Booklet on

HARMONISED METHODS

Version: June 2022

available on www.sara-project.info

Authors:

Claudia Stange[1], Johannes Ho[1], Anicet R. Blanch[2], Daniel Toribio-Avedillo[2], Elisenda Ballesté[2], Cristina Garcia-Aljaro[2], Ricardo Santos[3], Silvia Monteiro[3], Abidelfatah Nasser[4], Timothy M. Vogel[5], Concepcion Sanchez-Cid Torres[5], Magnus Simonsson[6] and Andreas Tiehm[1]

[1]TZW: DVGW-Technologiezentrum Wasser, Department of Water Microbiology
[2]Universitat de Barcelona, Departament de Genètica, Microbiologia i Estadística
[3]Universidade Lisboa, Instituto Superior Tecnico, Laboratorio Analises
[4]Ecole Centrale de Lyon, Laboratoire Ampere
[5]Ministry of Health, National Public Health Laboratory
[6]Swedish Food Agency, Biology Department, European Union Reference, Laboratory for Foodborne Viruses

Contents

1 Introduction

Wastewater treatment plants (WWTPs) are designed to reduce the concentration of pollutants and to avoid the direct discharge of wastewater into rivers and oceans. However, conventional wastewater treatment is not sufficient to entirely remove biological contaminants (Alexander et al., 2015; Hembach et al., 2017). Faecal microorganisms, including pathogens, are released by WWTP effluents into rivers and possibly subjected to inactivation while being transported downstream (Agulló-Barceló et al., 2013; Pascual-Benito et al., 2020). During dry periods, reduction in water flow can lead to higher concentrations of these pollutants as WWTP effluents constitute a greater proportion of the flow in intermittent streams (Muñoz et al., 2009; Pascual-Benito et al., 2020). Extreme rainfall events are also associated with a higher concentration of waterborne pathogens due to the re-mobilization of river sediments (García-Aljaro et al., 2017; Martín-Díaz et al., 2017), the inflow of untreated wastewater from overflow of combined sewer systems and oversaturation and disruption of WWTP performance (Curriero et al., 2001). Increases in microbial pollutant concentrations may result in human health risks due to pathogen exposure (Curriero et al., 2001; Tornevi et al., 2014), thereby, compromising water usability. This is especially important in the current scenario of climate change. The average annual temperature is gradually increasing and droughts will become more frequent and the frequency of heavy rains will increase at the same time (World Health Organization, 2011).

Waterborne pathogen contamination of surface waters and the transmission of infectious diseases via contaminated water are of heighten public health concern (Pandey et al., 2014). The analysis of the microbiological quality of water is an essential step for identifying the sanitary and environmental risks caused by faecal contamination. Assessment of the entire range of pathogen microorganisms is difficult and expensive, so microbial indicators (*E. coli* and enterococci) are typically used for water quality management (García-Aljaro et al., 2019). However, research has shown that monitoring faecal indicator bacteria (FIB) may not provide sufficient public health protection given that viruses have different persistence, survival and transport rates in water bodies (Edberg et al., 2000). Thus, water management protocols need to include somatic coliphages (Jofre et al., 2016; McMinn et al., 2017) and/or pathogenic viruses as viral faecal indicators.

Enteric viruses reach the aquatic environment through the discharge of WWTP effluents (Lee and Kim, 2002; Lipp et al., 2002). Enteric viruses are shed in high numbers in the faeces of infected individuals (10^5-10^{12} virus particles per gram of stool) (Atmar et al., 2008);. While norovirus is known to be involved in many food- and waterborne outbreaks (Amarasiri et al., 2018), also other enteric viruses are widely distributed in the aquatic ecosystem (Varela et al., 2018). The detection of enteric viruses using tissue-culture techniques are usually time-consuming and labour-intensive (Hodinka, 2013; Jofre and Blanch, 2010), although these methods could provide information about infectivity. Additionally, for most enteric viruses, no permissive cell culture systems for isolation on a routine basis are available. An alternative approach is offered by molecular methods based on qPCR detection (Ho et al., 2016; Karthe et al., 2016; Pei et al., 2012) and metagenomics (Sanguino et al., 2015). Virus levels in water are typically too low for detection by direct analysis; thus, the detection often involves viral

enrichment or concentration. Viruses can be concentrated from environmental samples via different methods such as filtration through electronegative or electropositive filters, ultrafiltration, viral flocculation/precipitation (Pandey et al., 2014)with organic/inorganic flocculants, and ultracentrifugation (Bofill-Mas and Rusiñol, 2020; Hamza and Bibby, 2019). Similar to concentration methods, a high number of methods and kits are available for nucleic acid extraction with varying efficiency and inhibitors removal capacity. The choice of concentration and extraction method can affect the results and the limit of detection and quantification.

A complete and routine analysis of all potential water-borne pathogens is unsustainable and not viable in cost and practical technological implementation in water management. Since more than a century, bacterial indicators have been defined as those microorganisms associated to the presence of faecal pollution and the potential presence of pathogens in water. However, the analysis of bacterial indicators by culture-based methods has limitations as they display different fate and transport characteristics in aquatic environments when compared to that of viral pathogens. The use of coliphages as indicated for different regulatory agencies and health organizations (United States Environmental Protection Agency, 2015; World Health Organization, 2017) is more appropriate and they have been included in national and international directives and regulations (*i.e.* recently in the EU regulation for water reuse (Regulation 2020/741, 05.06.2020) and the new EU directive on the quality of water intended for human consumption (COM/2017/0753 final - 2017/0332) already adopted by the European Council (23.10.2020) and pending for final adoption by the European Parliament in coming months. In the new EU drinking water directive (EU DWD, 98/83/EC) a risk-based approach is required and specific implementation plans have to be developed on a national level. The SARA project will provide data to facilitate the risk-based implementation until 2022 as foreseen in the DWD regulations.

In addition to viruses, wastewater contain a variety of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) that can be transmitted into the environment through the discharge of WWTP effluents (Berendonk et al., 2015; Chu et al., 2018; Petrovich et al., 2018). In recent years, the fate of ARBs and ARGs discharged from urban WWTPs and their prevalence in the aquatic ecosystem have received increasing attention (Berendonk et al., 2015; Calero-Cáceres et al., 2017; Jia et al., 2017; Lorenzo et al., 2018; Sabri et al., 2020; Stange et al., 2016; Stange et al., 2019; Voigt et al., 2020). There is a worldwide consensus that raw municipal wastewater and treated effluents are reservoirs of ARB and ARGs (Ghosh et al., 2009; Zhang et al., 2009) and hotspots for the evolution and spread of antibiotic resistance (Guo et al., 2017; Rizzo et al., 2013). Although treated wastewater contains significantly lower amounts of ARGs than raw wastewater, various researchers have demonstrated that the discharge of treated municipal wastewater increases the quantities of ARGs in the receiving water bodies (Amos et al., 2018; Jäger et al., 2018; Nasser et al., 2019; Pruden et al., 2012).

Next generation sequencing techniques, such as metagenomics, offer the ability to identify all known antibiotic resistance genes within a sample, providing a new approach for the environmental monitoring of antibiotic resistance (Nesme et al., 2014; Nesme and Simonet, 2015). These techniques have greatly increased the knowledge of the diversity and abundance of ARGs in WWTPs (Bengtsson-Palme et al., 2016; Ju et al., 2019).

Complementary to water quality monitoring using FIB, microbial source tracking (MST) methods provide strategies for the determination of faecal pollution sources in environmental waters. MST methods rely on the association of certain faecal microorganisms with a particular host (human or animal). Various microbiological and genotypic methods have been proposed for MST whereby culture-independent and library independent methods in particular have prevailed (Ballesté et al., 2020; Bernhard and Field, 2000; Gómez-Doñate et al., 2016). Environmental samples have been analysed by PCR for either defined bacterial DNA regions (*e.g*., *Bacteroidales* or *Bifidobacterium* spp. sequences) or viral genomes (*e.g.,* CrAssphage) associated to human or animal faeces. Besides of the qualitative determination of the faecal source, quantitative analysis using real-time PCR is also possible and provides information on the contribution of different sources when analysing samples containing a mixture of faecal contents from different origin (Ballesté et al., 2020; Gómez-Doñate et al., 2016). MST methods allow a better management of polluted water areas as they can determine the source of faecal pollution, allowing effective restoration measures on the origin. Thus, the strategy for water quality improvement is changing from a Public Health to a One Health approach (Ballesté et al., 2019; Sánchez-Alfonso et al., 2020). Attending to the scope of this project, human host-specific markers are considered related to human pathogens and antibiotic resistances mainly coming through wastewater from cities and hospitals.

Appropriate methods for wastewater-based epidemiology (WBE) and a better understanding of the fate of pathogenic viruses and antibiotic-resistant bacteria from the sources to river basins and estuaries are urgently required. Our project will determine the prevalence of pathogenic viruses (including SARS-CoV-2), microbial indicators, antibiotic resistance, and microbial source tracking (MST) markers in wastewater, surface water, coastal sea waters, sediment and bivalve molluscan shellfish (BMS) in catchments located in different climate areas (Sweden, Germany, France, Spain, Portugal, Israel, Mozambique, and Uganda). The project aims are inter alia: (i) acquisition of microbiological indicators; (ii) enteric viruses, antibiotic resistances and MST markers monitoring in aquatic environments, (iii) determination of the impact of climate and extreme weather events, and (iv) microbial risk assessment for water resources. Results and recommendations will be transferred to the scientific community

A harmonisation of sampling and sample handling is critical in order to obtain comparable data. During the first project phase of the SARA project, the procedures used by all partners were harmonised. This includes the cultural detection of faecal indicator bacteria, bacteriophages and ESBL *E. coli* as indicator for ARB. The harmonisation of methods also includes the sample preparation for molecular biological analyses. For the molecular biological analyses, nucleic acids have to be extracted during the first step. After their extraction, the nucleic acids can be stabilised and easily exchanged.

1.1 Literature

Agulló-Barceló, M., Oliva, F., Lucena, F., 2013. Alternative indicators for monitoring Cryptosporidium oocysts in reclaimed water. Environmental science and pollution research international 20 (7), 4448–4454.

Alexander, J., Bollmann, A., Seitz, W., Schwartz, T., 2015. Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria. The Science of the total environment 512-513, 316–325.

Amarasiri, M., Kitajima, M., Miyamura, A., Santos, R., Monteiro, S., Miura, T., Kazama, S., Okabe, S., Sano, D., 2018. Reverse transcription-quantitative PCR assays for genotype-specific detection of human noroviruses in clinical and environmental samples. International journal of hygiene and environmental health 221 (3), 578–585.

Amos, G.C.A., Ploumakis, S., Zhang, L., Hawkey, P.M., Gaze, W.H., Wellington, E.M.H., 2018. The widespread dissemination of integrons throughout bacterial communities in a riverine system. The ISME journal 12 (3), 681–691.

Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Graham, D.Y., 2008. Norwalk virus shedding after experimental human infection. Emerging infectious diseases 14 (10), 1553–1557.

Ballesté, E., Belanche-Muñoz, L.A., Farnleitner, A.H., Linke, R., Sommer, R., Santos, R., Monteiro, S., Maunula, L., Oristo, S., Tiehm A, A., Stange, C., Blanch, A.R., 2020. Improving the identification of the source of faecal pollution in water using a modelling approach: From multi-source to aged and diluted samples. Water research 171, 115392.

Ballesté, E., Pascual-Benito, M., Martín-Díaz, J., Blanch, A.R., Lucena, F., Muniesa, M., Jofre, J., García-Aljaro, C., 2019. Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments. Water research 155, 233–244.

Bengtsson-Palme, J., Hammarén, R., Pal, C., Östman, M., Björlenius, B., Flach, C.-F., Fick, J., Kristiansson, E., Tysklind, M., Larsson, D.G.J., 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. The Science of the total environment 572, 697–712.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: The environmental framework. Nature reviews. Microbiology 13 (5), 310–317.

Bernhard, A.E., Field, K.G., 2000. Identification of nonpoint sources of faecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from faecal anaerobes. Applied and environmental microbiology 66 (4), 1587–1594.

Bofill-Mas, S., Rusiñol, M., 2020. Recent trends on methods for the concentration of viruses from water samples. Current Opinion in Environmental Science & Health 16, 7–13.

Calero-Cáceres, W., Méndez, J., Martín-Díaz, J., Muniesa, M., 2017. The occurrence of antibiotic resistance genes in a Mediterranean river and their persistence in the riverbed sediment. Environmental pollution (Barking, Essex : 1987) 223, 384–394.

Chu, B.T.T., Petrovich, M.L., Chaudhary, A., Wright, D., Murphy, B., Wells, G., Poretsky, R., 2018. Metagenomics Reveals the Impact of Wastewater Treatment Plants on the Dispersal of Microorganisms and Genes in Aquatic Sediments. Applied and environmental microbiology 84 (5).

Curriero, F.C., Patz, J.A., Rose, J.B., Lele, S., 2001. The association between extreme precipitation and waterborne disease outbreaks in the United States, 1948-1994. American journal of public health 91 (8), 1194–1199.

Edberg, S.C., Rice, E.W., Karlin, R.J., Allen, M.J., 2000. Escherichia coli: The best biological drinking water indicator for public health protection. Symposium series (Society for Applied Microbiology) (29), 106S-116S.

García-Aljaro, C., Blanch, A.R., Campos, C., Jofre, J., Lucena, F., 2019. Pathogens, faecal indicators and human-specific microbial source-tracking markers in sewage. Journal of applied microbiology 126 (3), 701–717.

García-Aljaro, C., Martín-Díaz, J., Viñas-Balada, E., Calero-Cáceres, W., Lucena, F., Blanch, A.R., 2017. Mobilisation of microbial indicators, microbial source tracking markers and pathogens after rainfall events. Water research 112, 248–253.

Ghosh, S., Ramsden, S.J., LaPara, T.M., 2009. The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. Applied microbiology and biotechnology 84 (4), 791–796.

Gómez-Doñate, M., Casanovas-Massana, A., Muniesa, M., Blanch, A.R., 2016. Development of new host-specific Bacteroides qPCRs for the identification of faecal contamination sources in water. MicrobiologyOpen 5 (1), 83–94.

Guo, J., Li, J., Chen, H., Bond, P.L., Yuan, Z., 2017. Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. Water research 123, 468–478.

Hamza, I.A., Bibby, K., 2019. Critical issues in application of molecular methods to environmental virology. Journal of virological methods 266, 11–24.

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., Schwartz, T., 2017. Occurrence of the mcr-1 Colistin Resistance Gene and other Clinically Relevant Antibiotic Resistance Genes in Microbial Populations at Different Municipal Wastewater Treatment Plants in Germany. Frontiers in microbiology 8, 1282.

Ho, J., Seidel, M., Niessner, R., Eggers, J., Tiehm, A., 2016. Long amplicon (LA)-qPCR for the discrimination of infectious and noninfectious phix174 bacteriophages after UV inactivation. Water research 103, 141–148.

Hodinka, R.L., 2013. Point: Is the era of viral culture over in the clinical microbiology laboratory? Journal of clinical microbiology 51 (1), 2–4.

Jäger, T., Hembach, N., Elpers, C., Wieland, A., Alexander, J., Hiller, C., Krauter, G., Schwartz, T., 2018. Reduction of Antibiotic Resistant Bacteria During Conventional and Advanced Wastewater Treatment, and the Disseminated Loads Released to the Environment. Frontiers in microbiology 9, 2599.

Jia, S., Zhang, X.-X., Miao, Y., Zhao, Y., Ye, L., Li, B., Zhang, T., 2017. Fate of antibiotic resistance genes and their associations with bacterial community in livestock breeding wastewater and its receiving river water. Water research 124, 259–268.

Jofre, J., Blanch, A.R., 2010. Feasibility of methods based on nucleic acid amplification techniques to fulfil the requirements for microbiological analysis of water quality. Journal of applied microbiology 109 (6), 1853–1867.

Jofre, J., Lucena, F., Blanch, A., Muniesa, M., 2016. Coliphages as Model Organisms in the Characterization and Management of Water Resources. Water 8 (5), 199.

Ju, F., Beck, K., Yin, X., Maccagnan, A., McArdell, C.S., Singer, H.P., Johnson, D.R., Zhang, T., Bürgmann, H., 2019. Wastewater treatment plant resistomes are shaped by bacterial composition, genetic exchange, and upregulated expression in the effluent microbiomes. The ISME journal 13 (2), 346–360.

Karthe, D., Behrmann, O., Blättel, V., Elsässer, D., Heese, C., Hügle, M., Hufert, F., Kunze, A., Niessner, R., Ho, J., Scharaw, B., Spoo, M., Tiehm, A., Urban, G., Vosseler, S., Westerhoff, T., Dame, G., Seidel, M., 2016. Modular development of an inline monitoring system for waterborne pathogens in raw and drinking water. Environ Earth Sci 75 (23), e71642.

Lee, S.-H., Kim, S.-J., 2002. Detection of infectious enteroviruses and adenoviruses in tap water in urban areas in Korea. Water research 36 (1), 248–256.

Lipp, E.K., Jarrell, J.L., Griffin, D.W., Lukasik, J., Jacukiewicz, J., Rose, J.B., 2002. Preliminary evidence for human faecal contamination in corals of the Florida Keys, USA. Marine pollution bulletin 44 (7), 666–670.

Lorenzo, P., Adriana, A., Jessica, S., Carles, B., Marinella, F., Marta, L., Luis, B.J., Pierre, S., 2018. Antibiotic resistance in urban and hospital wastewaters and their impact on a receiving freshwater ecosystem. Chemosphere 206, 70–82.

Martín-Díaz, J., García-Aljaro, C., Pascual-Benito, M., Galofré, B., Blanch, A.R., Lucena, F., 2017. Microcosms for evaluating microbial indicator persistence and mobilization in fluvial sediments during rainfall events. Water research 123, 623–631.

McMinn, B.R., Huff, E.M., Rhodes, E.R., Korajkic, A., 2017. Concentration and quantification of somatic and F+ coliphages from recreational waters. Journal of virological methods 249, 58–65.

Muñoz, I., López-Doval, J.C., Ricart, M., Villagrasa, M., Brix, R., Geiszinger, A., Ginebreda, A., Guasch, H., Alda, M.J.L. de, Romaní, A.M., Sabater, S., Barceló, D., 2009. Bridging levels of pharmaceuticals in river water with biological community structure in the Llobregat River basin (northeast Spain). Environmental toxicology and chemistry 28 (12), 2706–2714.

Nasser, A.M., Fawaqa, H., Nitzan, Y., 2019. The Role of Wastewater Treatment Plants in the Environmental Dissemination of Antibiotic Resistant Bacteria (ARB) and Resistance Genes (ARG). JWARP 11 (08), 981–994.

Nesme, J., Cécillon, S., Delmont, T.O., Monier, J.-M., Vogel, T.M., Simonet, P., 2014. Large-scale metagenomic-based study of antibiotic resistance in the environment. Current biology : CB 24 (10), 1096–1100.

