Aquavalens Project

"Protecting the health of Europeans by improving methods for the detection of pathogens in drinking water and water used in food preparation."

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Deliverable D7.6

Standardised method for detection of molecular targets.

Authors: Jakob Ottoson, Swedish University of Agricultural Sciences (SLU), Albert Bosch, University of Barcelona (UB) and Antonio Martinez Murcia, Genetic PCR Solutions™ (GPS™).

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1. Background

Technological platforms for the study of pathogens in water can be described as technologies enabling the translation of lab-bench protocols to automated protocols. Automated protocols have the advantages of: 1) accelerated diagnostics procedures leading to higher through-put of samples, 2) improved repeatability within and between labs and 3) increased safety, with less probability of cross-contamination between samples. Platforms may encompass sample preparation, detection and analysis, or may provide only one or several of these tasks. Although some platforms have been successfully commercialised for other applications, few have been specifically studied and adapted for the detection of pathogens in water. Furthermore, practically no platform has been adopted by water companies or companies dealing with water quality control. The reasons for this limited take up by industry are multifactorial and include: costs of analysis, lack of robustness of automated systems, poor understanding of the market sectors and absence of regulatory requirements.

The development of a platform in Activity 7.1 aimed to suggest a rapid and robust solution for the standardised detection of molecular targets. The platform should further be suitable for automation, thereby reducing the cost of such analysis, and eliminating some of the human handling time and potential for contamination. In AQUAVALENS we have evaluated platforms according to the following criteria: (1) limit of detection, (2) potential for speciation, (3) robustness of detection, (4) manufacturing and operational costs, (5) potential for simultaneous analysis of multiple pathogens, (6) market demand, (7) fit with existing water management systems, (8) system reliability, (9) size and portability of the system and (10) potential for automation. Whereas some of the promising selected platforms will perform well over a large range of these criteria, others will excel in a specific subset resulting in different levels of integration, dealing with specific classes of target organisms and for specific market applications. This report describes the route towards a standardised platform for the detection of nucleic acid targets taking into account sensitivity, specificity and robustness as input for the integration D8.6 report which has a broader scope. The detection platform requires extracted nucleic acids, however a task covered in work package 6.

The fact that waterborne pathogens have the potential to cause disease and outbreaks even when present in low numbers, the starting point for this activity has been the need for amplification of the target in question. Since the 80's a wide range of PCR and real-time PCR systems have been developed and applied to the diagnosis of infectious diseases as well as the detection of pathogens in food and water (Pinto & Bosch, 2008). Various platforms (Taqman, LightCycler, PriProEt, etc.) have shown high specificity and sensitivity in the detection of pathogens (Belak & Thoren, 2001). Compared with the classical gel-based single, nested and ELISA-based test systems, real-time PCR assays offer: (i) faster and higher throughput, (ii) one-step amplification set-up, (iii) on-line detection of the product minimising the risk of lab contamination and consequently (iv) higher automation potential (Belak*, et al.*, 2009). Further, the specificity is increased by the use of specific probes.

Some other amplification systems such as NASBA, LAMP, and RPA do not require thermal denaturation of templates and can operate at a low and constant temperature. They therefore offer an easy and affordable technology for the integration in combined filtration, detection and output signal devices relative to other amplification methods. The downside is that different annealing temperatures cannot be deployed as a means to mitigate amplification bias in single tube systems.

Multiplex detection uses multiple primers to allow amplification of multiple templates within a single reaction. By broadening one analysis to several targets, such as the most commonly waterborne viruses in a water sample, the speed and cost of diagnostics can be decreased.

The specific aims of Task 7.1 were:

1. To provide background data in order to suggest which platform to include for integration in WP8, thermocycler for qPCR detection or heater for isothermal RPA detection?

2. To provide background data for the suggestion of multi- or monoplex protocols for detection of nucleic targets?

2. Detection platform to be used

The preliminary results on aim 1 were already forwarded within the project internally as Milestone 14 ("Detection platform for integration in WP8") in order to provide necessary input for the integration workpackage (WP8) as well as a functional technology (*e.g.* ready-to-use kits) for the field studies in cluster 3 (Annex 1).

