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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Method for multiplex detection of targets identified in cluster 1 on Luminex and by qPCR.

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1. Background

Merging and remerging water-borne pathogens, such as viruses, bacteria and parasites, continue to cause disease outbreak globally and remain to be a major public health concern. Rapid detection of those pathogens is essential to prevent the transmission of the pathogens from water to humans. Molecular detection assays like real-time PCR have been widely accepted due to its rapidness and high sensitivity. However, when it comes to detection of pathogen from water-related sources, two important factors must be considered: 1) the high volume of the original samples despite of concentration; 2) the abundance of polymerase inhibitors in them. Therefore, a procedure which enriches for target nucleic acid and removes inhibiter is preferred. Moreover, some of the RNA viruses pose a specific challenge for sequence-based detection, namely a great variation. Many of the water-borne viruses are of this kind.

1.1. VOCMA-rationale

VOCMA is concept for creation of variation tolerant capture multiplex nucleic assays. VOCMA maximises hybridisation between primers and probe versus target, in the presence of substantial target variation, leading to variation tolerance. The tolerance can further be increased by inclusion of several alternative primer probes according to a haplo-grouping, performed in the design program ConSort (J Blomberg, unpublished). VOCMA also provides for target enrichment (Capture) via the avidly binding primer-probes. Due to the low concentration of primer-probes VOCMA can be multiplexed to a greater extent than other PCR systems, because unwanted primer-primer interactions are minimized (table 1).

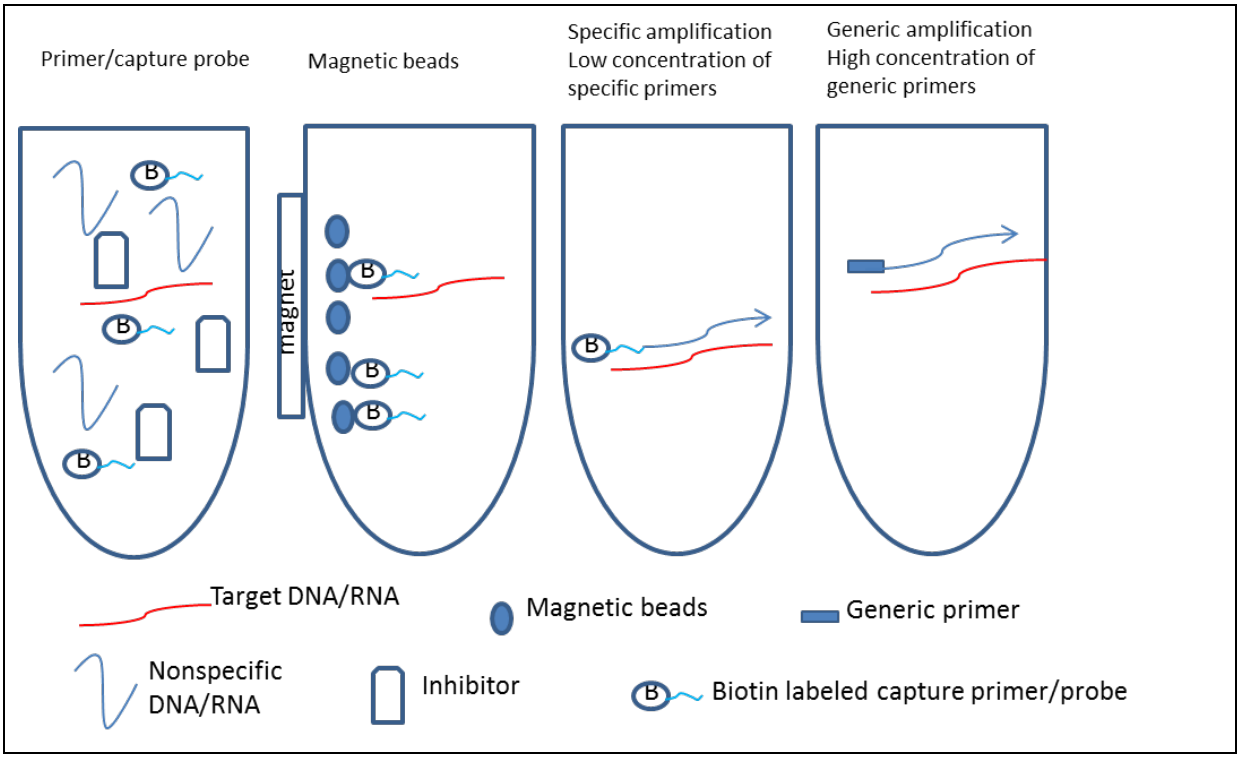
Feature	Technique	Effect	Outcome
Capture	First specific VT primer-probe coupled to magnetic bead	Removal of inhibitors	less inhibition of PCR
		Enrichment of target	Increases sensitivity and specificity
Variation tolerance	Primer-probes/detection probes designed according NucZip algorithm*	Increased mismatch tolerance	Detection of variable targets e.g RNA virus Detection of whole groups of genes e.g. Antibiotic resistance
	Long target specific part in the 3´end of the primer-probes	Increase the likelihood of long perfectly matching regions (i.e.nucleation sites)	Stronger hybridization Improved variation tolerance
Multiplexity	Usage of both specific and generic primers	Increased specificity	Increased specificity
	Low concentration of target specific primer-probes	Less primer-dimers	High multiplexability, increased specificity and sensitivity
	Few cycles in PCR program at high temp	Limited specific amplification with the long primer-probes	Increased specificity
	High concentration of generic primers	Less primer-dimers	Increased specificity and sensitivity
	High number of cycles in PCR program at low temp	High degree of generic amplification with the short generic primer	Increased specificity and sensitivity
Asymmetric amplification	Unequal conc. of generic primers with biotinylated generic primer in excess	Excess of single stranded biotinylated amplified target	Increased MFI signals
Biotinylation of the second generic primer	The first and second specific VT primer-probes and first generic primer are used in a sequential order to create the ”third copy”		Increased specificity
Single tube amplification	Temperature switch in the PCR-program	Transition from specific to generic primer usage	All primers in same mix Contamination risk minimized
One-step amplification	iScript one-step RT-PCR with both RT Pol and DNA Pol in same reaction	No transfer from Reverse transcription step to the PCR amplification	All enzymes in same mix Contamination risk minimized
Specific detection probe	Probe target region positioned in between primer-probe target regions	The probe introduce a third specificity step	Increased specificity
		No detection of wrongly hybridised and elongated second generic biotinylated primer	Increased specificity

1.2. VOCMA principle

Variation tolerance is achieved by use of much longer primers and probes than is normally used. This leads to a higher binding strength (high Tm) between target and primers and probe, which allows tolerance for nonmatching nucleotides. A key feature is to include long (>6 nt) perfectly matching stretches, as many as possible. Such stretches can be created by use of a few degenerated nucleotide positions together with a judicious use of universal bases like inosine. Our data show that hybridization then can occur in spite of profound target variation which would normally be considered impossible {Ohrmalm, 2012 #8}.