Nesme, J., Simonet, P., 2015. The soil resistome: A critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environmental microbiology 17 (4), 913–930.

Pandey, P.K., Kass, P.H., Soupir, M.L., Biswas, S., Singh, V.P., 2014. Contamination of water resources by pathogenic bacteria. AMB Express 4, 51.

Pascual-Benito, M., Ballesté, E., Monleón-Getino, T., Urmeneta, J., Blanch, A.R., García-Aljaro, C., Lucena, F., 2020. Impact of treated sewage effluent on the bacterial community composition in an intermittent mediterranean stream. Environmental pollution (Barking, Essex : 1987) 266 (Pt 1), 115254.

Pei, L., Rieger, M., Lengger, S., Ott, S., Zawadsky, C., Hartmann, N.M., Selinka, H.-C., Tiehm, A., Niessner, R., Seidel, M., 2012. Combination of crossflow ultrafiltration, monolithic affinity filtration, and quantitative reverse transcriptase PCR for rapid concentration and

quantification of model viruses in water. Environmental science & technology 46 (18), 10073–10080.

Petrovich, M., Chu, B., Wright, D., Griffin, J., Elfeki, M., Murphy, B.T., Poretsky, R., Wells, G., 2018. Antibiotic resistance genes show enhanced mobilization through suspended growth and biofilm-based wastewater treatment processes. FEMS microbiology ecology 94 (5).

Pruden, A., Arabi, M., Storteboom, H.N., 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environmental science & technology 46 (21), 11541–11549.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. The Science of the total environment 447, 345–360.

Sabri, N.A., Schmitt, H., van der Zaan, B., Gerritsen, H.W., Zuidema, T., Rijnaarts, H.H.M., Langenhoff, A.A.M., 2020. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. Journal of Environmental Chemical Engineering 8 (1), 102245.

Sánchez-Alfonso, A.C., Venegas, C., Díez, H., Méndez, J., Blanch, A.R., Jofre, J., Campos, C., 2020. Microbial indicators and molecular markers used to differentiate the source of faecal pollution in the Bogotá River (Colombia). International journal of hygiene and environmental health 225, 113450.

Sanguino, L., Franqueville, L., Vogel, T.M., Larose, C., 2015. Linking environmental prokaryotic viruses and their host through CRISPRs. FEMS microbiology ecology 91 (5).

Stange, C., Sidhu, J.P.S., Tiehm, A., Toze, S., 2016. Antibiotic resistance and virulence genes in coliform water isolates. International journal of hygiene and environmental health 219 (8), 823–831.

Stange, C., Yin, D., Xu, T., Guo, X., Schäfer, C., Tiehm, A., 2019. Distribution of clinically relevant antibiotic resistance genes in Lake Tai, China. The Science of the total environment 655, 337–346.

Tornevi, A., Bergstedt, O., Forsberg, B., 2014. Precipitation effects on microbial pollution in a river: Lag structures and seasonal effect modification. PloS one 9 (5), e98546.

United States Environmental Protection Agency, 2015. Review of coliphages as possible indicators of faecal contamination for ambient water quality: No. 820-R-15-098, Washington, D.C. https://www.epa.gov/sites/default/files/2016-07/documents/review_of_coliphages_as_possible_indicators_of_faecal_contamination_for_ambient_water_quality.pdf (accessed 14.02.2023).

Varela, M.F., Monteiro, S., Rivadulla, E., Santos, R., Romalde, J.L., 2018. Development of a novel digital RT-PCR method for detection of human sapovirus in different matrices. Journal of virological methods 254, 21–24.

Voigt, A.M., Zacharias, N., Timm, C., Wasser, F., Sib, E., Skutlarek, D., Parcina, M., Schmithausen, R.M., Schwartz, T., Hembach, N., Tiehm, A., Stange, C., Engelhart, S., Bierbaum, G., Kistemann, T., Exner, M., Faerber, H.A., Schreiber, C., 2020. Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater - An evaluation of clinical influences. Chemosphere 241, 125032.

World Health Organization, 2011. Guidance on Water Supply and Sanitation in Extreme Weather Events, Geneva.

World Health Organization, 2017. Potable reuse - Guidance for producing safe drinking-water. World Health Organization, Geneva, Switzerland.

Zhang, X.-X., Zhang, T., Fang, H.H.P., 2009. Antibiotic resistance genes in water environment. Applied microbiology and biotechnology 82 (3), 397–414.

2 Designation of the samples

Each sample is given a sample ID, which is composed as follows:

Country code_Number of sampling campaign_Number of sample_Type of sampling campaign_[Type of extract)		
Example: D_01_01_N		
Country code:	Germany	D
	Spain	E
	Portugal	P
	Sweden	S
	Israel	I
	France	F
	Mozambique	M
	Uganda	U
Number of sampling campaign:	Two digits	e.g., 01
Number of sample:	Two digits	e.g., 01
Type of sampling campaign	Normal	N
	Extreme event	E
Type of extract:	Bacterial extract	BacNA
	Viral DNA extract	VirDNA
	Viral RNA extract	VirRNA
	Sediment extract	Sed
	Bivalve molluscan shellfish extract	BMS

In addition, each partner is provided with a template for an Excel file. In this table, the partners will record the sample ID and sample details:

- Country
- Partner
- Type of extract
- Number of sampling campaign
- Type of sampling campaign
- Date of sampling
- Sampling time
- Sampling site
- Type of sample
- Specification of sampling site
- Air Temperature during sampling
- Water temperature during sampling
- Conductivity
- pH
- Volume concentrated
- Elution volume

Furthermore, results of the culture procedures will be reported in the table.
After each sample campaign, each partner will send an updated file to Claudia Stange (Claudia.Stange@tzw.de) and Johannes Ho (Johannes.Ho@tzw.de). TZW is responsible for merging all SARA data into one Excel file. This file will be saved on the SARA drive, where it is also regularly updated. In parallel, a backup file is always stored on the TZW server.

3 Overview of analytical parameters and protocols

In the first phase of the SARA project, protocols for sampling and microbiological analyses (culture method and RT-qPCR) were harmonised. The use of harmonised protocols will decrease variability in the analysis of the different matrices among partners. The SARA partners take samples in their respective area of investigation and carry out the culture procedures in their laboratory. In addition, the samples are prepared for molecular biological analysis and the nucleic acids are extracted. These nucleic acids are conserved and distributed to the different laboratory for analysis of each specific parameter. Therefore, the following protocols were harmonised:

- enumeration of *Escherichia coli*;
- enumeration of extended spectrum-β-lactamase producing *Escherichia coli;*
- enumeration of F-specific and somatic coliphages;
- concentration and extraction of bacteria;
- concentration and extraction of viruses;
- sample conservation and shipment;
- Enumeration of the human-specific microbial source tracking markers (*Bacteroidetes* marker HF183, *Bifidobacterium* marker HMBif, crAssphage).

4 Sampling

Sampling situations vary widely and therefore, no universal sampling procedure can be recommended. Sampling locations are to be determined and evaluated prior to the start of sampling. Sampling campaigns will be performed at model sites in Sweden, Germany, France, Spain, Portugal, Israel, Uganda, and Mozambique (see figure 1). Overall, the following sampling points should be considered:

- hospital wastewater
- influent of municipal sewage treatment plant
- effluent of secondary wastewater treatment
- surface water upstream and downstream of the treatment plant
- coastal sea water

Each partner is responsible for sampling at one site. The study sites represent different geographical regions and climate zones.

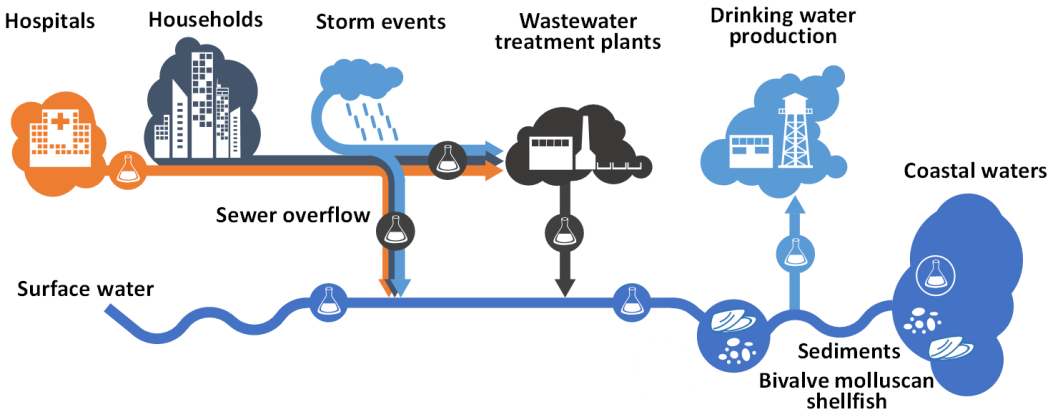


Figure 1: Sampling sites

4.1 Water sampling

For streams, rivers, and other surface waters, the direct method may be utilised to collect grab water samples. These samples will typically be collected either by directly filling the container from the surface water body being sampled or by decanting the water from a collection device such as a stainless steel scoop or other device:

- For the direct filling of the containers, remove the lid and invert the sample jar and lower the container beneath the surface. If surface debris or film is present, the container lid can be removed underwater if possible. Tilt the container in the direction of water flow and allow the container to fill and then quickly return to the surface. Secure the cap, label and immediately cool.
- Using a collection device, the container should be rinsed with surface water and the water discard downstream of the location. The intermediate container should be filled by following the direct sampling method. Once the intermediate container is filled, minimise agitation, and then carefully fill the sample containers. Secure the cap, label and immediately cool.

More specification on sampling can be found in ISO 8199 and ISO 5667-1-3. Get the samples and deliver them to the laboratory as quickly as possible. Use disposable gloves or sterile poles or forceps for subsurface sampling, to minimise the infection risk for the sampling personnel. Clean the outer surface of the flask.

Field parameters

For all samples, the pH value, the temperature at sampling and the conductivity are determined.

pH: The pH meter should be calibrated according to the manufacturers requirements, using a two or three point calibration with buffers of known concentration. The selection of the calibration standards to be used is dependent on the expected nature of the water to be sampled, with strongly acidic or basic waters requiring calibration with standards appropriate to the anticipated range. Calibration standards should be stored appropriately to ensure their accuracy

Temperature: The temperature should be measured and recorded directly after sample collection.

Conductivity: The electrical conductivity (EC) of the water is a measure of the concentration of chemical ions in the water. As with pH measurement, calibration of conductivity meters should be performed using standards of a known concentration appropriate to the anticipated range of conductivity of the water to be sampled. Calibration standards should be stored appropriately to ensure their accuracy.

Sampling volumes and processed volumes

Different sample volumes are required for the analysis of the different parameters. The following table gives an overview of the required volumes per parameter and sample type as well as the total sample volume to be taken.

Parameter/ Procedure	Hospital wastewater	Wastewater treatment plant influent	Wastewater treatment plant effluent (after secondary treatment)	Surface water	Coastal water
E. coli	1-100 µL*	1-100 µL*	1-100 mL	100 mL	100 mL
ESBL *E. coli*	1-100 µL*	1-100 µL*	1-100 mL	100 mL	100 mL
Somatic and F-specific coliphages	1-100 µL*	1-100 µL*	1-100 mL	100 mL	100 mL
Concentration of bacteria	50-100 mL	50-100 mL	250-500 mL	1-2 L	1-2L
Concentration of viruses	1 L	1 L	5 L	>20-100 L	50 L
Sampling volume	**1,5 L**	**1,5 L**	**6 L**	**103 L**	**53 L**

* Prepare decimal serial dilutions; use a minimum volume of 1 mL from the sample or dilution.

Sample transportation and maximum time until analysis

Cool samples at a temperature of 1-6 °C during transport to the laboratory. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water from melted ice during transit or storage. Analyse samples as soon as possible after collection. Maximum time until analysis for samples varies depending on the test.

Parameter / Procedure	**Maximum time until analysis**
E. coli and coliform bacteria	24 h
ESBL *E. coli*	24 h
F-specific and somatic coliphages	48 h
Concentration of bacteria	24 h
Concentration of viruses	24 h

4.2 Sediment sampling

Sediment samples are taken at each sampling site (if possible) from a depth of 0-5 cm. The samples are fill in sterile 50 mL centrifuge tubes or plastic bags and transported to the laboratory in a cooling box. After arrival in the laboratory the sediments are stored at -20 °C until shipment.

5 Method for the enumeration of ESBL-producing *Escherichia coli*

5.1 Scope

Chromogenic medium and membrane filter procudure are used for overnight detection of gram-negative bacteria producing extended spectrum beta-lactamase.

5.2 Definitions

ESBL (Extended Spectrum β-Lactamases) are enzymes that mediate resistance to penicillins, extended-spectrum third generation cephalosporins (C3G) and monobactams. ESBL-producing Enterobacteriaceae started to appear in the 80s, and have emerged as some of the most significant hospital-acquired infections with *Escherichia coli* and *Klebsiella* spp. being the main actors, but other gram-negative species have also been observed. Therefore, the early detection of ESBL-producing bacteria carriers is important to minimise their impact .

5.3 Principle and modification for environmental samples

This methods provides a direct count of ESBL *E. coli* in ambient water or wastewater based on the development of colonies that grow on the surface of a membrane filter placed on chromogenic media containing antibiotics. A sample is filtered through the membrane, which retains the bacteria. After filtration, the membrane is placed on a selective and differential medium and incubated. The target colonies on modified mTEC agar are red or magenta in color after the incubation period.

The agar plates are designed for clinical samples. Here, a significantly stronger interference of the detection due to high growth of background flora is shown than with samples from drinking water or human material. The original methodology according to the manufacturer's specifications was therefore modified with regard to the incubation temperature and additional tests for differentiation, especially with regard to growth suppression of the accompanying flora. The basis for this are the results of various preliminary tests for process optimisation with aquatic environmental samples (Schreiber et al. 2021).

5.4 Reagents and culture media

- CHROMagar™ ESBL
- Chromocult Coliform AGAR
- Bactident® oxidase test strips, Company: Merck, Art.-No.: 1.13300.0001
    - Catalase test or test for indole formation
    - CHROMagar ESBL plate

Use ready-to-use plates or prepare plates using dry medium according to manufacturers´ instructions:

- Disperse slowly 33 g of powder base in 1 L of purified water.
- Stir until agar is well thickened.

- Heat and bring to boiling (100 °C) while swirling or stirring regularly.

Advice 1: For enhanced growth, add 0.5 g/L of Tween 80 to the previous preparation mix.
Advice 2: For the 100 °C heating step, mixture may also be brought to a boil in a microwave oven: after initial boiling, remove from oven, stir gently, then return to oven for short repeated bursts of heating until complete fusion of the agar grains has taken place (large bubbles replacing foam).

- Autoclave at 121 °C during 15 min.
- Cool in a water bath to 45/50 °C, swirling or stirring gently

- Weight 570 mg of the required supplement powder.
- Add 10 mL of purified sterile water to this powder to make a supplement solution.

Warning 1: This step may require several minutes of stirring to obtain a good and homogenous suspension: opaque yellowish appearance.
Warning 2: Reconstituted supplement solution must be used the same day.
Warning 3: Do not store and re-use a supplement solution.

- Vortex this supplement to homogenise and add this supplement solution to melted CHROMagar™ Orientation cooled at 45/50 °C to prepare CHROMagar™ ESBL.
- Stir.

- Pour into sterile Petri dishes.
- Let it solidify and dry.

- Store in the dark before use.
- Prepared media plates can be kept for one day at room temperature.
- Plates can be stored for up to 1 month under refrigeration (2/8 °C) if properly prepared and protected from light and dehydration.

5.5 Apparatus

- Incubator
- Hand tally or electronic counting device
- Pipets, sterile, glass or plastic, of appropriate volume
- Pipet container, stainless steel, aluminum or borosilicate glass, for glass pipets
- Sterile graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper
- Sterile membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with
- aluminum foil or kraft paper
- Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source (In an emergency or in thefield, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used)
- Filter flask, vacuum, usually 1 L, with appropriate tubing
- Filter manifold to hold a number of filter bases (optional)

- Flask for safety trap placed between the filter flask and the vacuum source
- Forceps, straight or curved, with smooth tips to handle filters without damage
- Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps
- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles
- Petri dishes, sterile, plastic, 9 × 50 mm, with tight-fitting lids; and 15 × 100 mm with loose fitting lids
- Bottles
- Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 µm pore size
- Platinum wire inoculation loops, at least 3 mm diameter in suitable holders; or sterile plastic loops
- Water bath
- Autoclave

5.6 Procedure

The sample bottle is shaken before sample preparation and each sampling. Volumes > 1 mL are filtered and the filter is placed on the respective selective CHROMagar™ without air bubbles. If a higher load is expected, 1 mL or 0.1 mL sample or their dilutions can be plated out directly. At least three different volumes of each sample are examined in order to obtain at least one evaluable sample set.

Incubation is carried out at 42 ± 1 °C for 24 ± 3 h under aerobic conditions.

