The Use of Factorial Design, Image Analysis, and an Efficiency Calculation for Multiplex PCR Optimization

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Background: The quality of multiplex polymerase chain reaction (PCR) assays depends on several factors. Therefore, it is important to establish the optimal conditions to achieve efficient amplification. The objective of this study was to implement a 5×4 factorial design combined with image analysis using agarose gels and an efficiency calculation to optimize a multiplex PCR assays for the detection of *Salmonella enterica* serovar typhimurium. **Methods:** We used 12 ng of *Salmonella* DNA obtained from pure cultures and applied different annealing temperatures (65°C, 64.5°C, 63.3°C, 61.4°C, or 59°C) and different $MgCl_2$ concentrations (1 mM, 1.5 mM, 2 mM, or

2.5 mM) to amplify regions of the *fliC*, *rfbJ*, and *fliB* genes. The 5×4 factorial design was performed using Statgraphics Plus software version 5.1, and the images were analyzed using Image Lab™ software. **Results:** Superior amplification was obtained using an annealing temperature of 65°C and 2 mM $MgCl_2$. This finding was confirmed by calculating the efficiency of multiplex PCR assays (6.1%) at these conditions. **Conclusion:** We propose the application of factorial design and image analysis to determine the most suitable conditions for multiplex PCR optimization. J. Clin. Lab. Anal. 27:249–252, 2013. © 2013 Wiley Periodicals, Inc.

Key words: factorial design; PCR; optimization; *Salmonella*; diagnosis

INTRODUCTION

Salmonella is one of the main causative agents of food poisoning worldwide (1). This bacterium is usually isolated and identified from contaminated food and clinical specimens using traditional microbiological methods. These methods are relatively inefficient and expensive because they have low specificity and sensitivity, are time-consuming, and do not detect *Salmonella* spp. in food with low numbers of bacterial cells (2).

Analysis using the polymerase chain reaction (PCR) represents a superior alternative to the traditional microbiological detection methods because it avoids the aforementioned detection problems. PCR has proved to be a suitable method for the rapid detection of *Salmonella* via the in vitro amplification of one or more segments of *Salmonella* DNA. This technique, aside from being simple, reproducible, fast, and flexible, offers such alternatives as real-time quantitative PCR (RT-qPCR) (3) and multiplex PCR (mPCR) that facilitate, for both clinical and industrial purposes, the molecular diagnosis of pathogenic microorganisms in various types of samples (4–6).

During PCR, the amplified DNA accumulates exponentially relative to the initial amount of template DNA, the number of cycles applied, the concentration and annealing temperature of the primers, the concentration of the deoxynucleotide triphosphates (dNTP), and the concentration of $MgCl_2$. If one knows the initial DNA template copy number, the number of applied cycles, and the final concentration of amplified DNA, then it is possible to calculate the efficiency of the PCR as an indicator of its quality. However, because the efficiency of mPCR depends on several factors, it is important to establish the most suitable conditions using an optimization process (7). The factorial design is an excellent option for determining the optimal experimental conditions for mPCR

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amplification because it allows one to examine the effect of different factors on one or more quality indicators. In addition, the factorial design can identify possible combinations or different concentrations of the input factors, thereby resulting in a combination of variables that generate an efficient mPCR. This optimization requires a minimum number of tests and can be performed at a low cost (8). The objective of this study was to detect *Salmonella enterica* serovar *typhimurium* using PCR by applying a factorial design, imaging by agarose gels, and calculating the efficiency of the mPCR.

MATERIALS AND METHODS

Growth Conditions and Biomass Production

The *Salmonella enterica* serovar *typhimurium* strain was grown in Brain Heart Infusion broth (BHI; Oxoid, Solaar House, 19 Mercers Row, Cambridge, CB5 8BZ, UK) for 24 hours (h) at 37°C, as per the manufacturer's recommendations. To obtain the biomass, several aliquots of cultured cells were centrifuged at 13,000 revolutions per minute (rpm) for 10 minutes (min) at 4°C. After adding glycerol to a final concentration of 30%, the aliquots were stored at -70°C.

DNA Extraction

DNA was extracted using the phenol:chloroform:isoamyl alcohol method (9). The biomass was resuspended in 600 µl of 1x Tris-EDTA (TE) buffer containing 0.5% SDS (w/v) and 2 mg/ml of Lysozyme A. This mixture was incubated for 30 min at 37°C. To remove residual lipids and peptides, 80 µl of 5 M NaCl and 100 µl of 3 M ammonium acetate were added, and the mix was incubated for 10 min at 65°C. The resulting suspension was treated with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1). Once the phases were separated by centrifugation, the aqueous phase was treated with absolute ethanol. The precipitated DNA was washed with 70% ethanol (v/v), dried for 1 h at 56°C, and resuspended in 20 µl of water.

