



Isothermal Two color fluorimeter

Claremont BioSolutions (CBS), a spin-off company out of the Bioengineering and Microfluidics Laboratories at the Keck Graduate Institute (KGI), has been developing a variety of compact components that are amenable for integration into pathogen detection systems. One such component, Claremont BioSolutions has developed a real time isothermal incubator with two detection channels called the Rabbit™ molecular analyzer. Two versions of this instrument are shown in Figure 1. This device has the same sensitivity as commercial real time PCR instruments, uses commercial off the shelf (COTS) components, and has a cost of goods of ~\$1,500. It consists of an aluminum block (Figure 15) with 8 tube wells spaced at 9 mm intervals therefore compatible with 200ul 8-well microtiter strips. Each well has two input ports and two output ports to allow for two excitation and two emissions light paths positioned at a right angle. It relies on LEDs for transmission and photodiodes for detection. Pressure from the lid presses the tubes firmly into the wells providing consistent thermal contact. The configuration of this instrument is such that all light emission and detection occurs "below" the wells rather than through the lid. This provides advantages of incorporation into a deck of a robotic workstation or for adapting an integrated system for sample processing and detection. Filters can be changed for color options. In the 5.2 version the device's temperature is set using a keypad on the device, and data is sent through a USB port to a computer for analysis. In the 6.1 version temperature protocols can be programmed by the user from a personal computer. Both versions can operate from 12-volt power supplies that plug into a wall outlet. The version 6.1 can also run from an auto adaptor and from an internal battery. Lid and well temperatures can be controlled independently. Version 6.2 is capable of melt-curve analysis and can heat from 65° to 95° in 30 seconds and cool from 95° to 40° within 90 seconds.



Figure 1. Two version of the Rabbit Fluorimeter. Version 5.2 (left) and version 6.1 (right). On board display and controls are available.

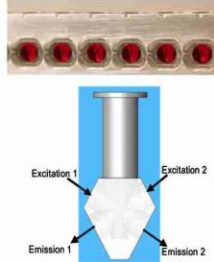


Figure 2. 8 well strip aluminum block.

HDA™ Assay Tested in the Rabbit Fluorimeter

The Helicase Dependent Assay (HDA™) is an isothermal amplification assay developed by Biohelix Corporation. The HDA™ assay for HSV I has been tested in version 5.2 of the Rabbit™ Fluorimeter with a serial dilution of a plasmid positive control, containing the HSV-1 target sequence ranging from 10³ to 10⁵ copies. Incubations were at 65°C for up to 2 hours and 13 minutes. Relative fluorescence units (RFU) were plotted against time in seconds (Figure 3). This version has eight wells for 200 ul tubes in microtiter format. Excitation light is transmitted at 470 ± 20 nm and emission detection occurs at 530 ± 25nm. The HDA™ HSV 1 assay employs an excess one of the two primers enabling the real-time detection with a labeled detection probe. The probe is dual-labeled with a fluorescent reporter and a quencher. Fluorescence is activated (de-quenched) when it is bound to its complementary target DNA. All three titers, 10³, 10⁴, and 10⁵ were easily detected above the no-target control.

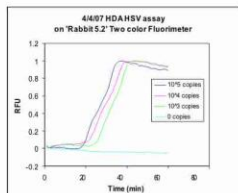


Figure 3. Real-time HDA detection of HSV. The HDA reaction was conducted at 65° for 60 minutes. Excitation light is transmitted at 470 ± 20 nm and emission detection occurs at 530 ± 25nm. The HDA is performed with one primer in molar excess of the other to generate a surplus of sense strand amplicon. As the excess strand is generated it is bound by a FAM labeled probe.

Micro-Bead Beaters (µBB)

Another component developed by CBS & KGI which is amenable for integration into pathogen detection systems, is the Micro Bead-Beater™ (µBB™). The µBB™ is a compact device that is capable of ultra-rapid lysis (~90% lysis in 15 seconds) of micro volumes (~80ul) of *Bacillus* spores in a continuous-flow format (batch mode) or in a disposable single-tube format. The µBB™ is also capable of processing much larger volumes (milliliters) of spores or vegetative cells using a continuous-flow mode. DNA quantification results using dsDNA binding fluorescence dyes and real-time PCR have shown that, when comparing the lysis of *Bacillus subtilis* (*Bs*) spores, µBB™ compares very favorably versus other well-known spore lysis techniques.

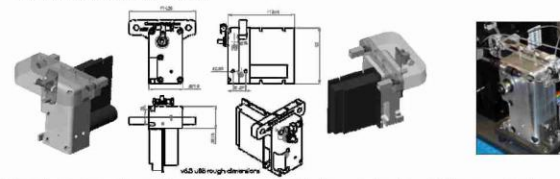


Figure 4: Representations of the micro bead beater and its incorporation into a fluidic system (right).

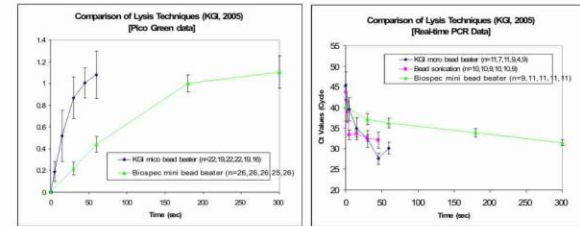


Figure 5: PicoGreen (left) and real-time PCR (right) assays comparing the lysis performance of µBB to the Biospec™ and bead sonication on *Bacillus subtilis* spores.

