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Recombinase-Based Isothermal Amplification of Nucleic Acids with Self-Avoiding Molecular Recognition Systems (SAMRS)

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Recombinase polymerase amplification (RPA) is an isothermal method to amplify nucleic acid sequences without the temperature cycling that classical PCR uses. Instead of using heat to denature the DNA duplex, RPA uses recombination enzymes to swap single-stranded primers into the duplex DNA product; these are then extended using a strand-displacing polymerase to complete the cycle. Because RPA runs at low temperatures, it never forces the system to recreate base-pairs following Watson–Crick rules, and therefore it produces undesired products that impede the amplification of the desired product, complicating downstream analysis. Herein, we show that most of these undesired side products can be avoided if the primers contain components of a self-avoiding molecular recognition system (SAMRS). Given the precision that is necessary in the recombination systems for them to function biologically, it is surprising that they accept SAMRS. SAMRS-RPA is expected to be a powerful tool within the range of amplification techniques available to scientists.

Introduction

Assays that target DNA and RNA (collectively xNA) are often viewed as “gold standards” for biomolecular analysis, in part, because they directly detect the species presumed to control the biological phenomena of interest. For infectious diseases, xNA sequences define the pathogen; furthermore, they can characterize the genotype of (for example) a drug-resistant phenotype. For genetic diseases, cancers, and other conditions that arise from genetic mutations, sequence analysis can directly detect etiology. Additionally, xNA-targeted assays can be used for monitoring the environment and public health, especially when they used to survey public spaces for pathogens (e.g. cruise ships that have suffered norovirus outbreaks or urinals).

xNA-targeted assays are especially useful because a DNA sequence in the causal organism can be amplified using the polymerase chain reaction (PCR).[1] With this method, a small number of xNA molecules can direct the synthesis of more identical ones, with the amount of amplification depending (in principle) only on the number of primers introduced into the PCR mixture. The resulting amplicons can then be easily detected, even (in some cases) by simple visual inspection.

As compelling as these reasons are for doing xNA-targeted tests in the clinic, they are equally compelling for tests intended to be used at points-of-care, in the field, or even in the home. Unfortunately, standard PCR instruments are difficult to adapt for use outside of a clinical laboratory. They consume considerable amounts of power, especially during the cooling cycle. This, in turn, requires that they have a certain weight, which makes them inconvenient to carry.

Accordingly, numerous techniques have been developed over the past two decades to allow for the isothermal amplification of nucleic acids, including those known as helicase-dependent amplification (HDA), rolling-circle amplification (RCA), nucleic acid sequence based amplification (NASBA), and loop-mediated amplification (LAMP), among others.[2] These are known as isothermal amplification methods.[3]

One especially useful isothermal amplification method is known as recombinase polymerase amplification (RPA). In RPA, the ability of enzymes involved in the normal homologous recombination processes to accept (as their natural substrate) duplex DNA is coupled with relatively long primers (~30–35 nucleotides) that can be recombined into the target duplex or amplicons from the previous round of amplification.[4] A strand-displacing polymerase then continues the primer extension, to create, after a second cycle, more double-stranded products. These then serve as the starting point for the next cycle of recombination (Figure 1).[4] RPA has been developed and used to detect several viral, bacterial, and parasitic diseases, to detect food-borne pathogens, and to screen anti-cancer drugs.[5]

RPA can be performed in a real-time PCR machine by using a DNA-binding dye (e.g., SYBR Green or EvaGreen) to detect the increase in the desired product.[6] In principle, formation of the correct amplicon can be validated by performing thermal denaturation studies on the products. In practice, however, RPA invariably forms large amounts of side products that, although normally not analyzed, are called primer-dimers.[4] Additional artifacts, generally not characterized, are also formed, to add to the background signal.[7] This is a common problem with isothermal amplification procedures, because the system