Recombinase Polymerase Amplification (RPA) is an isothermal system for nucleic acid amplification which employs prokaryotic enzymes, so-called recombinases, to guide synthetic oligonucleotide primers to target sites in sample nucleic acids. In this process, exponential amplification of the desired sequence occurs by reiterative oligonucleotide-primed DNA synthesis without a need for bulk melting of DNA to facilitate access of the primers, hence avoiding the requirement for any heating/cooling steps. As RPA operates at constant temperatures ranging 24 – 45 °C (optimum of 37ºC), sophisticated and power-demanding heating sources are not required to implement the technique. RPA was investigated as an alternative to the well-established qPCR in activity 7.1.

The main problem for this technology to be used is related to the requirements to design primers. First of all, primers should be > 30 bp long, as the ability of recombinase proteins to stimulate and complete recombination/priming with shorter oligonucleotides decreases sharply with size. Very often however, the finding of conserved specific sequences (inclusive for strains of target-taxon, exclusive for related taxa) is hard and these possibilities decrease with the length of signatures. Consequently, the chances to design species-specific primers longer than 30 bp are very low, many times not possible.

Even in the case of finding sequences that meet both requirements, the primers may fail to work with RPA. As indicated by the RPA experts (Piepenburg et al, 2006), selection of primers for RPA should be made by screening a number of candidates in a three-step process (Figure 1):

1. First RPA screening by using several primer combinations flanking the target sequence.
2. Once one or two combinations have shown to work, further experimental tests should be performed with primers differing by 1 bp increments around the best primary screen primers
3. Finally, a third RPA screen is needed by using primers differing in length by 1 bp increments at the 3'-end of the best secondary selected primers.

Obviously, this recommended manner to select primers optimized to work in RPA is a highly random process that makes the designing very complex, remaining the specificity of the test highly committed, if not very poor and too time-consuming in order to provide a functional technology for the field studies. Further, recombinase polymerase has been reported to be inhibited by background DNA (Rohrman & Richards-Kortum, 2015).

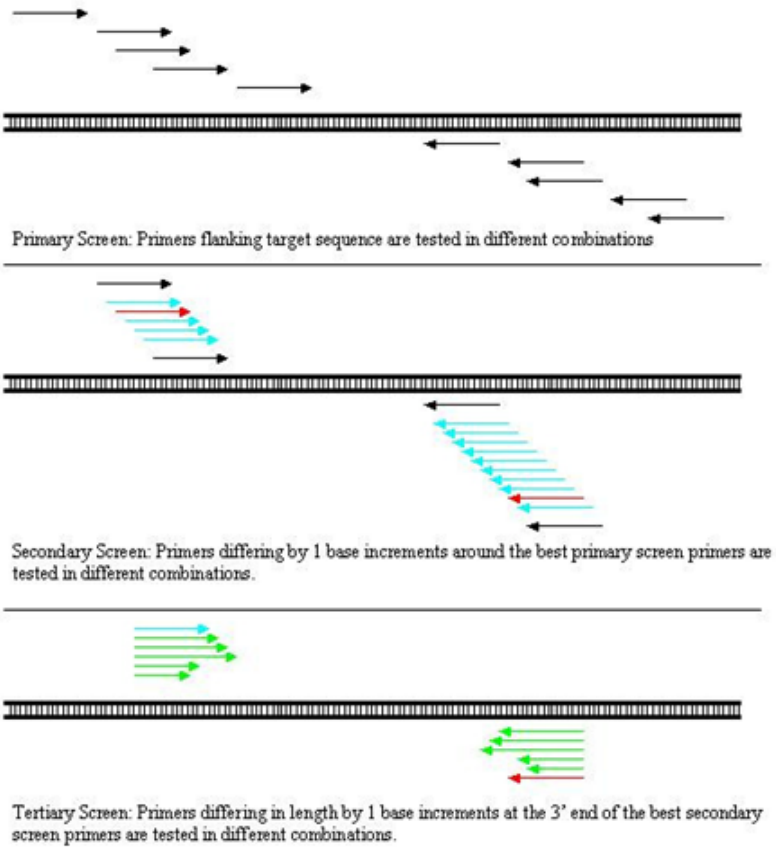

Figure 1. Experimental strategy recommended to design and optimize specific primers for RPA (http://www.twistdx.co.uk).

