steps 4.1.8 to 4.1.10.

## 475 **REPRESENTATIVE RESULTS:**

### 476 **Method development**

477  
478 This procedure has been developed in the Laboratory of Viruses Contaminants of Water and Food  
479 with the collaboration of GenIUL and Oxfam Intermón. It comprises of three different steps. The  
480 first one, the viral particle concentration, is an adaptation of a skimmed milk flocculation method  
481 previously described<sup>12,17,18</sup>. The original method was modified as to be independent of a power  
482 supply, simpler, and without centrifugation steps.  
483  
484

The recovery of the VirWaTest concentration method was tested in HAdV and MS2 bacteriophage-spiked groundwater samples. The viral recovery of the VirWaTest concentration and extraction method was estimated to be 3.01% to 18.02% for MS2 and 17.52% to 44.22% for HAdV. These recoveries were calculated from the values obtained by quantitative PCR during the development of the method compared to the initial concentrations of HAdV and MS2 in the water samples after spiking them with known concentrations of these viral stocks.

The VirWaTest magnetic nucleic acid extraction was compared to a commercial RNA mini kit (e.g., QIAamp Viral RNA Mini Kit), a column-based extraction method used in the laboratory, by testing 33 river and groundwater samples spiked with HAdV and MS2 and concentrated by skimmed milk flocculation. The comparison results showed that the VirWaTest method recovery was higher in 23/33 cases for HAdV, as well as for MS2 (**Table 4**). A Wilcoxon test showed *p*-values of 0.0005569 for HAdV and 0.02791 for MS2. VirWaTest nucleic acid extraction provides significantly higher viral recoveries than the commercial one.

#### **Detection of HAdV in environmental water samples concentrated by the VirWaTest method**

To test the developed method for viral concentration in the field, the Oxfam Water, Sanitation and Hygiene (WASH) team, located in Banghi (RCA) in March 2017, collected and concentrated viruses from five well water samples.

Also, in the area of Pedernales (Ecuador), which was affected by earthquakes in 2016 and 2017, six well water samples were collected by an Oxfam WASH team in February 2017, and its viruses were concentrated by the VirWaTest concentration method. Viral concentrates from both settings were sent to the laboratory in Barcelona for VirWaTest nucleic acid extraction and viral quantification.

In Ecuador, naturally occurring HAdV were detected in six out of the six samples analyzed, with concentration values ranging from  $3.27 \times 10^1$  to  $1.80 \times 10^2$  GC/L, whereas one out of the five samples collected and concentrated in Banghi (RCA) tested positive for HAdV, at a concentration of  $3.46 \times 10^2$  GC/L (**Table 5**).

MS2, added to all tested samples as internal process control, was detected in all samples tested, showing that the method was correctly performed from concentration to detection.

#### **FIGURE AND TABLE LEGENDS:**

**Figure 1: VirWaTest method.** Overview of the steps the VirWaTest method is composed of.

**Table 1: VirWaTest contents.** Equipment, consumables, and reagents that have to be prepared for the concentration, extraction, and detection of viruses in two water samples/replicates.

**Table 2: Oligonucleotides for quantitative PCR experiments.** Primers and probe designed to bind to nucleic acid sequences of HAdV and MS2.

**Table 3: Thermal conditions for quantitative PCR experiments.** Temperature, time, and cycles used for HAdV and MS2 amplification.

**Table 4: VirWaTest nucleic acid extraction development.** Median, mean, and SD values obtained when comparing the Qiagen and VirWaTest extraction methods in 33 groundwater samples for HAdV and MS2.

**Table 5: Quantification of HAdV using the VirWaTest Method.** Quantitative PCR results, expressed in GC/liter, for HAdV quantified in water samples collected and concentrated from RCA and Ecuador.

#### **DISCUSSION:**

The VirWaTest method enables the concentration of viruses and nucleic acid extraction from water samples at the point of use by non-experienced users. It is an affordable, rapid, and simple protocol. The concentration is based on the principle of organic flocculation using skimmed milk, by which the low pH and high conductivity conditions make skimmed milk proteins aggregate into flocs the viruses adsorb to. When the flocs sediment, it is easy to collect them, making it possible to concentrate 10 L of water, whereas traditional ultracentrifugation cannot deal with big water volumes.