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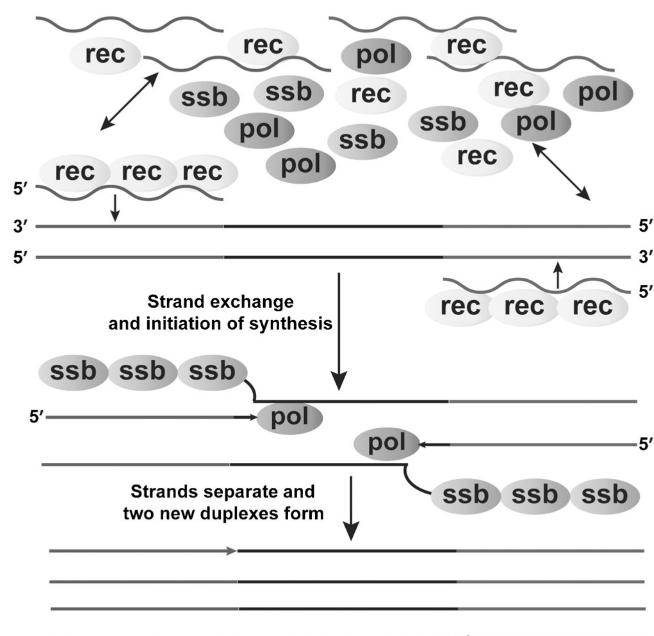

Figure 1. Scheme of the recombinase polymerase assay (RPA). A complex of primers (wavy lines) and DNA recombinase enzymes (rec) scans the double-stranded DNA template for complimentary sequences. Single-strand binding proteins (ssb) displace the complimentary strands and allow the polymerase (pol) to extend the primers and generate another copy of the template, which eventually results in an exponential amplification of the template DNA. Adapted from Piepenburg et al.[4]

is not forced (at any point in the cycle) to reform the Watson–Crick base-pairs by slowly annealing from high to low temperatures.

Recently, we reported the unexpected discovery that several high temperature (but not ultrahigh temperature) DNA polymerases could support standard PCR amplification by using primers that contained components of a self-avoiding molecular recognition system (SAMRS).[8] In a SAMRS oligonucleotide, A, T, G, and C nucleobases are substituted at some (but not necessarily all) sites.[9] In these oligonucleotides, the hydrogen-bonding units are strategically substituted with four replacement nucleotides (A*, T*, G*, and C*, Figure 2). SAMRS A* pairs with natural T, SAMRS T* pairs with natural A, SAMRS G* pairs with natural C, and SAMRS C* pairs with natural G. However, A*:T* and C*:G* base-pairs do not contribute to the stability of the helix, because they either form one or 1.5 bonds (Figure 2). This means that oligonucleotides built from SAMRS components do not interact with each other, no matter what their concentration, which allows them to bind solely to their intended targets. Indeed, in a variety of standard PCR methods, we found that by placing SAMRS nucleotides in the 3′-end of PCR primers, primer-dimers could mostly be avoided.[8–9] This was true even when the primers were extremely poorly designed. For example, Hoshika et al. demonstrated how SAMRS components could prevent primer-dimer formation even when the 3′-sequences of the primers form perfect formal matches in a 9 base-pair duplex.[8]

These results prompted us to ask whether SAMRS components, when added to the primers at their 3′-ends, would support RPA more broadly than primers composed only of natural nucleotides, and avoid the off-target interactions that make RPA difficult to detect by fluorescent dye binding. In particular, because SAMRS:standard base-pairs are joined by only two hydrogen bonds, their contribution to duplex stability is similar to the weaker natural A:T base-pair, and we were concerned that this would prevent RPA from working with SAMRS-containing primers. Furthermore, enzymes for recombination have evolved for billions of years to be highly specific for DNA; these general considerations predict that recombinases might not accept structurally altered nucleotides. Herein, we report data showing that SAMRS is compatible with RPA and reverse transcriptase RPA (RT-RPA).

Results and Discussion

Choice and placement of the SAMRS components

In this implementation of the SAMRS concept, the SAMRS adenine analogue A* was chosen to be 2-aminopurine, the SAMRS guanine analogue G* was hypoxanthine, the SAMRS thymine analogue T* was 2-thiothymine, and the SAMRS cytosine analogue C* was N^4-ethylcytosine (Figure 2). The pairing between SAMRS components and their complementary natural nucleotides (C*:G, G*C, T*:A, and A*:T) are all weak relative to the