A further bio-informatic feature is embodied in the Consort variation analysis program (Blomberg, unpublished). It can decompose target variation into several haplotype sequences, each of which has a smaller variation than the original consensus sequence would have. This is because variants at several sites are often coupled to each other in the form of haplotypes. This allows the number of the desired long uninterrupted perfectly matching stretches to be maximized.

The original VOCMA paper {Ohrmalm, 2012 #8} used bead-bound probes. After hybridization of the probe to the amplimers, the results were registered in the Luminex flow meter. A disadvantage of this system is that the tubes have to be opened, creating the possibility of contamination. We therefore wanted to transfer VOCMA to a real-time format in which the readout comes during amplification, without letting he amplimers free after the run. Hence we have adapted the VOCMA system with its advantages to a more sensitive qPCR-platform, however, losing some of the multiplex potential offered by detection in the Luminex. Another advantage is that VOCMA primers now can be biotinylated. This was not possible before, because biotin was used later in the hybridisation procedure. With biotinylated primers, the capture process can be made more efficient, letting biotinylated primers catch the target nucleic acids, then capturing them on streptavidin-coated magnetic beads.



2. Material and methods

2.1. Primer probe design

Target sequences or whole genomes were collected in BLASTN (NCBI, NIH) were aligned using ConSort, visualising the frequency of variation of nucleotides in each base position. Oligo primer-probe design was evaluated *in silico* using Visual OMP (DNA Software, Ann Arbor, MI, USA) to predict hybridisation efficiency. NucZip scores were also used as tools to predict hybridization efficiency and to optimize variation tolerance. NucZip simulates nucleation, the initial contact between segments of a perfect match, followed by "zipping" the two strands upstream and downstream (Ohrmalm C, et al. 2010).

2.2. Detection on Luminex

The panel of seven gastrointestinal viruses was built up stepwise in a recursive loop involving re-evaluation and redesign in order to realise an optimal primer-probe design, with as few cross-reactions as possible and minimised primer competition. The synthetic targets used for sensitivity analyses had a natural primer-probe and detection probe complementary sequence derived from GenBank (Tables 2 and 4). Synthetic ssDNA was serially diluted from $0.5 * 10^7$ to $0.5 * 10^0$ targets/µL in DEPC water (Ambion). Patient samples were provided anonymously according to Uppsala University hospital rules and extracted using the EasyMAG system (Bio-Merieux) according to the manufacturer's instructions.

The primers and probes used for detection using Luminex are included in table 2 and the synthetic targets in table 3. Two µL of synthetic ssDNA target was added to a 23- µL one-step reverse transcriptase PCR (RT-PCR) master mix containing a final concentration of 1 x iScript buffer (BioRad), 0.5 µL iScript reverse transcriptase, 300 nM generic first primer, 500 nM biotinylated generic second primer and 50 nM each of the 14 first and second specific VT primers-probes (table 2) to create the 7-plex VOCMA mixture. Amplification was carried out according to Öhrmalm et al. (2012) with PCR conditions as follow: 50°C 20 min, 95°C 5 min followed by 10 cycles of 95°C for 15 s, ramping at 0,1°C/s from 75°C to 65°C, 65°C for 1 min, followed by 40 cycles of 95°C for 15 s, 52°C 30 s, 60°C 30 s, followed by 60°C for 5 min, 95°C for 1 min and 4°C until the hybridisation step.

Hybridisation: Five microliters of biotin-labelled VOCMA amplified target were mixed with hybridisation buffer and probe-coupled xMAP beads. The mixture was heated to 95°C for 2 min followed by hybridisation at 50°C for 30 min with shaking at 600 rpm on a Thermostar microplate incubator (BMG LabTech). After centrifugation, 38 µL buffer was mixed with 2 µL streptavidin-R-phycoerythrin (Qiagen) and incubated for another 15 min at 50°C before analysis on the Luminex 200 flow meter (Luminex corporation). The quantity of the target was measured using the median fluorescent intensity (MFI).

Table 2. Oligonucleotides used in the Gastro VOCMA

Target	Primer/Probe	Modification	Sequence 5´to 3´
Generic first			TTGGATAAGTGGGATA
Generic second		Biotin	AAGATATCGTAAGGAT
Norovirus GG1	Second		AAGATATCGTAAGGATAAGACAGGGGGICTTGAAATGTATGTICCAGGITGGCAGGCCATGTTCCGITGGATGCG
	First	AminoC12	TTGGATAAGTGGGATACCGGCGCCAITIGCGCCATCIRIGITTGGIGTIGCGTCCTTAGACGCCAT
	Detection	AminoC12	CATCATCATTTACIAATTCGGGCAGIAGATTGCGATCTCCTGTCCACA
Norovirus GG2	Second		AAGATATCGTAAGGATAGATACAAGTACCACTATGATGCIGAITACTCTCGGTGGGA
	First	AminoC12	TTGGATAAGTGGGATATCAGAGAGCGCACAGAGAGTGAGAAGCCAGTGGGCGATGGAGTTCCATTGGGAGGTGCAGGGC ACCCCAGA
	Detection	AminoC12	CCCTCGTTGATTGATATTGTGAAGTCACCCACATCCACCACGCTAGGAGAAAGAAGGTCTTCTGC
Adenovirus	Second		AAGATATCGTAAGGATTTTGCCATGGAIATIAATITGGCGGCIA+AII+TIT+GGIGIAGCTTCTTITAYTCMAATGTAGC
	First	AminoC12	TTGGATAAGTGGGATAATGTTTACGTAGGTATCIAGGGCGCTIGGIACIGCIACCCGACCGTTCATGTAGGCATA
	Detection	AminoC12	TTGTTKTCGGGIAIIGTAATGTTITCIGGIGTIATITTITAIGAGTCAGGCAA
Rotavirus A	Second		AAGATATCGTAAGGATTATTCITTGTCAAAAACTCTTAAAGATGCTAGGG+AIA+ARATTGTYG+AAGG
	First	AminoC12	TTGGATAAGTGGGATACCAAAATCAAAAGTCCAA+TTTCTAATIGGTAGAT+TGCCAA+TTCCTCCAGT
	Detection	AminoC12	CCIATICCTCCAGTYTGRAAHTCATTYCCATTCATRGTAAYTATCATTTGRTTAAAITGITGAATIA
Sapovirus	Second		AAGATATCGTAAGGATGGTACAGTACITGACCIAGIGGGTCICACTGAAGGIACCCICAAAWTAGTGTTTGAGATGGA
	First	AminoC12	TTGGATAAGTGGGATAGCAAAGCAGTTGCGTATTGCITCAGGGACATTKGATTGGATTGCACCIGTIGCAACAGCCA
	Detection	AminoC12	TCCAGGCGYTGIGCGGSCCCAITGGGTTGITCIGGATTAGCAACAACAACGTGGGATGTGGTCGGICC

