

Biochemical solutions for portable nucleic acid testing

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INFO



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For the analysis of biological materials, nucleic acid tests (NATs) offer key advantages over alternative approaches (e.g. protein detection). NATs are capable of exquisite sensitivity (single molecule) and can be engineered to be extremely specific. Current laboratory-based applications include the

detection of pathogens, forensic analysis (such as DNA «fingerprinting»), assessing the polymorphic nature of genes («genotyping») and monitoring gene expression. Over the last decade the market for NATs has grown to a value in excess of a billion dollars, and this trend continues significantly year on year.

Most NATs rely on a technology invented over 20 years ago, the Polymerase Chain Reaction (PCR). Initially in the research sector, and then in medical and environmental applications, it provided the possibility of amplifying, and then detecting, minute quantities of nucleic acids. However, the PCR method does have significant practical drawbacks for the portable testing market. This article will look at how practical limitations of this approach, particularly with respect to power and engineering demands, may be remedied by emerging alternative nucleic acid amplification strategies.

The basic work-flow of NATs

NATs combine three essential steps (see figure 1):

- The nucleic acid is isolated, or made accessible, from the biological sample under investigation.
- The specific target within the nucleic acid is amplified.
- The amplification event is detected.

The isolation of material suitable for NATs (step 1) is a problem with diverse, largely application-specific solutions, and shall not concern us here. It suffices to say, that a number of approaches are on the market and each of these has to address the requirements shared by all NAT configurations: to separate sufficient quantities of target nucleic acids from contaminants and inhibitors, and to deliver these templates in manageable volumes to the next stage of the procedure.

Aspects of the amplification «read-out» (step 3) will be discussed further below. As in the case of sample preparation, the detection stage of a practical NAT configuration has to be compatible with the particular specifications of the technology at the heart of the workflow scheme: the amplification of the target nucleic acid (step 2). This central stage of the NAT workflow scheme is the main focus of this article. While a number of amplification technologies exist (and will be discussed later on), the foremost approach and «gold standard», PCR, massively

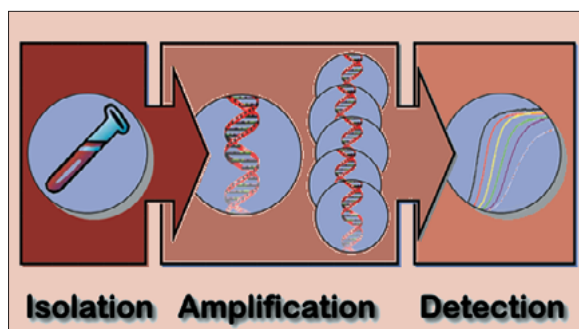


Figure 1: Nucleic acid testing procedures can be broken down into three steps. During sample preparation DNA (or RNA) is purified from the material under investigation (left). The template is then amplified by appropriate biochemical processes (middle). Finally, various read-out technologies allow to identify positive samples.

amplifies DNA fragments by a factor of up to 10^{11} to 10^{13} via a cyclic process that repeats rounds of thermally driven DNA melting at high temperatures, matching of synthetic oligonucleotide primers to target sites at reduced temperature and polymerase-dependant DNA synthesis at an intermediate third temperature. This simple, but beautiful process is performed in a special heating/cooling device («thermocycler») which is required to very rapidly change between large temperature differences, up to 120 times in a single amplification reaction. While

develop small and light (possibly handheld) devices. Attempts have resulted in either 'suitcase-sized' equipment which requires large and heavy batteries, or in highly miniaturised PCR systems with reduced power demands. However, implementing the latter option as robust commercially-viable platforms remains challenging, as significantly reducing reaction volumes creates new difficulties. Thermal characteristics across a thermoblock can be difficult to reliably maintain, microfluidics are required to deliver sample to the reaction chamber, and sealing of the small

