ORIGINAL ARTICLE

Amplification of viral RNA from drinking water using TransPlex™ whole-transcriptome amplification

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Abstract

Aims: Viral pathogens in environmental media are generally highly diffuse, yet small quantities of pathogens may pose a health risk. This study evaluates the ability of TransPlex™ whole transcriptome amplification (WTA) to amplify small quantities of RNA viruses from complex environmental matrices containing background nucleic acids.

Methods and Results: DNA extracts from mock drinking water samples containing mixed microbial populations were spiked with small quantities of echovirus type 13 (EV) RNA. Samples were amplified using a Transplex™ WTA kit, and EV-specific quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to quantify target pathogens before and after application of WTA. Samples amplified by WTA demonstrated a decreased limit of detection. The log-linear relationship between serial dilutions was maintained following amplification by WTA.

Conclusions: WTA is able to increase the quantity of target organism RNA in mixed populations, while maintaining log linearity of amplification across different target concentrations.

Significance and Impact of the Study: WTA may serve as an effective preamplification step to increase the levels of RNA prior to detection by other molecular methods such as PCR, microarrays and sequencing.

Introduction

Whole-transcriptome amplification (WTA) is an emerging technology designed to representatively amplify entire transcriptomes from limited quantities of starting RNA. WTA increases the total amount of RNA so that greater quantities of nucleic acids are available for downstream applications. This may be particularly useful for technologies limited by insufficient quantities of quality target RNA, such as microarray-based gene expression studies and RT-PCR. Several WTA methods (both PCR-based amplification and non-PCR-based linear amplification methods) have been described (Lauss *et al.* 2007) including *in vitro* transcription (IVT)-based amplification (Van Gelder *et al.* 1990), multiple displacement amplification (MDA) and universally primed PCR. Recently, a novel approach to WTA was developed which avoids primer and template biases associated with PCR and alleviates the need for a poly-A tail (which is absent in bacterial mRNA). The TransPlex™ WTA kit (Sigma-Aldrich Co., St Louis, MO, USA) relies on an initial round of MDA, followed by PCR-based amplification using universal primers to create WTA product. In TransPlex™ WTA, RNA is first incubated with reverse transcriptase and nonself-complementary primers with a quasi-random 3′ end and a universal 5′ end. As the annealed primers extend, they displace single strands that serve as new template. The resulting collection of random, overlapping fragments, each containing a universal end sequence, is then amplified using universal primers to produce WTA product. TransPlex™ WTA may be superior to IVT because it does not require the presence of a poly-A tail, which would necessitate oligo(dT) priming to generate cDNA, leading to 3′ bias (Tomlins *et al.* 2006). It may also be more successful in amplifying small quantities of RNA because of the combination of techniques.

To date, application of WTA to detection or characterization of microbial RNA has been limited because of the relative novelty of WTA technologies and difficulty of adapting existing methods to microbial RNA. IVT was successfully adapted to artificial and environmental mixed bacterial community samples by attaching random hexamers to the T7 RNA promoter sequence in lieu of oligo(dT) (Gao et al. 2007). However, microarray analysis of WTA product from these mixed bacterial communities showed representative detection was dependent upon the amount of starting RNA. In another study, Rift Valley fever virus from brain biopsies was successfully amplified by WTA in the presence of *Staphylococcus aureus* DNA (Berthet et al. 2008), but starting amounts of RNA were high, ranging from 10^4 to 10^6 copies. More recently, TransPlex^{TM} WTA has been used on cultures of coronavirus to generate sufficient nucleic acid for sequencing new human viruses when propagation in cell culture is inefficient (Banach et al. 2009).