Factorial Design

To find the best amplification conditions for mPCR and to maintain statistical confidence, six replicates were performed using a 5 × 4 factorial design with two variables: the annealing temperature of the oligonucleotides (65°C, 64.5°C, 63.3°C, 61.4°C, or 59°C), and MgCl₂ concentration (1 mM, 1.5 mM, 2 mM, or 2.5 mM) (10). The experimental design was achieved using Statgraphics Plus software version 5.1, Reference Manual; Manugistics: Rockville, MD.

Multiplex PCR

Regions of the *fliC*, *rflB*, and *fljB* genes of *Salmonella enterica* serovar *typhimurium* were amplified using the oligonucleotides 5'ATAGCCATCTTTACCAGTTCCCCC3' and 5'GCTGCAACTGTTACAGGATATGCC3'; 5'CCCGCACCAGTTCCAACCTTGATAC3' and 5'GGCTTCCGGCTTTATTGGTAAGCA3'; and 5'ACGAATGGTACGGCTTCTGTAACC3' and 5'TACCGTCGATAGTAACGACTTCGG3', respectively. These primers yielded products of 183 basepairs (bp), 663 bp, and 526 bp for *fliC*, *rflB*, and *fljB*, respectively (11–13). The PCR was performed in a final volume of 20 µl containing 1x PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), MgCl₂ (1 mM, 1.5 mM, 2 mM, or 2.5 mM, according to the factorial design), 0.2 mM dNTPs, 0.5 µM of each oligonucleotide, 1.5 U of recombinant *Taq* DNA polymerase (Invitrogen® Life Technologies Corporation, California, United States), and 0.6 ng/µl of DNA. Using a C1000 thermal cycler (BIO-RAD Laboratories, Inc.), the following amplification conditions were employed. First, an initial denaturation step was performed for 2 min at 95°C followed by 30 cycles that included 1 min at 95°C, 1 min at an annealing temperature gradient (65°C, 64.5°C, 63.3°C, 61.4°C, and 59°C), 1 min at 72°C, and a final step of 5 min at 72°C. Ten microliters of each PCR product and 5 µl of the HyperLadder IV™ (Bioline, USA Inc.) molecular weight marker were subjected to electrophoresis in a 1% agarose gel for 2 hh at 60 V. The agarose gel was stained with ethidium bromide at a final concentration of 0.5 µg/ml.

Imaging Analysis

The photographic capture of band intensities was performed using a UV illumination of the agarose gels in a transilluminator (BIO-RAD Laboratories, Inc.). Image analysis was performed using the Image Lab™ software (BIO-RAD Laboratories, Inc., California, United States). The relative fluorescence (Rf) units of the three bands were averaged, and the 300-bp band of the molecular weight marker HyperLadder IV™ (Bioline, London, United Kingdom), which contains 30 ng of DNA according to the manufacturer, was used as the reference.

Efficiency (ξ) of mPCR

The efficiencies of the various mPCR assays were calculated using the equation $N_f = N_0(1 + \xi)^n$ (14, 15). N_f is the final copy number expressed in nanograms, N_0 is the initial copy number, ξ is the PCR efficiency, and n is the number of cycles.

The defined independent variable was N_f . The constants were $N_0 = 12$ ng and $n = 30$ cycles. The dependent variable

Fig. 1. Effect of the possible combinations or treatments using the different variations of annealing temperature or MgCl_2 on the amplification of the *Salmonella enterica serovar typhimurium fliC*, *rfbJ*, and *fliB* genes. Samples were resolved by electrophoresis using a 1% (w/v) agarose gel followed by ethidium bromide staining. MWM: molecular weight marker (Hyperladder IV). The 300-bp (R) molecular weight marker band (30 ng) was used as a reference in the image analysis. The arrow in lane 3 illustrates that the optimal PCR condition uses the combination of 2 mM MgCl_2 and a 65°C annealing temperature; 207 × 145 mm (300 × 300 DPI).

was ξ (PCR efficiency $\xi \in (0,1)$). From equation (1), PCR efficiency is obtained:

$$\xi = \sqrt[n]{\frac{N_f}{N_o}} - 1 \quad (1)$$

and next,

$$\xi = \frac{30}{\sqrt[12]{\frac{N_f}{N_o}}} - 1 \quad (2)$$

where $N_f > 0$, as determined by image analysis. Finally, ξ percentage is calculated using the equation: $\xi \% = \xi \times 100$.