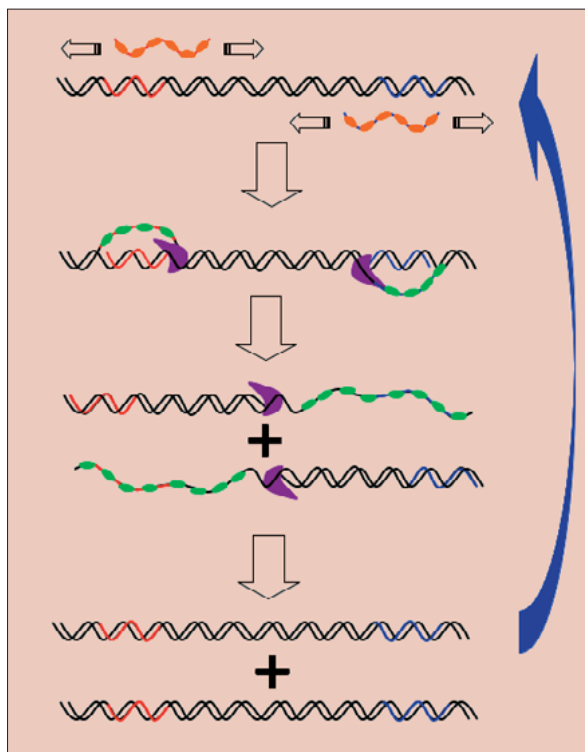


Figure 2: The Recombinase Polymerase Amplification technology uses recombinases (orange) to guide oligonucleotides to cognate sequences within the template (red and blue). Suitable polymerases (purple) extend the opposing primers and single-strand binding proteins (green) bind the displaced strands. Each primer-pair binding/elongation event results in the duplication of the target sequence. Reiteration of the process leads to exponential amplification.

extremely effective, the integration of this approach into a practical portable or point-of-care (PoC), NAT system has proven difficult. Thermocyclers are so power-demanding that it is difficult to

NATs into portable and point-of-use settings may be more easily enabled by switching away from PCR and adopting amplification strategies that eliminate the need for thermocycling. This reduces the demand on

equipment engineering and the need to manipulate and deliver very small volumes is necessary in order to prevent rapid evaporation of the entire sample. Perhaps more importantly, a need to concentrate the sample into a tiny volume is non-trivial and can lead to decreased sensitivity of the overall system. In summary, the apparently simple concept of portable thermal cyclers has been dogged by complications which have impeded penetration of the point-of-use market.

Isothermal amplification – biochemistry to the rescue

Penetration of

TABLE 1

Speed	Single molecule detection in 20 minutes
Sensitivity	Single molecule
Target complexity	Large complex genomes
Target nature	DNA and RNA
Detection	Gels, fluorescent probes, sandwich assays
User handling	Direct addition to pre-configured reagents
Temperature	24-45 °C, Rate optimal at ~37 °C
Amplicon sizes	80-1500 base pairs

equipment engineering and the need to manipulate and deliver very small volumes in environments that in practice demand as much simplicity as possible. Isothermal nucleic acid amplification technologies, working at a constant temperature, offer a natural solution to this problem. Such approaches have co-existed with PCR for some time and include the commercially used Transcription Mediated Amplification (TMA, Genprobe), Loop Mediated Amplification (LAMP, Eiken Chemical) and Strand Displacement Amplification (SDA, Becton Dickinson). However, despite success in a few notable areas when embedded in large platform systems, such alternative methods have, rather surprisingly, not driven significant development in the portable or PoC markets to date. The reason for this limited success is that these technologies – similar to PCR – lack the necessary specifications to be easily integrated in a practical workflow scheme for a portable NAT as outlined above. In order to meet the demands of portable or PoC testing, an amplification technology should provide the following:

- High sensitivity
- High specificity and ability to operate with complex nucleic acid samples
- Capacity to operate with multiple targets simultaneously («multiplexing»)
- High speed (detection in under an hour or less)
- sequence specific detection

approaches, preferably in real-time (e.g. «probe-based») equivalent to PCR gold standard

- Low, constant, temperature operation
- Robustness to off-temperature events
- Application to broad target range (DNA and RNA)
- No requirement for initial thermal or chemical melting or other complex handling before isothermal phase

Combining these properties in a single technology has proven to be challenging. The various merits and limitations of each reported approach will not be dissected in detail here, It suffices to say that there remain major stumbling blocks on the road to the implementation of most amplification strategies in a non-lab-

extensive sample handling (e.g. initial heating steps) and an absence of effective sequence specific detection approaches (i.e. probe systems). However, a novel technology, developed at ASM Scientific Ltd in the United Kingdom, combines the requirements outlined above in a single package and has the potential to enable the leap of NATs from the centralized laboratory to PoC environments. It provides a robust «amplification engine» to make fully integrated portable testing systems possible. This technology, the recently-described Recombinase Polymerase Amplification (RPA) [1], is described in more detail below.

