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CRITICAL REVIEW

# Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review

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Nucleic Acid Testing (NAT) promises rapid, sensitive and specific diagnosis of infectious, inherited and genetic disease. The next generation of diagnostic devices will interrogate the genetic determinants of such conditions at the point-of-care, affording clinicians prompt reliable diagnosis from which to guide more effective treatment. The complex biochemical nature of clinical samples, the low abundance of nucleic acid targets in the majority of clinical samples and existing biosensor technology indicate that some form of nucleic acid amplification will be required to obtain clinically relevant sensitivities from the small samples used in point-of-care testing (POCT). This publication provides an overview and thorough review of existing technologies for nucleic acid amplification. The different methods are compared and their suitability for POCT adaptation are discussed. Current commercial products employing isothermal amplification strategies are also investigated. In conclusion we identify the factors impeding the integration of the methods discussed in fully automated, sample-to-answer POCT devices.



## Introduction

### Nucleic acid testing (NAT)

The discovery of the structure of DNA,[1] elucidation of the molecular mechanisms of genetics and the development of the PCR[2] has given rise to powerful methods for the diagnosis of genetic and infectious disease.[3–5] Despite these advances, the gold standard of microbial identification remains the culture and subsequent phenotypic differentiation of the causative pathogen. This process usually takes in the order of 1–5 days, thus retarding effective treatment. This delay has a major impact on the morbidity and mortality of infectious disease. Accurate identification of the causative organism is time-critical. Inappropriate antimicrobial therapy has been shown to result in a five-fold reduction in survival for serious infections. Moreover, existing culture methods only identify, by definition,

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organisms which will grow on culture media. Fastidious or culture negative pathogens cannot be cultured *in vitro* and thus cannot be detected by the existing 'gold standard' method. They are, however, liable to identification by molecular methods.[6] Therefore a real need exists for more rapid, sensitive and specific diagnostic technologies for infectious disease to replace the time-consuming and limited culture methods.

Nucleic acid testing (NAT) techniques interrogate DNA sequences directly, allowing further clinically pertinent information to be garnered from either patient or pathogen. Antimicrobial resistance, virulence biomarkers and highly specific typing can be identified rapidly to allow optimal therapy and therapeutic intervention to proceed without delay. Quantitative assessment of pathogen load, which has shown to have powerful prognostic value,[7] can be determined using real-time amplification methods within a single assay. In addition to pathogen diagnostics, the emerging fields of theranostics, genomic medicine and companion diagnostics are demanding rapid, near patient gene detection technology to be employed as an adjunct to pharmacotherapy. Several PCR assays for the detection of genetic markers exist, such as Her2, EGFR and KRAS, which can be used to predict drug (Herceptin, Erbitux, Vectibix respectively) responses in patients.[8] Whilst no isothermal amplification based companion diagnostics are currently available, this market seems particularly amenable to rapid, near patient diagnostics.

There is no question that molecular technology offers very powerful diagnostic tools. The diagnostic industry, having recognised the manifold benefits of nucleic acid testing, is investing heavily in molecular diagnostics R&D. In 2007 the molecular diagnostics market saw revenues of $3.21 billion, expected to reach $5.42 billion by 2012.[9] Thus, as a corollary of the socio-medical benefits, there are also financial incentives for pursuing nucleic acid based diagnostics R&D.

Recent advancements have seen miniaturisation of NAT instrumentation; Genie II (OptiGene Horsham, UK) shown in Fig. 1 and Twista (TwistDx, Cambridge, UK) shown in Fig. 2 demonstrate the possibility for more portable NAT equipment. These developments notwithstanding, the need for skilled operators and the cost associated with their operation limit much of the existing technology to centralised, well-funded, urban laboratories with access to trained staff. These factors prevent the distributed delivery of these benefits to resource limited settings and those in geographically isolated areas. Thus, technology offering the benefits of molecular diagnosis without the current limitations of cost, complexity, and portability is widely desired to reduce both personal health impact of disease and the resultant public health burden. Table 1 compares existing, commercially available technology employing isothermal nucleic acid amplification.

One role for which NAT will prove especially powerful is in the rapid differentiation of pathogens whose prodromal presentation is similar, but may require significantly divergent therapeutic strategies. This is particularly evident in the diagnosis of respiratory viruses where overlapping clinical presentation of the varied upper respiratory tract infections (URTI) and lower respiratory tract infections (LRTI) necessitate laboratory diagnosis to identify the causative agent.[10] A salient example of this was the 2003 emergence of the SARS-CoV,


**Fig. 1** Genie II (OptiGene, Horsham, UK) platform for fluorescence detection of isothermal amplification reactions allows isothermal reactions to be run in parallel with real-time fluorometric detection. (Image used with permission of OptiGene).

which offered front line clinicians no pathognomonic signs or symptoms to differentiate it from other, less virulent, causes of community or hospital acquired pneumonia, thus complicating treatment plans and confounding the allocation of resources during an episode where health services were severely stretched.

### Nucleic acid testing formats

The goal of NAT, either laboratory based or POCT, is to identify and potentially quantify specific nucleic acid sequences from clinical samples as indicators of infectious or genetic disease presence, progression and prognosis or, in the case of genomic medicine, suitability for a tailored therapy. There are several formats which can be employed to effect sequence specific detection as shown below (Fig. 3).

### Separate amplification & detection

These methods involve sequential steps. Firstly amplification, typically PCR, of NA isolated from a clinical sample, followed


**Fig. 2** The Twista device from TwistDX (Cambridge, UK) allows portable, 2 channel fluorometric detection of 8 RPA reactions. Image reproduced with permission of TwistDx.

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**Table 1** Commercially available devices employing isothermal nucleic acid amplification technology

| Product | Amplification Method | Samples per run | Automated/integrated sample preparation | Detection method | Diagnostic use (regulatory body clearance) | Available Tests | Test Duration (sample→Result) | Cost (approx. US$) | Size (cm) | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| Twista (TwistDX, UK) | RPA | 8 | No | 2 channel Fluorescence | No (Assays in development) | User designed | 10–15 min (amplification & detection) | $4552 Instrument | 19 × 18 | 11 |
| Genie II (OptiGene, UK) | LAMP | 16 | No | 1 channel Fluorescence | No (Assays in development) | Compatible with most fluorescence based isothermal assays | Assay dependent | $13 000 Instrument | 20 × 21 × 30 | 12 |
| Illumigene Meridian Bioscience | LAMP | 10 | No | Fluorescence | Yes (FDA & CE) | *C. difficile* Group B Streptococcus | 60 min | POA | 21 × 29 × 9 | 13 |
| OligoC-TesT CorisBioConcept, Belgium | NASBA | 1 | No | Lateral flow | Yes | *Leishmania&T. Cruzi* | ~100 min | $26/test | Single Use Handheld dipstick | 14–16 |
| NucliSENSEasyQ Bio Merieux France | NASBA | 8–48 | Yes | Multichannel Fluorescence | Yes (CE) | HPV HSV RSV MRSA HIV-1 viral load Enterovirus | 120 min | $50 000 | 42 × 42 × 22 | 17,18 |
| APTIMA/Tigris Gen-Probe USA | TMA | 100 samples/Hour | Yes | Chemiluminescent DNA probe | Yes (FDA & CE) | NG CT HPV TV | 3.5 h | POA | 175 × 91 × 183 | 19 |
| BESt Cassette Type II BioHelix USA | HDA | 1 | No | Lateral flow | Yes (FDA) | HSV 1&2 | 90 min | $10/test | Single use Handheld cassette | 20–22 |
| ProbeTec Becton Dickinson & Co USA | SDA | 46 | Yes | Fluorescence | YES (FDA & CE) | NG CT HSV 1&2 | 60 min | POA | 55 × 72 × 72 | 23 |

by detection of the amplification product. This two part process allows analytical sensitivity and specificity to be temporally and spatially separated. Sensitivity can be achieved by powerful amplification with little regard to specificity, which can be later implemented through careful detection design to eliminate non-specific signals. Such methods have been well characterized, extensively used, and widely applied across genetic determination and infectious diseases. Amplification and detection tests therefore provide a robust format for the development of further NATs but may be limited by the co-amplification of non-specific targets which compete for limited reaction components. With regard to POCT applications, the requirement of multiple procedural steps for amplification and separate detection has driven the development formats that simplify the assay procedure and reduce the time-to-result. The development of simplified assays is limited by the need to maintain analytical sensitivity and specificity in a protocol consisting of fewer steps.

### Combined amplification & detection

The advent of fluorescent DNA probes and intercalating dyes[24,25] has allowed the real-time quantification of amplification products in both PCR and isothermal amplification reactions.

Highly specific isothermal amplification reactions (LAMP, SMAP2, HDA) allow for assays in which the non-specific detection of amplicon accumulation is sufficient to indicate the presence of an initial template allowing real-time fluorescent detection, using an intercalating DNA dye such as EvaGreen or SYBR Green I. The LAMP reaction has the added benefit of an insoluble reaction by-product, which can be easily quantified turbimetrically.[26]The limiting factor of such systems is the need to maintain analytical sensitivity to the target whilst developing reaction conditions with sufficiently high analytical specificity to eliminate non-specific products accumulating and producing a false positive result. The use of sequence-specific fluorescent probes such as TaqMan and molecular beacons offers the benefits of combined amplification and detection in reactions lacking the specificity for non- specific detection methods. Probe-based real-time detections also allow for multiplexing using different probe fluorophores. The simplicity of combined amplification and detection in a single step is desirable and will hasten the time-to-result.

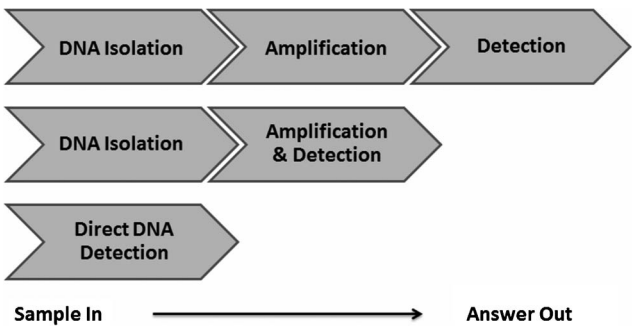


**Fig. 3** Nucleic acid testing formats.

### Amplification-free direct nucleic acid detection

Such techniques use highly sensitive detection strategies to identify target sequences in a sample without the need for nucleic acid amplification. This offers the potential for simplified protocols, reduced reagent consumption and simplified operating platforms. The success of such systems is entirely dependent on the development of robust biosensors with analytical sensitivity sufficient to detect the very low abundance NA in complex clinical samples whilst maintaining appropriate clinical specificity for diagnostic use. Non-POCT systems incorporating direct NA detection are commercially available. For example, NanoString (Seattle, USA) offers a bench-top instrument using molecular barcodes for gene analysis. Portable systems for direct detection, such as those being developed by Genefluidics (Irwindale, USA) using electrochemical detection and ExoCyte (Reading, UK) employing silicon nanowire, carbon nanotube and quantum dot technologies, have yet to be cleared for diagnostic use. Direct detection technologies are expected to enter the global testing market within the next 10 years a have the potential to displace numerous methods currently in use.

### Point-of-care nucleic acid amplification

Whilst biosensors for direct detection of nucleic acids without amplification have been described, an integrated system capable of reliable detection with relevant clinical sensitivity and specificity direct from complex samples has yet to reach the market. It is thus likely that some form of target or signal amplification must be performed, given existing detection technology.

The advent of PCR revolutionized genetics and molecular diagnosis providing a simple and elegant method for nucleic acid amplification using thermostable polymerase enzymes and a cyclic heating and cooling to obtain strand separation and annealing, respectively. This powerful technology has been well characterized and is widely employed for molecular diagnosis, biomedical and life science research.

The last two decades have seen many microfluidic PCR devices described.[27] These have taken a variety of forms with some displaying greater performance than existing PCR.[28] Unfortunately, the precise and repeated heating cycles required for PCR necessitate a complex, power consuming and bulky design, making micro-scale PCR an imperfect solution for incorporation into POCT platforms. Confining a PCR reaction within an enclosed microfluidic system requires additional engineering considerations not limited to the thermal constraints of the materials used, heat sensitivity of many of the enzymes used in diagnostics, unwanted evaporation of water and complex thermal control mechanisms.

In order to circumvent the limitations of traditional PCR in the amplification stages of POCT molecular diagnostics, recent research has turned towards the isothermal methods for nucleic acid amplification. Isothermal amplification techniques make use of enzymes to perform strand separation that would otherwise require repeated heating to achieve. These techniques offer those developing POCT diagnostic platforms a powerful tool to amplify nucleic acids without the need for the additional complexity of thermal cycling steps and the associated control mechanisms.

