Supporting information

A Multifunctional Reactor with Dry-Stored Reagents for Enzymatic Amplification of Nucleic Acids

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Section S1: Fabrication of the Multifunctional Reaction Chamber

The basic structure of our dry-storage chip is depicted in **Fig.1** and, in more details, in **Fig. S1**. The plastic chip was fabricated using rapid prototyping, layered-manufacturing. The 46 mm x 36 mm x 3.50 mm cassette consists of three layers: a top cover made of 250-μm thick, poly [methyl methacrylate (PMMA) 'acrylic'] film; a 3-mm thick, PMMA cassette body; and a 250-μm thick, PCR Sealers (Finnzymes™, ThermoFisher Scientific) tape bottom. For our rapid prototypes, both the top and bottom cover films were cut with a 30-W CO_2 laser (Universal Laser Systems, Scottsdale, AZ). The cassette body was milled with a precision, computer-controlled milling machine (HAAS Automation Inc., Oxnard, CA) to form two independent reactors (many more are possible, if desired).

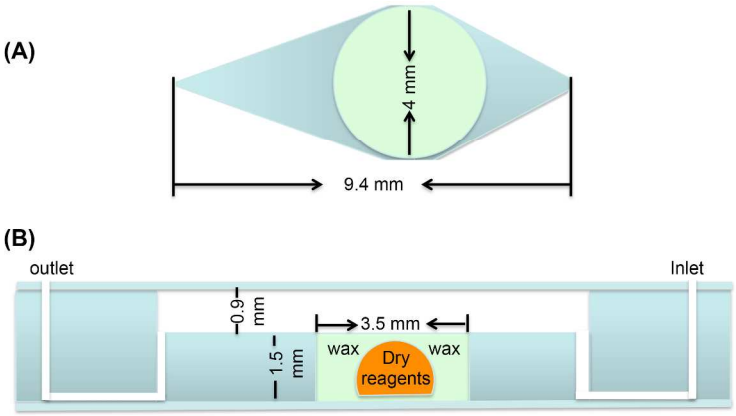

Figure S1: Top view (A) and cross-sectional view (B) of the multifunctional amplification reactor with dry-stored reagents (nucleic acid isolation membrane is not shown).

Each reaction chamber is connected to an inlet port and an exit port with 500 μm-wide and 200 μm-deep rectangular-cross section conduits. Each reactor has a flow-through Qiagen silica membrane (QiaAmp Viral RNA mini kit, Germantown, MD), seated on a ledge, at its entry port. See **Fig. 1** in the main text. The rhombus-shaped reaction chamber is 9.4 mm in length, 4.0 mm in maximum width, and 0.9 mm in depth. To accommodate the storage of dry reagents, a 1.5-mm deep, 3.5-mm diameter (~15μL) well was milled at the floor of the reaction chamber (**Figs. 1A** and **S1)**.

We lyophilized the reaction mix (BIOLYPH, MN), including enzymes (OmniAmp™ Polymerase with both reverse transcriptase and strand displacement activities, Lucigen), target-specific primers, and dye (EvaGreen), for long-term storage. The lyophilized reagents were then encapsulated with paraffin (Docosane ($C_{22}H_{46}$), Sigma, 134457-25G, melting temperature 42-45°C). The paraffin filled the reagent storage well, leaving a reaction chamber with 40 μL volume. The chip was then solvent bonded to a PMMA film. Since in our design, the reagents are pre-

stored in the amplification chamber, the paraffin encapsulation is needed to protect the reagents when sample and wash solutions are transmitted through the reaction chamber.

The lyophilized reagents can be encapsulated with paraffin prior to their introduction into the cassette or after their introduction into the cassette, during cassette fabrication process. The first approach offers flexibility, allowing one to maintain a library of beads for diverse targets and customize generic cassettes as needed. The second approach provides convenience since the cassette is supplied ready for use. Here we describe the latter approach.