3. Multi- or monoplex protocols?

A wide variety of human enteric viruses may be found as food and water contaminants. However, with few exceptions most well characterized food- and waterborne viral outbreaks are restricted to human norovirus (NoV), and hepatitis A virus (HAV). In this context, reliable and affordable methodologies for the detection of human NoV and HAV in water and food are needed. In the present study, a multiplex (quadruplex) for the quantitative detection of HAV, NoV GI and NoV GII, including mengovirus as process control, in food and water was developed. The efficiency of the based on four monoplex reactions was compared with the quadruplex format in which all viruses are detected in a single reaction (Fuentes*, et al.*, 2014).

As expected, the quadruplex assay showed a certain loss of sensitivity in comparison with the monoplex counterpart which however was negligible when naturally-contaminated samples were assayed (Table 1). The theoretical detection limit for NoV GII was similar in the mono- and quadruplex assays, while for HAV and NoV GI the detection limits were 1-log higher in the quadruplex format. (Figure 2). Nevertheless, the advantage of lower reagent costs and less time-consuming labour still makes the multiplex assay a valuable tool for the screening of viruses in water.

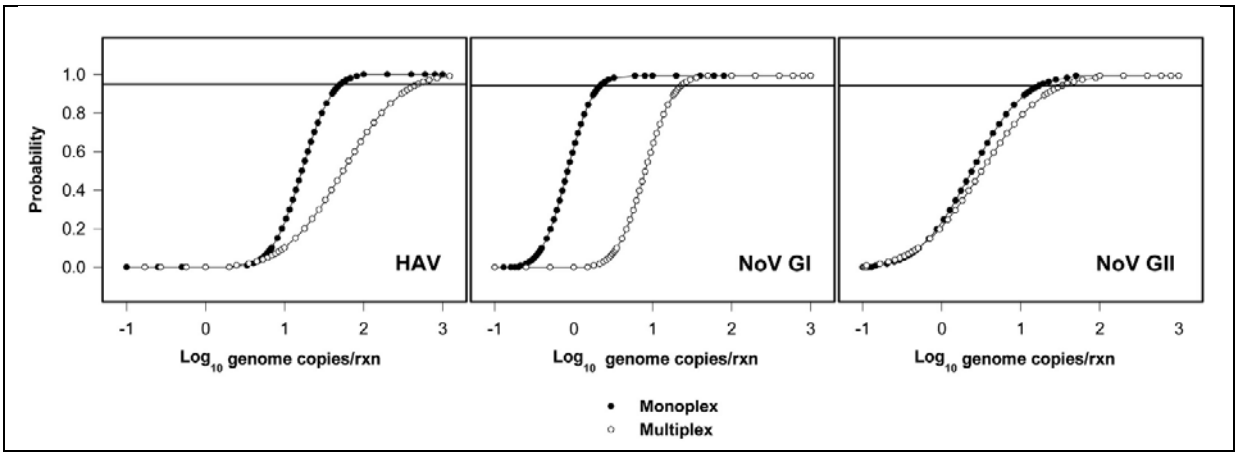

Figure 2. Probability of detection curves depending on target numbers for Hepatitis A virus (HAV), Norovirus genogroup 1 (NoV GI) and Norovirus genogroup 2 (NoV GII) with the monoplex (black dots) and multiplex (white dots) assays (Fuentes, et al., 2014).

The sensitivity was also compromised to some degree for the other amplification platform evaluated in AQUAVALENS, *e.g.* Variation tolerant capture multiplex analysis (VOCMA) where the intended four-plex gastrointestinal panel was split into two duplex ones. However; as for the quadroplex foodborne virus panel described above, detection of norovirus genogroup II was not compromised in the multiplex systems (D7.5. Method for multiplex detection of targets identified in cluster 1 on Luminex and by qPCR; Xia et al., 2016).

