The LightCycler™: A Microvolume Multisample Fluorimeter with Rapid Temperature Control

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ABSTRACT

Experimental and commercial microvolume fluorimeters with rapid temperature control are described. Fluorescence optics adopted from flow cytometry were used to interrogate 1–10- μ L samples in glass capillaries. Homogeneous temperature control and rapid change of sample temperatures (10°C/s) were obtained by a circulating air vortex. A prototype 2-color, 32-sample version was constructed with a xenon arc for excitation, separate excitation and emission paths, and photomultiplier tubes for detection. The commercial LightCycler[®], a 3-color, 24-sample instrument, uses a blue light-emitting diode for excitation, paraxial epi-illumination through the capillary tip and photodiodes for detection. Applications include analyte quantification and nucleic acid melting curves with fluorescent dyes, enzyme assays with fluorescent substrates and techniques that use fluorescence resonance energy transfer. Microvolume capability allows analysis of very small or expensive samples. As an example of one application, rapid cycle DNA amplification was continuously monitored by three different fluorescence techniques, which included using the double-stranded DNA dye SYBR[®] Green I, a dual-labeled 5' exonuclease hydrolysis probe, and adjacent fluorescein and Cy5[®]-labeled hybridization probes. Complete amplification and analysis requires only 10–15 min.

INTRODUCTION

Fluorimetry is a sensitive and versatile technique with many applications in molecular biology. Fluorescent dyes can be used to quantify nucleic acids (17) and to monitor hybridization and denaturation (13,30). Fluorogenic substrates can be used in many enzyme assays, including assays for proteases (12), restriction enzymes (6) and helicases (9). Fluorescence resonance energy transfer is a powerful technique for analysis of macromolecular structure, including protein-protein interactions (29), protein-nucleic acid complexes (15) and nucleic acid structure (20). One limitation encountered is the volume of sample required for conventional solution fluorimetry. When reagent costs are high, or when the interaction of expensive components occurs only at high concentrations, a fluorimeter with microliter instead of milliliter volume requirements would be very useful.

Fluorescence techniques have recently been applied to *in vitro* nucleic acid amplification. Nucleic acid products from strand displacement amplification (21), the ligase chain reaction (4) and the polymerase chain reaction (PCR) (11) have all been analyzed by fluorescence after amplification. Fluorescent probes can also be added before amplification is begun to monitor product accumulation during amplification (7,8,10,16,27,28).

Rapid cycle DNA amplification is a technique for amplification of specific DNA sequences in 10–15 min (23–28). Thirty temperature cycles can be completed routinely in <15 min when using small samples in glass capillaries and forced air heating. By minimizing denaturation and annealing times,

specificity and yield are improved (27). Rapid amplification is particularly useful for methods development and diagnostics (1,2,14,19). In addition to facilitating rapid heat transfer, glass capillaries are optically clear and make natural cuvettes for fluorescence analysis.

In this article, we describe experimental and commercial versions of a microvolume fluorimeter with rapid temperature control. The instruments combine rapid temperature cycling capability (24,25,27) with fluorescence optics modeled after flow cytometry (18). Specific applications are as diverse as the many uses of fluorimetry. The utility of the LightCycler™ (Idaho Technology, Idaho Falls, ID, USA) is demonstrated by focusing on one specific application: fluorescent monitoring of rapid cycle DNA amplification. Further details on template quantification (28), product analysis by melting curves (16) and hybridization monitoring within temperature cycles (26) are presented elsewhere.

MATERIALS AND METHODS

Prototype Instrument

The prototype microvolume fluorimeter with rapid temperature control is diagrammed in Figure 1. Initially, we were uncertain of the sensitivity required for various applications. Therefore, a high-power xenon arc was used for excitation, highly sensitive photomultiplier tubes were used for detection and optical collection efficiency was maximized through 5-cm optics. Excitation and emission were along separate paths, both perpendicular to the capillary tube axis. A 450–490-nm interference filter was used for excitation. For SYBR® Green I (Molecular Probes, Eugene, OR, USA) emission, a 520–580-nm filter was used. For fluorescein and rhodamine, a 560-nm dichroic filter separated the fluorescein (520–550 nm) and rhodamine (580–620 nm) emissions. For fluorescein

and Cy5™ (Pharmacia Biotech, Piscataway, NJ, USA), a 590-nm dichroic filter separated fluorescein from Cy5 (660–680 nm) emissions. Up to 32 samples were placed vertically on a 14-cm-diameter carousel that was rotated by a microstepper.