A potential new application of WTA is to improve detection of RNA viruses in environmental media where they may pose a risk to human health. RNA viruses may occur in very small concentrations in drinking water and other environmental media, making them difficult to detect with molecular methods. Additionally, there are numerous types of RNA viruses (e.g. enteroviruses, noroviruses, astroviruses) that may be present in environmental media, further complicating detection because they do not share a common gene, analogous to the conserved 16S gene in bacteria, which may be targeted for detection by a singleplex or easily multiplexed PCR. To determine whether WTA can improve molecular detection by amplifying viral RNA from environmental media, this study assessed the ability of WTA to maintain log-linear amplification over a range of initial RNA concentrations, to efficiently amplify RNA from environmental matrices and to overcome inhibition by substances in these matrices.

Echovirus 13 was chosen as a representative RNA virus for this study, based on the listing of echoviruses on the EPA's Drinking Water Candidate Contaminant List 2 (CCL2) (USEPA 2005). Although echovirus is not often associated with known outbreaks, it is a putative agent of waterborne disease (Leclerc et al. 2002). One concern with the application of WTA to low numbers of pathogens is the requirement for a specific amount of starting RNA, as quantities suggested for WTA are higher than the amount of target RNA which would be anticipated in a typical environmental sample. This study is one of the first to assess the ability of WTA to improve the detection of environmentally relevant quantities of human pathogens in environmental media and to test TransPlex^{TM} WTA on environmental pathogens.

Materials and methods

Echovirus

Echovirus 13 strain Del Carmen (ATCC # VR-43) (EV) was produced in buffalo green monkey kidney (BGMK) cells grown in minimum essential medium (MEM) with 10% foetal bovine serum. When CPE was complete (c. 5 days), infected cells were freeze–thawed once at −80°C, virus was purified with a chloroform extraction, and the EV viral stock was stored at −80°C. Titre of viral lysate was assayed in 24-well plates of confluent cells. Wells were scored as positive or negative for the evidence of CPE, and titre was calculated (Reed and Muench 1938) in units of tissue culture infectious dose (TCID$_{50}$). RNA extractions for EV were performed on sample volumes of 140 μl using the QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA, USA) and eluted into final volumes of 60 μl.

WTA reactions

WTA was conducted using the TransPlex^{TM} Whole Transcriptome Amplification kit (Sigma-Aldrich), following the specified protocol. Sample volumes of 10 μl were incubated with reverse transcriptase and primers in a strand displacement reaction to create the TransPlex^{TM} cDNA library of target fragments with a universal end sequence. The library was then amplified with universal primers for 25 cycles. Incubations and amplifications for WTA were performed on a DNA Engine Dyad Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA).

To determine the optimal amplification cycle number for WTA, initial runs were monitored with SYBR Green qRT-PCR on a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories) to visualize the amplification plateau. An average optimal cycle number of 25 cycles was selected.

qRT-PCR

Previously described enterovirus primers and probe (Schwab et al. 1995) were used to target EV: Pan-E5′ (CCT CCG GCC CCT GAA TG), Pan-E3′ (ACC GGA TGG CCA ATC CAA) and fluorescently labelled TaqMan Pan-EP (6FAM - TAC TTT GGG TGT CCG TGT TTC - BHQ1). These primers target the conserved 5′untranslated region of enteroviruses. Initially, during optimization of WTA reactions, qRT-PCRs for EV were performed using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad Laboratories). SYBR Green–based reagents were later replaced with the iScript One-Step RT-PCR kit for Probes (Bio-Rad Laboratories) for use with target-specific

TaqMan probes, which were used for all final qRT-PCR results. All qRT-PCRs were conducted in 25 μl volumes containing 0·2 μmol l^{-1} primers and 0·15 μmol l^{-1} probe. Either 1 or 2 μl template RNA was added to each reaction. Cycling parameters were as follows: 50°C for 10 min, 95°C for 5 min, 40 cycles of 95°C for 10 s and 55 for 30 s. Each set of qRT-PCRs included an EV 4-point standard curve for quantification of sample concentrations and a no-template negative control (NC) reaction. Standards were quantified relative to EV tissue culture titres in units of TCID$_{50}$.

