National Research Programme “to mitigate consequences of COVID-19”

Project “Integration of reliable technologies for protection against Covid-19 in healthcare and high-risk areas”

Deliverable of Task 5.1

Technology proposal for rapid analysis methods

Riga,
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Summary

Virus detection on surfaces and in environmental samples is still a challenge in respect to protocol time, complexity and specific methodology. Within this task a technological proposal for rapid, non-target viral presence method is described and tested. Average sample preparation time – 15 minutes. The protocol is based on immobilization of virus particles on a specific membranes and fluorescence staining with subsequent signal detection. The estimated resultant technology readiness level is TRL2.

Background

Viruses are the most abundant biological entities on earth, more than archaea and bacteria combined. They represent the largest and most genetically diverse group of nucleic acid that can infect all forms of life, including bacteria and protozoa. Currently in virus surveillance, collected samples (non-clinical) are subjected to a series of time-consuming steps, such as ultracentrifugation and subsequent cell culture, to enrich virus particles or amplify virus titers. Such approach is not suitable for surfaces, since often non-targeted analysis with unknown initial concentration is required.

Up till now, virus density, expressed as the number of virions or number of virus equivalents per unit surface area, has been measured only in limited studies[1]. Many viruses are not easily culturable, extraction and molecular techniques are not sensitive enough and bias is often introduced during amplification, leading to artifacts in the sequence data. Furthermore, environmental matrix can affect cell cultures/signal amplifications, thus, leading to faulty results. Thus, need for more sensitive and simultaneously robust sampling methods and detection assays are needed[2]. Existing technologies, such as immune-based and molecular assays [e.g., enzyme-linked immunosorbent assay (ELISA) and PCR], provide relatively sensitive detection for the identification of viruses but require prior knowledge of the strains, thus, inappropriate to be used in general viral presence monitoring. Deep sequencing techniques, such as next-generation sequencing (NGS) are powerful tools in virus surveillance but are not applicable on daily routine assays. One of the currently proposed rapid technologies - VIRRION platform employing carbon nanotubes and RAMAN[3] shows to be promising, however, still lack accuracy and are expensive when used in monitoring purposes.

At the same time, epifluorescence microscopy has been recognized as a rapid and accurate method to determine the abundance of microbial particles. Despite the small size of virions and certain limitations, research has shown the superiority of the technique when compared to transmission electron microscopy (TEM)[4]. The aim of the research was to design a technological proposal for rapid viral presence detection from environmental samples. To achieve the aim, classical viral fluorescence staining was selected as the method of choice[5].

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[1] Ibfelt T, Frandsen T, Permin A, Andersen LP, Schultz AC, 2016. Test and validation of methods to sample and detect human virus from environmental surfaces using norovirus as a model virus. J Hosp Infect, 92(4), 378-84.

[2] Julian TR, Tamayo FJ, Leckie JO, Boehm AB, 2011. Comparison of surface sampling methods for virus recovery from fomites. Applied and Environmental Microbiology, 77(19), 6918-6925.

[3] Yeh YT, Gulino K, Zhang YH, Sabestien A, Chou TW, Zhou B, Lin Z, Albert I, Lu H, Swaminathan V, Ghedin E, Terrones M., 2020. A rapid and label-free platform for virus capture and identification from clinical samples, Proceedings of the National Academy of Sciences, 117(2), 895-901.

[4] Ferris MM, Stoffel CL, Maurer TT, Rowlen KL, 2002. Quantitative intercomparison of transmission electron microscopy, flow cytometry, and epifluorescence microscopy for nanometric particle analysis. Anal. Biochem., 304, 249-256.

[5] Ortmann AC, Suttle CA, 2009. Determination of virus abundance by epifluorescence microscopy. In Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization and Interactions, vol 501, Humana Press, 87-95.