Table 1. Comparative quantitative detection through monoplex and multiplex assays in river water used as source of potable water. Mean and standard error of the log10 genome copies of HAV and NoV GI and GII per liter of water are depicted. Values corresponding to raw or corrected data, taken into consideration the extraction and RT-PCR efficiencies provided they were both over 1 %, are shown. The estimated limit of detection, expressed as the log10 genome copies per liter of water, is also shown

Sample (Volume)	Virus	Monoplex			Multiplex		
		Raw	Corrected	Limit of Detection	Raw	Corrected	Limit of Detection
1 (5 l)	HAV	ND[a]	ND	3.41	ND	ND	4.39
	GI	ND	ND	2.03	ND	ND	3.06
	GII	2.06 ± 0.28	2.10 ± 0.28	2.93	ND	ND	3.22
2 (5 l)	HAV	ND	ND	3.41	ND	ND	4.39
	GI	ND	ND	2.03	ND	ND	3.06
	GII	2.19 ± 0.14	2.30 ± 0.14	2.93	1.942	1.982	3.22
3 (500 l)	HAV	ND	ND	1.41	ND	ND	2.39
	GI	ND	ND	0.03	ND	ND	1.06
	GII	0.482[b]	0.492	0.93	-0.272	-0.262	1.22
4 (500 l)	HAV	ND	ND	1.41	ND	ND	2.39
	GI	ND	ND	0.03	ND	ND	1.06
	GII	1.44 ± 0.31	1.44 ± 0.31	0.93	0.852	0.862	1.22
5 (500 l)	HAV	ND	ND	1.41	ND	ND	2.39
	GI	ND	ND	0.03	ND	ND	1.06
	GII	0.44 ± 0.01	0.69 ± 0.01	0.93	0.332	0.672	1.22
6 (500 l)	HAV	ND	ND	1.41	ND	ND	2.39
	GI	ND	ND	0.03	ND	ND	1.06
	GII	1.652	1.652	0.93	0.592	0.592	1.22
7 (500 l)	HAV	ND	ND	1.41	ND	ND	2.39
	GI	ND	ND	0.03	ND	ND	1.06
	GII	0.942	0.942	0.93	0.062	0.142	1.22
8 (500 l)	HAV	4.29 ± 0.05	4.29 ± 0.05	1.41	4.15 ± 0.11	4.15 ± 0.11	2.39
	GI	ND	ND	0.03	ND	ND	1.06
	GII	-0.122	0.062	0.93	ND	ND	1.22
9 (500 l)	HAV	1.70 ± 0.04	1.70 ± 0.04	1.41	1.13 ± 0.33	1.13 ± 0.33	2.39
	GI	ND	ND	0.03	ND	ND	1.06
	GII	0.072	0.202	0.93	0.21 ± 0.11	0.21 ± 0.11	1.22

[a]ND: Not detected

[b]When intervals are not shown, only one replica was positive

GPS™ have further detected and reported some drawbacks with qPCR when performed multiplex even when the thermodynamics of single-plex are compatible to be used in the same tube, several unpredictable circumstances related to the sample may provide false results. These drawbacks do not only depend on the matrix of the sample and inhibitor compounds, but can also depend on the relative amounts of the different targets in the sample. Figure 3 below illustrate a case we found for a false positive when combining different qPCRs specific for targets PathA (red), PathB (green), and PathC (blue). All these three qPCR were assayed in single-plex and calibrated by using DNA standard ten-fold dilutions from 10 to 10^6 copies and results were according to expected. The duplex qPCR combinations PathA/PathB and PathA/PathC did not show significant differences to the plots obtained for the three independent single-plexes, when using the same amounts, or very similar, of DNA standards of corresponding targets combined. However, if the relative quantity of the two targets combined was different, the signal obtained for the PathA qPCR decreased dramatically when combined in duplex with PathC qPCR, to the point to obtain a false negative if that relative difference (quantities) becomes higher (i.e., 3 logs or more). However this did not occur when the same PathA qPCR was combined in duplex with PathB qPCR.

