Use of Signal-Mediated Amplification of RNA Technology (SMART) to Detect Marine Cyanophage DNA

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ABSTRACT

Here, we describe the application of an isothermal nucleic acid amplification assay, signal-mediated amplification of RNA technology (SMART), to detect DNA extracted from marine cyanophages known to infect unicellular cyanobacteria from the genus Synechococcus*. The SMART assay is based on the target-dependent production of multiple copies of an RNA signal, which is measured by an enzyme-linked oligosorbent assay. SMART was able to detect both synthetic oligonucleotide targets and genomic cyanophage DNA using probes designed against the portal vertex gene (g20). Specific signals were obtained for each cyanophage strain (S-PM2 and S-BnM1). Nonspecific genomic DNA did not produce false signals or inhibit the detection of a specific target. In addition, we found that extensive purification of target DNA may not be required since signals were obtained from crude cyanophage lysates. This is the first report of the SMART assay being used to discriminate between two similar target sequences.*

INTRODUCTION

Signal-mediated amplification of RNA technology (SMART) is an isothermal nucleic acid amplification assay that is rapid and simple to perform, relies on signal amplification, and does not require thermal cycling or involve copying long target sequences (14). The assay has further advantages in that it can be used for either DNA or RNA targets. The signal generated is highly target-dependent (i.e., a signal is only produced when a specific target is present), and discrimination of base changes within the target sequence is possible.

The SMART assay consists of two single-stranded oligonucleotide probes (extension and template); each probe includes one region that can hybridize to the target and another, much shorter, region that hybridizes to the other probe. The two probes are designed so that they can only anneal to each other in the presence of the specific target, so forming a structure called a three-way junction (Figure 1A, 3WJ).

Following three-way junction formation, DNA polymerase extends the short (extension) probe and transforms a single-stranded promoter sequence on the template probe into a double-stranded functional promoter (Figure 1B). The assay relies on the fact that only the double-stranded promoter is functional and allows the RNA polymerase to generate multiple copies of an RNA signal. Since the DNA and RNA polymerases are capable of functioning under the same reaction conditions, the reaction takes place in a single tube. The RNA signal generated may be increased further using additional rounds of extension and transcription before detection and quantification by an enzyme-linked oligosorbent assay (ELOSA) (Figure 1B). The end detection method uses a 96-well format, measuring color change in a standard plate reader. Therefore, multiple samples may be quantified simultaneously with no need for gel analysis.

The SMART process is based on the amplification of a signal—the nucleic acid target sequence is not amplified. The SMART signal is single-stranded and has the same sequence regardless of the target being detected, which facilitates hybridization-based detection techniques (i.e., gel analysis is not required). The system is easily adapted for the detection of different target sequences. Minimal probe changes are required, and the assay and signal-detection conditions remain the same.

In the current study, we describe the application of SMART for use in the detection of viruses that infect marine *Synechococcus* (cyanophages). Cyanophages are ubiquitous in both marine and freshwater aquatic systems (8). They are extremely numerous in surface seawater and are genetically diverse (9,10,12,16,17). The cyanophage hosts used in this study, *Synechococcus* spp., are marine cyanobacteria with a widespread distribution through the world's oceans and, in some regions, are thought to contribute up to 25% of primary productivity (13). Previously, a conserved region from the cyanophage genome was identified in three geneti-

cally distinct cyanophages, and sequence analysis revealed significant similarity to a gene encoding a portal vertex protein (gp20) from the enteric coliphage T4 (3). These cyanophage sequence data were used to develop cyanophage-specific SMART probes to test the ability of this technology to detect marine cyanophages.

Previous trials showed that the SMART assay could generate signals from sequences in genomic DNA and total RNA, using *E. coli* as a model target organism (14). Here, we report the use of the SMART technology with a viral target and the first use of the SMART assay to discriminate between two similar target sequences.