Protocol proposal

Materials, equipment, and reagents

SYBR Gold stain (1:400 dilution in sterile distilled water)
Filtration equipment able to use 25 mm filters
Materials for sample collection, e.g. swabs, and recovery, tubes with neutralizer
Cellulose filter discs
0.02 µm pore size membranes (recommended – inorganic Anodisc from Cytiva)
0.2 µm pore size membranes (recommended – Track-etched)
50:50 mix of PBS:glycerol
Cover glass and cover slip
Immersion oil
Epifluorescence microscope (Filter set: Ex. 450-40 nm; Em. > 515 nm; 100x immersion oil objective with NA 1.4)
Mechanical pipettes and tips, Eppendorf tubes, tweezers

Protocol

1. Collect environmental samples (swabs, liquids) according to existing standards, e.g., EN ISO 18593:2018.
2. Prepare decimal dilutions of the sample in sterile distilled water.
3. Place cellulose filter on filtration unit, moisture with distilled water, place a 0.02 µm pore size filter (if sample is suspected to contain bacteria, 0.2 µm membrane is placed on top).
4. Add 0.5 ml of the diluted sample and turn on filtration unit, then again add 0.5 ml of the same sample (total volume on the membrane should be 1 ml).
5. Depending on the number of samples and filtration unit, either remove the membrane or leave on the filtration unit.
6. Stain with 0.1 ml SYBR Gold for 5 minutes.
7. Remove the excess liquid by either turning on the filtration unit (if staining is performed on the unit). Add 2 ml of sterile distilled water to remove excess dye.
8. Airdry the membrane.
9. Put 45 µl of PBS:glycerol as antifade on a glass slide, place the membrane on and 1 drop of oil and cover with cover glass.
10. Observe under epifluorescence microscope under recommended wavelength.