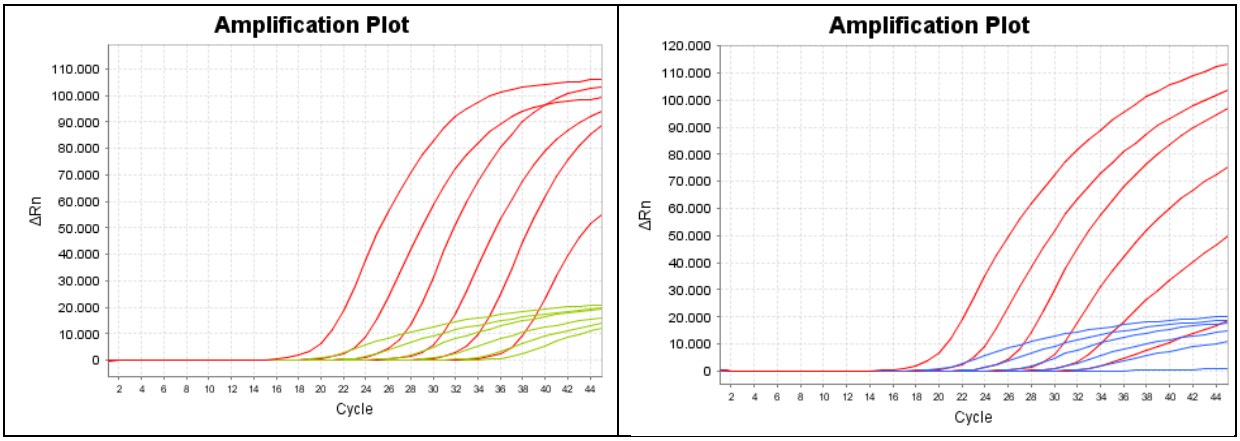

Figure 3. Duplex qPCR combinations PathA/PathB (red/green curves) and PathA/PathC (red/blue curves) results by using ten-fold dilutions standard DNA (10^1 to 10^6 copies).

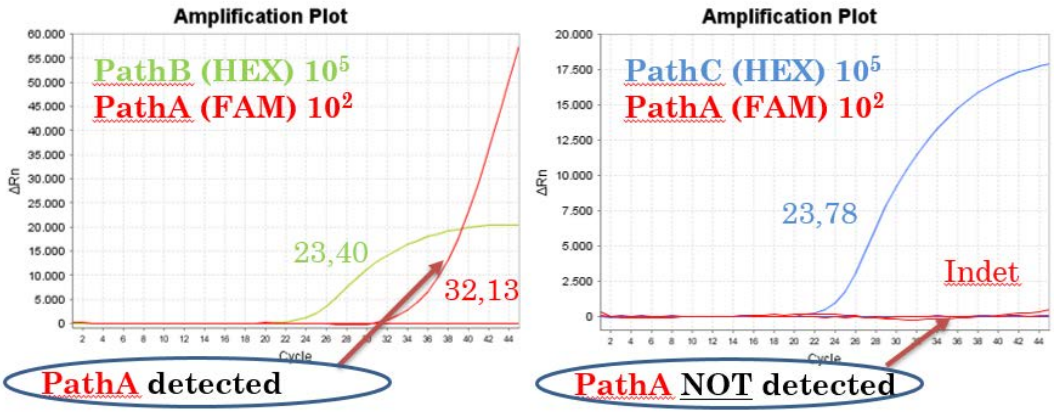

Figure 4. Duplex qPCR PathA/PathB (red/green curves) using and PathA/PathC (red/blue curves) results by using 10^2:10^5 ratios of the corresponding standard DNA templates.

GPS™ has explored the reasons why the qPCR of PathA is dependent of the accompanying qPCR of PathC in the duplex. Changes on the PathA qPCR, including primary structure modifications of primers, did not produced significant changes. Finally we found that some changes in the sequences of primers and probe designed for the PathC qPCR could reduce this problem enabling the combination A + C to work well independently of relative amounts of templates, even at a 10^1:10^4 ratio, as shown in Figure 5.

