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Specific detection of DNA and RNA targets using a novel isothermal nucleic acid amplification assay based on the formation of a three-way junction structure

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ABSTRACT

The formation of DNA three-way junction (3WJ) structures has been utilised to develop a novel isothermal nucleic acid amplification assay (SMART) for the detection of specific DNA or RNA targets. The assay consists of two oligonucleotide probes that hybridise to a specific target sequence and, only then, to each other forming a 3WJ structure. One probe (template for the RNA signal) contains a non-functional single-stranded T7 RNA polymerase promoter sequence. This promoter sequence is made double-stranded (hence functional) by DNA polymerase, allowing T7 RNA polymerase to generate a target-dependent RNA signal which is measured by an enzyme-linked oligosorbent assay (ELOSA). The sequence of the RNA signal is always the same, regardless of the original target sequence. The SMART assay was successfully tested in model systems with several single-stranded synthetic targets, both DNA and RNA. The assay could also detect specific target sequences in both genomic DNA and total RNA from *Escherichia coli*. It was also possible to generate signal from *E.coli* samples without prior extraction of nucleic acid, showing that for some targets, sample purification may not be required. The assay is simple to perform and easily adaptable to different targets.

INTRODUCTION

There is a need for simple, rapid and cheap isothermal assays, particularly in the diagnosis of diseases caused by bacteria, viruses and protozoa. Successful treatment of infected patients relies on the rapid and reliable identification of the infectious agent, and may also require the simultaneous identification of antibiotic resistance genes and other pathogenicity determinants such as toxin genes. The traditional method of culturing the organisms can be slow and, in the case of infectious agents, dangerous, but these problems may be avoided by the direct detection of pathogen-specific nucleic acid sequences. Many of the existing nucleic acid amplification assays are time consuming and require highly skilled personnel to set up the assays and analyse the results, hence the need for new, simple assays. Additional problems may be caused by the presence of non-viable organisms, which could give positive test results. Current tests for viability, which may include infection tests in animal models, can be costly and lack sensitivity and reproducibility (1). Since RNA is relatively unstable compared to DNA (which may persist when organisms no longer pose a risk of infection), the detection of RNA is a better indicator of viability (1–3). New assays should therefore be capable of detecting both DNA and RNA targets.

A number of RNA amplification technologies have been developed. Two major categories of these are: (i) those that utilise thermal cycling such as RT–PCR and (ii) isothermal assays such as nucleic acid sequence-based amplification (NASBA) (4,5) and transcription-mediated amplification (TMA) (6). Isothermal assays may be sub-divided, based on whether: (i) they copy and amplify the target sequence, such as TMA, NASBA and self-sustained sequence replication (3SR) (7,8); for review see 9), or (ii) they generate a target-dependent signal which can be further amplified, e.g. invader assays (10,11). There are various other amplification technologies that do not fit readily into these categories, such as Q beta replicase (12) and branched DNA (13,14). Owing to the problems associated with current screening systems, it is widely accepted that test results should be confirmed by using more than one type of assay. More technologies are therefore needed, to complement those already available.

We describe a novel dual enzyme technology, termed signal-mediated amplification of RNA technology (SMART), which is based on the formation of a three-way junction (3WJ) structure. This assay relies on signal amplification and does not require thermal cycling or involve the copying of target

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sequences. The assay generates a signal that is highly target-dependent and is appropriate for the detection of different DNA or RNA targets.

The SMART assay consists of two single-stranded oligonucleotide probes (extension and template): each probe includes one region that can hybridise to the target (at adjacent sites) and another, much shorter, region that hybridises to the other probe. The two probes are designed such that they can only anneal to each other in the presence of the specific target, so forming a 3WJ (Fig. 1A). Following 3WJ formation, *Bst* DNA polymerase extends the short (extension) probe by copying the opposing template probe to produce a double-stranded T7 RNA polymerase promoter sequence (Fig. 1B). The assay relies on the fact that only the double-stranded promoter is fully functional, and allows T7 RNA polymerase to generate multiple copies of an RNA signal. The signal is therefore target dependent, being produced only when a specific target is present to allow 3WJ formation. The RNA signal may itself be amplified. The RNA signal binds to a second template oligonucleotide (probe for RNA amplification) and is extended by DNA polymerase to generate a double-stranded promoter, leading to transcription which increases the RNA yield, so improving the sensitivity of the assay (Fig. 1B). The entire process requires two enzymes (*Bst* DNA polymerase and T7 RNA polymerase), which are capable of functioning under the same reaction conditions; hence the reaction can be performed in a single tube.

The RNA signal is detected and quantified by an enzyme-linked oligosorbent assay (ELOSA) (Fig. 1C). This end detection method involves specific capture of the RNA signal, together with the binding of an alkaline phosphatase-labelled probe. Breakdown of the alkaline phosphatase substrate is measured, by colour change, using a standard 96-well plate reader; therefore, multiple samples may be quantified simultaneously and gel analysis is not required. The signal-specificity of the end detection system provides an extra control step to ensure that signal generated by the assay is target-dependent.

