ABSTRACT

A biochemical kit has been developed compatible with a low-cost Real-Time PCR (qPCR) instrument. Further optimization of the biochemical kit was provided in the form of cosolvent tests and suggested additives for qPCR enhancement constitute a novel master mix catered for the budget device. Development of this kit was targeted towards the education sector as current technologies are not within the budget of American high schools. This new qPCR instrument developed in 2007/2008 is two orders of magnitude lower in cost than traditional qPCR machines and the accompanying kit, about half the market cost of the nearest competitor. Additional refinement of this package could result in widespread use of this product in biomedical and pharmaceutical research, providing a cheap tool for rapid diagnosis and prognosis in pre-clinical and field studies settings.

INTRODUCTION

The NUS Team was tasked to provide a viable and robust biochemical kit which would demonstrate the basic principles of qPCR on the prototype built last year. For the biochemical kit, a specific budget was not given to the NUS Team however the main components (enzymes, fluorescent dyes) were implied to be existing products of Applied Biosystems. Current protocols would be assessed and the addition of cosolvents to improve various aspects of the qPCR would be analyzed as part of kit optimization. Finally, as a measure of marketability, the final cost of the NUS Team’s biochemical kit would be assessed against Applied Biosystems’ main competitors to evaluate market feasibility of the product. The NUS Team was also tasked to review the optical detection system’s functionality.

MATERIALS AND METHODS

The optical subsystem was tested with SYBR Green Dye for LOD calculation. For the biochemical kit, existing protocols were reviewed and the concentrations of critical components in qPCR was reviewed (enzyme, DNA template, primer) together with thermal cycling conditions. The effects of cosolvents known to improve various aspects of qPCR was also investigated in context of this kit.
RESULTS

Primer Optimization

After establishment of the optimal enzyme and DNA template concentration, a range of primer concentrations from 0.5µM to 4.5µM was tested for qPCR product formation. Low concentrations of primers (up to 1.5µM) seemed to produce unsuccessful qPCR reactions whereas higher primer concentrations produced similar quantities of the desired product (3.0µM to 4.5µM).

Cosolvent Selection

In the three cosolvents tested in this series (there are nine in total), betaine displayed the most pronounced improvements in qPCR rate of reaction. DMSO and glycerol had minimal effects. Furthermore, results suggest that all three will affect the melting temperature of the amplicon without affecting specificity.

DISCUSSION

Optical Subsystem Verification

In the prototype, the energy emitted by the blue LED light source was insufficient to excite the SYBR Green dye to produce sufficient photons to trigger a response in the photodiode. The photon energy input from the excited SYBR Green-DNA complex would need to be boosted a thousand fold, or the detector’s sensitivity increased by the same magnitude, in order to translate to a discernable difference in reading from background noise. As such, the optical subsystem design is currently being replaced from the photodiode to a more sensitive photoresistor system.

Primer optimization

In qPCR, signal saturation could also be due to primer quantities (Kubista et al., 2001). Other important variables are the quantities of reporter dye and dNTPs. Although the Tm value of the amplicon was much lower than the theoretical 91.3°C with 3.0µM and 4.5µM of primers, the band fragment reflected by the agarose conformational gel suggests that the correct amplicon was produced. Also, primer concentrations of 3.0µM and 4.5µM express very similar cycling profiling whereas lower concentrations translate to very poor amplification similar to NTC values. This observation suggests that there is a threshold value for primer concentration between a successful and failed amplification. However, the large standard deviation in Tm values obtained at 1.5µM primer concentration suggests that with increasing concentrations of primers, increasing amount of the desired amplicons are produced.

Addition of cosolvents

Although betaine displayed the highest improvement in quality of qPCR (specificity and rate of reaction), it is the most costly working cosolvent in context of this kit. Also, three other cosolvents exhibit significant improvement to qPCR. Combined, the four improve qPCR in three major aspects, reducing secondary structure formation of amplicons due to GC-rich regions (Betaine (Henke et al., 1997)), stabilising the enzyme, increasing its durability within the PCR reaction (Bovine serum albumin (BSA) (Paabo et al., 1988), Tween-20 (Smith et al., 1990)) and improving the specificity of the PCR product (Formamide (Sarker et al., 1990)). For future
works, combining cosolvents resulting in the cumulative effect of small molecules such as betaine, Tween-20 and formamide could potentially be very effective in improving overall qPCR.

HMC profiling

The PCR profile of the biochemical kit is dependent on the functionality of the prototype. Thermal fluctuations and optical insensitivity inherent in the prototype ultimately influences whether a successful experiment is detected as a positive one or not. As such, the thermal profile of the HMC prototype has to be tested against the biochemical kit developed to determine the feasibility of the reaction and that has to be verified against the optical detection system to establish the resolution of the interpreted results.

REFERENCES


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