Gill & Ghaemi published the seminal review on isothermal amplification[29] outlining existing methods and more recent reviews have been presented by others.[30,31] The present review is not intended to duplicate these works. It presents an overview of the current state-of-the-art technologies for isothermal amplification with a focus on the critical assessment of their suitability for integration into POCT molecular diagnostic devices and briefly including the market landscape of isothermal amplification methods and products.

## Considerations regarding isothermal amplification technologies for POCT integration

**Amplification template.** The foremost consideration in assay design will be the assay target. The various amplification strategies described herein amplify varied targets, dsDNA, ssDNA and RNA. DNA being more stable is often preferred, where appropriate, and will likely increase clinical sensitivity in samples stored or transported in suboptimal conditions[32] due to the persistence of this molecule. RNA, being labile, allows detection restricted to viable targets. RNA will degrade following cell death whereas the more persistent DNA will remain detectable even once the pathogen is no longer viable.[33] This false positive effect can confound clinical management, especially following antimicrobial therapy.[34] RNA will often be present in concentrations up to 1000 times[35] greater than genomic or plasmid DNA, thus clinical sensitivity may be increased.[32] As a result, this may confound quantification of organisms as target RNA expression levels may vary. DNA assays can be modified with a reverse transcriptase step to allow detection of RNA, however, this will increase complexity.

**Operating temperature.** As with the PCR, many isothermal techniques (ICAN, RCA, SDA) require an initial heating step ($\sim$95 °C) to separate the dsDNA. This additional heating step will complicated control mechanisms and increase power demand. A number of isothermal techniques can act directly on dsDNA without a heating step (HDA, RPA, SMAP2). The reaction temperature itself is also a consideration, the techniques describe here vary from as low as 30 °C to 65 °C. An effect of higher temperatures is an increased power demand, this pitfall may be offset by a favourable increase in both the reaction kinetics and the stringency of the amplification reaction at higher temperatures. Higher temperatures can positively affect the specificity of the reaction[36,37] by reducing non-specific primer annealing.

**Procedural simplicity.** In order to reduce cost, prevent malfunction and achieve robust, reliable operation within a simple package an amplification technology will preferably be a "single tube" reaction with a minimal volume, employing few reagents and few fluidic manipulations.

**Multiplex capability.** To increase efficiency and clinical utility, the ability to amplify DNA from multiple targets within a single reaction volume is highly desirable. Furthermore, multiplex capability allows for the incorporation of both quality and quantification controls[38] to ensure accurate testing. Unfortunately multiplex amplification, be it PCR or isothermal, has pitfalls. It has been noted that multiplex assays can be less sensitive than equivalent simplex methods[34] on account of the interactions between additional primer sets, amplification bias[39,40] and masking of low abundance targets by higher abundance targets sequestering reaction components. Therefore, ensuring reliability of multiplex amplification assays in highly variable clinical samples will prove challenging and will require empirical evaluation for each assay.

**Tolerance to crude samples.** Current molecular diagnostic technologies require high purity nucleic acid samples for accurate diagnosis. Unprocessed clinical samples of blood, urine, sputum and mucous swabs contain many identified or potential inhibitors of PCR amplification including, heme, immunoglobulin G, lactoferrin, heparin, urea and acidic polysaccharides,[41] all of which will confound the design of sensitive NATs. Isothermal amplification techniques have been shown to be tolerant to many of these inhibitors, potentially reducing the procedural complexity in pre-amplification stages of extraction and purification. Assay design will favour technologies able to effect reliable amplification independent of the presence of cell detritus, background human genomic DNA and contaminants, which may otherwise act as inhibitors of amplification.

**High sensitivity.** Microfluidic dynamics afford many advantages.[42] To capitalize on these advantages the fluid volumes handled must be kept as low as possible. At such low volumes the amount of analyte nucleic acid present will be extremely low. An isothermal amplification technology must perform well at low analyte concentrations. The analytical sensitivity of each assay must be matched to the initial sample volume, analyte concentration amplification sensitivity, power and the detection limit of the biosensing mechanism being employed to ensure relevant diagnostic sensitivity. Thus the design process must consider these parameters from the outset to ensure reliable performance across clinically relevant concentrations.

**Specific amplification.** Protocols must offer sufficient specificity to amplify target DNA in the presence of background human genomic DNA, co-infecting pathogens and resident flora which will often exist in concentrations several orders of magnitude higher than the target.[43–45] Specific amplification is imperative when non-specific nucleic acid detection is being employed such as EvaGreen or SYBR intercalating dyes, which will fluoresce with any dsDNA amplicon as opposed to sequence-specific fluorescent probes such as molecular beacons or hydrolysis probes.

**Robust & reliable performance.** In order to provide a clinically useful diagnosis, the amplification must proceed in a predictable manner, which can be quantitatively profiled, ideally in real-time, to reveal amplification kinetics from which pathogen load can be determined. Pathogen load is increasingly being employed to direct treatment, evaluate therapy and predict outcomes and is thus a desirable feature.

**Rapid assay design.** A key asset of NAT is that following target and sample-specific nucleic acid isolation the amplification and detection steps can be applied to any target with minimal

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**Table 2** Existing isothermal nucleic acid amplification technologies

| Method | Preferred Template | Amplicon type | Performance (upper) | Analytical Sensitivity | Reaction Temperature (initial temp) (°C) | Primers required | Interventions (temperature/ reagent additions) | Multiplex demonstrated | Detection | Intellectual property | Key Publication |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NASBA | RNA | RNA | Fluorescent detection within 60 min | 1 copy | 41 (65/95) | 2 | 2 | yes | Fluorescence | Cangene Corp, Winnipeg, Canada | Compton, J. (1991) Nucleic acid sequence-based amplification. Nature, 350(6313), 91–2 (original paper)[46] |
| HDA | dsDNA | dsDNA | $10^{10}$-fold amplification in ~100 min | 1 copy | 64 | 2 | 1 | 2 plex plus Internal control | Intercalating DNA dye, Fluorescence probe, lateral flow strip | Biohelix,USA | Vincent, M. *et al.* (2004). Helicase-dependent isothermal DNA amplification. EMBO reports, 5(8), 795–800. (original paper)[79] |
| ICAN | ssDNA | DNA | Detection within 60 min | ~10–100 copies | 55 (95) | 2 DNA/ RNA chimeric primers | 2 | 2 plex | Fluorescence | Takara Bio (Otsu, Japan) | Uemori, T. *et al.* (2007) Investigation of the molecular mechanism of ICAN, a novel gene amplification method. Journal of Biochemistry, 142(2), 283–92. (Description of mechanism)[202] |
| LAMP | ssDNA | Concatenated DNA | $10^{9}$-fold in 60 min | ~5 copy | 60–65(95) | 4–6 | 2 | Yes | Intercalating DNA dye , Fluorescence probe, turbimetric | Eiken Chemical Co, Japan | Notomi, T *et al.* (2000). Loop-mediated isothermal amplification of DNA. Nucleic acids research, 28(12), E63. (original paper)[96] |
| NEAR | dsDNA | ssDNA | $10^{9}$-fold in 5 min with single nucleotide discrimination | >10 copies | 55 | 2 DNA/ RNA chimeric primers | 1 | 5 plex | Fluorescence | Ionian Technologies, San Diego, USA | Maples, B.K. *et al.* (2009) Ionian Technologies Inc. Nicking and extension amplification reaction for the exponential amplification of nucleic acids. US2009081670-A1 (Patent)[197] |
| RCA | Circular ssDNA | Concatenated ssDNA | $10^{9}$– fold in 90 min | 10 copies | 30–65(95) | 1 | 2 | — | Fluorescence | Various | Demidov, V. V. (2002). Rolling-circle amplification in DNA diagnostics: the power of simplicity. Expert Review of Molecular Diagnostics, 2(6), 542–8. (review)[172] |

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**Table 2** (*Continued*)

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| RPA | dsDNA | DNA | Detection in 20 min | 1 copy | 37–42 | 2 | 1 | — | Proprietary fluorescence probe | TwistDX, UK | Piepenburg, O. *et al.* (2006). DNA detection using recombination proteins. PLoS biology, 4(7), e204. (original paper)[91] |
| SDA | ssDNA | dsDNA | $10^7$-fold in 2 h | 10 copies | 37 (95) | 2 DNA/RNA chimeric primers + 2 bumper primers | 2 | — | Fluorescence | Becton, Dickinson & Company, Franklin Lakes, N.J, USA | Walker, G. T *et al.* (1992). Strand displacement amplification-an isothermal , *in vitro* DNA amplification technique. Nucleic Acids Research, 20(7), 1691. Oxford Univ Press. (original paper)[190] |
| SMAP2 | ssDNA | Concatemeric dsDNA | Detection in 15–30 min | 3 copy | 60 | 4 | 1 | — | Fluorescence | DNAForm /RIKEN (Japan) | Mitani, Y. *et al.* (2007). Rapid SNP diagnostics using asymmetric isothermal amplification and a new mismatch-suppression technology. Nature Methods, 4(3), 3000–3000. (original paper)[186] |
| SPIA | RNA/DNA | DNA | 4 h protocol for WGA | WGA from as little as 500 pg | 45–50 | RNA/DNA chimeric Primer | 1 | Whole genome amplification | Next-Gen sequencing, Microarray &7qPCR | NuGEN Technologies, Inc., San Carlos, CA | Kurn, N. *et al.* (2005). Novel isothermal, linear nucleic acid amplification systems for highly multiplexed applications. Clinical Chemistry, 51(10), 1973–81. Am AssocClin Chem. (original paper)[176] |

redesign. This is central to the success of the PCR. The ability to develop new assays rapidly with only a change of primers makes this a highly adaptable technique. The same flexibility in an isothermal method with simple design and evaluation is highly desirable. The techniques described herein vary in design complexity from simple primer pair modifications seen in RPA and HDA to multiple primer sets seen in LAMP and SMAP2.

**Amplification product produced.** The products of the methods outlined herein vary from ss/dsDNA (SMAP2, RPA, HDA) as per the PCR to concatenated (LAMP, RCA) replicons of the target and amplicons containing modified terminals (SDA, NEAR). Thus the product obtained during amplification constitutes a fundamental consideration when developing fully integrated POCT devices, as products must be compatible with, and optimized for, the detection strategy being employed. Table 2 shows the comparison of existing isothermal amplification technologies with respect to performance, analytical sensitivity, reaction temperature, number of primers required for the reaction, detection technology and multiplex capability. The following sections will describe the technologies and critically comment on their applicability in microfluidic-based POCT devices.

## Existing isothermal amplification technologies

### Nucleic acid sequence based amplification – NASBA

The NASBA and related methods, transcription mediated amplification (TMA) and self-sustained sequence replication (3SR) mimic *in vivo* retroviral replication mechanisms to produce RNA amplicons from an RNA template. The amplification process[46] forms a modified cDNA from an RNA template, which is then rapidly amplified into RNA amplicons in a process mediated by T7 RNA Polymerase (T7RNAP). Whilst the whole process forms a complex set of asynchronous reactions, the reaction itself is a single step and proceeds in a single volume with a ssRNA product suited for direct use with hybridization probes[47] making NASBA very appealing for POCT use (Fig. 4).

The direct and preferential amplification of RNA without the need for a reverse transcriptase step makes NASBA a desirable method for RNA virus diagnostics, viable target detection[33] and transcriptome analysis. The caveat of RNA detection being its labile nature which, whilst enabling viability studies,[33] may adversely affect assay reliability and performance if there are extended delays in the sampling-to-assay time during which RNA may degrade. If used in a near-patient device this will not be of great concern, as the sample will often be tested promptly following sample collection.

For POCT adaptation, the low incubation temperature (41 °C) is desirable in reducing power consumption and thermal control complexity but may result in a low stringency reaction environment and allow non-specific amplification,[36] making robust primer design and assay evaluation crucial. Furthermore, an initial 95 °C strand separation step is required if dsDNA is to be targeted whilst RNA amplification requires a 65 °C step[48] to remove secondary structures. These temperature steps will be an engineering consideration in POCT devices where precise thermal control and associated increased power consumption will be disadvantageous.

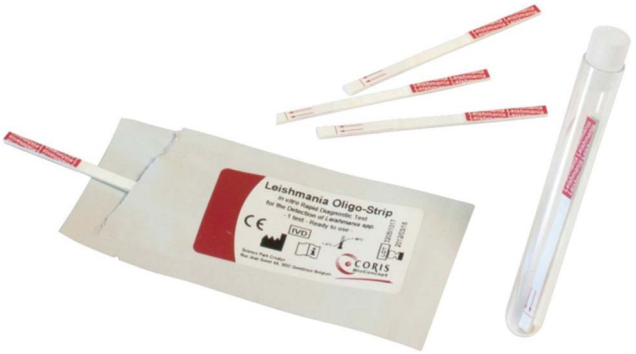


**Fig. 4** Leishmania OligoC-Test from Coris BioConcept. A NASBA-based, point-of-care amplification reaction and dipstick oligochromatographic detection test for Leishmania species (reproduced with permission CorisBioConcept).