Suspect colonies (dark pink to reddish) with the same morphology are grouped together. At least one colony per group must be further investigated for confirmation. The bacteria can be distinguished as follows:

Colonies with a dark pink/purple to reddish colouration are counted as suspected *E. coli*. Confirmation possible via:

- Typical growth at 37 ± 1 °C overnight on selective culture medium for *E. coli*, e.g. Chromocult Coliform (CC) agar, as an alternative Violet Red Bile (VRB) agar or Endo (Lactose-Fuchsin Sulphite) agar can be used. Colonies of *E. coli* are purple on CC agar, red with reddish halo on VRB agar and deep purple with fuchsin lance on Endo agar.
- Physiological reactions: *E. coli* is oxidase negative, catalase positive, indole positive.
- MALDI TOF Mass Spectra analysis

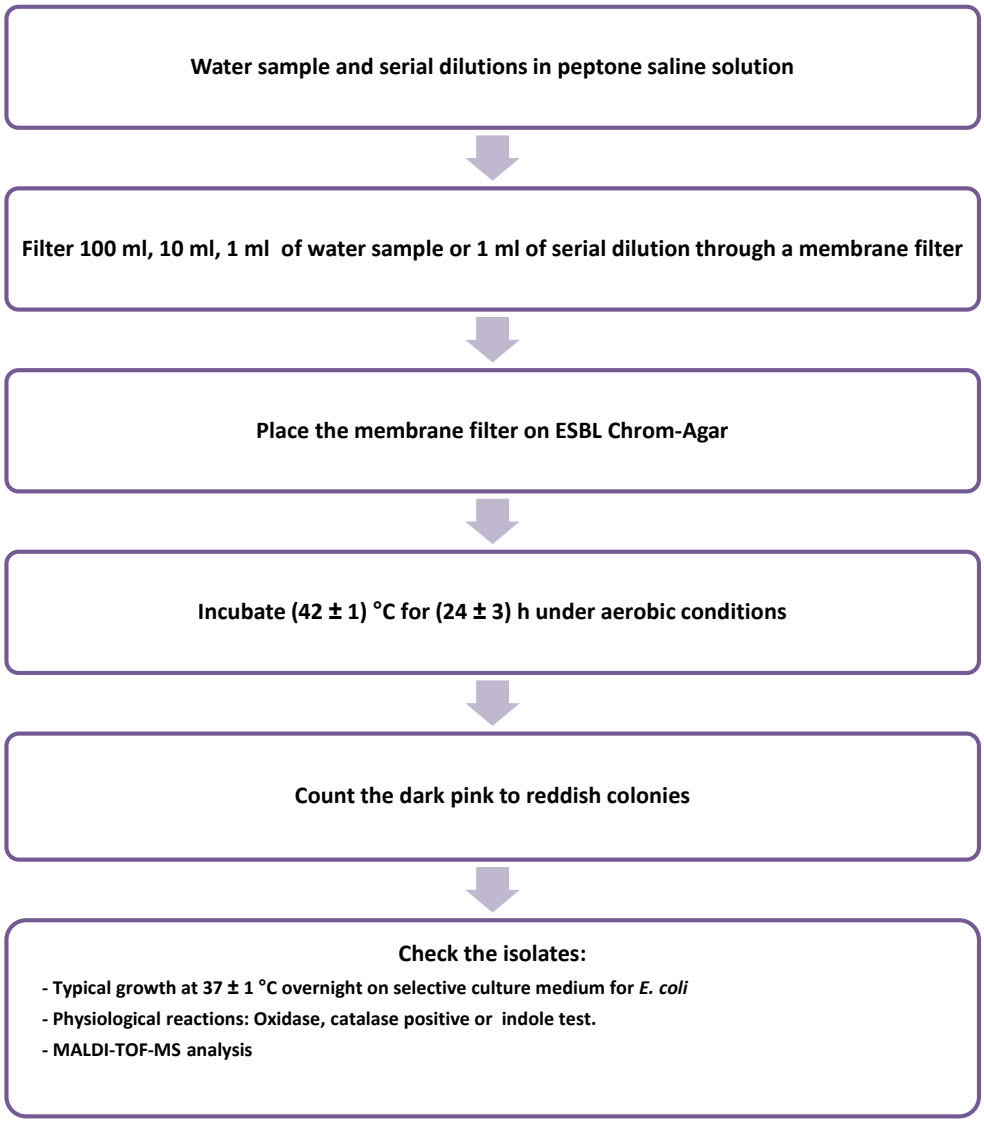


Figure 2: Scheme of the method for the detection and enumeration of ESBL E. Coli

5.7 Quality assurance measures

A positive and negative control is performed prior to the use of each new batch of chemicals or culture media. The controls are documented and the released batches are labelled accordingly.

Positive controls:

- ESBL *E. coli* CIP 103982
- ESBL *K. pneumoniae* ATCC® 700603

Possible negative controls:

- *E. faecalis* ATCC® 29212
- *P. aeruginosa* ATCC® 10145
- *E. coli* ATCC® 25922
- *C. albicans* ATCC® 60193
- *S. aureus* ATCC® 25923

Sterility control:

- Uninoculated CHROMagarTM ESBL plate.

5.8 Literature

Schreiber, C., Zacharias, N., Essert, S.M., Wasser, F., Müller, H., Sib, E., Precht, T., Parcina, M., Bierbaum, G., Schmithausen, R.M., Kistemann, T., Exner, M., 2021. Clinically relevant antibiotic-resistant bacteria in aquatic environments – An optimised culture-based approach. The science of the total environment 750, 142265.

6 Method for the enumeration of *Escherichia coli* (Colilert, IDEXX)

6.1 Scope

This standard operating procedure describes the test method for the collection and analysis of water samples for the enumeration of Escherichia coli (*E. coli*) and total coliform bacteria.

6.2 Definitions

- IDEXX: Manufacturer of technology-based products and services for veterinary, food and water applications including IDEXX Colilert® Test Kit and IDEXX QuantiTray/2000.
- **Total coliforms** include bacteria that are found in the soil, in water that has been influenced by surface water, and in human or animal waste.
- **Faecal coliforms** are the group of the total coliforms that are considered to be present specifically in the gut and feces of warm-blooded animals. Because the origins of faecal coliforms are more specific than the origins of the more general total coliform group of bacteria, faecal coliforms are considered a more accurate indication of animal or human waste than the total coliforms.
- ***Escherichia coli* (*E. coli*)** is the major species in the faecal coliform group. Of the five general groups of bacteria that comprise the total coliforms, only E. coli is generally not found growing and reproducing in the environment. Consequently, E. coli is considered to be the species of coliform bacteria that is the best indicator of faecal pollution and the possible presence of pathogens.

6.3 Principle

The Colilert® reagent is added directly to the 100 mL undiluted or diluted sample. It is mixed thoroughly to dissolve the reagent. The sample is transferred to QuantiTrays®/2000 and sealed using the Quanti-Tray sealer. Samples are incubated at (35.0 ± 0.5) °C for 24 hours. Results are reported as MPN/100mL.

6.4 Reagents and materials

- Colilert® reagent, IDEXX Laboratories, catalog number WP100I.
- Quanti-Tray®/2000, , IDEXX Laboratories, catalog number WQT-2K
- Sterile, shrink-wrapped 100 mL IDEXX bottles
- Quanti-Tray Sealer®: catalog number WQTS2X-115. IDEXX Laboratories
- Incubator

6.5 Procedure

The 100 mL duplicate water sample is shaken well just prior to preparation for analysis. Open a Colilert ampule and pour contents into either the diluted sample or undiluted sample. Repeat for the remaining sample.

Mix thoroughly, making sure the Colilert reagent is completely dissolved. Follow manufacturer's instructions for preparation of QuantiTray/2000 and use of the Quanti-Tray Sealer.

Allow bubbles to settle or dissipate. Failure to do this may result in the wells filling or sealing improperly.

Record the sample's site code on the back of the well for identification purposes. Record the lot number of the reagents and the wells used on the bench sheet in the comments section.

Incubate at (35.0 ± 0.5)°C for 24 hours.

Count the number of small and large positive wells and refer to the MPN table to find the most probable number for total coliform bacteria. *E. coli* results are obtained by placing the wells under a black light and counting the number of fluorescent wells. Refer to the MPN table to determine the *E. coli* concentration.

Perform data acquisition, calculations, and reporting. For each sample analysed, including quality control samples, record the number of small and large positive wells and the MPN in the appropriate places on the bench sheet (see below). Calculate precision for duplicate analyses.

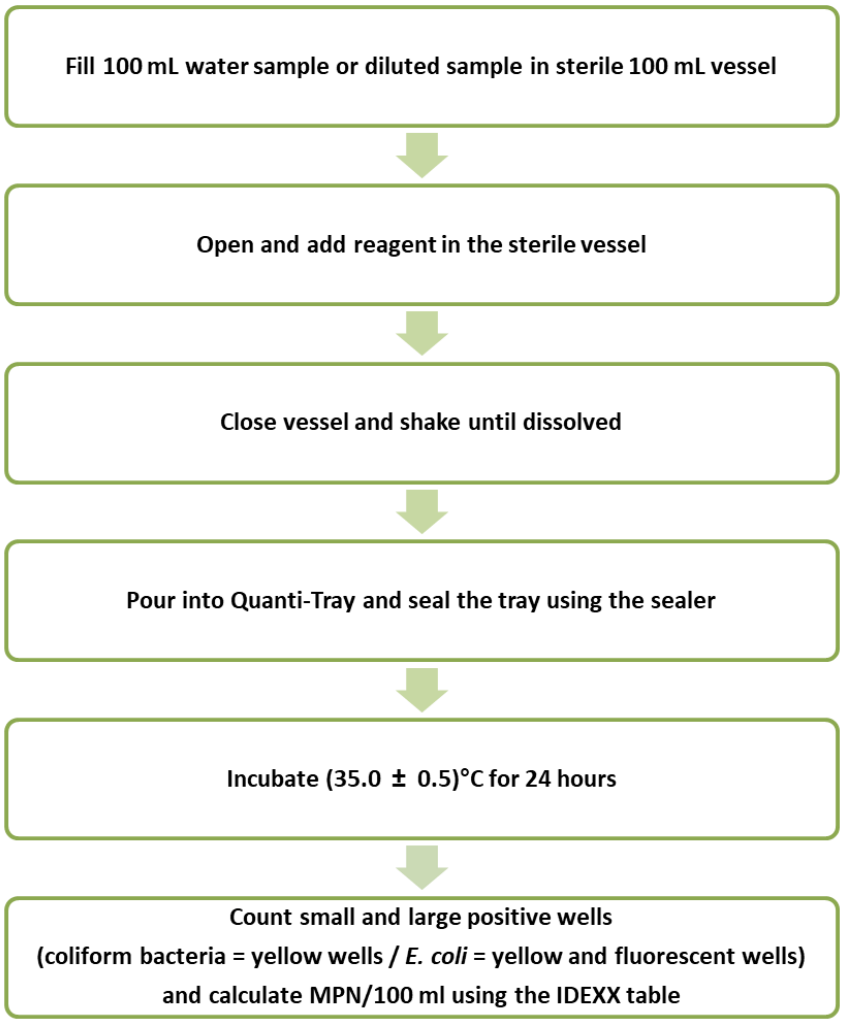


Figure 3: Scheme of the method for the detection and enumeration of coliform bacteria and E. coli

IDEXX Quanti-Tray®/2000 MPN Table (per 100ml)

Small Wells Positive

# Large Wells Positive	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
0	<1	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0	14.1	15.1	16.1	17.1	18.1	19.1	20.2	21.2	22.2	23.3	24.3
1	1.0	2.0	3.0	4.0	5.0	6.0	7.1	8.1	9.1	10.1	11.1	12.1	13.2	14.2	15.2	16.2	17.3	18.3	19.3	20.4	21.4	22.4	23.5	24.5	25.6
2	2.0	3.0	4.1	5.1	6.1	7.1	8.1	9.2	10.2	11.2	12.2	13.3	14.3	15.4	16.4	17.4	18.5	19.5	20.6	21.6	22.7	23.7	24.8	25.8	26.9
3	3.1	4.1	5.1	6.1	7.2	8.2	9.2	10.3	11.3	12.4	13.4	14.5	15.5	16.5	17.6	18.6	19.7	20.8	21.8	22.9	23.9	25.0	26.1	27.1	28.2
4	4.1	5.2	6.2	7.2	8.3	9.3	10.4	11.4	12.5	13.5	14.6	15.6	16.7	17.8	18.8	19.9	21.0	22.0	23.1	24.2	25.3	26.3	27.4	28.5	29.6
5	5.2	6.3	7.3	8.4	9.4	10.5	11.5	12.6	13.7	14.7	15.8	16.9	17.9	19.0	20.1	21.2	22.2	23.3	24.4	25.5	26.6	27.7	28.8	29.9	31.0
6	6.3	7.4	8.4	9.5	10.6	11.6	12.7	13.8	14.9	16.0	17.0	18.1	19.2	20.3	21.4	22.5	23.6	24.7	25.8	26.9	28.0	29.1	30.2	31.3	32.4
7	7.5	8.5	9.6	10.7	11.8	12.8	13.9	15.0	16.1	17.2	18.3	19.4	20.5	21.6	22.7	23.8	24.9	26.0	27.1	28.3	29.4	30.5	31.6	32.8	33.9
8	8.6	9.7	10.8	11.9	13.0	14.1	15.2	16.3	17.4	18.5	19.6	20.7	21.8	22.9	24.1	25.2	26.3	27.4	28.6	29.7	30.8	32.0	33.1	34.3	35.4
9	9.8	10.9	12.0	13.1	14.2	15.3	16.4	17.6	18.7	19.8	20.9	22.0	23.2	24.3	25.4	26.6	27.7	28.9	30.0	31.2	32.3	33.5	34.6	35.8	37.0
10	11.0	12.1	13.2	14.4	15.5	16.6	17.7	18.9	20.0	21.1	22.3	23.4	24.6	25.7	26.9	28.0	29.2	30.3	31.5	32.7	33.8	35.0	36.2	37.4	38.6
11	12.2	13.4	14.5	15.6	16.8	17.9	19.1	20.2	21.4	22.5	23.7	24.8	26.0	27.2	28.3	29.5	30.7	31.9	33.0	34.2	35.4	36.6	37.8	39.0	40.2
12	13.5	14.6	15.8	16.9	18.1	19.3	20.4	21.6	22.8	23.9	25.1	26.3	27.5	28.6	29.8	31.0	32.2	33.4	34.6	35.8	37.0	38.2	39.5	40.7	41.9
13	14.8	16.0	17.1	18.3	19.5	20.6	21.8	23.0	24.2	25.4	26.6	27.8	29.0	30.2	31.4	32.6	33.8	35.0	36.2	37.5	38.7	39.9	41.2	42.4	43.6
14	16.1	17.3	18.5	19.7	20.9	22.1	23.3	24.5	25.7	26.9	28.1	29.3	30.5	31.7	33.0	34.2	35.4	36.7	37.9	39.1	40.4	41.6	42.9	44.2	45.4
15	17.5	18.7	19.9	21.1	22.3	23.5	24.7	25.9	27.2	28.4	29.6	30.9	32.1	33.3	34.6	35.8	37.1	38.4	39.6	40.9	42.2	43.4	44.7	46.0	47.3
16	18.9	20.1	21.3	22.6	23.8	25.0	26.2	27.5	28.7	30.0	31.2	32.5	33.7	35.0	36.3	37.5	38.8	40.1	41.4	42.7	44.0	45.3	46.6	47.9	49.2
17	20.3	21.6	22.8	24.1	25.3	26.6	27.8	29.1	30.3	31.6	32.9	34.1	35.4	36.7	38.0	39.3	40.6	41.9	43.2	44.5	45.9	47.2	48.5	49.8	51.2
18	21.8	23.1	24.3	25.6	26.9	28.1	29.4	30.7	32.0	33.3	34.6	35.9	37.2	38.5	39.8	41.1	42.4	43.8	45.1	46.5	47.8	49.2	50.5	51.9	53.2
19	23.3	24.6	25.9	27.2	28.5	29.8	31.1	32.4	33.7	35.0	36.3	37.6	39.0	40.3	41.6	43.0	44.3	45.7	47.1	48.4	49.8	51.2	52.6	54.0	55.4
20	24.9	26.2	27.5	28.8	30.1	31.5	32.8	34.1	35.4	36.8	38.1	39.5	40.8	42.2	43.6	44.9	46.3	47.7	49.1	50.5	51.9	53.3	54.7	56.1	57.6
21	26.5	27.9	29.2	30.5	31.8	33.2	34.5	35.9	37.3	38.6	40.0	41.4	42.8	44.1	45.5	46.9	48.4	49.8	51.2	52.6	54.1	55.5	56.9	58.4	59.9
22	28.2	29.5	30.9	32.3	33.6	35.0	36.4	37.7	39.1	40.5	41.9	43.3	44.8	46.2	47.6	49.0	50.5	51.9	53.4	54.8	56.3	57.8	59.3	60.8	62.3
23	29.9	31.3	32.7	34.1	35.5	36.8	38.3	39.7	41.1	42.5	43.9	45.4	46.8	48.3	49.7	51.2	52.7	54.2	55.6	57.1	58.6	60.2	61.7	63.2	64.7
24	31.7	33.1	34.5	35.9	37.3	38.8	40.2	41.7	43.1	44.6	46.0	47.5	49.0	50.5	52.0	53.5	55.0	56.5	58.0	59.5	61.1	62.6	64.2	65.8	67.3
25	33.6	35.0	36.4	37.9	39.3	40.8	42.2	43.7	45.2	46.7	48.2	49.7	51.2	52.7	54.3	55.8	57.3	58.9	60.5	62.0	63.6	65.2	66.8	68.4	70.0
26	35.5	36.9	38.4	39.9	41.4	42.8	44.3	45.9	47.4	48.9	50.4	52.0	53.5	55.1	56.7	58.2	59.8	61.4	63.0	64.7	66.3	67.9	69.6	71.2	72.9
27	37.4	38.9	40.4	42.0	43.5	45.0	46.5	48.1	49.6	51.2	52.8	54.4	56.0	57.6	59.2	60.8	62.4	64.1	65.7	67.4	69.1	70.8	72.5	74.2	75.9
28	39.5	41.0	42.6	44.1	45.7	47.3	48.8	50.4	52.0	53.6	55.2	56.9	58.5	60.2	61.8	63.5	65.2	66.9	68.6	70.3	72.0	73.7	75.5	77.3	79.0
29	41.7	43.2	44.8	46.4	48.0	49.6	51.2	52.8	54.5	56.1	57.8	59.5	61.2	62.9	64.6	66.3	68.0	69.8	71.5	73.3	75.1	76.9	78.7	80.5	82.4
30	43.9	45.5	47.1	48.7	50.4	52.0	53.7	55.4	57.1	58.8	60.5	62.2	64.0	65.7	67.5	69.3	71.0	72.9	74.7	76.5	78.3	80.2	82.1	84.0	85.9
31	46.2	47.9	49.5	51.2	52.9	54.6	56.3	58.1	59.8	61.6	63.3	65.1	66.9	68.7	70.5	72.4	74.2	76.1	78.0	79.9	81.8	83.7	85.7	87.6	89.6
32	48.7	50.4	52.1	53.8	55.6	57.3	59.1	60.9	62.7	64.5	66.3	68.2	70.0	71.9	73.8	75.7	77.6	79.5	81.5	83.5	85.4	87.5	89.5	91.5	93.6
33	51.2	53.0	54.8	56.5	58.3	60.2	62.0	63.8	65.7	67.6	69.5	71.4	73.3	75.2	77.2	79.2	81.2	83.2	85.2	87.3	89.3	91.4	93.6	95.7	97.8
34	53.9	55.7	57.6	59.4	61.3	63.1	65.0	67.0	68.9	70.8	72.8	74.8	76.8	78.8	80.8	82.9	85.0	87.1	89.2	91.4	93.5	95.7	97.9	100.2	102.4
35	56.8	58.6	60.5	62.4	64.4	66.3	68.3	70.3	72.3	74.3	76.3	78.4	80.5	82.6	84.7	86.9	89.1	91.3	93.5	95.7	98.0	100.3	102.6	105.0	107.3
36	59.8	61.7	63.7	65.7	67.7	69.7	71.7	73.8	75.9	78.0	80.1	82.3	84.5	86.7	88.9	91.2	93.5	95.8	98.1	100.5	102.9	105.3	107.7	110.2	112.7
37	62.9	65.0	67.0	69.1	71.2	73.3	75.4	77.6	79.8	82.0	84.2	86.5	88.8	91.1	93.4	95.8	98.2	100.6	103.1	105.6	108.1	110.7	113.3	115.9	118.6
38	66.3	68.4	70.6	72.7	74.9	77.1	79.4	81.6	83.9	86.2	88.6	91.0	93.4	95.8	98.3	100.8	103.4	105.9	108.6	111.2	113.9	116.6	119.4	122.2	125.0
39	70.0	72.2	74.4	76.7	78.9	81.3	83.6	86.0	88.4	90.9	93.4	95.9	98.4	101.0	103.6	106.3	109.0	111.8	114.6	117.4	120.3	123.2	126.1	129.2	132.2
40	73.8	76.2	78.5	80.9	83.3	85.7	88.2	90.8	93.3	95.9	98.5	101.2	103.9	106.7	109.5	112.4	115.3	118.2	121.2	124.3	127.4	130.5	133.7	137.0	140.3
41	78.0	80.5	83.0	85.5	88.0	90.6	93.3	95.9	98.7	101.4	104.3	107.1	110.0	113.0	116.0	119.1	122.2	125.4	128.7	132.0	135.4	138.8	142.3	145.9	149.5
42	82.6	85.2	87.8	90.5	93.2	96.0	98.8	101.7	104.6	107.6	110.6	113.7	116.9	120.1	123.4	126.7	130.1	133.6	137.2	140.8	144.5	148.3	152.2	156.1	160.2
43	87.6	90.4	93.2	96.0	99.0	101.9	105.0	108.1	111.2	114.5	117.8	121.1	124.6	128.1	131.7	135.4	139.1	143.0	147.0	151.0	155.2	159.4	163.8	168.2	172.8
44	93.1	96.1	99.1	102.2	105.4	108.6	111.9	115.3	118.7	122.3	125.9	129.6	133.4	137.4	141.4	145.5	149.7	154.1	158.5	163.1	167.9	172.7	177.7	182.9	188.2
45	99.3	102.5	105.8	109.2	112.6	116.2	119.8	123.6	127.4	131.4	135.4	139.6	143.9	148.3	152.9	157.6	162.4	167.4	172.6	178.0	183.5	189.2	195.1	201.2	207.5
46	106.3	109.8	113.4	117.2	121.0	125.0	129.1	133.3	137.6	142.1	146.7	151.5	156.5	161.6	167.0	172.5	178.2	184.2	190.4	196.8	203.5	210.5	217.8	225.4	233.3
47	114.3	118.3	122.4	126.6	130.9	135.4	140.1	145.0	150.0	155.3	160.7	166.4	172.3	178.5	185.0	191.8	198.9	206.4	214.2	222.4	231.0	240.0	249.5	259.5	270.0
48	123.9	128.4	133.1	137.9	143.0	148.3	153.9	159.7	165.8	172.2	178.9	186.0	193.5	201.4	209.8	218.7	228.2	238.2	248.9	260.3	272.3	285.1	298.7	313.0	328.2
49	135.5	140.8	146.4	152.3	158.5	165.0	172.0	179.3	187.2	195.6	204.6	214.3	224.7	235.9	248.1	261.3	275.5	290.9	307.6	325.5	344.8	365.4	387.3	410.6	435.2