The method has been modified for being practicable at the point of sampling without using any electric equipment except for a battery-operated magnetic stirrer. Some other approaches based on water filtration have been adapted to the concentration of viruses and can also be performed at the point of use. However, suspended material present in water samples often clogs the filters; thus, the small volume that may be concentrated with these systems is a serious limitation.

This is the first description of a method for concentrating viruses from water samples at the point of use, regardless of the turbidity of the sample. The VirWaTest concentration method allows several samples to be processed simultaneously if the appropriate material is available. Moreover, skimmed milk flocculation has been shown to be useful for bacteria and parasite concentration<sup>19</sup>.

The preparation of the appropriate material is crucial for performing the procedure properly. Before moving the samples to where they are needed, their number should be considered, for the preparation of the reagents and material. Several samples can be processed at the same time if the appropriate amount of material is available.

Before starting the concentration of a water sample, a known concentration of a viral stock will be used to spike the sample as process control. This method uses a dried viral stock which is rehydrated with distilled water before being added to the water sample at the point of use. This is useful to rule out false-negative results at the end of the procedure and gives an indication of the performance of the method.

Viral concentrates obtained with the VirWaTest may be further tested in the field applying the

VirWaTest nucleic acid extraction and detection methods or, alternatively, concentrates may be sent to a reference laboratory at room temperature. The preservative solution added to the concentrate enables viruses to remain stable for up to 2 weeks (unpublished results).

VirWaTest nucleic acid extraction is a magnetic particle-based method. It is easy and fast and allows several samples to be processed at the same time and shows equivalent and even better recovery efficiency than methods currently used for viral nucleic acid extractions. The nucleic acids may be sent to reference labs at room temperature or, if users are confident in performing molecular detection, a quantitative PCR assay can be performed at the point of use of the original water sample.

Also, if a small laboratory facility is available, the VirWaTest concentration protocol may be coupled to standard nucleic acid extraction kits that depend on a centrifuge and standard PCR-based methods that require a freezer to maintain the reagents but that use standard thermocyclers, which are less expensive than battery-operated ones.

However, since a power supply is sometimes not available, we have optimized detection assays for MS2 bacteriophages as process control and HAdV as a viral fecal indicator, and the methodology can be customized for any other virus of interest, such as hepatitis viruses, RoV, NoV, or others, to be run in the field without needing a freezer for maintenance of the reagents, nor conventional thermocyclers but only a battery-operated one. Several battery-operated thermocyclers are commercially available. Alternatively, if a power supply is available, other conventional qPCR equipment may be used. If an eight-tube thermocycler is used, up to two nucleic acid extractions (two different samples or two replicates from the same sample) can be tested in the same PCR assay. For each nucleic acid extraction, two different quantities will be tested to rule out enzymatic inhibition originated by the sample. The adaptation is based on the previous preparation of PCR tubes by air-drying primers, probes, and standard suspensions. Several lyophilized commercial qPCR solutions exist that could be used by applying the same procedure as described here. We have described one possibility that we considered to be easy to perform.

Comparison assays performed during the development of the method showed that the VirWaTest methods of concentration, extraction, and detection are efficient for the quantification of viruses in water samples.

The limit of detection (LOD) of the described procedure is variable since the volume collected after concentration is variable. Also, the LOD can be slightly different for different viruses. If a volume of approximately 10 mL is collected, the LOD for HAdV would be around  $1 \times 10^2$  viral GC/L. So, relatively small concentrations of HAdV can be detected by the VirWaTest method. In Pedernales (Ecuador), six out of the six samples tested presented HAdV, in concentrations close to the LOD, ranging from  $3.27 \times 10^1$  to  $1.80 \times 10^2$  GC/L. In Banghi (RCA), HAdV were detected in one out of the five samples analyzed, at a concentration of  $3.46 \times 10^2$  GC/L. The presence of HAdV, as well as the presence of MS2 as the control process in the tested samples, shows the method is useful for the recovery of viral particles from water samples, even when performed by

nonexperienced users.