The SMART process is based on the amplification of a signal: the nucleic acid target sequence is not itself amplified. The advantage of this is that the system is easily adapted for the detection of different target sequences since probes for signal amplification, capture and detection, and all reaction conditions remain the same.

In this work, we used various single-stranded synthetic oligonucleotides as targets to test the SMART assay. The assay was then used to detect targets in complex genetic material. We present data showing the generation of a signal from both genomic DNA and total RNA, using *Escherichia coli* sequences as model systems. Signal was also generated from *E.coli* without prior nucleic acid extraction, showing that target sample purification is not always required.

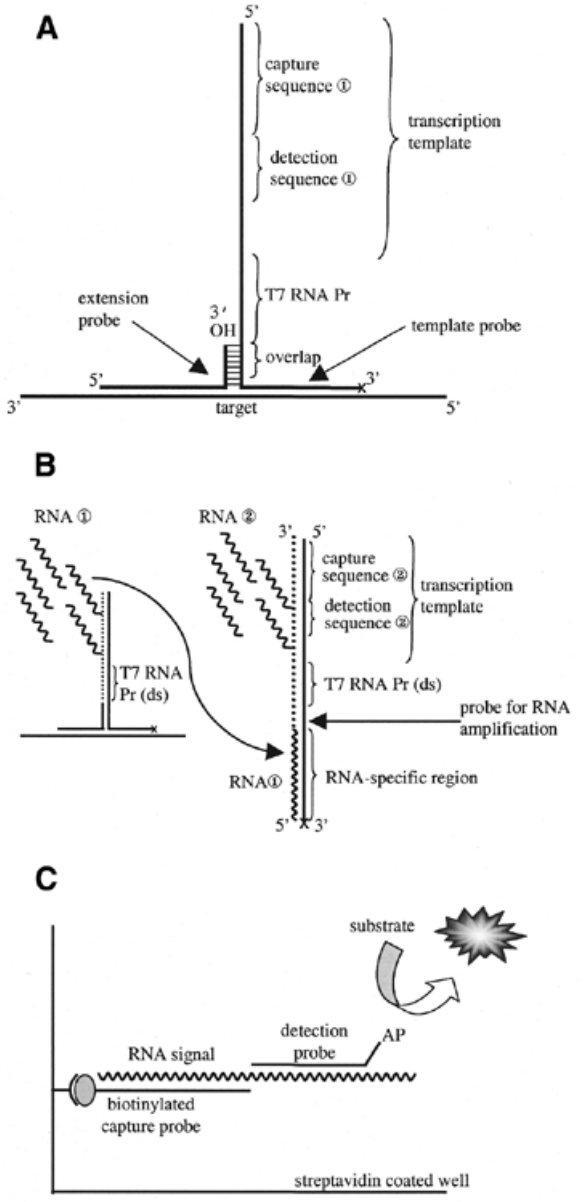

Figure 1. The SMART assay. (**A**) Formation of a 3WJ. Extension and template probes anneal to the target, and only then to each other (overlap between the two probes is only 8 bp). The short extension probe has a free 3′-OH to allow extension. The template probe includes a single-stranded (non-functional) T7 RNA polymerase promoter (Pr) and sequences to allow the capture and detection of the RNA signal. The 3′ end of the template probe is blocked (x) by phosphorylation to prevent extension. (**B**) Extension and transcription generate an RNA signal. *Bst* DNA polymerase extension of the extension probe generates a double-stranded (ds), hence functional, T7 RNA polymerase promoter (Pr), allowing transcription of multiple copies of an RNA signal (RNA1) by T7 RNA polymerase. If required, RNA1 anneals to a second template (probe for RNA amplification), leading to further extension and transcription by the DNA and RNA polymerases to generate increased amounts of a second RNA signal (RNA2). (**C**) Detection and quantification of the RNA signal by ELOSA. Specific sequences included in RNA signals 1 and 2 allow capture, via a biotinylated probe, onto the streptavidin-coated well of a microtitre plate and detection and quantification via an alkaline phosphatase (AP)-linked probe. Wash steps remove unbound probe and the colour change of AP substrate (4-nitrophenyl phosphate) is followed for 30 min (37°C) at 405 nm. By comparing the AP activities of different samples with a standard curve, the relative amounts of signal may be calculated.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotide probes, synthesised by phosphoramidite chemistry using an Applied Biosystems 380A synthesiser, and HPLC or FPLC purified using standard techniques, were obtained from Oswel Research Products Ltd, UK. The synthetic target sequences are shown in Table 1. The probes

Table 1. Synthetic target sequences

Target	Sequence of synthetic target
CFTR	5′-GTTGGCATGCTTTGATGACGCTTCTGTATCTATATTCATCATAGGAAACACC↓AAAGATGATATTTTCTTTAATGGTGCCAGGC-ATAATCCAGGAAAACTGAGAACAGAATGAAATTCTTC-3′
23S	5′-TGGGCGCTGGAGAACTGAGGGGGGCTGCTCCTAGTACGAGAGGACCGGAGTGGACGCATCACTGGTGTTCGGGTTGTCATGCC↓AATGGCACTGCCCGGTAGCTAAATGCGGAAGAGATAAGTGCTGAAAGCATCTAAGC-3′
Mtb	5′-CGAGACGATCAACGGCCTATACAAGACCGAGCT↓GATCAAACCCGGCAAGCCCTGGCG-3′
HBV	5′-TCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCT↓CTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCT-3′