09-63235-01

Figure 4: IDEXX evaluation table – part I

Figure 5: IDEXX evaluation table – part II

IDEXX Quanti-Tray®/2000 MPN Table (per 100ml)

# Large Wells Positive	# Small Wells Positive																							
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
0	25.3	26.4	27.4	28.4	29.5	30.5	31.5	32.6	33.6	34.7	35.7	36.8	37.8	38.9	40.0	41.0	42.1	43.1	44.2	45.3	46.3	47.4	48.5	49.5
1	26.6	27.7	28.7	29.8	30.8	31.9	32.9	34.0	35.0	36.1	37.2	38.2	39.3	40.4	41.4	42.5	43.6	44.7	45.7	46.8	47.9	49.0	50.1	51.2
2	27.9	29.0	30.0	31.1	32.2	33.2	34.3	35.4	36.5	37.5	38.6	39.7	40.8	41.9	43.0	44.0	45.1	46.2	47.3	48.4	49.5	50.6	51.7	52.8
3	29.3	30.4	31.4	32.5	33.6	34.7	35.8	36.8	37.9	39.0	40.1	41.2	42.3	43.4	44.5	45.6	46.7	47.8	48.9	50.0	51.2	52.3	53.4	54.5
4	30.7	31.8	32.8	33.9	35.0	36.1	37.2	38.3	39.4	40.5	41.6	42.8	43.9	45.0	46.1	47.2	48.3	49.5	50.6	51.7	52.9	54.0	55.1	56.3
5	32.1	33.2	34.3	35.4	36.5	37.6	38.7	39.9	41.0	42.1	43.2	44.4	45.5	46.6	47.7	48.9	50.0	51.2	52.3	53.5	54.6	55.8	56.9	58.1
6	33.5	34.7	35.8	36.9	38.0	39.2	40.3	41.4	42.6	43.7	44.8	46.0	47.1	48.3	49.4	50.6	51.7	52.9	54.1	55.2	56.4	57.6	58.7	59.9
7	35.0	36.2	37.3	38.4	39.6	40.7	41.9	43.0	44.2	45.3	46.5	47.7	48.8	50.0	51.2	52.3	53.5	54.7	55.9	57.1	58.3	59.4	60.6	61.8
8	36.6	37.7	38.9	40.0	41.2	42.3	43.5	44.7	45.9	47.0	48.2	49.4	50.6	51.8	53.0	54.1	55.3	56.5	57.7	59.0	60.2	61.4	62.6	63.8
9	38.1	39.3	40.5	41.6	42.8	44.0	45.2	46.4	47.6	48.8	50.0	51.2	52.4	53.6	54.8	56.0	57.2	58.4	59.7	60.9	62.1	63.4	64.6	65.8
10	39.7	40.9	42.1	43.3	44.5	45.7	46.9	48.1	49.3	50.6	51.8	53.0	54.2	55.5	56.7	57.9	59.2	60.4	61.7	62.9	64.2	65.4	66.7	67.9
11	41.4	42.6	43.8	45.0	46.3	47.5	48.7	49.9	51.2	52.4	53.7	54.9	56.1	57.4	58.6	59.9	61.2	62.4	63.7	65.0	66.3	67.5	68.8	70.1
12	43.1	44.3	45.6	46.8	48.1	49.3	50.6	51.8	53.1	54.3	55.6	56.8	58.1	59.4	60.7	62.0	63.2	64.5	65.8	67.1	68.4	69.7	71.0	72.4
13	44.9	46.1	47.4	48.6	49.9	51.2	52.5	53.7	55.0	56.3	57.6	58.9	60.2	61.5	62.8	64.1	65.4	66.7	68.0	69.3	70.7	72.0	73.3	74.7
14	46.7	48.0	49.3	50.5	51.8	53.1	54.4	55.7	57.0	58.3	59.6	60.9	62.3	63.6	64.9	66.3	67.6	68.9	70.3	71.6	73.0	74.4	75.7	77.1
15	48.6	49.9	51.2	52.5	53.8	55.1	56.4	57.8	59.1	60.4	61.8	63.1	64.5	65.8	67.2	68.5	69.9	71.3	72.6	74.0	75.4	76.8	78.2	79.6
16	50.5	51.8	53.2	54.5	55.8	57.2	58.5	59.9	61.2	62.6	64.0	65.3	66.7	68.1	69.5	70.9	72.3	73.7	75.1	76.5	77.9	79.3	80.8	82.2
17	52.5	53.9	55.2	56.6	58.0	59.3	60.7	62.1	63.5	64.9	66.3	67.7	69.1	70.5	71.9	73.3	74.8	76.2	77.6	79.1	80.5	82.0	83.5	84.9
18	54.6	56.0	57.4	58.8	60.2	61.6	63.0	64.4	65.8	67.2	68.6	70.1	71.5	73.0	74.4	75.9	77.3	78.8	80.3	81.8	83.3	84.8	86.3	87.8
19	56.8	58.2	59.6	61.0	62.4	63.9	65.3	66.8	68.2	69.7	71.1	72.6	74.1	75.5	77.0	78.5	80.0	81.5	83.1	84.6	86.1	87.6	89.2	90.7
20	59.0	60.4	61.9	63.3	64.8	66.3	67.7	69.2	70.7	72.2	73.7	75.2	76.7	78.2	79.8	81.3	82.8	84.4	85.9	87.5	89.1	90.7	92.2	93.8
21	61.3	62.8	64.3	65.8	67.3	68.8	70.3	71.8	73.3	74.9	76.4	77.9	79.5	81.1	82.6	84.2	85.8	87.4	89.0	90.6	92.2	93.8	95.4	97.1
22	63.8	65.3	66.8	68.3	69.8	71.4	72.9	74.5	76.1	77.6	79.2	80.8	82.4	84.0	85.6	87.2	88.9	90.5	92.1	93.8	95.5	97.1	98.8	100.5
23	66.3	67.8	69.4	71.0	72.5	74.1	75.7	77.3	78.9	80.5	82.2	83.8	85.4	87.1	88.7	90.4	92.1	93.8	95.5	97.2	98.9	100.6	102.4	104.1
24	68.9	70.5	72.1	73.7	75.3	77.0	78.6	80.3	81.9	83.6	85.2	86.9	88.6	90.3	92.0	93.8	95.5	97.2	99.0	100.7	102.5	104.3	106.1	107.9
25	71.7	73.3	75.0	76.6	78.3	80.0	81.7	83.3	85.1	86.8	88.5	90.2	92.0	93.7	95.5	97.3	99.1	100.9	102.7	104.5	106.3	108.2	110.0	111.9
26	74.6	76.3	78.0	79.7	81.4	83.1	84.8	86.6	88.4	90.1	91.9	93.7	95.5	97.3	99.2	101.0	102.9	104.7	106.6	108.5	110.4	112.3	114.2	116.2
27	77.6	79.4	81.1	82.9	84.6	86.4	88.2	90.0	91.9	93.7	95.5	97.4	99.3	101.2	103.1	105.0	106.9	108.8	110.8	112.7	114.7	116.7	118.7	120.7
28	80.8	82.6	84.4	86.3	88.1	89.9	91.8	93.7	95.6	97.5	99.4	101.3	103.3	105.2	107.2	109.2	111.2	113.2	115.2	117.3	119.3	121.4	123.5	125.6
29	84.2	86.1	87.9	89.8	91.7	93.7	95.6	97.5	99.5	101.5	103.5	105.5	107.5	109.5	111.6	113.7	115.7	117.8	120.0	122.1	124.2	126.4	128.6	130.8
30	87.8	89.7	91.7	93.6	95.6	97.6	99.6	101.6	103.7	105.7	107.8	109.9	112.0	114.2	116.3	118.5	120.6	122.8	125.1	127.3	129.5	131.8	134.1	136.4
31	91.6	93.6	95.6	97.7	99.7	101.8	103.9	106.0	108.2	110.3	112.5	114.7	116.9	119.1	121.4	123.6	125.9	128.2	130.5	132.9	135.3	137.7	140.1	142.5
32	95.7	97.8	99.9	102.0	104.2	106.3	108.5	110.7	113.0	115.2	117.5	119.8	122.1	124.5	126.8	129.2	131.6	134.0	136.5	139.0	141.5	144.0	146.6	149.1
33	100.0	102.2	104.4	106.6	108.9	111.2	113.5	115.8	118.2	120.5	122.9	125.4	127.8	130.3	132.8	135.3	137.8	140.4	143.0	145.6	148.3	150.9	153.7	156.4
34	104.7	107.0	109.3	111.7	114.0	116.4	118.9	121.3	123.8	126.3	128.8	131.4	134.0	136.6	139.2	141.9	144.6	147.4	150.1	152.9	155.7	158.6	161.5	164.4
35	109.7	112.2	114.6	117.1	119.6	122.2	124.7	127.3	129.9	132.6	135.3	138.0	140.8	143.6	146.4	149.2	152.1	155.0	158.0	161.0	164.0	167.1	170.2	173.3
36	115.2	117.8	120.4	123.0	125.7	128.4	131.1	133.9	136.7	139.5	142.4	145.3	148.3	151.3	154.3	157.3	160.5	163.6	166.8	170.0	173.3	176.6	179.9	183.3
37	121.3	124.0	126.8	129.6	132.4	135.3	138.2	141.2	144.2	147.3	150.3	153.5	156.7	159.9	163.1	166.5	169.8	173.2	176.7	180.2	183.7	187.3	191.0	194.7
38	127.9	130.8	133.8	136.8	139.9	143.0	146.2	149.4	152.6	155.9	159.2	162.6	166.1	169.6	173.2	176.8	180.4	184.2	188.0	191.8	195.7	199.7	203.7	207.7
39	135.3	138.5	141.7	145.0	148.3	151.7	155.1	158.6	162.1	165.7	169.4	173.1	176.9	180.7	184.7	188.7	192.7	196.8	201.0	205.3	209.6	214.0	218.5	223.0
40	143.7	147.1	150.6	154.2	157.8	161.5	165.3	169.1	173.0	177.0	181.1	185.2	189.4	193.7	198.1	202.5	207.1	211.7	216.4	221.1	226.0	231.0	236.0	241.1
41	153.2	157.0	160.9	164.8	168.9	173.0	177.2	181.5	185.8	190.3	194.8	199.5	204.2	209.1	214.0	219.1	224.2	229.4	234.8	240.2	245.8	251.5	257.2	263.1
42	164.3	168.6	172.9	177.3	181.9	186.5	191.3	196.1	201.1	206.2	211.4	216.7	222.2	227.7	233.4	239.2	245.2	251.3	257.5	263.8	270.3	276.9	283.6	290.5
43	177.5	182.3	187.3	192.4	197.6	202.9	208.4	214.0	219.8	225.8	231.8	238.1	244.5	251.0	257.7	264.6	271.7	278.9	286.3	293.8	301.5	309.4	317.4	325.7
44	193.6	199.3	205.1	211.0	217.2	223.5	230.0	236.7	243.6	250.8	258.1	265.6	273.3	281.2	289.4	297.8	306.3	315.1	324.1	333.3	342.8	352.4	362.3	372.4
45	214.1	220.9	227.9	235.2	242.7	250.4	258.4	266.7	275.3	284.1	293.3	302.6	312.3	322.3	332.5	343.0	353.8	364.9	376.2	387.9	399.8	412.0	424.5	437.4
46	241.5	250.0	258.9	268.2	277.8	287.8	298.1	308.8	319.9	331.4	343.3	355.5	368.1	381.1	394.5	408.3	422.5	437.1	452.0	467.4	483.3	499.6	516.3	533.5
47	280.9	292.4	304.4	316.9	330.0	343.6	357.8	372.5	387.7	403.4	419.8	436.6	454.1	472.1	490.7	509.9	529.8	550.4	571.7	593.8	616.7	640.5	665.3	691.0
48	344.1	360.9	378.4	396.8	416.0	436.0	456.9	478.6	501.2	524.7	549.3	574.8	601.5	629.4	658.6	689.3	721.5	755.6	791.5	829.7	870.4	913.9	960.6	1011.2
49	461.1	488.4	517.2	547.5	579.4	613.1	648.8	686.7	727.0	770.1	816.4	866.4	920.8	980.4	1046.2	1119.9	1203.3	1299.7	1413.6	1553.1	1732.9	1986.3	2419.6	>2419.6

09-63235-01

7 Detection and enumeration of bacteriophages – Part 1: Enumeration of F-specific RNA bacteriophages (adapted from ISO 10705-1:2000)

7.1 Scope

This protocol specifies a method for the detection and enumeration of F-specific RNA bacteriophages by incubating the sample with an appropriate host strain. The method is applicable to all kinds of water.

7.2 Principle

A culture of host strain is added to the sample that was previously mixed with a small volume of semi-solid nutrient medium and plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. The results are expressed as the number of plaque-forming particles, pfp (also termed plaque-forming units, pfu), *per* unit of sample volume.

7.3 Safety precautions

The host strain used is a *Salmonella typhimurium* mutant of low pathogenicity. Use only by microbiologically trained personnel. F-specific RNA bacteriophages are not pathogenic to humans and animals, but are highly resistant to desiccation. Appropriate precautions should therefore be taken to prevent cross-contamination of test materials, especially when testing and handling cultures that have a high titre or when inoculating host stock cultures. Such procedures must be performed under a safety cabinet or in a segregated laboratory area.
Possible impairments

In samples with very high background bacterial flora, it is possible that the addition of nalidixic acid is not sufficient to suppress them. The evaluation of such samples is then not possible.
If the number of phages per plate is >1000-10000, it is no longer possible to detect plaques. The plate resembles a negative control and thus false-negative results may occur.

7.4 Definitions and abbreviations

- F-specific bacteriophage: The F-specific bacteriophage group is composed of multiple groups of bacteriophages that infect bacteria via the F-specific pili (F-pili) and contain members with single-stranded DNA genomes (F-specific DNA [F-DNA] bacteriophages) or RNA genomes (F-specific RNA [F-RNA] bacteriophages).
- *S. typhimurium*: *Salmonella typhimurium*
- WG49: *Salmonella typhimurium* mutant WG49

7.5 Apparatus

Usual microbiological laboratory equipment, including:

- Hot-air oven for dry-heat sterilization and an autoclave. All the apparatus shall be sterilised according to the instructions given in ISO 8199 except the ones supplied sterile;
- Incubator or water bath thermostatically controlled at (36 ± 2) °C;
- Incubator or water bath, thermostatically controlled at (36 ± 2) °C and equipped with a shaking device, at (100 ± 10) r/min;
- Water bath or heating block, thermostatically controlled at (45 ± 1) °C;
- Water bath or equivalent device for melting of agar media;
- pH meter;
- Counting apparatus;
- Deep freezer, thermostatically controlled at (-20 ± 5) °C;
- Deep freezer, thermostatically controlled at (-70 ± 10) °C or liquid nitrogen storage vessel;
- Spectrophotometer, capable of holding cuvettes of 1 cm optical path length or side-arm of nephelometric flasks and equipped with a filter for the range 500 nm to 650 nm with a maximum bandwidth of ± 10 nm;
- Refrigerator, temperature set at (5 ± 3) °C;
- Petri dishes of 9 cm or 14 cm to 15 cm diameter, vented;
- Graduated pipettes of 0,1 mL, 1 mL, 5 mL and 10 mL capacity;
- Pasteur pipettes;
- Glass bottles;
- Culture tubes with caps or suitable alternative;
- Measuring cylinders;
- Conical flasks of 250 mL to 300 mL capacity, with cotton wool plugs or suitable alternative;
- Cuvettes of optical path length 10 mm or nephelometric conical flasks with cylindrical side-arms which fit in the spectrophotometer (capacity 250 mL to 300 mL with cotton wool plugs or suitable alternative)
- Membrane filter units, pore size 0, 2 µm for decontamination.
- Plastics vials, with lid, of 1,5 mL to 3 mL capacity.

7.6 Reagents and culture media

Use ingredients of uniform quality and chemicals of analytical grade (other grades of chemicals is permissible providing they are shown to be of equal performance in the test) for the preparation of culture media and reagents, alternatively, use dehydrated complete media and follow strictly the manufacturer's instructions.

For the preparation of media, use glass-distilled water or deioniced water free from substances, which might inhibit bacterial growth under the conditions of the test, and complying with ISO 3696.

Calcium-Glucose Solution

Dissolve 3 g $CaCl_2*2H_2O$ and 10 g glucose in 100 mL distilled water and sterile filter through a 0.2 µm membrane filter under the clean room workbench.
Store in the dark at (4 ± 2)°C for not more than 6 months.