Enterovirus	Second		AAGATATCGTAAGGATGGTGGGAAGAGGCTATTGAGCTACITGIGAGTCCTCCGGCCCCTGAATGCGGCTAATCC
	First	AminoC12	TTGGATAAGTGGGATACACCGGATGGCCAATCCAATIGCTITATGGTAACAATCTITGIATTGTCACCATAAGCAGCCA
	Detection	AminoC12	ACGGACACCCAAAGTAGTCGGTTCCGCYGCAGAITTGCICGTTACGACAIGCIACICACTGGTTTGTG
Astrovirus	Second		AAGATATCGTAAGGATGAATCICTCCATGGGIAICTCCTITGTTATCAGTTGC+TYK+CTG+CITTTATGG
	First	AminoC12	TTGGATAAGTGGGATACTAGCCATTGCITTTTTTTGGTCCTCCCCTCCAGATGGRATGGAGTTGCTCTTC
	Detection	AminoC12	CCAGAITCACGAAGCTGCTTIGCAGTCCKIGAIAGGCAGTGITCIAIGTA
	Seq_Fw_GG2		TAGATACAAGTACCACTATGATGC
	Seq_Rev_GG2		CAGAGAGTGAGAAGCCAGTG

Table 3. Sequences for synthetic targets for VOCMA on Luminex platform

Target	Origin	Sequence 5´to 3´ of synthetic target
Norovirus GG1	M87661	AAGACTGGTGGATTGGAAATGTATGTCCCAGGATGGCAGGCCATGTTCCGCTGGATGCGCTTCCATGACCTCGGATTGTGGACAGGAGATCGCGATCTTCTGCCCGAATTCGTA AATGATGATGGCGTCTAAGGACGCTACATCAAGCGTGGATGGCGCTAGTGGCGCTGG
Norovirus GG2	Consensus	AGATACAAGTACCACTATGATGCGGAGTACTCUCGGTGGGAGCAGAAGACCTTCTTUCTCCTAGCGTGGTGGATGTGGGTGACTUCACAATATCAATCAACGAGGGTCTGGGG TGCCCTGCACCTCCCAAUGGAACTCCATCGCCCACTGGCTTCTCACTCTCTGTGCGCTCTCTGA
Adenovirus	Ad 41	TTTGCCATGGAAATCAATTTGGCGGCCAATCTCTGGCGCAGCTTCTTATACTCCAATGTAGCTTTGTACTTGCCTGACTCATACAAGATTACGCCAGACAACATTACACTGCCCGA AAACAAGAACACCTATGCCTACATGAACGGTCGGGTGGCGGTTCCTAGCGCCCTCGATACCTACGTAAACAT
Rotavirus A	K02086	TACTCACTGTCAAAAACTCTTAAAGATGCTAGGGACAAAATTGTTGAAGGTACATTATATTCTAATGTTAGCGATCTTATTCAGCAATTCAATCAAATGATAGTAACTATGAATGG AAATGATTTTCAGACTGGAGGAATTGGTAATTTACCTGTTAGAAATTGGACTTTCGATTTTGG
Sapovirus	NC_006269	GGTACAGTACCTGACCCAGTGGGTCACACTGAAGGAACCCACAAAATAGTGTTTGAGATGGAGAGGTCCGACCACATCCCACGTTGTTGTTGCTAATCCGGAGCAACCCAATGG GGCCGCACAGCGCCTGGATTGGCTGTTGCCACTGGTGCAATCCAATCCAATGTCCCTGAGGCAATACGCAACTGCTTTGC
Enterovirus	Poliovirus NC_002058	GGTGTGAAGAGCCTATTGAGCTACATAAGAATCCTCCGGCCCCTGAATGCGGCTAATCCCACAAACCAGTGATTGGCCTGTCGTAACGCGCAAGTCCGTGGCGGAACCGACTAC TTTGGGTGTCCGTTGGCTGCTTATGGTGACAATCACAGATTGTTATCATAAAGCGAATTGGATTGGCCATCCGGTG
Astrovirus	FJ755402	GAATCACTCCATGGGGAGCTCCTATGCTATCAGTTGCTTGCTGCGTTCATGGCAGAAGATCACCCTTTTAAGGTGTATGTAGAACACTGCCTATCACGGACTGCGAAGCAGCTTC GTGACTCTGGCCTTCCGGCCAGACTCACAGAAGAGCAACTCCATCGCATTTGGAGGGGAGGACCAAAGAAGTGTGATGGCTAG

2.3. Detection using (RT)-qPCR

The design of oligo primer–probes used in the qPCR was performed as described above. The sequences of primers and probes used in Real-Time PCR were shown in table 4 and synthetic targets were in table 5. In order to maintain the variation tolerance, the specific probes in VOCMA are often in the range of 50-70 nucleotides (nt), which makes them unsuitable as conventional TaqMan® end-labelled probes for real-time PCR. Therefore, using norovirus genogrup II (GII) as template, we first compared and evaluated the probes with different labelling methods or with variation of quenchers (Xia et al., 2016). Afterwards, all the probes except probe for HEV had one Zen internal quencher at position 9nt from the 5´ end as internal quencher and one conventional quencher at 3´ end.

In order to test whether the primers and probes can tolerate variation of the target sequences, Variation of Noro GII target sequences, which had different hybridization efficiencies with variation of NucZip scores from 596 to 528) or nearest neighbour scores from -71 to -19 kcal/mol, were synthesized and used in Noro GII real-time PCR assay.

Several real-time PCR assays were developed: 1) Singleplex assay for detection of norovirus GGII; 2) Duplex assays for detection of hepatitis A virus (HAV) and hepatitis E virus (HEV); 3) A real-time PCR panel for simultaneous detection of norovirus GI/norovirus GII in one reaction, and rotavirus/sapovirus in another reaction. For all the assays, the synthetic targets were used for optimization of the real-PCR condition and tested for sensitivity of the assays. A 10-fold serial dilution of 10^6 to 10^0 copies of the synthetic targets was used in the sensitivity test.

In the real-time PCR assay for detection of norovirus GGII, a total of 86 clinical patient samples were used when evaluating various probes of norovirus GII. Of these samples, 46 samples were norovirus genogroup II, 23 samples were genogroup I and 20 samples tested negative for genogroup I and II, as determined by routine diagnostics at the Clinical Microbiology Laboratory of Uppsala University Hospital based on a specific norovirus genogroup I and II real-time PCR assay {Hohne, 2004 #10}. The samples were sequenced if they did not agree with the result from routine diagnostic lab. For the duplex HAV/HEV assay, a total of 32 samples were tested. Among those samples, 16 samples were HAV positive by ELISA and the remaining 16 samples were tested by real-time PCR at the Swedish Veterinary Institute. In the real-time PCR panel for norovirus GG I, norovirus GG II, rotavirus and sapovirus, 126 samples were included. Among those samples, 33 samples were norovirus GG I positive ; 46 samples were norovirus GG II positive, 27 samples were sapovirus positive and 20 samples were rotavirus positive.

The real-time PCR was performed on a CFX 96 touch real-time PCR detection system (Bio-Rad) using Path-ID™ Multiplex One-Step RT-PCR Kit. The concentrations of the genotype II specific primer, generic primers and probes were 40 nM, 300 nM and 100 nM, respectively, if not mentioned specifically. The concentrations of the specific primers for HEV and norovirus GGI were 20nM and 80nM, respectively. Real-time PCR conditions were: 45°C 10 min, 95°C 10 min followed by 15-20 cycles of 95°C 15 sec, 61°C 45 sec and 40 cycles of 95°C 15 sec, 52°C 45 sec.