RESULTS

mPCR and Image Analysis Using Agarose Gels

In this study, a factorial design using two variables (i.e., MgCl_2 and annealing temperature) was applied. The MgCl_2 and annealing temperature variables were tested at four and five different conditions, respectively. The averages of the bands products were averaged because the reaction is multiplex.

A total of six assays were performed; however, amplification was only obtained in five. Figure 1 displays the result of one of the assays.

In Table 1, the Rf value averages of five mPCR assays are displayed. The highest amplification average was obtained at an annealing temperature of 65°C and an MgCl_2 concentration of 2 mM.

TABLE 1. Rf Value Averages Expressed in Nanograms (ng)

MgCl_2 concentration (mM)	Temperature (°C)				
	59.0	61.4	63.3	64.5	65.0
1.0	41.3	52.1	53.3	53.4	56.7
1.5	49.6	46.8	54.7	57.4	57.7
2.0	54.7	64.5	54.4	70.7	73.2
2.5	54.7	59.4	60.1	60.6	59.1

Note: Rf value averages obtained from five mPCR assays for the diagnosis of *Salmonella enterica serovar typhimurium*.

After performing PCR using all possible combinations of the different factors' levels, we can conclude that a temperature of 65°C and a concentration of 2 mM MgCl_2 yields the highest PCR efficiency. Although the Rf values analyzed using Image Lab™ software decreased with lower annealing temperatures and MgCl_2 concentrations (Table 1), the combination of the different levels of temperature and MgCl_2 concentration ($P = 0.9$, $P > 0.5$) do not significantly influence the results. This finding can be partially explained by the proximity of the annealing temperatures, which generated a wide, homogeneous operating area, at least when the 65°C, 64.5°C, 63.3°C, and 61.4°C temperatures were applied.

mPCR Efficiency (ξ)

Table 2 displays the percent efficiency of the mPCR for each of the combinations of the oligonucleotide annealing temperatures and MgCl_2 concentrations. The highest efficiency (6.2%) is observed at a temperature of 65°C and 2 mM MgCl_2 . This finding is consistent with the average amplification (Table 1).

When an annealing temperature of 59°C and 1 mM MgCl_2 were used, a low mPCR percent efficiency (4.2%) was obtained. These results might be explained by the fact that temperature and MgCl_2 concentration values are farthest from the ideal annealing temperature found for the oligonucleotides used and because the calculated percent efficiency was outside of the homogeneous operational range. Additionally, it is known that *Taq* DNA polymerase has little activity at low MgCl_2 concentrations (16).

TABLE 2. Percent Efficiency of Various mPCR Assays for the Diagnosis of *Salmonella enterica serovar typhimurium*

MgCl_2 concentration (mM)	Temperature (°C)				
	59.0	61.4	63.3	64.5	65.0
1.0	4.2%	5.0%	5.1%	5.1%	5.3%
1.5	4.8%	4.6%	5.2%	5.4%	5.4%
2.0	5.2%	5.8%	5.2%	6.1%	6.2%
2.5	5.2%	5.5%	5.5%	5.5%	5.5%

DISCUSSION

Determining the optimal amplification conditions for an mPCR is a time-consuming process because it involves the development of experimental strategies of trial and error until one of the combinations of variables tested generates the desired effect. However, this task can be simplified using the factorial design strategy because it can determine in advance the possible combinations of the different variables. From this information, it is possible to then study the variables' effect on one or more qualitative characteristics. In addition, it is possible to calculate the PCR efficiency (14, 15).

While the greatest amplification efficiency was reached at a temperature of 65°C and 2 mM MgCl₂, using the temperature and MgCl₂ concentration close to the optimal values achieved satisfactory results. This result indicates that it is possible to reduce the concentration of such factors as MgCl₂. This conclusion is consistent with the findings of Niens et al. who determined that small changes in the variables do not significantly alter mPCR efficiency (17).

Previous studies by Boleda et al. demonstrated the advantage of applying two types of experimental design techniques. However, the parameters studied included extrinsic variables, such as DNA extraction methods or autoclaving the sample. This group neither calculated the PCR efficiency, nor were quality assessments determined by measuring band intensities using a densitometer (18). In our study, quantitative results were obtained using the Image LabTM software, which is based on the quantification of the Rf values extracted from agarose gel imaging. As a result, mPCR efficiency was estimated, demonstrating that this approach can be an ideal tool for objectively determining the best combination for the mPCR conditions. Small changes in the variables do not considerably affect the mPCR efficiency. To optimize the mPCR assay, we propose the application of factorial design to select the most suitable conditions, to evaluate the quality responses using agarose gel image analysis, and to calculate PCR efficiency. Additionally, the application of factorial design and image analysis can be used as tools for optimizing other PCR methods.

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CONFLICTS OF INTEREST

The authors declare no competing interests.

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