Feedback obtained until now indicates that viral concentration is an easy procedure to perform, with no major problems when applied at the point of use of the samples, although 8 h and 5 h steps are required. It is important to note that these steps occur without any human assistance. Moreover, a faster method based on filtration is being developed as an alternative for nonturbid waters. However, flocculation seems to be, until now, the unique method that allows the concentration of samples presenting high turbidity. Viral concentrates may be then sent to any laboratory around the world at room temperature, which also makes it easier to test viruses since sampling areas do not always have good transportation services providing cooling conditions. Alternatively, the extraction and detection protocols presented here allow testing for viral detection at the point of use of the samples.

As far as we know, this is the first method reported to be useful for the concentration and testing for the presence of viruses in water samples in the field. Further efforts should be conducted to apply the procedure to the evaluation of the presence of human viral pathogens of interest in several other humanitarian crisis scenarios. Also, the user's feedback will be necessary to provide insights into the potential implementation to make the procedure friendlier.

#### **ACKNOWLEDGMENTS:**

VirWaTest was a research project funded by the HIF (Humanitarian Innovation Funds) program of ELHRA (Enhancing Learning & Research for Humanitarian Assistance). The authors acknowledge the WASH teams who kindly collaborated in this study. The analysis of samples in Ecuador was funded by Oxfam Ecuador and Dirección de Investigaciones de la Universidad de las Americas (AMB.BRT.17.01). S. Bofill-Mas is a Serra-Hunter fellow at the University of Barcelona.

#### **DISCLOSURES:**

The authors have nothing to disclose.

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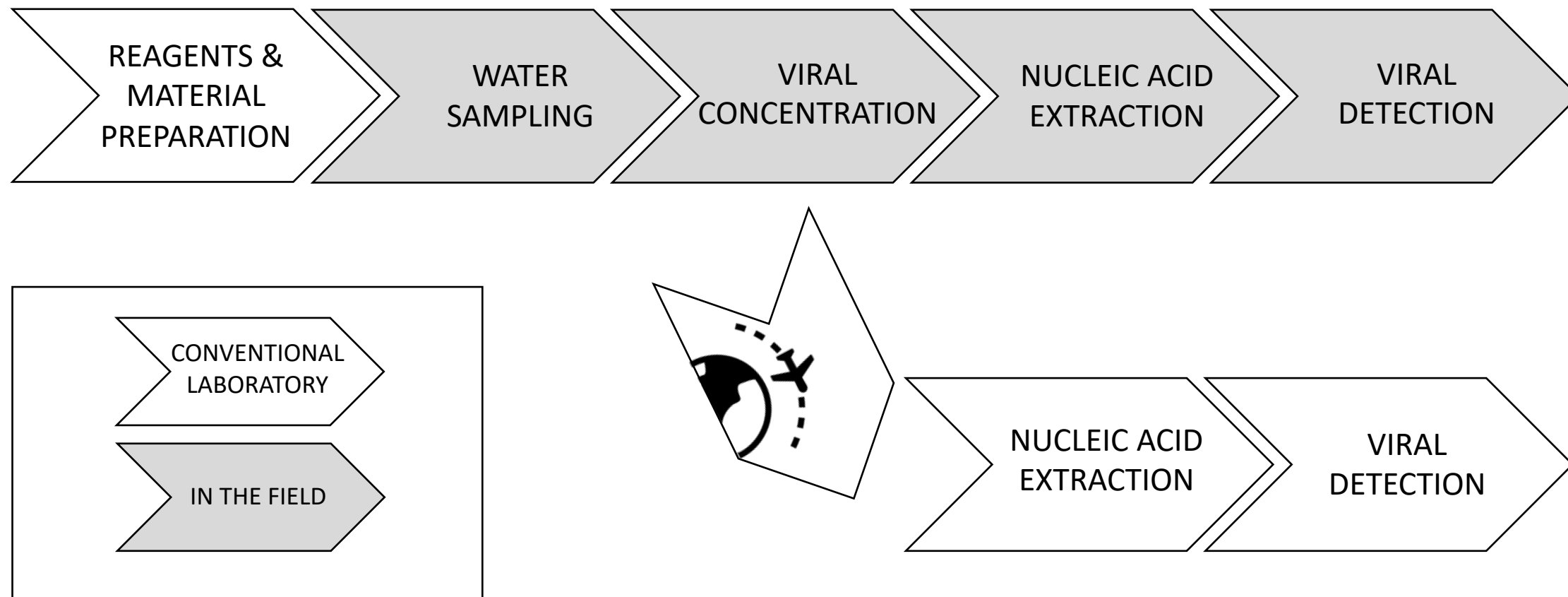