CFTR, human cystic fibrosis transmembrane conductance regulator gene (GenBank accession no. 6995995); 23S, *E.coli* K12 MG1655 23S rRNA gene sequence (GenBank accession no. 2367276); Mtb, *M.tuberculosis* IS6110 sequence (GenBank accession no. 6523392); HBV, Hepatitis B virus small surface antigen (GenBank accession no. 6684102). SMART probe hybridising regions are underlined. 3WJ sites (where target-binding regions of extension and template probes meet) are indicated by arrows.

Table 2. Specific probes to form 3WJ with 23S target

3WJ probe	Sequence
23S-ext	5′-GCATTTAGCTACCGGGCAGTGCCATT*TTCGAAAT*-3′
23S-tem	5′-TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGCTCTCTCTCCCTATAGTGAGTCGTATTA*ATTTCGAA*LGGCATGACAACCCGAAC-ACCAGTGATx-3′

Target-hybridising regions are underlined. Positions of a chemical linker molecule (L), when included, and 3′ phosphorylation to block extension (x) are indicated. The region of overlap between the two probes (italics) and the template sequence for transcription, optimised during this study, were kept the same when detecting any target. Target-hybridising regions were altered to be complementary to each target listed in Table 1.

Table 3. Probes for further amplification and/or end detection of the RNA signal generated from a 3WJ

Probe	No further amplification of RNA signal (detect RNA1)	With further amplification of RNA signal (detect RNA2)
Probe for RNA amplification	N/A	5′-TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATA-GTGAGTCGTATTAATTTCTCGTCTTCCLGGTCTCTCCTCTCAAGCCTCAGC-GCTCTCTCTCCCx-3′
Capture probe	5′Bio-TCTGCTCGTCTTCCGGTC-TCTCCTC-3′	5′ Bio-TCTGCTGCCTGCTTGTCTGCGTTCT-3′
Detection probe	5′-TCAAGCCTCAGC-3′AP	5′-GGATATCACCCG-3′AP
Synthetic signal for ELOSA standard curve	5′-GGGAGAGAGAGCGCTGAGGC-TTGAGAGGAGAGACCGGAAGA-CGA-3′	5′-GGAAGCGAGAACTCGGGTGATATCCAGAACGCAGACAAGCAGGCA-3′

Chemical linker molecule (L) and 3′ phosphorylation (x) are indicated. Bio, biotin; AP, alkaline phosphatase.

used to generate a target-specific 3WJ for the *E.coli* K12 23S target are listed in Table 2. The non-specific background signal was reduced by including a non-nucleosidic linker, such as a hexaethylene glycol in the template probe at the position of the 3WJ (indicated L in Table 2). Probes to detect synthetic targets based on the human cystic fibrosis transmembrane conductance regulator (CFTR) gene, *Mycobacterium tuberculosis* (Mtb) and Hepatitis B virus (HBV) sequences were designed in the same way, by changing the target-hybridising regions only. In this paper, the probes will be referred to by the target name, followed by -ext or -tem; e.g. CFTR-ext is the extension probe for the CFTR target and CFTR-tem the template probe. Probes for the amplification and end detection of RNA signal, generated from any target, are shown in Table 3.

Probe design

Template probes included the T7 RNA polymerase promoter sequence (15) and surrounding sequences that favour efficient transcription (16). Target sequences, probes and RNA signals were designed to include as little potential secondary structure as possible, according to the *mfold* computer program (17–19). Probe/probe and probe/target melting temperatures were calculated using the Tm computer program (20).

Extension and transcription off a 3WJ to produce an RNA signal

Target DNA was added to a mixture containing 2 µl 10× transcription buffer (Ambion), extension probe and template probe (used at varying concentrations, depending on the amount of

target to be detected, see below), 1 mM spermine and ultrapure, sterile, RNase-free H_2O to give a final volume of 14 µl. Samples were mixed, heated at 95°C for 5 min (PTC-200 Peltier thermal cycler, MJ Research), then ramped down to 41°C (0.1°C/s). When the samples had been at 41°C for 1 h, 6 µl of a solution containing dNTPs [5 µM final for each dNTP (A-, C-, G- and T-)], NTPs [2 mM final for each NTP (A-, C-, G- and U-)] (dNTPs and NTPs from Amersham Pharmacia Biotech), 4 U *Bst* (3′–5′ exo⁻) DNA polymerase (New England Biolabs) and 240 U T7 RNA polymerase (Ambion) were added and the reaction was incubated at 41°C for 3 h. (Ambion unit definition: 1 U catalyses the incorporation of 1 nmol NTP into acid-insoluble material in 60 min. This differs from the definition commonly used by other manufacturers: 1 U catalyses the incorporation of 5 nmol NTP in 60 min.) Samples could be stored at –20°C or the signals were quantified immediately by ELOSA. Alternatively, when greater sensitivity was required, the RNA signal was amplified further before quantification (see below). Optimum probe concentrations for 3WJ reactions depended on the target concentration. The favoured concentrations for 50 fmol synthetic target were 60 fmol extension and 50 fmol template, whereas for 1 fmol target, 50 fmol extension and 10 fmol template were used.

