Analytical Biochemistry 448 (2014) 75–81

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Fluorescence-based temperature control for polymerase chain reaction ☆

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ARTICLE INFO

Article history:
Received 14 October 2013
Received in revised form 20 November 2013
Accepted 22 November 2013
Available online 28 November 2013

Keywords:
Polymerase chain reaction (PCR)
Fluorescence
Temperature monitoring
High-resolution melting

ABSTRACT

The ability to accurately monitor solution temperature is important for the polymerase chain reaction (PCR). Robust amplification during PCR is contingent on the solution reaching denaturation and annealing temperatures. By correlating temperature to the fluorescence of a passive dye, noninvasive monitoring of solution temperatures is possible. The temperature sensitivity of 22 fluorescent dyes was assessed. Emission spectra were monitored and the change in fluorescence between 45 and 95 °C was quantified. Seven dyes decreased in intensity as the temperature increased, and 15 were variable depending on the excitation wavelength. Sulforhodamine B (monosodium salt) exhibited a fold change in fluorescence of 2.85. Faster PCR minimizes cycling times and improves turnaround time, throughput, and specificity. If temperature measurements are accurate, no holding period is required even at rapid speeds. A custom instrument using fluorescence-based temperature monitoring with dynamic feedback control for temperature cycling amplified a fragment surrounding rs917118 from genomic DNA in 3 min and 45 s using 35 cycles, allowing subsequent genotyping by high-resolution melting analysis. Gold-standard thermocouple readings and fluorescence-based temperature differences were 0.29 ± 0.17 and 0.96 ± 0.26 °C at annealing and denaturation, respectively. This new method for temperature cycling may allow faster speeds for PCR than currently considered possible.

The ability to accurately monitor and control solution temperature during PCR[1] and melting analysis is crucial for successful amplification and analysis of high-resolution melting curves. Due to practical limitations, temperature measurements are typically made externally to the sample solution. This produces significant solution-instrument temperature mismatches [1] which are exacerbated during rapid temperature transitions. One proposed solution for ameliorating solution-instrument temperature discrepancies is to correlate the fluorescence of a passive dye (one that does not interact with DNA) to solution temperature for noninvasive monitoring in real time. This approach has been successfully demonstrated in harsh environment flow-field applications [2,3], to make temperature maps of microfluidic systems [4], and more recently, in commercial PCR instruments [1] to assess the differences in temperature recorded by the instrument and actual PCR solution temperatures.

The ability to noninvasively monitor solution temperatures during PCR may provide more accurate sample temperatures and allow for faster cycling. Furthermore, the ability to dynamically control thermal cycling based on actual solution (and not external) temperature is enabled. The idea of utilizing fluorescence to control PCR temperature cycling was first suggested in 1994 [5], and some progress has been made toward that goal. Fluorescence monitoring has been used to control extension times during PCR [6]. This allowed for heating (toward denaturation) to begin as soon as extension was complete, instead of waiting for a prespecified time interval to pass. However, this approach used a fluorescent dye that also interacts with DNA, potentially confounding the fluorescent signals for temperature measurement and real-time PCR monitoring.

An alternative approach is to use a temperature-sensitive dye that does not interact with DNA (and that does not inhibit the PCR), but responds to changes in temperature with altered emission intensity. For instance, the fluorescence of a temperature-sensitive dye has been used to adjust the power of a laser used to heat PCR mixtures from annealing to denaturation temperatures in a droplet-based PCR system [7] to demonstrate fluorescence-based heating control. However, complete thermal cycling control during PCR using fluorescence measurements requires the ability to control all parts of PCR including heating, cooling, and holding times.

We begin with an examination of 22 alternative dyes for fluorescence-based temperature monitoring, evaluating temperature

☆ Partial support for this research was provided by BioFire Diagnostics and Canon US Life Sciences.

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[1] *Abbreviation used:* PCR, polymerase chain reaction.

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http://dx.doi.org/10.1016/j.ab.2013.11.027

stability, fluorescence quenching, and the magnitude of the calibration constant that relates temperature to fluorescence. Then, we select one of these dyes to noninvasively monitor solution temperatures, and finally use the fluorescence-based temperature to control PCR cycling with simultaneous real-time amplification output and subsequent melting analysis for genotyping single nucleotide variants.

Materials and methods

Fluorescent dye temperature sensitivity

The temperature sensitivity of 22 fluorescent dyes was examined on a custom multicolor fluorimeter with xenon excitation, spectral dispersion (405–590 nm) on a grating, and focusing onto a fiber optic (delta RAM, Photon Technology International). The fiber optic illuminated the end of a glass capillary (LightCycler, Roche Applied Science) placed within a heating unit (HR-1, BioFire Diagnostics). Fluorescent emission was collected by another fiber optic at a right angle to the capillary, delivering light onto a CCD spectrometer (DV420-OE, Andor Technology), and collecting 1024 bins between 400 and 850 nm. A J-type miniature thermocouple (5SRTC-TT-J-40-36, Omega) was inserted into the sample capillary for physical temperature measurements.