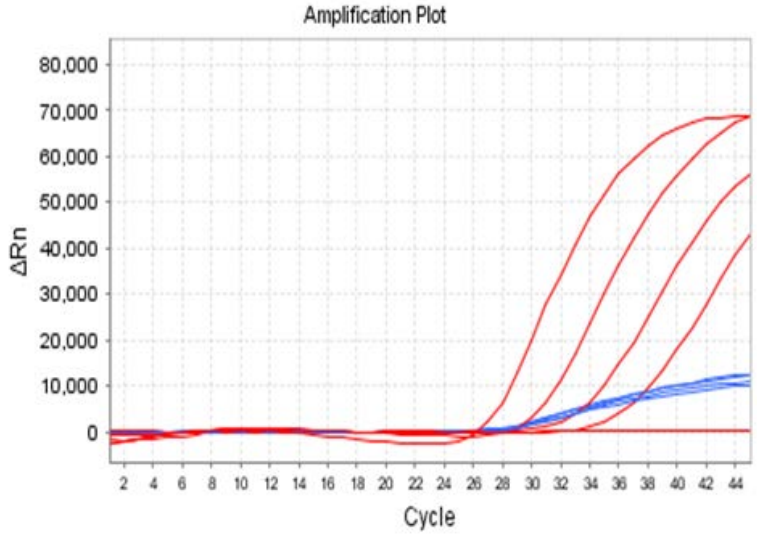

Figure 5. Duplex qPCR PathA/PathC (red/blue curves) results by using 10^1:10^4, 10^2:10^4, 10^3:10^4 and 10^4:10^4 ratios of the corresponding standard DNA templates. * The sequences of primers and probe designed for the PathC qPCR was partially modified.*

As an alternative, GPS has suggested the use of panels with several single-plex qPCR to be run at the same time. With this aim, all qPCR have been designed for reactions to run with the same protocol of Tª/time regime (Figure 6).

Figure 6. Schematic prototype of a panel designed for pathogens A, B, C, D, and E, by simultaneous single-plex qPCR.

Another advantageous innovation developed within task 7.1, with the purpose to propose these panels, was the format so-called "MONODOSE", which contains all reagents, dehydrated and ready to use, for each of the targeted pathogens. The MONODOSE format simplifies the operational procedures (only the sample template should be added to each tube), minimising possible cross contamination and deterioration of enzyme/fluorophores by freezing/thawing. The whole cartridge may be transported at room temperature, which is really appreciated for marketing. Furthermore, with only one step, *e.g.* sample addition, the possibility to automate the procedure is simplified.

Figure 7. MONODOSE dtec-qPCR Test KIT. The bag contains individual ready-to-use tubes containing all the components needed for detection of a specific pathogen.

Despite limitations on designing functional multi-plex systems using iso-thermal PCR a recently published paper reported a successful multiplex RPA protocol for the detection of three parasites on the genus level (Crannell et al., 2016). However, only inclusivity was tested (using synthetic DNA) but not exclusivity. The LOD was roughly 1.5 orders of magnitude higher in the multiplex compared to the respective singleplex assays whereas the loss was about 0.4 log genome copy numbers in the developed viral quadroplex (Fuentes et al, 2014).

4. Prioritised pathogens and suggested kits to be used

The internal workshop "selection of target organims" (MS10) held in Alicante 19th November 2014 resulted in the prioritasion of target pathogens (D16.3 report "Workshop for the determination of which developments of Cluster 1 will be taken forward into Cluster 2"; Bouzid, 2015). The kit manufacterers GPS and Ceeram have developed ready to use kits for detection of all these target organisms (Table 2).