Table 4. Primers and Probes used for Real-Time PCR assays

Primers/Probes	Sequences (5’ to 3’)
Generic first	TTGGATAAGTGGGATA
Generic second	AAGATATCGTAAGGAT
NoroGG1_3_fw_s	AAGATATCGTAAGGATAAGACAGGGGGICTTGAAATGTATGTICCAGGITGGCAGGCCATGTTCCGITGGATGCG
NoroGGI R	TTGGATAAGTGGGATACCGGCGCCAITIGCGCCATCIRIGITTGGIGTIGCGTCCTTAGACGC
NoroGGIprobe	5/HEX/TCATCATTT/zen/ACRIAT+TCGGGCAGVAGATTGCGATCTCCTGTC/3IABKFQ/

Noro GII NV Fw	AAGATATCGTAAGGATAATYAGYAARYTRGTYATTGCAGAGYTDAARGARGGTGGYATGGATTTTTAC
Noro GII NVRev	TTGGATAAGTGGGATAAYTCGACGCCATCTTCATTCACAAARCTGGGAGCCAGATTGCGATCGCCCTC
Noro GII NVprobe1	FAM/CCACGTGCTCAGRTCYGAGA/iBHQ1/ATCTCATCCAYCTRAACATYGGCTCTTGYCTGGGCAC/3Spacer C3
Noro GII NVprobe2	FAM/CCACGTGCTCAGRTCYGAGAA[T(BHQ-1)]CTCATCCAYCTRAACATYGGCTCTTGYCTGGGCAC/spacer C3
Noro GII NVprobe3	FAM/CCACGTGCT/zen/CAGRTCYGAGAATCTCATCCAYCTRAACATYGGCTCTTGYCTGGGCAC/3lowa black RQ-Sp
Sapo F	AAGATATCGTAAGGATCGTTGACCCGCCTGGCACAACAGGTCCGACCACAIICCACGTTGTTGTTGCTA
Sapo R	TTGGATAAGTGGGATAGGCATCCTGTCGTTCCAAGCAAAAGTACGAAAGACTGCAAA
Sapoprobe	5/5Cy5/ATCCGGAGC/zen/AACCCAATGGGGCCGCACAGCGCCTGGAGTTGG/3IAbRQSp
Rota_FW_16gs_s	AAGATATCGTAAGGATTATTCITTGTCAAAAACTCTTAAAGATGCTAGGG+AIA+ARATTGTYG+AAGG
Rota_Rev_16gs_as	TTGGATAAGTGGGATACCAAAATCAAAAGTCCAA+TTTCTAATIGGTAGAT+TGCCAA+TTCCTCCAGT
Rota probe	5/RHO101N/TGA+AARTCA/ilB-QB/TTYCCRT+TCATRGTHACTATC/ilB-QB/ATTTGATTRAATTGRTGAAT/3 AbRQSp
Noro GII NVprobe4	FAM/CCACGTGCT/zen/CAGRTCYGAGAATCTCATCC/zen/AYCTRAACATYGGCTCTTGYCTGGGCAC/3lowa black RQ-Sp
HAVF	AAGATATCGTAAGGATAGTCTAAATTGGGGACRCAGATGTTKGRRACGTCRCCTTGCAGTGTTAAC
HAVR	TTGGATAAGTGGGATATCACCAATATCCGCCGCTGTTACCCTATCCAARGCATCTCTTCATAG
HAVprobe	5/TET/CATGAABCT/ZEN/CTTTGATCTTCCACAAGRGGTAGGCTACGGGTGAAACCYCTTARGCTARTAC/3/lowa BlackFQ
HEVF	AAGATATCGTAAGGATCGTGGT+TTC+TGGGGTGAC
HEVR	TTGGATAAGTGGGATAAGGGGT+TGGT+TGGAT+GAA
HEVprobe	5/FAM/TGAT+TCTCAGCCCT+TCGC\BHQ1

Table 5. Synthetic targets used for Real-Time PCR assays

Synthetic targets	Sequences (5’ to 3’)
Norovirus GGI	aagactggtggattggaaatgtatgtcccaggatggcaggccatgttccgctggatgcgcttccatgacctcggattgtggacaggagatcgcgatcttctgcccgaattcgtaaatgatgatggcgtctaaggacgctacatcaagcgtggatggcgctagtggcgctgg
Norovirus GGII	AATTAGCAAGCTAGTCATTGCAGAACTGAAGGAAGGTGGCATGGATTTTTACGTGCCCAGACAAGAGCCAATGTTCAGATGGATGAGATTCTCAGATCTGAGCACGTGGGAGGGCGATCGCAATCTGGCTCCCAGCTTTGTGAATGAAGATGGCGTCGAAT
Sapo	CGTTGACCCGCCTGGCACAACAGGTCCGACCACATCCCACGTTGTTGTTGCTAATCCGGAGCAACCCAATGGGGCCGCACAGCGCCTGGAGTTGGCTGTTGCCACTGGTGCAATCCAATCCAATGTCCCTGAGGCAATACGCAACTGCTTTGCAGTCTTTCGTACTTTTGCTTGGAACGACAGGATGCC
Rota	TACTCACTGTCAAAAACTCTTAAAGATGCTAGGGACAAAATTGTTGAAGGTACATTATATTCTAATGTTAGCGATCTTATTCAGCAATTCAATCAAATGATAGTAACTATGAATGGAAATGATTTTCAGACTGGAGGAATTGGTAATTTACCTGTTAGAAATTGGACTTTCGATTTTGG
HAV	AGTCTAAATTGGGGACACAGATGTTTGGAACGTCACCTTGCAGTGTTAACTTGGCTTTCATGAATCTCTTTGATCTTCCACAAGGGGTAGGCTACGGGTGAAACCTCTTAGGCTAATACTTCTATGAAGAGATGCCTTGGATAGGGTAACAGCGGCGGATATTGGTGA
HEV	GCGGTGGTTTCTGGGGTGACCGGGTTGATTCTCAGCCCTTCGCMMTCCCCTATATTCATCCAACCAACCCCTTCGCCCCCGATGTCACCGCTGCGGCCGGGGCTGGACCTCGTGTTCGCCAACCCGCCCGACCACTCGGCTCCGCTTGGCGTGACCAGGCCCAGCG

3. Results

3.1. Detection on Luminex

The sensitivity and specificity of the assay was determined using synthetic target. The VOCMA assay on Luminex format showed high specificity with no cross-hybridization (figure 1).