MATERIALS AND METHODS

Cyanophages, Host Strain, and Media

The two genetically distinct marine cyanophage isolates that were propagated on *Synechococcus* sp. WH7803 were previously isolated by plaque assay (strain S-PM2) from coastal water off Plymouth, UK (17) and a bay adjacent to Raunefjorden, 20 km south of Bergen, Norway (S-BnM1) (3). *Synechococcus* sp. WH7803 (formerly designated DC2) is a member of marine cluster A (11) and originates from the North Atlantic (13). *Synechococcus* sp. WH7803 was routinely grown in 100-mL batch cultures in 250-mL conical flasks under constant illumination at total volume of 5–36 µmol quanta, 25°C in artificial seawater (ASW) medium (15). Cyanophage stocks with concentrations that ranged from 5×10^7 to 5×10^8 pfu/mL were prepared by adding a clonal cyanophage suspension to a 50-mL culture of exponentially growing host cells to an MOI of approximately 4×10^{-3}. The infected culture was incubated at 25°C under constant illumination (5–36 µmol quanta). Once cell lysis was observed (usually within a week), the lysate was passed through a 0.2-µm Acrodisc® Supor syringe filter (Pall Gelman Laboratory, Ann Arbor, MI, USA) (to remove host-cell debris) into a sterile bottle. The filtered lysates were then stored in the dark at 4°C.