Visual representation of the protocol

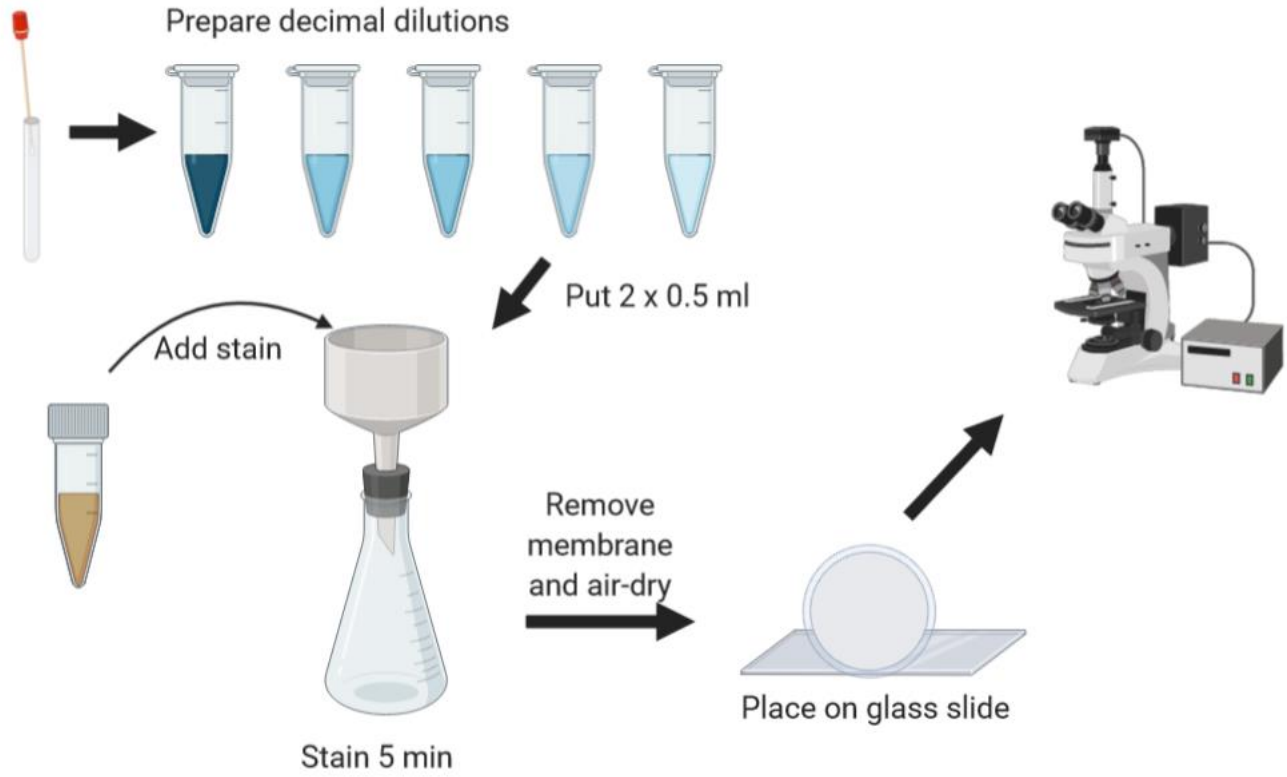

Figure 1. Schematic representation of sample preparation for rapid staining protocol

Technological development and novel approaches introduced in fluorescent staining protocols

	Method	Modifications	Comments
Stain	SYBR Gold SYBR Green DAPI		SYBR Gold was selected as optimal; 1:400 dilution sufficient
Support filters	Cellulose		Needs to be moist prior addition of membrane
Membranes	Inorganic (Al_2O_3) with 0.02 µm pore size		Placed on top of cellulose filter. If sample potentially contains bacteria, 0.2 µm membrane is placed on top.
Viral sample dilution	Fixation with 25% glutaraldehyde for 30 min	No fixation	Essential to reduce background fluorescence

Staining	Staining for 15 min from the bottom	Staining for 5 min and stain is placed on top	Membrane is left on the filtration unit to reduce risk of breaking and cross-contamination
Washing	Remove the excess stain by vacuum	Filter through 2 mL of sterile distilled water to remove excess stain	
Draying	By using dry wipes	Air-dry	No wipes are used, to reduce presence of fluorescent particles
Antifade solution	10% p-phenylenediamine + (50:50) PBS+ glycerol	Only PBS+glycerol (50:50)	1% p-phenylenediamine is toxic, no visual difference was detected in samples

Protocol development results

During the development of the protocol, bacteriophage phi6 was used as surrogate virus. Phi6 is a member of the family *Cystoviridae* and is among the few bacteriophages that have a lipid envelope, has a size of 75 nm[6] and are characterized by large, enveloped, single-stranded RNA with genomes ranging from 13.5-35 kbp[7]. Phi6 was the first bacteriophage with a lipoprotein envelope to be isolated and well described, which is why it has historically been chosen as an enveloped virus surrogate[6]. Phi 6 is easier to work with than other enveloped viruses and can be propagated to high titers[8].

As the base for the protocol development,t existing staining approach was used. This methodology was both simplified and evaluated for the selected task: surface testing and intense signal generation.

During the protocol development multiple factors were assessed:

1. Selection of stain and its concentration.

Multiple stains known for their ability to bind to genetic information were evaluated. SYBR Gold was estimated as the most suitable

2. Fixation of the sample

Available classical protocols employ fixation step. During the development we observed that fixatives (formaldehyde, glutaraldehyde) generate high background fluorescence, that hinder detection of viral particles (Fig 2). If long term storage of the samples is not required, removal of

[6] Carvalho NA, Stachler EN, Cimabue N, Bibby K, 2017. Evaluation of Phi6 Persistence and Suitability as an Enveloped Virus Surrogate. Environmental Science and Technology, 51: 8692−8700.
[7] Kaufer AM, Theis T, Lau KA, Gray JL, Rawlinson WD, 2020. Laboratory biosafety measures involving SARS-CoV-2 and the classification as a Risk Group 3 biological agent. Pathology, 52(7): 790–795.
[8] Ye Y., Chang P. H., Hartert J., Wigginton K.R. 2018. Reactivity of enveloped virus genome, proteins, and lipids with free chlorine and UV254. Environmental Science and Technology, 52: 7698–7708.

fixation, not only reduces background fluorescence but also reduces sample preparation time by 30 minutes.

