CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Repositorio da Producao Cientifica e Intelectual da Unicamp

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

TALITA SIGNORETI GRAZIANO

DESENVOLVIMENTO DE UMA MISTURA REACIONAL DE BAIXO CUSTO PARA PCR QUANTITATIVA BASEADA EM *SYBR® GOLD*

DEVELOPMENT OF A LOW-COST BUFFER SYSTEM FOR QUANTITATIVE PCR BASED IN SYBR® GOLD

PIRACICABA
2018

Talita Signoreti Graziano

DEVELOPMENT OF A LOW-COST BUFFER SYSTEM FOR QUANTITATIVE PCR BASED IN SYBR® GOLD

DESENVOLVIMENTO DE UMA MISTURA REACIONAL DE BAIXO CUSTO PARA PCR QUANTITATIVA BASEADA EM *SYBR® GOLD*

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Oral Biology, in the Microbiology and Immunology area.

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Biologia Buco-dental, área de Microbiologia e Imunologia.

Orientador: Prof. Dr. Rafael Nobrega Stipp

Este exemplar corresponde à versão final da tese defendida pela aluna Talita Signoreti Graziano e orientada pelo Prof. Dr. Rafael Nobrega Stipp.

PIRACICABA

2018

Agência(s) de fomento e nº(s) de processo(s): CNPq, 154620/2014-9; FAPESP, 2017/03263-3

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

G796d

Graziano, Talita Signoreti, 1988-
Development of a low-cost buffer system for quantitative PCR based in SYBR® Gold / Talita Signoreti Graziano. – Piracicaba, SP : [s.n.], 2018.

Orientador: Rafael Nobrega Stipp.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Biologia molecular. 2. Reação em cadeia da polimerase em tempo real. I. Stipp, Rafael Nobrega, 1982-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Desenvolvimento de uma mistura reacional de baixo custo para PCR quantitativa baseada em SYBR® Gold
Palavras-chave em inglês:
Molecular biology
Real-time polymerase chain reaction
Área de concentração: Microbiologia e Imunologia
Titulação: Doutora em Biologia Buco-Dental
Banca examinadora:
Rafael Nobrega Stipp [Orientador]
Bruna Benso
Luiz Eduardo Nunes Ferreira
Marcelo Rocha Marques
Natália Leal Vizoto
Data de defesa: 28-08-2018
Programa de Pós-Graduação: Biologia Buco-Dental

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 28 de Agosto de 2018, considerou a candidata TALITA SIGNORETI GRAZIANO aprovada.

PROF. DR. RAFAEL NOBREGA STIPP

PROFª. DRª. BRUNA BENSO

PROF. DR. LUIZ EDUARDO NUNES FERREIRA

PROF. DR. MARCELO ROCHA MARQUES

PROFª. DRª. NATÁLIA LEAL VIZOTO

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

Dedico este trabalho aos meus pais, **Nelida** e **Jaime**; aos meu irmãos, **Jackson e Bruno**; à minha cunhada **Carolina**; pelo incentivo, amor e carinho em todos os momentos.

AGRADECIMENTOS

À Universidade Estadual de Campinas, na pessoa do seu Magnífico Reitor, **Prof. Dr. Marcelo Knobel.**

À Faculdade de Odontologia de Piracicaba, na pessoa do Diretor, **Prof. Dr. Guilherme Elias Pessanha Henriques**.

À **Profa. Dra. Karina Gonzales Silvério Ruiz**, Coordenadora Geral da Pós-Graduação da FOP-UNICAMP.

À **Profa. Dra. Ana Paula de Souza**, Coordenadora do Programa de Pós-Graduação em Biologia Buco-dental.

Ao meu orientador **Prof. Dr. Rafael Nobrega Stipp** pelos ensinamentos e por permitir a realização desse trabalho.

Aos Professores do Programa de Pós-Graduação, em especial aos da área de Microbiologia e Imunologia e da área de Farmacologia, Anestesiologia e Terapêutica pela participação na minha formação e por sempre incentivarem a busca do conhecimento e aprimoramento acadêmico.

Aos **profs. Drs. José Franscisco Hofling, Marcelo Gomes Boriollo e Antônio Pedro Ricomini Filho** pelas sugestões e contribuições no exame de qualificação.

Aos responsáveis pelo laboratório de Microbiologia e Imunologia da FOP-UNICAMP, **Valéria DeFavari Franco** e **Anderson Laerte Teixeira**, pela amizade e colaboração durante todos estes anos.

À minha família por todo carinho, por sempre estarem ao meu lado me incentivando e me apoiando durante todos esses anos. E em especial a minha

mãe **Nelida**, cuja dedicação, apoio, incentivo e amor incondicional tornou tudo isso possível.

À **Bruna Benso, Juliana Botelho e Josiane Neres**, pelo carinho, colaboração, apoio e principalmente pela amizade.

À **Livia Alves, Giovana Boni, Simone Busato, Janaina Barbosa, Jeferson Silva, Rodrigo Bassi, Felipe Joia, Filipe Matheus, Marcele Buso e Thais Oliveira** pela convivência diária e por proporcionarem momentos agradáveis.

Ao **Conselho Nacional de Pesquisa (CNPQ)** pela concessão da bolsa de doutorado e a **Fundação de Amparo à Pesquisa (FAPESP)**, pela concessão do auxílio à pesquisa.

Às **Sras. Ana Paula Carone**, **Érica Alessandra Pinho Sinhoreti** e **Raquel Quintana Marcondes Cesar Sacchi** por todas as orientações e indispensável ajuda.

Meu eterno reconhecimento a todos que de alguma forma contribuíram para a realização deste trabalho.

RESUMO

A PCR em tempo real ou quantitativa (qPCR) é amplamente utilizada para quantificação de DNA e apresenta um alto custo nas pesquisas das áreas biológicas. O objetivo foi desenvolver uma mistura reacional para a qPCR com especificidade, eficiência e sensibilidade equivalente ao produto industrializado importado. Desse modo, foram preparados sistemas tampões que variavam em relação às concentrações de *SYBR® Green* ou *SYBR® Gold*, $MgCl_2$ e *Taq DNA Polimerase Brazil*. Para potencializar a reação, aditivos como: albumina sérica bovina, dimetil sulfóxido, formamida, polietileno glicol 400, Triton x-100, trealose e Tween-20 foram utilizados em variadas concentrações de forma separada ou combinada. O DNA genômico de *Porphyromonas gingivalis* (300 a 0,003 ng) e *primers* espécie-específico foram utilizados para a comparação inicial entre as misturas. Para garantir a aplicabilidade, as misturas com melhores resultados foram avaliadas contra DNA de outras espécies: *Streptococcus sanguinis*, *Mus Musculus, Homo sapiens* e *Candida albicans*. Além disso, foram submetidas à testes de estabilidade. As misturas foram equiparadas ao reagente comercial *Power SYBR Green qPCR Master Mix Kit©* através dos valores de *Cycle threshold* e análises das curvas de *melting*. A mistura com melhor eficiência, especificidade e estabilidade foi chamada de *Master Mix* para qPCR não comercial. A eficiência do *Master Mix* para qPCR não comercial foi até 28% superior ao produto comercial, variando de acordo com a concentração, origem e qualidade do material genético. O preço atual do consumível da reação de qPCR pode ser reduzido em até 80% empregando a mistura reacional proposta. Em conclusão, foi desenvolvida uma mistura reacional para PCR, com alta eficiência e especificidade, e um baixo custo. A mistura permite alterações individuais, uma vez que, sua composição e concentração são conhecidas. Além disso, foi o primeiro relato do uso do *SYBR Gold* como corante fluorescente em reações de qPCR. A patente da mistura reacional desenvolvida foi depositada no Instituto Nacional de Propriedade Industrial (INPI), número de processo BR 10 2017 022164 4.

Palavras-chave: Reação em Cadeia da Polimerase. *Sybr Gold*. Biologia Molecular.

ABSTRACT

Quantitative PCR (qPCR), or real-time PCR, is widely used for DNA quantification and the consumable for the reactions represents a high cost for users. This study aimed to develop a low-cost reaction mixture for qPCR with specificity, efficiency, stability, and sensitivity equivalent to the commercial consumable. Mixtures were assembled with various concentrations of *SYBR® Green* or *SYBR® Gold*, $MgCl_2$ and *Taq DNA Polymerase Brazil* (core reagents). The additives bovine serum albumin, betaine, dimethyl sulfoxide, formamide, polyethylene glycol 400, polyoxyethylene-octyl-phenyl-ether, trehalose and polysorbate-20 were added alone or in combination and evaluated as possible qPCR reactions enhancers. During the additives evaluation, reactions were conducted with genomic DNA (300 to 0.003 ng) of *Porphyromonas gingivalis* and species-specific primers. To ensure applicability, the mixtures with best results were evaluated against DNA or cDNA from other species with species-specific primers: *Homo sapiens; Candida albicans; Mus musculus* and *Streptococcus sanguinis*. Cycle threshold values and melting curves were compared to the commercial *Power SYBR Green qPCR MasterMix Kit®*. The reaction mixture that showed best results has its stability evaluated. The mixture with better efficiency, sensitivity, specificity, and stability was called as *non-commercial qPCR Master Mix*. The efficiency of the *non-commercial qPCR Master Mix* was up to 28% higher than the commercial Mastermix, varying according to the concentration, origin, and purity of the genetic material. The price of the *non-commercial qPCR Master Mix* was about 80% than the commercial one. In conclusion, a consumable for qPCR reactions was developed, with high efficiency, sensitivity, specificity, and stability and at low cost. The consumable developed allows individual changes, since its composition and its concentration are known. In addition, it was the first report of using *SYBR Gold* as a fluorescent dye for qPCR consumable. The developed reaction mixture was patented in the National Institute of Industrial Property, process number BR 10 2017 022164 4.

Key-words: Polymerase Chain Reaction. SYBR Gold. Molecular Biology.