To store reagents in the chip, we follow the operations described below (see also **Video S1**)

(1) Heat paraffin wax (Docosane ($C_{22}H_{46}$), Sigma, 134457-25G, mp: 42-45 °C) to ~200 °C and coat the bottom of the reagent well with 5 µL of molten wax.

(2) Lyophilize 1x LAMP reaction mixture (including labels) using an industry standard biological sample stabilization process (e.g., Biolyph, Hopkins, MN) or a conventional laboratory lyophilization unit (with similar results). For the experiments described here, the mix includes: OmniAmp™ Polymerase with both reverse transcriptase and strand displacement activities (Lucigen Corporation, Middleton, WI), primers (IDT, Commercial Park Coralville, IA), and EvaGreen™ dye (Biotium, Hayward, CA). The dried reagents were deposited on top of the wax layer in the well and compacted with a glass rod.

(3) Pipette 10 µL of molten wax to completely encapsulate the reagents and fill the reagent storage well.

(4) Solvent bond the top PMMA film with acetonitrile at room temperature to cap the reaction chambers.

To study dry reagents release and hydration, 2 µL of 10-fold diluted liquid food coloring (McCormick food and egg coloring dye, Amazon) was dried and wax-encapsulated to mimic the lyophilized LAMP reagents. See **Video S2**.

Section S2: Processor[1,2]

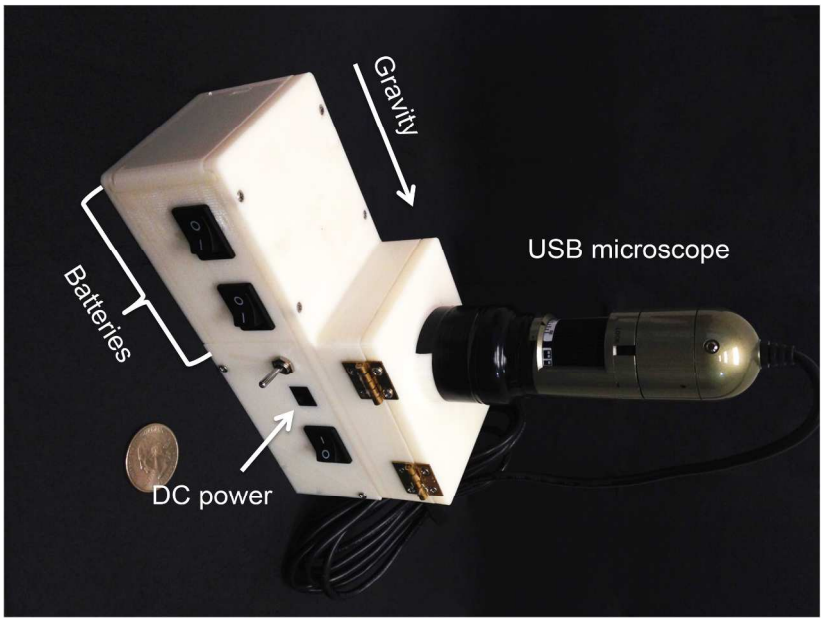

Figure S2: The UPenn custom-made, portable processor for nucleic acid isothermal amplification and detection[1,2].

Our custom-made, portable processor[1,2] is shown in **Fig. S2.** In the above and the remainder of this supplement, superscripts with the prefix "S" refer to the electronic supplement's references. Our processor includes an USB-based, fluorescence microscope (AM4113T-GFBW Dino-Lite Premier, AnMo Electronics, Taipei, Taiwan), a flexible, polyimide-based, thin film heater (Model HK5572R7.5L23A, Minco Products, Inc., Minneapolis, MN), a thermocouple, and a micro-controller for closed-loop temperature control.

Our processor can be powered either with four AA batteries or grid power. The fluorescence image of the amplification reactors is directly displayed on a computer screen and analyzed with a home-written Matlab™ - based program. When desired, the USB microscope can be replaced with a smartphone, wherein the fluorescent is excited with the smartphone flashlight, and the fluorescence emission detected with the smartphone camera and analyzed with custom application written for this purpose[3].