Experiments with mixed populations of micro-organisms

The background (BG) population of micro-organisms was composed of equal amounts of DNA extracted from cultures of *Escherichia coli* (EC) and *Bacillus subtilis* (BS). The surface water (SW) population of micro-organisms was composed of DNA extracted from SW collected from the north side of Portage Bay, Seattle, Washington off of NE Boat Street. Both BG and SW populations also included small seeded quantities of the pathogens Adenovirus 41 (AdV), *Aeromonas hydrophila* (AH) and *Mycobacterium avium* (MAC) (3·6 ng ml^{-1} total pathogen DNA in 'EV A' sample described below, data not shown).

DNA extractions were performed using the PowerSoil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). For the BG population, 1 ml aliquots of overnight EC and BS cultures was centrifuged for 10 min at 9000 *g*. The pellet was resuspended in 100 μl PBS and extracted. For the SW population, 2 l of water was filtered through replicate 47-mm-glass fibre prefilters, Type APFB, 1 μm pore size (Millipore, Billerica, MA, USA), and frozen at −80°C in sterile microtubes. Frozen SW filters were torn

into pieces using sterile forceps and divided to yield two extractions per filter.

The Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA) was used to quantify dsDNA in the extracted samples using the NanoDrop ND-3300 Fluorospectrometer (NanoDrop Technologies, Wilmington, DE, USA). Total EV RNA concentrations were too small to be quantified by Nanodrop fluorometric measurements but are estimated to be orders of magnitude <5 ng, based on the mass of the EV genome and an assumed particle to infectivity ratio of 1 : 1 (Table 1).

RNA concentrations ranging from *c.* 0.01×10^{-6} to 3×10^{-6} μg were spiked into DNA from different backgrounds of mixed populations of micro-organisms (laboratory-grown and environmental micro-organisms) and WTA was applied (Fig. 1). After WTA, side-by-side qRT-PCR detection of pre-WTA and post-WTA samples was performed to quantify the impact of WTA on target nucleic acid concentration and WTA:pre-WTA ratios were determined. Triplicate experiments were conducted with low concentrations of EV spiked into the BG population and the SW population. Pre-WTA samples created for each experiment included four EV samples (EV), two BG or SW samples and eight samples containing

Table 1 Approximate EV concentrations in samples

Sample	EV concentration [TCID$_{50}$ μl^{-1}]	EV RNA [ng μl^{-1}]
EV A	110	2·5 E-06
EV B	11	2·5 E-07
EV C	1·1	2·5 E-08
EV D	0·55	1·3 E-08
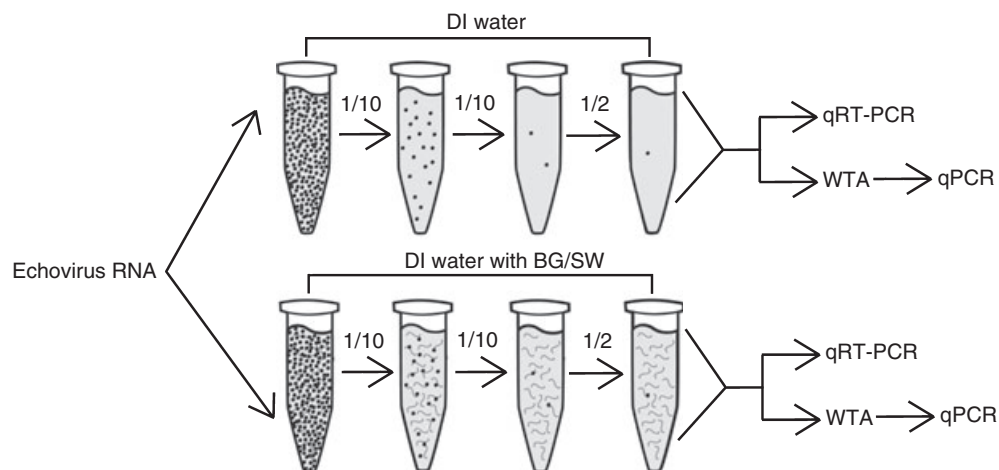
Figure 1 Experimental design.