Reagents

Oligonucleotide probes synthesized by phosporamidite chemistry using a

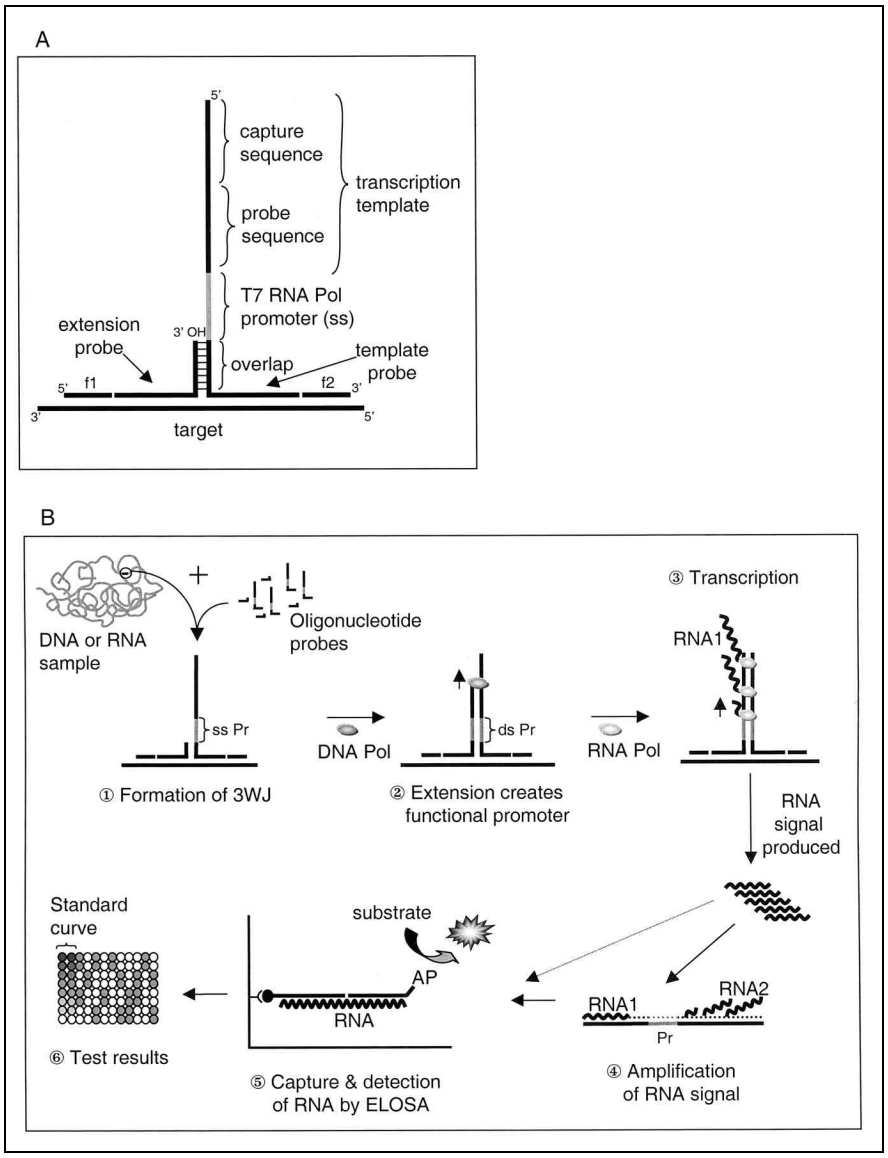

Figure 1. The SMART assay. (A) Structure of a three-way junction. Extension and template probes anneal to a specific target sequence, and then to each other (the overlap between the two probes is only 8 bp). The short extension probe has a free 3′-OH to allow extension. The template probe includes a single-stranded (ss) T7 RNA polymerase (Pol) promoter (nonfunctional) and sequences to allow the capture and detection of the RNA signal. Two facilitator probes (f1 and f2) anneal to target sequences adjacent to the regions hybridizing to the extension and template probes, which improves the efficiency of three-way junction formation with double-stranded targets (unpublished data). The 3′ ends of the templates and facilitators are blocked to prevent extension. (B) Schematic representation of the SMART assay. A three-way junction forms only if the specific target sequence is present in the nucleic acid sample (①). Extension from the 3′OH of the extension probe by *Bst* DNA polymerase creates a double-stranded (ds), functional, T7 RNA Pol promoter (Pr) (②). T7 RNA polymerase generates a signal, RNA1 (③), which anneals to a second template (RNA amplification probe). This leads to further extension and transcription by the DNA and RNA polymerases to generate increased amounts of a second signal, RNA2 (④). The RNA signal is detected and quantified by an ELOSA (⑤). Specific sequences included in the RNA signals allow capture, via a biotinylated probe, onto the streptavidin-coated well of a microplate and detection and quantification via an alkaline phosphatase (AP)-linked probe. Wash steps remove unbound probe, and the color change of the alkaline phosphatase substrate (4-nitrophenyl phosphate) is followed at 37°C for 30 min at 405 nm. The relative amounts of signal may be calculated from the test results (⑥) by comparing the alkaline phosphatase activities of different samples with a standard curve.

Table 1. Oligonucleotide Probe Sequences Used in This Study

	Cyanophage Strain	
	S-PM2 (5′→3′)	**S-BnM1 (5′→3′)**
Synthetic target[a]	TCTATATTGATGTTGGTAATCTACC AAAGGTAAAGGCAGAACAATACT TGCGTGATGTAATGTCACGT↓TAC AGAAACAAGCTTGTTTACGATGG TCAAACAGGAGAGATT	TTCTACATTGATGTTGGCAATCT TCCAAAGGTAAAAGCAGAACAAT ACCTCAGAGAGGTAATGGGACGT ↓TACCGCAACAAACTTGTTTATG ATGCAAACACAGGTGAAATCAAGGA CGACAAGAAACATATGTCGATGCTT
Extension probe	TGACCATCGTAAACAAGCTTGTTTC TGTAT TCGAAAT	GTTTGCATCATAAACAAGTTTGTTGC GGTATTCGAAAT
Template probe	TCGTCTTCCGGTCTCTCCTCTCAA GCCTCAGCGCTCTCTCTCCCTATAG TGAGTCGTATTAATTTCGAAhACGTG ACATTACATCACGCAAGTATTGTTh	TCGTCTTCCGGTCTCTCCTCTCAAGC CTCAGCGCTCTCTCTCCCTATAGTGAG TCGTATTAATTTCGAAhACGTCCCATTA CCTCTCTGAGGTATTGh
Facilitator 1	TGCTTTTTATCATCACGAATCTCTC CTGTTx	TGTTTCTTGTCGTCCTTGATTTCACC TGTx
Facilitator 2	CTGCCTTTACCTTTGGTAGATTA CCAACAx	TTCTGCTTTTACCTTTGGAAGATTGC CAACx
RNA amplification probe	TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGTGAGTC GTATTAATTTCTCGTCTTCChGGTCTCTCCTCTCAAGCCTCAGCGCTCTCTCTCCCx	
Capture probe	Bio-TCTGCTGCCTGCTTGTCTGCGTTCT	
Detection probe	GGATATCACCCG AP	
Synthetic product for standard curve	GGGACTGACGATTCGGGTGATATCCAGAACGCAGACAAGCAGGCA	