Section S3: Apparent Paraffin self-Fluorescence

During fluorescence microscopy with a green filter, the intact paraffin film appears green (**Fig. 2A** and **Video S4**). We believe that the paraffin's green color results from the reflection of the camera's excitation light. We are using an inexpensive camera with an imperfect separation (filtering) between the excitation and emission spectra. When higher quality filters are used, such as in a fluorescent microscope (Olympus BX51), no fluorescence was observed, and the paraffin film is not visible. **Video S4** shows the melting of a paraffin patch in the absence of reagents. Once the paraffin melted, the green color disappears.

Section S4: HPV-16 LAMP primer set design and HPV-16 DNA Extraction, Concentration, and Detection with Our Multifunctional Reaction Chamber

Loop Mediated Isothermal Amplification (LAMP) developed by Notomi et al.[4] is, perhaps, the most widely used NA amplification method for point-of-care diagnostics due to its high sensitivity and specificity. In this work, we designed HPV-16 LAMP primers (**Table S1**) using standard procedures as we have previously described before[5], but accounting for the higher operating temperature (66~74°C) of the OmniAmp polymerase (Lucigen) that we are using in this work. Briefly, we aligned and analyzed complete genome sequences of various HPV strains to identify sequences that are conserved for HPV-16, but divergent from other HPV strains. The six LAMP primer-set was designed with the PrimerExplorer V4 software[6] (Eiken Chemical Co. Ltd). A BLAST search of the GenBank nucleotide database was carried out for the selected primers' sequences to verify specificity. The LAMP sequences were synthesized by a commercial vendor (IDT, Coralville, IA) and documented in **Table S1** together with the concentrations used in our reaction mixtures.

To test chip performance, we prepared simulated saliva samples. Saliva (50 µL) donated by healthy individuals was spiked with 10^4, 10^3, and 10^2 copies of HPV gDNA and mixed with 50 µL chaotropic salt (6M guanidinium chloride), lysis buffer (QIAamp® MinElute Virus Spin Kit, Qiagen, Germantown, MD) and 62.5 µL ethanol. The mixture was then pipetted into the amplification reactor through the nucleic acid immobilization membrane. In the presence of the high salt concentrations and low pH, nucleic acids in the sample bind to the silica membrane. Subsequent to sample introduction, following manufacturer's protocol, 150 µL of ethanol-based wash buffer 1 (AW1, Qiagen, Germantown, MD) was pipetted into the chip to remove any bound proteins from the membrane. Then, the silica membrane was washed again with 150 µL of wash buffer 2 containing 70% ethanol (AW2, Qiagen, Germantown, MD) to remove salt, followed by air-drying for 30s at room temperature to remove any residual ethanol. Next, each reaction chamber was filled with 40 µL DNA grade water, injected through the inlet port and the immobilization membrane. The various operations were carried out manually with pipettes (**Fig. S3**). In the future, we will replace the pipettes with blisters filled with the various solutions.