EV spiked into BG or SW (Mix). Dilutions for the four EV samples were chosen so that two (EV A and EV B) were above and 2 (EV C and EV D) were below the estimated EV qRT-PCR limit of detection (LOD) (Table 1). Final concentrations of BG/SW in Mix samples were BG $10^3\times/10^4\times$ or SW $10^4\times/10^5\times$ (values relate to EV A concentration, e.g. DNA in $10^3\times$ is *c.* 1000 times the concentration of target in EV A). Exact BG/SW DNA concentrations were 10, 100 and 1000 ng ml^{-1} for $10^3\times$, $10^4\times$ and $10^5\times$, respectively.

WTA samples were produced from the 14 pre-WTA samples. EV and Mix samples were amplified with WTA in triplicate and BG/SW samples (WTA negative controls) in singlicate. All samples (pre-WTA and WTA samples) were analysed with qRT-PCR in triplicate to quantify target pathogens before and after application of WTA. WTA products were diluted 1/100 prior to qRT-PCR, which was factored into final calculations of WTA sample concentration.

Statistical analyses

Linear regressions of pre-WTA and WTA cycle threshold (C_t) values were performed for each set of experiments (BG and SW) using EV A and EV B samples (samples with reliably detectable pre-WTA EV concentrations). Linear regression equations were generated, with slope and R^2 values reported as measures of efficiency and goodness of fit/variance, respectively. For EV C and EV D samples (samples near/below the estimated qRT-PCR LOD prior to WTA), Fisher's exact test was used to test for a significant difference between numbers of positive pre-WTA samples *vs* positive WTA samples.

Equivalent TCID$_{50}$ values were estimated using the EV standard curves for each qRT-PCR run. Data were log transformed, and average and median TCID$_{50}$ values were calculated for data points. For reactions resulting in nondetects, values equal to the approximate LOD were imputed for statistical analyses. Descriptive statistics and linear regressions for all data were conducted using Microsoft® Office Excel 2003.

Results

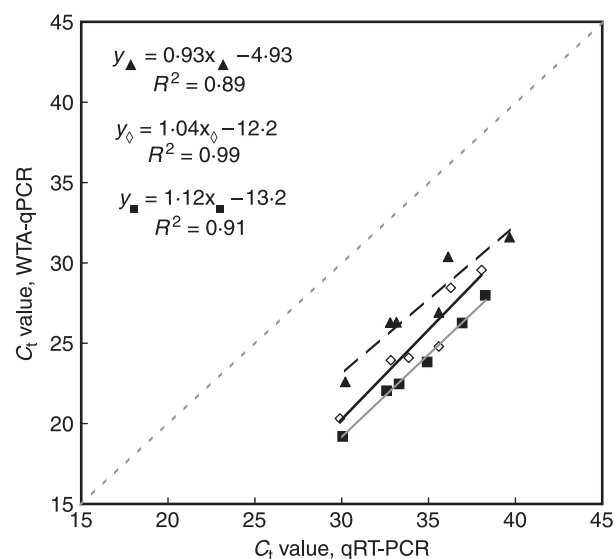#### Optimization of WTA and qRT-PCR detection of WTA product