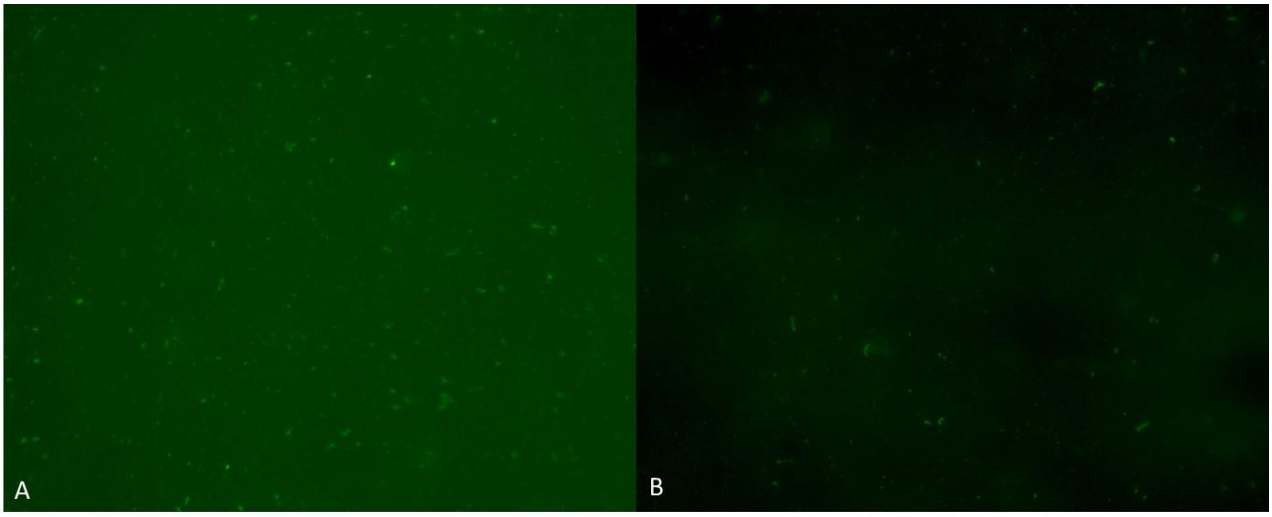

Figure 2. Sample staining with (A) and without (B) fixation with glutaraldehyde.

3. Removal of reagents that might cause background fluorescence

Further impact and need for all other buffers and reagents were evaluated. As a result we excluded the use of buffers for washing and dilution, replaced them with sterile distilled water and removed the toxic p-phenylenediamine from the antifade mix. The result demonstrated acceptable target fluorescence intensity and optimal background (Fig 3)

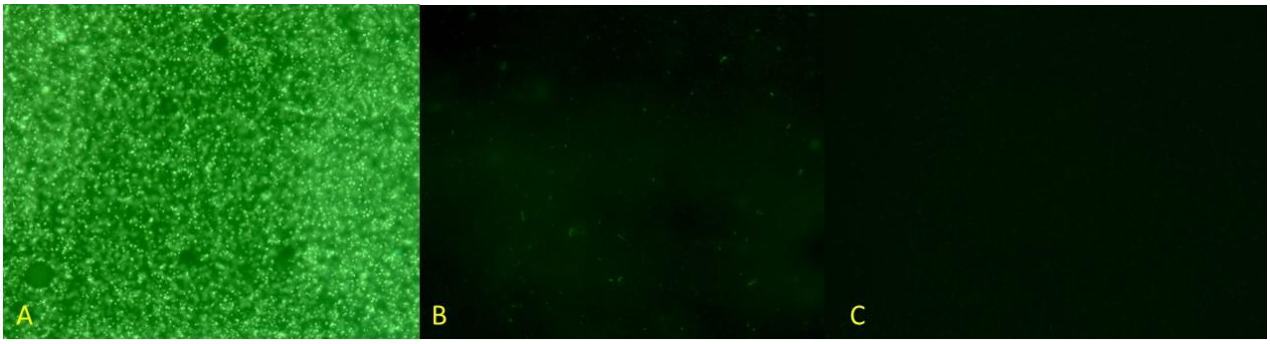

Figure 3. Fluorescently stained virus (bacteriophage phi6 as surrogate for SARS-COV-2) particles (A) and pre-filtered (0,1µm filter) water (B) and sterile buffer (C)

4. Removal of bacterial and other potential contaminants

In environmental samples it is expected that many particles might be present, thus, hindering virus detection and competing with available stain (Fig 4). To minimize this risk, use of second membrane to collect bacteria was introduced.