Recombinase Polymerase Amplification (RPA)

RPA is a nucleic acid amplification system which employs prokaryotic enzymes, so-called recombinases, to guide synthetic oligonucleotide primers to target sites in sample nucleic acids. In a process that may be likened to PCR in some ways, exponential amplification of the desired sequence occurs by reiterative oligonucleotide-primed DNA synthesis (see figure 2). The analogy with PCR ends here: critically, there is no need for bulk melting of DNA to facilitate access of the primers, hence avoiding the requirement for any heating/cooling steps. This elegant advance means that similar specifications to PCR can be delivered at low and constant temperatures. RPA will operate from ambient temperatures (24 °C) to as high as 45 °C (with an optimum of 37 °C), tolerating fluctuations within these bounds. Thus, sophisticated and power-demanding heating sources are not required to implement the technique successfully (in fact, body temperature itself is ideal) and the optimal rate behaviour can be easily delivered by low power equipment, providing a unique advantage compared to other systems.

A second unique advantage of RPA is that the process begins operating the moment that a sample is contacted to

the reagents: there is no requirement to first melt double-stranded DNA (or to heat RNA to unfold it) and it comprises a true one-step isothermal amplification system with no loss of sensitivity. These two features set RPA aside from other isothermal amplification processes.

RPA – an amplification engine for portable diagnostics

RPA delivers similar specifications to PCR over a wide variety of key metrics [1]. It demonstrates extremely rapid kinetics and can attain detection of amplification from a single target molecule within 20 minutes. A few thousand target molecules can be registered in just a little over 10 minutes in recent studies (see figure 4 in «ESE's Miniature Fluorescence Technology», this edition). The template nucleic acid can be either DNA, or, with minor modifications, RNA, making RPA suitable for the detection of RNA-based viruses as well.

As pointed out earlier, the complete integration of a NAT requires that the amplification technology is interfaced with detection approaches. To satisfy this need, ASM Scientific has developed oligonucleotide-probe based detection formats. While other read-out methods are possible (gel-electrophoresis, dyes intercalating in real-time, etc.) the sequence specific probe system can be modified to deliver either fluorescence detection in real-time, at endpoint, or in simple «sandwich-assays». These and other properties of RPA are summarised in the table 1.

Finally, a key requirement for the development of useful diagnostic tools is the provision of internal controls in the reaction setup. Both, the amplification approach and the detection method combined in a NAT must hence be able to tolerate multiple targets simultaneously (i.e. cope with «multiplexing»). The RPA technology amply demonstrates this capability in the prototype test for the common «hospital bug» Methicillin-resistant *Staphylococcus aureus* (MRSA) [2]. In this case a multiplex

