Chemically-heated non-instrumented nucleic-acid amplification assay platform

Weigl BH, Domingo GJ, Gerlach J, Wilmoth J, Tang D, Harvey D, Talwar N, Fichtenholz A, van Lew B, LaBarre P.
PATH, Seattle, WA.

Concept

PATH is developing a diagnostic platform based on nucleic-acid (NA) amplification that will require no instrumentation. It has the dual purpose of providing molecular diagnostics at the point of care as well as stabilizing NA specimens for further analysis via a centralized surveillance system. PATH is developing a diagnostic platform based on nucleic-acid (NA) amplification that will require no instrumentation. It has the dual purpose of providing molecular diagnostics at the point of care as well as stabilizing NA specimens for further analysis via a centralized surveillance system. Such assays are especially useful in low-resource settings (Figure 1).

Characteristics of this platform include:

- Ease of use in low-resource settings by minimally trained health workers.
- No need for additional instrumentation.
- Almost as rapid and simple as a lateral flow strip test
- The sensitivity and specificity of NA amplification tests.

The low-cost integrated device has three functional components:

(1) a manual sample-processing subunit that generates clean and stabilized DNA from raw samples containing NAs.

(2) an exothermically heated NA amplification subunit.

(3) a visual amplicon detection sub-unit.

We aim to integrate manual sample prep, chemical exothermic heating, temperature stabilization using phase-change materials, isothermal NA amplification, and visual amplicon detection in a single low-cost disposable.

Introduction

Current NA based assays require instrumentation

Assays based on nucleic NA amplification (e.g., PCR, TMA, LAMP, NASBA, etc.) have found many applications in medical diagnostics, forensics, biodefense, biotechnological research, and other fields. Such assays generally require skilled users, a laboratory, and sophisticated instrumentation. Those instruments need to heat or heat-cycle the samples, and also in most cases, to detect the amplified NA material. While many attempts have been made to simplify and integrate such assays (e.g., through the use of lab-on-a-chip technologies), all methods described so far still do require, at a minimum, a thermocycler or heat controller, and many require a detection unit as well as a centrifuge for sample processing.

Figure 1. Lab supply transport and a typical laboratory in a mid-level health care center in the developing world. In many cases conditions and instrumentation for NA assays can be difficult to support.

Exothermic heating for NA amplification

In this poster we describe present concepts and first experimental results from combining exothermic chemical heating (currently in use in "ready-to-eat" meals and hand warmers for campers) and nucleic NA amplification technologies. Exothermic heating replaces the need for heating instruments typically used to drive the reactions necessary for NA amplification.

Initial Targets: Influenza and Malaria

Influenza A virus (an RNA virus)—to demonstrate RNA stabilization through generation of cDNA.

Malaria—the amplification and visual detection of the DNA of Plasmodium falciparum (a protozoon).

Influenza

As of May 31, 2007, 309 human cases of avian influenza H5N1 resulting in 187 fatalities had been reported by WHO, with Indonesia and Vietnam reporting the most cases. Access to laboratories or testing facilities that provide reliable diagnosis of influenza of any type was limited. The lack of inexpensive, simple, and accurate testing methods at the point of care limits effective surveillance, early treatment of patients, and tracing of outbreaks. Figure 1 emphasizes the wide range of conditions found in mid-level health care centers in the developing world.

Unlike DNA, RNA (as present in the influenza virus) is labile to hydrolysis and susceptible to prevalent RNAses. As a consequence, RNA stabilization requires the use of stabilizing agents and refrigeration and/or freezing. The current stabilization protocols such as RNALater from Ambion require refrigeration for long-term preservation of specimens and sample transfer from, for example, a clinical site to a clinical laboratory or a surveillance site.

Malaria

More than 300 million new cases of malaria continue to occur each year, resulting in more than a million deaths worldwide. Microscopy is still the standard method for diagnosis in many parts of the developing world, but it is time consuming, labor intensive, and can only be performed by expert microscopists in specialty clinics or higher levels of the health care system.

Despite their many very positive attributes for low-resource situations, lateral flow strips frequently have limited sensitivity and, sometimes, specificity. Although some lateral flow assays can achieve sensitivity and selectivity in the range of 90% or higher, others, even commercialized assays, have a much lower sensitivity (between 50% and 70%).

Background

Loop-mediated isothermal amplification (LAMP)

- Relies on a single enzyme—DNA polymerase (unlike widely used isothermal NA amplification technologies such as NASBA, TMA, or SDA).
- Demonstrates sensitivity comparable to PCR.
- May be less prone to nonspecific amplification.