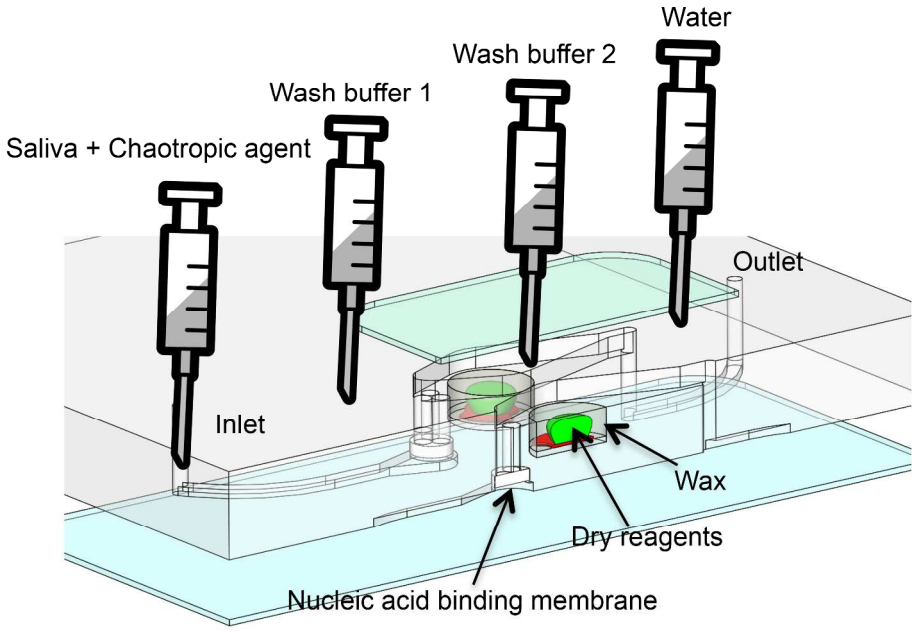

Figure S3: Workflow with the dry-storage chip.

Subsequent to the filling of the reaction chamber with water, the inlet and outlet ports were sealed with a transparent tape (3M, Scotch™ brand cellophane tape, St. Paul, MN) to minimize evaporation during the amplification process.

The chip was vertically positioned in our custom-made, portable, battery-heated processor that houses a temperature-controller and USB-based fluorescent camera system[1,2] (**Fig. S2**). The vertical orientation of the chip allowed the molten paraffin (which is lighter than water) and any bubbles migrate to the far (upper) end of the chamber, preventing their interference with monitoring of the amplification reaction. The advection in the chamber induced by the paraffin motion assisted in stirring the hydrated reagents. The heater temperature was, respectively, set to 66°C and 68°C for the DNA and RNA targets.

The camera was connected to a laptop. A custom program was written with Matlab™ to acquire images at 1-second intervals for the first 3 minutes and at 60-seconds intervals thereafter. The MatLab™ program processed and analyzed the images to produce a real-time fluorescence emission intensity curves as functions of time, similar to real time PCR machine.

Section S5: RT-LAMP with our Multifunctional Reactor

In **section S4**, we examined the operation of our multifunctional reaction chamber with a DNA target. In this section, we discuss the processing of a RNA target. Since the enzyme that we are using (OmniAmp, Lucigen) has both reverse-transcriptase and strand-displacement activities, we can process RNA targets in a single step without a need for a separate transcription step prior to amplification. To demonstrate single step RT-LAMP with our multifunctional reactor, we used both HIV clade C and MS2 bacteriophage RNA as targets.

We detected HIV virions in plasma. HIV-1 subtype C Viral strain (originated from Zambia, ZAM18, GenBank: L03705.1, SeraCare Life Science) was spiked into 140 µL human plasma at various concentrations. The plasma was then mixed with 560 µL of lysis buffer (QIAamp® Viral RNA Mini Kit, Qiagen) and 560 µL ethanol, and pipetted into the amplification reactors. The sample introduction was followed by two wash steps, amplification, and detection – similar to the processing of HPV DNA (**Section S4**). In this process, we used our custom-designed universal (strain-independent) HIV LAMP primer set ACeIN-26 (**Table S1**) that we have recently developed to detect HIV subtypes A, B, C, D, and G[7].

Section S6: HPV-16 DNA Detection with Lyophilized Reagents and Benchtop Equipment

In this section, we describe the results of benchtop experiments carried out to test the performance of the lyophilized reagents (same as the ones used in our chip experiments, but without any paraffin cladding) and our newly-designed primers for HPV-16 DNA (**Table S1**). The lyophilized reagent bead was hydrated in 25 µL of water laden with various numbers of template copies. The amplification was carried out with BioRad Real Time PCR machine at 66°C. Fluorescence emission intensity data were collected once every minute for 120 min. Tests with 100 copies per reaction were readily detected.