With experience, we found that the xenon arc was usually too bright and typically required a neutral-density filter to mask 90%–99% of the exciting light in order to prevent photobleaching. Similarly, the photomultiplier tubes were often too sensitive and therefore driven at low voltages. Sensitivity was usually limited by background fluorescence, most of which came from the probes, not the detection system.

Commercial LightCycler

The high signal levels observed with most fluorescent probes allowed us to design a relatively inexpensive commercial instrument (Figure 2). A blue light-emitting diode is used for excitation instead of a xenon arc or a laser, and silicon photodiodes are used for detection instead of photomultiplier tubes. While the prototype was built on a large optical bench, the LightCycler occupies approximately 1 cubic foot (24 cm × 28 cm × 45 cm).

Up to 24 samples can be loaded onto the circular carousel around a cylindrical sample chamber. The temperature is controlled with a heating cartridge and a motor that drives a chamber fan. For heating, the cartridge is proportionally controlled, and the fan is run at a low speed. For cooling, the heater is disabled, and the fan is run at a high speed. The centrifugal force from the fan forces air in the central opening, past the heating cartridge and out the exit ports. The heating and cooling elements are symmetric around the central axis. The 8-cm-diameter carousel is positioned with a microstepper (14 000 steps per revolution).

The optical design is based on paraxial epi-illumination of the capillary tip. Filtered excitation light (450–490 nm) is reflected from a 505-nm epi-illumination dichroic filter and focused on the capillary tip. Much of the excitation light is piped up the capillary by total internal reflection at the glass-air surface. Similarly, emitted light is piped down the capillary, then leaves the tip and passes through the dichroic filter and a 515-nm long-pass glass filter. For 3-color acquisition of fluorescein, rhodamine and Cy5, a 560-nm dichroic reflects fluorescein emissions, a 630-nm dichroic reflects rhodamine emissions and a 650–690-nm bandpass filter is used for Cy5 collection. The filters and excitation source are modular to allow a variety of excitation and emission wavelengths.

Sample Handling

For the prototype, samples of 5–10 µL were loaded into glass capillary tubes and sealed with a butane microtorch (27). A more sophisticated sample handling system was developed for the LightCycler (Figure 3). Five-microliter samples were loaded into composite plastic/glass containers and centrifuged to form a 1-cm fluid column at the capillary tip (0.8 mm i.d., 1.0 mm o.d.). The entrance ports were sealed with a plastic plug and placed in the instrument for fluorescence monitoring. Samples can be prepared and centrifuged in a 96-well format.

Instrument Control and Data Acquisition

The graphical programming language LabView (National Instruments, Austin, TX, USA) was used for instrument con-

trol and data acquisition with a 12-bit multifunction input/output card in a 120-MHz Pentium® microcomputer (Intel, Santa Clara, CA, USA). Signals were obtained once each cycle during extension by sequentially positioning at each tube for 20–100 ms. Quantitative display of fluorescence vs. cycle number for all samples was continually updated each cycle. Generated data can optionally be stored as a text file for off-line analysis. Batch analysis for initial template quantification and melting curve analysis are available as post-amplification software routines.

RESULTS

The temperature response of the fluorescence cyclers are similar to previous rapid cycle designs (24,27) with 20–30 s cycles (30 cycles in 10–15 min, Figure 1). Repeated positioning and fluorescence acquisition gives coefficients of variation ranging from 0.5%–1.4% for individual tubes. Despite design differences, the functional characteristics of the prototype and the LightCycler are practically identical. The sample handling system of the LightCycler greatly simplifies the capillary format and allows samples to be loaded in 96-well format without needing to fuse the capillary tips.