A typical reaction involves heating the reaction mixture to 95°C for 5 minutes then incubating for 60 minutes at 65°C.

Visual detection of positive reaction

Recently, Poon, et al.[1] demonstrated visual detection of amplification in the reaction tube with no additional processing. During a dramatically simplified NA amplification test for malaria in which blood is heat treated at 99°C, then added directly to the LAMP reaction mix, a white precipitate, visible as turbidity to the naked eye, forms.

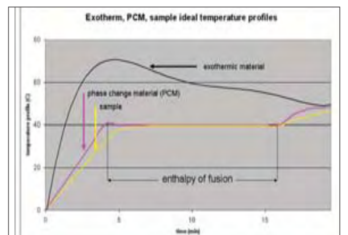

Figure 2. Model diagram showing how a phase-change material can be used to control temperature.

Chemical reactions as heat source

Chemical reactions can provide significant and controllable amounts of heat. We apply exothermic reactions, in combination with phase-change materials, to provide temperature-controlled heat for performing NA amplification assays.

Common exothermic methods:

- Saturated sodium acetate trihydrate
- Oxidation reaction

Exothermic materials can be activated in various ways suitable for a simple disposable device. For example, fine iron powder generates heat when it starts oxidizing upon exposure to moist air, whereas a supersaturated sodium acetate trihydrate generates heat after crystallization if triggered by a nucleating agent, which can be provided by a physical process such as the shockwaves emanating from the sharp and sudden bending of a metal disc.

Phase-change materials to maintain temperature

In these phase-change materials a specific amount of energy, the enthalpy of fusion, as shown in Figure 2, can transfer into and from the material while the material is at the phase transition temperature. This chemical property allows energy to be stored in substances like paraffin. Just as two-phase ice water maintains 0°C regardless of surrounding temperature, the proper application of an exothermic reaction, temperature moderating phase-change materials, and inexpensive insulating materials can maintain a constant diagnostic sample temperature. This feature is critical because it allows the assay to be used in a wide range of ambient temperatures.

Results to Date

Design Requirements

Exothermic heating applications in disposable isothermal devices need to:

- Provide sufficient heat.
- Achieve a set duration.
- Have relatively little reagent volume.
- Be relatively benign (neither explosive, extremely caustic, nor toxic).

Phase-change materials

Some materials have performed better than others. We have found that parafins tend to have a wide (+/- 5C) range of melting temperatures, due to the variable length of polymer chains, as opposed to the precise melting temperatures of alloys or ice.

Most promising phase-change materials

- Cerro shield, an inexpensive alloy with a high melting enthalpy per unit volume and a phase transition temperature of 95°C, is most promising for high temperature reactions.
- RT 65, a product of Rubitherm® Technologies GmbH, is our leading candidate for the LAMP amplification temperatures.
- Finally, we have demonstrated a combination of two different mixtures to generate a short heat spike (to melt DNA) followed by a lower, constant temperature (to perform isothermal amplification).

Exothermic reactions

Three promising exothermic reactions investigated by PATH are

(1) Catalyzed oxidation of iron (providing up to 69°C).

(2) Reaction of calcium oxide and diluted hydrochloric acid (generates >100°C).

(3) Reaction of magnesium and copper sulfate which can generate similar temperatures.

(4) Crystallization of salt solutions can also generate stable exothermic heating by combining the characteristics of exothermic materials with phase change materials.

Figure 3 shows the temperature profile of a saturated sodium acetate solution seeded with a crystal, a process that can be easily replicated in a disposable cassette and can generate useful temperatures for RT and cDNA generation processes. If more precise temperatures are needed, we have demonstrated (Figure 4) that supersaturated salts solution crystallization temperatures can be customized through the addition of a precise amount of water. The sodium acetate reaction produces 250 J/g of sodium acetate.

For these experiments, the sodium acetate was packaged in a trilaminate foil pouch which maintains the liquid mixture in a clean, stable environment and also prevents evaporative losses. We initiated crystallization and heat formation by puncturing the pouches. However, we foresee that employing a mechanism that will snap a metal disc inside the pouch will be a more robust solution and allow for reuse of the pouch and sodium acetate as chemical heater.

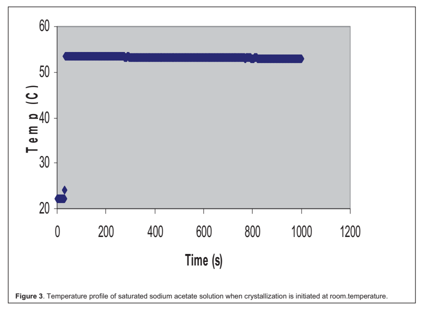

Figure 3. Temperature profile of saturated sodium acetate solution when crystallization is initiated at room.temperature.