Cyanophage-specific probes are listed, followed by oligonucleotides used for further amplification and detection of the RNA signal.
[a]S-PM2 GenBank® accession no. AFO16384, bases 836–945. S-BnM1 GenBank accession no. AFO16386, bases 823–963.
↓ indicates the position of the three-way junction site (i.e., position at which the extension and template probes meet).
h indicates the position of HEG molecules.
x indicates the position of phosphorylation to prevent extension.
Bio indicates the position of Biotin.
AP indicates alkaline phosphatase.

model 380A synthesizer (Applied Biosystems, Foster City, CA, USA) and purified using standard HPLC or FPLC techniques were obtained from Oswel Research Products Ltd. (Southhampton, UK). Table 1 lists synthetic target sequences and probes used to generate target-specific, three-way junctions. The template probes include a hexaethylene glycol (HEG) linker molecule (Table 1, h) to reduce nonspecific background signal. Table 1 also lists the probes for amplification and end detection of the RNA signal generated from any target.

Extraction of Viral DNA

A 2-mL sample of cyanophage lysate was centrifuged for 10 min at 6240× *g* to remove cellular debris. The supernatant, containing the cyanophages, was combined with lysis buffer containing a final concentration of 0.05 M EDTA, 1% SDS. DNA was extracted using a DNeasy™ Tissue Kit, according to the manufacturer's instructions (Qiagen, West Sussex, UK) for crude lysates, eluting in a final volume of 100 µL. The DNA was stored in aliquots at -20°C before being used in SMART reactions.

Probe Design

The sequences of all targets, probes, and RNA signals (Table 1) were analyzed for potential secondary structure using the *mfold* computer program (5,7,18) to choose sequences and design probes with as little folding as possible. Probe/probe and probe/target melting temperatures were calculated using the Tm computer program (1).

The SMART Assay: Extension and Transcription from a Three-Way Junction to Produce an RNA Signal

Our protocol was adapted from one that has been described previously (14). Target DNA was added to a mixture containing 2 µL 10× transcription buffer (Ambion, Austin, TX, USA), extension probe (5 fmol), template probe (1 fmol),

facilitator probes 1 and 2 (100 fmol each for assays on double-stranded target only), and ultra-pure, sterile, RNase-free water to a final volume of 15 µL. The samples were mixed, heated at 90°C for 3 min on a PTC-200™ thermal cycler (MJ Research, Waltham, MA, USA), ramped down to 41°C (0.1°C/s), and held at this temperature for 1 h. A 5-µL volume of a solution containing dNTPs (5 µM each), NTPs (2 mM each) (both from Amersham Biosciences, Aylesbury, UK), 4 U *Bst* (3′→5′ exo$^-$) DNA polymerase (New England Biolabs, Beverly, MA, USA), and 240 U T7 RNA polymerase (Ambion) was then added, and the reaction was incubated at 41°C for an additional 2 h.

To amplify the RNA signal further, the samples were brought to room temperature before the addition of 20 fmol RNA amplification probe, followed by a mixture containing 4.5 µL 10× transcription buffer, dNTPs (50 µM each dNTP), NTPs (2 mM each NTP), 4 U *Bst* (3′→5′ exo$^-$) DNA polymerase, 160 U T7 RNA polymerase, and ultra-pure, sterile, RNase-free water to give a final volume of 17 µL. The samples were mixed and then incubated at 37°C for 2 h. The samples could be stored at -20°C before the signals were quantified.