SUMÁRIO

1.INTRODUÇÃO 11

2. ARTIGO: DEVELOPMENT OF A LOW-COST BUFFER SYSTEM FOR QUANTITATIVE PCR BASED IN SYBR® GOLD 18

3. CONCLUSÃO. 44

REFERÊNCIAS 45

1. INTRODUÇÃO

A descoberta do DNA foi uma um dos grandes marcos na ciência, revolucionando a mesma, ao possibilitar uma melhor compreensão dos mecanismos relacionados a vida. O estudo do genoma tem permitido uma melhor análise e compreensão dos processos hereditários, da variabilidade inter e intra-específica, da evolução das espécies, controle de doenças, entre outros aspectos (Pelt-Verkuil et al., 2008; Oliveira, 2010). A necessidade de estudar mais afundo todos os fenômenos relacionados aos ácidos nucleicos deu origem a uma série de técnicas que permitem a identificação e quantificação do material genético.

A Reação em Cadeia da Polimerase (PCR) é uma técnica que permite a amplificação do material genético (Farell & Alexandre 2012). A técnica baseia-se na repetição de ciclos de duplicação, que aumentam de forma exponencial a quantidade de DNA. Ocorre a síntese artificial de DNA num processo em cadeia que imita a replicação natural do DNA (Bustin, 2009; VanGuilder et al 2008; Quan et al., 2018). A criação da PCR permitiu um aumento exponencial da quantidade de DNA de forma rápida e segura, sem necessitar de microrganismos vivos. Essa técnica revolucionou as áreas de patologia, medicina forense, botânica, farmácia, biologia evolutiva, dentre outras. Sua aplicabilidade vai desde de mapeamento genético, clonagem de genes, testes de paternidade, identificação de microrganismos até construção de árvores filogenéticas (Mackay et al., 2007; Pelt-Verkuil et al 2008; Farell & Alexandre 2012).

A PCR pode ser dividida em 3 fases: desnaturação, anelamento e extensão. Na fase de desnaturação ocorre a separação da dupla fita de DNA por aquecimento a uma temperatura entre 94 °C – 96 °C. Na fase seguinte, ocorre o anelamento dos *primers* à fita simples (50 °C – 54 °C). Após a ligação do *primer* à sequência complementar e especifica do DNA alvo, inicia-se a fase de extensão. Nessa fase, a enzima DNA polimerase se liga ao sítio *primer*-DNA a uma temperatura que varia entre 72 °C a 76 °C. Após a ligação, a enzima inicia o processo de síntese da nova fita de DNA, complementar a do DNA alvo (Oliveira, 2010; Quan et al., 2018). Ao final do ciclo obtém-se duas novas fitas

simples de DNA. Desse modo, com o decorrer dos ciclos ocorre um crescimento exponencial da quantidade de DNA. O produto da PCR é normalmente visualizado através da eletroforese em gel (Bustin et al., 2004; VanGuilder et al., 2008; Pelt-Verkuil et al 2008). Desse modo, se trata de uma técnica de caráter qualitativo, em que ao final do processo se avalia o tamanho dos fragmentos amplificados. Diante da necessidade de se realizar análises mais profundas e de caráter quantitativa, foram surgindo outras variantes da técnica de PCR.

A PCR em tempo real ou PCR quantitativa (qPCR) é uma variação da técnica de PCR, que permite a quantificação do material genético estudado. O procedimento é semelhante ao do PCR convencional, com a diferença de detectar o DNA amplificado ao final de cada ciclo, realizando a quantificação do mesmo ao final do processo (Oliveira, 2010; Quan et al., 2018). Desse modo, a diferença para a PCR convencional, é a presença de um sistema de detecção de fluorescência que permita o monitoramento da quantidade de produto amplificado (Bustin et al., 2009; VanGuilder et al., 2008; Pelt-Verkuil et al., 2008). Os sistemas de detecção para qPCR, podem ser divididos em dois grupos: corantes fluorescentes e sondas de sequência específica. Enquanto que o primeiro envolve uma detecção inespecífica, uma vez que, os corantes se ligam a qualquer segmento de DNA dupla fita, o segundo envolve uma detecção específica, se ligando apenas ao gene ou fragmento de interesse. Apesar da vantagem apresentada pelas sondas de sequência especifica, por se tratar de uma técnica de maior custo é menos utilizada que os corantes fluorescentes (Bustin et al., 2009; VanGuilder et al., 2008; Pelt-Verkuil et al., 2008).

Para a realização da PCR, além do DNA alvo, é necessária uma mistura de reagentes denominada *Master Mix* (Oliveira, 2010). Essa mistura é composta por um sistema tampão, $MgCl_2$, enzima DNA polimerase, desoxirribonucleótideos trifosfatados (dNTP’s), os *primers* e o corante fluorescente (Chen e Janes, 2002; Pelt-Verkuil et al 2008). O tampão é responsável por manter o pH estável durante a reação, garantindo dessa forma a ativida da enzima *Taq polimerase* (Chen e Janes, 2002; Pelt-Verkuil et al 2008). O $MgCl_2$ funciona como um co-fator para a enzima *Taq polimerase*. Os íons Mg^{+2},

catalisam a formação da ligação fosfodiéster entre o 3'-OH de um *primer* e o grupo fosfato de um dNTP, permitindo desse modo, à incorporação de dNTP durante a extensão (Pellissier et al., 2006). Além disso, o Mg^{+2} participa da formação do complexo *primers*-DNA alvo, estabilizando as cargas negativas das cadeias de fosfato do ácido nucleico (Chen e Janes, 2002; Oliveira, 2010). A DNA polimerase é a enzima responsável pela atividade de polimerização da nova fita de DNA. A *Taq polimerase* é a enzima mais utilizada por possuir uma boa estabilidade, se mantendo ativa até o final dos ciclos de amplificação (Chen e Janes, 2002; Yazd et al., 2009; Oliveira, 2010). Os dNTP's são nucleotídeos de DNA formados por um açúcar, um fostato e uma das 4 bases: adenina, citosina, guanina e timina. São utilizados durante o processo de construção da nova fita de DNA (Chen e Janes, 2002; Pelt-Verkuil et al 2008). Já os *primers* são oligonucleotideos responsáveis por iniciar a reação de PCR e seu tamanho e sequência são de suma importância para o correto emparelhamento e sucesso da amplificação (Oliveira, 2010; Thornton & Basu, 2015).

O principal corante fluorescente utilizado na qPCR é o *SYBR Green*, uma molécula com capacidade de se ligar na região do *minor groover* do DNA. Após se ligar a dupla fita, exibe um aumento de cerca de 1000x na sua intensidade (Oliveira, 2010). Esse complexo DNA-corante possui como característica absorver luz azul (λ_{max} = 497 nm) e emitir luz verde (λ_{max} = 520 nm) (Navarro et al, 2015). Esse corante apesenta como vantagens uma alta sensibilidade, um reduzido custo, fácil manuseio e uma maior segurança que o seu antecessor brometo de etídio (Navarro et al., 2015). Como principal desvantagem, apresenta a possibilidade de ligação a todo DNA dupla fita presente na amostra, incluindo dímeros de primers e produtos não específicos que possam ter sido formados durante a reação (Mackay et al, 2007; Pelt-Verkuil et al, 2008; Oliveira, 2010). O *SYBR Gold* assim como o *SYBR Green* também pertence à classe dos corantes cianinas. Possui como principal característica uma maior sensibilidade na detecção de ácidos nucleicos, principalmente nos comprimentos de onda 300 nm e 495 nm (Tuma et al., 1999). Até o momento sua aplicação tem sido exclusiva na detecção de ácidos nucleicos em géis de eletroforese, não existindo relatos de seu uso em qPCR.

Durante o processo de anelamento e extensão, o corante fluorescente se liga a dupla fita, aumentando a intensidade do sinal de fluorescência. Ao final de cada ciclo, um sistema óptico para excitação e recolha da emissão de fluorescência detecta esse sinal emitido, que é diretamente proporcional a quantidade de duplas fitas presentes na amostra. Ao final do processo obtém-se a chamada curva de amplificação, que representa a fluorescência obtida ao longo dos ciclos (Mackay et al., 2007; Pelt-Verkuil et al 2008; Quan et al., 2018).

A curva de amplificação pode ser dividida em 3 fases: crescimento exponencial, crescimento linear e fase estacionária (Oliveira, 2010). A primeira fase é considerada a melhor para se estudar a reação devido a sua elevada eficiência e especificidade. Na fase de crescimento linear os produtos da reação são consumidos, e a taxa de produção de fitas novas diminui. A fase estacionaria ou platô, corresponde a fase final em que ocorre depleção dos reagentes, e consequentemente a fluorescência se mantem constante, uma vez que se tem ausência de formação de novas fitas (Oliveira, 2010; Quan et al., 2018). Para auxiliar a interpretação e análise dos resultados obtidos existem alguns conceitos referentes as curvas de amplificação. Um desses conceitos é o de *baseline*, que se refere ao limiar mínimo de detecção de fluorescência do instrumento, é determinada pelo próprio aparelho e os sinais detectados anteriores a esse valor são considerados “ruído” (Oliveira, 2010). Já o *threshold* é o valor selecionado quando a PCR inicia a fase exponencial, sendo definido de forma automática e arbitrária pelo *software* do aparelho em função do baseline. O ponto de intersecção entre o *threshold*, ou seja, o valor de fluorescência que representa o início da fase exponencial, e o número de ciclos, é chamado de C_T ou *Cycle Threshold*. Desse modo, o valor de C_T representa o número de ciclos necessários para que a fluorescência atinja a fase exponencial (Bustin,S.A 2009; VanGuilder HD et al 2008; Pelt-Verkuil et al 2008).

Além da curva de amplificação, o aparelho de PCR também fornece a chamada curva de dissociação ou curva de *melting*. Essa curva é gerada após o término dos ciclos da reação de PCR, através de aumento sucessivos da temperatura até a perda de fluorescência devido a desnaturação do DNA (Chen

e Janes, 2002; Gudnason et al., 2007; Oliveira, 2010). Desse modo, relaciona a temperatura em função da fluorescência. Durante o processo de desnaturação do DNA, ao atingir a chamada temperatura de *melting* (Tm), que é quando 50% do DNA se encontra em dupla fita e 50% em fita simples, ocorre uma quebra abrupta da fluorescência (Pelt-Verkuil et al 2008). Desse modo, diminuições adicionais de fluorescência indicam a presença de outras duplas fitas que não sejam o DNA alvo. Isso porque, a temperatura de *melting* depende da composição e tamanho do fragmento estudado (Monis et al., 2005; Gudnason et al., 2007). Desse modo, a análise da curva de *melting* permite a distinção de diferentes produtos de PCR, diferenciando produtos específicos e não-específicos, verificando a especificidade da reação (Oliveira, 2010).