Table 1

Materials	Concentration	Nucleic Acid Extraction	Detection
Equipment	Magnetic Stirrer (2 Units)	Magnetic Pipette (1 Unit)	Thermocycler (1 Unit)
	Battery (2 Units)	Solar Rotary Platform (1 Unit)	Battery (1 Unit)
	Battery/Stirrer Connectors (2 Units)	Multi-Size Tube Rack (1 Unit)	Computer
	Power Adapters (2 Units)	Timer (1 Unit)	Tube Rack (1 Unit)
	Bucket Support (2 Units)		0.5 µL - 10 µL Micropipette (1 Unit)
	Rope (1 Unit)		2 µL - 20 µL Micropipette (1 Unit)
	Stirring Magnet (3 Units)		
	Tweezers (1 Unit)		
	Silicon Tubing (1 Unit)		
	Pipette Controller (1 Unit)		
	Marker (1 Unit)		
Consumables and Reagents (for every 2 water samples/replicates)	Bucket (3 Units)	Magnetic Pipette Tips (2 Units)	10 µL Micropipette Tips (1 box)
	pH Indicator Strips	Pasteur Pipette (2 Units)	20 µL Micropipette Tips (1 box)
	Tape-End 10 mL Pipette (2 Units)	Binding Buffer (2 Tubes)	DNA qPCR Mix (1 Tube)
	Open-Tip 10 mL Pipette (2 Units)	Washing Buffer 1 (2 Tubes)	RNA qPCR Mix (1 Tube)
	Tape-End 50 mL Pipette (2 Units)	Washing Buffer 2 (2 Tubes)	Reverse Transcriptase Enzyme (1 Tube)
	Tape-End 100 mL Pipette (2 Units)	Washing Buffer 3 (2 Tubes)	Molecular Biology Water (1 Tube)
	Pasteur Pipette (4 Units)	Elution Buffer (2 Tubes)	HAdV Tube Strip with primers, probe and standard (1 Unit)
	Stand-Up Plastic Bag (4 Units)	Gloves (6 Pairs)	MS2 Tube Strip with primers, probe and standard (1 Unit)
	Plastic Container with 25 mL of distilled water (1 Unit)		Nucleic Acid Remover
	Plastic Container with 50 mL of distilled water (1 Unit)		Gloves (6 Pairs)
	100 mL Empty Container (1 Unit)		
	Gloves (6 Pairs)		
	Process Control (2 Tubes)		
	Tube with 10 mL of distilled water (2 Units)		
	Citric Acid (1 Tube)		
	Sodium Hydroxide (1 Tube)		
	Skimmed Milk (1 Tube)		
	Citric Acid Sachet (1 Unit)		
	Distilled Water (1 500 mL Bottle)		
	Sample Conditioning (2 Sachets)		
	Preservative Solution (2 Tubes)		
	Neutralizing Agent (4 Sachets)		

