Designing of Biochemical Kit for Novel Real-Time Polymerase Chain Reaction Instrument Aimed at Education Sector

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ABSTRACT

The 2008-2009 Global Clinic Programme, involving members of Harvey Mudd College, California, and National University of Singapore, is sponsored by Applied Biosystems, Foster City, California. The aim of the programme is to optimise the low cost real-time polymerase chain reaction prototype aimed at secondary schools developed by the 2007-2008 Global Clinic team, as well as to design a user-friendly interface and a robust biochemical kit for the operation of the instrument. Optimisation of the PCR prototype involved increasing the rate of heating and cooling of the thermal cycler, and improving the sensitivity of the optical detector. NUS students designed the biochemical kit while HMC students worked on the prototype and the interface. This report detailed the selection tests on the determination of DNA concentration and cycling profile, and the effects of tetramethylammonium chloride, tetramethylammonium oxalate and formamide on PCR. The kit is able to demonstrate the underlying principles of real-time PCR and the reaction can be completed within a secondary school class of 40 minutes. The kit costs S$3.66 per reaction, less than the current market prices which can range from S$4.05 to S$10.78.

INTRODUCTION

The real-time PCR instrument is used to amplify and simultaneously quantify a targeted DNA molecule. There are three main components of the instrument, the thermal cycler, the optical detection subsystem and the chemistry. Following a market research conducted in 2007 by Keck Graduate Institute which reflected that the secondary education market cannot afford current PCR instruments and commercial kits, AB engaged HMC and NUS to develop a low cost real-time PCR instrument for the education sector in 2007-2008. As a continuation of that project, the objective of the 2008-2009 Global Clinic Programme is to optimise the prototype, as well as to design a user-friendly interface and a biochemical kit for the operation of the instrument. Optimisation of the PCR prototype involved increasing the rate of heating and cooling of the thermal cycler, and improving the sensitivity of the optical detector.

In particular, NUS is tasked to incorporate a compatible and robust biochemical protocol for the real-time PCR prototype. The biochemical components would be limited to those provided by AB. The kit should be inexpensive, since the educational market has a very limited budget; educational, promoting student interest in biotechnology and enabling students’ understanding of the underlying principles of PCR and real-time PCR; and efficient, such that the reaction can be completed within a secondary class period of 40 minutes. Though the work was

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collaborative, the scope of each individual was divided at each stage of the project. This report covers the work on varying the PCR cycling profiles and determining the suitable DNA concentration during the initial phase, testing out the effects of adding three (of the selected nine) co-solvents at different concentrations for the final PCR protocol, and modifying the annealing and extension temperature to correspond to the parameters of the prototype.

**MATERIALS AND METHODS**

The approach to this project was separated into three main phases:

1. **Selection tests** for basic PCR protocol to determine the plasmid and its complementary primers, the enzyme, PCR cycling profiles, DNA concentration, primer concentration, and enzyme concentration;

2. **Selection tests** for final PCR protocol to select co-solvents which enhance DNA amplification and establishes their concomitant concentrations; and

3. **Optimisation tests** of varying the annealing and extension time and temperature to work within the limitations of the thermal cycler prototype, and considering the freeze/thaw cycling and standing time viability to measure the robustness of the kit. However, this phase is yet completed due to insufficient time.

**Materials**

All the reactions were carried out using AB StepOnePlus™ Real-Time PCR System with MicroAmp™ Fast Optical 96-Well Reaction Plate and MicroAmp™ Optical Adhesive Film. The results were analysed using StepOne software v2.0. TaqMan® RNase P 96-Well Instrument Verification Plate was used to verify the performance of the StepOnePlus™ system before using the instrument. 1% agarose (Sigma-Aldrich, St. Louis, Missouri) gel electrophoresis was used to verify the desired product and its purity on a different platform. All apparatus and chemicals were obtained from AB unless otherwise specified.

The basic chemicals used include 0.3 ng/μL pEmGF P-Bsd plasmid (DNA template), forward (TTA AAC GGG GGA GGC TAA CT) and reverse primers (GGT GGG GAA AAG GAA GAA A) specific to the gene of interest at 1.0 μM each, 0.050 U/μL SuperTaq™ Polymerase (Cloned) (enzyme), 1X Complete PCR Buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂), PCR dNTP Mix (200 μM each) and 1X SYBR® Green (reporter dye). The plasmid was transformed in *Escherichia coli* (DH5α host strain), using ampicillin (marker) and Luria-Bertani broth (culture medium). The primers were obtained from 1st BASE, Singapore.

In the selection tests for the final PCR protocol, the effects of TMA chloride, TMA oxalate and formamide on PCR were experimented. TMA oxalate was prepared by adding oxalic acid to 50 mM TMA hydroxide solution until pH 8.0 was reached. All co-solvents were obtained from Sigma-Aldrich. The pH of all additives was adjusted to 8.5 – 9.0 using NaOH.