Fluorescent dyes were tested in a "mock" (no polymerase) PCR solution consisting of 50 mM Tris (pH 8.3), 2 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (Roche), 500 μg/ml bovine serum albumin (Sigma), and 0.08% (v/v) glycerol. Final concentrations and excitation wavelengths for each dye are listed in Supplemental Table 1. Spectra for temperature-sensitivity profiles of 22 dyes were recorded at 4 excitation wavelengths selected from 405, 455, 470, 490, and 530 nm. Twenty-five microliter sample volumes were heated from 45 to 95 °C (in discrete 10 °C increments, with 100 points averaged at each step). The ramp rate between steps during heating was approximately 0.4 °C/s. The entire emission spectrum was recorded and the fold change in fluorescence (45–95 °C) calculated over selected bandwidths.

Fluorescent dye degradation

The system described above was used to assess the amount of dye degradation after a single heating and cooling cycle of approximately 20 min. Dye concentrations and excitation wavelengths are shown in Table 1. After the sample temperature reached 95 °C, samples were cooled to 45 °C (using passive cooling). The spectrum was taken at 45 °C before and after heating and the intensity of peak spectral bands compared.

Fluorescence-based and thermocouple temperature comparisons during melting

Six dyes exhibiting the greatest sensitivity to temperature were further examined. Dye concentrations and excitation wavelengths are detailed in Table 2. Calibration constants (see Table 2) for each dye were calculated from temperature and fluorescence data acquired from 45 to 95 °C in 10 °C increments. Using a second sample with a 30 μL final volume (25 μL sample with 5 μL oil overlay), an initial holding period at 50 °C was used to determine reference temperature and intensity values. The sample was then heated to 95 °C at an approximate rate of 0.05 °C/s. Fluorescence-based solution temperatures were calculated for both single-dye/single-color and single-dye/two-color configurations using sulforhodamine B (acid form). Solution temperatures using the single-dye/two-color method were calculated as described previously [8]. Fluorescence-based temperatures were compared to sample thermocouple readings.

Instrumentation for fluorescence-based temperature cycling control

Fluorescence-based temperatures were calculated by monitoring sulforhodamine B (monosodium salt, 600 μM, Sigma–Aldrich) fluorescence. A LightCycler 24 (BioFire Diagnostics) was modified to accept voltage inputs from both standard thermocouples and fluorescence-based equivalents. Additional hardware is listed in Supplemental Table 2. A (5SRTC-TT-J-40-36) thermocouple was placed in an adjacent capillary for the initial calibration to determine reference values and for comparison to fluorescence-based temperatures during cycling.

PCR

Three forensic single-nucleotide variants [9] were amplified using the following primers: rs876724 (5′-CCACTGCACTGAAGTATAAGT-3′ and 5′-TTAGCAGAGTGTGACAAAAAA-3′), rs917118 (5′

Table 1
Dye and spectral band selection.

Dye	Concentration (μM)	Excitation wavelength (nm)	Degradation (Fold-change)[a]	Temperature–sensitive band (nm)	Temperature–insensitive band (nm)
Ethyl eosin	800	455	0.76	535–600	490–515
Ethyl eosin	800	470	0.83	535–590	490–515
Merocyanine 540	40	470	0.41	580–650	500–525
Merocyanine 540	40	490	0.54	575–625	525–550
Merocyanine 540	40	530	0.53	575–700	530–550
Rhodamine B	4	470	0.99	570–610	525–550
Rhodamine B	4	490	0.98	570–610	525–550
Rhodamine B	4	530	0.98	560–590	514–540
Snarf-1	160	455	0.96	630–680	500–525 or 580–600
Snarf-1	160	470	0.97	630–680	500–525 or 560–600
Snarf-1	160	490	0.89	625–675	525–575
Sulforhodamine B (acid form)	8	455	0.85	575–610	500–550
Sulforhodamine B (acid form)	8	470	0.93	575–620	500–550
Sulforhodamine B (acid form)	8	490	0.95	575–620	525–550
Sulforhodamine B (acid form)	8	530	0.86	575–620	525–550
Sulforhodamine B (monosodium salt)	4000	470	1.0	575–610	516–545
Sulforhodamine B (monosodium salt)	2400	490	0.98	575–610	516–545
Sulforhodamine B (monosodium salt)	2400	530	0.97	575–610	516–545

[a] Samples were heated from 45 to 95 °C and then cooled to 45 °C. Values were calculated at the emission peak wavelength. The fluorescence at the second measurement was divided by the fluorescence at the first measurement. Thus, a value of 1 indicates that no degradation occurred.