Further amplification of the RNA signal to increase sensitivity

Reactions were set up as described above, except that the samples were incubated for only 2 h at 41°C after the addition of the enzyme mix. Samples were then brought to room temperature and 20 fmol probe for RNA amplification, followed by a mix containing 4.5 µl 10× transcription buffer, dNTPs (to give 50 µM final concentration), NTPs (2 mM final), 4 U *Bst* DNA polymerase, 160 U T7 RNA polymerase (see unit definition above) and ultrapure, sterile, RNase-free H_2O to give a final reaction volume of 45 µl, were added. Samples were incubated at 37°C for 2 h. Sample storage and signal quantification were as described for standard 3WJ reactions. For reactions involving further amplification of signal, the favoured probe concentrations for the 3WJ step were 5 fmol extension and 1 fmol template.

End detection of the RNA signal

The RNA signal, generated directly from a 3WJ or following further amplification, was assayed by ELOSA. The RNA signal sequence includes regions for capture, via a biotinylated probe, and detection using a further probe linked to alkaline phosphatase (Fig. 1; Table 3). Biotinylated capture probe (0.9 pmol) and alkaline phosphatase-labelled detection probe (6 pmol) were added to each well of a streptavidin-coated Combiplate (Life Sciences International), in hybridisation buffer (50 mM Tris–HCl pH 8.0, 1 M NaCl, 20 mM EDTA and 1% w/v BSA). A sample of the reaction mixture to be quantified (5–20 µl from 3WJ reaction or 5–40 µl from 3WJ amplified further) was then added, bringing the total volume to 150 µl per well. Samples were incubated at room temperature on a platform shaker at 300 r.p.m. for 1 h. Unbound material was removed from wells by washing four times with 200 µl wash buffer (1× TBS/0.1% Tween-20), then once with 200 µl alkaline phosphatase substrate buffer (Boehringer Mannheim). Substrate [4-nitrophenyl phosphate (Boehringer Mannheim) at 5 mg/ml in substrate buffer] was added (180 µl/well) and alkaline phosphatase activity was measured by reading absorbance at 405 nm every 2 min for 30 min at 37°C (Labsystems integrated EIA Management system). Increase in absorbance per minute for each sample was compared to a standard curve, generated using dilutions of a synthetic signal oligonucleotide. This allowed the relative amount of RNA produced in each extension/transcription reaction to be estimated.

Growth of *E.coli* and extraction of nucleic acid

Escherichia coli K12 MG1655 was grown in 5 ml batches in universal tubes at 37°C, shaking at 200 r.p.m. Cells for direct addition to SMART assays were grown to mid log phase in tryptone broth (10 g tryptone, 0.5 g NaCl per l) and used immediately. Samples of culture (1–2 µl, neat or diluted in sterile tryptone broth) were added to 3WJ reactions, set up as described above. The number of cells added to each assay was estimated by plating out serial dilutions of the culture and performing colony counts.

Nucleic acid extractions were performed on fresh, mid log phase cells grown in L-Broth (10 g tryptone, 5 g yeast extract, 0.5 g NaCl per l). Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions, eluting DNA from 1.5 ml mid log culture in a final volume of 100 µl. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, eluting RNA from 1.5 ml mid log culture in a final volume of 50 µl. Nucleic acid concentrations were determined by standard techniques.

RESULTS

Detection of different synthetic target sequences

Several synthetic target oligonucleotides, matching sequences from the human CFTR gene (GenBank accession no. 6995995), the IS6110 sequence from Mtb (GenBank accession no. 6523392) and the small surface antigen gene of HBV, isolate rbo11 (GenBank accession no. 6684102) (Table 1) were detected using specific SMART probes (Table 2). For detection of the different targets, only the target-hybridising regions of the probes were changed to make them specific for each new target. The sequence of the RNA signal, determined by the template probe sequence, was identical for each of the different target sequences, meaning that the same capture and detection conditions could be used for all the targets. Results from these experiments, using 50 fmol of each synthetic target under conditions without further signal amplification, are shown in Figure 2A. In each case, target-dependent signal was generated by the assay. The sensitivity was improved by lowering the probe concentrations, and a specific signal was obtained from 1 fmol CFTR target (Fig. 2B).

Detection of DNA and RNA synthetic targets

Two single-stranded synthetic CFTR target oligonucleotides (Table 1) were used as targets in the SMART assay. The targets were identical to each other except that one was DNA and the other RNA. Using the same conditions for each target (60 fmol CFTR-ext and 50 fmol CFTR-tem for 50 fmol target), both targets were successfully detected without further signal amplification (Fig. 3).