**PCR Profile**

The optimised PCR conditions used was: 95.0 °C for one minute, followed by 40 cycles of five seconds at 95.0 °C, 10 seconds at 55.0 °C and 20 seconds at 74.0 °C. After the cycling stage, the melt curve stage (95.0 °C, 15 seconds; 60.0 °C one minute; 0.3 °C increment till 95.0 °C, 15 seconds) generates the melt curve to verify the amplified product and reveal the presence of non-specific products. This stage is not included in the protocol for the operation of the real-time PCR prototype as the instrument does not have the facility to run a melt curve stage.
RESULTS AND DISCUSSION

Selection Tests for Basic PCR Protocol

Optimising PCR profile  Optimal performance of the PCR process is influenced by the choice of temperature, the duration of each step, and the length of time between temperatures for each step in the cycle. A typical cycle consists of a melting step (usually at about 94 ºC to 96 ºC), a primer annealing step (37 ºC to 70 ºC, depending on the target and primers), and an extension step (at a temperature dependent on the enzyme used). As a general guideline, the optimal annealing temperature is at least 5 ºC lower than the melting temperature, \( T_m \), of the primers. The extension temperature follows the optimal temperature for DNA polymerase activity since the complementary DNA strand is synthesised by the enzyme during the extension step.

An experiment using 0.3 ng/µL and 0.6 ng/µL pEmGFP-Bsd was conducted to investigate the effect of annealing temperatures on PCR. Annealing temperature at 55.0 ºC resulted in lower threshold cycle (\( C_T \)) as compared to that at 50.0 ºC. The duration of each step in the optimised profile was selected based on that of the RNase P Verification Plate.

DNA concentration  A standard curve was constructed by running pEmGFP-Bsd at 4.8 ng/µL, 1.2 ng/µL, 0.3 ng/µL, 0.075 ng/µL, 0.02 ng/µL, 0.01 ng/µL and 0.005 ng/µL. An amplification with relatively high normalised reporter (\( R_n \)), and with \( C_T \) value at around 10 corresponded to 0.3 ng/µL DNA. Hence, pEmGFP-Bsd at 0.3 ng/µL was chosen for the basic PCR protocol.

Selection Tests for Final PCR Protocol

TMA chloride  5 mM, 10 mM and 20 mM TMA chloride was added to the reaction mixture and tested. Results showed no improvements in PCR amplification upon addition of TMA chloride. It was thus concluded that TMA chloride of concentration up to 20 mM was not useful to be included in the final protocol.

TMA oxalate  The effects of 1 mM, 2 mM and 3 mM TMA oxalate on PCR were experimented. Results showed that the addition of this co-solvent decreased the rate of amplification. This was probably because the reagents and cycling conditions used in this report were different from that in literature (Kovarova, 2000). TMA oxalate could have directly inhibited, instead of facilitated, the DNA polymerase activity in this case.

Formamide  1.0%, 2.5%, 5.0% and 7.5% formamide were tested. Significant decrease in \( C_T \) of 0.6 and doubling of the final \( R_n \) was observed at 2.5% formamide. The reaction mixtures supplemented with 1.0% formamide showed no changes in PCR while 7.5% formamide resulted in no amplification. At 5.0% formamide, final \( R_n \) was doubled but \( C_T \) was increased by 2.3. As such, further investigation was performed on formamide at lower cycling temperatures as an attempt to work within the limitations of the thermal cycler prototype.

Cycling Temperature Variation

The experiment on formamide was repeated with the denaturation temperature at 90.0 ºC and extension temperature at 60 ºC. Results showed that the addition of formamide facilitated PCR
amplification at lower cycling temperatures. Amplification occurred in all reaction mixtures supplemented with formamide. However, all the C_T values (with and without formamide) were much higher than that of the control under optimised cycling conditions.

CONCLUSION

The basic PCR components and their concomitant concentrations were optimised: 0.3 ng/μL pEmGFP-Bsd, forward and reverse primers at 1.0 μM each, dNTPs at 200 μM each, 0.050 U/μL SuperTaq™ Polymerase (Cloned) with its associated 1X Complete PCR Buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂), and SYBR® Green reporter dye at 1X concentration. The optimum PCR conditions (without melt curve stage) was established: 95.0 °C for one minute, followed by 40 cycles of five seconds at 95.0 °C, 10 seconds at 55.0 °C and 20 seconds at 74.0 °C.

The effects of individual co-solvents on DNA amplification were investigated. Formamide showed improvements in the efficiency of PCR which were not observed in the experiments on TMA chloride and TMA oxalate. The best results were obtained when the PCR mixture was supplemented with 2.5% formamide under optimum cycling conditions.

According to these parameters, the PCR amplification can be completed within a secondary school class of 40 minutes. The biochemical kit for the basic PCR protocol costs S$3.66 per reaction and the expected cost of the kit for the final PCR protocol is S$3.84 per reaction, which is less than the current market prices ranging from S$4.05 to S$10.78. Thus the objective of designing a biochemical kit for real-time PCR aimed at secondary schools was met.

FUTURE WORKS

The effects of adding combinations of several co-solvents to the reaction mixture will be tested as studies (Musso, 2006) have shown that combinatorial selection of additives can enhance PCR amplification. The components of the biochemical kit will then be finalised. After which, robustness of the kit will be examined by running experiments on freeze/thaw cycling and standing time viability, and cycling conditions of the PCR protocol will be modified to fit the parameters of the thermal cycler prototype.

ACKNOWLEDGEMENT

I acknowledge Mr Ronian Siew, Mr Jee Soo Yao and Ms Poh Qiu Yun for their invaluable assistance, and I thank Applied Biosystems for funding 2008-2009 Global Clinic Programme.

REFERENCE