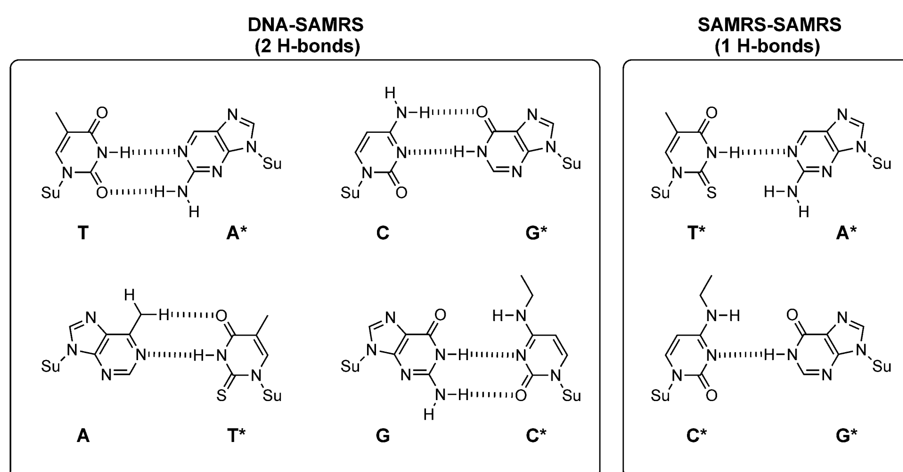

Figure 2. Chemical formulas of the self-avoiding molecular-recognition system (SAMRS). Natural nucleotides (T, A, C, and G) pair with SAMRS nucleotides (A*, T*, G*, and C*) following the Watson–Crick complementarity rules and allowing for a stable double-helix (left). SAMRS nucleotides pair with each other with less stability (right). Hashed lines = hydrogen bonds. Su = sugar backbone of xNA.

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standard C:G pair and comparable to the strength of the A:T pair (because they are joined by just two hydrogen bonds or, in the case of C*:G, a weakened set of three hydrogen bonds). Therefore, we found in practice it is best not to place SAMRS components at all sites in a primer. Rather, we recommend that SAMRS nucleotides be placed near the 3′-ends of the primers, generally at sites $n-1$, $n-2$, $n-3$, and $n-4$, where n is the 3′-terminal (last) site of the oligonucleotide primer. This allows the primer to be synthesized on an oligonucleotide support beginning from a standard nucleotide (which reduces costs). Table 1 shows the primers used in this work obtained by solid-phase synthesis based on this design.

Table 1. Primers for RPA and RT-RPA.

Organism (gene)	Name	Sequence[c]	% of genomes
Influenza A (*M*)[a]	InfA_F1	CTTGAGGCTCTCATGGAATGGCTAAAGACAagacC	70
	InfA_R1	CATTTTGGACAAAGCGTCTACGCTGCagtcC	
M. tuberculosis (*rpoB*)[a]	RpoB_F1	GGTGGTCGCCGCGATCAAGGAGTTCttcgG	96
	RpoB_R1	GACAGTCGGCGCTTGTGGGTCAACCccgaC	
MERS* (*ORF1a*)[b]	*MERS1_F1*	GGTATGTTCTTCCCTTATGACATCGAACTTGTCacagG	100
	MERS1_R1	CAAGAGGTGTTGTCTCGCTCATAGTGGaatgG	

[a] Plasmid DNA. [b] Full-length viral RNA. [c] Primers contain either standard nucleotides (upper case) or SAMRS nucleotides (lower case).

SAMRS RPA provides excellent sensitivity

With standard nucleotides, RPA primers work idiosyncratically, and currently no rules exist to reliably predict which primers will perform best and which might not perform at all. Furthermore, RPA reactions generate background noise; the products that generate this noise are rarely sequenced, but are usually thought to arise from undesired interactions between or within the primers. These artifacts interfere with the desired amplification through the unproductive consumption of primers (tying up the resources of the assay), which produces duplexes that are also stained by the DNA dyes and impedes the amplification of the preferred product. Therefore, it is usually necessary to screen many primers to identify a set for any specific RPA target that work, minimize primer noise, and provide acceptable sensitivity for the assay.

Because SAMRS components that are placed strategically in primers have been shown to prevent the formation of primer-dimers in standard PCR,[8] we hypothesized that these components might also suppress noise in RPA. We did preliminary tests confirm this hypothesis. In our initial experiment, one pair of primers (30 and 32 nucleotides) was designed to amplify a region of the M segment gene of influenza A; analysis of a recent viral genomic database using the in-house developed StrainTargeter software package suggested that the primers would recognize ~70% of the influenza A subtypes recently in circulation. These primers were built with either entirely standard nucleotides (STD primers, for comparison) or with SAMRS nucleotides placed near their 3′-ends (SAMRS primers, Table 1).