A diverse body of research exists on NASBA which will encourage confidence in its uptake and can hasten development and integration time in novel applications. Having been first described in 1991[46] NASBA was promptly applied to pathogen detection and diagnostics,[49–52] especially RNA viruses[53,54] to which NASBA is particularly suited. Early research focused on HIV diagnosis[55,56] coinciding with the growth of the AIDS epidemic of the early 1990s and the need for more rapid and reliable diagnostics to replace the existing immunoassays. NASBA has since was shown to also have benefits over RT-PCR[57] in HIV diagnosis.

Since its development, NASBA has been widely demonstrated in the detection and analysis of RNA across genomic,[58,59] messenger,[60,61] ribosomal[62,63] and tmRNA targets,[64] including several quantitative real-time assays[58,65–67] which incorporate molecular beacon detection. More recently, NASBA has been shown to outperform both ELISA and RT-PCR when employed to diagnose respiratory tract infections[68,69] where rapid, near-patient, discrimination between virulent and avirulent variants is of great clinically utility.

In addition to a diverse research history, miniaturisation and integration of NASBA into lab-on-a-chip systems has been described at sensitivities comparable to laboratory-based NASBA in both nano and microliter volumes with molecular beacon detection[70,71,64] on centrifugal microfluidic devices[72] and as a detection mechanism in novel pathogen capture technology.[73] These devices form a robust proof-of-concept for the integration of NASBA technology into POCT/NAT devices. However, truly portable 'sample to answer' devices with 'walk-away' functionality have, thus far, not been described. CorisBioconcept (Gembloux, Belgium) have incorporated NASBA (or PCR) amplification into a simple molecular assay for the diagnosis of *Trypanosomabrucei/cruzi*[74] and *Leishmania*[14] species a lateral flow format termed "Oligochromatography "(OC) (patent granted in USA, Europe, Japan), offering instrument free testing. The simple NASBA-OC or PCR-OC assay displayed 1–10 parasite analytical sensitivity, far exceeding that of microscopy, the standard diagnostic procedure in developing countries where the cost and bulk of the more sensitive PCR assays is prohibitive.

Multiplex NASBA was demonstrated as early as 1999[75] but appears to be subject to a reduction in sensitivity compared to

equivalent single-plex assays,[34,76,69] an effect also seen when multiplexing PCR assays.[77] This reduction, however, may be modest with detection limits of > 1 PCR-detectable unit being reported elsewhere.[36] These effects will be dependent on assay design optimisation and will vary considerably between assays.

Real-time NASBA has been suggested as being a suitable alternative to the ubiquitous PCR[48] and performs favourably in comparison.[76,32] BioMerieux (Marcy l'Etoile, France) has developed the NucliSENSEasyQ automated NASBA amplification and real-time molecular beacon detection (NucliSENSEasyQ), processing 48 samples in 1.5 to 3 h,[18] with a range of optimized assays available and 'home brew' kits for user-designed assays, thus allowing research use for novel devices. The NucliSENS system is a laboratory-based instrument aimed at centralised laboratories and is currently employed to monitor HIV-1 viral load in patients on ART (antiretroviral therapy) in the South African public sector.[78] Gen-Probe[19] (San Diego, USA) offers a range of diagnostic nucleic acid tests based on their proprietary version of NASBA called TMA amplification for *Neiserriagonorrhoea*, *Chlamydiatrachomatis*, HPV, HIV, *Trichomonasvaginalis* and *M.tuberculosis*. TMA includes an acridinium ester-labelled DNA probe for chemilluminescent detection without the need for fluorescence optics. These tests are compatible with the Tigris instrument allowing high throughput laboratory automation.

### Helicase dependent amplification (HDA)

HDA is an elegant method for DNA amplification, employing helicase enzymes to effect strand separation, as opposed to thermal strand separation in PCR. The developers have created a simple and not unfamiliar reaction scheme for isothermal amplification that more closely resembles *in vivo* DNA replication.

The process initiates with helicase unwinding of dsDNA to which forward and reverse primers can bind, followed by polymerase mediated elongation. Following elongation, helicase can again act on the freshly synthesized dsDNA and the cycle asynchronously repeats, with similar amplification kinetics to existing PCR, at 60–65 °C without further temperature steps.

The existing HDA protocol is typically 60–120 min for low copy number targets.[21] This shortcoming, however, appears to be responsive to optimisation and modifications,[21] including the use of restriction endonucleases targeting regions upstream of the target sequence to enhance helicase activity in the target region, addition of crowding agents and increasing enzyme concentrations.

The primary appeal of HDA for POCT applications is the relative simplicity of the reaction. A single set of primers, two enzymes (three for reverse transcription-HDA) and compatibility with existing fluorescent detection chemistries[37,20] make this method familiar to those familiar with PCR. Since its first description in 2004,[79] HDA has been the focus of considerable development, optimisation and research including the development of TaqMan assays for biothreat detection,[20] quantitative reverse transcriptase-tHDA protocol for RNA detection[80] and solid-phase amplification using immobilised primers.[81] Importantly, HDA has also been shown to be effective across sample types, urine,[21] stool,[20] blood[82] and plasma.[22] HDA technology has been shown to be tolerant to crude bacterial samples with only a 1-fold reduction in sensitivity compared to purified genomic DNA.[79]


**Fig. 5** BESt$^{TM}$ Cassette - Type II (BioHelix, Beverly USA) . Allows portable detection of amplicons from HDA or PCR reactions in a vertical flow testing format. (reproduced with permission of BioHelix).

Various microfluidic devices using HDA are discussed in the literature. Mahalanabis *et al.* describe a disposable device integrating sample preparation and amplification with a $10^2$ copy limit of detection for *E. coli* O157:H7. This device however is not significantly faster than an equivalent PCR protocol.[83,84] Ramalingam *et al.* describe microfluidic chips with 5 μL reaction volumes capable of PCR equivalent amplification in 30 min using real time detection in a real-time instrument[37] and Zhang *et al.* present a novel droplet microfluidic technique for DNA isolation, HDA amplification and detection on an open chip using silica superparamagnetic particles as the actuation mechanism for fluidic movement.[85]

Thus HDA appears to be an appealing method for POCT devices. The simplicity of the two primer reaction allows rapid optimisation compared to other methods, which require the redesign and evaluation of multiple primers, thus complicating the assay design. The speed of HDA has been identified a limitation when working with samples containing less than < 100 copies.[21] This may be remedied with continued optimisation of the HDA process as a whole or optimisation of specific assays. A further consideration is the 10-fold increase in sensitivity, which results from dividing the single tube reaction into a two-step process whereby primers anneal separately from the enzyme mix.[86] This performance increase makes a two-step process highly desirable when working with low abundance targets and/or small sample volumes, despite the increased complexity that it may demand on microfluidic platforms.

The developers of HDA (BioHelix, Beverly, MA) offer research kits containing all the required components to carry out one or two step, real-time or end-point HDA amplification. In conjunction with this they offer the BESt Cassette Type II[87] for qualitative amplicon detection on a lateral flow platform in 5–10 min. The BESt cassette has been applied to a range of targets: *Neisseria gonorrhoeae*,[88] *Clostridium difficile*,[20] *Staphylococcus aureus*[89] and HIV-1.[82,22] An HSV assay, including the BESt cassette recently became the first HDA product to receive FDA 501(k) approval.

### Recombinase polymerase amplification (RPA)

RPA is a single tube, single temperature (37–42 °C) amplification method. The key to the amplification process is the formation of a recombinase filament, a complex combining a target-specific primer and a recombinase enzyme. When the target specific sequence is encountered by the recombinase filament it performs strand exchange, inserting the primer onto the target. The displaced d-loop formed is stabilized by ssDNA binding proteins (gp32) to prevent re-annealing. Spontaneous disassembly of the recombinase filament upon strand exchange leaves the primer/target hybrid open to extension by strand-displacing polymerase activity. Repetition of the cycle leads to geometric amplification.

Favourable thermal requirements, procedural simplicity and very rapid amplification (20–40 min[90]) make this recently developed process[91] a leading technology for integration into POCT devices. The added 'off-temperature' ability of RPA to proceed at a variety of temperatures is of great appeal for field applications where precise temperature control is often technically challenging and will allow for instrument-free amplification.

The biochemistry of RPA is incompatible with existing intercalating dyes, molecular beacons and TaqMan® technology. Alternative fluorescent probe detection strategies, TwistAmpexo and TwistAmp fpg, have been developed to allow single tube fluorescent detection using sequence-specific probes.

As an emerging technology, there is comparatively little in the published literature regarding RPA technology, RPA primer/probe design and its integration with POCT devices. These considerations notwithstanding, what is present in the literature hints toward one of the fastest amplification techniques on the market offering some promising attributes for POCT applications. A centrifugal disc format for RPA-based fluorescent detection integrating lyophilised reagents and silica-based DNA isolation has been demonstrated,[92,93] allowing detection of $< 10$ copies in $\sim$30 min within a commercial rotor PCR instrument (Rotor-Gene 2000, Qiagen, Hilden). Additional studies have shown rapid amplification, $< 10$ min, starting with 100 copies of *N. gonorrhoeae*.[94] Ismagilov *et al.* describe a digital slip chip employing 1550 9 nL RPA reaction on a single SlipChip[95] to effect a 300 copies/mL sensitivity and dynamic range of 1400–1 000 000 copies/mL, clearly demonstrating the efficacy and sensitivity of this technique in low volumes.

A valuable feature of the RPA system is the commercial availability (TwistDX, Cambridge, UK) of a range of ready-to-use kits containing the reagents required for the development of both end-point assays (gel electrophoresis detection/lateral flow) and real-time fluorescent probe detection[11] allowing rapid integration for bench-top, portable device and instrument free use. There are currently no RPA products cleared for diagnostic use.

### Loop mediated isothermal amplification (LAMP)

Among the isothermal nucleic acid methods currently available, loop mediated isothermal amplification[96] (LAMP) is the most widely researched and has been well characterized offering significant support during the development process.

LAMP is a rapid amplification method employing a strand-displacing Bst DNA polymerase and 4–6 primers, two of which are 'fold back' primers[97] which form stem-loops motifs with self-priming capability. This results in an amplification scheme where the priming sequence is copied with each round of replication and remains tethered to the previous amplicon resulting in a concatenated product of alternating sense/anti-sense repeats of varied length. Detailed schematic and animation of the complex amplification scheme can be found on-line.[98] Subsequent studies have found the use of additional 'loop primers', which bind to the loop structures, can greatly reduce the reaction times[99] resulting in a total of 6 primers.

The 60–65 °C reaction temperature combined with a minimum of 4 primers makes LAMP a highly specific reaction allowing an 'amplification is detection' scheme. This specificity has allowed the insoluble pyrophosphate reaction by-product[100] to be employed in a tubimetric detection strategy for both qualitative visual indication[101] or real-time quantitative turbidimetry,[26] which offers a very simple, robust detection strategy for POCT integration. Whilst a 95 °C initial strand separation step is not essential,[102] it has been shown to increase analytical sensitivity.[103,104]

LAMP has been well characterized and widely employed for pathogen detection. Bacterial,[105–112] viral,[113–120] fungal[121–123] and parasite[104,124–127] assays have all been described performing equal to or better than equivalent PCR, immunoassay or culture based diagnostic methods. Analytical sensitivity has been shown to exceed that of equivalent PCR assays[118] with detection limits as low as 5 copies.[128] Specificity has been demonstrated in several studies to be robust with the ability to discriminate between viral variants,[129] SNP,[130,131] human gene copy number[132] and human RNA expression.[133,134] Within these examples, existing descriptions of LAMP assays using little or no sample preparation from serum,[129] CSF[104] swabs[135] and heat treated blood[101] can be found, indicating that LAMP is a robust amplification strategy displaying a tolerance to substances which would inhibit the PCR. These attributes make LAMP well suited for adaptation into microfluidic and POCT assays. Several such assays have been described,[136–139] including integrated devices for purification, amplification and detection within a single assembly[140] and devices using pocket warmers as the heat source driving the reaction.[141,142] However, all of these devices use optical detection, either fluorescence or turbidity, which limits their use to single target detection (turbidity) or a few targets (fluorescence) per reaction. This may limit integration with internal controls and multiplexing.

