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Short Communication

How to speed up the polymerase chain reaction

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ABSTRACT

Reducing the time taken to run qPCR assays on today's qPCR cyclers is rather straightforward and requires no specialised reagents or instruments. As the first article in a new series of short technical reports, I demonstrate that it is possible to reduce significantly both denaturation temperatures and cycling times, whilst retaining sensitivity and specificity of the original qPCR conditions.

1. Introduction

Most users of the polymerase chain reaction (PCR) would describe it as a fairly fast technique, taking about 45 min to an hour to complete 40 cycles, depending on the particular protocol and instrument used. The first description of a PCR reaction carried out using thermostable DNA polymerase amplified targets ranging from 110 to 408 base pairs (bp) with one-minute denaturation, two-minute annealing and 30 s polymerisation steps [1]. Since then, there have been numerous and significant improvements to both instruments and reagents. These include the introduction of real-time (qPCR) and digital PCR (dPCR) technologies, new fluorophores, specialist master mixes and fast thermal cyclers, leading to an enormous expansion of practical uses, not least high throughput applications.

Despite these improvements, what has not changed a great deal are the protocols used to run mainstream qPCR reactions. A quick perusal of the peer-reviewed literature reveals that current standard methods, if they are published at all, include near universal denaturation temperatures and times of 95 °C and 15–30 s, respectively, annealing/polymerisation times of 15 s–1 min and optional separate polymerisation times of 45 s–2 min. These add up to around an hour for a typical 40-cycle reaction. In addition to these times, the speed of a PCR run is also dependent on ramp rates, i.e. how fast the PCR instrument can change from one temperature to another and especially how fast the cooling process works. Hence any attempt to reduce significantly PCR run times must look at reducing both cycle times and minimising the differences in temperatures for the various steps.

There have been several reports of PCR methods that significantly increase PCR reactions times significantly over those in mainstream use. However, whilst these can reduce PCR cycle times to a few minutes or even seconds, they use specialised equipment and procedures and generally compromise PCR efficiency [2–6]. Interestingly, a recent a series of publications by Carl Wittwer's group has investigated the kinetics of PCR and its conclusions suggest that it is possible to use significantly modified protocols to achieve faster PCR results without compromising the sensitivity or specificity of the PCR assay. Although the extension rates of native Taq polymerases ranged from 10 to 45 nucleotides/second, some polymerases achieved up to 155 nucleotides/second [7]. Maximum extension rates were achieved by using optimised extension temperatures (T_m −5 °C) on somewhat G/C-rich templates (around 60%) without secondary structures [8] in the presence of minimal monovalent cations [9]. More recently, super-fast PCR reaction times were achieved by increasing primer and polymerase concentrations to around 20-fold above typical concentrations, increasing annealing/extension temperatures to around 75 °C and reducing denaturation temperatures to below 90 °C, so allowing amplification of short PCR amplicons less than 15 s [10].

Clearly, there is potential to reduce the time taken to complete standard qPCR reaction times using regular reagents and instruments. Therefore, if the speed of a PCR reaction is an important consideration, it is worth modifying legacy PCR procedures to incorporate these findings into a mainstream fast PCR protocol. This technical note describes such a modification, which reduces PCR reaction times on standard PCR instruments without compromising either its sensitivity or specificity.

2. Materials and methods

All pipetting was carried out using 0.1–3 μL Biohit mLine (Sartorius) manual pipettes for volumes up to 3 μL, 0.5–10 μL pipettes for volumes

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Table 1
qPCR assay features. All six assays were designed using Beacon Designer (Premier Biosoft).

Targets		Primers	Size (bp)	Accession no	Start	End	CG	AT	%GC
UBC2	F:	AGGAAAAGTAGTCCCTTCTC	177	NM_021009	295	471	99	78	56%
	R:	CGAAGATCTGCATTGTCAAG							
IGF1	F:	CCTTTCAAGCCACCCATTGAC	100	NM_000376	1166	1265	62	38	62%
	R:	AGCAGCGGGTACAAGATAAATATCC							
TP53I3	F:	CTGCTGCCGGTTCTGGAC	96	NM_004881	1778	1873	51	45	53%
	R:	CAGGACGATCTTGCCTATGTT							
GAPDH	F:	GCACAAGAGGAAGAGAGAGACC	84	NM_000875	1089	1172	52	32	62%
	R:	AGGGGAGATTCAGTGTGGTG							
VDR1	F:	ATCTGCATCGTCTCCCCAGAT	104	NM_001111283	6731	6834	44	60	42%
	R:	AGCGGATGTACGTCTGCAGTG							
IGF1R	F:	CTCCTGTTTCTCTCCGCCG	78	NM_002046	1225	1302	47	31	60%
	R:	ATAGTCGTTGCGGATGTCGAT							