Virus	Primers	Genbank Accession No.	Position	Sequence (5'–3')	Length	Reference
Human Adenovirus (HAdV)	AdF	J01917.1	18869–18887	CWTACATGCACATCKCSGG	19	Hernroth et al., 2002 <sup>15</sup>
	AdR		18919–18937	CRCGGGCRAAYTGCACCAG	19	
	AdP1		18890–18916	6-FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BMN-Q535	27	
MS2 Bacteriophage (MS2)	pecson-2F	NC_001417.2	344–363	AAGGTGCCTACAAGCGAAGT	20	Pecson et al., 2009 <sup>16</sup>
	pecson-2R		659–678	TTCGTTTAGGGCAAGGTAGC	20	
	PecP-2		369–388	6-FAM-ATCGTGGGGTCGCCGTACG-BHQ-1	20	

HAdV			MS2		
Temperature	Time	Cycles	Temperature	Time	Cycles
95 °C	12 min	1	55 °C	15 min	1
			95 °C	10 min	1
95 °C	15 s	40	95 °C	15 s	40
60 °C	1 min		60 °C	1 min	

	HAdV			MS2	
	QIAgen	VirWaTest		QIAgen	VirWaTest
Median	2.53 x 10 <sup>3</sup>	2.43 x 10 <sup>3</sup>		4.74 x 10 <sup>4</sup>	5.13 x 10 <sup>4</sup>
Mean	1.74 x 10 <sup>4</sup>	3.12 x 10 <sup>4</sup>		4.24 x 10 <sup>4</sup>	5.97 x 10 <sup>4</sup>
SD	5.66 x 10 <sup>5</sup>	2.23 x 10 <sup>6</sup>		4.49 x 10 <sup>4</sup>	1.63 x 10 <sup>5</sup>
	p-value (Wilcoxon) for HAdV: 0.0005569				
	p-value (Wilcoxon) for MS2: 0.02791				
Virus tested	mples Tested	QIAgen	VirWa Test		
HAdv	33	10	23		
MS2	33	10	23		

[illegible]

Table 5

Country	Site	Sample	HAdV GC/L
RCA	Eau de la SODECA	1	ND
	Ile Bongossoua, Village 1	2	ND
	Ile Bongossoua, Village 3	3	ND
	Bangui, Puits Quartier Fondo	4	ND
	Bangui, Puits Quartier Yambassa	5	$3.64 \times 10^2$
Ecuador	Borehole A	6	$7.96 \times 10^1$
	Borehole B	7	$1.80 \times 10^2$
	Borehole C	8	$8.88 \times 10^1$
	Well water A	9	$6.06 \times 10^1$
	Well water B	10	$1.12 \times 10^2$
	Well water C	11	$3.27 \times 10^1$



Name of Material/ Equipment	Company	Catalog Number
5x HOT FIREPol Probe qPCR Mix Plus (ROX)	Solis BioDyne	08-14-00001
8-Microtube Strips with Caps	dD Biolab	840637
Aquagenx CBT E. coli Kit	Aquagenx, LLC	ECCBT10
Batteries and Power Adapters for Magnetic Stirrer	GenIUL	900011674
Bucket Support	GenIUL	900011648
Bucket, 10 L	Cater4You	10LTR
Centrifuge Tube, 50 mL	LabBox	CTSP-E50-050
Citric Acid 1-Hydrate, 500 g	PanReac AppliChem	1410181211
Clear PET Bottle	LabBox	FPET-500-088
Difco Skim Milk, 500 g	Becton Dickinson	232100
DNA/RNA Shield, 250 mL	Zymo Research	R1100-250
Easy9 Pipette Controller	LabBox	EAS9-001-001
Eppendorf Tube, 0.5 mL	Eppendorf	0030121023
Eppendorf Tube, 2 mL	Eppendorf	0030120094
Eppendorf Tube, 5 mL	dD Biolab	999542
Ethanol 96% V/V, 1 L	Panreac AppliChem	361085-1611
Laboratory Tweezers	LabBox	FORS-007-002
Magnetic Stirrer	GenIUL	900017000
Marker	dD Biolab	929203
Micro Rota-Rack for Microtubes	dD Biolab	37782
Mini8 Real-Time PCR Cycler	Coyote Biosciences, China	Mini-8
NucliSens Lysis Buffer	Biomerieux	200292
Open Tip Serological Pipette, 10 mL	Deltalab	900136N
PE Screw Cap PP28	LabBox	TP28-004-020
pH Indicator Strip	LabBox	WSPH-001-001
Plastic Test Tube	Quimikals	300913