With the STD primers applied to an influenza target, the isothermal amplification reactions failed. Analysis by gel electrophoresis showed that the desired product was not formed. Rather, the DNA products appeared as smeared bands. Furthermore, products were seen even in the absence of target DNA (Figure 3 A).

In contrast, with the primers containing SAMRS components, clean amplification products (amplicons) of the correct identity (as judged by length) were produced if the reaction mixture contained the target DNA (Figure 3 A, lane 2). Also, the primers that contained SAMRS components produced no signal by gel

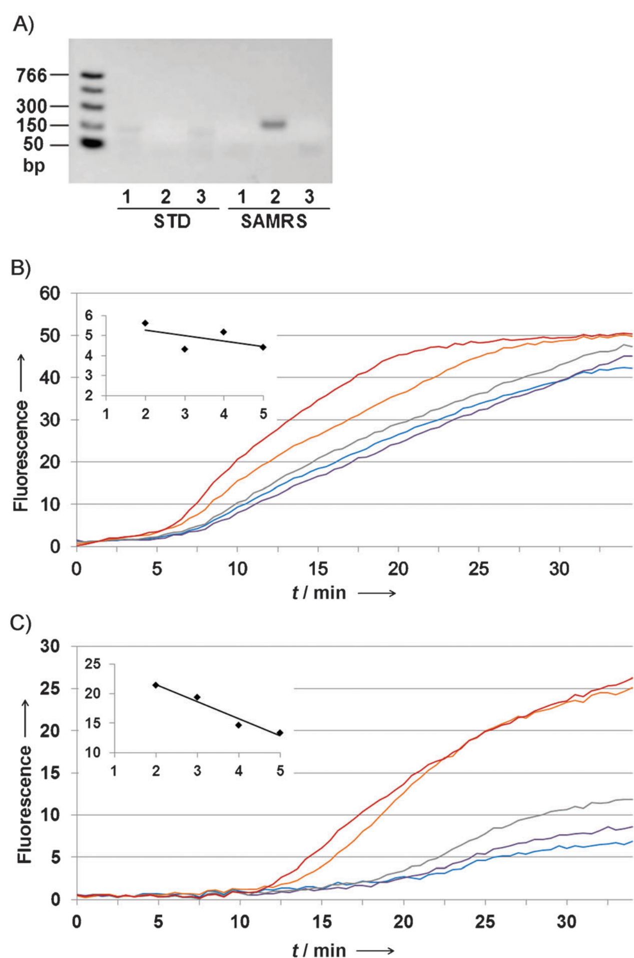

Figure 3. Sensitive amplification of DNA targets with SAMRS primers compared to STD primers. A) Agarose gel electrophoresis of RPA products generated by using influenza A primers containing standard nucleotides (STD) or primers containing standard and SAMRS nucleotides (SAMRS). Templates in these reactions: lane 1: water; lane 2: 10^3 copies of influenza A DNA; lane 3: 10^3 copies of non-specific DNA. The expected size of the product was 133 base-pairs. B–C) Real-time RPA using STD (B) and SAMRS primers (C) for influenza A. Increase in fluorescence was detected over a period of 40 min. DNA templates in these reactions were 0 copies (blue), 10^2 copies (purple), 10^3 copies (gray), 10^4 copies (orange), and 10^5 copies (red). Insets: correlation between C_p [crossing point (*y*-axis, time in minutes)] versus the logarithm of the input template copy number (*x*-axis).

electrophoresis in the negative control experiments that lacked the target DNA (Figure 3 A, lane 3).

The time course of RPA reactions was also examined in real-time by an increase in fluorescence from EvaGreen. In these reactions, varying concentrations of the influenza A DNA template was used with STD or SAMRS primers. As shown in Figure 3 B, real-time amplification with STD primers resulted in a nonspecific signal. The noise arising from this nonspecific signal made it difficult to identify a signal that correlated with the amount of target DNA added (Figure 3 B). In contrast, sensitive and exponential amplification of DNA template was observed by using the SAMRS primers and EvaGreen dye (Figure 3 C). The amplification detection threshold (C_p) depends linearly on the copy number of the input template when SAMRS primers were used (insets, Figures 3 B,C). Thus, by allowing for a simpler detection method and avoiding the need for gel electrophoresis, SAMRS primers simplified the assay. This means that SAMRS primers improve RPA as an amplification method and are potentially useful for point-of-care and low-resource-environment applications. The background signal is not completely eliminated by using SAMRS primers, however, they do reduce noise sufficiently to allow the detection of as few as 1000 molecules of target DNA. Although we explored (without optimization) only one SAMRS primer design, with just four SAMRS components placed on the penultimate four nucleotides, other designs may further reduce the background signal and further increase the sensitivity of these assays.