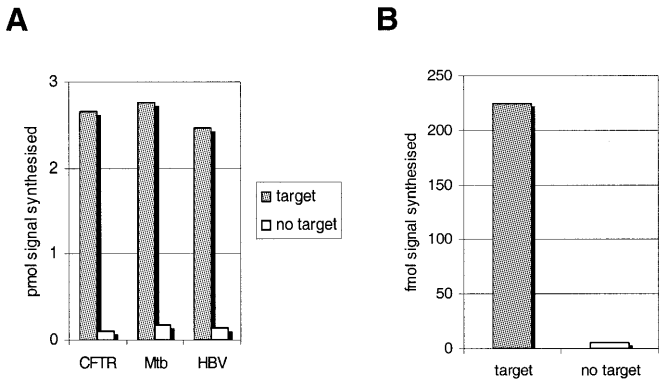

Figure 2. (**A**) Comparison of signals from different synthetic targets. Synthetic target sequences were designed for: CFTR, human cystic fibrosis transmembrane conductance regulator gene (GenBank accession no. 6995995); Mtb, the IS6110 sequence from *M.tuberculosis* (GenBank accession no. 6523392); and HBV, the small surface antigen of Hepatitis B virus, isolate rbo11 (GenBank accession no. 6684102). Extension and template probes were identical, except that the target-hybridising regions were altered, making them specific for the different targets. Probes were added at 60 fmol extension and 50 fmol template for 50 fmol target. Negative controls contained no target. The amount of RNA signal produced was determined by ELOSA (detecting RNA1). (**B**) Signal generated from 1 fmol target. CFTR probes were added at 50 fmol extension and 10 fmol template for 1 fmol target. Other reaction conditions were unaltered. Negative controls contained no target.

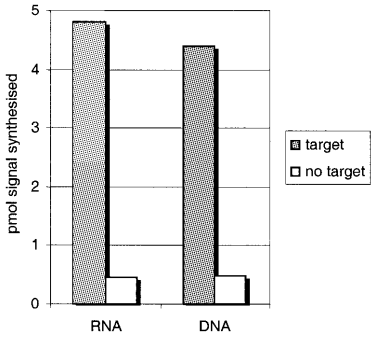

Figure 3. Comparison of the signals generated from DNA or RNA targets. Both targets (single-stranded, synthetic oligonucleotides) had the CFTR sequence (GenBank accession no. 6995995), identical except for the T or U residues and the dNTPs or NTPs. The same probes were used for both targets, added at 60 fmol CFTR-ext and 50 fmol CFTR-tem for 50 fmol target. Negative controls contained no target oligonucleotide. The amount of RNA signal produced was determined by ELOSA (detecting RNA1).

Effect of a linker molecule in the template probe

23S template probes with and without a hexaethylene glycol linker molecule (Table 2) were compared, using the single-stranded 23S target oligonucleotide (Table 1). The linker molecule improved the signal:noise ratio by both increasing signal and reducing background. The benefit of the linker was very marked when further amplification of the RNA signal was performed and specific signal was successfully generated from 50 amol synthetic target (Fig. 4).

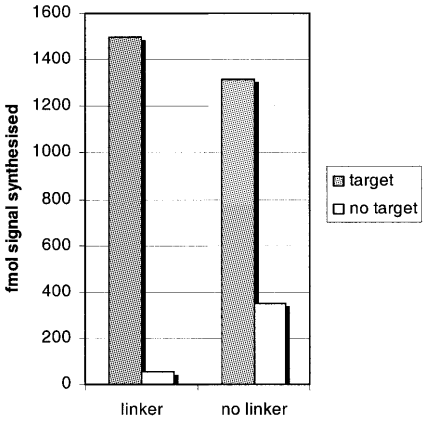

Figure 4. Effect of a linker molecule in the template probe. 23S-tem probes were used with or without a hexaethylene glycol linker at the 3WJ site. All other probes were identical in the different reaction samples. Negative controls contained no target oligonucleotide. All 3WJ reaction samples, including the no target controls, contained 100 ng non-target genomic DNA (*M.lysodeikticus*). The RNA signal produced was amplified further before signal detection and quantification by ELOSA (detecting RNA2). Probe concentrations were 5 fmol 23S-ext and 1 fmol 23S-tem with 20 fmol probe for RNA amplification for 50 amol 23S synthetic target.

Probing genomic DNA and total RNA from *E.coli*

Nucleic acid extracted from *E.coli* K12 was quantified and known amounts of either genomic DNA or total RNA were probed for the 23S target sequence using 23S-ext and 23S-tem probes (Table 2). The RNA signal from the 3WJ was amplified further before quantification by ELOSA. Signals were successfully generated from both types of complex target material (Fig. 5), even in the presence of excess amounts of non-target genomic material (DNA from *Micrococcus lysodeikticus*). Very little signal was generated from the non-target genomic DNA, showing that the assay is specific. The 23S sequence was detected from 10 ng genomic DNA, and from 0.1 ng total RNA.