According to the Transplex™ WTA protocol, optimal amplification, estimated to be 17 cycles, is reached 2–3 cycles into the amplification plateau, at which point the reaction can be stopped. Optimization of the WTA library amplification step by real-time visualization with SYBR Green showed that, for samples containing RNA at low

concentrations of interest, most samples had reached the amplification plateau by 25 amplification cycles. Therefore, 25 cycles were selected as an appropriate number of amplification cycles for all future WTA reactions.
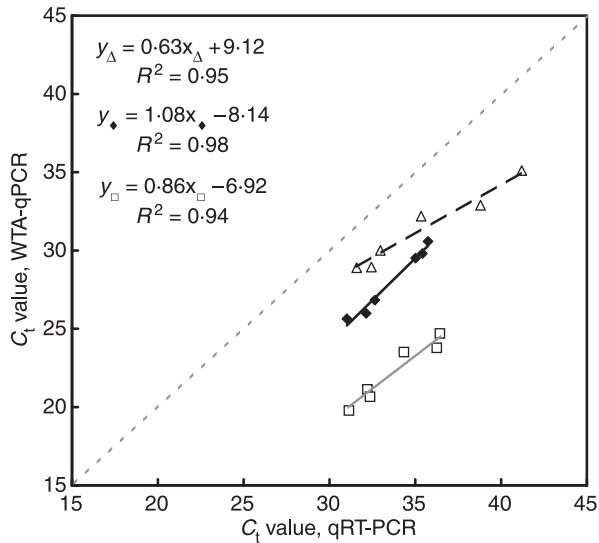
Melting curve analysis of SYBR Green–based qRT-PCR product revealed multiple nonspecific product peaks for WTA samples, indicating detection of products other than the target EV amplicon (data not shown). TaqMan qRT-PCR of the same samples eliminated detection of false positives and was selected for final qRT-PCR analyses. However, TaqMan qRT-PCR analysis of WTA product initially resulted in fewer positive results than anticipated based on pre-WTA sample concentrations, and positive WTA samples had low fluorescence (≤ 0.2) and a large amount of probe signal drift, where fluorescence gradually increases over time but is not exponential. It was hypothesized that reagents were being utilized prior to successful amplification because of the presence of excess WTA product. Therefore, 1/100 dilution of WTA product was required prior to analysis to eliminate false negatives and maintain efficient exponential amplification.

Linear regressions of pre-WTA and WTA C_t values

Linear regressions of pre-WTA and WTA C_t values demonstrate the effective amplification of WTA over several orders of magnitude at low initial copy number concentrations (Figs 2 and 3). Regressions are highly linear, with R^2 values ranging from 0·89 to 0·99 despite

$$y_{\blacktriangle} = 0.93x_{\blacktriangle} - 4.93$$
$$R^2 = 0.89$$
$$y_{\diamond} = 1.04x_{\diamond} - 12.2$$
$$R^2 = 0.99$$
$$y_{\blacksquare} = 1.12x_{\blacksquare} - 13.2$$
$$R^2 = 0.91$$

Figure 2 Linear regressions of average echovirus pre-WTA and post-WTA C_t values for background experiments. A 1 : 1 C_t ratio line is shown for reference. ▲ BG $10^4\times$; ◇ BG $10^3\times$; ■ Control (no competing nucleic acid).

$$y_\triangle = 0.63x_\triangle + 9.12$$
$$R^2 = 0.95$$

$$y_\blacklozenge = 1.08x_\blacklozenge - 8.14$$
$$R^2 = 0.98$$

$$y_\square = 0.86x_\square - 6.92$$
$$R^2 = 0.94$$

Figure 3 Linear regressions of average echovirus pre-WTA and post-WTA C_t values for surface water experiments. A 1 : 1 C_t ratio line is shown for reference. △ SW $10^5\times$; ◆ SW $10^4\times$; □ Control (no competing nucleic acid).

the variability in the highly sensitive qRT-PCR assays and potential RNA degradation/variation between experiments. As C_t values correspond to the logarithm of the initial copy number, results show that log linearity of amplification is preserved over a range of environmentally significant concentrations. This suggests the ability to accurately quantify initial copy number, given a sample amplified by WTA.