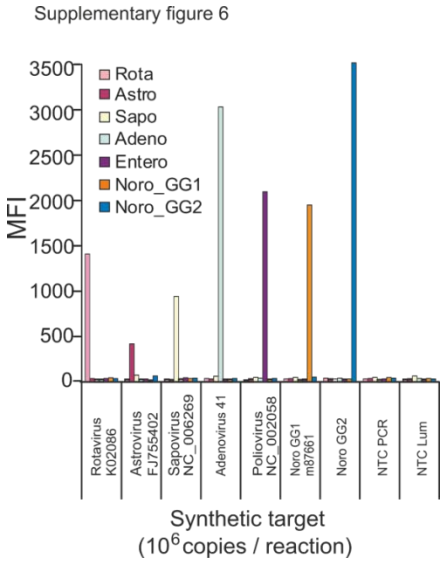


Figure 1. A 7-plex VOCMA detecting different synthetic ssDNA targets (10^6 copies / reaction). The synthetic targets (Table 3) have different degrees of mismatch towards the primer-probes and the detecting probes. The panel of seven detection probes is indicated by the different colours in the figure. The X-axis displays the different targets, with seven differently coloured bars for the indicated detection probes. The Y-axis displays the Median Fluorescent Intensity, MFI. Signals are specific, with a very low background.

The analytic sensitivity of the assay for rotavirus and adenovirus is 1-10 copies/reaction, 100 copies/reaction for astrovirus and poliovirus, 100-1000 copies/reaction for norovirus genogroup II, and 10000 copies/reaction for norovirus genogroup I and sapovirus (Figure 2).

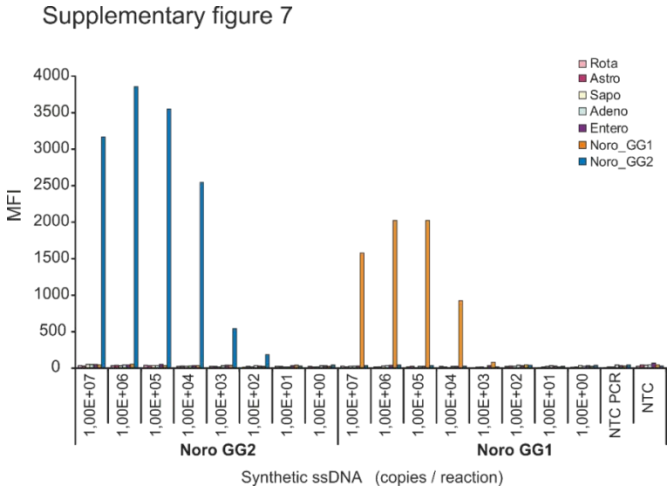


Figure 2. A 7-plex VOCMA detecting synthetic ssDNA targets of norovirus genotype 1 (orange bars) and genotype 2 (dark blue bars) in titres ranging from 10^7 to 10^0 copies per sample. The panel of the seven detection probes is indicated by the different colours in the figure. The X-axis displays the different targets, with seven differently coloured bars for the indicated detection probes. The Y-axis displays the Median fluorescent intensity, MFI.

3.2. Detection with real-time PCR

3.2.1 Development of single-tube nested real-time PCR assays with long internally quenched probes for detection of norovirus GG II

The sensitivity of the assays was tested using a dilution series of 10^6 to 10^0 copies of synthetic oligonucleotide. The Ct values from the second round of real-time PCR assays were used to plot standard curves (Fig.3). A linear relationship in the range 10 - 1 000,000 copies/reaction for the standard curves was observed when Noprobe3 and Noprobe4 were used in real-time PCR, with a detection limit of 10 copies/reaction. However, signals from 1-3 of six parallel wells containing a single copy were also seen. The detection limit for Noprobe1 was 1000 copies/reaction, while the detection limit for Noprobe2 was 100 copies / reaction.

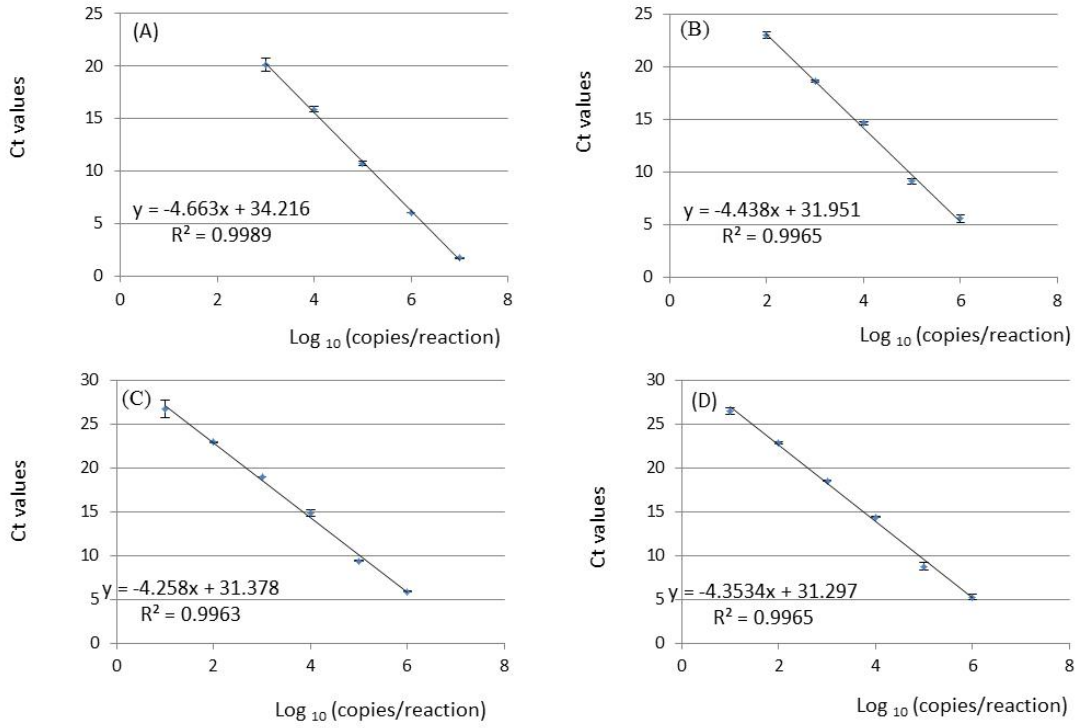


Figure 3 Standard curves for the norovirus GII real-time PCR using 10-fold serial dilutions of synthetic target. A. Noprobe1, B. Noprobe2, C. Noprobe3, and D. Noprobe4. The assays were performed in duplicate and the figure was based on the averages. Only the cycle numbers in the second of PCR were counted in Ct values.

The specificity of the real-time PCR assays was evaluated by testing 46 norovirus GG II samples, 23 norovirus GG I samples and 20 samples testing negative for both norovirus GG I and GG II. In all assays, no cross-reactivity was observed for RNA from the 23 norovirus GG I samples and RNA from the 20 norovirus GG I and II negative samples. Among the 46 norovirus GG II samples, 44 samples were determined as norovirus GG II positive by all assays. The PCR products of the two remaining samples, which were false negative compared with the routine PCR assays, were run in agarose gel electrophoresis. A clear band with the expected size could be seen on the agarose gel (results not shown). This indicated that there might be a mismatch between target and probe. To clarify the sequence variation within the probe region, the PCR products of these two samples were cloned into a pCR™4-TOPO vector, followed by sequencing. A long stretch of sequence deletion within the probe region was observed for both samples (results not shown). Sequencing results for sample 6363

indicated that a mixture of at least two virus variants was present, with deletions of 51 and 39 nucleotides, respectively. We thus obtained an explanation for the absence of signal in these two samples: the probe could not bind to the target region due to a long stretch of sequence deletion in the samples.