SAMRS prevent primer-dimer formation and other assay artifacts in RPA reactions

To further understand this improved performance of SAMRS primers for RPA amplification reactions, we tested other targets to assess its reproducibility. For the *rpoB* gene of *Mycobacterium tuberculosis* H37Rv, a set of STD and SAMRS primers (>30 nucleotides) were designed. A section of the *rpoB* gene cloned into the TOPO 2.1 DNA plasmid was used as a template in the RPA reactions.

Like in Figure 3 A, gel electrophoresis of the products of RPA amplification with STD primers showed many bands, mostly lower molecular weight primer-dimers as well as some unknown higher molecular weight bands that are artifacts of the assay. In contrast, the SAMRS primers generated only a single band of cleanly amplified product. SAMRS components in the primers not only prevented the generation of primer-dimers and other unwanted assay artifacts, but also promoted the amplification of the desired products (Figure 4 A).

Real-time amplification with STD primers resulted in a nonspecific signal that does not depend on the amount of target template added to the reaction mixtures (Figure 4 B, left). The amplification threshold does not show any particular correlation with the amount of template added (see inset Figure 4 B, left). In contrast, when SAMRS primers were added to the reactions, the amplification was both sensitive (limit of detection,

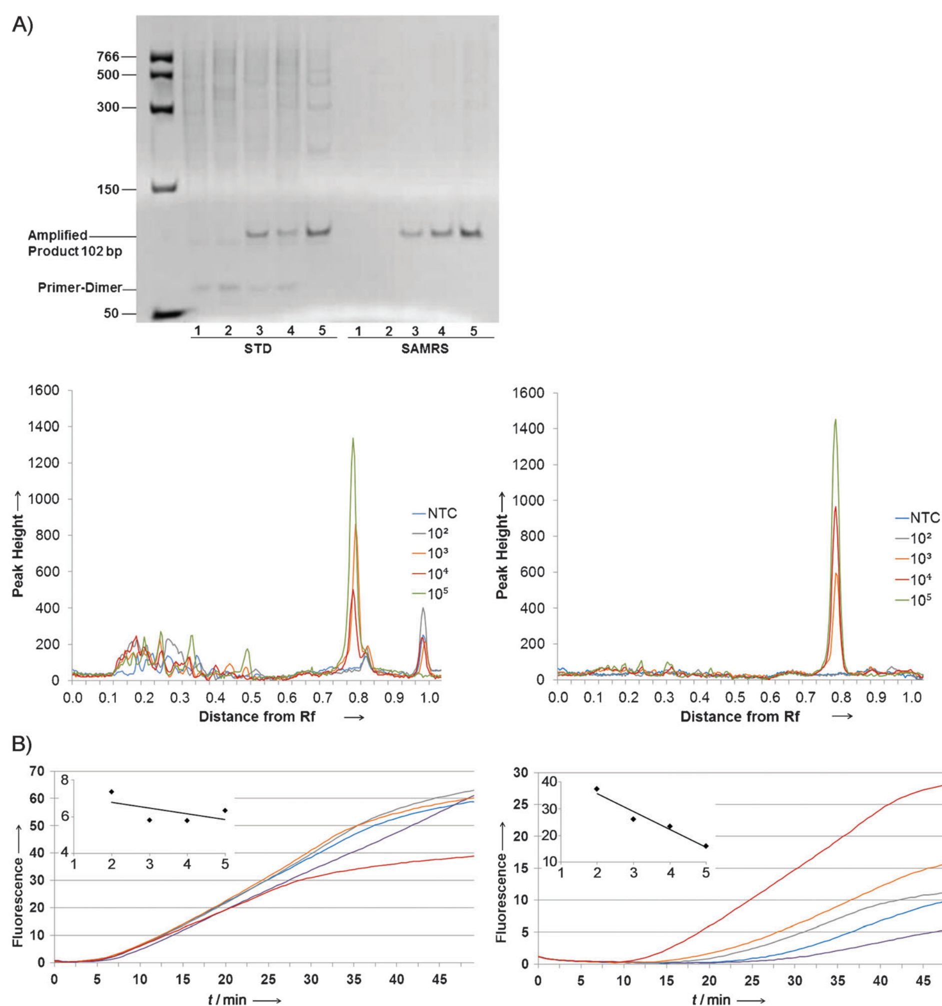

Figure 4. RPA with SAMRS primers. A) Native PAGE of RPA products generated by using primers for *rpoB* containing standard nucleotides (STD) or primers containing standard and SAMRS nucleotides (SAMRS). Lane 1: water; lane 2: 10^2 copies; lane 3: 10^3 copies; lane 4: 10^4 copies; lane 5: 10^5 copies of a plasmid coding for RpoB served as templates in these reactions. The expected size of the product was 102 base-pairs. PAGE analysis clearly shows the noise in the amplified products with STD primers (A, bottom left) versus SAMRS primers (A, bottom right). B) Real-time RPA using STD (left) and SAMRS primers (right) for *rpoB* gene. Fluorescence upon binding of EvaGreen to the amplified product was detected over 50 min. DNA template: 0 copies (blue), 10 copies (purple), 10^2 copies (gray), 10^3 copies (orange), 10^4 copies (red). Insets: correlation between C_p (*y*-axis, time in minutes) and the logarithm of the input template copy number (*x*-axis).

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LOD ≈ 10^3 copies) and correlated to the amount of target DNA added (Figure 4B, right). A linear correlation was observed between the threshold of amplification and the amount of template DNA added when the SAMRS primers were used (see inset Figure 4B, right)