As with some other isothermal methods (SMAP, NEAR, SDA), LAMP is highly dependent on the careful design of multiple complex primers[97] and has been cited as a reason for

researchers avoiding the use of LAMP.[143] To address this issue, web-based software is available for designing candidate LAMP primers and loop primers.[144]

The LAMP method was developed, and is marketed, by the Eiken Chemical Company (Tokyo). LAMP amplification kits for both DNA and RNA amplification for assay development in research settings are available for purchase. In addition, Eiken Chemical Company also supply food and environmental inspection kits for major human pathogens. Eiken Chemical Company are currently undertaking in-house R&D projects in such fields as medicine, agriculture, food production and processing, animal husbandry, and environmental protection.[145] Recently they have announced two major developments. Firstly, the release of Loop amp® Tuberculosis Complex Detection Reagent Kit, which is capable of direct detection of Tuberculosis from untreated sputum samples in 50 min. Secondly, the design and development of a field test for Human African Trypanosomiasis (HAT) has been completed and is currently entering multiple site field trials in Uganda and Congo with clinical availability expected in 2012. LAMP technology is also employed by the Illumigene *C. difficile* assay (Meridian Bioscience, Cincinnati, OH) for rapid (< 1 h) diagnosis from a stool sample, using a proprietary instrument.

The popularity of LAMP assays is evident in the rich body of literature that exists and unlike several other amplification methods, a large number of commercial test kits are already available.[145] This offers engineers, scientists and clinicians a wealth of knowledge to call upon during research and development to reduce the development complexity, time and cost of research programs. This solid knowledge base combined with the favourable speed, analytical specificity and detection simplicity demonstrated for LAMP indicate that it is likely to be seen in POCT devices in the future.

### Rolling circle amplification technology (RCA)

RCA[146] is a powerful technique which exploits the strand displacement and highly processive polymerase activity of the Phi29 bacteriophage DNA polymerase (φ29DNAP)[147] acting on circular DNA targets. The basic RCA reaction (linear RCA or single primer RCA) is initiated by a primer annealing to a circular ssDNA. The φ29DNAP can elongate a new strand of the circular template eventually completing a loop and reaching the point of initiation. Strand displacement activity allows the newly forming strand to continuously displace the previously generated strand as polymerisation advances. Generation of a continuous catenated ssDNA of up to 0.5 megabases[148] has been reported and continues until an external factor, such as nucleotide depletion, halts the reaction. This continuous catenated product attached to the template allows *in situ*[149,150] or localised amplification,[151] which can be used to concentrate labels within a small detection area and enumerating single DNA molecules.[152] The original RCA protocol[146] has been applied and optimised[153] for detection of pathogens[154–156] and has been integrated with various detection strategies including intercalating dyes,[157] real time fluorescent probes[158] and molecular beacons.[159]

Low (30–60 °C) temperature requirements[160] make these techniques attractive for POCT and have been successfully demonstrated with bacterial,[161] viral[162,163] and fungal[164–166] targets in traditional laboratory assays.

The requirement for a circular template for amplification makes RCA a leading candidate for amplification of DNA molecules which exist *in vivo* as circular molecules such as plasmids and certain phage, virus and viroid genomes. Padlock probes,[167] molecular inversion probes for SNP detection[168] and connector inversion probes[169] all yield circular structures from linear DNA to allow RCA amplification with the additional effect of greatly increasing specificity.[170] This circularization process demands additional enzymes and procedural complexity,[149] which may limit its value in POCT devices where procedural simplicity is preferred.

In addition to the linear RCA kinetics originally described in a ~4 h amplification, more powerful variants of RCA amplification have been developed capable of generating detectable levels of product in 30–90 min[164,149] with sensitivity as low as 10 copies.[171] Variously referred to as geometric, hyperbranched, ramification or cascade RCA,[172] these methods employ secondary primers which target the amplification product. As the initial ssDNA product is elongated from the circular DNA, these additional primers bind at regular intervals along the repeating strand and initiate additional primer elongation events. The multiple elongating strands now displace downstream strands resulting in further exposed sites for primer binding, resulting in a hyperbranched, self-propagating, geometric amplification pattern of primer-extension and strand-displacement.

Isothermal multiple displacement amplification (IMDA) is a whole genome amplification method developed from a modified RCA protocol.[173] Random thiophosphorylated hexamer primers are added to complex genomic samples and serve as elongation points for φ29 DNAP. The random priming and high processivity of the φ29 DNA generate long amplicons and ensure overlapping sequences thus providing whole genome amplification (WGA).

Commercially, no RCA based diagnostic products have yet reached the market. The Illustra TempliPhi DNA amplification kit (GE HealthCare, Buckinghamshire, UK) is an RCA based technique[174] using random primers to generate sequencing templates from any circular DNA, thus eliminating the need for overnight cultures and plasmid preparations.[175]

### Single primer isothermal amplification (SPIA)

SPIA is a linear amplification technology for DNA based on repeated replication of target sequences[176] enabled by the use of a chimeric RNA/DNA primers, which bind target regions and initiate polymerisation. The RNA/DNA primer is engineered in such a way that RNase H degradation of the RNA portion of the chimeric primer will re-expose the binding site to allow a subsequent primer to anneal. Strand displacement activity of the polymerase removes the previously generated strand. This repeated cycle continuously generates new amplicons until reagents or primers are depleted. A similar method, Ribo-SPIA, developed to amplify total mRNA, replicates only the original transcripts and not copies, resulting in a high-fidelity product. Additionally, SPIA methods have been modified to allow random priming for whole genome amplification/whole transcriptome amplification.[176]

NuGen (San Carlos, USA) market several SPIA products for WGA and whole transcriptome amplification (WTA) from as little as 10 ng of DNA and 5 ng of RNA,[177] which lends them toward the sequencing market more so than infectious disease diagnostics. Consequently, SPIA products are targeted at laboratory and high throughput genomic analysis[176] applications. There is no description in the literature of SPIA being used for single gene or POCT application, possible due to low specificity of the chimeric primers or the relatively long (~4 h) incubation resulting from the linear kinetics. Whilst there is no description of SPIA in the literature for POCT diagnostics, it may become more widespread as genomic analysis enters the point-of-care domain.

### Smart amplification process version 2 (SMAP2/SmartAmp2)

This nascent amplification technology (not to be confused with the signal amplification method SMART, signal mediated amplification of RNA technology) employs similar enzymes and self-priming loop motifs to LAMP. In contrast to the symmetrical primers of LAMP, SMAP2 primers are designed asymmetrically with different tail motifs in the two target flanking primers. This serves to reduce the formation of background products from mis-amplification.

The amplification process occurs in two steps: an initial 'key intermediate' step forming a target sequence flanked 3′ and 5′ with fold-back domains to provide self-priming ability; and a second amplification step where the key intermediates undergo repeated self-priming and rapid target amplification resulting in concatenated, primer inclusive amplification products. This process is well described and animated online.[178]

In addition to this amplification format, SMAP2 employs background suppression technology to increase specificity and permit an 'amplification is detection' assay. Ultra high specificity is achieved by employing *Thermusaquaticus* MutS[179] to identify mismatched primer/target hybrids. MutS scans dsDNA and will irreversibly bind to any mismatch duplex with single nucleotide sensitivity. Bound MutS prevents polymerisation thus checking amplification of non-specific sequences resulting in complete inhibition of non-specific amplification.

The incorporation of MutS and asymmetric primer design permits single nucleotide discrimination, making SMAP2 particularly useful for SNP identification. Capitalising on this, much of the research conducted to date has been in the rapid identification of SNPs for genotyping.[180–183] Little research exists regarding the application of SMAP2 to infectious disease diagnosis. Kawai *et al.*[184] have developed a simple reverse-transcriptase (RT) SMAP2 assay for pandemic flu that is sufficiently sensitive to allow early detection (< 12 h post infection). This study also identified an, as yet unidentified, inhibitor of the RT-SMAP2 reaction. A noteworthy reminder that this technology is in its infancy.

The conspicuous drawback of SMAP2 is the requirement for meticulous design of folding primers and evaluation in order to achieve desired clinical specificity and sensitivity. Whilst software has been developed[185] to assist with the complex SMAP2 primer design, empirical evaluation is recommended[186] in addition to careful consideration and design of turn-back primers[97] and folding-primer tail design.[187]

Unlike more established isothermal amplification methods, LAMP, SDA, NASBA , which have been widely used and well characterized, there is relatively little research published on SMAP2[182,183,184,186,188] and kits for either research or diagnostic use are not available. Whilst SMAP2 has been shown to proceed slower than LAMP[186] the SMAP2 technique's very high specificity, high sensitivity (3 copies),[186] powerful amplification (> 100 fold larger than PCR)[187] combined with the developers (DNAFORM & RIKEN, Yokohama, Japan) reporting of specific detection from crude cell lysate, makes this a promising development and potentially powerful tool for POCT devices.

### Strand displacement amplification (SDA)

This method was first described in 1992 by Walker *et al.*[189,190] and relies on bifunctional primers incorporating both target recognition and endonucleases target regions. Following strand separation, these bifunctional primers extend incorporating the restriction target into the amplicon. Bumper primers, which bind and extend upstream, release this amplicon. Successive rounds of primer binding generate dsDNA incorporating restriction sites, which can then be acted upon by the restriction endonucleases to nick a single strand of the newly formed duplex. This nicking allows the polymerase to displace the existing strand and incorporate a new amplicon. This nick and run scheme is repeated to effect exponential amplification. Single strand nicking is effected by the incorporation of a modified adenine nucleotide, dATP αS (5′-*O*-l-thiotriphosphate), which is resistant to the endonuclease activity. Thus only the newly incorporated primer will be nicked leaving the amplified strand to repeatedly act as a template for primer binding.

The complex asynchronous reactions occur concurrently and user interventions are limited to an initial heat denaturation with primers followed by addition of polymerase and restriction enzymes at a 37 °C[190] incubation, a protocol which is by no means complex and appears amenable to POCT use. However, there is little mention of SDA point-of-care devices in the literature. This may be due to the original two hour amplification process[190] being seen as too long for a POCT or the SDA reactions sensitivity to background DNA,[190] which will co-amplify following non-specific primer binding, possibly as a result of reduced stringency conditions present at 37 °C. Co-amplified product will compete for limited reaction components and reduce the total target amplification. Interference from background (human) DNA is of significant consequence for nucleic acid based clinical diagnostics[43–45] as human DNA may be several orders of magnitude more abundant than the desired target. In addition to reducing total amplification yield, this non-specific product will necessitate the use of a specific detection strategy such as electrophoresis, molecular beacon[191] or hybridisation based sensor[192] technology to differentiate the amplification product from non-specific product, thus making non-specific detection methods such as intercalating DNA dyes inappropriate.

Commercially the BD ProbeTec™ ET System[23] (Becton Dickinson Microbiology Systems, Sparks, Md.) has been available since 1999 and offers a 1 h, CE mark/FDA cleared, SDA based assay for the diagnosis of CT, NG and HSV from urogenital specimens on a high throughput platform

(96 microwell) using real-time molecular beacon detection.[191] Although not portable, the semiautomated platform is suitable for use in primary care settings as well as central laboratories and has been shown to be a valuable tool in the diagnosis of these pathogens[193] and perform favourably compared to PCR.[194] No 'home-brew' research kits for SDA are available.

### Nicking and extension amplification reaction (NEAR)

NEAR is a recent development of the earlier described EXPAR[195] reaction. Capitalizing on nicking-enzymes to expose binding sites for primers, the EXPAR displays excellent reaction kinetics and $10^6$–$10^9$-fold amplification in a few minutes. However, EXPAR is limited to amplification of sequences adjacent to native nicking-enzyme recognition sites[196] within the target genome. NEAR is a refinement of EXPAR to allow amplification of any target by inserting nicking-enzyme recognition sites adjacent to target regions

The two-stage NEAR reaction proceeds in a similar manner to the SDA reaction exploiting nicking-enzymes to generate a site from which polymerase elongation can initiate. In contrast to SDA, the nicking enzyme employed in NEAR will only nick a single side of a duplex, removing the need, as seen in SDA, for strand modification of the duplex to prevent double stranded cleavage. Significantly, for POCT development, the thermal denaturation of dsDNA prior to primer binding is not required as the primers can bind during the normal breathing of DNA molecules or *via* nicks generated in the target genome.[197] This reduces overall process complexity. The NEAR reaction is carried out above the melting temperature of the primer–target hybrid, making their annealing a transient event[197] which does not require the strand displacing bumper primers seen in SDA. This is made possible on account of the chosen polymerases which stabilize the duplex during elongation at temperatures above the melting temperature ($T_m$) presumably resulting in the NEAR reaction being most efficient with short (21–28 nt) oligonucleotides.[197]

With the NEAR amplification duplex formed, nicking enzymes act on their nicking site between the promoter region and the target on both the sense and antisense strands. This divides the duplex into two parts, each part having single stranded 3′ overhangs identical to the target and a short double stranded section comprising the nicking enzyme promoter region. Polymerase activity converts the overhands to dsDNA, in doing so replicating the target. Further nicking enzyme activity displaces the newly formed strand which is identical to the initial target. This can then bind free template and re-enter the NEAR reaction resulting in geometric amplification.