Slope is indicative of the average ratio of WTA:pre-WTA target over all of the samples (Figs 2 and 3). If slope = 1,

amplification efficiency is the same across concentrations. Variation in the slope (variation in amplification efficiency) is seen depending on the amount and type of mixed population addition. Slopes are close to 1, but decrease somewhat with increasing addition of BG/SW, showing that amplification is less efficient for the smallest concentrations when inhibiting substances are present. Position of samples along the y-axis is also indicative of amplification efficiency. Compared with the 1 : 1 reference regression line, all samples are positioned lower on the y-axis, so concentrations are improved by application of WTA. Samples with the highest concentrations of BG/SW are positioned higher on the y-axis, indicating decreased amplification because of the mixed DNA addition. On average, EV was amplified c. 2–4 logs by WTA.

WTA:pre-WTA ratios

Ratios of WTA:pre-WTA concentrations decrease with decreasing initial target concentration, based on both medians and averages (Table 2). For nine replicates of low target concentration, there is a high probability that ≥½ of WTA samples will be ND, resulting in medians that are also ND, while averages may be detectable. WTA:pre-WTA ratios also show that increasing addition of BG or SW decreases efficiency of amplification.

Numbers of qRT-PCR-positive samples before and after WTA

The number of EV C and EV D samples detected as positive before and after application of WTA was tabulated (Table 3). Results show that there was an increase in the

Table 2 WTA:pre-WTA ratios* for background (BG) and surface water (SW) experiments. Medians and averages are shown ($n = 9$)

BG experiments	Median	Average	SW experiments	Median	Average
EV A	3264	2149	EV A	12 683	14 740
EV B	3493	1915	EV B	10 812	12 810
EV C	ND	2915	EV C	>1115	8134
EV D	ND	949	EV D	>1255	16 255
EV A BG $10^3\times$	1136	1097	EV A SW $10^4\times$	282	364
EV B BG $10^3\times$	1530	1017	EV B SW $10^4\times$	296	269
EV C BG $10^3\times$	ND	1237	EV C SW $10^4\times$	680	780
EV D BG $10^3\times$	ND	874	EV D SW $10^4\times$	ND	100
EV A BG $10^4\times$	483	402	EV A SW $10^5\times$	52	68
EV B BG $10^4\times$	246	222	EV B SW $10^5\times$	261	114
EV C BG $10^4\times$	>368	293	EV C SW $10^5\times$	>6	2824
EV D BG $10^4\times$	ND	316	EV D SW $10^5\times$	ND	>1·1
BG $10^3\times$	ND	ND	SW $10^4\times$	ND	ND
BG $10^4\times$	ND	ND	SW $10^5\times$	ND	ND

LOD, limit of detection.
*Ratios calculated using estimated qRT-PCR LOD of 1·1 TCID$_{50}$ (as imputed from standard curve) in place of nondetects (ND).

Table 3 Samples positive by qRT-PCR before and after WTA. Determined for EV C and EV D samples for all experiments. Fisher's exact test for combined C and D samples is significant ($P = 0.0280$)

		pre-WTA		WTA	
		Each	Combined	Each	Combined
+	EV C	20	39	33	56
	EV D	19		23	
−	EV C	34	69	21	52
	EV D	35		31	

WTA, whole transcriptome amplification.

number of EV C and EV D samples positive for EV after WTA. Results from Fisher's exact test showed that the difference between the number of samples positive before *vs* after WTA was significant (two-tailed, $P = 0.0280$).

Discussion

TransPlex^TM WTA for small quantities of pathogens

The ultimate goal of this study was to evaluate WTA for successful amplification of environmentally relevant amounts of RNA, which are less than the amounts of RNA previously suggested for successful WTA. Trans-Plex^TM WTA, which had not been applied to microbial RNA in the presence of background nucleic acids prior to this study, successfully amplified viral RNA from lower levels of starting material than suggested for the kit.