3.2.2 Development of single-tube nested real-time PCR duplex assays for detection of HAV and HEV

Using a dilution series of 10^6 to 10^0 copies of synthetic oligonucleotide as template, the sensitivity of the assay was tested. The Ct values from the second round of real-time PCR assays were used to plot standard curves (Fig.4). In a duplex assay, both HAV and HEV showed linear relationship in the range 10 - 1 00,000 copies/reaction, with a detection limit of less than 10 copies/reaction. When it came to clinical samples, the established duplex real-PCR could detect 13 HAV samples as positive among 16 positive samples. And only half of the samples among 16 HEV positive samples were detected by the assay.

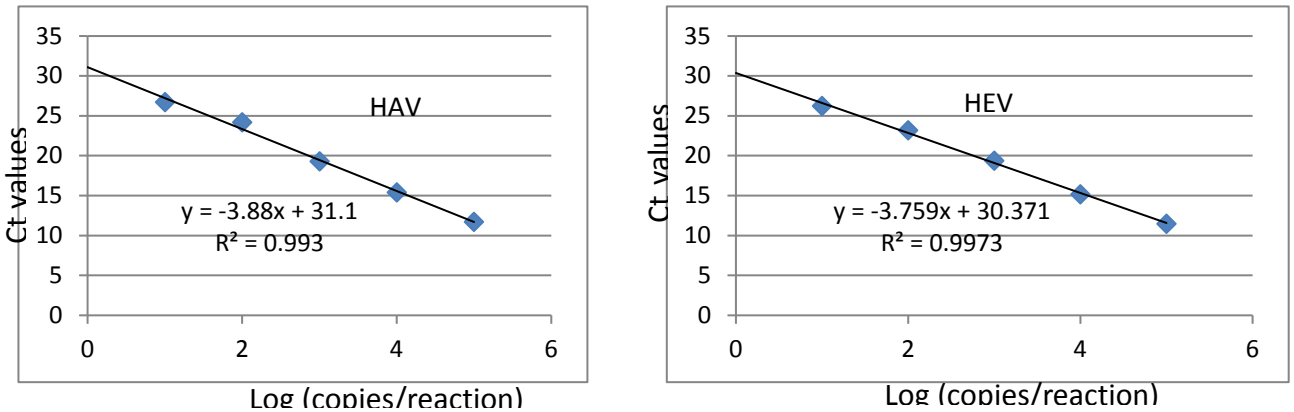


Figure 4. Standard curves of duplex real-time PCR assay for detection of HAV and HEV. The assay was performed in duplicate and the figure was based on the averages. Only the cycle numbers in the second of PCR were counted in Ct values.

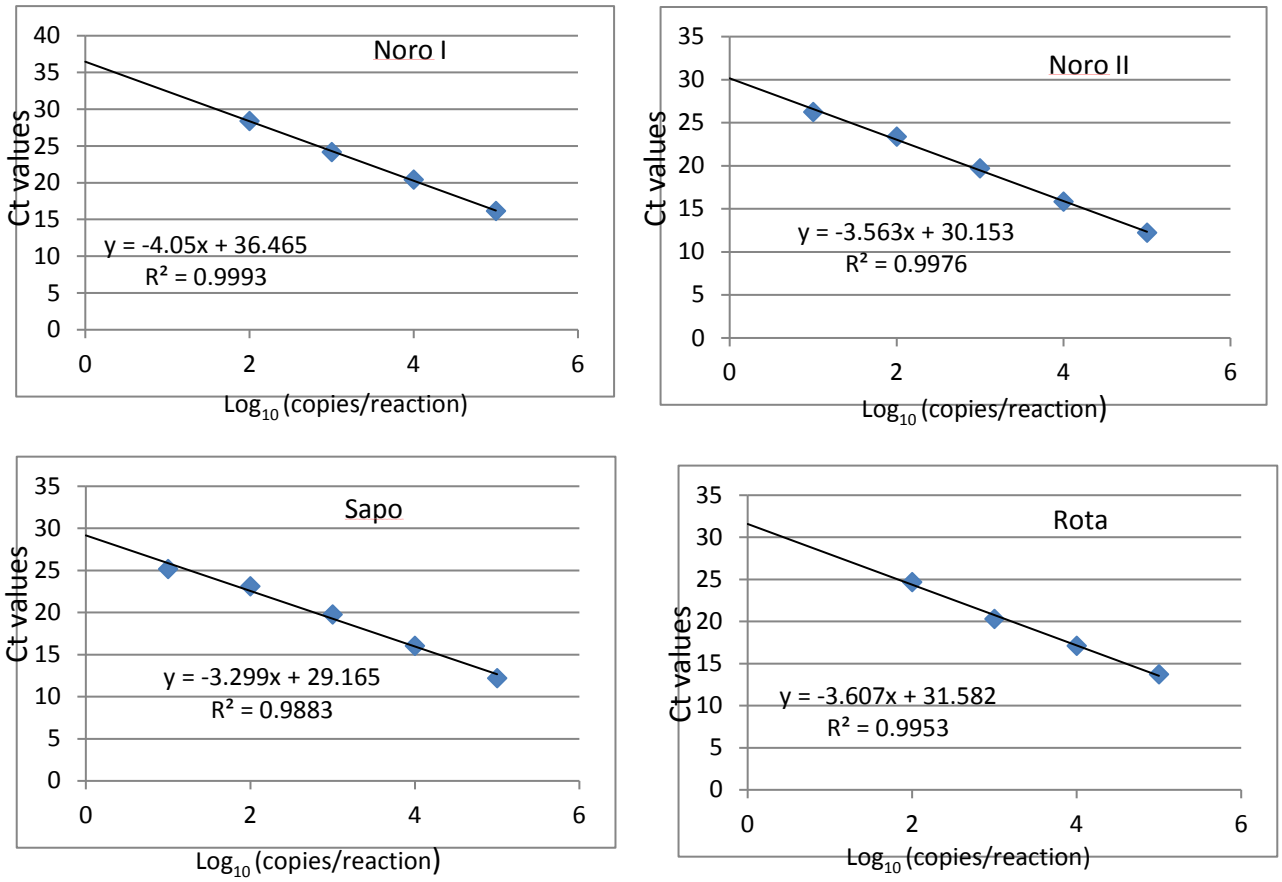


Figure 5. Standard curves of real-time PCR panel for detection of Noro I, Noro II, Sapovirus and Rotavirus. The assay was performed in duplicate and the figure was based on the averages. Only the cycle numbers in the second of PCR were counted in Ct values.