Whilst there is little in the literature regarding this emergent technique, what information has been released into the public domain points toward a rapid, sensitive and specific technique well suited for integration into POCT devices. Ionian technologies (San Diego, USA), have reported[198] a 5 min assay for *Neisseria gonorrhoeae* with a 10 copy LOD, a *Chlamydia trachomatis* assay capable of 10 copy LOD and are presently developing assays for a number of biothreat agents across viral, bacterial and eukaryotic genomic samples in collaboration with private and governmental partners.

EnviroLogix (Portland ME, USA) have developed rapid, specific molecular tests for two important plant pathogens, *Clavibactermichiganesis* and *Ralstoniasolanacearum*, with a 50 copy LOD in 10 min.[199] This research showed significant performance benefits (specificity and sensitivity) in the 10 min NEAR assay over the 2 h real-time PCR assay from a crude sample input.

The ability to directly accept genitourinary/nasopharyngeal swabs, urine without purification and blood with 'minimal sample processing[197] suggests a tolerance to inhibition superior to PCR. For downstream detection the short DNA amplicons generated by NEAR, with minimal flanking regions, make them amenable to multiple existing detection strategies including hybridization, (real-time) fluorescence and lateral flow. The current 5-plex limit is still better than many existing isothermal methods.

In summary, NEAR is a promising technique with a significant development pipeline currently underway. The reported performance[197] of the technique is competitive with other isothermal techniques and PCR in simplicity, sensitivity and specificity and a clear leader with regard to time-to-answer. However there is very little published on this technology and it has yet to reach the market in any form.

### Isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN)

ICAN[200] is a simple scheme for DNA amplification at 55 °C using relatively few reagents: a pair of 5′-DNA–RNA-3′ chimeric primers, thermostable RNaseH and a strand-displacing DNA polymerase.

Following initial heat denaturation of the target dsDNA, the chimeric primer binds to the template and is elongated by *Bca*BEST DNA polymerase. The newly formed strand is nicked by thermostable RNaseH action, not at the 3′ border of the chimeric primer as initially thought[201] but at the penultimate 3′ RNA residue, allowing the strand displacing DNA polymerase to release a newly synthesized strand with a single 5′ RNA residue, leaving the template with a truncated primer, which is still sufficient to prime elongation. The cycle repeats until the chimeric primer is sufficiently shortened, allowing a new, free chimeric primer to anneal preferentially, recommencing the cycle. In addition to this multi-priming model a template-switching mode of amplification has been identified.[202] Template-switching amplification occurs when both forward and reverse primer bind to the same dsDNA target and proceed to elongate toward one another, eventually switching the template from using the original template to using the newly synthesized strand elongating from the opposite primer as the template. This displaces both parent strands, forming a dsDNA of two daughter strands consisting of the target flanked by the primer regions with incorporated chimeric primer on one strand. This dsDNA of daughter strands is acted upon by RNaseH, which introduces a nick in the RNA region and polymerase elongation can commence. If both forward and reverse elongation reactions occur simultaneously, the template switching cycle will recommence with the parent strands being displaced and the chimeric primer bound strands becoming the dsDNA product. If the nicking and elongation occurs asynchronously, a single stranded product is formed, still having an incorporated chimeric primer. This single strand with primer can enter the

multi-priming amplification cycle, thus there is no dead-end product and amplification will be sustained until the reagents become exhausted. The frequency of the template-switching reaction appears variable dependent of reaction conditions, most significantly with the polymerase used, with *Bca*BEST DNA polymerase showing a higher frequency of template switching than Taq DNA polymerase.

Initial research has shown ICAN to specifically amplify DNA sequences (70–110 bp) from complex bacterial DNA (*Mycobacterium bovis*),[200] human genomic DNA and cDNA/RNA hybrids (Chrysanthemum stunt virus).[201] The same research also demonstrated the ability to amplify sequences of 250–300 bp from *Arabidopsis thaliana* and to produce real-time quantification of viral copies. Sensitivity of ICAN assays has been reported to be 25 times greater than equivalent PCR based assays.[203] Thus on the face of it, ICAN demonstrates some very appealing characteristics across varied samples.

ICAN has been incorporated into several POCT assays: a semi-automated, field deployable endospore detection system for biological warfare agents[204] including real-time fluorescent detection, a highly sensitive chromatostrip *SalmonellainvA* detection kit,[205] *Mycobacterium* detection,[200] a highly specific fluorescence detection 2-plex CTNG detection system[206] and a *Neisseria gonorrhoeae* fluoroquinolone resistance assay.[207] A commercial version of the *Neisseria gonorrhoeae* test kit, the ICAN NG-QR, was previously marketed by Takara Bio (Otsu, Japan) but has since been discontinued. Currently there are no ICAN products available for research or diagnostic use.

## Discussion

As shown in Table 1 above, there currently exists a diverse range of isothermal amplification technologies each with application specific pros and cons to be considered. The initial consideration is the amplification template and amplicon type. Of the existing methods, most produce a DNA amplicon from a ssDNA template. The notable exception being NASBA and the similar methods TMA/3SR, which directly amplify RNA without a reverse transcriptase step, making them highly desirable for RNA virus detection. The desired detection strategy will dictate which amplicon type is preferred. Of the above methods, most produce discrete strands of DNA, as produced in PCR, and are thus compatible with most PCR detection strategies. LAMP, RCA and SMAP2 conversely produce a concatemeric product allowing the amplicon to be localised, allowing *in situ* detection for both single cell and multiplex applications.

A fair comparison of performance data for amplification methods is problematic due the multitude of variables (target type, sample pre-concentration, detection strategy), which must be considered in addition to the differential metrics used for performance quantification. This is confounded by the relative scarcity of literature on certain methods such as NEAR, SMAP2 and ICAN compared to the abundance of, often variant, data for more widely published methods such as LAMP and NASBA. Consequently, a valid performance comparison is challenging and must be seen as a guide and not a definitive figure. Furthermore, whilst all but two methods state a 1–10 copy sensitivity and several state a single copy sensitivity, the stochastic nature of such low concentration analyte samples make these figures uncertain.

RPA and SDA both offer a 37 °C degree reaction temperature, highly desirable for low consumption POCT devices, although SDA does require a 95 °C strand denaturation step which is also seen in several of the other methods. Furthermore, the reduced stringency conditions present in a low temperature annealing may result in non-specific binding, which highlights the need for each assay to be evaluated empirically for clinical specificity, sensitivity and performance.

The assay design process will also be a consideration for many groups wanting to integrate isothermal amplification technology into novel POCT devices. NASBA, HDA and RPA methods rely on two primers with a design, evaluation and optimisation process similar to PCR, which will accelerate the uptake and integration of such methods. Other methods utilise up to 6 primers and others include RNA/DNA chimeric primers. The design and evaluation of more complex primers will complicate the assay design process. The use of such additional primers can, however, greatly improve specificity of the assay, as seen in SMAP2 reactions, and can increase the speed of the reaction, as seen with the addition of loop primers in LAMP.

The ability to detect and potentially quantify multiple targets would be an invaluable asset to any amplification strategy allowing higher throughput, multiple and differential pathogen detection, greatly increasing overall diagnostic utility. Equally important is the ability to integrate one or more control reactions in a multiplex system. Specifically, extraction and amplification controls provide robust, real-time assay validation within a single reaction volume. As with traditional PCR, the multiplexing of isothermal amplification reactions results in a highly complex interplay of factors not limited to primer competition, primer interaction, amplification bias and product interactions. For these reasons, the isothermal methods outlined above currently have limited multiplex capability with NEAR reporting the maximum multiplex capability and several other proprietors currently working toward improving multiplex performance.

## Conclusion

Many of the isothermal amplification methods described above display speed, amplification power, analytical and diagnostic specificity and sensitivity equal to, and often in excess of, existing molecular techniques based on real-time PCR/qPCR. Given this excellent performance and their suitability for miniaturisation, it is highly likely that isothermal amplification strategies will become commonplace in the next generation of point-of-care diagnostic devices, facilitating the distributed delivery of personalised healthcare within mobile and electronic healthcare networks (mHealth/eHealth).

Despite great potential, a ~20 year history and a diverse range of powerful amplification strategies, the field of nucleic acid-based POCT is still in its infancy and has yet to see any killer application reach the market. Given the above stated examples, it seems that this discrepancy results not from a lack of suitable amplification technologies but from the complex engineering challenges, which must be overcome in integrating and automating a chosen amplification method with both the upstream and downstream processes to provide sample-in to answer-out functionality in a portable device. The realisation of

a truly portable, rapid nucleic acid-based POCT device is not solely a molecular biology problem but a complex multi-disciplinary problem, which requires a systems engineering approach, calling on multidisciplinary knowledge to overcome the obstacles which currently retard the development of such devices. An issue which will undoubtedly become more pertinent as POCT devices become integrated within wider mHealth and eHealth infrastructure.

Intellectual property (IP) concerns and regulatory hurdles also seem to hinder advancement in this field. Understandably, in order to protect their IP and investments, the major stakeholders in the isothermal amplification arena prevent, limit or regulate access to their technology. This is evidenced by the notable absence of research kits for many leading amplification methods, thus complicating the application of these techniques by other groups possessing complimentary technology wishing to adopt these methods. Furthermore, the complex regulatory frameworks relating to diagnostic technology, which exist in the leading markets of North America and Europe, pose a non-trivial obstacles in the path to market which many universities and small enterprises are not well suited to undertake unassisted.

In summary the authors have made the following three broad conclusions:

1. There now exist several powerful, rapid isothermal nucleic acid amplification techniques, which have been shown to perform as well as, and often better than existing PCR-based assays.

2. The limiting factor in the nucleic acid POCT product development pipeline is not the availability of suitable techniques but the integration of existing amplification techniques with upstream sample processing and NA isolation methods with downstream detection schemes. Proprietors of specific techniques may lack the expertise and resources to fully integrate these steps. This will require a multidisciplinary systems engineering approach making industry/industry and industry/academia partnerships invaluable.

3. The proprietors of said technology may be reluctant to partner, collaborate and licence their technology for fear of diluting their intellectual property rights, which will undoubtedly retard progress.

## Abbreviations

| | |
|---|---|
| 3SR | Self sustained sequence replication |
| CE | Conformité Européenne |
| CSF | Cerebrospinal Fluid |
| CT | *Chlamydia trachomatis* |
| cDNA | Complementary DNA |
| DNA | Deoxyribose Nucleic Acid |
| dsDNA | Double Stranded Deoxyribose Nucleic Acid |
| EGFR | Epidermal Growth Factor Receptor |
| EXPAR | Exponential Amplification Reaction |
| FDA | Food and Drug Administration |
| HAT | Human African Tripanosomiasis |
| HDA | Helicase Dependent Amplification |
| Her2 | Human Epidermal Growth Factor Receptor 2 |
| HIV | Human Immunodeficiency Virus |
| HPV | Human Papilloma Virus |
| ICAN | Isothermal and Chimeric primer-initiated Amplification of Nucleic acids |
| IP | Intellectual Property |
| KRAS | Kirsten Rat Sarcoma Viral Oncogene Homolog |
| LAMP | Loop mediated Isothermal Amplification |
| LOD | Limit of Detection |
| LRTI | Lower Respiratory Tract Infection |
| mHealth | Mobile Health |
| NA | Nucleic Acid |
| NASBA | Nucleic Acid Sequence Based Amplification |
| NAT | Nucleic Acid Test |
| NEAR | Nicking and Extension Amplification Reaction |
| NG | *Neisseria gonorrhoeae* |
| PCR | Polymerase Chain Reaction |
| POCT | Point of Care Test(ing) |
| qPCR | Quantitative Polymerase Chain Reaction |
| RCA | Rolling Circle Amplification |
| RPA | Recombinase Polymerase Amplification |
| RT-PCR | Real-Time Polymerase Chain Reaction |
| SARS-CoV | Severe Acute Respiratory Syndrome Coronavirus |
| SDA | Strand Displacement Amplification |
| SMAP2 | Smart Amplification Process version 2 |
| SNP | Single Nucleotide Polymorphisms |
| SPIA | Single Primer Isothermal Amplification |
| ssDNA | Single Stranded Deoxyribose Nucleic Acid |
| T7RNAP | T7 RNA Polymerase |
| TMA | Transcription Mediated Amplification |
| TV | Trichomonas Vaginalis |
| URTI | Upper Respiratory Tract Infection |
| WGA | Whole Genome Amplification |
| WTA | Whole Transcriptome Amplification |

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## References

1 J. D. Watson and F. H. C. Crick, *Nature*, 1953, **171**, 964–967.
2 R. Saiki, D. Gelfand, S. Stoffel and S. Scharf, *Science*, 1988, **239**, 487–491.
3 U. Reischl and R. P. Schmitz, *European Infectious Disease*, 2011, **5**, 44–46.
4 T. Suntoke, A. Hardick, A. Tobian, B. Mpoza, O. Laeyendecker, D. Serwadda, P. Opendi, C. Gaydos, R. Gray and M. Wawer, and others, *Sex. Transm. Infect.*, 2009, **85**, 97.
5 S. Park, Y. Zhang and S. Lin, *Biotechnol. Adv.*, 2011, **29**, 830–839.
6 S. S. K. Rampini, G. V. Bloemberg, P. M. Keller, A. C. Büchler, G. Dollenmaier, R. F. Speck and E. C. Böttger, *Clin. Infect. Dis.*, 2011, **53**, 1245–1251.
7 L. M. Luft, M. . Gill and D. Church, *Int. J. Infect. Dis.*, 2011, **15**, 661–70.
8 F. Eibl, *IVD Technology*, 2011, **17**, 50–53.
9 R. Park, *IVD Technology*, 2011, **17**, 8.
10 J. B. Mahony, *Clin. Microbiol. Rev.*, 2008, **21**, 716–47.
11 www.twistdx.co.uk.