In previous studies, other WTA methods have not been able to efficiently amplify low copy number samples. For IVT of bacterial mRNA, 50–100 ng RNA is needed for representative detection (Gao *et al.* 2007), and amplification fidelity decreases when <100 ng RNA is used, particularly if the target transcripts are underrepresented in the sample (Wang 2005). This suggests that the sensitivity of IVT would be inadequate for detection of viruses in environmental samples. TransPlex^TM WTA recommends input of 50 ng RNA, but notes that <5 ng is usable. Total EV RNA concentrations in this study are estimated to be orders of magnitude <5 ng (Table 1).

Modification of the WTA protocol was required for successful WTA under the conditions of this study. TransPlex^TM WTA was designed to amplify human RNA and had not previously been optimized for the amplification of microbial nucleic acids or low copy number samples. Increasing the number of amplification cycles greatly improved amplification and was necessary to maximize product formation.

Transplex^TM WTA combines MDA and universally primed PCR. MDA typically has higher product yield

than previously described universally primed methods (Bergen *et al.* 2005; Sorensen *et al.* 2007; Uda *et al.* 2007), but hyperbranching products may obscure the target site (Vora *et al.* 2004). In TransPlex^TM WTA, MDA creates random, overlapping strands of variable size that are flanked by universal binding regions. These strands are then amplified by universally primed PCR. This takes advantage of the benefits of each technology, yielding superior results. Independent researchers have previously combined MDA and universally primed PCR for DNA amplification, yielding improvements in detection (Vora *et al.* 2004; Breitbart and Rohwer 2005; Panelli *et al.* 2005).

Increases in sensitivity and total sample volume with WTA

Using WTA as a preamplification step can successfully increase the sensitivity of downstream molecular analyses. In this study, increases in EV target copy number of *c.* 2–4 logs after application of WTA resulted in significant improvements in detection by qRT-PCR, as evidenced by the statistically significant increase in the total number of positively detected low copy number (at or near the LOD) samples. However, it is difficult to accurately quantify improvements in sensitivity from application of WTA (other than positive/negative detection), because consistently obtaining an aliquot positive for virus when the sample concentration is below a certain limit is improbable (Chen *et al.* 2007). In this study, when target concentrations are at or below the estimated LOD, the probability of successful WTA is decreased because of decreased probability of target being added to the WTA reaction. Additionally, as formation of WTA template is a random process, metatranscriptomes produced from replicate amplifications can differ, particularly for low copy number samples. Although WTA of low copy number samples may result in infrequent amplification because of these factors, when a target is successfully amplified, improvements in concentration are significant, as demonstrated by pre-WTA:WTA ratios that are higher for averages than medians (Table 1).

Another benefit of WTA is to increase the total volume of sample available for analysis. Improvements in detection from WTA have been described on a concentration basis, but it is also useful to describe gains in terms of volume. TransPlex^TM WTA increases an initial sample of 10–375 μl of WTA product. Access to a larger amount of product makes it easier to run multiple reactions for multiple pathogens or conduct assays where a larger volume of high-quality sample is needed such as microarray analysis. There is more room for assay error or to run the assay multiple times if the quantity after amplification is

near the LOD. Volume may no longer be a limiting factor for some types of molecular assays, which is beneficial even if no significant gains are seen in terms of target concentration.

The ability to quantify the original target number of samples amplified by WTA is another important feature of this study. Because the dilution series stayed consistent post-WTA application, resulting in log-linear amplification, environmental samples amplified by WTA may not be limited to a presence/absence interpretation of data. Samples can instead be quantified based on known standard curves. This demonstrates that increases in sensitivity and total sample volume gained using WTA are not at the expense of sample quantitation.

WTA for detection in mixed microbial communities

The second major goal of this research was to determine whether WTA can be successfully applied to environmental samples containing mixed populations of microorganisms. WTA was able to amplify target RNA despite competition from background nucleic acids. Moderate concentrations of nucleic acids had a minor effect on amplification efficiency, while higher concentrations had a greater impact. As background nucleic acids increase, there is likely a decreased chance of amplification of the target as nontarget nucleic acids out-compete low concentration targets during the random amplification. Although EV WTA:pre-WTA ratios were lower for WTA of EV added to mixed nucleic acids (*c.* 1–3 log increase in target) compared with WTA of EV only (*c.* 2–4 log increase in target), the ratios still show significant amplification of EV targets were gained despite the presence of competing nucleic acids.