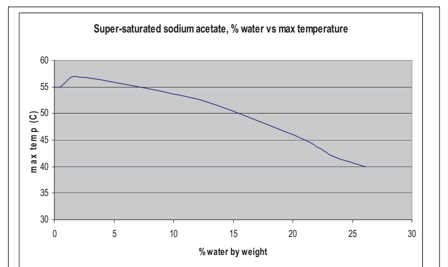

Figure 4. Empirically determined relationship between % water in supersaturated sodium acetate mixture and peak temperature.

Nucleic-acid extraction and cDNA generation using exothermic heating

Our current protocol has a similar efficiency to the Qiagen Qiamp (#52904) viral RNA kit (within a log value) and much higher efficiency than the Qiagen mini-elute RNA + DNA virus spin kit.

Protocol

- Described by Boom, et al.[2,3]
- Guanidinium thiocynate lysis buffer to develop a kit that extracts RNA from clinical samples without the use of centrifugation or vacuum pumps.

Pathogens

- Cultured dengue virus in plasma.
- Influenza A virus from nasal swabs and nasal lavages.

Lyophilized RT master mix

We are also developing a lyophilized RT master mix that is stable outside of the cold chain. Trehalose is primarily used to stabilize the dried master mix but has the added benefit of increasing the active temperature range of the RT.

Initial experiments were performed to determine the effects of varying concentrations of trehalose on RT activity. Moloney Murine Leukemia Virus RT activity in 0, 5, 10, and 20% trehalose was determined over four temperatures: 37, 45, 50, and 60°C. As shown in previous literature our data shows a broad temperature range tolerance by the RT in the presence of trehalose.

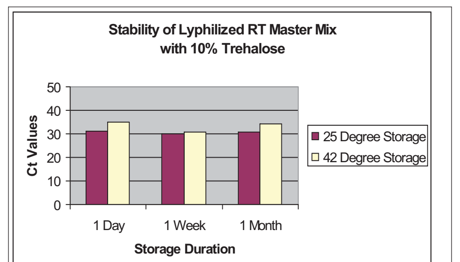

Figure 5. Comparison of QPCR results from lyophilized reverse transcription master mix over time.

RT, in conjunction with all the components required to perform an RT reaction, was lyophilized in the presence of 5, 10, 20% trehalose. Pre-aliquoted, dried master mix was stored desiccated in foil pouches (representing common packaging conditions) at 25°C and 42°C. Stability was assessed at various time points by performing reverse transcription of an RNA solution and then performing qPCR to determine relative amounts of template DNA generated during reverse transcription. Superior results were observed for samples lyophilized with 10% trehalose which indicates stability for at least 1 month at 42°C (Figures 5 and 6).

We performed a study comparing the stability of cDNA two other common RNA stabilization methodologies (preservation of RNA in RNALater® and preservation of whole virus in viral transport medium. Experiments were performed with dengue virus.

The preserved samples were stored at five temperatures, -20, 4, 25, 37, and 42°C and tested for stability at time points ranging from 1 day to 1 month. Stability was assessed by performing one-step qRT-PCR. Samples stored at -20°C were established as the baseline value for stability, and samples at other temperatures were compared. The change in Ct values compared to baseline were plotted (Figure 6). Comparing change in Ct value over time, it is evident that cDNA is more stable than whole virus stored in viral transport medium or RNA preserved in RNALater®.

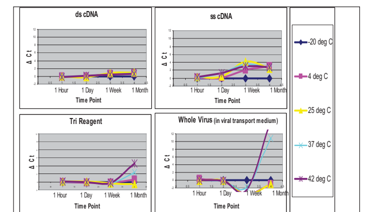

Figure 6. The stability of different NA stabilization methods. All sample types were stored at five temperatures ranging from -20°C to 42°C. All samples were assumed to be 100% stable at -20°C. Thus, for each method, the Ct values for samples stored at higher temperatures were compared to the Ct value at -20°C. Delta Ct (Δ Ct) is defined as the Ct of an elevated temperature sample minus the Ct of the -20°C. A Δ Ct of 0 indicates 100% stability whereas a ΔCt above 0 indicates that degradation has occurred.

Figure 7. Macroscopic two-chamber exothermic heater for the development of exothermically driven NA amplification methods. The outer (white) chamber contains an exothermic material, while the inner chamber (shown disassembled in front of the heater) contains the phase-change material. The lid has receptacles for inserting centrifuge tubes containing NA material and reaction mixtures. The components of the heater will be miniaturized and integrated with the assay cartridge.