Após a amplificação e detecção do DNA é possível quantifica-lo, existindo duas formas de quantificação: absoluta (método da curva padrão) ou relativa (método da comparação do limiar da fase exponencial) (Ma et al., 2006; Pelt-Verkuil et al 2008; Svec et al., 2015). A quantificação absoluta determina o numero exato de moléculas de DNA. Nesse método, é feita a determinação da concentração inicial de uma amostra de concentração desconhecida a partir de uma curva padrão de concentração conhecida (Oliveira, 2010). Para isso, se extrapola a concentração de DNA a partir da projeção do valor de C_T da amostra, num gráfico do C_T em função do logaritmo da concentração de DNA obtido a partir da curva padrão (Ma et al., 2006; Svec et al., 2015). Já o método de quantificação relativa utiliza a comparação do limiar da fase exponencial, ou seja, do *threshold*, sem a utilização da curva padrão. A comparação ocorre entre os valores de C_T das amostras com um controle, que são normalizados por um gene endógeno apropriado (Oliveira, 2010). Esse método é aplicado na quantificação da expressão gênica, comparando os genes alvo com um gene que não se altere nas condições do estudo. Desse modo, ao invés de fornecer o número exato de moléculas, avalia em que proporção o gene alvo se encontra expresso nas condições do estudo (Ma et al., 2006; Svec et al., 2015).

A eficiência da amplificação depende de uma série de fatores: concentração dos reagentes do *Master Mix*, comprimento do DNA alvo,

existência de estruturas secundárias ou contaminantes na amostra, qualidade dos *primers*, procedimentos laboratoriais incorretos, presença de inibidores da reação de PCR, entre outros (Pelt-Verkuil et al 2008; Thornton & Basu, 2015). Reagentes em excesso ou em escassez podem prejudicar a atividade da enzima *Taq polimerase*, a quantidade e qualidade do produto formado, assim como, influenciar na formação de produtos inespecíficos. Por exemplo, concentrações elevadas de dNTP's e de $MgCl_2$ resultam em erros de incorporação durante a amplificação e formação de produtos não-específicos, já baixas concentrações diminuem o rendimento da reação (Karsai et al., 2012).

Para aumentar a eficiência e especificidade da reação existem alguns aditivos que podem ser acrescentados ao *Master Mix* da qPCR (Simonović et al, 2012). Essas substâncias podem atuar contribuindo para a estabilidade da reação, melhorando a ligação entre o DNA alvo e os *primers* e reduzindo a formação de estruturas secundárias nas regiões ricas em CG (Simonović et al, 2012; Jensen et al., 2009). Como exemplo temos a formamida, uma amida derivada do ácido fórmico, capaz de desestabilizar as regiões ricas em CG, facilitando o anelamento do primer e a extensão da reação (Zeng et al., 2006). Já betaína, um aminoácido natural que participa do metabolismo vegetal e animal, consegue equilibrar a diferença da Tm entre as regiões ricas em AT e CG (Jensen et al., 2009). Existem ainda outros aditivos que contribuem com a melhora da reação como: polietileno glicol 400 (PEG-400), polissorbato 20 (Tween-20), Triton X-100, albumina sérica bovina (BSA), dimetilsulfóxido (DMSO) e trealose (Jung et al., 2001; Chakrabarti et al., 2001; Spiess et al., 2004).

A técnica de qPCR tem sido amplamente utilizada em análises de expressão gênica, identificação e quantificação de microrganismos, controle de qualidade e validação de ensaios, quantificação viral, detecção de agentes patogênicos, toxicologia forense, análise de danos no DNA, genotipagem, determinação pré-natal do sexo, oncologia clínica, quantificação de proteínas, quantificação de DNA mitocondrial e de DNA nuclear, qualidade do DNA, estudo da presença de níveis de OGM nos alimentos, certificação, entre outras (Farell

and Alexandre 2012; Navarro et al., 2015). Isso porque, se trata de uma técnica simples, rápida, de fácil quantificação e com elevada especificidade e sensibilidade (Navarro et al., 2015). Em contrapartida, apresenta um elevado custo, principalmente por empregar reagentes importados.

O mercado dispõe de diversos *Master Mix* como o *Power SYBR Green PCR Master Mix Kit (Applied Biosystems), iTaq SYBR Green Supermix with Rox Kit (BioRad), Perfecta SYBR Green Super Mix (Quanta), Real RT2 Real-Time SYBR Green/Rox PCR Master Mix kit (SA Biosciences).* Esses produtos contém todos os reagentes necessários para a PCR, com exceção dos *primers*, que são específicos para a amostra a ser utilizada. Apresentam como características uma boa especificidade, reprodutibilidade, sensibilidade, linearidade e eficiência. Entretanto, além dos altos custos, possuem composição e concentrações desconhecidas, o que impossibilita alterações individuais de acordo com a necessidade da sua amostra (Ralser et al. 2006). Desse modo, o objetivo do presente estudo foi desenvolver uma mistura reacional (*Master Mix*) de baixo custo para PCR quantitativa, com eficiência, estabilidade e sensibilidade comparável ao reagente comercial *Power SYBR Green qPCR 2 × Master Mix* (Applied Biosystems, USA, #4367659).

2. ARTIGO

DEVELOPMENT OF A LOW-COST BUFFER SYSTEM FOR QUANTITATIVE PCR BASED IN SYBR® GOLD

Graziano, T.S.[a]

Hofling, J.F.[a]

Mattos-Granner, R.O.[a]

Stipp, R.N.[a]

[a] Department of Oral Diagnosis, Piracicaba Dental School, University of Campinas-UNICAMP, SP, Brazil. E-mail: rafaelns@fop.unicamp.br

Corresponding author:

Rafael Nobrega Stipp

Av. Limeira, 901, 13414-903, Piracicaba, SP, Brazil

Phone/Fax: (19) 2106-5707

Running title: Low-cost Mastermix for qPCR

Keywords: RT-PCR, *SYBR Gold*, Molecular biology

ABSTRACT

Quantitative PCR (qPCR), or real-time PCR, is widely used for DNA quantification and the consumable for the reactions represents a high cost for users. This study aimed to develop a low-cost reaction mixture for qPCR with specificity, efficiency, stability, and sensitivity equivalent to the commercial consumable. Mixtures were assembled with various concentrations of *SYBR® Green* or *SYBR® Gold*, $MgCl_2$ and *Taq DNA Polymerase Brazil* (core reagents). The additives bovine serum albumin, betaine, dimethyl sulfoxide, formamide, polyethylene glycol 400, polyoxyethylene-octyl-phenyl-ether, trehalose and polysorbate-20 were added alone or in combination and evaluated as possible qPCR reactions enhancers. During the additives evaluation, reactions were conducted with genomic DNA (300 to 0.003 ng) of *Porphyromonas gingivalis* and species-specific primers. To ensure applicability, the mixtures with best results were evaluated against DNA or cDNA from other species with species-specific primers: *Homo sapiens; Candida albicans; Mus musculus* and *Streptococcus sanguinis*. Cycle threshold values and melting curves were compared to the commercial *Power SYBR Green qPCR MasterMix Kit®*. The reaction mixture that showed best results has its stability evaluated by 20× cycles of freezing and thawing. Results: The *non-commercial qPCR Mastermix* was a blend of formamide, betaine and PEG-400 and core reagents with SYBR® Gold and showed efficiency up to 28% higher than the commercial product. The price of the *non-commercial qPCR Master Mix* developed was about 80% than the commercial one. In conclusion, a consumable for qPCR reactions was developed, with high efficiency, sensitivity, specificity, and stability and at low cost. The consumable developed allows individual changes, since its composition and its concentration are known. In addition, it was the first report of using *SYBR Gold* as a fluorescent dye for qPCR consumable. The developed reaction mixture was patented in the National Institute of Industrial Property, process number BR 10 2017 022164 4

Key-words: qPCR. SYBR Gold. SYBR Green.

INTRODUCTION

Quantitative PCR (qPCR) is widely used in molecular biology (1). Although qPCR machine prices have decreased over time, the cost of the consumable for the reaction is still high. The high price of the *qPCR Master Mix* affects the popularization and assay use. To solve this, here we present a low-cost *qPCR Master Mix*.

The *qPCR Master Mix* reagent is based on many compounds that allow qPCR and hence quantification (2). The most important substance for qPCR is the fluorescent dye (2). The amount of fluorescence produced allow quantification of the samples. Fluorescent dyes bound themselves within the nucleic acid chains and release a fluorescence that is thousand times brighter than the dye in in an unbound state (3).

Currently, most commercial reactional mixtures for qPCR are SYBR® Green based (4). However, several studies have shown that SYBR® Green can reduce the efficiency of qPCR (5, 6). Other alternative dyes, such as BEBO (7), YO-PRO-1 (8), LC Green (9), SYTO-9 (10, 11), SYTO-13 (12) and SYTO-82 (12) have been evaluated. Given that Taq Polymerase enzyme and fluorescent dyes represents one of the most expensive components of qPCR reactional mixtures; a dye with a lower cost and same effectiveness is an interesting alternative, making the price per reaction more accessible. SYBR Gold, a dye with higher sensitivity than SYBR green, is half the price (13), but no records have been found of its use in qPCR.

Specificity, efficiency, stability and sensibility of the reactions are three essential requirements for successful qPCR (14). To enhance the production of specific PCR products, a variety of additives have been evaluated alone or in combination (14). These include bovine serum albumin (BSA), dimethyl sulfoxide (DMSO) (15, 16), N,N,N-trimethylglycine monohydrate (betaine) (17, 18), formamide (19-21), glycerol (22), trehalose (23, 24), *polyethylene glycol 400 (*PEG-400), polysorbate 20 and Triton X-100 (22-24). Most of these additives have been described as having beneficial effects on PCR amplifications, contributing to the stability of the reaction, improving the bond between the DNA

target and the primers, and reducing the formation of secondary structures in GC-rich regions (25-27).

