Monitoring Temperature by Fluorescence during PCR and Melting Analysis

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Introduction

Successful Polymerase Chain Reaction (PCR) depends on reaching denaturation and annealing temperatures. Temperature measurements made through direct contact with the sample are problematic, forcing measurements to be made externally to the sample. This compromises temperature accuracy, particularly during temperature transitions.

As cycling rates increase, the ability to measure and control sample temperatures becomes more difficult. Solution temperatures and instrument readings rarely coincide, reducing temperature accuracy and affecting PCR performance.

To better monitor solution temperature, a passive reference dye may be added to the PCR reaction. Alterations in fluorescence are then non-invasively correlated to changes in temperature.

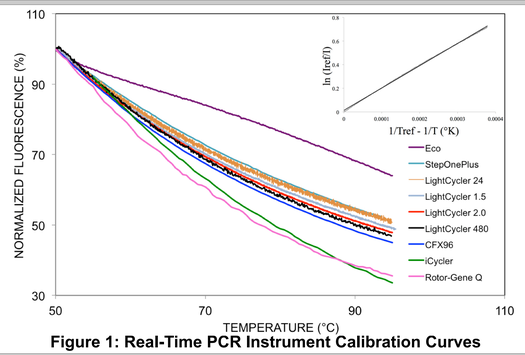

Figure 1: Real-Time PCR Instrument Calibration Curves

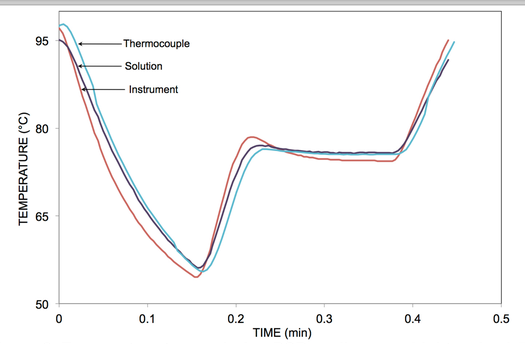

Figure 2: Temperature traces during PCR cycling on a Lightcycler 1.5.

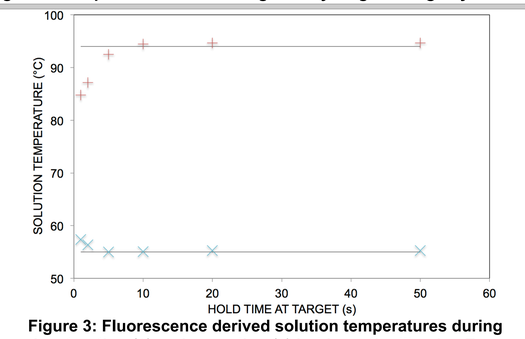

Figure 3: Fluorescence derived solution temperatures during denaturation (+) and annealing (x) holds on the Illumina Eco.

Materials and Methods

Calibration curves were used to correlate the fluorescence of sulforhodamine B with temperature on nine real-time PCR instruments. Calibration curves were generated by heating (at 0.018 – 0.1°C/s) between 50 and 95°C. Instrument characteristics and experimental conditions are summarized in Table 1.

Temperature may be correlated to fluorescence through the use of a calibration constant[1]: $C = \ln\left(\frac{I}{I_{ref}}\right) / \left(\frac{1}{T} - \frac{1}{T_{ref}}\right)$

The calibration constant, reference values (I_{ref} and T_{ref}), and measured fluorescence intensity are used to calculate solution temperature[1]: $T = \frac{1}{\frac{\ln(I/I_{ref})}{C} + (1/T_{ref})}$

When possible, micro-thermocouple measurements were compared to solution and instrument temperatures during cycling and heating/cooling transitions.

Lambda DNA melting curves were generated on the LightCycler 480 at two rates (0.14°C/s and 0.01°C/s). Melting curves using either the instrument or solution temperatures were compared.