Capture and Detection of RNA Signal From an Amplification Reaction

The RNA signal was assayed by an ELOSA. The RNA sequence includes regions for capture via a biotinylated probe and detection using another probe linked to alkaline phosphatase (Figure 1B). The biotinylated capture probe (0.9 pmol) and alkaline phosphatase-labeled probe (6 pmol) were added to each well of a streptavidin-coated Combiplate® (Thermo Life Sciences, Hampshire, UK) in hybridization buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA, and 1% BSA). An aliquot (5–20 µL) of the sample to be quantified was then added, bringing the total volume to 150 µL per well. The samples were incubated at room temperature on a platform shaker at 300 rpm for 1 h. Unbound material was removed from wells by washing four times with 200 µL wash buffer (1× TBS/0.1% Tween® 20) and then once with 200 µL alkaline phosphatase substrate buffer (Scil Diagnostics, Martinsried, Germany). Substrate (4-Nitrophenyl phosphate; Boehringer-Mannheim UK, Sussex, UK), at 5 mg/mL in substrate buffer, was then added (180 µL/well), and alkaline phosphatase activity was measured using a plate reader (Labsystems integrated EIA Management system; Thermo Life Sciences) pre-warmed at 37°C, reading

absorbance at 405 nm every 2 min for 30 min. The rates of alkaline phosphatase activity for each sample were compared to a standard curve that was generated using dilutions of a synthetic DNA oligonucleotide with the same sequence as the RNA product. This allowed the amount of RNA produced in each extension/transcription reaction to be calculated.

RESULTS

Generation of Signals From Synthetic Target

Synthetic cyanophage DNA was detected by specific cyanophage extension and template probes (Table 1). Each reaction was carried out using 100 amol either specific or nonspecific single-stranded synthetic oligonucleotide target to determine if a target-dependent signal could be achieved. No facilitator probes were used. Each set of cyanophage probes was shown to be specific and only produced a signal from the corresponding synthetic cyanophage target (i.e., S-PM2 probes only detected S-PM2 synthetic DNA and S-BnM1 probes only detected S-BnM1 synthetic DNA) (data not shown).

Specific Detection of Genomic DNA

Genomic cyanophage DNA extracted from fresh lysates was detected using the same extension and template probes as those used for synthetic target, together with facilitator probes (Table 1). Using 270 ng either specific or nonspecific genomic cyanophage DNA, target-specific signals were gen-

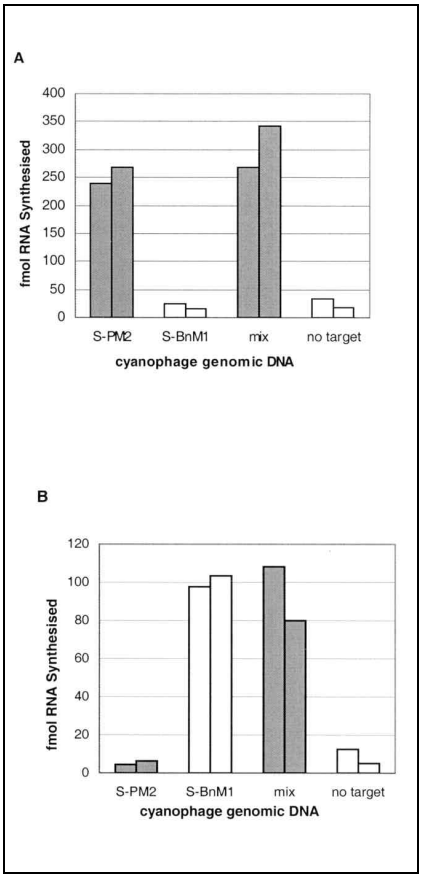

Figure 2. Detection of cyanophage purified genomic DNA targets using specific SMART probes. (A) Cyanophage strain S-PM2-specific probes detected S-PM2 genomic DNA added separately or when mixed with S-BnM1 genomic DNA, but they did not detect S-BnM1 genomic DNA. (B) Cyanophage strain S-BnM1-specific probes detected S-BnM1 genomic DNA added separately or when mixed with S-PM2 genomic DNA, but they did not detect S-PM2 genomic DNA. Genomic DNA (270 ng) was used as a target in each duplicate reaction, and a 1:1 ratio (270 ng each) of genomic DNA from both strains was used in the S-PM2/S-BnM1 genomic DNA mixtures. Graphs show the amount of RNA signal (fmol) generated from each target, as determined by an ELOSA.