Tryptone Yeast Extract Glucose Broth (TYGB)

Basic medium:

Tryptone	10 g
Yeast extract	1 g
NaCl	8 g
Distilled water	1 000 mL

Dissolve the ingredients in hot water. Adjust the pH to 7.2 ± 0,2 at (45 ± 3) °C so that after sterilization it will be 7.2 ± 0.5. Distribute the medium in bottles in volumes of 200 mL and sterilise in the autoclave at (121 ± 3) °C for 15 min. Store in the dark at (5 ± 3) °C until 6 months.

Complete medium:

Add aseptically calcium glucose solution in a ratio of 1:100 to the basic medium and mix well. Store in the dark at (5 ± 3) °C for not more than 6 months.

Tryptone Yeast Extract Glucose Agar (TYGA)

Basal medium:

Tryptone	10 g
Yeast extract	1 g
NaCl	8 g
Agar-Agar	15 g
Distilled water	1 000 mL

Dissolve the ingredients in hot water. Adjust the pH to 7.2 ± 0,2 at (45 ± 3) °C so that after sterilization it will be 7.2 ± 0.5. Distribute the medium in bottles in volumes of 200 mL and sterilise in the autoclave at (121 ± 3) °C for 15 min. Store in the dark at (5 ± 3) °C until 6 months.

Complete medium:

Melt the basal medium and cool to between 45 °C and 50 °C. Aseptically, add the calcium chloride solution in a ratio of 1:100 and mix well. Allow to solidify and store in the dark at (5 ± 3) °°C for not more than 6 months.

Tryptone yeast extract glucose soft agar (ssTYGA)

Basal medium:

Tryptone	10 g
Yeast extract	1 g
NaCl	8 g
Agar-Agar	8 g
Distilled water	1 000 mL

Dissolve ingredients by heating in a water bath. Adjust the pH to 7.3 ± 0.1 at (25 ± 3) °C so that after sterilization it will be 7.2 ± 0.1. Autoclave at (121 ± 3) °C for 15 min. Store in the dark at (5 ± 3) °C for no longer than 6 months.

Complete medium:

On the day of plaque assay, melt the basal medium and cool to (45-50) °C in the water bath. Aseptically, add the calcium chloride solution in a ratio of 1:100, mix well and store in the water bath at (45±1) °C until using.

Tryptone Yeast Extract Glucose Soft Agar Double Concentrated (dssTYGA)

Basal medium:

Tryptone	20 g
Yeast extract	2 g
NaCl	16 g
Agar-Agar	16 g
Distilled water	1 000 mL

Dissolve ingredients by heating in a water bath. Adjust the pH to 7.3 ± 0.1 at (25 ± 3) °C so that after sterilization it will be 7.2 ± 0.1. Autoclave at (121 ± 3) °C for 15 min. Store in the dark at (4±2) °C for no longer than 6 months. Store in the dark at (5 ± 3) °C for no longer than 6 months.

Complete medium:

On the day of plaque assay, melt the basal medium and cool to (45-50) °C in the water bath. Aseptically, add the calcium chloride solution in a ratio of 1:100, mix well and store in the water bath at (45±1) °C until using.

Nalidixic acid solution

Nalidixic acid	250 mg
NaOH solution (1M)	2 mL
Distilled water	8 mL

Dissolve the nalidixic acid in the NaOH solution after add distilled water and mix well. Filter through a membrane filter of 0.2 µm pore size, or sterilise in the autoclave at (121 ± 3) °C for 15 min. Store at (5 ± 3) °C for not longer than 8 h or at (-20 ± 3) °C for not longer than 6 months.

RNase solution

Dissolve 100 mg RNase in 100 mL deionate water by heating at 100°C for 10 minutes. Distribute 1.5 mL in cryotubes and store at (-20 ± 3) °C for a maximum of 1 year. Thaw at room temperature before use.

Glycerine (sterile)

Portion glycerine and autoclave at 121°C for 20 min. Store in the dark for a maximum of 1 year.

McConkey agar

Peptone	20 g
Lactose	10 g
Bile salts	5 g
Neutral red	75 mg
Agar	12 g to 20 g
Distilled water	1 000 mL

Dissolve the ingredients in boiling water. Adjust pH so that after sterilization it will be 7.4 ± 0,1 at (25 ± 3) °C and distribute the medium in bottles in volumes of 200 mL. Sterilise in the autoclave at (121 ± 3) °C for 15 min.
Cool to between 45 °C and 50 °C and pour 20 mL in Petri dishes of 9 cm diameter. Allow to solidify and store in the dark at (5 ± 3) °C for not longer than 6 months.
Alternatively, use commercially available dehydrated complete medium and reagents prepared and used according to the manufacturer's instructions.

Peptone saline solution

Peptone	1.0 g
NaCl	8.5 g
Distilled water	1 000 mL

Dissolve the ingredients in hot water. Adjust the pH to 7.2 ± 0.2 at (45 ± 3) °C so that after sterilization it will be 7.2 ± 0.5. Dispense in convenient volumes and autoclave at (121 ± 3) °C for 15 min. Store in the dark for not longer than 6 months.

7.7 Reference organisms

- Salmonella typhimurium strain WG49, phage type 3 Nalr (F'42 lac: Tn5), NCTC 12484
- Bacteriophage MS2, NCTC 12487

7.8 Preparation of test materials

Culture and maintenance of host strains

For the culture of the host strains it is advisable that they will be gently shaking. This will increase the growth rate of bacteria and ensures that all the cells are actively growing and no stationary-

phase cells which could decrease the efficiency of plating. Inoculum cultures should be repeatedly shacked by hand if a shaker is not available.
The culture and maintenance of host strains contains several steps, summarised in following figure.

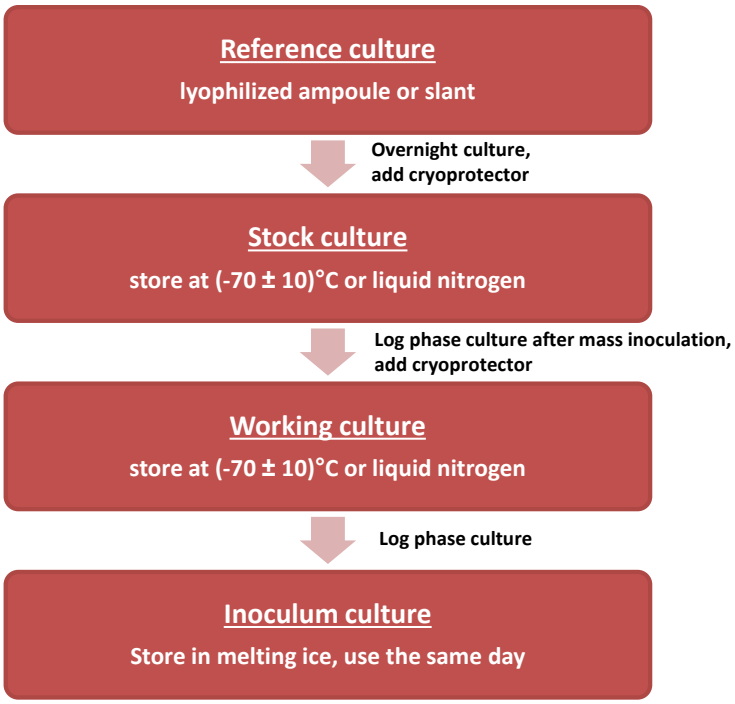


Figure 6: Overview of the cultures needed for the enumeration of F-specific RNA bacteriophages

Stock cultures

Rehydrate a reference culture of the host strains in a small amount (2 mL) TYGB using a Pasteur pipette. Transfer this suspension to a 300 mL conical flask containing (50 ± 5) mL of TYGB. Incubate for (20 ± 4) h at (36 ± 2) °C with agitation (100 ± 10) rpm, using an incubator or water bath. After this period, add 10 mL of sterile glycerol and mix well. Distribute into cryogenic vials in 0.5 mL aliquots and store at (-70 ± 10) °C or in liquid nitrogen.

Preparation of working cultures

Take a vial of stock culture; allow the temperature to equilibrate to room temperature (between 15 °C and 30 °C) and inoculate on a plate of McConkey agar in a way that single colonies are obtained. Incubate at (36 ± 2) °C for (20 ± 4) h. The remaining content of the vial of stock culture can be used to inoculate more plates on the same working day, if necessary. Otherwise it should be treated as contaminated waste.
Add (50 ± 5) mL of TYGB to a flask. Select three to five lactose-positive colonies from the McConkey agar and inoculate material from each of these colonies in the flask with TYGB. Incubate for (5 ± 1) h at (36 ± 2) °C while gently shaking using an incubator. Add 10 mL of sterile glycerol mix well and distribute in plastics vials, in 1.2 mL aliquots. Store the aliquots in a deep freezer at (-70 ± 10) °C for a maximum of two years.

Calibration of absorbance measurements for counts of viable microorganisms

Remove a vial of working culture from the deep freeze and allow equilibrating to room temperature. Add (50 ± 5) mL of TYGB to a plain conical flask and aseptically transfer a portion to a cuvette. Using this cuvette, adjust the spectrophotometer reading to zero. Discard the broth transferred to the cuvettes used to measure absorbance. Inoculate TYGB with 0.5 mL of working culture. Incubate at (36 ± 2) °C with gentle shaking in an incubator or water bath for up to 3.5 h. Every 30 min measure absorbance as indicated above and withdraw a 1 mL aliquot for viable counts, ensuring that the flask is removed from the incubator for as short a time as possible.
Dilute aliquots to 10^{-7} and count colony-forming units (CFU) in 1 mL volumes of the 10^{-5}, 10^{-6} and 10^{-7} dilutions by the standard pour-plate procedure in TGYA, in duplicate.
Alternatively, perform membrane filtration with the same volume (1 mL) and the same dilutions and count CFU by the standard membrane filter procedure on TGYA. Do this in duplicate. Incubate at (36 ± 2) °C for (20 ± 4) h.
Count the total number of colonies of each plate yielding between 30 and 300 colonies and calculate the number of CFU/mL.

Repeat this procedure several times (approx. two to three times) to establish the relationship between absorbance measurements and colony counts. After sufficient data have been obtained, further work can then be based only on absorbance measurements.

Preparation of the MS2 standard and stock solution

Inoculate 50 mL TYGB with 0.5 mL working culture. Incubate for (18 ± 2) h at (37 ± 1) °C, while shaking at (100 ± 10) rpm.
Pre-heat 50 mL TYGB to 35°C to 37°C and inoculate with 0.5mL of the overnight culture. Incubate for 90min as indicated above.
Add MS2 from stock solution at a final concentration of approximately 10^7 pfp/mL and incubate for 4h to 5h, as indicated above.
Add 5mL of chloroform, mix well and store overnight at (4 ± 2) °C. Pour off the aqueous phase into centrifuge tubes and centrifuge at 3500 g for 20 min. Carefully pipette off the supernatant and store at (4 ± 2)°C.
To determine the phage concentration, prepare a dilution series of the supernatant in steps of 10, plate out like explained later. Add glycerol at a concentration of 4.1mL/L and mix. Portion sensibly and store at (-70 ± 10) °C.
After incubation of the plated phages, count the number of plaques of each dilution step and calculate the MS2 concentration.

7.9 Procedure

Inoculum of cultures

Allow a vial of working culture of WG49 to equilibrate to room temperature (15 °C to 30 °C). Add (50 ± 5) mL of TYGB to a culture flask, and pre warm to at least room temperature. Adjust the spectrophotometer reading to zero. Inoculate 0.5 mL of working culture into TYGB. Incubate at (36 ± 2) °C with gentle shaking in an incubator or water bath. Measure absorbance every 30 min. At an absorbance corresponding to a cell density of approximately 10^8 CFU/mL (based on data

obtained previously), take the inoculum culture from the incubator and quickly cool the culture by placing it in melting ice. Use this inoculum culture within the same working day.

Standard procedure

Prepare an inoculum culture as described before. Melt bottles of 50 mL ssTYGA in a boiling water bath or microwave and place in a water bath at (45 ± 1) ^{o}C. Aseptically add calcium chloride solution pre warmed at room temperature and distribute 2.5 mL aliquots into culture tubes with caps, placed in a water bath at (45 ± 1) ^{o}C.

To each culture tube, add 1 mL of the original sample (or concentrated or diluted sample) pre warmed at room temperature. Examine each aliquot at least in duplicate. Add 1 mL of inoculum culture to each culture tube containing the aliquots of sample and ssTYGA, mix carefully. Avoid the formation of air bubbles and pour the contents on a layer of complete TYGA in a 9 cm Petri dish. Distribute evenly and allow to solidify on a horizontal, cool surface. Dry the plates, then cover and incubate the plates upside-down at (36 ± 2) ^{o}C for (18 ± 2) h. Do not stack more than 6 plates.

Count the number of plaques on each plate within 4 h after finishing incubation, using indirect oblique light.

If a great number of tests are to be performed, several conical flasks can be inoculated in parallel. In this case the contents of the different flasks should be mixed together and homogenised before.

If necessary, plates can be read after 6 h of incubation. This may be useful if a preliminary count is required and also if high background of contaminating bacterial colonies is expected. If a reading is taken after 6 h, this should be noted when expressing the results.

Figure 7: Scheme of the method for the detection and enumeration of F-specific bacteriophages

Samples with high bacterial background flora

Proceed according to 0. Add nalidixic acid to ssTGYA to give a final concentration of 250 µg/mL.

Samples with low phage counts

Proceed according to 1.4.10.2 but modify the procedure. Use 10 mL of ssTGYA, 60 µL of calcium chloride solution, 1 mL of host culture and 5 mL of sample in duplicate.

Pour over 50 mL of complete TGYA in a 14 cm to 15 cm diameter Petri dish (or use two 9 cm diameter Petri dishes, each containing 20 mL of TGYA).

This procedure will allow for the detection of one plaque-forming particle in 50 mL or 100 mL, if 10 or 20 plates are inoculated in parallel. Due to the high consumption of culture media, it may be advisable to use concentration methods which will also be necessary for even lower counts.

7.10 Expression of results

Select plates with well-separated, and preferably more than 30, plaques whenever present. If there are only counts below 30 per plate, select the ones inoculated with the largest volume of sample.
From the number of plaques counted, calculate the number X of plaque-forming particles of F-specific RNA bacteriophages in 1 mL of the sample as follows:

$$X = \frac{N}{[(n_1 V_1 F_1) + (n_2 V_2 F_2)]}$$

Where:
X = number of plaque-forming particles of F-specific RNA bacteriophages per millilitre (pfp/mL)
N = total number of plaques counted on plates (1.4.10.2, 1.4.10.3 or 1.4.10.4)
n_1, n_2 = number of replicates counted for dilution F_1, F_2
V_1, V_2 = test volume, in millilitres, used with dilution F_1, F_2
F_1, F_2 = the dilution or concentration factor used for the test portion V_1, V_2 (F = 1 for an undiluted sample, F = 0.1 for a ten-fold dilution, F = 10 for a ten-fold concentrate, etc.).

If only one dilution/concentrate is counted, simplify the formula to:

$$X = \frac{N}{nVF}$$

7.11 Quality assurance

For each inoculation culture vessel of the host strain, the MS2 standard is carried in duplicate as a positive control and a sterile peptone salt sample as a negative control. If the RNase confirmatory test is performed, additionally include the MS2 standard in duplicate as explained before

If the host strain loses sensitivity to the MS2 phage (this is unusual but can happen very suddenly and completely), prepare a new working culture.

Ensure incubators and refrigerators are in good working order by keeping temperature control charts.

7.12 Disposal

Incubated and contaminated material

In all cases, thermal disinfection of the material in an autoclave.

7.13 Literature

Havelaar, A.H. and Hogeboom, W.M., 1983. Factors affecting the enumeration of coliphages in sewage and sewage-polluted waters. Antonie van Leeuwenhoek 49: 387-397.

Grabow, W.O.K. and Coubrough, P., 1986. Practical direct plaque assay for coliphages in 100-mL samples of drinking water. Applied and environmental microbiology 52, 430-433

8 Detection and enumeration of bacteriophages – Part 2: Enumeration of somatic coliphages (adapted from ISO 10705-2:2000)

8.1 Scope

This protocol specifies a method for the detection and enumeration of somatic coliphages by incubating the sample with an appropriate host strain. The method is applicable to all kinds of water.

8.2 Principle

A culture of host strain is added to the sample that was previously mixed with a small volume of semi-solid nutrient medium and plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. The results are expressed as the number of plaque-forming particles, pfp (also termed plaque-forming units, pfu), *per* unit of sample volume.

8.3 Safety precautions

The host strain used is non-pathogenic for humans and animals, handling should be done according to the usual safety regulations for bacteriological laboratories. Somatic coliphages are also non-pathogenic, but some types are very resistant to desiccation. Appropriate precautions should therefore be taken to prevent cross-contamination of test materials, especially when testing and handling cultures that have a high titre or when inoculating host strain cultures. Such procedures must be performed under a safety workbench or in a segregated laboratory area.

8.4 Possible impairments

In samples with very high background bacterial flora, it is possible that the addition of nalidixic acid is not sufficient to suppress them. The evaluation of such samples is then not possible.
If the number of phages per plate is >1000-10000, it is no longer possible to detect plaques. The plate resembles a negative control and thus false-negative results may occur.

8.5 Definitions and abbreviations

- Somatic coliphage: Somatic coliphages are viruses that infect bacteria and are capable of infecting selected E. coli host strains by attaching to the bacterial cell wall as the first step in the infectious process and producing visible plaques in a confluent lawn of the bacterial host grown under suitable conditions.
- *E. coli*: *Escherichia coli*
- WG5: *E. coli* WG5

8.6 Apparatus

Usual microbiological laboratory equipment, including:

- Hot-air oven for dry-heat sterilization and an autoclave. All the apparatus shall be sterilised according to the instructions given in ISO 8199 except the ones supplied sterile;
- Incubator or water bath thermostatically controlled at (36 ± 2) °C;
- Incubator or water bath, thermostatically controlled at (36 ± 2) °C and equipped with a shaking device, at (100 ± 10) r/min;
- Water bath or heating block, thermostatically controlled at (45 ± 1) °C;
- Water bath or equivalent device for melting of agar media;
- pH meter;
- Counting apparatus;
- Deep freezer, thermostatically controlled at (-20 ± 5) °C;
- Deep freezer, thermostatically controlled at (-70 ± 10) °C or liquid nitrogen storage vessel;
- Spectrophotometer, capable of holding cuvettes of 1 cm optical path length or side-arm of nephelometric flasks and equipped with a filter for the range 500 nm to 650 nm with a maximum bandwidth of ± 10 nm;
- Refrigerator, temperature set at (5 ± 3) °C;
- Petri dishes of 9 cm or 14 cm to 15 cm diameter, vented;
- Graduated pipettes of 0,1 mL, 1 mL, 5 mL and 10 mL capacity;
- Pasteur pipettes;
- Glass bottles;
- Culture tubes with caps or suitable alternative;
- Measuring cylinders;
- Conical flasks of 250 mL to 300 mL capacity, with cotton wool plugs or suitable alternative;
- Cuvettes of optical path length 10 mm or nephelometric conical flasks with cylindrical side-arms which fit in the spectrophotometer (capacity 250 mL to 300 mL with cotton wool plugs or suitable alternative)
- Membrane filter units, pore size 0, 2 µm for decontamination.
- Plastics vials, with lid, of 1,5 mL to 3 mL capacity.