Kit to detect the RNA virus causing Middle Eastern Respiratory Syndrome (MERS)

As this project was nearing completion, we were asked develop an assay to detect RNA arising from 2cEMC, the coronavirus that is the causative agent for MERS.[10] For the design of primers and probes to specifically identify 2cEMC coronavirus, all of the nine complete viral genomes for closely related viral species were downloaded from ViPR (http://www.viprbrc.org; Table 2). Using USEARCH, these sequences were clustered with other *Coronavirdae* genomes to identify all closely related species.[11] All sequences within the 2cEMC viral cluster (such as bat coronavirus HKU5 genomes) were then input into MUSCLE to create a multiple sequence alignment (MSA).[12] This MSA was used as the input for StrainTargeter, which found sets of amplification primers and probes that captured all 2cEMC genomes while avoiding hits to other RNA virus species within the MSA. Ten sets of primers and probes where chosen to be specific to multiple regions throughout the genome. These sets were then compared against NCBI's RNA virus (ftp://ftp.ncbi.nih.gov/genomes/Viruses), Ensembl's human genome (ftp://ftp.ensembl.org/pub/release-76/fasta/homo_sapiens/dna/), and The Broad Institute mosquito genome databases (https://olive.broadinstitute.org/collections/anopheles.1) to ensure that no combination of chosen primers and probes from any of the sets would cross-react with other RNA viruses or human genomes. Two primers were synthesized both with SAMRS components and, for comparison, with entirely standard nucleotides.

Table 2. Strains of MERS coronavirus used for primer design.

GenBank accession number	Strain name	GenBank accession number	Strain name
JX869059	EMC/2012 HCoV-EMC	KC164505	EMC/2012 England 1
KC667074	EMC/2012 England/Qatar/2012	KC776174	EMC/2012 Jordan-N3/2012
NC_019843	EMC/2012 England 1	KF186564	Al-Hasa_4_2013
KF186565	Al-Hasa_3_2013	KF186566	Al-Hasa_2_2013
KF186567	Al-Hasa_1_2013	KF192507	Munich

To serve as a target, a synthetic DNA molecule encoding the amplicon region was obtained from Integrated DNA Technologies. An RNA copy was prepared from this by transcription with T7 RNA polymerase. Separately, Lisa Hensley and Reed Johnson of the National Institute of Allergy and Infectious Diseases, Ft. Detrick, Frederick, MD provided a Trizol-inactivated sample of full-length RNA from the MERS virus. All data shown were obtained with the sample of full-length viral RNA. The first step of RT-RPA involves the use of SAMRS-containing oligonucleotides as primers, and then as templates, for reverse transcription.