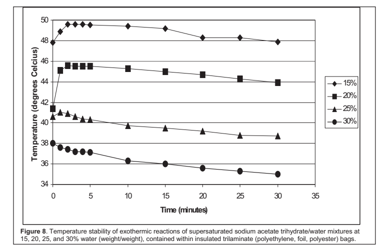

Figure 8. Temperature stability of exothermic reactions of supersaturated sodium acetate trihydrate/water mixtures at 15, 20, 25, and 30% water (weight/weight), contained within insulated trilaminate (polyethylene, foil, polyester) bags.

We also assessed the ability of sodium acetate/water mixtures to sustain a constant temperature over 30 minutes using small trilaminate bags containing 30 g of sodium acetate plus water added to make up 15, 20, 25, 30% weight/weight water sodium acetate mixtures. The mixtures were melted, and stability of the primed mixtures at room temperature was confirmed. The exothermic crystallization reaction was initiated by puncturing the bags. The temperatures of the mixtures were maintained for 30 minutes within 3°C (Figure 8).

We used a sodium acetate-based exothermic heater to extract RNA using the PATH method and then generate cDNA from nasal lavages spiked with influenza A RNA. This method was compared to a method utilizing Qiagen reagents for RNA extraction and a heat block for reverse transcription (Table 1).

	Sodium Acetate Sachet Heating	Heat Block
PATH purification	21.7	21.4
Qiagen Purification	20.7	20.2

Table 1. A comparison of Ct values generated by QPCR of cDNA prepared with varying RNA purification methods and reverse transcription heat sources.

Next Steps

Demonstration of isothermal amplification with a LAMP assay protocol for malaria

We are developing the prototype of a simple, single-use, plastic device that performs all steps outlined in the Poon et al. paper (Figure 9). It will be approximately the size of a centrifuge tube, with caps at both ends, and will use PCM-stabilized exothermic heating in place of an outside heat source. It combines the steps of sample preparation, LAMP amplification, and visual detection of amplification. We intend the device to be as easy to use as a lateral flow immunoassay (strip test) and as sensitive as a PCR assay. The device will consist of two compartments heated by exothermic reactions moderated by PCMs.

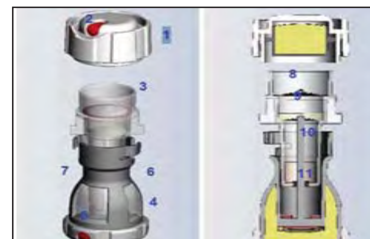

Figure 9. Preliminary design of a POC NAAT for Malaria. (Left—outside. Right—cross-section). Length approx. 40 mm. (1) Top screw cap containing exothermic material and PCM for generating 99°C for several minutes, activated by the screwing action. (2) Color temperature indicator showing when the heating step is complete. (3) Reagent tube containing sample (top), buffer (bottom), and LAMP reagents and primers in dry form (this can be in the sample compartment, or, optionally, in a separate compartment). (4) Bottom screw cap, containing exothermic material and PCM for generating 65°C for 0 to 60 minutes, also activated by the screwing action. (5) Color temperature indicator showing when the 65°C heating step is complete. (6) Viewing window and (7) daylight inlet to observe presence of turbidity, indicating positive reaction. (8) Open sample compartment inside reagent tube, to receive the blood drop. (9) Optional LAMP reagent compartment in reagent tube. (10) Buffer compartment in reagent tube. (11) Spike for isolating/fracturing membranes that separate reagent tube compartments.

Conclusions

From our research, we believe that it is possible to design a NA amplification assay that is entirely disposable and requires no instrumentation, power, or electronics. So far we have demonstrated chemical heating and heat stabilization using phase-change materials. We have also demonstrated chemically heated reverse transcription to generate cDNA from RNA which will allow the stabilization of labile viral RNA from pathogens such as Influenza A in field settings. We have also outlined concepts that should allow exothermically driven isothermal amplification and visual detection of the amplified NA. We are continuing work in all these areas and will report results as they become available.

Citations

1. Poon LL, Wong BW, Ma EH, et al. Sensitive and inexpensive molecular test for falciparum malaria: detecting Plasmodium falciparum DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clinical Chemistry. 2006;52(2):303–306.)

2. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der NJ. Rapid and simple method for purification of NAs. Journal of Clinical Microbiology. 1990;28(3):495–503.

3. Jean J, Blais B, Darveau A, Fliss I. Detection of hepatitis A virus by the NA sequence-based amplification technique and comparison with reverse transcription-PCR. Applied and Environmental Microbiology. 2001;67(12):5593–5600.

PATH
A catalyst for global health