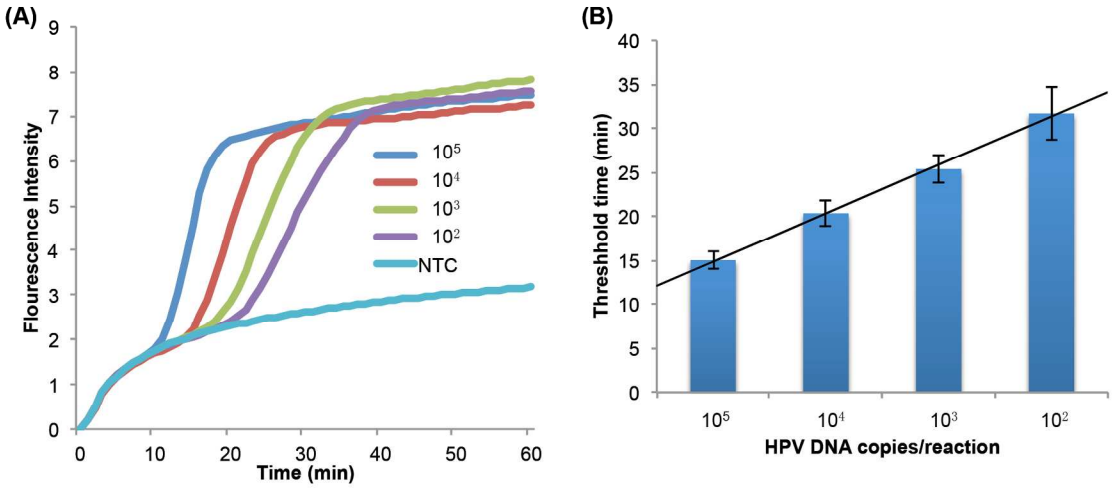

Figure S4: (**A**) LAMP amplification curves in the presence of 10^5, 10^4, 10^3, 10^2, and 0 (NTC) copies of HPV DNA per reaction. (**B**) The threshold time (in minutes) as a function of HPV DNA copies per reaction (N=3).

Section S7. HPV-16 Detection with a Rapid Sample Preparation Method

To compare the performance of our chip with rapid detection methods, we carried out a rapid test[8] for HPV LAMP amplification. We spiked 6×10^5, 6×10^4, 6×10^3, and 6×10^2 copies of HPV gDNA into 25μL saliva donated by healthy individuals. The spiked saliva was mixed with 475 μL dilution buffer. The mixture was then filtered with an SQ Easy filter (pore size, 10 μm; Porex, Georgia), collected in a clean 1.5 mL tube, and incubated at 90°C for 3 min. After cooling, 40 μL of filtrate was used to hydrate lyophilized reagents. The workflow of the rapid test is detailed in **Fig. S5**. The reaction mixture was amplified with a real-time thermocycler (BioRad, Model CFD3240), programmed to operate at a fixed temperature (66^{o}C) and monitored at 1-minute intervals for 60 minutes. In the rapid test, only 8% of the targets that were present in the initial sample are available for amplification. In contrast, our multifunctional reactor operates with nearly 100% of the targets present in the sample.