12 www.optigene.co.uk.
13 www.meridianbioscience.com/illumigene.
14 www.corisbio.com/Products/Leishmania.php.
15 S. Deborggraeve, F. Claes, T. Laurent, P. Mertens, T. Leclipteux, J. C. Dujardin, P. Herdewijn and P. Büscher, *J. Clin. Microbiol.*, 2006, **44**, 2884–9.
16 F. Claes, S. Deborggraeve, D. Verloo, P. Mertens, J. R. Crowther, T. Leclipteux and P. Büscher, *J. Clin. Microbiol.*, 2007, **45**, 3785–7.
17 S. E. Capaul and M. Gorgievski-Hrisoho, *J. Clin. Virol.*, 2005, 236–240.
18 www.biomerieux-diagnostics.com.
19 www.gen-probe.com/products-services.
20 W. H. a Chow, C. McCloskey, Y. Tong, L. Hu, Q. You, C. P. Kelly, H. Kong, Y.-W. Tang and W. Tang, *J. Mol. Diagn.*, 2008, **10**, 452–8.
21 Y. Tong, B. Lemieux and H. Kong, *BMC Biotechnol.*, 2011, **11**, 50.
22 W. Tang, W. H. A. Chow, L. Ying, H. Kong, Y. W. Tang and B. Lemieux, *J. Infect. Dis.*, 2010, **201**, S46.
23 http://bd.com/ds/productCenter/MD-ProbetecEt.asp.
24 F. Vitzthum and J. Bernhagen, *Recent Research Developments in Analytical Biochemistry*, 2002, **2**, 65–93.
25 P. M. Holland, R. D. Abramson, R. Watson and D. H. Gelfand, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 7276.
26 Y. Mori, M. Kitao, N. Tomita and T. Notomi, *J. Biochem. Biophys. Methods*, 2004, **59**, 145–157.
27 Y. Zhang and P. Ozdemir, *Anal. Chim. Acta*, 2009, **638**, 115–25.
28 A. Musyanovych, V. Mailänder and K. Landfester, *Biomacromolecules*, 2005, **6**, 1824–8.
29 P. Gill and A. Ghaemi, *Nucleosides, Nucleotides Nucleic Acids*, 2008, **27**, 224–43.
30 P. J. Asiello and A. J. Baeumner, *Lab Chip*, 2011, **11**, 1420–30.
31 A. Niemz, T. M. Ferguson and D. S. Boyle, *Trends in Biotechnology*, 2011, 1–11.
32 K. Loens, T. Beck, D. Ursi, M. Overdijk, P. Sillekens, H. Goossens and M. Ieven, *J. Microbiol. Methods*, 2008, **73**, 257–62.
33 J. Keer and L. Birch, *J. Microbiol. Methods*, 2003, **53**, 175–183.
34 Y. Zhao, S. Park, B. Kreiswirth, C. Ginocchio, R. Veyret, A. Laayoun, A. Troesch and D. Perlin, *J. Clin. Microbiol.*, 2009, **47**, 2067–78.
35 R. Emmadi, J. B. Boonyaratanakornkit, R. Selvarangan, V. Shyamala, B. L. Zimmer, L. Williams, B. Bryant, T. Schutzbank, M. M. Schoonmaker, J. A. A. Wilson, L. Hall, P. Pancholi and K. Bernard, *J. Mol. Diagn.*, 2011, **13**, 583–604.
36 J. Jean, D. H. D'Souza and L.-A. Jaykus, *Appl. Environ. Microbiol.*, 2004, **70**, 6603–10.
37 N. Ramalingam, T. C. San, T. J. Kai, M. Y. M. Mak and H.-Q. Gong, *Microfluid. Nanofluid.*, 2009, **7**, 325–336.
38 J. Hoorfar, B. Malorny, A. Abdulmawjood, N. Cook, M. Wagner and P. Fach, *J. Clin. Microbiol.*, 2004, **42**, 1863–1868.
39 T. Kanagawa, *Journal of Bioscience and Bioengineering*, 2003, **96**, 317–323.
40 M. F. Polz and C. M. Cavanaugh, *Applied and Environmental Microbiology*, 1998, **64**, 3724.
41 H. H. Kessler, *Molecular Diagnostics of Infectious Diseases*, Walter de Gruyter & Co, 2010.
42 G. M. Whitesides, *Nature*, 2006, **442**, 368–373.
43 S. Sachse, E. Straube, M. Lehmann, M. Bauer, S. Russwurm and K.-H. Schmidt, *J. Clin. Microbiol.*, 2009, **47**, 1050–7.
44 F. B. Cogswell, C. E. Bantar, T. G. Hughes, Y. Gu and M. T. Philipp, *Journal of Clinical Microbiology*, 1996, **34**, 980.
45 M. Handschur, H. Karlic, C. Hertel, M. Pfeilstöcker and A. G. Haslberger, *Comp. Immunol., Microbiol. Infect. Dis.*, 2009, **32**, 207–219.
46 J. Compton, *Nature*, 1991, **350**, 91–2.
47 B. Deiman, P. van Aarle and P. Sillekens, *Mol. Biotechnol.*, 2002, **20**, 163–79.
48 J. Logan, K. Edwards, N. Saunders, *Real-time PCR: Current Technology and Applications*, Horizon Scientific Press, 2009..
49 G. V. der Vliet, R. R. A. F. Schukkink, M. Gabrielle and B. van Gemen, and others, *Journal of General Microbiology*, 1993, **139**, 2423.
50 R. Sooknanan, L. Malek, X. Wang, T. Siebert and A. Keating, and others, *Experimental Hematology*, 1993, **21**, 1719.
51 M. Uyttendaele, R. Schukkink, B. van Gemen and J. Debevere, *Applied and Environmental Microbiology*, 1995, **61**, 1341–1347.
52 H. H. L. Smits, B. V. Gemen, R. Schukkink, B. Van Gemen, S. P. Tjong-a-hung and J. Ter Schegget, and others, *J. Virol. Methods*, 1995, **54**, 75–81.
53 J. Romano, R. Shurtliff, R. Pal, M. Sarngadharan and M. Kaplan, *Second National Conference on Human Retroviruses and Related Infections*, 1995, 119.
54 R. C. Hollingsworth, P. Sillekens, P. van Deursen, K. R. Neal, W. L. Irving and H. Trent, *J. Hepatol.*, 1996, **25**, 301–306.
55 B. van Gemen, T. Kievits, P. Nara, B. van Gemen, H. G. Huisman, S. Jurriaans, J. Goudsmit and P. Lens, and others, *AIDS*, 1993, **7**, S107.
56 A. Vandamme, S. V. Dooren and W. Kok, *Conference on Aids*, 1994.
57 A. M. Vandamme, S. Van Dooren, W. Kok, P. Goubau, K. Fransen, T. Kievits, J. C. Schmit, E. De Clercq and J. Desmyter, *J. Virol. Methods*, 1995, **52**, 121–32.
58 C. Moore, S. Corden, J. Sinha and R. Jones, *J. Virol. Methods*, 2008, **153**, 84–9.
59 S. Wacharapluesadee and T. Hemachudha, *Lancet*, 2001, **358**, 892–893.
60 B. S. N. Blank, P. L. Meenhorst, W. Pauw, J. W. Mulder, W. C. van Dijk, P. H. M. Smits, F. Roeles, J. M. Middeldorp and J. M. A. Lange, *J. Clin. Virol.*, 2002, **25**, 29–38.
61 A. Heim, I. M. M. Grumbach, S. Zeuke and B. Top, *Nucleic Acids Res.*, 1998, **26**, 2250–1.
62 S. A. Morré, P. Sillekens, M. Jacobs, P. van Aarle, S. de Blok, B. van Gemen, J. M. Walboomers, C. J. Meijer and A. J. van den Brule, *Journal of Clinical Microbiology*, 1996, **34**, 3108–3114.
63 S. Condon, F. Zhang, C. Ginocchio, in *43rd Interscience Conference on Antimicrobial Agents and Chemotherapy*. Chicago, IL, USA, 2003, pp. 13–17.
64 I. K. Dimov, J. L. Garcia-Cordero, J. O'Grady, C. R. Poulsen, C. Viguier, L. Kent, P. Daly, B. Lincoln, M. Maher, R. O'Kennedy, T. J. Smith, A. J. Ricco and L. P. Lee, *Lab Chip*, 2008, **8**, 2071–8.
65 J.-N. Telles, K. Le Roux, P. Grivard, G. Vernet and A. Michault, *J. Med. Microbiol.*, 2009, **58**, 1168–72.
66 D. Rodríguez-Lázaro, J. Lloyd, A. Herrewegh, J. Ikonomopoulos, M. D'Agostino, M. Pla and N. Cook, *FEMS Microbiology Letters*, 2004, **237**, 119–26.
67 G. Leone, H. van Schijndel, B. van Gemen, F. R. Kramer and C. D. Schoen, *Nucleic Acids Res.*, 1998, **26**, 2150–5.
68 R. L. Tillmann, A. Simon, A. Müller and O. Schildgen, *PLoS One*, 2007, **2**, e1357.
69 L. T. Lau, X. Y. Feng, T. Y. Lam, H. K. Hui and A. C. H. Yu, *J. Virol. Methods*, 2010, **168**, 251–4.
70 A. Gulliksen, L. Solli, F. Karlsen, H. Rogne, E. Hovig, T. Nordstrom and R. Sirevag, *Anal. Chem.*, 2004, **76**, 9–14.
71 A. Gulliksen, L. A. Solli, K. S. Drese, O. Sörensen, F. Karlsen, H. Rogne, E. Hovig and R. Sirevåg, *Lab Chip*, 2005, **5**, 416–20.
72 I. K. K. Dimov, J. O. Grady, J. Ducreel, T. Barry, A. J. J. Ricco, J. L. Garcia-Cordero, J. O'Grady and J. Ducree, *IEEE 22nd International Conference on Micro Electro Mechanical Systems, 2009. MEMS 2009*, 2009, 356–359.
73 J. Y. Won, J. Min and J.-H. Park, *Biosens. Bioelectron.*, 2010, **26**, 1763–1767.
74 C. M. Mugasa, T. Laurent, G. J. Schoone, P. A. Kager, G. W. Lubega and H. D. F. H. Schallig, *J. Clin. Microbiol.*, 2009, **47**, 630–5.
75 P. B. van Deursen, A. W. Gunther, C. C. van Riel, M. M. van der Eijnden, H. L. Vos, B. van Gemen, D. a van Strijp, N. M. Tackent and R. M. Bertina, *Nucleic Acids Res.*, 1999, **27**, e15.
76 K. Loens, T. Beck, D. Ursi, M. Overdijk, P. Sillekens, H. Goossens and M. Ieven, *J. Clin. Microbiol.*, 2008, **46**, 185–91.
77 C. Y. W. Tong, C. Donnelly, G. Harvey and M. Sillis, *J. Clin. Pathol.*, 1999, 257–263.
78 G. U. van Zyl, S. N. J. Korsman, L. Maree and W. Preiser, *J. Virol. Methods*, 2010, **165**, 318–9.
79 M. Vincent, Y. Xu and H. Kong, *EMBO Rep.*, 2004, **5**, 795–800.
80 J. Goldmeyer, H. Kong and W. Tang, *J. Mol. Diagn.*, 2007, **9**, 639–44.
81 D. Andresen, M. von Nickisch-Rosenegk and F. F. Bier, *Clin. Chim. Acta*, 2009, **403**, 244–8.
82 J. A. Jordan, O. C. Ibe, G. Simon, in 2010 HIV Diagnostics Conference, Orlando, 2010.