A low-cost qPCR mix was developed with SYBR® Gold as fluorescent dye and combining the additives: betaine, formamide and peg400. This proposed non-commercial *qPCR Master Mix* is easy to prepare, have low-cost, highly reproducible, stable and its assembly was highly detailed. It may be useful for research, clinical diagnosis laboratories and industry, etc. The rights to sell the mixture are protected by patent in Brazil, but unless commercializing it, the mixture is free for those preparing it for their own use.

METHODS

DNA and Primers

Table 1 shows the genes and primers used. The nucleic acid was purified according to Stipp et al., 2013 (28) using the phenol-chloroform method. The purification ratio was established by absorbance $A_{260}/A_{280} \geq 1.99$ and $A_{260}/A_{230} \geq 1.99$. DNA integrity was confirmed on agarose gel.

Table 1. Species, primers, annealing temperature and the amount of CG of each fragment are showed. Ta = temperature annealing of primer.

Organisms	Genes	*Primer* (*forward* and *reverse)*	Ta (°C)	%CG fragment
Porphyromonas gingivalis ATCC® BAA-308™/W83	16S-rRNA	5'AGGCAGCTTGCCATACTGCG3' 5'ACTGTTAGCAACTACCGATGT3'	57	51.6
Streptococcus sanguinis ATCC® BAA-1455™	SSA_1234	5’TAGTTGTAGATGCGGGTGATG3’ 5’CATTGTTGGCGTAGGTGTTAG3’	57	46.41
Streptococcus sanguinis ATCC® BAA-1455™	SSA_0250	5’CGTTTTCACACCAGATGGA3’ 5’CCGAAGGAGTTAGCATTGG3’	57	40.71
Streptococcus sanguinis ATCC® BAA-1455™	SSA_0263	5’ACTTATTTCCCCTTTGGTGTG3’ 5’ATCCTTGATTTCCTCTTCTGC3’	57	39.9
Mus musculus - lineage *MDPC-23 (ATCC® CRL-2537™)*	GAPDH	5’GTGCTGAGTATGTCGTGGAGT3’ 5’TTGTCATATTTCTCGTGGTTCA3’	55	54.47
Mus musculus - lineage *MDPC-23 (ATCC® CRL-2537™)*	DNMT 3B	5’CGAGTCCTGTCATTGTTTGATG3’ 5’GCGACGTACTTTCCTACCTTTAT3	55	61.2
Candida albicans ATCC® 5314™	ACT	5’AGCTCCAGAAGCTTTGTTCAGACC3’ 5’TGCATACGTTCAGCAATACCTGGG3’	57	33.47

Master Mix CORE reagents

To prepare the non-commercial *qPCR Master Mix*, the concentration of the reagents necessary for any PCR (CORE reagents) was determined. The reagents included in the mix and their concentrations are found in Table 2. The *Master Mix* was prepared with 2 × the final concentration, which is a concentration similar to the commercial product *Power SYBR Green qPCR 2 × Master Mix* (Applied Biosystems, USA). The mixture with the best concentration combination of these core reagents was called the *Master Mix Core reagents* (without additives).

Table 2. Reagents and concentration tested for the determination of the *Master Mix CORE reagents*.

Reagents	Stock solution	Concentrations tested	2x Concentration
H_2O	------	-------	-------
PCR Buffer *	10 ×	1 ×	2 ×
$MgCl_2$ *	50 mM	2; 3.5 and 7 mM	4; 7 and 14 mM
dNTP Nucleotides (Thermo Fisher Scientific - Invitrogen, #10297018)	10 mM	0.2 mM/dNTP	0.4 mM/dNTP
SYBR^{TM} Green I nucleic acid gel stain (Invitrogen, #S7563) or *SYBR^{TM} Gold nucleic acid gel stain* (Invitrogen, #11494)	10 ×	0.1 to 0.9 ×	0.2 to 1.8 ×
Reference Dye qPCR (Sigma-Aldrich R4526-.3ML)	100 ×	1 ×	2x
Taq DNA Polymerase Brazil (Thermo Fisher Scientific Invitrogen, #11615010)	5 U/ µl	0.05 and 0.1 U/µl	0.1 and 0.2 U/µl

*Comes with the *Taq Polymerase* (Thermo Fisher Scientific Invitrogen, #11615010)

Additives

The additives described in Table 3 were tested as reaction enhancers. Once the *Master Mix CORE reagents* had been established, the additives were added to this mixture alone or in combination with each other. The mixtures containing the additives were compared to the *Master Mix CORE reagents* and the commercial product. The best combinations were subjected to stability tests. The best mixture after the stability tests was called the non-commercial *qPCR Master Mix* (Core reagents + additives).

Table 3. Additives tested with the *Master Mix CORE reagents*.

Additives	Concentrations tested	Reference
Bovine serum albumin (BSA) (Sigma, #A1933)	50 ng/ml, 100ng/ml e 200ng/ml	29
Dimethylsulfoxide (DMSO) (Sigma, #D2650)	1%, 2%, 3%, 4% e 5%	30 - 32
Formamide (Sigma, #F9037)	0.5%; 1%; 1.25% e 2.5%	15
N,N,N-trimethylglycine monohydrate (Betaine) (Sigma, #61962)	1M; 1.35M; 1.7M; 2M; 2.2M; 2.5M	30 e 33
*Polyethylene glycol 400 (*PEG-400) (Sigma, #202398)	1%, 3% e 5%	33
Polysorbate 20 (Tween 20) (Sigma, #P1379)	0.1%; 0.5% e 1%	32 e 33
Trehalose (Sigma, #PHR1344)	0.3M e 0.6M	33
Triton X-100 (Sigma, #T8787)	1% e 1.5%	33

PCR conditions

Experiments were conducted with a StepOne™ Real-Time PCR Systems Machine (*Applied Biosystems Real-Time PCR Instruments, Thermo Fisher Scientific, USA*). Reactions were performed in 48-well plates with each well containing: 3.4 µl of ultrapure H_2O, 0.3 µl of each primer (at 10 µM), 1 µl of DNA or cDNA template and 5 µl of *qPCR Master Mix* (2×). The non-commercial mixture was compared to the commercial product *Power SYBR™ Green qPCR 2× Master Mix* (*Applied Biosystems Real-Time PCR Instruments, Thermo Fisher Scientific, USA*) as a positive control.

Cycling conditions were as follows: 95°C for 5 minutes, 40 cycles of denaturation at 94°C for 15 seconds, annealing temperature according to each primer (Table 1) for 15 seconds and extension to 72°C for 30 seconds. Reference curves from 300 ng to 0.003 ng at 10-time dilutions were performed (28).

Analysis of efficiency and specificity

The amplification and efficiency were evaluated using cycle threshold (C_T) values: the lower the C_T value, the more efficient the reaction. Therefore, a threshold value was set at 0.45 and the C_T values were compared. For the calculation of the efficiency, the C_T value of the commercial product was set at 100% and the ratio inversely calculated for the C_T values of the mixtures.

Moreover, the melting curve of the dissociation of the amplified PCR products after the qPCR cycles was observed. A single fluorescence peak shows specificity, whereas more than one peak reveals non-specificity (1).

Stability and durability tests

The stability and durability of the non-commercial *qPCR Master Mix* was evaluated by freezing and thawing cycles. Considering regular usage of the commercial product that comes with 5 ml, 20 cycles of -20°C freezing and thawing is commonly used. Thus, the mixture was prepared, and an aliquot was frozen (t = 0 cycles). The remainder of the mixture was completely frozen (-20°C, 20 minutes). Then, the flask was kept at room temperature protected from light until completely thawed (20 minutes). This process was repeated 20 times and then compared by PCR in the same assay (t = 5, 10, 15 and 20 cycles).

Stability was also evaluated at room temperature. The mixture was prepared, and an aliquot was frozen (t = 0 h). The remainder of the mixture was kept overnight at room temperature. After 16 hours, both were subjected to PCR (t = 16h) in the same assay. The commercial reagent underwent the same tests.

The PCR master mix with the best stability was called the non-commercial *qPCR Master Mix* (CORE reagents + additives).

Quantify of a microorganism from mixed species infection.

The ability of the non-commercial *qPCR Master Mix* to amplify an authentic target DNA from a mixed species infection was evaluated. A sample of a mixed infection from a periodontitis pocket was obtained from a non-traceable personal identity (from Microbiology stock lab), so there was no need for Ethics Committee approval. The sample had all DNA bacteria purified (28). The qPCR conditions were the same as previously described but with 1 µl of DNA of mixed DNA and 1 µl of DNA from *Porphyromonas gingivalis* ATCC® BAA-308™ at concentrations from 300 ng to 0.03 ng.

RESULTS

The non-commercial *qPCR Master Mix* is more efficient than the commercial product

The concentration and composition of the non-commercial *qPCR Master Mix* are described in table 4.

Table 4. Composition and concentration of the non-commercial *qPCR Master Mix*.

Reagents	Stock solution	Preparing 5 ml	Working concentration (1 ×)	Final concentration (2 ×)
H_2O	-	800 µl	-------	-------
PCR Buffer	10 ×	1000 µl	1 ×	2 ×
$MgCl_2$	50 mM	700 µl	3.5 mM	7mM
Nucleotides dNTP	10 mM	200 µl	0.2 mM/dNTP	0.4 mM/dNTP
SYBR Gold nucleic acid gel stain	10 ×	700 µl	0.7 ×	1.4 ×
Reference Dye qPCR	100 ×	100 µl	1 ×	2 ×
Betaine	10 M	1100 µl	2.2 M	4.4 M
PEG 400	50 %	200 µl	1 %	2 %
Formamide	100 %	100 µl	1 %	2 %
Taq DNA Polymerase	5 U/µl	100 µl	0.1 U/µl	0.2 U/µl

In Figure 1, the amplification curves of genomic DNA of *P. gingivalis* are compared at concentrations from 300 to 0.03 ng. qPCR is quantified based on a standard curve consisting of a serial dilution of known concentrations of the molecular target. The amplification curve follows a pattern of increase of the generated product, resulting in three phases: exponential, linear and plateau. The non-commercial *qPCR Master Mix* amplification curve showed a behavior comparable to the commercial product.