Experiments with mixed microbial communities were also used to determine the ability of WTA to overcome inhibition of qRT-PCR from background nucleic acids and other contaminating substances. There is evidence that whole-genome amplification (WGA) can improve detection in the presence of inhibitors by amplifying nucleic acids to levels which dilute the inhibiting substances (Gonzalez *et al.* 2005). This effect could also overcome decreased sensitivity caused by the background metagenome. Although inhibition was difficult to quantify, WTA successfully amplified targets of low concentration despite the presence of large of amounts of competing nucleic acids from a natural water sample that probably contained substances that typically interfere with PCR. This suggests that WTA would be a good replacement for the initial round of nested PCR, which has been used to improve detection of low copy number samples, particularly those with inhibiting substances.

SYBR green for qRT-PCR of WTA product

SYBR Green may not be able to effectively serve as a fluorescent label for qRT-PCR analysis of TransPlex™ WTA product. Although SYBR Green qRT-PCR detection of pre-WTA samples with the Schwab *et al.* (1995) EV primer set was effective, it was not effective for analysis of WTA product. Detection of multiple product peaks in melting curve analyses of SYBR Green–based qRT-PCR indicates that multiple products were formed during WTA, a by-product of template shearing during the nonspecific, nonprimer-directed amplification. The multiple nontarget products ultimately interfered with detection of the target EV amplicon. Also, TransPlex™ WTA creates both single-stranded and double-stranded cDNA product. Because SYBR Green is an intercalating dye, which indiscriminately binds between all dsDNA molecules, the high background fluorescence can probably be attributed to the large quantities of nontarget dsDNA produced during WTA. TaqMan probes increase the specificity of qRT-PCR by binding only with the target amplicon. However, product information from Sigma-Aldrich now states that cDNA from WTA should be purified prior to use because some components may interfere with downstream applications. This information was not provided in the pre-release version of the kit used in these experiments, but could perhaps improve the use of SYBR Green in this situation.

Application of WTA to environmental pathogens

WTA has not previously been assessed as a tool for improving molecular detection of low levels of pathogens in environmental samples. Results from these experiments demonstrate that WTA improves molecular detection of small quantities of RNA from small quantities of viral pathogens. WTA can be effectively applied to mixed microbial communities, such as those that might be found in drinking water. WTA also increases the quantity of nucleic acids so that volume is not the limiting factor to multiple detection assays for multiple pathogens. The potential applications for WTA as a preamplification step are diverse and include any molecular analyses which could benefit from an increase in target copy number (e.g. qRT-PCR), increase in sample volume (e.g. microarray analysis), dilution of inhibiting substances, a replacement for more inferior preamplification methods (e.g. nested PCR), immediate preservation of unstable samples (i.e. conversion to cDNA for future use) and/or a generally higher-quality, higher-concentration nucleic acid sample (e.g. for sequencing, microsatellite analysis). For some downstream applications, WTA product may need additional processing steps prior to analysis (e.g. whole-genome sequencing might require an additional

concentration step, such as magnetic bead-based separation, prior to sequencing).

Analyses of samples before and after WTA indicate that linear regressions of these concentrations remain relatively stable despite the addition of different types and concentrations of microbial community DNA. This suggests that standard curves may be used to estimate starting copy numbers of target RNA and quantify unknown amounts of pathogens. Based on the results of this study, it is recommended that, for samples where low quantities of pathogens are anticipated, replicate reactions containing larger amounts of template should be performed to increase the chances of detecting low copy number pathogens. The implications for improving detection of pathogens in drinking water are promising. It is essential to test a variety of other human pathogens present in actual environmental samples such as this to further validate WTA.

Acknowledgements

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