3.2.3. Development of a real-time PCR panel for detection of gastro-enteric viruses

Both individual real-time PCR assays and a fourplex assay were optimized for detection of norovirus GGI and GGII, rotavirus and sapovirus. Using synthetic oligonucleotides (oligos) as templates, the sensitivity of the assays were determined. In monoplex assays, the analytical sensitivities of the real-time PCR assays for all the viruses are less than 10 copies per reaction. In the fourplex assay, the analytical sensitivities of viruses were reduced 100-1000 times with the exception of Noro GGII (results not shown). With the goal of high sensitivity and multiplex capacity, we developed a real-time PCR panel: two duplex assays (Noro GGI and GGII in one well, Rota and Sapo in the other well) with same real-time PCR condition in a single run. The panel consisting of the duplex assays did not compromise the detection limits of the Noro GGII and Sapo comparing with monoplex assays (Figure 5). The analytical sensitivities of Rota and Noro GI were at 100 copies per reaction. Considering the high number of those two viruses in the clinical samples, the detection limit of 100 copies was judged acceptable.

When it came to clinical samples, the panel could detect norovirus GGII and rotavirus with diagostic sensitivity of 95% and 80%, respectively. However, the panel showed limitations for detection of norovirus GGI and sapovirus. Among the tested 33 norovirus GGI positive samples, 16 samples were detected as positive by our panel and among the 27 sapovirus positive samples, we could only detect 8 samples. These VOCMA components need further optimization.

3.2.4 Sequence variation toleration

Using Noro II, which has high sequence variation, as a model, we tested whether the developed assay could tolorate sequence variation. According to the predicted hybridization efficiency between primers, probes and targets (Figure 6), seven different Noro II sequences with different hybridization scores were synthesized. The result showed that the duplex assay detection of Noro I and noro II could detect all the variation of the synthetic Noro II targets. The CT values correlated well with the NucZip scores and Nearest Neighbor scores(Figure 7). In this limited test, NucZip scores had a higher correlation with Ct value than the Nearest Neighbor score. It accurately predicted hybridization even for the most deviating target sequences (shown as "..virus genomic RNA" in the figure.

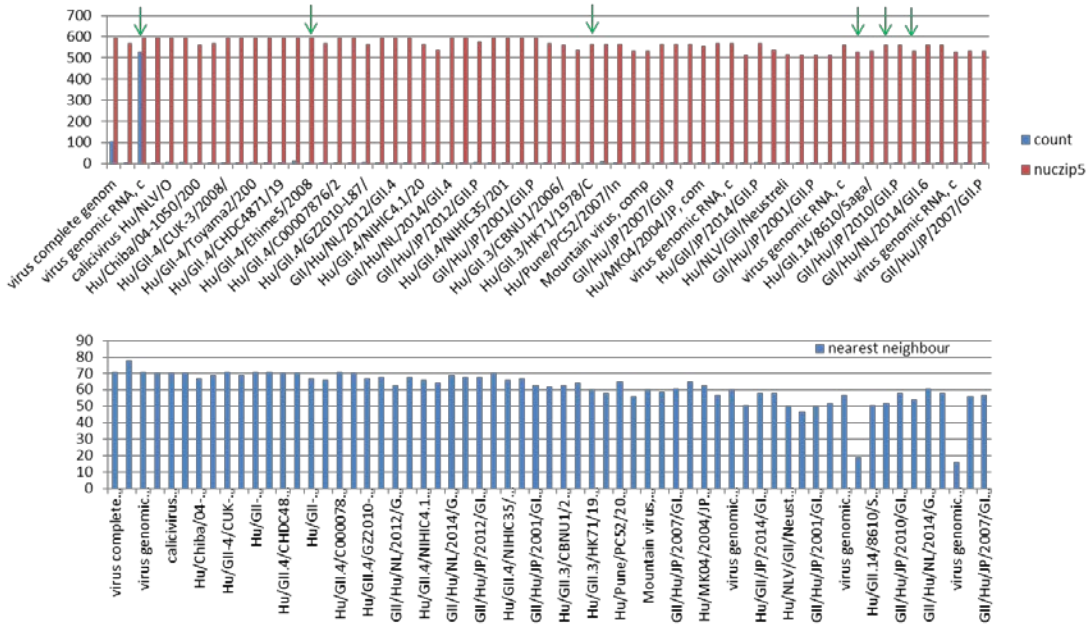


Figure 6 Predicted hybridization of probes with variant of Noro GII according to NucZip (red, score) and Nearest Neighbor algorithms (blue, -kcal/mol), both with 5´preference scoring. The green arrows indicate targets which were synthetized and PCR tested.

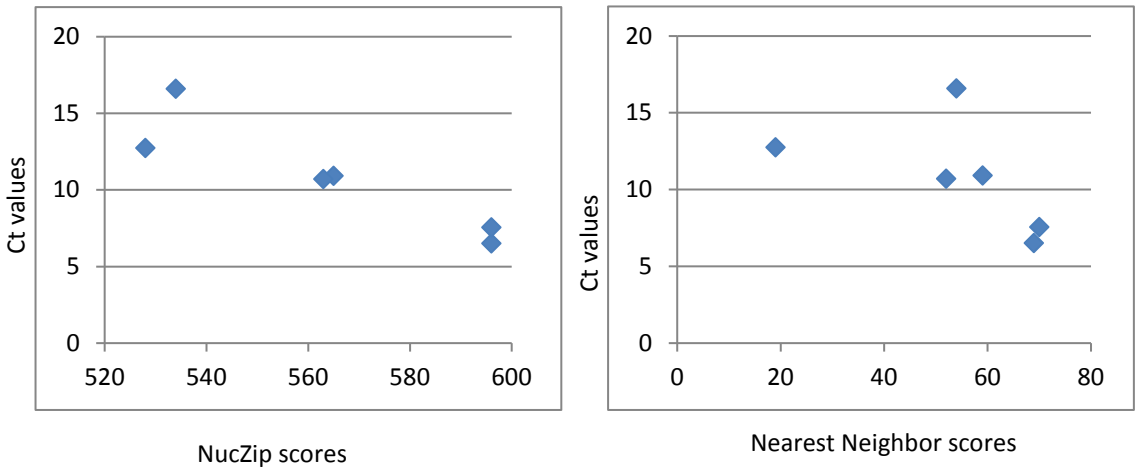


Figure 7. The relationship between Cq values by various Noro GII targets and the NucZip scores and Nearest neighbour scores. Target concentration: 100,000 copies/reaction.

3.2.5. Possibilities for real-time PCR panels for other targets

In our original VOCMA, 22-plex for diagnosis of sepsis and 7-plex for diagnosis of gastroenteritis were developed. As the gastroenteritic pathogens cause the most important water transmitted diseases, adaption of gastro VOCMA panel directly to VOCMA real-time PCR assays became a priority. In the original VOCMA, probes were used in post-PCR reaction, and were not tested in silico using ConSort. In the real-time PCR assays, the probes had to be re-evaluated and redesigned. However, in most cases, we found that the probes in original VOCMA could be easily adapted to VOCMA real-time PCR. Based on the result s from the gastro VOCMA real-time PCR panel, we tried to use the designs from the original VOCMA 22-plex format in the VOCMA real-time PCR panel with the exception of the probes which had to be re-evaluated *in Silico*. This opens the possibility to include antibiotic resistance genes, which were included in original VOCMA panel to the suggested bacterial and parasite panels suggested in Table 6.