83 M. Mahalanabis, J. Do, H. ALMuayad, J. Y. Zhang and C. M. Klapperich, *Biomed. Microdevices*, 2010, **12**, 353–359.
84 M. Mahalanabis, J. Do, H. Almuayad, J. Y. Zhang and C. M. Klapperich, *Biomed. Microdevices*, 2011, **12**, 353–9.
85 Y. Zhang, S. Park, K. Liu, J. Tsuan, S. Yang and T.-H. Wang, *Lab Chip*, 2011, **11**, 398–406.
86 BioHelix Corporation, IsoAmp® III Universal tHDA Kit, Instruction Manual.2010.
87 BioHelix Corporation, www.biohelix.com, 2011, BESt$^{TM}$ Cassette eDataCard..
88 J. Goldmeyer, H. Li, M. McCormac, S. Cook, C. Stratton, B. Lemieux, H. Kong, W. Tang and Y.-W. Tang, *J. Clin. Microbiol.*, 2008, **46**, 1534–1536.
89 S. T. W. Tang, K. Cooper, T. Pack, N. Nasser, T. Ranalli, R. Lollar, H. Kong, in *Abstracts of 21st ECCMID / 27th ICC*, Milan, 2011.
90 TwistDx, Appendix to the TwistAmp TM reaction kit manuals, 20th edn, 2009.
91 O. Piepenburg, C. Williams, D. Stemple and N. Armes, *PLoS Biol.*, 2006, **4**, e204.
92 S. Lutz, P. Weber, M. Focke, B. Faltin, J. Hoffmann, C. Müller, D. Mark, G. Roth, P. Munday, N. Armes, O. Piepenburg, R. Zengerle and F. von Stetten, *Lab Chip*, 2010, **10**, 887–93.
93 D. Mark, M. Focke, S. Lutz, J. Burger, M. Müller, L. Riegger, M. Rombach, J. Hoffmann, G. Roth and O. Piepenburg, *Procedia Eng.*, 2010, **5**, 444–447.
94 *Alessandra Vinelli, Doctoral Thesis*, Università di Bologna, 2011.
95 F. Shen, E. K. Davydova, W. Du, J. E. Kreutz, O. Piepenburg and R. F. Ismagilov, *Anal. Chem.*, 2011, **83**, 3533–40.
96 T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T. Hase, *Nucleic Acids Res.*, 2000, **28**, 63e.
97 Y. Kimura, M. J. L. de Hoon, S. Aoki, Y. Ishizu, Y. Kawai, Y. Kogo, C. O. Daub, A. Lezhava, E. Arner and Y. Hayashizaki, *Nucleic Acids Res.*, 2011, **39**, e59.
98 http://loopamp.eiken.co.jp/e/lamp/principle.html.
99 K. Nagamine, T. Hase and T. Notomi, *Mol. Cell. Probes*, 2002, **16**, 223–229.
100 Y. Mori, K. Nagamine, N. Tomita and T. Notomi, *Biochem. Biophys. Res. Commun.*, 2001, **289**, 150–154.
101 L. L. M. Poon, B. W. Y. Wong, E. H. T. Ma, K. H. Chan, L. Chow, W. Abeyewickreme, N. Tangpukdee, K. Y. Yuen, Y. Guan and S. Looareesuwan, and others, *Clin. Chem.*, 2006, **52**, 303.
102 K. Nagamine, K. Watanabe, K. Ohtsuka, T. Hase and T. Notomi, *Clinical Chemistry*, 2001, **47**, 1742–3.
103 R. Suzuki, M. Ihira, Y. Enomoto, H. Yano, F. Maruyama, N. Emi, Y. Asano and T. Yoshikawa, *Microbiology and Immunology*, 2010, **54**, 466–70.
104 Z. K. Njiru, A. S. J. Mikosza, E. Matovu, J. C. K. Enyaru, J. O. Ouma, S. N. Kibona, R. C. Thompson and J. M. Ndung'u, *Int. J. Parasitol.*, 2008, **38**, 589–99.
105 V. Lalande, L. Barrault, S. Wadel, C. Eckert, J. C. Petit and F. Barbut, *J. Clin. Microbiol.*, 2011, **49**, 2714–6.
106 J. P. J. McKenna, D. J. D. Fairley, M. D. M. Shields, S. L. S. Cosby, E. Wyatt, C. McCaughey, P. V. Coyle and D. E. Wyatt, *Diagn. Microbiol. Infect. Dis.*, 2011, **69**, 137–144.
107 I. Neonakis, D. Spandidos and E. Petinaki, *Eur. J. Clin. Microbiol. Infect. Dis.*, 2011, **30**, 937–942.
108 K. I. Hanaki, J. I. Sekiguchi, K. Shimada, A. Sato, H. Watari, T. Kojima, T. Miyoshi-Akiyama and T. Kirikae, *J. Microbiol. Methods*, 2010, **84**, 251–254.
109 G.-Z. Lin, F.-Y. Zheng, J.-Z. Zhou, X.-W. Gong, G.-H. Wang, X.-A. Cao and C.-Q. Qiu, *Mol. Cell. Probes*, 2011, **25**, 126–9.
110 Y. Koide, H. Maeda, K. Yamabe, K. Naruishi, T. Yamamoto, S. Kokeguchi and S. Takashiba, *Lett. Appl. Microbiol.*, 2010, **50**, 386–92.
111 W. Yamazaki, K. Seto, M. Taguchi, M. Ishibashi and K. Inoue, *BMC Microbiology*, 2008, 8.
112 W. Yamazaki, M. Taguchi, M. Ishibashi, M. Nukina, N. Misawa and K. Inoue, *Vet. Microbiol.*, 2009, **136**, 393–394.
113 D. T. Dinh, M. Thi, Q. Le, C. D. Vuong and F. Hasebe, *Tropical Medicine and Health*, 2011, **39**, 3–7.
114 M. Parida, J. Shukla, S. Sharma, S. Ranghia Santhosh, V. Ravi, R. Mani, M. Thomas, S. Khare, A. Rai, R. Kant Ratho, S. Pujari, B. Mishra, P. V. Lakshmana Rao and R. Vijayarahhavan, *J. Mol. Diagn.*, 2011, **13**, 100–7.
115 B. Hatano, M. Goto, H. Fukumoto, T. Obara, T. Maki, G. Suzuki, T. Yamamoto, K. Hagisawa, Y. Matsushita and T. Fujii, and others, *J. Med. Virol.*, 2011, **83**, 568–573.
116 Y. Kurosaki, A. Grolla, A. Fukuma, H. Feldmann and J. Yasuda, *Journal of Clinical Microbiology*, 2010, **11**, 197.
117 M. Imai, A. Ninomiya, H. Minekawa, T. Notomi, T. Ishizaki, M. Tashiro and T. Odagiri, *Vaccine*, 2006, **24**, 6679–82.
118 M. Fujino, N. Yoshida, S. Yamaguchi, N. Hosaka, Y. Ota, T. Notomi and T. Nakayama, *J. Med. Virol.*, 2005, **76**, 406–413.
119 M. Hagiwara, H. Sasaki, K. Matsuo, M. Honda, M. Kawase and H. Nakagawa, *J. Med. Virol.*, 2007, **79**, 605–615.
120 T. Okafuji, N. Yoshida, M. Fujino, Y. Motegi, T. Ihara, Y. Ota, T. Notomi and T. Nakayama, *J. Clin. Microbiol.*, 2005, **43**, 1625–1631.
121 J. Sun, M. Najafzadeh, V. Vicente, L. Xi and G. De Hoog, *J. Microbiol. Methods*, 2010, **80**, 19–24.
122 L. Niessen and R. F. Vogel, *Int. J. Food Microbiol.*, 2010, **140**, 183–91.
123 S. Lucas, M. da Luz Martins, O. Flores, W. Meyer, I. Spencer-Martins and J. Inácio, *Clin. Microbiol. Infect.*, 2010, **16**, 711–714.
124 E. Matovu, I. Kuepfer, A. Boobo, S. Kibona and C. Burri, *J. Clin. Microbiol.*, 2010, **48**, 2087–2090.
125 Y. L. Y. L. Lau, P. Meganathan, P. Sonaimuthu, G. Thiruvengadam, V. Nissapatorn and Y. Chen, *J. Clin. Microbiol.*, 2010, **48**, 3698.
126 M. A. M. Bakheit, D. Torra, L. A. Palomino, O. M. M. Thekisoe, P. A. Mbati, J. Ongerth and P. Karanis, *Vet. Parasitol.*, 2008, **158**, 11–22.
127 A. Nkouawa, Y. Sako, T. Li, X. Chen, T. Wandra, I. K. Swastika, M. Nakao, T. Yanagida, K. Nakaya and D. Qiu, and others, *J. Clin. Microbiol.*, 2010, **48**, 3350.
128 T. Iwamoto, T. Sonobe and K. Hayashi, *J. Clin. Microbiol.*, 2003, **41**, 2616–2622.
129 M. Ihira, H. Sugiyama, Y. Enomoto, Y. Higashimoto, K. Sugata, Y. Asano and T. Yoshikawa, *J. Virol. Methods*, 2010, **167**, 103–6.
130 M. Iwasaki, T. Yonekawa, K. Otsuka, W. Suzuki, K. Nagamine, T. Hase, K. I. Tatsumi, T. Horigome, T. Notomi and H. Kanda, *Genome Lett.*, 2003, **2**, 119–126.
131 N. Nakamura, K. Ito, M. Takahashi, K. Hashimoto, M. Kawamoto, M. Yamanaka, A. Taniguchi, N. Kamatani and N. Gemma, *Anal. Chem.*, 2007, **79**, 9484–9493.
132 N. Nakamura, T. Fukuda, S. Nonen, K. Hashimoto, J. Azuma and N. Gemma, *Clin. Chim. Acta*, 2010, **411**, 568–573.
133 T. Osako, T. Iwase, K. Kimura, K. Yamashita, R. Horii, A. Yanagisawa and F. Akiyama, *Cancer*, 2011, **117**, 4365–74.
134 D. Horibe, T. Ochiai, H. Shimada, T. Tomonaga, F. Nomura, M. Gun, T. Tanizawa and H. Hayashi, *Int. J. Cancer*, 2007, **120**, 1063–1069.
135 Y. Enomoto, T. Yoshikawa, M. Ihira, S. Akimoto, F. Miyake, C. Usui, S. Suga, K. Suzuki, T. Kawana and Y. Nishiyama, *J. Clin. Microbiol.*, 2005, **43**, 951–955.
136 C. Liu, M. G. Mauk and H. H. Bau, *Microfluid. Nanofluid.*, 2011, **11**, 1–12.
137 X. Fang, Y. Liu, J. Kong and X. Jiang, *Anal. Chem.*, 2010, **82**, 3002–3006.
138 Z. Fang, J. Huang, P. Lie, Z. Xiao, C. Ouyang, Q. Wu, Y. Wu, G. Liu and L. Zeng, *Chem. Commun.*, 2010, **46**, 9043–5.
139 L. Lam, S. Sakakihara and K. Ishizuka, *Biomed. Microdevices*, 2008, **10**, 539–546.
140 Q. Wu, W. Jin, C. Zhou, S. Han, W. Yang, Q. Zhu, Q. Jin and Y. Mu, *Analytical Chemistry*, 2011, **83**, 3336–3342.
141 B. Hatano, M. Goto and H. Fukumoto, *Journal of medical*, 2011, **573**, 568–573.
142 B. Hatano, T. Maki, T. Obara, H. Fukumoto, K. Hagisawa, Y. Matsushita, A. Okutani, B. Bazartseren, S. Inoue and T. Sata, and others, *Japanese Journal of Infectious Diseases*, 2010, **63**, 36–40.
143 T. Notomi and M. Yasuyoshi, *Journal of Infection and Chemotherapy*, 2009, 62–69.
144 http://primerexplorer.jp/*A Guide to LAMP primer designing*, Eiken Chemical Company, Tokyo, Japan.
145 http://www.eiken.co.jp/en/product/index.html.
146 A. Fire, S. Q. Xu, *Proceedings of the National Academy of Sciences* (USA), 1995, **92**, 4641–5..
147 L. Blanco, A. Bernad, J. M. Lázaro, G. Martin, C. Garmendia and M. Salas, *Journal of Biological Chemistry*, 1989, **264**, 8935.