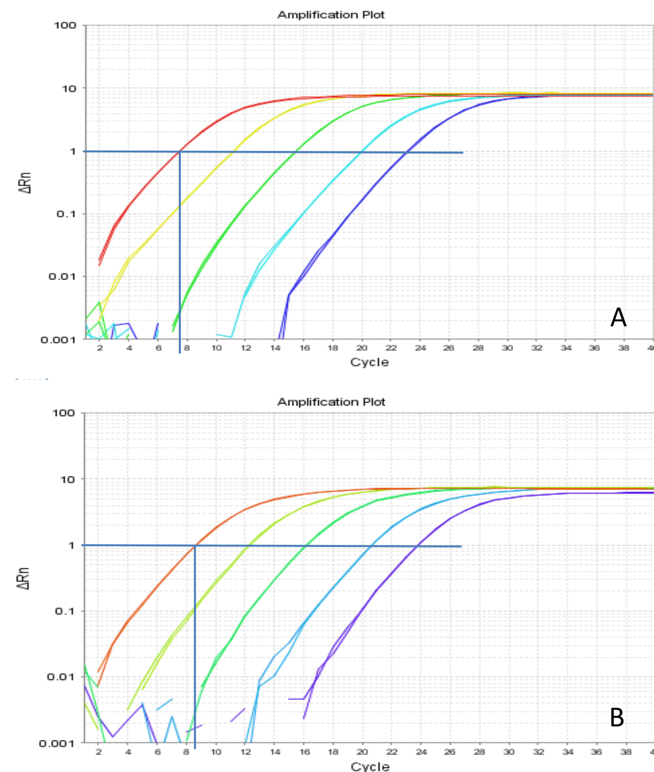

Figure 1. Amplification curves of genomic DNA of *P. gingivalis* at concentrations of 300 to 0.03 ng, using (A) non-commercial *qPCR Master Mix* and (B) the commercial product and the (B).

Table 5 shows the C_T values of the commercial product and the non-commercial *qPCR Master Mix*. The non-commercial *qPCR Master Mix* presented lower C_T values than the commercial product and consequently higher efficiency. For prokaryotic DNA this increase is observed in all concentrations tested: 300 ng to 0.03 ng (data not shown for concentrations 30 and 0.03). The results for eukaryotic DNA varied more widely than for the prokaryotic DNA. For the species *Mus musculus* the efficiency is higher for the concentrations 2.5 ng (data not shown) and 0.25 ng. For *Candida albicans* the amplification is detectable at 30 ng for the non-commercial *qPCR Master Mix* and 3 ng for the commercial product. Neither *Master Mix* could be detected at the 300 ng concentration. The efficiency is similar for commercial product and the non-commercial *qPCR Master Mix* for concentrations below 30 ng. Thus, the increase in efficiency ranged from 5% to 28% according to the origin and concentration of the nucleic acid.

Table 5. C_T value, standard deviation, and efficiency of the commercial product and the non-commercial *qPCR Master Mix*. The efficiency of the commercial product was set at 100%.

Organism	Gene	Concentration	Commercial Master Mix	Non-commercial *qPCR Master Mix*
Porphyromonas gingivalis ATCC® BAA-308™/W83	16S	300 ng	6.7 ±0.07 - 100%	5.99 ±0.08 112%
		3 ng	12.88 ±0.01 100%	12.55 ±0.04 102%
Streptococcus sanguinis ATCC® BAA-1455™/SK36	SSA_1234	300 ng	10.94 ±0.00 100%	10.23 ±0.01 107%
		3 ng	19.03 ±0.01 100%	18.0 ±0.00 105%
Streptococcus sanguinis ATCC® BAA-1455™/SK36	SSA_0250	300 ng	10.90 ±0.05 100%	9.96 ±0.06 109%
		3 ng	18.99 ±0.05 100%	16.01 ±0.05 118%
Streptococcus sanguinis ATCC® BAA-1455™/SK36	SSA_0263	300 ng	10.97 ± 0.04 100%	10.00 ± 0.00 110%
		3 ng	18.06 ± 0.04 100%	16.77 ± 0.05 108%
Mus musculus - lineage MDPC-23 (ATCC® CRL-2537™)	Gapdh	25 ng	14.98 ± 0.04 100%	16.00 ± 0.04 93%
		0,25 ng	32.03 ± 0.04 100%	24.00 ± 0.04 128%
Candida albicans ATCC® 5314™	ATC	30 ng	------------	19.45 ± 0.02 100%
		3 ng	22.20 ± 0.05 100%	22.21 ± 0.04 100%
Homo sapiens – HEK 293T (ATCC® CRL-3216™)	Dnmt 3B	2,5 ng	20.63 ± 0.01 100%	20.41 ± 0.02 101%
		0,25 ng	24.75 ± 0.07 100%	24.95 ± 0.07 99%

***SYBR gold* as intercalating dye for qPCR**

In this study, the fluorophores *SYBR Green*, *SYBR Gold* and *SYTO*-82 (data not shown) were tested. Figure 2 illustrates the melting curve of the commercial product, the *Master Mix* CORE reagents with *SYBR GREEN* and the *Master Mix* CORE reagents with *SYBR GOLD*. After amplification of *S. sanguinis* DNA at 300 ng, *SYBR GOLD* presented less formation of secondary structures. Thus, it has a higher specificity to the DNA target than SYBR Green. Therefore, for the *Master Mix* CORE reagents and the non-commercial *qPCR Master Mix* we chose *SYBR GOLD* as the fluorescent dye.

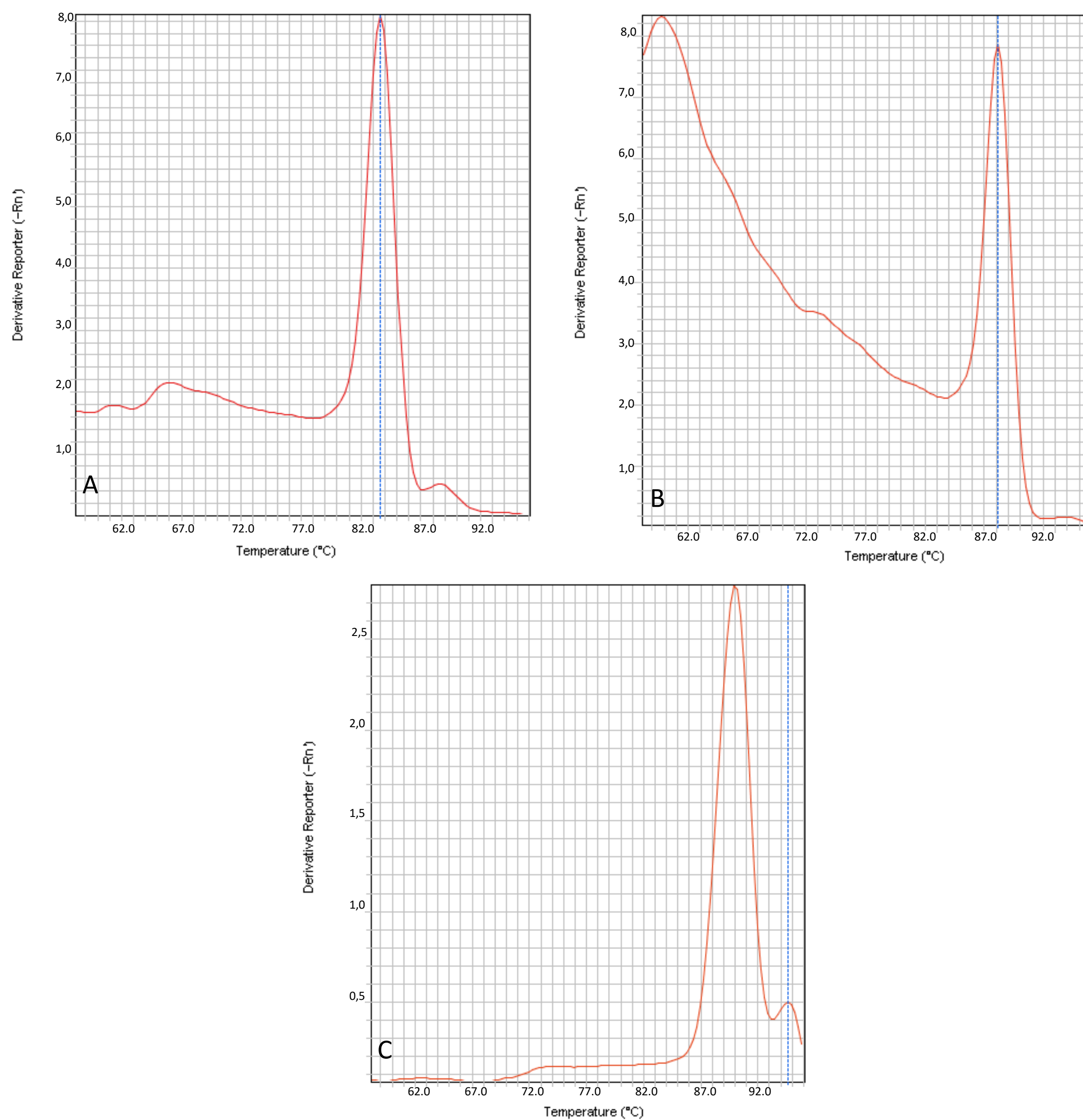

Figure 2. Melting curve for (A) *Power SYBR Green qPCR 2 × Master Mix*, (B) *Master Mix* CORE reagents with *SYBR Green* and (C) *Master Mix* CORE reagents with *SYBR Gold*. The curves were obtained after the amplification of *S sanguinis* DNA at 300 ng. Additional peaks indicate formation of non-specific product.