Polyethylene Pasteur Pipette	LabBox	PIPP-003-500
Polypropylene Screw Flask With Screw Cap, 150 mL	Deltalab	409726
Polypropylene Screw Flask With Screw Cap, 60 mL	Deltalab	409526G
Powder Powder Detergent	-	-
Power Cables for Magnetic Stirrer	GenIUL	900011692
QuickPick Magnetic Tool	BioNobile	24001
QuickPick Tips in Box	BioNobile	24296
QuickPick XL gDNA Magnetic Particles	BioNobile	SN51100
Sea Salts	Sigma-Aldrich	S9883-500G
Silicone Tubing	LabBox	SILT-006-005
Sodium Hydroxide Pellets, 98.5 - 100.5%	VWR Chemicals	28244295
Solar Rotary Platform	SOL-EXPERT Group	70020
SOLIScript 1-step Probe Kit	Solis BioDyne	08-57-00250
SPEEDTOOLS RNA Virus Extraction Kit	BioTools	21.141-4197
SpinBar Octahedral Stirring Magnet	dD Biolab	045926
Tape-End Serological Pipette, 10 mL	Deltalab	PN10E1
Tape-End Serological Pipette, 50 mL	Deltalab	900043
Termi-DNA-Tor - Nucleic Acid Remover	BioTools	22001-4291
Water Molecular Biology Reagent, 1L	Sigma-Aldrich	W4502-1L
Whirl-Pak Bag, 540 mL	Deltalab	200361
Zip Lock Plain Bag	LabBox	BZIP-080-100

Comments/Description
Includes Solis Biodyne's 5x HOT FIREPol Probe qPCR Mix Plus (qPCR Mix), 50 Reactions
Low Profile, Thin Walls, Adapted for Quantitative and Qualitative PCR
10 Tests per Kit
Includes 12V car power adapter
Aluminium support
Polypropylene, Tamperproof, Clear color
Polypropylene, Sterile, Graduated, With Skirt
Pure, Pharma Grade, 1 Kilogram
Clear Color, PET, Cap Not Included
Dehydrated
DNA/RNA Preservation Medium, 250 mL
0.3 µm filter, Pipettes from 0.1 to 100 mL, Autoclavable silicone pipette holder
Polypropylene, Safe-Lock
Polypropylene, Safe-Lock
Polypropylene, Sterile, Graduated
For UV, IR and HPLC
Thin, Curved End, L= 120 mm
Battery-powered
Black, Extra fine Tip, Water Resistant, Fast Drying, For Plastic and Glassware
4 Modules, L x W x H= 208 x 100 x 100 mm
Portable, Works with 12V Power Supplies or External Batteries, Two channels, Capacity for 8 Tubes
Reagents for up to 48 Isolations, Store at Ambient Temperature
Sterile, Individually Wrapped (Paper/Plastic)
For PET Bottles
Range pH 2.8 to pH 4.4, 50 Strips per Pack
Includes Cap

Graduated, 7 mL Overall Volume, Non-Sterile
Screw cap, Sterile, graduated up to 100 mL
Screw cap, Sterile, Graduated up to 50 mL
Regular Powder Soap for washing clothes
Connection between batteries and magnetic stirrers
Hand-held tool for magnetic particles
RNase-Free, Autoclaved, 96 Units
3.2 mL
An artificial salt mixture closely resembling the composition of the dissolved salts of ocean water
Roll of 5 Meters, Inner $\varnothing$ x Outer $\varnothing$ = 6 x 10 mm
Pellets, 1 Kg
Acrylic Plate, 10 RPM, Supports up to 300 Grams
Includes Solis Biodyne's 5x One-Step Probe Mix (qPCR Mix) and 40x One-Step SOLIScript Mix (Reverse Transcriptase Enzyme), 250 Reactions
Includes BioTools's BAW Buffer (Washing Buffer 1), BAV3 Buffer (Washing Buffer 2 and 3) and BRE Buffer (Elution Buffer).
Pivot Ring, L x $\varnothing$ = 38 x 8 mm, Blue
Sterile, Individually Wrapped (Paper/Plastic)
Sterile, Individually Wrapped (Paper/Plastic)
Remover of nucleic acids, bacteria, fungi and mycoplasma from material and surfaces, 450 mL
Nuclease and Protease Free, 0.1 $\mu$ m Filtered
Stable bottom
Polyethylene, L x W= 120 x 80 mm