148 J. Banér, M. Nilsson, M. Mendel-Hartvig and U. Landegren, *Nucleic Acids Res.*, 1998, **26**, 5073–5078.
149 P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas and D. C. Ward, *Nat. Genet.*, 1998, **19**, 225–32.
150 S. Henriksson, A. L. Blomstrom, L. Fuxler, C. Fossum, M. Berg and M. Nilsson, *Virology Journal*, 2011, 8.
151 Y. Tanaka, H. Xi, K. Sato, K. Mawatari, B. Renberg, M. Nilsson and T. Kitamori, *Analytical Chemistry*, 2011, **83**, 3352–3357.
152 J. Melin, J. Jarvius, J. Goransson and M. Nilsson, *Anal. Biochem.*, 2007, **368**, 230–238.
153 T. Murakami, J. Sumaoka and M. Komiyama, *Nucleic Acids Res.*, 2009, **37**, e19.
154 F. Li, Z. Chunyan, Z. Wandi, C. Shenghui, M. Jianghong, J. Wu and D. Y. Zhang, *J. Clin. Microbiol.*, 2005, **43**, 6086–6090.
155 W. Zhang, M. Cohenford, B. Lentrichia, H. D. Isenberg, E. Simson, H. Li, J. Yi and D. Y. Zhang, *J. Clin. Microbiol.*, 2002, **40**, 128–132.
156 E. Schopf, Y. Liu, J. C. Deng, S. Yang, G. Cheng and Y. Chen, *Anal. Methods*, 2011, **3**, 267–273.
157 D. A. Di Giusto, W. A. Wlassoff, J. Gooding, B. Messerle and G. C. King, *Nucleic Acids Res.*, 2005, **33**, e64.
158 J. Yi, W. Zhang and D. Zhang, *Nucleic Acids Res.*, 2006, **34**, e81.
159 M. Nilsson, M. Gullberg, F. Dahl, K. Szuhai and A. K. Raap, *Pharmacia*, 2002, **30**, 1–7.
160 W. Zhao, M. M. Ali, M.A. Brook, Y. Li, *Angewandte Chemie* (International ed. in English), 2008, **47**, pp. 6330–7..
161 Z. Tong, F. Kong, B. Wang, X. Zeng and G. L. Gilbert, *J. Microbiol. Methods*, 2007, **70**, 39–44.
162 D. Haible, S. Kober and H. Jeske, *J. Virol. Methods*, 2006, **135**, 9–16.
163 B. Wang, S. Potter, Y. Lin, A. Cunningham, D. Dwyer, Y. Su, X. Ma, Y. Hou and N. Saksena, *J. Clin. Microbiol.*, 2005, **43**, 2339–2344.
164 S. Kaocharoen, B. Wang, K. M. Tsui, L. Trilles, F. Kong and W. Meyer, *Electrophoresis*, 2008, **29**, 3183–3191.
165 M. Najafzadeh, J. Sun, V. A. Vicente and G. S. DeHoog, *Mycoses*, 2011, **54**, 577–82.
166 X. Zhou, F. Kong, T. Sorrell, H. Wang, Y. Duan and S. Chen, *J. Clin. Microbiol.*, 2008, **46**, 2423–2427.
167 M. Nilsson, H. Malmgren, M. Samiotaki, M. Kwiatkowski, B. Chowdhary and U. Landegren, *Science*, 1994, **265**, 2085–2088.
168 P. Hardenbol, J. Banér, M. Jain, M. Nilsson, E. Namsaraev, G. Karlin-Neumann, H. Fakhrai-Rad, M. Ronaghi, T. Willis, U. Landegren and R. W. David, *Nat. Biotechnol.*, 2003, **21**, 673–678.
169 M. Akhras, M. Unemo, S. Thiyagarajan, P. Nyren, R. Davis, A. Fire and N. Pourmand, *PLoS One*, 2007, **2**, e915.
170 U. Landegren, R. Kaiser, J. Sanders, L. Hood, *Science* (New York, N.Y.), 1988, **241**, 1077–80..
171 D. Y. Zhang, W. Zhang, X. Li and Y. Konomi, *Gene*, 2001, **274**, 209–16.
172 V. V. Demidov, *Expert Rev. Mol. Diagn.*, 2002, **2**, 542–8.
173 F. B. Dean, S. Hosono, L. Fang, X. Wu, A. F. Faruqi, P. Bray-Ward, Z. Sun, Q. Zong, Y. Du, J. Du, M. Driscoll, W. Song, S. F. Kingsmore, M. Egholm, R. S. Lasken, *Proceedings of the National Academy of Sciences* (USA), 2002, **99**, 5261–6..
174 M. J. Reagin, T. L. Giesler, A. L. Merla, J. M. Resetar-Gerke, K. M. Kapolka and J. A. Mamone, *Journal of Biomolecular Techniques*, 2003, **14**, 143.
175 J. R. Nelson, Y. C. Cai, T. L. Giesler, J. W. Farchaus, S. T. Sundaram, M. Ortiz-Rivera, L. P. Hosta, P. L. Hewitt, J. A. Mamone, C. Palaniappan and C. W. Fuller, *BioTechniques*, 2002, 44–7.
176 N. Kurn, P. Chen, J. . Heath, A. Kopf-Sill, K. M. K. M. Stephens and S. Wang, *Clin. Chem.*, 2005, **51**, 1973–81.
177 A. Dafforn, P. Chen, G. Deng, M. Herrler, D. Iglehart, S. Koritala, S. Lato, S. Pillarisetty, R. Purohit and M. Wang, and others, *Biotechniques*, 2004, **37**, 854–857.
178 DNAform, Japan. http://www.dnaform.jp/smartamp/index_e.html/.
179 I. Biswas and P. Hsieh, *J. Biol. Chem.*, 1996, **271**, 5040–5048.
180 J. Watanabe, Y. Mitani, Y. Kawai, T. Kikuchi, Y. Kogo, A. Oguchi-Katayama, H. Kanamori, K. Usui, M. Itoh and P. E. Cizdziel, and others, *BioTechniques*, 2007, **43**, 479.
181 K. Tatsumi, Y. Mitani, J. Watanabe, H. Takakura, K. Hoshi, Y. Kawai, T. Kikuchi, Y. Kogo, A. Oguchi-Katayama and Y. Tomaru, and others, *J. Mol. Diagn.*, 2008, **10**, 520.
182 K. Azuma, A. Lezhava, M. Shimizu, Y. Kimura, Y. Ishizu, T. Ishikawa, T. Kamataki, Y. Hayashizaki and H. Yamazaki, *Clin. Chim. Acta*, 2011, **412**, 1249–51.
183 T. Araki, K. Shimizu, K. Nakamura, T. Nakamura, Y. Mitani, K. Obayashi, Y. Fujita, S. Kakegawa, Y. Miyamae, K. Kaira, T. Ishidao, A. Lezhava, Y. Hayashizaki, I. Takeyoshi and K. Yamamoto, *J. Mol. Diagn.*, 2010, **12**, 118–24.
184 Y. Kawai, J.-E. Morlighem, Y. Kimura, H. Kanamori, T. Ishidao, Y. Mitani, Y. Kogo, T. Hanami, T. Soma, Y. Ishizu, M. Hanami, S. Aoki, A. Katayama, H. Kinoshita, Y. Tanaka, A. Lezhava, T. Ishikawa, Y. Hayashizaki, R. Omics, *Miniaturized Systems for Chemistry and Life Sciences 3 - 7 October 2010*, Groningen, The Netherlands 14th International Conference on, 2010, 1493–1495.
185 http://www.smapdna.com/.
186 Y. Mitani, A. Lezhava, Y. Kawai, T. Kikuchi, A. Oguchi-katayama, Y. Kogo, M. Itoh, T. Miyagi, H. Takakura, K. Hoshi, C. Kato, T. Arakawa, K. Shibata, K. Fukui, R. Masui, S. Kuramitsu, K. Kiyotani, A. Chalk, K. Tsunekawa, M. Murakami, T. Kamataki, T. Oka, H. Shimada, P. E. Cizdziel and Y. Hayashizaki, *Nat. Methods*, 2007, **4**, 3000–3000.
187 Y. Kimura, A. Oguchi-katayama, Y. Kawai, K. Naito, Y. Mitani, J.-étienne Morlighem, Y. Hayashizaki and A. Lezhava, *Biochem. Biophys. Res. Commun.*, 2009, **383**, 455–459.
188 Y. Inoue, T. Mori, Y. Toyoda, A. Sakurai, T. Ishikawa, Y. Mitani, Y. Hayashizaki, Y. Yoshimura, H. Kurahashi and Y. Sakai, *J. Plast., Reconstr. Aesthetic Surg.*, 2010, **63**, 1369–1374.
189 G. T. Walker, M. C. Little, J. G. Nadeau, D. D. Shank, *Proceedings of the National Academy of Sciences* (USA), 1992, **89**, 392–6..
190 G. T. Walker, M. S. Fraiser, J. L. Schram, M. C. Little, J. G. Nadeau and D. P. Malinowski, *Nucleic Acids Res.*, 1992, **20**, 1691.
191 M. C. Little, J. Andrews, R. Moore, S. Bustos, L. Jones, C. Embres, G. Durmowicz, J. Harris, D. Berger and K. Yanson, and others, *Clinical Chemistry*, 1999, **45**, 777.
192 Q. Chen, Z. Bian, M. Chen, X. Hua, C. Yao, H. Xia, H. Kuang, X. Zhang, J. Huang and G. Cai, and others, *Biosens. Bioelectron.*, 2009, **24**, 3412–3418.
193 D. Akduman, J. M. M. Ehret, K. Messina, S. Ragsdale and F. N. N. Judson, *J. Clin. Microbiol.*, 2002, **40**, 281–283.
194 R. P. Verkooyen, G. T. Noordhoek, P. E. Klapper, J. Reid, J. Schirm, G. M. Cleator, M. Ieven and G. Hoddevik, *J. Clin. Microbiol.*, 2003, **41**, 3013.
195 J. Van Ness, L. K. Van Ness and D. J. Galas, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 4504–9.
196 H. Jia, Z. Li, C. Liu, Y. Cheng, *Angewandte Chemie* (International ed. in English), 2010, **49**, pp. 5498–501..
197 B. K. Maples, R. C. Holmberg, A. P. Miller, J. Provins, R. B. Roth, J. Mandell, 2007, 12/173020 *US Patent Application*, 20090081670.
198 U. Roth, B. Richard. San Diego, CA, in *Lab Automation 2008, Conference*, 2008.
199 K. V. Spenlinhauer T., M. Estock, T. McFadd, S. Kovacs, G. Hoyos, V. Perez, B. Maples, J. Provins and R. Roth, *American Phytopathological Society Annual meeting*, 2009.
200 M. Shimada, F. Hino, H. Sagawa, H. Mukai, K. Asada and I. Kato, *Rinsho byori. The Japanese Journal of Clinical Pathology*, 2002, **50**, 528.
201 H. Mukai, T. Uemori, O. Takeda, E. Kobayashi, J. Yamamoto, K. Nishiwaki, T. Enoki, H. Sagawa, K. Asada and I. Kato, *J. Biochem.*, 2007, **142**, 273–81.
202 T. Uemori, H. Mukai, O. Takeda, M. Moriyama, Y. Sato, S. Hokazono, N. Takatsu, K. Asada and I. Kato, *J. Biochem.*, 2007, **142**, 283–92.
203 N. Urasaki, S. Kawano, H. Mukai, T. Uemori, O. Takeda and T. Sano, *IRCHLB Proceedings*, 2008, 124–127.
204 H. Inami, K. Tsuge, M. Matsuzawa, Y. Sasaki, S. Togashi, A. Komano and Y. Seto, *Biosens. Bioelectron.*, 2009, **24**, 3299–305.
205 E. Isogai, C. Makungu, J. Yabe, P. Sinkala, A. Nambota, H. Isogai, H. Fukushi, M. Silungwe, C. Mubita, M. Syakalima, B. M. Hang'ombe, S. Kozaki and J. Yasuda, *Comp. Immunol., Microbiol. Infect. Dis.*, 2005, **28**, 363–70.
206 M. Shimada, F. Hino, J. Yamamoto, H. Mukai, T. Hosobe, S. Onodera, S. Hosina and K. Machida, *Rinsho byori. The Japanese Journal of Clinical Pathology*, 2003, **51**, 1061–7.
207 T. Horii, A. Monji, K. Uemura and O. Nagura, *J. Microbiol. Methods*, 2006, **65**, 557–61.