Table 6. Primers and probes developed for the different panels for detection of waterborne pathogens and indicators

Panel	Agent	Primers/ probe	Nucleotide sequences (5' – 3')*
Bacteria	*Campylobacter*	Forward	GTAATGCATGCTTGCGGTCATGATGGACATACTACTTCTTTATTGCTTGCT
	jejuni	Reverse	CTATCCGAAGAAGCCATCATCGCACCTTTTTTAAGATAAAATTTCTTATC
		Probe	TTTGCCTTTTCTGGAGCACTTCCATGACCACCTCTTCCAATAACTTCAAT
	Salmonella	Forward	TAGGCAATATCGGTCATACTACGTAACGCCATTAACAATTCTTCCTGCCA
	enterica	Reverse	TCGCTATTGCAACAGCCACATGAAGTGGATTCGCTGTTAGCCGATATTAT
		Probe	ATATTTTATCAAGTATGTAAAGCCATACCCTCTTCACTCTTTTATGAAGA
	E. coli	Forward	Tgcctggtcattcgcctgtaaaaccgccaatggtaccgctatccctattg
		Reverse	Acacgccgccataagccgagcctcgttgcagtgtgacatagtctgtaatg
		Probe	Ggtggcagcgctaatgtttatgtaaaccttgcgcctgccgtgaatgtggg
	Pseudomonas aeruginosa	Forward	Tggcgagatttgcccatagagccatcacttatgactgcgacagacagaac
		Reverse	Gcgcagggagagcttgctgccgaccaacgcgccgttcctcgcatagatg
		Probe	Cattgaaatggctctgcctcggcaaccgtgatgcgaacgacggattcgag
Parasites	*Giardia*	Forward	ACATCGCGCTCTGGAAGTTCGAGACGAAGAAGTACATCGTCACGATCATC
	intestinalis	Reverse	TTGACCTGGCCGTCGTCCATCTTGTTGAYGCAGATGATCATCGTCTTGAT
		Probe	GCGACGTCIGCCTGGGACGTSCCCGTGATCATGTTCTTGATGAAGTCGCG
	Cryptosporidium Parvum/hominis	Forward	GTAATGCATGCTTGCGGTCATGATGGACATACTACTTCTTTATTGCTTGCT
		Reverse	TTTGCCTTTTCTGGAGCACTTCCATGACCACCTCTTCCAATAACTTCAAT
		Probe	CTATCCGAAGAAGCCATCATCGCACCTTTTTTAAGATAAAATTTCTTATC

* The forward primer starts with the generic second sequence and the reverse primers with the generic first ditto. The probes include internal quenchers; however, which and the positioning of these will be kept confidential until publication. I, R, T, M and Y stands for degenerations and dInosines at sites of target variation; + follows a locked nucleic acid (LNA) residue (to increase affinity) (Ohrmalm*, et al.*, 2010, Ohrmalm*, et al.*, 2012).

4. Discussion

As explained above the design of VOCMA primer-probes maximises hybridisation between primers and probe versus target, in the presence of substantial target variation, leading to variation tolerance. This fact also enables this PCR system to address more variable regions of the genomes and, hence, target genes involved in pathogenicity with minimised risk of false negative results. Further, due to the low concentration of primer-probes VOCMA can be multiplexed to a greater extent than other PCR systems, because unwanted primer interactions are minimized. Another advantage, not discussed above, is that the products generated are typically longer than for many other PCR systems. The products may therefore have enough information potentially of use for typing during outbreak situations without having to reanalyse your sample using another primer pair.

The original VOCMA was designed for detection using Luminex. This is advantageous because it provides a highly multiplex potential. One problem with this system, however, is the potential for contamination to occur when the PCR tubes were opened before the hybridization step. Due to the use of generic primers in the final amplification, such a contamination could ruin all VOCMA components which used the same generic primers. Despite this threat, it has not happened during two years of developmental work. The sensitivity could also be questioned, even though for some targets down to ten copies could be detected, for others almost a thousand copies in the original sample are needed. For these reasons, VOCMA has been developed to a real time PCR platform within Aquavalens.

VOCMA in the real time-PCR format proved to be highly sensitive, and still multi-plexable. The low concentration of the primary (virus specific) primers minimizes primer-primer interactions, a major cause of primer-dimers which diminishes sensitivity. According to our results, VOCMA in the real time format is also quantitative. Another novelty is that the VOCMA primers now can be biotinylated. This was not possible before, because biotin was used later in the hybridisation procedure. With biotinylated primers, the capture process can be made more efficient, letting biotinylated primers catch the target nucleic acids, then capturing them on streptavidin-coated magnetic beads. Capture efficiencies of >50% are feasible as an alternative secondary concentration procedure that also is efficient in removing inhibiting substances that do not bind as efficiently to magnetic beads as they do to silica.

5. Implications for the future

We have established several VOCMA assays for detection of gastro-enteric viruses, hepatitis A and hepatitis E virus. The assays had a high sensitivity, with detection limits of 10-100 copies/reaction. The developed assays can detect clinical samples containing norovirus GGII and rotavirus with high diagnostic sensitivities. Assays for Norovirus GGI and Sapoviruses still need to be optimized. Some of the assays have a potential to be commercialized as a ready-kit for rapid detection of water-borne viruses. Assays for detection of environmentally spread antibiotic resistance genes, food and waterborne bacteria and parasites are also possible, but needs to go through as thorough optimisation and validation processes as the developed viral panels. The potential for secondary concentration using magnetic beads is another advantage that can improve the diagnostic sensitivity in environmental samples containing inhibitors.

6. Implications of the results of Deliverable Report 7.5.

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 a complement to task 7.1 “Standardised molecular detection”, since the concept of VOCMA enables higher multiplexability compared to other PCR-methods. However, in order to deal with environmental samples further improvements needs to be done. The method is more likely to be used within clinical rather than environmental microbiology.

Implications of the results for this Cluster

This task didn’t have any dependencies within the cluster. The achievement with biotinylated primers coupled to magnetic beads for secondary concentration and inhibitor reduction could have been part of WP6 protocols (However, a bit late for that now).

Implications of the results for the whole project

Since the method didn’t reach the technology readiness level, *i.e.* ready to use kits, needed for inclusion in field sampling, VOCMA was not chosen from the portfolio. The method is more likely to be used within clinical rather than environmental microbiology. Hence, the results are of minor importance for the future of AQUAVALENS.

Indicate key external stakeholders interested in the results

The main end-users of the results are biotech companies interested to further develop the concept for commercialisation, for example as ready to use kits. **However, this can only be done after solving issues around Intellectual property.**

Which internal partners should your deliverable be sent to?
UEA, UB, HZI, GPS, CEERAM, HWU, DTU, NFA, DVGW

7. Acknowledgement

The expert advice and assistance from Christina Öhrmalm is gratefully acknowledged.

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