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Article Title: **VirWaTest: A Point-of-Use Method for the Detection of Viruses in Water Samples**

Signature:  Date: **30/11/2018**

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**1. This sentence is not needed here as it is already present in the Acknowledgment section.**

The sentence has been removed.

**2. This is not important in the abstract.**

The sentence has been removed.

**3. If this sentence is presented here, please provide results for the same. Both human adenovirus and Hepatitis E viruses, else just remove HEV details.**

The sentence has been removed.

**4. Please move this part to the Acknowledgment section.**

The sentence has been removed.

**5. What kind of feedback was provided? Maybe include (see discussions). Add in the discussion section.**

The feedback obtained was related to the performance of the concentration method in the field. We have added this in the discussions suggested: "Feedback obtained until now indicates that viral concentration is an easy procedure to perform with no major problems when applied in the point-of-use although an 8 and 5-hour steps are required. It is important to say that these steps occur without any human assistance."

**6. We cannot have non-numbered text in the protocol section. Moved here instead. Include a sentence somewhere in the introduction, that the protocol detailed below, uses HAdV as an example of virus indicator in the water sample and MS2 as spike control in the test to be performed at the point of use. Also reason out why. This will bring out further clarity in the protocol.**

We have added the following sentence: "A quantitative PCR assay to detect HAdV, excreted by humans and found in wastewater samples in high concentrations, has been adapted to be run in the point-of-use. HAdV are used as human fecal viral indicators. A PCR for quantification of MS2 bacteriophage has been also adapted since MS2 is used in VirWaTest as a process control. The method can be customized for the detection of any virus of interest."

**7. Please bring out homogeneity between written text and the protocol described. Please use numbered sentences in imperative tense as if describing someone how to perform your experiment.**

Sentences across the manuscript have been revised and rewritten when necessary to match the required format.

**8. Need to bring out clarity on MS2 use as spike control. Please include this detail somewhere in the introduction section to be clear.**

This has been detailed in the introduction.

**9. Needs rewording for clarity.**

This sentence has been rewritten.

**10. Extracted nucleic acid?**

This has been rewritten.

**Do you perform O>D check at 280/260?**

Measuring OD has not been considered, since it requires shipping additional equipment and power sources to perform this in the field.

**11. Please number the step. We cannot have non-numbered step in the protocol section.**

This has been removed and explained in the introduction.

**12. Needs rewording.**

This has been rewritten.

**13. Please reword for clarity.**

This sentence has been rewritten.

**14. How was this calculated? Please provide a short description.**

This explanation has been added to the paragraph to indicate how it was calculated: "These recoveries were calculated from the values obtained by quantitative PCR during the development of the method compared to the initial concentrations of HAdV and MS2 in the water samples after spiking them with known concentrations of these viral stocks."

**15. So basically, you only test one virus at one time? Or do you test a pool of viruses since you already have genomic DNA. It will be nice to show a figure containing all the viruses being tested in the sample. Please include this figure in the video as well.**

PCR is a technique that detects specifically one virus per reaction (unless you perform multiplex PCRs). You can detect any viruses as far as you have the primers to do it but you have to run a different PCR reaction for any virus of interest. HAdV is suggested as a human fecal contamination indicator (such as *E. coli* which is a fecal indicator bacteria).

**16. Is this spike control or real test of water? Please bring out clarity.**

This has been clarified. It was naturally occurring HAdV what was detected.