The addition of betaine, formamide and PEG400 to the *Master Mix* CORE reagents increased the stability, efficiency and specificity of the reaction compared to the commercial product.

The additives were first added alone to *Master Mix* CORE reagents. Those that achieved the best results were added in combination. The mixtures that obtained the best efficiency were subjected to stability tests. Table 6 shows the

C_T values for commercial product and the non-commercial *qPCR Master Mix* before (freshly prepared) and after 15 freezing and thawing cycles and being left overnight at room temperature as well the respective efficiencies.

Table 6. CT values for the commercial master mix and the non-commercial *qPCR Master Mix.* The values were obtained after amplification of *S. sanguinis* DNA at concentrations of 300 ng and 3 ng. The PCR was performed with the freshly prepared non-commercial *qPCR Master Mix*, after 15 freezing and thawing cycles (FTC) and after being left overnight at room temperature (ON).

Buffer systems		Commercial Master Mix	Non-commercial *qPCR Master Mix*
300 ng	Fresh Prepared	10.97 ± 0.04	10.00 ± 0.00
	Efficiency	100%	110%
	15 FTC	11.29 ± 0.04	10.10 ± 0.02
	Efficiency	97%	109%
	0vernight	17.00 ± 0.06	10.07 ± 0.03
	Efficiency	65%	109%
3 ng	Fresh Prepared	18.06 ± 0.04	16.77 ± 0.05
	Efficiency	100%	108%
	15 FTC	18.26 ± 0.01	16.84 ± 0.03
	Efficiency	99%	107%
	0vernight	23.31 ± 0.12	16.98 ± 0.01
	Efficiency	77%	106%

After freezing and thawing cycles and being left overnight at room temperature, the *Master Mix* CORE reagents with betaine, formamide and PEG 400 was more stable, with the lowest drop in efficiency for either DNA concentration. Thus, the mixture containing the CORE reagents and the

combination of these three additives was called the non-commercial *qPCR Master Mix.*

In addition, the presence of these 3 combined additives increases the specificity at the lower concentrations of DNA. Figure 3 shows the reduction of additional peaks on the melting curve for the non-commercial *qPCR Master Mix* compared to the commercial product and the *Master Mix* CORE reagents.

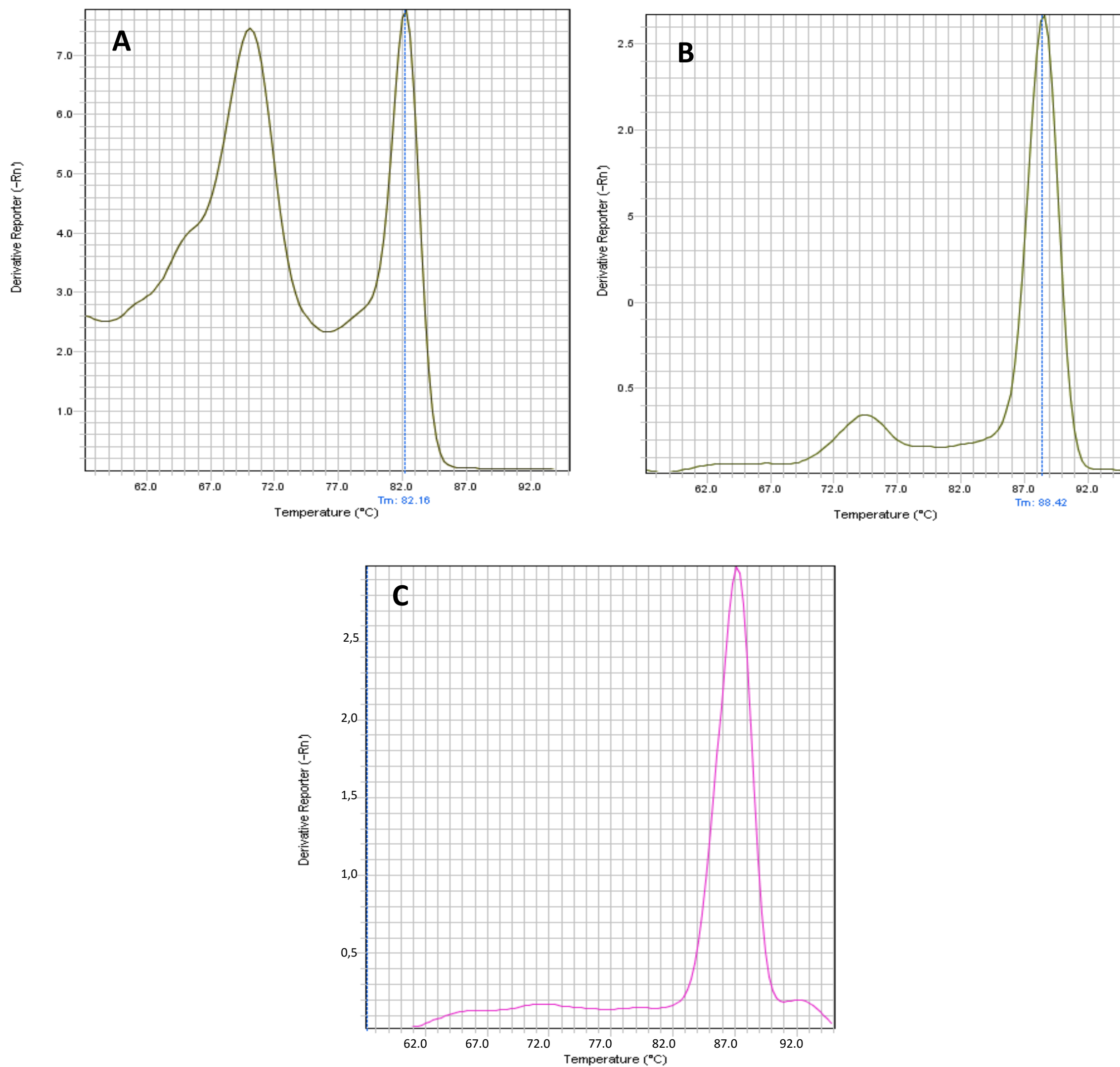

Figure 3. Melting curve for (A) the Power SYBR Green qPCR 2 × Master Mix, (B) the Master Mix CORE reagents and (C) the non-commercial *qPCR Master Mix*. The curves were obtained after amplification of *S sanguinis* DNA at 3 ng. Additional peaks indicate formation of non-specific product.

The non-commercial *qPCR Master Mix* is efficient and more sensitive to eukaryotic DNA than the commercial product

The *Master Mix* CORE reagents and the non-commercial *qPCR Master Mix* performed better in the amplification of the eukaryotic DNA than the commercial product. The amplification curves of *Mus Musculus* with the *Master Mix* CORE reagents and the non-commercial *qPCR Master Mix* were similar (Figure 4). However, when analyzing the melting curve, it was observed that the additives decreased the formation of secondary structure, which resulted in the non-commercial *qPCR Master Mix* being more specific than commercial one. In addition, for *C. albicans* the non-commercial *qPCR Master Mix* was able to amplify higher concentrations than the commercial product (Table 5).

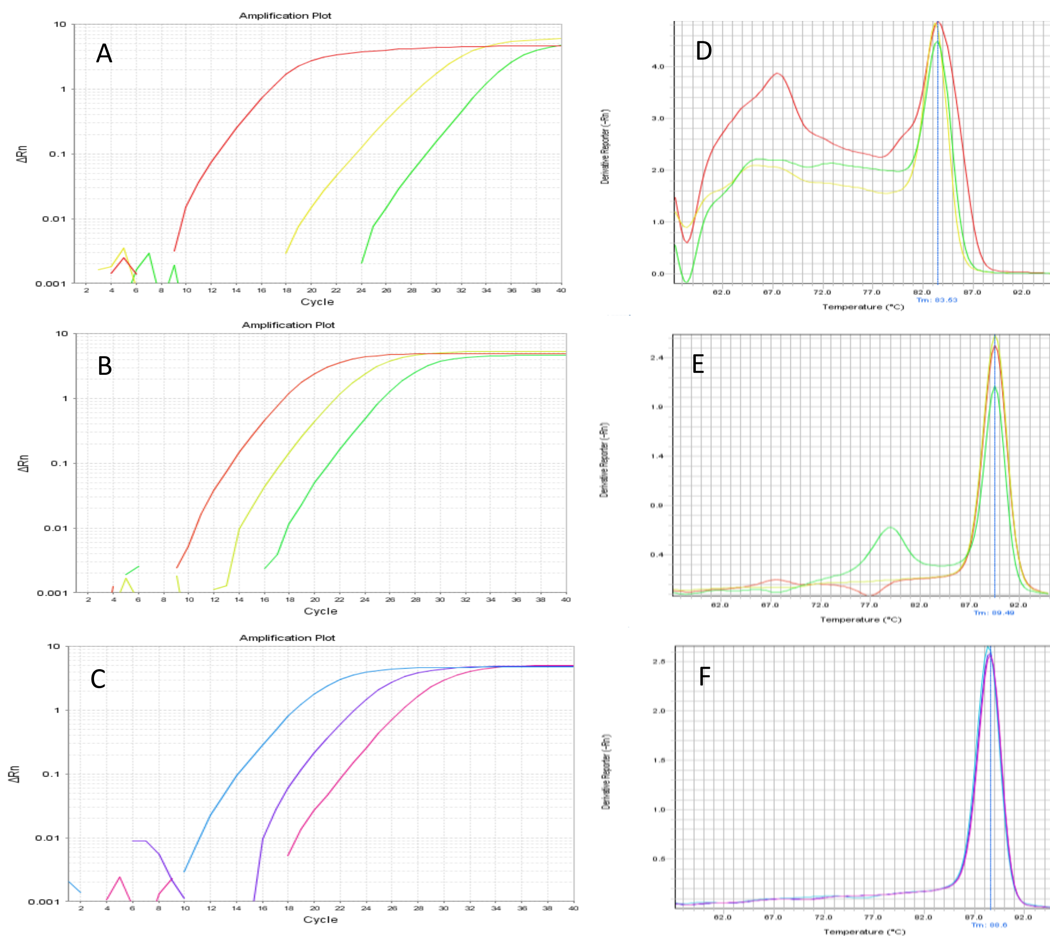

Figure 4. Amplification curves and melting curves of genomic DNA of eukaryotic DNA, using (A) (D) the commercial product, (B) (E) the *Master Mix* CORE reagents and (C) (D) the non-commercial *qPCR Master Mix*.