Probing crude *E.coli* cultures

Samples of mid log phase cultures of *E.coli* K12 were diluted and added to SMART reactions, without prior nucleic acid extraction, and probed for the 23S sequence using 23S-ext and 23S-tem probes. The RNA signal was further amplified before detection by ELOSA. Signals were successfully generated from the crude *E.coli* cells (down to 10^4 cells per assay), showing that extensive sample preparation may not be required for the SMART assay (Fig. 6).

DISCUSSION

We have designed a novel nucleic acid amplification assay (SMART) that does not involve thermal cycling. The isothermal system is based on the production of a functional T7 RNA polymerase promoter, leading to the production of a target-dependent RNA signal, which can be amplified further. The level of amplification currently achieved allowed us to generate signal from 50 amol single-stranded synthetic target,

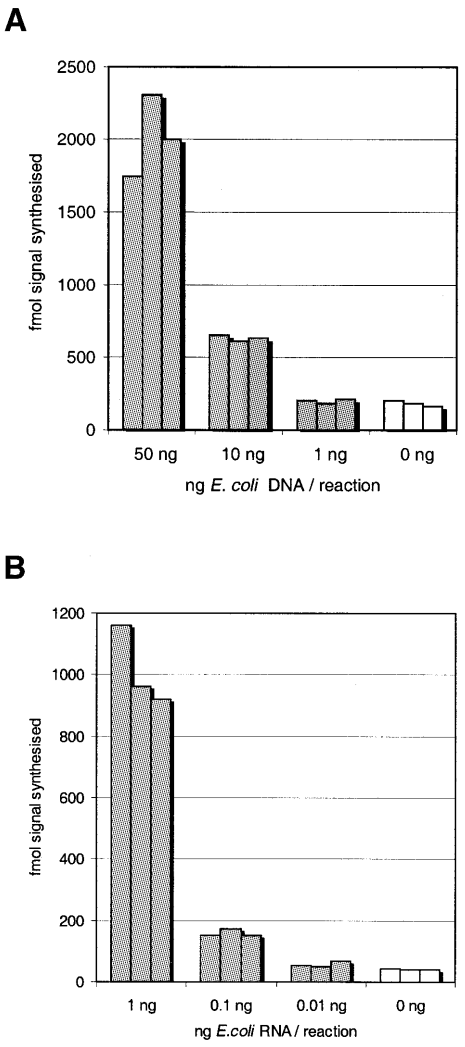

Figure 5. Generation of signals from complex targets. (**A**) Detection of the gene encoding 23S rRNA in genomic DNA from *E.coli* K12 MG1655. Genomic DNA extracted from mid log phase cells was quantified and used in SMART reactions. All 3WJ reaction samples, including those containing no *E.coli* DNA, were made up to a total of 50 ng genomic DNA with non-target (*Micrococcus* sp.) DNA. The RNA signal produced was amplified further before signal detection and quantification by ELOSA (detecting RNA2). Results of triplicate reactions are shown. (**B**) Detection of 23S rRNA from *E.coli* K12 MG1655 total RNA. Total RNA extracted from mid log phase cells was quantified and used in SMART reactions. All 3WJ reaction samples, including those containing no *E.coli* RNA, contained 100 ng non-target genomic DNA (*Micrococcus* sp.). The RNA signal produced was amplified further before signal detection and quantification by ELOSA (detecting RNA2). Results of triplicate reactions are shown. The same probes (23S-ext and 23S-tem) were used for the DNA and RNA targets: and were used at 5 fmol extension and 1 fmol template (containing linker molecule), with 20 fmol probe for RNA amplification.

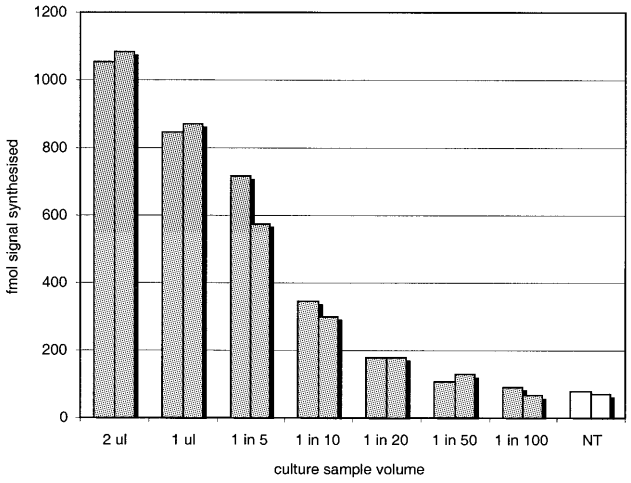

Figure 6. Detection of 23S target in a crude *E.coli* culture. *Escherichia coli* K12 MG1655 was grown to mid log phase in tryptone broth. SMART reactions were set up, using dilutions of a fresh *E.coli* culture (2 or 1 µl neat, or 1 µl of various dilutions in sterile tryptone broth) as the target. Cell counts were performed on the culture used. One microlitre of neat culture contained 3×10^5 cells. The no target control sample (NT) contained 1 µl sterile tryptone broth. All 3WJ reaction samples contained 100 ng non-target nucleic acid (*Micrococcus* sp. genomic DNA). Standard reaction conditions were used, amplifying the RNA signal produced from the 3WJ before signal detection and quantification by ELOSA (detecting RNA2). Results of duplicate reactions are shown. Probe concentrations were 5 fmol 23S-ext, 1 fmol 23S-tem (containing linker molecule) and 20 fmol probe for RNA amplification.