The sensitivity of the reverse transcriptase-RPA reactions was explored by increasing the concentrations of RNA template from 0 to 10^3 pfu in log unit steps (0, 1, 10, 10^2, 10^3 pfu). The RT-RPA reactions were performed using Superscript II reverse transcriptase. Once again, STD primers amplified unwanted products in the negative control (Figure 5A), giving a background signal that obscured the desired amplicons when lower concentrations of viral RNA template was used in the RT-RPA reaction. Only at a higher concentration of the viral RNA template (10^3 pfu) did STD primers give a visible product having the correct size (Figure 5A). In contrast, SAMRS primers gave no signal in the negative-control lane and produced increasing amounts of the correctly sized amplicons with increasing concentration of the MERS viral RNA template (Figure 5A).

Real-time analysis with EvaGreen also showed improvements upon introduction of SAMRS components into the oligonucleotide primers (Figure 4B). Increasing concentrations of viral RNA template did not give an easily correlated increase in fluorescence with STD primers (Figure 5B, left, inset). In contrast, with SAMRS primers, the RT-RPA was semi-quantitative with EvaGreen as the readout, allowing samples with increasing concentrations of viral RNA templates to be easily distinguished (Figure 5B, right, inset).

In addition to its technological significance, this work has scientific interest for biological chemists. We did not expect that SAMRS nucleotides would be accepted as components of a primer-recombinase complex. The duplex that SAMRS forms is weaker, and a SAMRS oligonucleotide contains some molecular structural features that the natural enzymes might regard as "alien". The fact that SAMRS components are accepted by the enzymes shows the versatility of this complex, as well as the effect of the primer–template complex on its function.

Conclusion

These experiments show that, at least for these three cases, adding SAMRS nucleotides to primers used in RPA isothermal amplification eliminates assay artifacts, allows for readout by using real-time fluorescence signal, and increases the robustness of the assay with respect to using different target sequences. Indeed, without SAMRS components, RPA amplifications generally fail. Thus, SAMRS converts RPA from a method suited only for the research laboratory into one that has practical value in the field, at points-of-care, and in low-resource environments. We expect that SAMRS will also make the creation of RPA-based diagnostics faster, by eliminating the need for primer optimization and allowing primers to be designed to match the biology (e.g. to target conserved/variable regions) rather than to just "get something that works". Our ability to develop a SAMRS-based assay for MERS RNA in just two weeks illustrates this.

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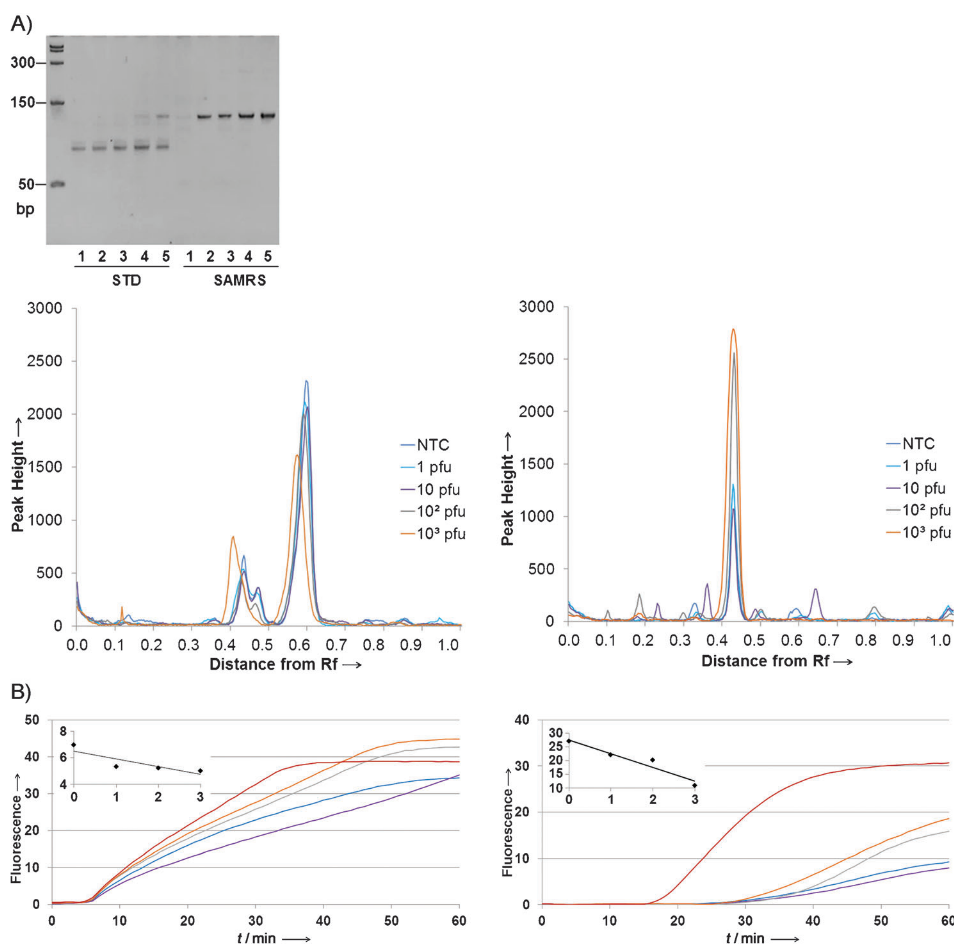