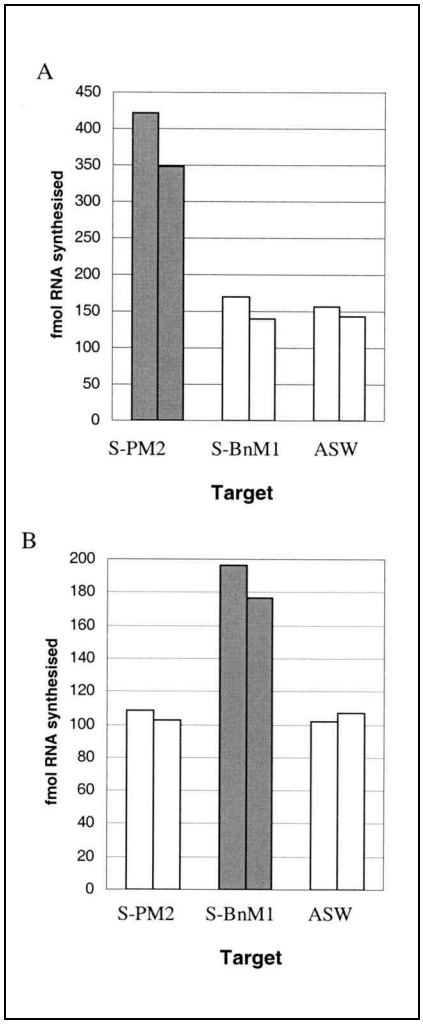

Figure 3. Detection of DNA from cyanophages in lysates using specific SMART probes. (A) Cyanophage strain S-PM2-specific probes generated signals from S-PM2 lysate. (B) Cyanophage strain S-BnM1-specific probes generated signals from S-BnM1 lysate. Approximately 10^6 cyanophage were detected, as determined by SYBR® Green 1 staining combined with epifluorescence microscopy (6). Graphs show the amount of RNA signal (fmol) generated from the cyanophage lysate, as determined by an ELOSA.

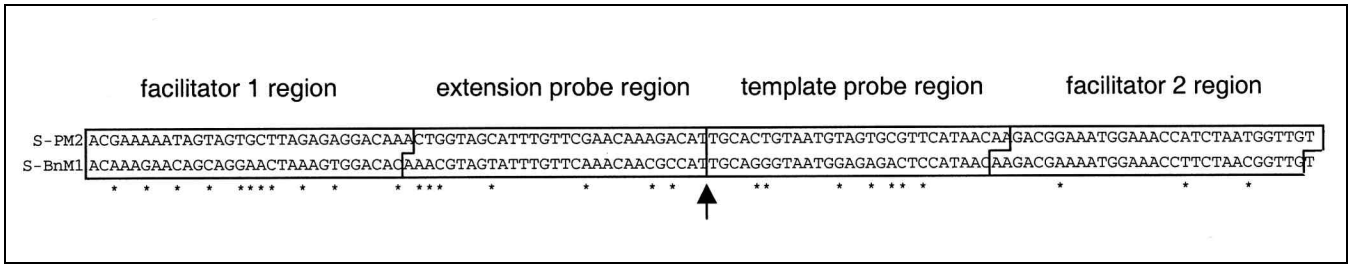

Figure 4. Comparison of the S-PM2 and S-BnM1 target sequences. The regions of the g20 sequence hybridizing to the various SMART probes (boxed) are shown for S-PM2 (GenBank AFO16384, bases 846–962) and S-BnM1 (GenBank AFO16386, bases 835–951). Note that the target sequences are written 3′→5′, as opposed to the conventional 5′→3′, to enable the positions of the probes to be marked, which correspond to earlier figures showing the three-way junction structure where the extension probe is written 5′→3′. An arrow indicates the position of the three-way junction site (i.e., where the extension and template probes meet). Mismatches between the two sequences are indicated (*).

erated (Figure 2). RNA signals were also detected when genomic DNA from the two cyanophage strains was mixed in a 1:1 ratio; hence, signals could be generated in the presence of nonspecific DNA. Moreover, the signals were similar to those generated when only specific genomic DNA was present.