8.7 Reagents and culture media

Use ingredients of uniform quality and chemicals of analytical grade (other grades of chemicals is permissible providing they are shown to be of equal performance in the test.) for the preparation of culture media and reagents, alternatively, use dehydrated complete media and follow strictly the manufacturer's instructions.

For the preparation of media, use glass-distilled water or deionised water free from substances, which might inhibit bacterial growth under the conditions of the test, and complying with ISO 3696.

$MgCl_2$ solution (4.14M)

Dissolve 100 g $MgCl_2*6H_2O$ in 50 mL distilled water.

$MgCl_2*6H_2O$ is very hygroscopic and it is not advisable to store it in the crystalline form once the container has been opened. Therefore, use the total contents of a container and dissolve in the appropriate amount of water.

The final concentration of $MgCl_2*6H_2O$ in this solution will be 4.14 M. Sterilise the solution by autoclaving and store at room temperature in the dark.

Na_2CO_3 (150 g/l)

Dissolve 150 g Na_2CO_3 150 g in 1000 mL distilled water.

Calcium chloride solution (1M)

Dissolve 14.9 g$CaCl_2*2H_2O$ in 100 mL distilled water.

Dissolve the calcium chloride in the water while heating gently. Cool to room temperature and filter-sterilise through a membrane filter of 0.2 µm pore size. Store the solution in the dark at (5 ± 3) ºC for not longer than 6 months.

Modified Scholtens' Broth (MSB)

Basal medium:

Peptone	10 g
Yeast extract	3 g
Meat extract	12 g
NaCl	3 g
Na_2CO_3 solution (150 g/l)	5 mL
$MgCl_2$ solution (100 g of $MgCl_2.6H_2O$ in 50ml water)	0.3 mL
Distilled water	1 000 mL

Dissolve the ingredients in hot water. Adjust the pH to 7.2 ± 0,2 at (45 ± 3) °C so that after sterilization it will be 7.2 ± 0.5. Distribute the medium in bottles in volumes of 200 mL and sterilise in the autoclave at (121 ± 3) ºC for 15 min. Store in the dark at (5 ± 3) ºC until 6 months.

Alternatively, use commercially available dehydrated complete medium and reagents prepared and used according to the manufacturer's instructions.

Modified Scholtens' Agar (MSA)

Basal medium:

Peptone	10 g
Yeast extract	3 g
Meat extract	12 g
NaCl	3 g
Na_2CO_3 solution (150 g/l)	5 mL
$MgCl_2$ solution (100 g of $MgCl_2.6H_2O$ in 50ml water)	0.3 mL
Agar	10 g to 20 g
Distilled water	1 000 mL

Dissolve all the ingredients in boiling water. Adjust the pH to 7.2 ± 0.2 at (55 ± 3) °C so that after sterilization it will be 7.2 ± 0.5. Distribute the medium in bottles in volumes of 200 mL and sterilise in the autoclave at (121 ± 3) ºC for 15 min. Store in the dark at (5 ± 3) ºC for not longer than 6 months.

Complete medium:

Melt the basal medium and cool to between 45 °C and 50 °C. Aseptically, add the calcium chloride solution in a ratio of 3:500 and mix well. Allow to solidify and store in the dark at (5±3)°C for not more than 6 months.

Semi-solid Modified Scholtens' Agar (ssMSA)

Basal medium:

Peptone	10 g
Yeast extract	3 g
Meat extract	12 g
NaCl	3 g
Na_2CO_3 solution (150 g/l)	5 mL
$MgCl_2$ solution (100 g of $MgCl_2.6H_2O$ in 50ml water)	0.3 mL
Agar	6-10 g
Distilled water	1 000 mL

Choose the agar concentration that produces highest plaque counts but also controls plaque size to reduce confluence. Distribute into bottles in volumes of 50 mL.

Dissolve all the ingredients in boiling water. Adjust the pH to 7.2 ± 0.2 at (55 ± 3) °C so that after sterilization it will be 7.2 ± 0.5. Distribute the medium in bottles in volumes of 200 mL and sterilize in the autoclave at (121 ± 3) ºC for 15 min. Store in the dark at (5 ± 3) °C for not longer than 6 months.

Complete medium:

On the day of plaque assay, melt the basal medium and cool to (45-50) °C in the water bath. Aseptically, add the calcium chloride solution in a ratio of 3:500, mix well and store in the water bath at (45 ± 1) °C until using.

Modified Scholtens' soft agar double concentrated (dssMSA)

Basal medium:

Peptone	20 g
Yeast extract	6 g
Meat extract	24 g
NaCI	6 g
Na_2CO_3 solution (150 g/l)	10 mL
$MgCl_2$ solution (100 g of $MgCl_2.6H_2O$ in 50ml water)	0.6 mL
Agar	14 g
Distilled water	1 000 mL

Dissolve all the ingredients in boiling water. Adjust the pH to 7.2 ± 0.2 at (55 ± 3) °C so that after sterilization it will be 7.2 ± 0.5. Distribute the medium in bottles in volumes of 200 mL and sterilize in the autoclave at (121 ± 3) °C for 15 min. Store in the dark at (5 ± 3) °C for not longer than 6 months.

Complete medium:

On the day of the assay, melt the basal medium and cool to (45-50)°C in the water bath. Aseptically, add the calcium chloride solution in a ratio of 3:500, mix well and store in the water bath at (45 ± 1)°C until using.

Nalidixic acid solution

Nalidixic acid	250 mg
NaOH solution (1M)	2 mL
Distilled water	8 mL

Dissolve the nalidixic acid in the NaOH solution after add distilled water and mix well. Filter through a membrane filter of 0.2 µm pore size, or sterilise in the autoclave at (121 ± 3) ^{o}C for 15 min. Store at (5 ± 3) ^{o}C for not longer than 8 h or at (-20 ± 3) ^{o}C for not longer than 6 months.

Glycerine (sterile)

Portion glycerine and autoclave at 121°C for 20 min. Store in the dark for a maximum of 1 year.

McConkey agar

Peptone	20 g
Lactose	10 g
Bile salts	5 g
Neutral red	75 mg
Agar	12 g to 20 g
Distilled water	1 000 mL

Dissolve the ingredients in boiling water. Adjust pH so that after sterilization it will be 7.4 ± 0,1 at (25 ± 3) °C and distribute the medium in bottles in volumes of 200 mL. Sterilise in the autoclave at (121 ± 3) °C for 15 min.
Cool to between 45 °C and 50 °C and pour 20 mL in Petri dishes of 9 cm diameter. Allow to solidify and store in the dark at (5 ± 3) °C for not longer than 6 months.
Alternatively, use commercially available dehydrated complete medium and reagents prepared and used according to the manufacturer's instructions.

Peptone saline solution

Peptone	1.0 g
NaCl	8.5 g
Distilled water	1 000 mL

Dissolve the ingredients in hot water. Adjust the pH to 7.2 ± 0.2 at (45 ± 3) °C so that after sterilization it will be 7.2 ± 0.5. Dispense in convenient volumes and autoclave at (121 ± 3) °C for 15 min. Store in the dark for not longer than 6 months.

8.8 Reference organisms

- *Escherichia coli* WG5, DSM 18455
- Bacteriophage phiX174, DSM 4497

8.9 Preparation of test materials

Culture and maintenance of host strains

For the culture of the host strains it is advisable that they will be gently shaking. This will increase the growth rate of bacteria and ensures that all the cells are actively growing and no stationary-phase cells which could decrease the efficiency of plating. Inoculum cultures should be repeatedly shacked by hand if a shaker is not available.
The culture and maintenance of host strains contains several steps, summarised in following figure.

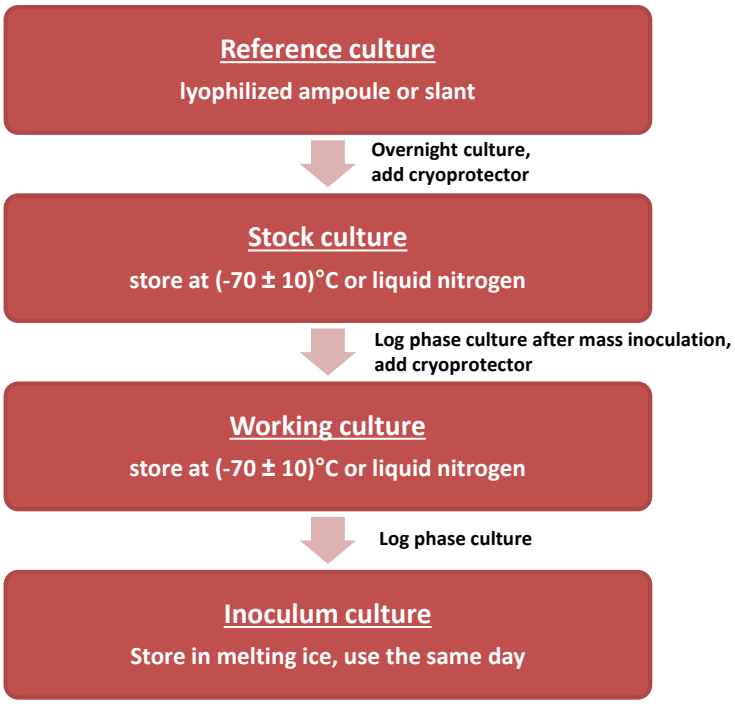


Figure 8: Overview of the cultures needed for the enumeration of somatic coliphages

Stock cultures

Rehydrate a reference culture of the host strains in a small amount (2 mL) of MSB using a Pasteur pipette. Transfer this suspension to a 300 mL conical flask containing (50 ± 5) mL of MSB. Incubate for (20 ± 4) h at (36 ± 2) °C with agitation, using an incubator or water bath. After this period, add 10 mL of sterile glycerol and mix well. Distribute into cryogenic vials in 0.5 mL aliquots and store at (-70 ± 10) °C or in liquid nitrogen.

Preparation of working cultures

Take a vial of stock culture; allow the temperature to equilibrate to room temperature (between 15 °C and 30 °C) and inoculate on a plate of McConkey agar or another lactose-containing medium in a way that single colonies are obtained. Incubate at (36 ± 2) °C for (20 ± 4) h. The remaining content of the vial of stock culture can be used to inoculate more plates on the same working day, if necessary. Otherwise it should be treated as contaminated waste.

Add (50 ± 5) mL of MSB to a flask. Select three to five lactose-positive colonies from the McConkey agar and inoculate material from each of these colonies in the flask with MSB. Incubate for (5 ± 1) h at (36 ± 2) °C while gently shaking using an incubator. Add 10 mL of sterile glycerol mix well and distribute in plastics vials, in 1.2 mL aliquots. Store the aliquots in a deep freezer at (-70 ± 10) °C for a maximum of two years.

Calibration of absorbance measurements for counts of viable microorganisms

Remove a vial of working culture from the deep freeze and allow equilibrating to room temperature. Add (50 ± 5) mL of MSB to a plain conical flask and aseptically transfer a portion to a cuvette. Using this cuvette, adjust the spectrophotometer reading to zero. Discard the broth transferred to the cuvettes used to measure absorbance. Inoculate MSB with 0.5 mL of working

culture. Incubate at (36 ± 2) °C with gentle shaking in an incubator or water bath for up to 3.5 h. Every 30 min measure absorbance as indicated above and withdraw a 1 mL aliquot for viable counts, ensuring that the flask is removed from the incubator for as short a time as possible.
Dilute aliquots to 10^{-7} and count colony-forming units (CFU) in 1 mL volumes of the 10^{-5}, 10^{-6} and 10^{-7} dilutions by the standard pour-plate procedure in nutrient agar or Modified Scholtens' Agar (MSA), in duplicate.
Alternatively, perform membrane filtration with the same volume (1 mL) and the same dilutions and count CFU by the standard membrane filter procedure on nutrient agar or MSA. Do in duplicate. Incubate at (36 ± 2) °C for (20 ± 4) h.
Count the total number of colonies of each plate yielding between 30 and 300 colonies and calculate the number of CFU/mL.

Repeat this procedure several times (approx. two to three times) to establish the relationship between absorbance measurements and colony counts. After sufficient data have been obtained, further work can then be based only on absorbance measurements.

Preparation of the ΦX174 standard and stock culture

Inoculate 50 mL MSB with 0.5 mL of a working culture. Incubate for (20 ± 4) h at (36 ± 2) °C, while shaking at (100 ± 10) rpm. Pre heat 50 mL MSB to 35°C to 37°C and inoculate with 5 mL of the overnight culture. Incubate for 90 min as indicated above.
Add ΦX174 from stock solution at a final concentration of at least 10^7 pfp/mL and incubate for 4 h to 5 h, as indicated above. If a higher titre is to be achieved, inoculate ΦX174 at a higher concentration.
Add 5 mL chloroform, shake for 15 min at 200 rpm and store overnight at (4 ± 2) °C. Pour off the aqueous phase into centrifuge tubes and centrifuge at 3500 g for 20 min. Carefully pipette off the supernatant and store at (4 ± 2) °C.
To determine the phage concentration, prepare a dilution series of the supernatant in steps of 10 and plate out. Add 5% (by volume) glycerol and mix. Portion sensibly and store at (-70 ± 10) °C. After incubation of the plated phages, count the number of plaques of each dilution step and calculate the ΦX174 concentration.

8.10 Procedure

Inoculum of cultures

Allow a vial of working culture to equilibrate to room temperature (15 °C to 30 °C). Add (50 ± 5) mL of MSB to a culture flask, and pre warm to at least room temperature. Adjust the spectrophotometer reading to zero. Inoculate 0.5 mL of working culture into MSB. Incubate at (36 ± 2) °C with gentle shaking in an incubator or water bath. Measure absorbance every 30 min. At an absorbance corresponding to a cell density of approximately 10^8 CFU/ml (based on data obtained previously), take the inoculum culture from the incubator and quickly cool the culture by placing it in melting ice. Use this inoculum culture within the same working day.

Standard procedure

Prepare an inoculum culture as described in 1.4.10.1. Melt bottles of 50 mL semi-solid Modified Scholtens' Agar (ssMSA) in a boiling water bath or microwave and place in a water bath at (45 ± 1) °C. Aseptically add 300 µL of a calcium chloride solution pre warmed at room temperature and distribute 2.5 mL aliquots into culture tubes with caps, placed in a water bath at (45 ± 1) °C. To each culture tube, add 1 mL of the original sample (or concentrated or diluted sample) pre warmed at room temperature. Examine each aliquot at least in duplicate. Add 1 mL of inoculum culture to each culture tube containing the aliquots of sample and ssMSA, mix carefully. Avoid the formation of air bubbles and pour the contents on a layer of complete MSA in a 9 cm Petri dish. Distribute evenly and allow to solidify on a horizontal, cool surface. Dry the plates, then cover and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) h. Do not stack more than 6 plates.

Count the number of plaques on each plate within 4 h after finishing incubation, using indirect oblique light.

If a great number of tests are to be performed, several conical flasks can be inoculated in parallel. In this case the contents of the different flasks should be mixed together and homogenised before.

If necessary, plates can be read after 6 h of incubation. This may be useful if a preliminary count is required and also if high background of contaminating bacterial colonies is expected. If a reading is taken after 6 h, this should be noted when expressing the results.

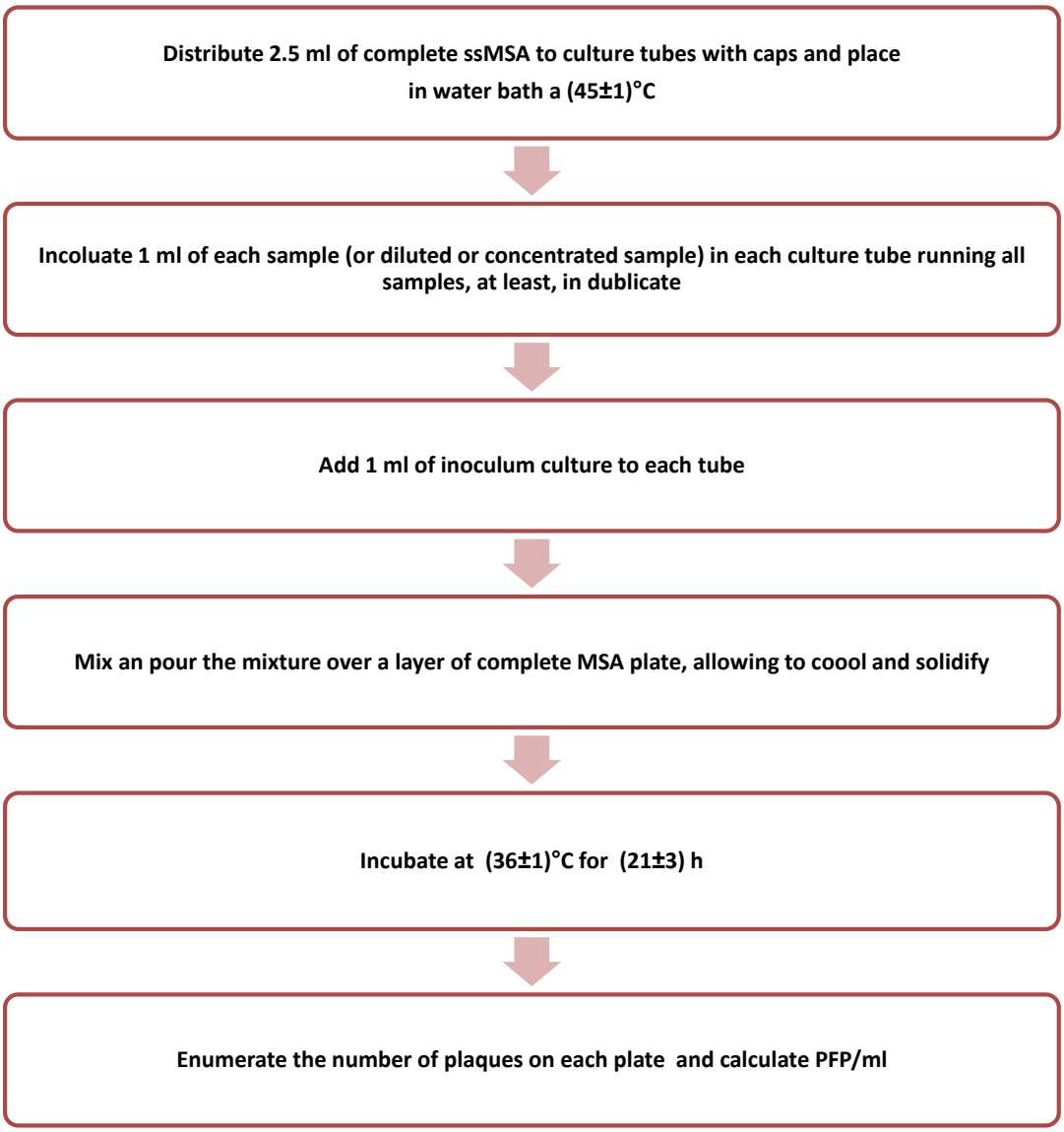


Figure 9: Scheme of the method for the detection and enumeration of somatic coliphages.