10 ng genomic DNA and 0.1 ng total RNA, when probing for the 23S sequence of *E.coli*, which was used as a model system for development purposes. We are now working to improve the level of amplification and hence sensitivity on other targets, more appropriate for diagnostic assays.

The assay is based on the interaction of two oligonucleotide probes with a specific target to form a 3WJ, generating a functional promoter via extension of one probe. The double-stranded promoter has to be generated by extension, because tests showed that if it was formed directly by sequences on the template probe and a longer extension probe, these came together too readily and produced signal in the absence of target. We chose to work with an extension/template probe overlap of 8 bp, although 6–8 bp were acceptable. With longer overlaps the signal would not be dependent on target, whereas shorter overlaps produce very low signals (data not shown). Even using a 6–8 bp overlap, some background signal (in the absence of target) was still observed, due to a low level of transcription off the single-stranded template probe. This background was reduced by optimisation of the template probe concentration and by incorporating a non-nucleosidic linker (such as a hexaethylene glycol) into the template probe at the junction site. The inclusion of a linker increased the signal:noise ratio (Fig. 4). Various linker molecules were tested: hexaethylene glycols and propane-, pentane- and octane-diols all had similar effects (data not shown). The linker moiety not only reduced the level of background transcription off the single-stranded template in the absence of target, but also increased the specific signal, probably by stabilising the 3WJ structure, as has been reported for 3WJs containing unpaired nucleotides (21). The benefit of the linker molecule was greatest in reactions when the RNA signal from the 3WJ was amplified further. This type of reaction has the potential for greater problems with background. Since both signal and background RNA would be amplified in the second step, controlling the background from the 3WJ is particularly important.

Generation of signal from a 3WJ relies on the ability of both DNA and RNA polymerases to function under the same

reaction conditions. The reaction conditions used were a compromise for both enzymes: the buffer conditions were optimised for T7 RNA polymerase, while allowing sufficient DNA polymerase activity. The choice of DNA polymerase was important, as not all those tested were active in the T7 transcription buffer. Both Klenow and *Bst* DNA polymerases were used successfully, but *Bst* was favoured, as it was more processive (data not shown). The transcription template sequence was optimised to increase the yield of RNA.

We tested the SMART assay using various targets as model systems. Using single-stranded synthetic target oligonucleotides, the assay was successfully adapted for the detection of different target sequences, either DNA or RNA without changing the reaction conditions. The assay was shown to specifically detect sequences in genomic DNA and total RNA from *E.coli*. Signals were also generated from crude *E.coli* cultures, without prior nucleic acid extraction. The addition of excess amounts of non-target genomic DNA did not interfere with the assay.

One clear advantage of SMART over standard PCR techniques is that the SMART signal is single-stranded and has the same sequence regardless of the target being detected. This facilitates hybridisation-based detection techniques, meaning that gel analysis is not required. Since the amount of RNA produced is dependent on the amount of target present, the assay has the potential to be quantitative and therefore ideal for the enumeration of bacteria or viruses from clinical samples.

Future work will focus on developing a system that has both amplification and end detection taking place in a single tube. Real time detection might be possible, by replacing the ELOSA capture and detection system with a molecular beacon (22,23). The sensitivity and specificity of the assay are still being improved. Initial work on single-stranded, synthetic targets has shown that the SMART assay is capable of detecting single nucleotide polymorphisms (SNPs) within the target sequence (unpublished data). Ultimately, following further optimisation of the assay for SNP detection in complex targets, we hope to have a system capable of detecting not only specific strains of infectious disease agents, but also human sequence polymorphisms associated with genetic disease.

CONCLUSIONS

SMART is a novel, isothermal nucleic acid amplification technology, which generates a quantifiable, target-dependent signal. Amplification does not involve copying the target sequence; instead an RNA signal with a specific sequence is produced which may be the same irrespective of the target sequence. Consequently, it is relatively simple to adapt the SMART assay for the detection of any target, without having to change reaction and detection conditions. Both DNA and RNA targets can be detected by the same method, which does not require reverse transcriptase or RNaseH. When fully developed, the system will have broad applicability, for example in the detection of infectious agents, genetic variant screening and gene expression analysis.