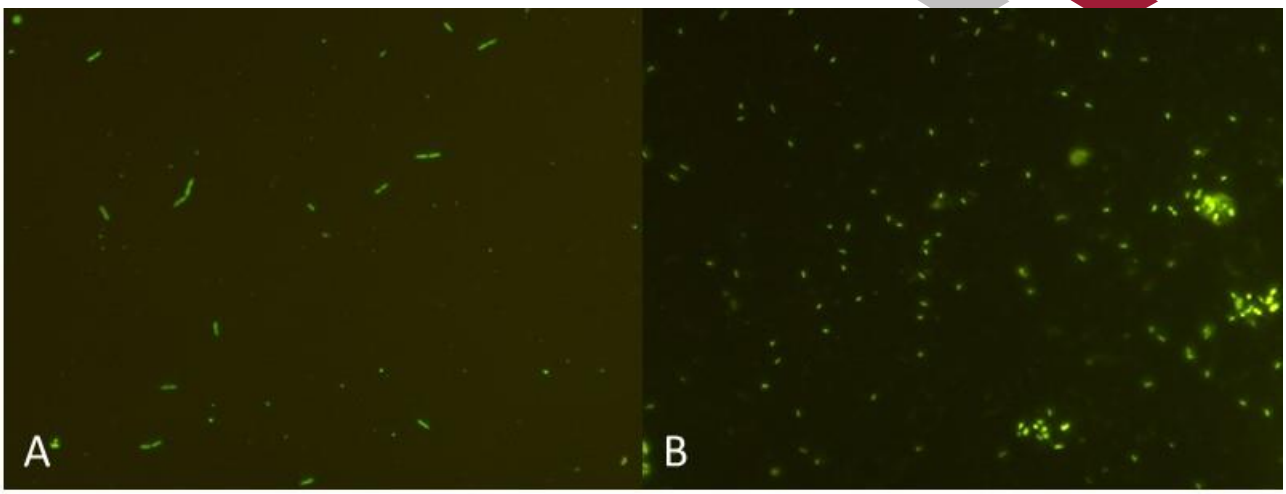

Figure 4. Viral sample contaminated with unknown bacteria (A) and bacteriophage phi6 with its host *Pseudomonas* (B) with lysis regions. Samples prepared on 0.02 µm membranes with no pre-filtration membrane.

5. Evaluation of virus concentration and signal intensity

Ideally the method should be able to enumerate the number of viral particles. During the development various concentrations were tested (Fig 5). In general, it was possible to detect individual virus particles, however, when the expected concentration is low higher resolution equipment might be necessary (or automated counting systems)