Samples with high bacterial background flora

Proceed like explained before. Add nalidixic acid to ssMSA to give a final concentration of 250 µg/mL and use *E. coli* CN as the inoculum culture.

Samples with low phage counts

Proceed like explained before but modify the procedure. Use10 mL of ssMSA, 60 µL of calcium chloride solution, 1 mL of host culture and 5 mL of sample in duplicate.

Pour over 50 mL of complete MSA in a 14 cm to 15 cm diameter Petri dish (or use two 9 cm diameter Petri dishes, each containing 20 mL of MSA).

This procedure will allow for the detection of one plaque-forming particle in 50 mL or 100 mL, if 10 or 20 plates are inoculated in parallel. Due to the high consumption of culture media, it may be advisable to use concentration methods which will also be necessary for even lower counts.

8.11 Expression of results

Select plates with well-separated, and preferably more than 30, plaques whenever present. If there are only counts below 30 per plate select the ones inoculated with the largest volume of sample.
From the number of plaques counted, calculate the number X of plaque-forming particles of somatic coliphages in 1 mL of the sample as follows:

$$X = \frac{N}{[(n_1 V_1 F_1) + (n_2 V_2 F_2)]}$$

Where:
X = number of plaque-forming particles of somatic coliphages per millilitre (pfp/mL)
N = total number of plaques counted on plates (1.4.10.2, 1.4.10.3 or 1.4.10.4)
n_1, n_2 = number of replicates counted for dilution F_1, F_2
V_1, V_2 = test volume, in millilitres, used with dilution F_1, F_2
F_1, F_2 = the dilution or concentration factor used for the test portion V_1, V_2 (F = 1 for an undiluted sample, F = 0.1 for a ten-fold dilution, F = 10 for a ten-fold concentrate, etc.).

If only one dilution/concentrate is counted, simplify the formula to:

$$X = \frac{N}{nVF}$$

8.12 Quality assurance

For each inoculation culture vessel of the host strain, the MS2 standard is carried in duplicate as a positive control and a sterile peptone salt sample as a negative control. If the RNase confirmatory test is performed, additionally include the MS2 standard in duplicate as explained before

If the host strain loses sensitivity to the MS2 phage (this is unusual but can happen very suddenly and completely), prepare a new working culture.

Ensure incubators and refrigerators are in good working order by keeping temperature control charts.

8.13 Disposal

Incubated and contaminated material

In all cases, thermal disinfection of the material in an autoclave.

8.14 Literature

Havelaar, A.H. and Hogeboom, W.M., 1983. Factors affecting the enumeration of coliphages in sewage and sewage-polluted waters. Antonie van Leeuwenhoek 49: 387-397.

Grabow, W.O.K. and Coubrough, P., 1986. Practical direct plaque assay for coliphages in 100-mL samples of drinking water. Applied and environmental microbiology 52, 430-433

.

9 Method for the concentration and DNA extraction of bacteria

9.1 Scope

The purpose of this protocol is to outline the procedure for concentrating of bacteria from water samples and extracting their genomic DNA. The nucleic acids are subsequently exchanged for analysis in the specialised partner laboratories.

9.2 Principle

A sample is filtered through a 0.22 µm membrane, which retains the bacteria. After filtration, the membrane is placed in a microcentrifuge tube, GITC buffer is added and the membrane is frozen at -20°C until DNA extraction. For DNA extraction the QIAamp DNA Mini kit is used. This kit is designed to extract total DNA from whole blood, buffy coat, cultured cell, body fluid, tissue and dried blood spot samples. The kit was demonstrated to be suitable for environmental samples (Gourmelon et al., 2007). Bacterial cells are lysed and DNA released. The released DNA is bound to a QIAamp column and purified using two washes that remove contaminants. The purified DNA is released from the column using buffer.

9.3 Reagents and equipment

- Forceps
- Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps
- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing forceps
- Membrane filters, 47 mm diameter, with 0.22 µm pore size (GSWP04700, SO-PAK, Millipore)
- Guanidine thiocyanate (e.g., Sigma, G6639-100G)
- EDTA (e.g., Merck, 1.08418.1000)
- N-Laurolylsarcosine Sodium salt (sarcosyl) (e.g., Sigma, L5777-50G)
- QIAamp DNA Blood Mini Kit (Qiagen)
- Ethanol (96-100%, molecular biology grade)
- 1.5 mL microcentrifuge tubes
- 2.0 mL collection tubes
- 1-20, 100-200 and 1000 µl single channel automatic pipettes
- Filter pipette tips for the above pipettes
- Paper towels or wipes
- Distilled water
- Timer

GITC buffer

5 M guanidine thiocyanate
100 mM EDTA [pH 8.0]
0.5% sarkosyl

9.4 Apparatus

- Sterile graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper
- Membrane filtration units (filter base and funnel), glass, plastic or stainless steel
- Electric vacuum pump, or waterjet pump for use as a vacuum source
- Filter flask, vacuum, usually 1 L, with appropriate tubing
- Filter manifold to hold a number of filter bases (optional)
- Flask for safety trap placed between the filter flask and the vacuum source
- Heating block or waterbath
- Microcentrifuge
- Vortex

9.5 Procedures

Collection of water/sewage samples

Water and sewage samples should be collected as aseptically as possible and stored at 4°C for not longer than 24 hours until processed.

- Collect the water/sewage in 1 litre, wide mouthed, sterile bottles. Samples should be labelled with the location and date of collection.
- Place the samples on ice for transport to the laboratory and process within 24 hours of sampling.

Concentration of bacteria

- Place a membrane filter (SO-PAK, Millipore, diameter: 45 mm, poresize: 0.22 µm) on the filter base and assemble a filtration
- Shake the sample bottle vigorously at least 10 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel.
- Filter each sample (adjust the appropriate volume according to the type of water) and remove the filter.
- Place the filter in 0.5 mL of GITC buffer that has been pre-aliquoted into sterile 15 mL tubes. Freeze at -20°C in lysis buffer until DNA extraction.
- Clean the filtration unit between samples rinsing thoroughly with sterile distilled water and sterilizing it (in autoclave).

Sample volumes

Sample type	Volume to concentrate
Hospital wastewater	100 mL
Wastewater treatment plant influent	100 mL
Wastewater treatment plant effluent (after secondary treatment)	500 mL
Combined sewer overflow	500 mL
Surface water	>1 L (as much as possible, exact volume must be recorded)
Coastal water	>1 L (as much as possible, exact volume must be recorded)

Extraction of DNA

Annotation: DNA is extracted using the QIAamp DNA Blood Mini Kit (Qiagen) according to manufacturers´ instructions with some modifications: the proteinase K step is omitted, and 700 µl of QIAGEN AL buffer is added to the filters placed in GITC buffer.

- Prepare wash buffers AW1 and AW2 (these may be kept at +15 ºC to +25 ºC for up to 1 year). Always label reagents with the date.
- Check buffer AL. If a precipitate has formed, dissolve by incubating at 56 ºC.
- Label an appropriate number of 1.5 mL microcentrifuge tubes (label both the lid and the side of the tube) with the sample ID.
- Remove the tubes containing the filters and buffer from the freezer and thaw.
- Add 500 µL of AL buffer to each tube containing a filter and vortex for 30 seconds. Invert the tubes 5 times and vortex for another 30 seconds.
- Allow the tubes to stand for 5 minutes, as a lot of foam is generated by the vortexing.
- Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.
- Add 500 µL ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.
- Carefully apply the mixture to the QIAamp Mini spin column without wetting the rim. Close the cap, and centrifuge at 6000 x g for 1 min. Discard flow through. Repeat until the entire volume has passed the column. Discard flow through.
- Add 500 µL of buffer AW1 to the column without wetting the rim and centrifuge at 6,000 x g for 1 minute. Discard flow through.
- Add 500 µL of buffer AW2 to the column and spin at full speed (20,000 x g; 14,000 rpm) for 3 minutes, to dry the column.
- Place the column in a new collection tube and centrifuge again at full speed. Discard the flow through and the tube.
- Place the column in a 1.5 mL collection tube and add 200 µl of buffer AE, the elution buffer.
- Let the columns stand at room temperature (15-25°C) for 1 minute and centrifuge at 6,000 x g (8,000 rpm) for 1 minute to recover the DNA.
- Aliquot the DNA for sending to the different partners.
- Store the DNA extracts at -80°C until shipment

9.6 Quality assurance

Filtration and DNA extraction controls are run together with the samples:

Always prepare at least one filtration negative sample per filtration run. Use the same volume of sterile distilled water as was used for the samples. Filtration and DNA extraction controls are run together with the samples. An extraction mixture containing only reagents should be included in each extraction run, to control for technique.

9.7 Literature

Gourmelon, M., Caprais, M.P., Ségura, R., et al., 2007. Evaluation of two library-independent microbial source tracking methods to identify sources of faecal contamination in French estuaries. Applied and environmental microbiology 73(15):4857-4866.

10 Method for the concentration and nucleic acid extraction of viruses

10.1 Scope

The purpose of this protocol is to outline the procedure for concentrating of viruses from water samples and extracting their genomic RNA or DNA. The nucleic acids are subsequently exchanged for analysis in the specialised partner laboratories.

10.2 Principle

Virus levels in water are typically too low for detection by direct analysis; thus, the detection often involves viral enrichment or concentration. Viruses can be concentrated from environmental samples via different methods such as filtration through electronegative or electropositive filters, ultrafiltration, viral flocculation/precipitation with organic/inorganic flocculants, and ultracentrifugation. Similar to concentration methods, a high number of methods and kits are available for nucleic acid extraction with varying efficiency and inhibitors removal capacity. The choice of concentration and extraction method can affect the results and the limit of detection and quantification. Within the SARA project a fist concentration using ultrafiltration followed by a second concentration by polyethylenglycol (PEG) precipitation is performed. For viral nucleic acid extraction, the Qiagen DNA Mini Kit and Qiagen Viral RNA are used. Viral capsids cells are lysed and nucleic released. The released DNA or RNA is bound to a silica gel column and purified using two washes that remove contaminants. The purified nucleic acids are released from the column using buffer.

10.3 Equipment

- Peristaltic pump
- Tubing (autoclavable) that fits the peristaltic pump being used
- Tips for micropipettes
- Sample containers (sterile)

10.4 Reagents

- Sodium Polyphosphate (NaPP, 10%)
- Antifoam A (1%) / Tween 80 (10%)
- Sterile reagent water
- 0.01 M Phosphate buffered saline (PBS) pH 7.4

Sample volumes

Sample type	Volume to concentrate
Hospital wastewater	1 L
Wastewater treatment plant influent	1 L
Wastewater treatment plant effluent (after secondary treatment)	5 L
Combined sewer overflow	5 L
Surface water	> 20 L-100 L (as much as possible, exact volume must be recorded)
Coastal water	50 L

10.5 Filters

Use hollow-fiber filter with a molecular weight cutoff of 30kDa, e.g. REXEED-25S (Asahi Kasei Kuraray Medical Co), FX80 (Fresenius), inuvai R180 (Inuvai), Diacap LO PS 15 (Braun) or Polyflux 17R (Gambro).

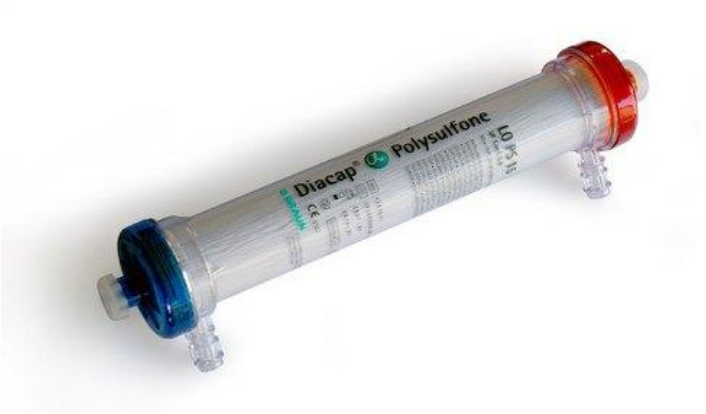


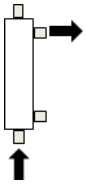
Figure 10: **Example of a hollow-fiber filter used for primary concentration of viruses**

10.6 Ultrafiltration

Pre-treatment of the filter

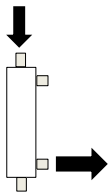
- Open the bottom and top parts of the filter (diagram 1) and fill it with a 0.9% NaCl

- do the same procedure for the exterior of the hollow fibers



Filtration

- Assemble the filter according to Figure 1: Connect the tube from the peristaltic pump to the top of the filter. Open the opposite side of the filter as an outlet from the filter.

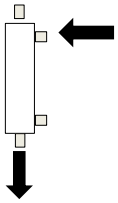


Connect the tubing to a 10 mL pipet with the cotton removed. Place the pipet in a bottle containing the sample.

- Remove the port cap at the bottom.
- Flush the filter at 250 mL/min until all the volume is filtered.
- Switch the pump off

Back-flush elution

- Assemble the filter set up as shown. Replace the inlet tubing by a port cap.



- Prepare a 0.001% Antifoam, 0.01% NaPP and 0.01% Tween80 solution by adding 0.5 mL 1% Antifoam/10% Tween 80 and 0,5 mL 10% NaPP to 500 mL sterile reagent grade water.

- Pump the solution through the filter at speed of 250 mL/min, collecting the eluate in a sterile container, until the system is empty.
- Carefully release the pressure on the system by letting air into the system by removing the tubing from the peristaltic wheel.
- Record the volume of the eluate.

10.7 Secondary Concentration – PEG precipitation

- Add to the eluate 2g/150mL Beef extract and mix by shaking until completely dissolved
- Add 5%PEG-8000 and mix by shaking
- Incubate at +4 °C overnight, ideally on a slow rotating platform or similar
- Centrifuge at 10 000xg for 30 minutes. Meanwhile prepare 10 mL PBS with 0.001% Antifoam A and 0.01% Tween80 by adding 10 µl 1% Antifoam/10% Tween 80 to 10 mL PBS.
- Aspirate off the supernatant and resuspend the pellet in 1-3 mL PBS with 0.001% Antifoam A and 0.01% Tween80 depending on the size of the pellet. Keep the volume as low as possible.
- Determine the total volume of the resuspended PEG-precipitate using a glass or plastic graduated 5 or 10 mL pipette.

10.8 Viral DNA/RNA Extraction

For the extraction use the kits according to manufacturer's instructions:

- DNA extraction: Qiagen DNA Mini Kit - protocol for DNA purification from blood or body fluids (spin, see page 26-29 in the handbook)
- RNA extraction: Qiagen Viral RNA Kit – protocol purification of viral RNA (spin, see page 26-29 in the handbook)

Viral DNA extraction

Note:

Starting volume for one extraction is 200 µL

Preparations:

- Buffer AW1 (store at room temperature, 15–25°C) Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle.
- Buffer AW2* (store at room temperature, 15–25°C) Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle.
- Heat water bath or heating block to 56°C

- If a precipitate has formed in Buffer AK, dissove by incubating at 56°C.

Protocol:

- Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 mL microcentrifuge tube. 2.
- Add 200 µl sample to the microcentrifuge tube. Note: It is possible to add QIAGEN Protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme
- Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
- Incubate at 56°C for 10 min.
- Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.
- Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.
- Carefully apply the mixture 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. Empty collection tube.
- 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. Empty collection tube.
- 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 (20,000 x g; 14,000 rpm) for 3 min. Place the QIAamp Mini spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
- Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge 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 (15–25°C) for 5 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.
- Store the DNA at -80°C

Viral RNA extraction

Note:

Starting volume for one extraction is 140 µL. To obtain sufficient RNA extract for all assays, perform at least **two extractions per sample**, each eluting in 100 µL.

Preparations:

- Add Buffer AVE to the tube containing lyophilised carrier RNA to obtain a solution of 1 µg/µL (i.e., add 310 µL Buffer AVE to 310 µg lyophilised carrier RNA, or 1550 µL Buffer AVE to 1550

µg lyophilised carrier RNA; check the tube label for content). Dissolve the carrier RNA thoroughly, divide it into conveniently sized aliquots, and store it at −30 to −15°C. Do not freeze–thaw the aliquots of carrier RNA more than 3 times.

- Addition of dissolved carrier RNA to Buffer AVL* Check Buffer AVL for precipitate, and if necessary incubate at 80°C until the precipitate is dissolved. Calculate the volume of Buffer AVL–carrier RNA mix needed per batch of samples by selecting the number of samples

No. samples	Buffer AVL (ml)	Carrier RNA–AVE (µl)	No. samples	Buffer AVL (ml)	Carrier RNA–AVE (µl)
1	0.56	5.6	13	7.28	72.8
2	1.12	11.2	14	7.84	78.4
3	1.68	16.8	15	8.4	84.0
4	2.24	22.4	16	8.96	89.6
5	2.80	28.0	17	9.52	95.2
6	3.36	33.6	18	10.08	100.8
7	3.92	39.2	19	10.64	106.4
8	4.48	44.8	20	11.20	112.0
9	5.04	50.4	21	11.76	117.6
10	5.60	56.0	22	12.32	123.2
11	6.16	61.6	23	12.88	128.8
12	6.72	67.2	24	13.44	134.4

- Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle
- Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle

Protocol:

- Pipet 560 µL prepared Buffer AVL containing carrier RNA into a 1.5 mL microcentrifuge tube.
- Add 140 µL plasma, serum, urine, cell-culture supernatant or cell-free body fluid to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.
- Incubate at room temperature for 10 min.
- Briefly centrifuge the tube to remove drops from the inside of the lid.
- Add 560 µL ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
- Carefully apply 630 µL of the solution from last step to the QIAamp Mini 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. Emty the 2 mL collection tube.
- Carefully open the QIAamp Mini column, and repeat the last step. If the sample volume was greater than 140 µL, repeat this step until all of the lysate has been loaded onto the spin column.
- Carefully open the QIAamp Mini column, and add 500 µL Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Empty 2 mL collection tube.
- Carefully open the QIAamp Mini column, and add 500 µL Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Place the QIAamp Mini column

in a new 2 mL collection tube, and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

- Place the QIAamp Mini column in a clean 1.5 mL microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µL Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.
- Centrifuge at 6000 x g (8000 rpm) for 1 min. A single elution with 100 µL Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini column.
- Store the RNA at -80°C

11 Method for sample conservation and shipment

11.1 Scope

For reasons of better comparability, certain molecular biological parameters are to be analysed in a respective defined partner laboratory. For this purpose, the nucleic acid extracts obtained must be exchanged between the partners. In order to ensure stability of the nucleic acids, this should be done under defined conditions, which are described in more detail below.