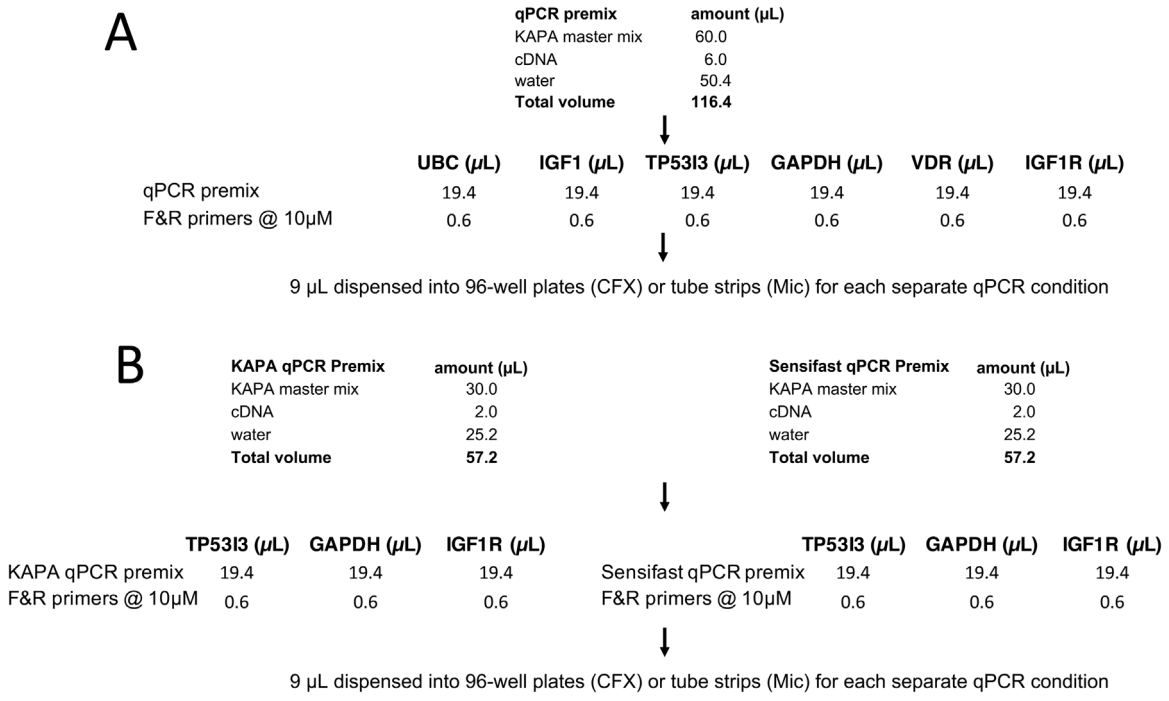

Fig. 1. qPCR workflow. All reaction components except for the primers were dispensed to individual microfuge tubes, to which target-specific forward and reverse primer mixes were added. Reaction mixtures were kept on ice until subjected to PCR. Sufficient qPCR premixes were prepared for each of the six targets to run six assays in duplicate. A. Workflow for initial investigation using KAPA master mix. B. Workflow for comparison between KAPA and SensiFast master mixes.

between 3 and 10 µL, 2–10 µL pipettes for volumes between 10 µL and 20 µL and 10–100 µL pipettes for volumes between 10 and 100 µL. qPCR reactions were carried out on the CFX Connect (Biorad) and Mic (Biomolecular Systems) qPCR cyclers.

2.1. cDNA synthesis

cDNA was prepared from 1 µg of RNA, which had been manually extracted from MCF 7 tissue culture cells using an RNeasy (Qiagen) RNA extraction kit with a DNase step according to the manufacturer's protocol. Total RNA quality was assessed using a Bioanalyser (Agilent) and purity was assessed using the SPUD assay [11]. Reverse transcription was carried out using random primers and Superscript IV (ThermoFisher) in three separate 20 µL reactions at 23 °C for 10 min and 55 °C for 10 min, followed by an incubation at 80 °C for 10 min. The three samples were pooled, diluted with 540 µL of water, aliquoted and stored at −80 °C.

2.2. qPCR

Six qPCR assays targeting Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), insulin-like growth factor 1 (IGF-1), insulin-like growth factor 1 receptor 1 (IGF-1R), tumour protein p53 inducible protein 3 (TP53IP3), Ubiquitin C (UBC) and Vitamin D receptor (VDR) were chosen at random from a selection of over 60 assays kept in the freezer and are described in Table 1. All primers were synthesised by Sigma-Aldrich and used at a final concentration of 300 nM. The effects of varying cycling times and temperatures were determined using KAPA (Sigma Aldrich), using the workflow shown in Fig. 1A. Three of the assays were chosen for comparison between SensiFAST (Bioline) and KAPA master mixes using the workflow shown in Fig. 1B.

2.3. Data analysis

Data were analysed using instrument default settings and quantification cycles (Cqs) were calculated automatically. On the Mic the data were analysed using the in-built dynamic algorithm option, whilst the CFX data were analysed using the regression method option, both without manual intervention.