Generation of Signals From Crude Lysate

Signals were obtained from fresh, filtered lysates of cyanophage strain S-PM2 using S-PM2-specific SMART probes and facilitators (Table 1) and from cyanophage strain S-BnM1 using S-BnM1 SMART probes. Cyanophages were added directly to the reaction without prior DNA extraction. The assay was capable of detecting approximately 10^6 cyanophages per reaction. The probes for S-PM2 and S-BnM1 could distinguish between crude lysates of the two strains (Figure 3).

DISCUSSION

SMART is currently being developed for the detection of specific nucleic acid sequences from clinical samples. Here, we report the first use of the SMART assay for viral targets and for distinguishing between two similar target sequences in the specific detection of DNA from strains of marine cyanophages.

The results from this study clearly demonstrate that the SMART assay can be used to specifically detect both synthetic (data not shown) and genomic (Figure 2) cyanophage DNA. Specific signals were obtained for each cyanophage strain, and nonspecific genomic DNA did not interfere with the assay. The identity of the two target DNA sequences over the probed region was approximately 75%, and Figure 4 illustrates the mismatches between the two target regions. Using synthetic targets, the technology is actually capable of discriminating between SNPs in different targets. The position of the SNP within the probed region is not critical because discrimination had been achieved for most changes tested using synthetic targets (unpublished data).

Specific signals were also generated from cyanophage lysates (Figure 3) without prior extraction and purification of target DNA, which indicated that detection could be achieved in the presence of contaminants, such as viral and host proteins, host nucleic acid, and growth media salts. This is an important first step for the SMART detection of cyanophages in natural samples.

Inevitably, SMART will be compared with other techniques currently available and used widely for virus detection. PCR is generally accepted as the gold standard of amplification technologies. PCR-based methods use variations in reaction temperature to achieve specificity. In contrast, SMART is an isothermal method, and specificity is achieved at a constant, low (41°C) reaction temperature. Another isothermal amplification technique is nucleic acid sequence-based amplification (NASBA), in which RNA targets are themselves amplified (2). NASBA has been used to study gene expression and cell viability (4). The amplification of signals, as opposed to targets, gives SMART the potential to be a simple and flexible alternative amplification technology. It is relatively straightforward to adapt SMART for the detection of different targets. For each set of probes designed, only the target-hybridizing regions (Figure 1A) need to be changed. Since the probe overlap and template sequences are not altered, the RNA signal, RNA amplification and detection probes, and assay and detection conditions all remain the same. For other amplification technologies, where the target sequence itself is amplified, reactions usually need to be re-optimized for each new target.

Currently, the detection limit of SMART, following DNA extraction of cyanophage lysates, is between 10^4 and 10^5 cyanophage particles per reaction, which is at least two orders of magni-

tude less sensitive than PCR [Fuller et al. (3) could detect down to 190 pfu/mL]. Current research is aimed at improving the sensitivity of the SMART assay. Cyanophage concentrations in the marine environment range from 0 to 10^6 mL^{-1} (9,12), and there is a wide range of commercially available ultra-filtration hardware that could be used to concentrate cyanophages to within current detection limits.

At first glance, the SMART assay may appear complicated to set up because each reaction involves the activities of two enzymes on a number of different oligonucleotide probes. However, in reality, a master mixture is added to the different targets, and amplification takes place in a single tube. The current end detection system measures color change in a standard 96-well format plate reader so that the signals from multiple samples may be measured simultaneously, with no need for agarose or acrylamide gel analysis. Indeed, the most up-to-date instruments can read 384 or 1536 sample wells simultaneously. This could ultimately allow very high throughput of samples and facilitate the detection of multiple targets by using a range of different probes.

SMART is a technique that can be used for the detection of specific viruses. In fact, it could potentially be used for many other applications including the detection of viral hosts. Further research will focus on improving the sensitivity of the assay. By developing high-throughput detection of multiple targets, the technology could be applied to the analysis of virus-host population dynamics in the marine environment.

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