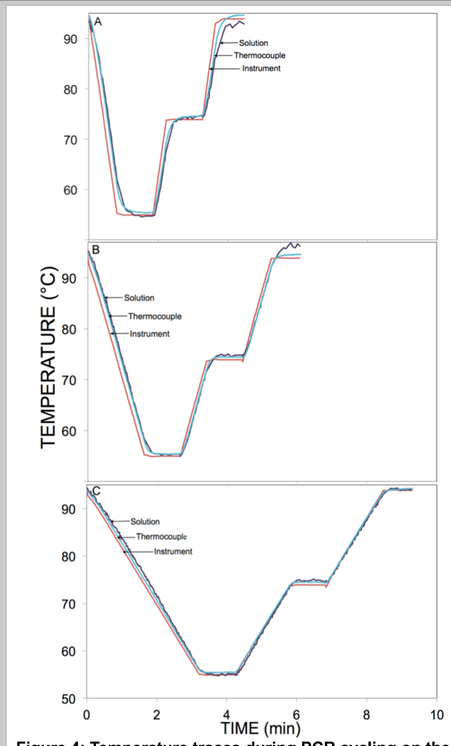

Figure 4: Temperature traces during PCR cycling on the LightCycler 480. (A) 50 µL at 0.57°C/s (B) 25 µL at 0.29°C/s (C) 10 µL at 0.14°C/s.

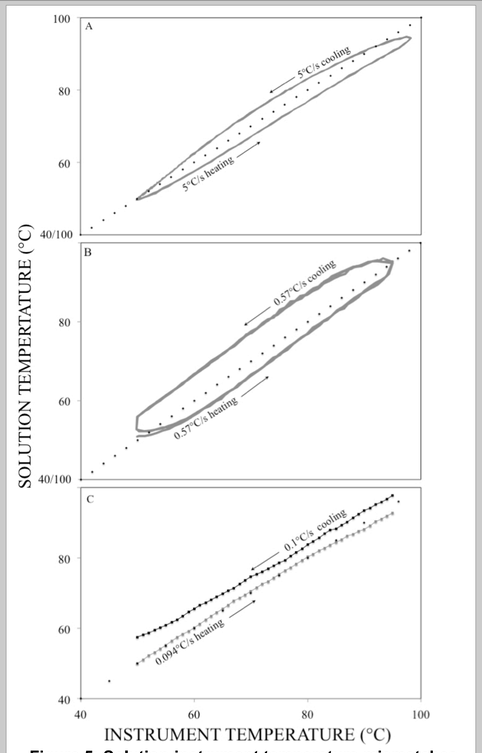

Figure 5: Solution-instrument temperature mismatches during heating and cooling. (A) LightCycler 1.5 (B) LightCycler 480 (C) Rotor-Gene Q.

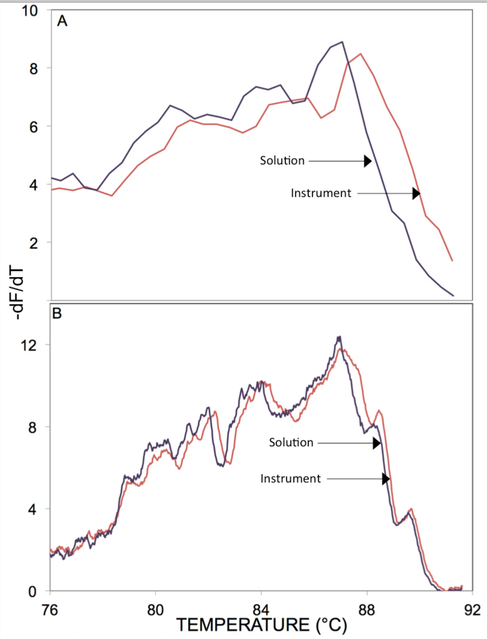

Figure 6: Lambda DNA melting curves generated on the LightCycler 480 at (A) 0.14°C/s and (B) 0.01°C/s.

Instrument	Solution Volume (µL)	Oil Overlay Volume (µL)	Calibration Constant	Cycle Time (s)	Hold Time (s)	Fluorescence Acquisition	
						During Cycling	During Ramping
LightCycler 24	10	2	1787	18	10	Continuous	
LightCycler 1.5	10	2	1890	27	10	Continuous	
LightCycler 2.0	10	2	1953	25	10	Continuous	
LightCycler 480	0-70	5-20	1977-2055	120	1, 2, 5, 10, 20, 50	Continuous	
Rotor-Gene Q	10	15	2831	85	1, 2, 5, 10, 20, 50	Once each hold	Heating or Cooling
iCycler	10	15	2661	120	5, 7, 10, 15, 20, 50	Once each hold	Heating or Cooling
CFX96	10	15	2122	80	1, 2, 5, 10, 20, 50	Once each hold	Heating or Cooling
StepOnePlus	10	15	1779	78	10, 15, 20, 30, 50	Once each hold	Heating
Eco	5	5	1097	66	1, 2, 5, 10, 20, 50	Once each cycle	Heating