Non-commercial *qPCR Master Mix* is more sensitive and efficient in amplification of DNA target in a mixed DNA sample

The amplification curves show that the non-commercial *qPCR Master Mix* can amplify the target DNA in a mixed DNA sample more satisfactorily than the commercial product. The non-commercial *qPCR Master Mix* was able to amplify all the target DNA added to the mixed sample resulting in a curve similar to the isolated target DNA (Figure 5).

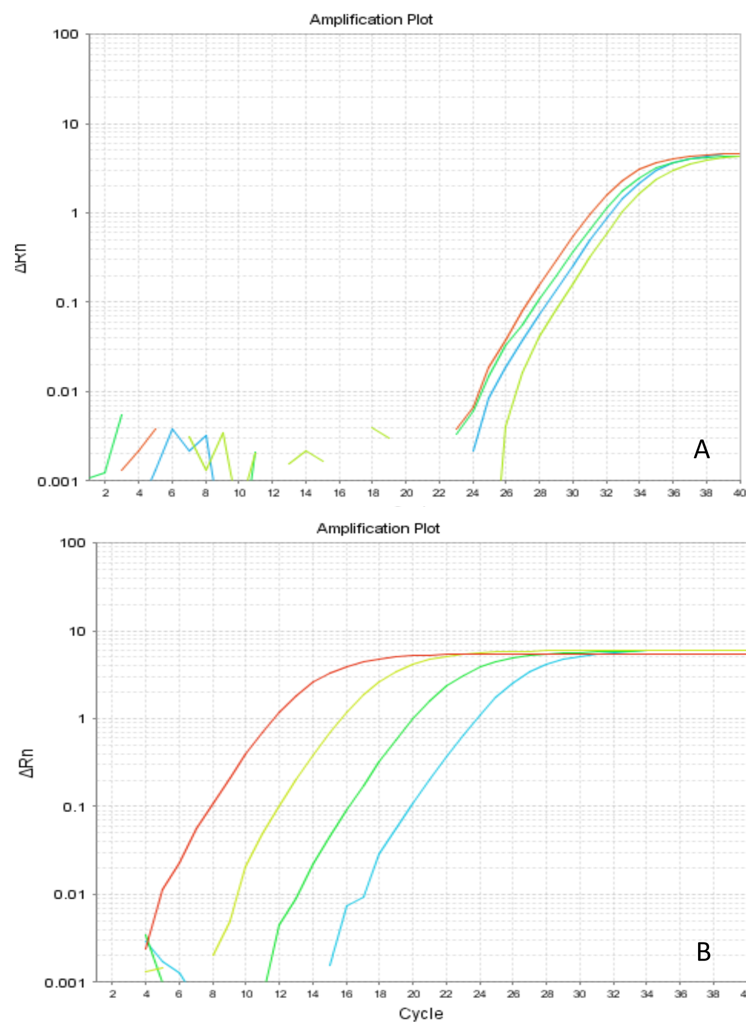

Figure 5. Amplification curves of genomic DNA of *P. gingivalis* in mixed DNA samples, using (A) the commercial product and (B) the non-commercial *qPCR Master Mix*.

DISCUSSION

qPCR is an essential technique in gene expression and other biotechnological studies (34). The commercial ready-to-use solutions have led to good results; however, they have the disadvantage of an unknown composition, lack of flexibility to adjust the concentration of individual components and high prices (35). In this work, a mixture was developed for qPCR with high efficiency, low cost and detailed composition.

The composition and concentration of reagents are determinant for the success of a qPCR. In-depth, $MgCl_2$ functions as an essential cofactor for the reaction. In general, the ion Mg^{+2} binds to dNTPS, to DNA polymerase and contributes to the primer binding to the DNA target (3). Thus, low concentrations of this ion result in low yield, whereas high concentrations result in the production of non-specific products. The concentration adopted for the homemade mixture was 2 mM (29). The salt concentration can range from 1.5 to 4.5 mM. The concentration of the $MgCl_2$ chosen for the non-commercial *qPCR Master Mix* was 3.5 mM, being close to those described.

The dye concentration also directly influences qPCR. *SYBR Green* appears to be inhibitory to PCR in a concentration-dependent manner. Concentrations higher than 2 × increase the melting temperature (Tm) and modify the peaks of the melting curve (10). The binding force between the dye and the DNA influences the Tm and the PCR inhibition (12). In general, dyes with higher affinity inhibit PCR to a higher degree than low-affinity dyes. However, the adopted concentration of 0.7 × did not cause inhibition of the PCR, even with the high affinity presented by *SYBR Gold* for double-stranded DNA. This higher affinity is probably associated with the higher Tm value exhibited by *SYBR Gold* compared to *SYBR Green*. The dyes present a certain stabilizing effect: the higher the affinity, the tighter the binding of the dye to the DNA; consequently, the temperature required to dissociate the double strand is higher (12). However, the higher Tm did not influence the efficiency of the PCR.

SYBR Gold is the most sensitive fluorescent stain available for detecting double or single-stranded DNA or RNA in electrophoretic gels, being more

sensitive than *SYBR Green* or ethidium bromide (13). A melting curve analysis showed that *SYBR Gold* presented a lower formation of secondary structures and was more sensitive to the target DNA. This greater sensitivity can also be seen in the detection of target DNA in a mixed DNA sample. By amplifying all the target DNA present in the mixed sample, it has been shown to be more applicable for this type of analysis.

The efficiency of qPCR depends on the structure and sequence of the target DNA. High GC content can form secondary structures (34, 36, 37). An alternative is the addition of additives (25) that may act in isolation (21) or synergistically (34, 35), increasing the specificity of the reaction. For example, formamide caused destabilization of GC rich regions, facilitating the annealing process and the extension of the reaction (26). Betaine is a natural amino acid that can balance the difference in Tm between regions rich in AT and GC and improve *de novo* synthesis of two GC-rich fragments without modifying the nucleotide composition (27). PEG400 is associated with the reversal of the PCR inhibitors (38).

The combination of these three additives proved to be effective in the reduction of secondary structures. It is a low-cost alternative and easily accessible for the improvement of the specificity of the reaction. But the choice of the additives was also made based on the stability they provided to the *Master Mix*. The freezing-thawing tests simulated routine laboratory use. The non-commercial *qPCR Master Mix* showed a lower drop in efficiency after the freezing-thawing cycles. This stability ensures more reliable results, since the efficiency will remain constant until the consumption of the whole volume of master mix. Furthermore, the enzyme *Taq polymerase* was able to remain more stable in the presence of these three additives after being left overnight at room temperature.

The optimization of amplification protocols for eukaryotic DNA can be a laborious process because of the GC-rich regions (34). The non-commercial *qPCR Master Mix* was effective in amplifying both eukaryotic and prokaryotic DNA. C_T values and the melting curve showed that the non-commercial *qPCR*

Master Mix has a higher specificity, predominantly higher efficiency and better stability than the *Power SYBR Green qPCR 2 × Master Mix*. The differences in efficiency found are closely related to the concentration, origin and quality of the nucleic acid, and the primer design.

This is the first report on the use of SYBR Gold as a fluorescent dye in qPCR analysis. The non-commercial *qPCR Mastermix* described was effective in amplifying eukaryotic and prokaryotic DNA. The master mix supports routine laboratory use and costs up to 80% less than the commercial product. The composition of the master mix is described in detail, can be recreated by any laboratory and allows for individual changes. The reaction mixture has been patented with the National Institute of Industrial Property, process number BR 10 2017 022164 4.

Acknowledgments

The authors thank Prof. Dr. Marcelo Rocha Marques, Dr. Gustavo Narvaes Guimarães and Prof. Dr. Natalia Leal Vizoto for contributing with purified DNA and primers to this work.

This work was supported by the São Paulo Research Foundation (FAPESP), Grant 2017/03263-3 and the Brazilian National Council for Scientific and Technological Development (CNPq) for the PhD Scholarship 154620/2014-9 to T.S.G.

REFERENCES

1. Bustin SA. A-Z of Quantitative PCR. International University Line, La Jolla. 2004
2. VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analyses. Biotechniques, 2008. 44(5): 619-26
3. Pelt-Verkuil E, Van Belkum A, Hays JP. Principles and technical aspects of PCR amplification. 2008
4. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem, 1997. 245: 154–60.
5. Nath K, Sarosy JW, HahnJ. Di Como CJ. Effects of ethidium bromide and SYBR (R) Green I on different polymerase chain reaction systems. J. Biochem. Biophys. Methods, 2000. 42, 15–29.
6. Giglio S, Monis PT., Saint CP. Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. Nucleic Acids Res, 2003. 32: 106
7. Bengtsson M, Karlsson HJ, Westman G, Kubista M. A new minor groove binding asymmetric cyanine reporter dye for real-time PCR. Nucleic Acids Res, 2003. 31: 45
8. Ishiguro T, Saitoh J, Yawata H, Yamagishi H, Iwasaki S, Mitoma Y. Homogeneous quantitative assay of hepatitis-C virus-RNA by polymerase chain-reaction in the presence of a fluorescent intercalater. Anal. Biochem, 1995. 229: 207–13.
9. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin. Chem, 2003. 49; 853–60.
10. Monis PT, Giglio S, Saint CP. Comparison of SYT09 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal. Biochem, 2005. 340: 24–34.