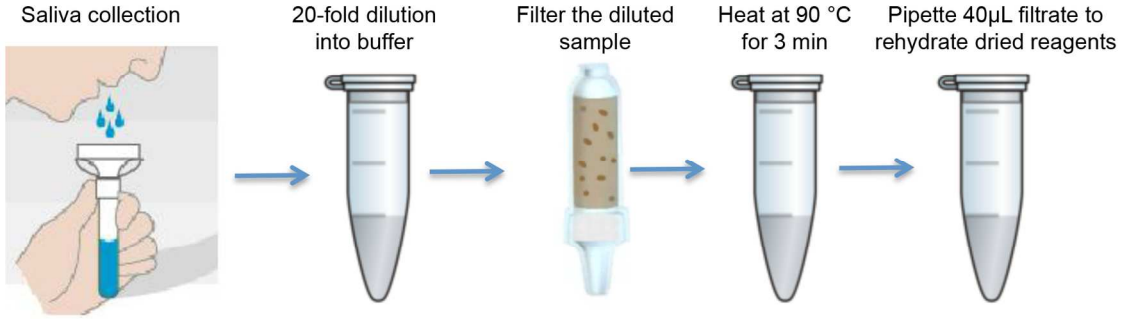

Figure S5: Workflow associated with the rapid test

Section S8: Dry-stored reagents exposed to ambient conditions in the absence of paraffin encapsulation

When lyophilized reagents were stored under ambient conditions (without paraffin encapsulation), the reagents absorbed condensate from the atmosphere and self-hydrated. **Fig. S6** shows images of initially dried reagents as a function of exposure to ambient conditions.

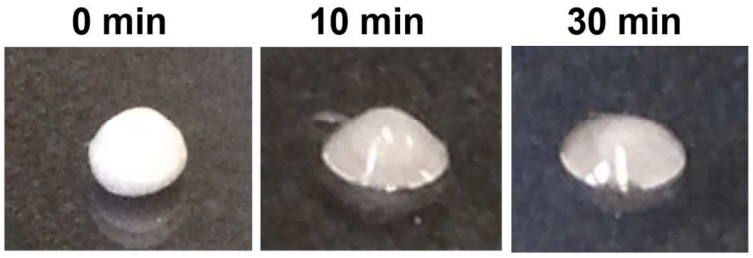

Figure S6: Lyophilized reagents exposed to ambient conditions. The lyophilized bead is exposed to air for 0, 10, 30 minutes at ambient temperature (21 °C) and ambient humidity (50~70%).

Table S1: Primers' Sequences and concentrations

Primer set	Primer name	Sequence (5' to 3')	Concentration (μM)
HPV-16	F3	CCGTTGTGTGATTTGTTAATTAGGT	0.2
	B3	AGAGATCAGTTGTCTCTGGTTG	0.2
	LF	TGCTTTTTGTCCAGATGTCTTTGCT	0.8
	LB	CAAGAACACGTAGAGAAACCCAGC	0.8
	FIP	TCCACCGACCCCTTATATTATGGAAAACTGTCAAAAGCCACTGTGT	1.6
	BIP	CGGTCGATGTATGTCTTGTTGCAGAGCAATGTAGGTGTATCTCCATGC	1.6
MS-2[9]	F3	TGTCATGGGATCCGGATGTT	0.2
	B3	CAATAGAGCCGCTCTCAGAG	0.2
	LF	CCAGAGAGGAGGTTGCCAA	0.8
	LB	TGCAGGATGCAGCGCCTTA	0.8
	FIP	GCCCAAACAACGACGATCGGTAAAACCAGCATCCGTAGCCT	1.6
	BIP	GCACGTTCTCCAACGGTGCTGGTTGCTTGTTCAGCGAACT	1.6
ACeIN-26[7]	F3_c	CCTATTTGGAAAGGACCAGC	0.2
	B3_a	TCTTTGAAAYATACATATGRTG	0.2
	B3_b	AACATACATATGRTGYTTTACTA	0.2
	FIP_e	CTTGGTACTACYTTTATGTCACTAAARCTACTCTGGAAAGGTG	0.8
	FIP_f	CTTGGCACTACYTTTATGTCACTAAARCTYCTCTGGAAAGGTG	0.8
	BIP	GGAYTATGGAAAACAGATGGCAGCCATGTTCTAATCYTCATCCTG	1.6
	LF	TCTTGTATTACTACTGCCCCTT	0.8
	LB	GTGATGATTGTGTGGCARGTAG	0.8

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