Figure 4 compares three different fluorescence techniques for once-per-cycle monitoring of rapid cycle DNA amplification. In Figure 4A, amplification is monitored by the fluorescence of the double-stranded DNA (dsDNA)-specific dye

Figure 2. Schematic of the LightCycler, a commercial microvolume fluorimeter with rapid temperature control. The sample chamber is on top and the optics for epi-illumination on the bottom. Samples are placed on a circular carousel that can position the samples over the optics by a stepper motor. The temperature is rapidly controlled by a central heater and the speed of the chamber fan. Air flows in the direction shown because of centrifugal force from the fan (27). The chamber fan ensures temperature homogeneity between samples. The samples are placed in composite black plastic/glass capillaries (Figure 3) and interrogated through the capillary tip by epi-illumination. The light source is a blue light-emitting diode that is filtered and reflected off a dichroic towards the sample. Light is focused to and collected from the capillary tip by an epi-illumination lens. Emitted light passes through the dichroic and is filtered as desired and focused to photodiodes.

SYBR Green I (16,26,28). With genomic DNA as template, fluorescence begins to increase around cycle 22 and plateaus by cycle 35. The fluorescence of the sample without template also increases, but begins later around cycle 30 showing reduced amplification efficiency (lower maximal slope) and does not plateau by cycle 45. Gel electrophoresis of the products shows that while specific product is made with genomic DNA as template, the fluorescence from the negative control arises from nonspecific amplification of multiple lower molecular weight products (data not shown).

In Figure 4B, amplification is monitored with a dual-labeled hydrolysis probe and is expressed as a ratio of fluorescein to rhodamine fluorescence (26,28). With genomic DNA as template, the fluorescence ratio begins to increase around cycle 22. While the fluorescence from dsDNA-specific dyes plateaus with excess cycling, the signal from hydrolysis probes continues to increase even after many cycles. Gel electrophoresis shows that no net product is being made during this continued probe hydrolysis (data not shown).

In Figure 4C, amplification is monitored using adjacent hybridization probes and is expressed as a ratio of Cy5 to fluorescein fluorescence (26,28). With genomic DNA as template, the fluorescence ratio begins to increase around cycle 23, peaks around cycle 35 and then begins to decrease. Gel electrophoresis confirms that specific product is made from the genomic DNA template, but does not show a decrease in product above cycle 35 (data not shown).

The sensitivity of the three fluorescence techniques appears similar, with initial detection of amplified product around cycles 22–23. To further assess sensitivity, the fluorescence signals were calibrated to either purified amplified product (Figure 4A) or by comparison of band intensities to standards after gel electrophoresis (Figure 4, B and C). In terms of amplified product, the sensitivity of each method also appears similar (ca. 1 pM product).

The fluorescence signal generated is not always proportional to the amount of specific product. This nonlinearity is a characteristic of the fluorescent probes, not the instrumentation. With SYBR Green I, both nonspecific amplification of alternative templates (Figure 4A) and limiting amounts of SYBR Green I at DNA concentrations greater than 20 ng

Figure 3. Sample handling system for the LightCycler. Four steps for loading samples into the LightCycler are shown. Samples are initially pipetted into composite glass/plastic sample holders. Then, caps are placed over each sample and they are centrifuged to place the fluid column at the bottom of each tube. An air tight seal is formed by pressing the cap down to plug the capillary. Finally, the samples are placed in the LightCycler for fluorescence monitoring. The first three steps of this process can be performed in 96-well format.

contribute to nonlinearity (26). With hydrolysis probes, fluorescence continues to increase after the plateau phase is reached (Figure 4B), whereas with hybridization probes, fluorescence decreases during the plateau phase (Figure 4C). Despite these limitations, initial template copy number can be quantified easily by measuring fluorescence each amplification cycle (26,28).