Figure 5. Reverse transcription and RPA (RT-RPA) with SAMRS primers of a small region in ORF1a of MERS viral RNA. A) Native PAGE of RPA products generated by using primers for MERS with STD primers or SAMRS primers. Lane 1: water; lane 2: 1 pfu; lane 3: 10 pfu; lane 4: 10^2 pfu; lane 5: 10^3 pfu of MERS viral RNA served as templates in these reactions. The expected size of the product was 122 base-pairs. PAGE analysis clearly depicts primer-dimers and other artifacts in the amplified products with STD primers (A, bottom left) but not in the assay with SAMRS primers (A, bottom right). B) Real-time RPA using STD (left) and SAMRS primers (right) for MERS viral RNA. Fluorescence upon binding of EvaGreen to amplified product was detected over 60 min. MERS viral RNA template: 0 pfu (blue), 10 pfu (purple), 10^2 pfu (gray), 10^3 pfu (orange), 10^4 pfu (red). Insets: correlation between C_p (*y*-axis, time in minutes) and the logarithm of the input template copy number (*x*-axis).

Experimental Section

Materials: Synthetic ultramer DNA (purified by PAGE) and all other standard nucleotide primers were obtained from Integrated DNA Technologies (IDT; Coralville, IA). JumpStart *Taq* DNA polymerase was obtained from Sigma–Aldrich. The RPA kit (TwistAmp Basic) was obtained from TwistDx Ltd. (Babraham, UK). EvaGreen was obtained from Biotium (Hayward, CA, USA). Plasmid DNA constructs containing a gene from influenza were ordered from Biobasic (Markham, Canada). The plasmid for RpoB in Topo 2.1 was a gift from the Ellington laboratory (University of Texas; Austin, TX). Full-length middle east respiratory syndrome (MERS) viral RNA (extracted from live virus particles and inactivated by using Trizol) was a gift from Lisa Hensley and Reed Johnson of the National Institute of Allergy and Infectious Diseases, Ft. Detrick, Frederick, MD.

Synthesis of SAMRS-containing oligonucleotides: Oligonucleotides containing SAMRS nucleotides were designed using StrainTargeter, an in-house software package. They were then synthesized using published methods[8] by solid-phase automated synthesis from the corresponding protected phosphoramidites (Glen Research; Sterling, VA).

Recombinase Polymerase Assay (RPA): RPA was performed in solution following the manufacturer's instructions. Briefly, rehydration buffer (29.5 μL), nuclease-free water (10 μL), and solutions of each of the forward and reverse primers (2.25 μL, 10 μM) were mixed and added to the lyophilized enzyme pellet provided in the kit. Varying copies of template were added to the reaction mixtures (0, 10, 10^2, 10^3, 10^4, 10^4 copies). Nuclease-free water was added to the reactions without template and used as no-template-controls (NTCs). To start the reactions, a solution of magnesium acetate (2.5 μL, 280 mM) was placed in the cap of each tube and the tubes were briefly centrifuged. The tubes were placed in a heat block at 37–38 °C for 40–60 min. The products were purified using standard nucleotide extraction procedures with phenol/chloroform/isoamyl alcohol (25:24:1), and a part of the reaction mixture was electrophoresed on an agarose gel (2.5 %) or a polyacrylamide gel (6–8 %). Products were visualized by staining with ethidium bromide.

Reverse transcription and RPA: To perform reverse transcription and RPA (RT-RPA) in the same reaction, SuperScript II reverse transcriptase (1 μL) was added to the RPA reaction (50 μL) and the reactions were incubated at 37–38 °C for 60 min. Products were purified and electrophoresed as described above.

Acknowledgements

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Keywords: isothermal amplification · nucleotide analogues · oligonucleotides · polymerase chain reaction · recombinase polymerases

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