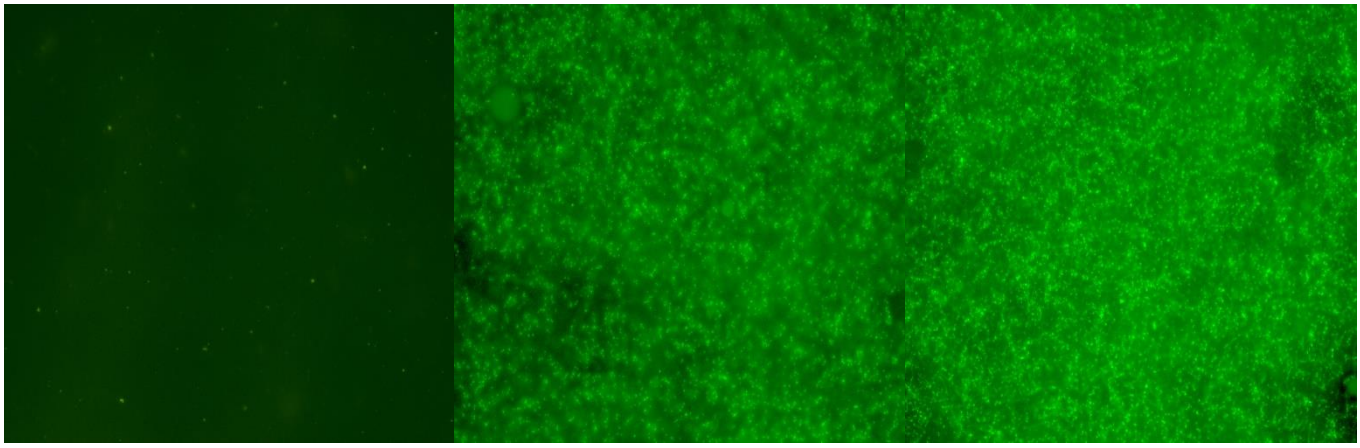

Figure 5. Samples with low (left), medium (middle) and high (right) bacteriophage phi6 concentration.

6. Enumeration of infective virus

Since surface contamination is most often described by the positivity rate, defined as the fraction of total samples collected on which the organism is detectable. However, the positivity rate does not provide an indication of general infection risk. To estimate the risk of infection, information about both virus quantity and infectivity is needed.

Due to this, an alternative approach for viral quantification *Pseudomonas* spp were infected with fluorescently labelled virus, incubated and then visualized. The results demonstrated that certain

bacterial cells demonstrate higher fluorescence intensity (Fig 6) and might be indirectly related to the presence of infective virus particles, if needed.

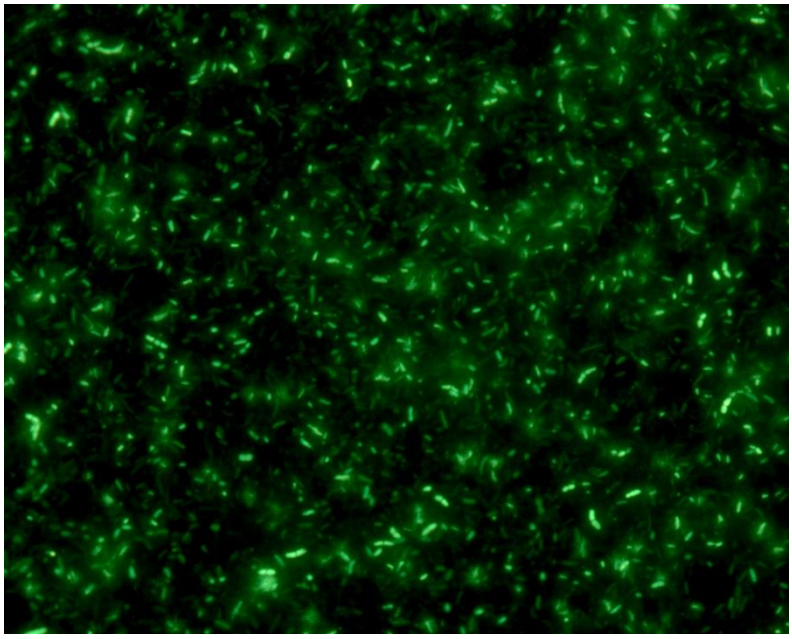

Figure 6. Bacterial cells infected with fluorescently labelled virus. Uninfected cells seen as weakly stained.

Conclusions

The developed technological protocol (result of project activity W5.1) is rapid (samples are prepared within 15 minutes), does not require use of carcinogenic antifading reagents and can detect individual virus particles (tested with 75 nm size virus). Quantitative studies demonstrated that a decrease in virion concentration resulted in reduced fluorescence intensity yielding countable particles and reaching an estimated TRL2. Infective studies demonstrated the potential use of the approach in detection not only total virus but also infective viral presence.

Future recommendations

1. To validate the protocol with other viruses
2. To introduce quantification by combining the protocol with higher resolution systems
3. To validate the protocol with a set of real samples