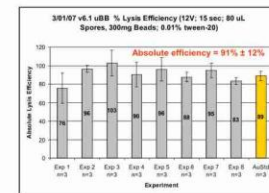
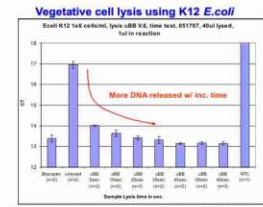


Figure 6: PicoGreen data showing absolute lysis efficiency of *Bacillus subtilis* spores using the µBB in flow-through batch mode (80ul samples).



Performed in batch mode using air injector to liberate sample

Figure 7: Real-time PCR data showing lysis of K12 *E. coli* efficiency using the µBB performed in flow-through batch mode (40ul samples).

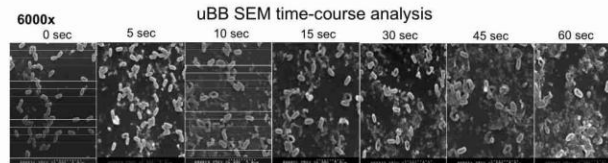


Figure 8: Lysis time-course of *Bacillus subtilis* spores visualized using a Hitachi S520 SEM.

Microfluidic Bead Blenders (BBL)

Micro Bead Blenders (BBL) have also been developed at CBS as an inexpensive and therefore disposable method of bead beating for lysis of spores. A low cost inexpensive disposable DC electric motor operates in contact with the sample fluid and drives a micro propeller mixing appropriately sized zirconium beads. The bead chamber is flanked by nylon meshes. The Bead Blender can process a 50 ul sample aliquots or by controlling a continuous flow of sample the Bead Blender is able to process a sample volume much larger than the bead beating chamber and deliver efficient lysis of spores. Figure 9 shows the version 2.0 of the BBL with intake and outflow in parallel positions. Figure 10 shows results for a time course study of BBL treatment of *B. subtilis* spores. Results were reported as a percent yield of results from treatment with the commercially available Biospec™ method that calls for three minutes of beating. Samples were measured for DNA content by available Biospec™ method that calls for three minutes of beating standard method is accomplished in 45 seconds by the bead blender. Titration of *B.s.* spores has been tested in 200 ul preparations in the bead beater down to a concentration of 100 copies of spore/ul and 1 ul of this sample gave positive results for PCR. (Data not shown).

The effect of bead blending was also tested on vegetative cells of Ecoli K12. Ten replicates of bead beating for 45 second were compared to five replicate of no bead-beating controls by PCR. On average the PCR reaction was accelerated by about 7 cycles (data not shown).

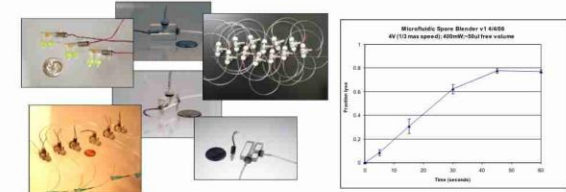


Figure 9. Micro Bead Blenders. Chambers are made of milled acrylic. Ports are fitted with custom filters that trap zirconium beads. A disposable 4mm diameter motor drives a laser-carved propeller. The motor is capable of running within the fluid without contaminating the sample.

Figure 10. Bead Blender Flow-through mode results show 50% lysis in under 30 sec and 75% in 45 sec. Fraction lysed as percentage yield of Biospec™ Bead Beating for 3 minutes. (Determined using PicoGreen assay).

Characterization of the effect of bead blending on gDNA shearing has been performed and the results are shown in Figure 11. *Calif* Thymus gDNA has been treated by Bead beating for 15 seconds and compared to shearing by sonication for 20 seconds. Lanes 10 and 11 show the untreated gDNA. The sonicated DNA is distributed at around the 1500 bp marker (lanes 7-9). Lanes 1-6 show the 15-second µBB treated samples. The majority of the smear is larger than 1500 bp marker, therefore above ~1,000 kDa.

Figure 12 shows electron micrographs of spore samples, revealing the obvious destruction of spore particles from µBB processing compared to untreated spores (panel C) and the comparable effectiveness of flow through processing (panel A) to static processing (Panel B)

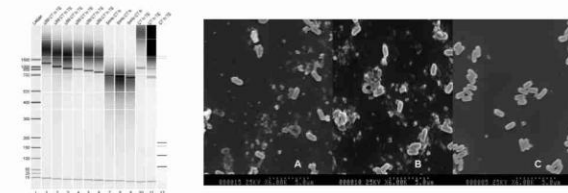


Figure 11. Agilent Bioanalyzer analysis of gDNA shearing. 20 ng/ul *Calif* Thymus gDNA in TE Lanes 1-6, bead beating for 15 seconds. Lanes 7-9 probe-sonicated for 20 seconds. Lanes 10-11 untreated 20 ng/ul. 1500bp marker included.

Figure 12. Electron micrographs of *B. subtilis* spores. Panel A; 1ml sample treated by Bead Blender in flow through mode at high velocity, for 90 sec (11ul/sec) Panel B; 80 ul aliquot treated for 40 second. Panel C. Untreated spores.