Table 2. Prioritised pathogens and kits provided for field studies

Target pathogen	Kit provider
Norovirus genogroup I	CEERAM
Norovirus genogroup II	CEERAM
Hepatitis A virus	CEERAM
Hepatitis E virus	CEERAM
Arcobacter butzleri	GPS
Campylobacter coli	GPS
Campylobacter jejuni	GPS
Vibrio cholerae	GPS
Pseudomonas aeruginosa	GPS
Salmonella enterica Typhi	GPS
Escherichia coli (O157:H7)	GPS
Legionella pneumophila[a]	GPS
Listeria monocytogenes[a]	GPS
Giardia intestinalis Ass A	CEERAM
Giardia intestinalis Ass B	CEERAM
Cryptosporidium hominis	CEERAM
Cryptosporidium parvum	CEERAM
Toxoplasma gondii	GPS

[a] Optional, outside the scope of AQUAVALENS

The next step was the validation of these kits for detection of HAV, NoV GI, NoV GII, *Escherichia coli*, *Campylobacter jejuni* and *Cryptosporidium parvum*. The first round of validation was performed during the spring 2015. In total, 30 samples per target were prepared in order to represent specific analyt/matrix/concentration combinations. The complexity of samples increased incrementally in four different categories:

1. General Test: DNA/RNA of one species (target) in different concentrations in DNase and RNase free water
2. Specificity/Cross-reactivity: Mixture of DNA/RNA of different species in different concentrations in DNase and RNase free water (different target/non-target ratios)
3. Inhibition: DNA/RNA of one species (target) in DNase and RNase free water with humic acids as inhibitory substances
4. Complex Matrix: DNA/RNA of one species (target) in nucleic acid extracts of surface water

Generally, the results were highly satisfactory with no false positives, few false negatives and insignificant variation between labs (D9.3 report "Report of validation with nucleic acids"; Stange & Tiehm, 2015).

5. Discussion

The starting point for the detection systems evaluated in task 7.1. has been the detection of molecular targets to provide insight into the occurrence and virulence of prioritised waterborne pathogens, irrespective of the agent domain (virus, bacteria or parasite). However; the possibility to be innovative and bring out new technologies within cluster 2 work packages has been limited due to: 1) outcomes from cluster 1 development could not be expected until the earliest two years into the project and 2) field studies were to start and cluster 3 preparations needed information on the respective technologies to take forward already before year three of the project. Hence, in order to provide a functional and validated technology to bring forward we had a gate decision already by month 24 on which platform to use (see Annex 1).

As already been addressed in the background, there are pros and cons with as well mono- as multiplex protocols. In the end we suggest a compromise– single tube systems but using the same time temperature regime for all targets. Using this strategy we actually get the best from both protocols. The downsides are that the extracted nucleic acids (template) will be divided into separate tubes and hence influencing the limit of detection (LOD) and that the costs for reagents and labour (*e.g.* in a non-automated system) will increase compared to multiplex assays (Fuentes*, et al.*, 2014). On the other hand, one major problem with amplification protocols from concentrates of large volumes of water is the influence of inhibitors making it necessary to dilute the template. Hence, in reality, the LOD is normally not compromised using a protocol with split template. Further, with an automated protocol, the time for labour will not be influenced. This automation procedure will be easier to develop with the new MONODOSE format since the only step needed is the addition of sample to the respective test tube.

In order to minimise inter- and intravariability between technicians and laboratories respectively we suggest that kits should be used in the field studies. The SMEs within AQUAVALENS, Genetic PCR Solutions™ and CEERAM, have developed and provided kits for the detection of prioritised pathogens for the field studies in due time. The kits are by this date not necessarily targeting the newest sites as of the different outcomes from work packages 2-4 in cluster 1. However, a decision was made during the fourth yearly meeting (2016-04-21) in Cassis to save extracted nucleic acids from all samples during the field studies. Hence it will be possible to compare the performance of the primers and probes developed later in the project with the ones presently used by cluster 3.

6. Final conclusions/outcomes

- Real-time PCR was the chosen standard for detection of target nucleic acids due to
    - Outcome sequences from cluster 1
    - SME partners' competences
    - Shortcomings of the isothermal platform evaluated and the time limit to be able to overcome them
- Single tube systems run at the same thermocycling programme are suggested, but
    - Multiplex systems can be used, however, these need to be thouroughly evaluated. This has been done for the food- and water viral quadroplex panel for the simultaneous detection of norovirus genogroup I and II, hepatitis A virus and mengovirus internal control
- AQUAVALENS SMEs GPS^{TM} and Ceeram (Biomerieux) have developed validated kits for all prioritised pathogens, enabling the first broad European study on waterborne pathogens providing comparable results
- The GPS^{TM} MONODOSE format simplifies automation and minimises possible cross contamination and deterioration of enzyme/fluorophores by freezing/thawing.
- The kits do not yet include primers addressing all of the latest targets suggested by cluster 1 work packages; however, saved nucleic acids enables the comparison of newer developments to the "gold standard" kits

7. References

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Annex 1. Milestone 14. Detection platform for integration in WP8 decided.