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REFERENCES

1. Abbaszadegan,M., Huber,M.S., Gerba,C.P. and Pepper,I.L. (1997) Detection of viable *Giardia* cysts by amplification of heat shock-induced mRNA. *Appl. Environ. Microbiol.*, **63**, 324–328.
2. Bej,A.K., Mahbubani,M.H. and Atlas,R.M. (1991) Detection of viable *Legionella pneumophila* in water by polymerase chain reaction and gene probe methods. *Appl. Environ. Microbiol.*, **57**, 597–600.
3. Hellyer,T.J., DesJardin,L.E., Hehman,G.L., Cave,M.D. and Eisenach,K.D. (1999) Quantitative analysis of mRNA as a marker for viability of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.*, **37**, 290–295.
4. Compton,J., (1991) Nucleic acid sequence-based amplification. *Nature*, **350**, 91–92.
5. Kievits,T., van Gemen,B., van Strijp,D., Schukkink,R., Dircks,M., Adriaanse,H., Malek,L., Sooknanan,R. and Lens,P. (1991) NASBA isothermal enzymatic *in vitro* nucleic acid amplification optimised for the diagnosis of HIV-1 infection. *J. Virol. Methods*, **35**, 273–286.
6. Hill,C.S. (1996) Molecular diagnostics for infectious disease. *J. Clin. Ligand Assay*, **19**, 43–51.
7. Guatelli,J.C., Whitfield,K.M., Kwoh,D.Y., Barringer,K.J., Richman,D.D. and Gingeras,T.R. (1990) Isothermal, *in vitro* amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. *Proc. Natl Acad. Sci. USA*, **87**, 1874–1878.
8. Chadwick,N., Bruce,I., Davies,M., van Gemen,B., Schukkink,R., Khan,K., Pounder,R. and Wakefield,A. (1998) A sensitive and robust method for measles RNA detection. *J. Virol. Methods*, **70**, 59–70.
9. Chan,A.B. and Fox,J.D. (1999) NASBA and other transcription-based amplification methods for research and diagnostic microbiology. *Rev. Med. Microbiol.*, **10**, 185–196.
10. Lyamichev,V., Mast,A.L., Hall,J.G., Prudent,J.R., Kaiser,M.W., Takova,T., Kwiatkowski,R.W., Sandere,T.J., de Arruda,M., Arco,D.A., Neri,B.P. and Brow,M.A.D. (1999) Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. *Nat. Biotechnol.*, **17**, 292–296.
11. Ryan,D., Nuccie,B. and Arvan,D. (1999) Non-PCR-dependent detection of the factor V Leiden mutation from genomic DNA using a homogeneous invader microtitre plate assay. *Mol. Diagn.*, **4**, 135–144.
12. Lizardi,P.M., Guerra,C.E., Lomeli,H., Tussie-Luna,I. and Kramer,F.R. (1988) Exponential amplification of recombinant-RNA hybridization probes. *Biotechnology*, **6**, 1197–1202.
13. Todd,J., Pachl,C., White,R., Yeghiazarian,T., Johnson,P., Taylor,B., Holodniy,M., Kern,D., Hamren,S., Chernoff,D. and Urdea,M. (1995) Performance characteristics for the quantitation of plasma HIV-1 RNA using branched DNA signal amplification technology. *J. AIDS Hum. Retrovirol.*, **10**, S35–S44.
14. Pawlotsky,J.-M., Martinot-Peignoux,M., Poveda,J.-D., Bastie,A., Le Breton,V., Darthuy,F., Rémiré,J., Erlinger,S., Dhumeaux,D. and Marcellin,P. (1999) Quantification of hepatitis C virus RNA in serum by branched DNA-based signal amplification assays. *J. Virol. Methods*, **79**, 227–235.
15. Milligan,J.F., Groebe,D.R, Witherell,G.W. and Uhlenbeck,O.C. (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.*, **15**, 8783–8798.
16. Ikeda,R.A., Ligman,C.M. and Warshamana,S. (1992) T7 promoter contacts essential for promoter activity *in vivo*. *Nucleic Acids Res.*, **20**, 2517–2524.
17. SantaLucia,J.Jr (1998) A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc. Natl Acad. Sci. USA.*, **95**, 1460–1465.
18. Zuker,M., Mathews,D.H. and Turner,D.H. (1999) Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide. In Barciszewski,J. and Clark,B.F.C. (eds), *A Practical Guide in RNA Biochemistry and Biotechnology*. NATO ASI Series, Kluwer Academic Publishers, pp. 11–43.
19. Mathews,D.H., Sabina,J., Zuker,M. and Turner,D.H. (1999) Expanded sequence dependence of thermodynamic parameters provides robust prediction of RNA secondary structure. *J. Mol. Biol.*, **288**, 911–940.

20. Breslauer,K.J., Frank,R., Blocker,H. and Marky,L.A. (1986) Predicting DNA duplex stability from the base sequence. *Proc. Natl Acad. Sci. USA*, **83**, 3746–3750.
21. Leontis,N.B., Kwok,W. and Newman,J.S. (1991) Stability and structure of 3-way DNA junctions containing unpaired nucleotides. *Nucleic Acids Res.*, **19**, 759–766.
22. Tyagi,S. and Kramer,F.R. (1996) Molecular beacons: probes that fluoresce upon hybridisation. *Nat. Biotechnol.*, **14**, 303–308.
23. Tyagi,S., Bratu,D.P. and Kramer,F.R. (1998) Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.*, **16**, 49–53.