DISCUSSION

We have designed, optimized and commercialized a capillary-based microvolume fluorimeter with rapid temperature control. The glass capillary format provides an optically clear sample container and small volume analysis dependent on the capillary diameter. Homogeneous sample temperature control up to 10°C/s is possible because of small sample volumes, the high surface area-to-volume ratio of capillaries, and the low heat capacity and ease of mixing air. This combination of factors allows precise control of sample temperatures at a speed not possible with other designs. For example, sample temperature vs. time plots in capillaries show sharp spikes at denaturation and annealing temperatures, whereas several seconds are required for all of the sample to reach equilibrium in conical plastic tubes (24,25,27). Although high surface area-to-volume ratios are possible on etched silicon or glass chips, reported cycle times have yet to approach those in capillaries (3,5), and it may be difficult to achieve temperature homo-

geneity over the large chip surface exposed to the sample.

The commercial LightCycler analyzes up to 24 samples of 1–10 μL each at a constant temperature (ambient to 100°C) or during temperature transitions ($<0.1^\circ$ to 10°C/s). Composite glass/plastic sample holders (Figure 3) were designed to simplify sample handling, a drawback for some users of prior capillary techniques (27). A generic microcomputer interface allows flexible programming for sample positioning and acquisition.

Although microfluorimetry has many uses, the utility of the LightCycler is demonstrated here by monitoring rapid cycle DNA amplification. This technique uses rapid temperature transitions for DNA amplification in 10–15 min (24,27). Three fluorescence techniques were used to monitor amplification once each cycle. The accumulation of dsDNA was followed with SYBR Green I (Figure 4A). Specific product formation was monitored with a dual-labeled 5'-exonuclease

Figure 4. Comparison of three different fluorescent probes for monitoring rapid cycle DNA amplification. Fluorescent probes were: (A) the dsDNA-specific dye SYBR Green I, (B) a fluorescein/rhodamine dual-labeled hydrolysis probe and (C) 3'-fluorescein and 5'-Cy5 adjacent hybridization probes. Either 25 ng of genomic DNA (◆) or no template (○) was added to each amplification. In Panel A, the target was a 536-bp fragment of the human β -globin gene. A 1:10 000 dilution of SYBR Green I was used, and each temperature cycle was 28 s long (95°C maximum, 61°C minimum, 15 s at 72°C, average rate between temperatures 5.2°C/s). The calibration points along the right axis are the fluorescence of 0, 5, 10 and 20 ng of purified 536-bp product/5 μL in an amplification reaction without polymerase. In Panel B, the target was a 295-bp fragment of the human β -actin gene, and 0.2 μM of the hydrolysis probe was used. Each temperature cycle was 26 s long (94°C maximum, 60°C for 15 s, average rate between temperatures 6.2°C/s). In Panel C, the target was a 110-bp fragment of the human β -globin gene, and 0.2 μM of each hybridization probe was used. Each temperature cycle was 30 s long (94°C maximum, 59°C for 20 s, average rate between temperatures 7.0°C/s). For Panels B and C, the calibration points along the right axes were estimated by gel electrophoresis of the product at 25 cycles.

hydrolysis probe (Figure 4B) or with two adjacent hybridization probes labeled individually with fluorescein and Cy5 (Figure 4C). All three fluorescence techniques appear to detect amplified product with about the same sensitivity (ca. 1 pM). All three techniques also show artifacts at later cycles that do not correlate to specific product accumulation. With SYBR Green I, negative control samples eventually develop fluorescence, while the hydrolysis and hybridization probes show artifactual increases or decreases in the fluorescence signal, respectively. These techniques and their applications are discussed in more detail elsewhere (16,26,28).

In addition to monitoring amplification once each cycle, hybridization can be followed with fluorescence during temperature cycling (26,28). Both amplification and hybridization are dependent on temperature changes. The ability to monitor hybridization with fluorescence during temperature cycling is a powerful tool. The melting temperature of products or sequence-specific probes can identify and discriminate products during amplification. For example, product melting curves can be obtained with the dsDNA dye SYBR Green I. These melting curves are characteristic of the products being denatured. Because of heterogeneity in size and GC content, different products melt over a 40°–50°C range (16). Other potential applications include mutation detection by monitoring the melting temperature of hybridization probes (26), competitive quantitative amplification by the analysis of product melting curves (16) and absolute product quantification by product-to-product annealing kinetics (28).

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