Lead: GPS™ (partner 20)

A platform for pathogen detection which adapts the best candidates systems developed in previous WPs should be selected for integration into automated multiplex systems in WP8.

Several strategies using PCR and isothermal amplification have been considered and evaluated. Finally, the quantitative “real-time PCR” (qPCR) has been considered the best platform as, in overall, it fits well within the relevant requirements: reliability of performance parameters. Above decision has been fully supported by the knowledge provide by expert partners at present project, AQUAVALENS, but also from published scientific data. Moreover, it has been motivated by the following facts:

1. Mostly, the outcome from cluster 1 provided to date comprises sequence detailed description of primers and probes for qPCR detection (for example see report D3.1.), which have been compared to commercially available qPCR-kits and subjected to validation. The developed methods should be integrated for multiplexing and ready for Cluster 3. Timing to develop alternative solutions has also been pointed.

2. Aquavalen´s partners Genetic Analysis Strategies (GPS) and Ceeram, both SMEs, shows a high expertise in developing qPCR kits for pathogens detection and their core business is based in this kind of solutions. They have offered qPCR kits for bacteria, virus, and parasite detection to all partners of Cluster 1 and 2 and have been used as reference qPCR methods at validation processes. Certified genomic standards developed by GPS and calibrated with their kits has also been provided for harmonization of standard calibration curves and spike water samples while testing sampling, extraction, and purification methods

3. Evaluated alternative isothermal platform using Recombinase Polymerase Amplification (RPA) was considered less useful as, because the length of primer sequence request (i.e. > 30 bp), the specificity of the test at the design stage seems highly compromised and very poor. Other issues are related to efficiency in multiplex format.

As a consequence, the platform suggested to be selected for integration in WP8 is thermocycler for real time (q)PCR detection.

Annex 2. Implications of the results of Deliverable Report 7.6.

Implications of the results for the Work Package

WP7 does not have any internal dependencies since the different tasks deals with different technologies as part of the whole WP7-portfolio of detection methods. This specific activity reported was however the most important since it was supposed to provide the main technology used in the field studies.

Implications of the results for this Cluster

The standardised detection method based on amplification of target sequences by PCR could benefit from automation and the results important to feed into WP8. Hence we had a gate decision already by month 24 on which platform and protocol to use. The development of ready-to-use-kits with only sample addition needed could further simplify automation. SME's kits and standards will further be valuable in the WP9 validation studies.

Implications of the results for the whole project

As the most likely method to be broadly used by all WPs in cluster 3 this task in WP7 could not fail. We have provided ready to use kits that presently undergoes validation in order for AQUAVALENS to provide comparable data on specific pathogen and virulence occurrence in European waters; data enabling refined quantitative microbial risk assessment in cluster 4, WP14. The detection methods will also be useful in the formation and evaluation of water safety plans (cluster 4, WP13).

Indicate key external stakeholders interested in the results

The detection method will in the short term be available in food and water laboratories, including laboratories at large water treatment plants. As technology develops and go down in price detection by qPCR will be a standard even for smaller water treatment plants; however, sample preparation is likely the bottle-neck, not detection. The key external stakeholder is the water manager but all organisations involved in (food and) water quality control are also important stakeholders, *e.g.* governmental agencies, county boards and municipalities.

Which internal partners should your deliverable be sent to?
UEA, UB, HZI, DTU, HWU, CEERAM, **DVGW, Cetaqua, IST, Teagasc, URV, Surrey, James Hutton, UI**, GPS, TU Wien, **NFA, Desing**, **Belgrade, Fruit and veggies, ENKR, Hlab, WRC, NV, City analysts** (End-users in bold)