11. Giglio S, Monis PT, Saint CP. *Legionella* confirmation using real-time PCR and SYTO9 is an alternative to current methodology. Appl. Environ. Microbiol, 2005. 71: 8944–48.
12. Gudnason H, Dufva M, Bang DD, Wolff A. Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. Nucleic Acids Res, 2007. 35 (19): 127
13. Thermo Fisher Website – Document https://goo.gl/KXxJFf – Immortalized in GDrive – Accessed in 07/29/2018.
14. Shaik GM, Draberova L, Draber P, Boubelik M, Draber P. Tetraalkylammonium derivatives as real-time PCR enhancers and stabilizers of the qPCR mixtures containing SYBR Green I. Nucleic Acids Res, 2008. 36 (15): 93
15. Sidhu MK, Liao MJ, Rashidbaigi A. Dimethyl sulfoxide improves RNA amplification. BioTechniques, 1996. 21: 44–7.
16. Jung M, Muche JM, Lukowsky A, Jung K, Loening SA. Dimethyl sulfoxide as additive in ready-to-use reaction mixtures for real-time polymerase chain reaction analysis with SYBR Green I dye. Anal. Biochem, 2001. 289: 292–95
17. Weissensteiner T and Lanchbury JS. Strategy for controlling preferential amplification and avoiding false negatives in PCR typing. BioTechniques, 1996. 21: 1102–08.
18. Hengen P N. Optimizing multiplex and LA-PCR with betaine. Trends Biochem. Sci, 1997. 22: 225–26.
19. Sarkar G, Kapelner S, Sommer SS. Formamide can dramatically improve the specificity of PCR. Nucleic Acids Res, 1990. 18: 7465.
20. Comey CT, Jung JM, Budowle B. Use of formamide to improve amplification of HLA DQ alpha sequences. BioTechniques, 1991. 10: 60–1.
21. Chakrabarti R and Schutt CE. The enhancement of PCR amplification by low molecular weight amides. Nucleic Acids Res, 2001. 29: 2377–81.

22. Varadaraj K and Skinner DM. Denaturants or cosolvents improve the specificity of PCR amplification of a G + C-rich DNA using genetically engineered DNA polymerases. Gene, 1994. 140: 1–5
23. Carninci P, Nishiyama Y, Westover A, Itoh M, Nagaoka S, Sasaki N, Okazaki Y, Muramatsu M, Hayashizaki Y. Thermostabilization and thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA. Proc. Natl Acad. Sci, 1998, 95: 520–24.
24. Spiess AN, Muellr N, Ivell R. Trehalose is a potent PCR enhancer: lowering of DNA melting temperature and thermal stabilization of Taq polymerase by the disaccharide trehalose. Clin Chem, 2004. 50 (7): 1256-9.
25. Simonović A, Trifunovic m, Raspor M, Cingel A, Bogdanovic M, Dragicevic M and Subotic A. Dimethyl sulfoxide improves sensitivity and specificity of RT-PCR and QRT-PCR amplification of lowexpressed transgenes. Arch Biol Sci, 2012. 64: 865-876
26. Chakrabarti R and Schutt C. 2010. Chemical PCR: Compositions for enhancing polynucleotide amplification reactions. U.S. Patent No. 7,772, 2010. 383.
27. Jensen M.A., Fukushima M., Davis R.W. DMSO and Betaine Greatly Improve Amplification of GC-Rich Constructs in De Novo Synthesis. PLoS ONE, 2010. 5(6): 11024
28. Stipp RN, Boisvert H, Smith DJ, Höfling JF, Duncan MJ, Mattos-Graner RO. CovR and VicRK regulate cell surface biogenesis genes required for biofilm formation in *Streptococcus mutans*. PLoS One 2013. 8(3): 58271
29. Karsai A., Muller S., Platz S., Hauser M.T. Evaluation of a homemade SYBR Green I reaction mixture for real-time PCR quantification of gene expression. Biotechniques, 2002. 4: 790-6.
30. Frackman S., Kobs G., Simpson D., Storts D. Betaine and DMSO enhancing agents for PCR. Promega notes, 1998. 65: 27.

31. Jung J.M., Muche A., Lukowsky K., Jung S.A. Dimethyl sulfoxide as additive in ready-to-use reaction mixtures for real-time polymerase chain reaction analysis with SYBR Green I dye. Anal. Biochem., 2001. 289: 292-295.
32. McPherson M., Moller S. PCR: The Basics. 2nd Edition, Garland Science, UK, 2007.
33. Pellissier F., Glogowski C.M., Heinemann S.F., Ballivet M., Ossipow V. Lab assembly of a low-cost, robust SYBR green buffer system for quantitative real-time polymerase chain reaction. Anal Biochem, 2006. 350 (2):310-2.
34. FarelL EM and Alexandre G. Bovine serum albumin further enhances the effects of organic solvents on increased yield of polymerase chain reaction of GC-rich templates. BMC Res Notes, 2012. 5: 1.
35. Ralser M, Querfurth R, Warnatz HJ, Lehrach H, Yaspo ML and Krobitsch S. An efficient and economic enhancer mix for PCR. Biochem Biophys Res Commun, 2006. 347: 747-751.
36. Zeng ZS, Yan HT, Zheng XD, Hu GZ, Chen Y and Ding M. High GC amplification: a comparative study of betaine, DMSO, formamide and glycerol as additives. Life Sci, 2006. 3: 67-71.
37. Mamedov TG, Pienaar E, Whitney SE, Termaat JR, Carvill G, Goliath R, Subramanian A and Viljoen HJ. A fundamental study of the PCR amplification of GC-rich DNA templates. Comput Biol Chem, 2008. 32: 452-457.
38. Demeke T, Adams RP. The effects of plant polysaccharides and buffer additives on PCR. Biotechniques, 1992. 12(3):332-4.

3. CONCLUSÃO

- A mistura reacional desenvolvida para qPCR, apresenta ótima eficiência, alta especificidade e menor custo que o produto comercial.

- *SYBR Gold* é um corante fluorescente aplicável em análises de qPCR.

- A Betaína, Formamida e PEG400 são aditivos eficientes que melhoram a reação de qPCR.

REFERÊNCIAS

1. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T.The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin.Chem, 2009. 55:611–622.

2. Chakrabarti R and Schutt C. 2010. Chemical PCR: Compositions for enhancing polynucleotide amplification reactions. U.S. Patent No. 7,772, 2010. 383.

3. Chen BT, Janes Hw. PCR cloning protocols. Second Edition. Human Press, 2002. 192: 439.

4. FarelL EM and Alexandre G. Bovine serum albumin further enhances the effects of organic solvents on increased yield of polymerase chain reaction of GC-rich templates. BMC Res Notes, 2012. 5: 1

5. Gudnason H, Dufva M, Bang DD, Wolff A. Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. Nucleic Acids research, 2007. 35(19):e117

6. Jensen M.A., Fukushima M., Davis R.W. DMSO and Betaine Greatly Improve Amplification of GC-Rich Constructs in De Novo Synthesis. PLoS ONE, 2010. 5(6): e11024

7. Jung J.M., Muche A., Lukowsky K., Jung S.A. Dimethyl sulfoxide as additive in ready-to-use reaction mixtures for real-time polymerase chain reaction analysis with SYBR Green I dye. Anal. Biochem., 2001. 289: 292-295.

8. Karsai A., Muller S., Platz S., Hauser M.T. Evaluation of a homemade SYBR Green I reaction mixture for real-time PCR quantification of gene expression. Biotechniques, 2002. 4: 790-6.

9. Ma H, Shieh KJ, Chen G, Qiao T, Chuang MY. Application of Real-time Polymerase Chain Reaction (RT-PCR). Journal of America Science, 2006. 2: 1-15.

10. Mackay IM, Mackay JF, Nissen MD, Sloots TP. Real-time PCR: History and Fluoregenic Chemistries. Caister Academic Press Norfolk, 2007. 1-40.

11. Monis P.T., Giglio S., Saint C.P. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the eVect of dye concentration on ampliWcation and DNA melting curve analysis. Analytical Biochemistry, 2005. 340, 24-34

12. Navarro E, Serrano-Heras G, Castano MJ, Solera J. Real-time PCR detection chemistry. Clinica Chimica Acta, 2015. 439: 231-50.
13. Oliveira TMS. PCR em tempo real: métodos e aplicações. Universidade de Aveiro. Departamento de Biologia. 2010.

14. Quan PL, Sauzade M, Brouzes E. dCR: A Technology Review. Sensors, 2018. 18 (4): 1271

15. Pellissier F., Glogowski C.M., Heinemann S.F., Ballivet M., Ossipow V. Lab assembly of a low-cost, robust SYBR green buffer system for quantitative real-time polymerase chain reaction. Anal Biochem, 2006. 350 (2):310-2.

16. Pelt-Verkuil, E., Van Belkum, A., Hays, J.P. Principles and technical aspects of PCR amplification. 2008

17. Ralser M, Querfurth R, Warnatz HJ, Lehrach H, Yaspo ML and Krobitsch S. An efficient and economic enhancer mix for PCR. Biochem Biophys Res Commun, 2006. 347: 747-751.

18. Simonović A, Trifunovic m, Raspor M, Cingel A, Bogdanovic M, Dragicevic M and Subotic A. Dimethyl sulfoxide improves sensitivity and specificity of RT-PCR and QRT-PCR amplification of lowexpressed transgenes. Arch Biol Sci, 2012. 64: 865-876

19. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. How good is a PCR efficiency estimate: recommendations for precise and robust qPCR efficiency assessments. Biomolecular Detection and Quantification, 2015. 3: 9-16.

20. Thornton B & Basu C. Rapid and simple method of qPCR primer design. Methods Mol Biol, 2015. 1275: 173-9.

21. Tuma RS, Beaudet MP, Jin X, Jones LJ, Cheung CY, Yue S, Singer V. Characterization of SYBR Gold Nucleic Acid Gel Stain: A Dye Optimized for Use with 300nm. Analytical Biochemistry, 1999. 268: 278-88.

22. VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analyses. Biotechniques, 2008. 44(5):619-26

23. Yazd EF, Sadeghizadeh M, Hosseinkhani S, Khalaj-kondori M, Emamzadeh R. Molecular cloning, expression and sequence analysis of DNA polymerase I from an Iranian thermophilic bacterium, Bacillus sp. Journal of the Iranian Chemical Society, 2006. 6: 831-37.

24. Zeng ZS, Yan HT, Zheng XD, Hu GZ, Chen Y and Ding M. High GC amplification: a comparative study of betaine, DMSO, formamide and glycerol as additives. Life Sci, 2006. 3: 67-71.