Table 1: Instrument characteristics and experimental conditions

	LC 1.5	LC480	LC480	LC480
Volume (sample + oil, µL)	10 + 2	5 + 5	10 + 15	30 + 20
Transition Rate (°C/s)	20[b]	0.14	0.29	0.57
Cycle Time (min)	0.44	9.3	6.1	4.5
Temperature Transition	Difference between Sample and Instrument Temperatures (°C, Mean ± SD)			
95 to 55°C	-3.8 ± 1.5	-1.3 ± 0.2	-3.1 ± 0.3	-5.1 ± 1.5
55 to 74°C	0.4 ± 2.3	0.2 ± 0.2	1.6 ± 0.6	4.5 ± 0.6
74 to 95°C	1.4 ± 1.5	0.1 ± 0.3	1.3 ± 0.7	3.4 ± 1.9
Temperature Transition	Time Delay (s, mean)			
95 to 55°C	0.5	6.1	7.7	6.1
55 to 74°C	0.6	1.4	4.4	4.8
74 to 95°C	0.3	2.0	4.1	8.3

Table 2: Sample and instrument temperature differences and time delays during temperature transitions

Results

Calibration curves were successfully generated on all instruments (Fig. 1). Due to optical constraints, fluorescein was used in place of sulforhodamine B on the Eco instrument. Calibration constants were derived by re-plotting calibration curve data (Fig. 1 inset) so that the slope of the linear line could be calculated. Calibration constant values were 1097 for fluorescein and 1787 – 2831 for sulforhodamine B.

During cycling, solution temperatures determined by fluorescence agreed well with thermocouple measurements but not to instrument temperature readings (Figs. 2 and 4). Solution temperatures lagged behind instrument readings by an average of 0.5 – 6.4 s, reaching a maximum delay of 8.3 s on the approach to denaturation (Table 2). Average solution-instrument temperature differences were 0.5 – 4.3°C, reaching a maximum of 5.1°C on the approach to annealing (Table 2). An example of data acquired during holds is shown in Fig. 3. For all instruments, solution temperatures lagged behind instrument temperatures by up to 8°C on the approach to denaturation, while initial readings during the annealing hold were 1.1 – 4.5°C too high. Five to 10 s were typically required for equilibration at target temperatures.

Differences between solution and instrument temperatures were also compared by examining temperature hysteresis (lag of the solution temperature to the instrument temperature) during heating and cooling (Fig. 5). Solution-instrument temperature hysteresis reached a maximum of 8.1°C. Deviation from the ideal solution-instrument correlation (denoted by a dotted line) indicates temperature mismatches.

Melting curves (Fig. 6) were derived and compared using either solution or instrument temperatures. The melting curve derived from instrument temperatures was 1.1°C higher when compared to the melting derived from the solution temperature at a rate of 0.14°C/s. At a slower rate (0.01°C/s) the solution temperature lag was reduced to 0.2°C.

Discussion

The use of fluorescence to monitor solution temperatures is a viable and non-intrusive method of addressing issues with temperature measurement during real-time PCR and melting analysis. Instrument-solution temperature mismatches have been presented, with solution temperatures lagging behind instrument temperature readings. Even with mandatory hold times, solution temperatures at annealing were found to be 1 - 5°C above the target temperature and more than 8°C below the target temperature at denaturation, discrepancies that could significantly impact PCR quality. Through using fluorescence to directly monitor solution temperature, inaccuracies at denaturation and annealing can be ascertained and correlated to efficiency, yield, and specificity. Temperature mismatches also produce melting curves that are artificially elevated in temperature, exceeding 1°C at common ramp rates (Fig. 6). High-resolution melting applications can also benefit from increased solution temperature accuracies, as genotyping[2] and variant scanning[3] are dependent on temperature accuracies of less than 1°C.

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Citation

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[2]M. Liew, R. Pryor, R. Palais, C. Meadows, M. Erali, E. Lyon, C. Wittwer, Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons, Clin. Chem. 50 (2004) 1156-64.

[3]M. Erali, C. Wittwer, High resolution melting analysis for gene scanning, Methods. 50 (2010) 250-61.