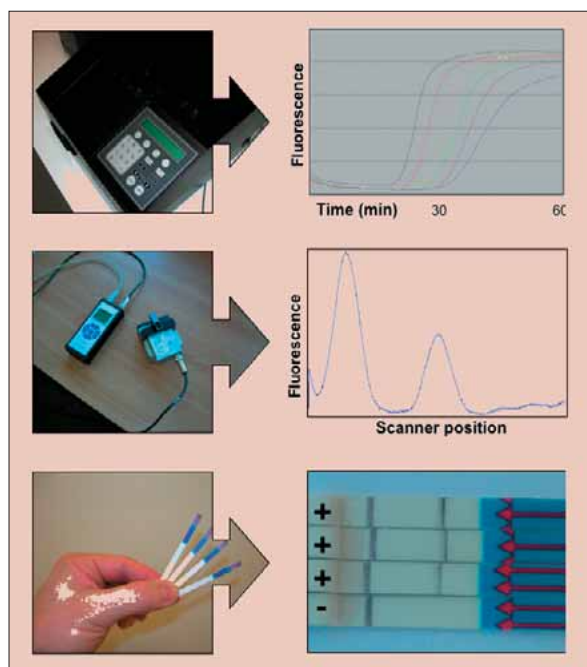


Figure 3: Various read-out technologies are compatible with RPA. Examples include laboratory-based fluorescence readers (top, SybrGreen detection of RPA reactions with 500'000, 50'000, 5'000, 500, 50 or 0 copies template), handheld scanning-fluorometers (middle, compare ESE article, SybrGreen detection of RPA reactions with 2000, 200 or 0 copies template after 26 minutes is shown) or disposable lateral-flow-strips (bottom, compare DCN article; signals of RPA reaction with 1'000, 100, 10 or 0 copies are shown).

laboratory setting, in particular the restriction to specific types of template (e.g. RNA), the requirement for

environment of amplification primers and probes had to be developed to permit detection of multiple forms of the complex MRSA pathogen and to include suitable internal controls.

Mobile detection– RPA meets portable detection devices

DNA amplification is the second part of the three sequential components required for an effective NAT. The first – sample preparation – is not dealt with in depth here, but is in general terms well-suited to isothermal strategies which obviate a need to highly concentrate samples to retain sensitivity. Switching to the final step in the NAT workflow relevant for portable use – detection – we shall now discuss the need for a small low power monitoring approaches. RPA has been well endowed in this respect by the development of interfacing probe systems which may be employed in homogeneous assays. Recent work has shown that these amplification/probe approaches work with excellent results in handheld fluorescence devices (such as those developed by ESE GmbH, see figure 3 and elsewhere in this special section) and allow ultra-rapid detection of pathogen DNA in a portable format. Currently the combination of RPA technology and ESE instrumentation is being further explored by developing and testing prototype handheld devices which include complete temperature control, battery operation and multiple tube scanning capabilities.

In addition to portable fluorescence-reading devices, the RPA amplification/probe system has been adapted to interface with simple disposable sandwich assays such as lateral flow strip technologies (see figure 3). In the latter case the amplified sample is applied at the end of the reaction to the sample pad of a strip and within a few minutes the result is shown by the presence or absence of a coloured line. This approach permits the development of disposable test formats which require little or no equip-

ment at all, and may be highly enabling for some applications, particularly in the PoC and home-testing markets.

A test case – rapid molecular test for MRSA

There are many applications, both clinical and in environmental testing, that could benefit from rapid, equipment-light and portable NATs. Mobile on-site tests for H5N1 influenza, for food pathogens and GMO markers, as well as biological warfare agents are obvious candidates. One clinical target that is equally well-suited is the drug-resistant MRSA pathogen. This *S. aureus* bacterium is the cause of a huge crisis in hospitals throughout the developed world and is responsible for many deaths and huge costs to the healthcare system [2]. Currently, the majority of tests performed to detect this organism are performed by culturing methods which take several days to reveal the result. To combat the spread of infection and manage costs it would be more efficient to screen all hospital patients at the point of entry. Consequently, the development of molecular tests which can be practiced by admission staff and which can diagnose the pathogen quickly (less than an hour) would be a huge benefit. The RPA technology does offer an opportunity to address the urgent need of the healthcare system for widely deployable, easy-to-use disposable or equipment-light tests.

Summary

Portable and equipment-light NATs are beginning to address unmet needs in diagnostics, forensics and environmental testing. In the near future, they will open up completely new market opportunities. The Recombinase Polymerase Amplification technology, embedded with suitable sample preparation and detection approaches, is ideally placed to attain these goals. Some challenges remain – the case-specific optimisation of sample preparation

processes and the optimisation of ambient-stable amplification reagents – however, a proliferation in the NAT market is very close on the horizon.

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References

1. Piepenburg O, et al. (2006): DNA Detection Using Recombination Proteins. *PLoS Biology* Vol. 4, No. 7: e204.
2. Enright MC, et al. (2002): The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc Natl Acad Sci USA*, 99: 7687–7692.

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