Extracted DNA and RNA samples are stored at -80°C until shipment. Sediment samples are stored at -20°C. Viral RNA extracts are on dry ice by European partners (exception IST). DNA extracts and sediments must not be send on dry ice – cooling is sufficient. For the African partners the samples will be exchanged during visits and meetings.

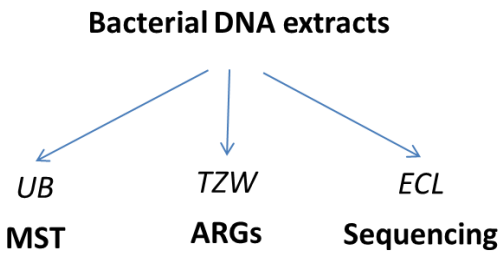


Figure 11: **Overview of sample transfer for bacterial DNA extracts**

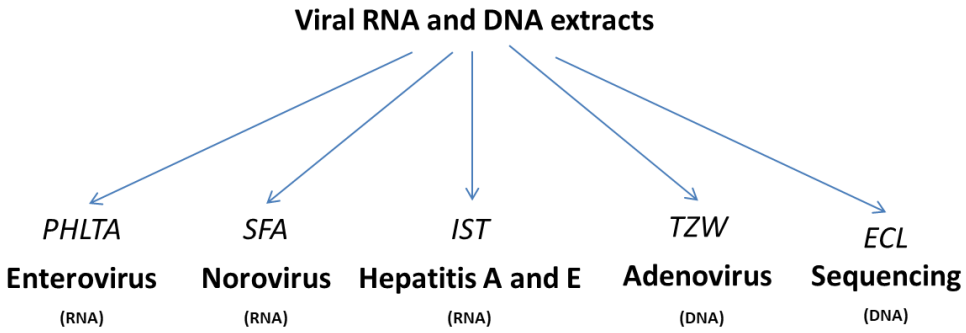


Figure 12: **Overview of sample transfer for viral nucleic acid extracts**

12 Contact persons and volumes to ship

Contact person and address	Bacterial DNA	Viral DNA	Viral RNA
TZW: DVGW-Technologiezentrum Wasser Claudia Stange Karlsruher Str. 84 76139 Karlsruhe, Germany Tel: +49 721 9678-134 Claudia.Stange@tzw.de (ARGs and adenovirus)	50 µL	15 µL	-
Daniel Toribio Dept. Genètica, Microbiologia i Estadística. Secció de Microbiologia, Virologia i Biotecnologia. Universitat de Barcelona Av/ Diagonal 643 08028 Barcelona, SPAIN (MST marker)	30-50 µL	-	-
Ricardo Santos / Silvia Monteiro Microbial Water Laboratory | Laboratorio de Análises de Água Instituto Superior Tecnico Universidade de Lisboa Av. Rovisco Pais 1049-001 Lisboa, PORTUGAL (HEV and HAV)	-	-	40 µL
Concepcion Sanchez-Cid Torres Laboratoire Ampère, Ecole Centrale de Lyon 36 avenue Guy de Collongue 69134 Ecully (France) (Metagenomics)	10 ng for Illumina and 1 µg for Nanopore	10 ng for Illumina and 1 µg for Nanopore	-
Magnus Simonsson European Union Reference Laboratory Foodborne Viruses Science Department Box 622, SE-751 26 UPPSALA, Sweden Tel. +46 18 17 14 64 Mobile +46 72-227 20 52 (Norovirus GI and GII, HAV, virus sequencing)	-		100 µL
Prof Abid Nasser Public Health Laboratory Ministry of Health Ben Zvi Rd 69 Tel Aviv, 6810416, Israel (Enterovirus)	-	-	20 µL

13 Methods for the enumeration of human Microbial Source Tracking (MST) Markers

13.1 Scope

This protocol specifies the methods for the detection and quantification of human-specific microbial source tracking markers using a quantitative polymerase chain reaction (qPCR). It specifies general methodological requirements, performance evaluation requirements, and quality control requirements.

13.2 Principle

Water samples are collected an extracted according to chapter 7 and 8. PCR assays, using host specific primers, are used to analyse the extracted DNA. PCR

13.3 Procedures

Method for enumeration of the human-specific Bifidobacterium marker HMBif

- Start cycler and computer
- Prepare and print plate setup, use different areas for negative controls, DNA samples and qPCR standards.
- Put DNA samples from -80°C or -20°C to 4°C
- For preparation of mastermix use DNA-free working space: Put on new gloves and clean DNA-free working space with DNA away
- Let reagents needed for assay thaw on DNA-free working space. WARNING: Transfer NOTHING from the normal lab to the DNA free working space!!!
- Prepare (DNA-free) pipettes, tips, reaction vials (for mastermix)
- Pipette mastermix (start with water; probe, primers and TaqMan® Environmental Master Mix last), vortex and spin down
- Put reagents back into the refrigerator (TaqMan® Environmental Master Mix) or to the freezer. Leave cleaned DNA-free working space (take nothing with you except mastermix) and go to working space where DNA samples/ qPCR standards are handled.
- Take qPCR standards out of the freezer to thaw and change gloves afterwards (use different working space and pipettes for handling qPCR standards and DNA samples, respectively)
- Vortex template DNAs well and spin them down
- Vortex qPCR standards well and spin them down
- Distribute the mastermix into the wells (15 µL per well). One of these wells should remain without sample in order to serve as PCR negative control
- Pipette 5 µL of each template DNA to the reactions and mix with pipette tip, starting with negative controls, then DNA samples and qPCR standards (highest dilution first)
- Take sealing film with clean forceps and seal the wellplate.
- Spin down wellplate in wellplate-centrifuge

- Place wellplate into cycler and start the run (check plate-setup, temperature profile, in case of IAC selection of correct channels, and folder or file storage)
- Put samples back to -80°C or -20°C.
- Clean used working space and pipettes with Termi-DNA-Tor solution
- While PCR is running, type plate setup in computer (or adapt an existing file)

Mastermix protocol for HMBif real-time PCR assay:

Reagent	End concentration	Volume for one 20 µl reaction
TaqMan® Environmental Master Mix (version 2.0)	1x	10 µl
Primers and Probe 10x Mix	1x	2 µl
PCR-grade water		3 µl
Sample/ Standard dilution		5 µl
Total volume		**20** µl

Primer and Probe 10x mix preparation example:

µM	Stock µM	Primer-Probe mix 10x µM	**µl to add for a 250 µl 10x mix**	qPCR reaction µM
Forward primer Bif-F	100	9	22.5	0.900
Reverse primer Bif-R	100	9	22.5	0.900
Probe HMBif (FAM)	100	2.5	6.25	0.250
PCR-grade water			198.75	

qPCR standards:

Standard gBlock for HMBif: (315 bp)
5‘GGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGGCCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGC
GGGATGGCGGCCTTCGGGTTGTAAACCGCTTTTGACTGGGAGCAAGCCCTTCGGGGTGAGTGTACCTT
TCGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAAGCGTTATCCGG
AATTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTG 3‘

Primers are colored in green and probe in blue.Regarding the serial dilutions of the standard, the standard dilution with the highest concentration should exceed the highest concentrations in the samples (sample concentration should be within the standard curve range). The standard with the lowest concentration should be in the 0.1 to 1 copy per reaction range. The minimum of useful standard dilution steps should span 5 orders of magnitude.

PCR program for HMBif amplification:

	Temperature	Time	Cycles
Initial Denaturation	50°C	2 min	
	95°C	10 min	
Denaturation	95°C	15 s	40
Annealing, Elongation	60°C	1 min	

qPCR data are collected at the annealing temperature. Amplification yields a product of **104 bp**.

Primers and probe sequences for the HMBif real-time PCR assay:

Name	Function	Sequence (5'-3')	Length (bp)
Bif-F	Forward primer	TTCGGGTTGTAAACCGCTTTT	21
Bif-R	Reverse Primer	TACGTATTACCGCGGCTGCT	19
Probe HMBif (FAM)	TaqMan probe	FAM-TCGGGGTGAGTGTACCT-MGB-NFQ	17

*FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher; MGB, minor groove binder

Suppliers of reagents:

Reagent	Supplier
TaqMan Environmental Real-Time PCR Master Mix 2.0	Applied Biosystems, Spain
Primers	Invitrogen, Thermo Fisher Scientific, Waltham, MA USA
Probes	Invitrogen, Thermo Fisher Scientific, Waltham, MA USA
gBlocks	Integrated DNA Technologies, Leuven, Belgium
Termi-DNA-tor solution	Biotools, B & M Labs, S.A, Madrid, Spain
Water for molecular biology	Applied Biosystems, Spain

Method for enumeration of the human-specific Bacteroidetes marker HF183

- Start cycler and computer.
- Prepare and print plate setup, use different areas for negative controls, DNA samples and qPCR standards.
- Put DNA samples from -80°C or -20°C to 4°C
- For preparation of mastermix use DNA-free working space: Put on new gloves and clean DNA-free working space with DNA away
- Let reagents needed for assay thaw on DNA-free working space. WARNING: Transfer NOTHING from the normal lab to the DNA free working space!!!
- Prepare (DNA-free) pipettes, tips, reaction vials (for mastermix)
- Pipette mastermix (start with water; probe, primers and TaqMan® Environmental Master Mix last), vortex and spin down
- Put reagents back into the refrigerator (TaqMan® Environmental Master Mix) or to the freezer. Leave cleaned DNA-free working space (take nothing with you except mastermix) and go to working space where DNA samples/ qPCR standards are handled.
- Take qPCR standards out of the freezer to thaw and change gloves afterwards (use different working space and pipettes for handling qPCR standards and DNA samples, respectively)
- Vortex template DNAs well and spin them down
- Vortex qPCR standards well and spin them down
- Distribute the mastermix into the wells (15 µL per well). One of these wells should remain without sample in order to serve as PCR negative control
- Pipette 5 µL of each template DNA to the reactions and mix with pipette tip, starting with negative controls, then DNA samples and qPCR standards (highest dilution first)
- Take sealing film with clean forceps and seal the well plate.
- Spin down well plate in well plate-centrifuge
- Place well plate into cycler and start the run (check plate-setup, temperature profile, in case of IAC selection of correct channels, and folder or file storage)
- Put samples back to -80°C or -20°C.
- Clean used working space and pipettes with Termi-DNA-Tor solution
- While PCR is running, type plate setup in computer (or adapt an existing file)

Mastermix protocol for HF183 real-time PCR assay:

Reagent	End concentration	Volume for one 20 µL reaction
TaqMan® Environmental Master Mix (version 2.0)	1x	10 µL
Primers and Probe 10x Mix	1x	2 µL
PCR-grade water		3 µL
Sample/ Standard dilution		5 µL
Total volume		**20** µL

Primer and Probe 10x mix preparation example

µM	Stock µM	Primer-Probe mix 10x µM	**µl to add for a 250 µl 10x mix**	qPCR reaction µM
Forward primer HF183	100	10	25	1
Reverse primer BacR287	100	10	25	1
Probe BacP234MGB (FAM)	100	0.8	2	0.08
PCR-grade water			198	

qPCR standards:

Standard gBlock for HF183: (416 bp)
5'ACAGGCTTAACACATGCAAGTCGAGGGGCAGCATGGTCTTAGCTTGCTAAGGCTGATGGCGACCGG
CGCACGGGTGAGTAACACGTATCCAACCTGCCGTCTACTCTTGGCCAGCCTTCTGAAAGGAAGATTAAT
CCAGGATGGGATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGATGC
GTTCCATTAGATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAA
GGTCCCCCACATTGGAACTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTC
AATGGGCGATGGCCTGAACCAGCCAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAAACTTCTTT
TATAAAGGAA 3'

Primers are colored in green and probe in blue.Regarding the serial dilutions of the standard, the standard dilution with the highest concentration should exceed the highest concentrations in the samples (sample concentration should be within the standard curve range). The standard with the lowest concentration should be in the 0.1 to 1 copy per reaction range. The minimum of useful standard dilution steps should span 5 orders of magnitude.

PCR program for HF183 amplification

	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	
Denaturation	95°C	15 s	45
Annealing, Elongation	60°C	60 s	

qPCR data are collected at the annealing temperature. Amplification yields a product of **132 bp**.

Primers and probe sequences for the HF183 real-time PCR assay

Name	Function	Sequence (5'-3')	Length (bp)
HF183	Forward primer	ATCATGAGTTCACATGTCCG	20
BacR287	Reverse Primer	CTTCCTCTCAGAACCCCTATCC	22
BacP234MGB (FAM)	TaqMan probe	[6-FAM]- CTAATGGAACGCATCCC -MGB	17

*FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher; MGB, minor groove binder

Suppliers of reagents

Reagent	Supplier
TaqMan Environmental Real-Time PCR Master Mix 2.0	Applied Biosystems, Spain
Primers	Invitrogen, Thermo Fisher Scientific, Waltham, MA USA
Probes	Invitrogen, Thermo Fisher Scientific, Waltham, MA USA
gBlocks	Integrated DNA Technologies, Leuven, Belgium
Termi-DNA-tor solution	Biotools, B & M Labs, S.A, Madrid, Spain
Water for molecular biology	Applied Biosystems, Spain

Method for enumeration of the human-specific microbial source tracking marker crAssphage

- Start cycler and computer
- Prepare and print plate setup, use different areas for negative controls, DNA samples and qPCR standards.
- Put DNA samples from -80°C or -20°C to 4°C
- For preparation of mastermix use DNA-free working space: Put on new gloves and clean DNA-free working space with DNA away
- Let reagents needed for assay thaw on DNA-free working space. WARNING: Transfer NOTHING from the normal lab to the DNA free working space!!!
- Prepare (DNA-free) pipettes, tips, reaction vials (for mastermix)
- Pipette mastermix (start with water; probe, primers and TaqMan® Environmental Master Mix last), vortex and spin down
- Put reagents back into the refrigerator (TaqMan® Environmental Master Mix) or to the freezer. Leave cleaned DNA-free working space (take nothing with you except mastermix) and go to working space where DNA samples/ qPCR standards are handled.
- Take qPCR standards out of the freezer to thaw and change gloves afterwards (use different working space and pipettes for handling qPCR standards and DNA samples, respectively)
- Vortex template DNAs well and spin them down
- Vortex qPCR standards well and spin them down
- Distribute the mastermix into the wells (15 µL per well). One of these wells should remain without sample in order to serve as PCR negative control

- Pipette 5 µL of each template DNA to the reactions and mix with pipette tip, starting with negative controls, then DNA samples and qPCR standards (highest dilution first)
- Take sealing film with clean forceps and seal the well plate.
- Spin down well plate in well plate-centrifuge
- Place well plate into cycler and start the run (check plate-setup, temperature profile, in case of IAC selection of correct channels, and folder or file storage)
- Put samples back to -80°C or -20°C.
- Clean used working space and pipettes with Termi-DNA-Tor solution
- While PCR is running, type plate setup in computer (or adapt an existing file)

Mastermix protocol for crassphage real-time PCR assay:

Reagent	**End concentration**	**Volume for one 20 µL reaction**
TaqMan® Environmental Master Mix (version 2.0)	1x	10 µL
Primers and Probe 10x Mix	1x	2 µL
PCR-grade water		3 µL
Sample/ Standard dilution		5 µL
Total volume		**20** µL

Primer and Probe 10x mix preparation example:

µM	Stock µM	Primer-Probe mix 10x µM	**µl to add for a 250 µL 10x mix**	qPCR reaction µM
Forward primer crAss-UB-F	100	9	22.5	0.9
Reverse primer crAss-UB-R	100	9	22.5	0.9
Probe crAss-UB-P (FAM)	100	2.5	6.25	0.250
PCR-grade water			198.75	

qPCR standards:

Standard gBlock for crassphage: (334 bp)
5'AACTACTAATATTCTTGATAAACTTTCTACTCCTCCATATAATTGTGATGGTAAAGATAGATGTTGTCA
ACTTATTATTAATAGTGCTCAGAGAATTACTTGGAAAGAAGTTAAATCTATTGAAGAATTAGGAGAAAG
TGAACGTGGAAACAAAGGATTTGGAGAAGGAACCGGTGGAGCAGCTAAAGCTTAAAGTTGGTGCTCG
TTATATTCATAATAAAACATCTAATGAATATATAATTATTAGTATTACTAAAATGAAACATCCAGATACA
GGTGAATGGATTCCTGCTGTTATTTATAAACTTAATGGACTTGAACCTTTATGGTGTAG3'

Primers are colored in green and probe in blue.Regarding the serial dilutions of the standard, the standard dilution with the highest concentration should exceed the highest concentrations in the samples (sample concentration should be within the standard curve range). The standard with the lowest concentration should be in the 0.1 to 1 copy per reaction range. The minimum of useful standard dilution steps should span 5 orders of magnitude.

PCR program for crassphage amplification:

	Temperature	**Time**	**Cycles**
Initial Denaturation	50 °C	2 min	
	95°C	10 min	
Denaturation	95°C	15 s	40
Annealing, Elongation	60°C	1 min	

qPCR data are collected at the annealing temperature. Amplification yields a product of **78 bp**.

Primers and probe sequences for the crassphage real-time PCR assay:

Name	**Function**	**Sequence (5'-3')**	**Length (bp)**
crAss-UB-F	Forward primer	AGGAGAAAGTGAACGTGGAAACA	23
crAss-UB-R	Reverse Primer	AACGAGCACCAACTTTAAGCTTTA	24
crAss-UB-P (FAM)	TaqMan probe	FAM-AGGATTTGGAGAAGGAA-MGB-NFQ	17

*FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher; MGB, minor groove binder

Suppliers of reagents:

Reagent	Supplier
TaqMan Environmental Real-Time PCR Master Mix 2.0	Applied Biosystems, Spain
Primers	Invitrogen, Thermo Fisher Scientific, Waltham, MA USA
Probes	Invitrogen, Thermo Fisher Scientific, Waltham, MA USA
gBlocks	Integrated DNA Technologies, Leuven, Belgium
Termi-DNA-tor solution	Biotools, B & M Labs, S.A, Madrid, Spain
Water for molecular biology	Applied Biosystems, Spain

13.4 Literature

Gómez-Doñate, M., Ballesté, E., Muniesa, M., & Blanch, A. R. (2012). New molecular quantitative PCR assay for detection of host-specific Bifidobacteriaceae suitable for microbial source tracking. Applied and environmental microbiology 78(16), 5788-5795.

Green, H.C., Haugland, R.A., Varma, M., Millen, H.T., Borchardt, M.A., Field, K.G., Walters, W.A., Knight, R., Sivaganesan, M., Kelty, C.A., Shanks, O.C., 2014. Improved HF183 quantitative real-time PCR assay for characterization of human faecal pollution in ambient surface water samples. Applied and environmental microbiology 80(10), 3086-3094.

García-Aljaro, C., Ballesté, E., Muniesa, M., Jofre, J., 2017. Determination of crAssphage in water samples and applicability for tracking human faecal pollution. Microbial Biotechnology 10(6), 1775-1780.