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OPTIMIZATION OF PCR CONDITIONS BY RECOMBINANT DNA POLYMERASE I ORIGINATING FROM *THERMUS SCOTODUCTUS* K1 STRAIN

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The optimization of PCR reaction components and cycling conditions for novel recombinant TsK1 polymerase originating from *Thermus scotoductus* K1 strain has been performed. It was shown that Mg^{2+} in final concentration of 3 mM was suitable for efficient PCR using TsK1, and higher concentrations inhibited the reaction. Addition of glycerol as enhancer was essential for PCR. Enzyme exhibited amplification activity in both mono- and multiplex PCR, and might become a promising tool for different molecular biological applications as well as in diagnostics.

DNA-polymerase – polymerase chain reaction – Thermus

Իրականացվել է ռեկոմբինանտ TsK1 պոլիմերազի կիրառմամբ ՊՇՌ ամպլիֆիկացման պայմանների բարելավում: Ցույց է տրվել, որ արդյունավետ ամպլիֆիկացիայի իրականացման համար բավարար է Mg^{2+} իոնների 3 մՄ կոնցենտրացիան, ընդ որում, ավելի բարձր կոնցենտրացիաները ճնշում են ՊՇՌ-ն: Ռեակցիայի իրականացման համար պարտադիր է գլիցերոլի ավելացումը որպես կայունացուցիչ: Ֆերմենտը ցուցաբերում է արդյունավետ ամպլիֆիկացիայի հնարավորություն ինչպես միակի, այնպես էլ՝ մուլտիպլեքս ՊՇՌ ռեակցիաներում և կարող է խոստումնալից գործիք հանդիսանալ տարաբնույթ մոլեկուլային գենետիկական կիրառություններում ինչպես հետազոտական, այնպես էլ՝ ախտորոշման ոլորտներում:

ԴՆԹ-պոլիմերազ – պոլիմերազային շղթայական ռեակցիա – Thermus

Была проведена оптимизация компонентов ПЦР реакции и условий амплификации с применением новой рекомбинантной полимеразы TsK1, происходящей от штамма *Thermus scotoductus* K1. Эффективный ПЦР осуществляется при наличии Mg^{2+} в конечной концентрации 3 мМ, а высокие концентрации подавляют реакцию. Также добавление глицерола в качестве усилителя крайне необходимо для ПЦР. Фермент проявляет высокую эффективность амплификации как в моно-, так и в мультиплексной ПЦР и может стать многообещающим инструментом для различных молекулярно-биологических применений в рутинных исследованиях, а также в диагностике.

ДНК-полимераза – полимеразная цепная реакция – Thermus

DNA polymerases "mined" from various organisms are widely used for in vitro DNA manipulation (e.g. DNA cloning, sequencing, labeling, mutagenesis, etc.) [7]. A variety of thermostable DNA polymerases have been isolated and identified from prokaryotic organisms. Detailed information about individual properties of these enzymes and their related applications have been recently reviewed [15, 16]. Each thermostable

DNA polymerase has different characteristics (e.g., thermostability, processivity, fidelity, specificity, resistance to contaminants and inhibitors) [5, 9]. The different properties of DNA polymerases may lead to the development of unique reagents, thus searching for novel DNA polymerase has been one of the major focuses in this research field. And protein engineering techniques to create artificial DNA polymerases have been successfully developing powerful DNA polymerases, suitable for specific purposes [6, 7, 15, 16]. However, all applications of novel polymerases depend upon an optimized PCR. Several variables, including $MgCl_2$ concentration, enhancing additives and other parameters can be extremely important, as every element of PCR can affect the outcome [8]. A variety of additives and enhancing agents can be included in PCR amplifications to increase yield, specificity and consistency (e.g. dimethyl sulfoxide (DMSO), N,N,N-trimethylglycine (betaine), formamide, glycerol, nonionic detergents, bovine serum albumin, polyethylene glycol, tetramethylammonium chloride spermidine, gelatin, tween-20, nonidet P40 (NP40) and Triton-X100) [3, 14]. These additives have beneficial effects on some PCR amplifications; however, it is not possible to predict which agents might be useful for a particular target [3].

This paper describes optimization of reaction parameters and cycling conditions for novel TsK1 polymerase which originates from *Thermus scotoductus* K1 strain isolated from Karvachar geothermal spring (Nagorno-Karabakh).

***Materials and methods*. DNA polymerase used in this study**

Pol 1 gene of *T. scotoductus* K1 previously was cloned and expressed in *E. coli* BL21 (DE3) competent cells [12, 13]. Desired protein designed as TsK1 was purified using HisTalon gravity column (Clontech Laboratories, Inc.) [13], and kept in storage buffer suggested by [4] at -20^0C.

DNA template and primers

For PCR optimization pUC19 plasmid (New England BioLabs, Inc.) was used as template to amplify 265 bp region of lacZ gene. Appropriate primers Puc19_f (5′-gcatgaaagcttgcatgcctgcaggtcgac-3′) and Puc19_r (5′-gcatgacatatgcggtgtgaaataccgcac-3′) were manually designed using Primer3 online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

PCR assay

PCR reactions were performed in reaction mixtures (tab. 1) varying in buffer composition: different amounts of $MgCl_2$ (0-4.5 mM final concentration) and enhancers (DMSO and glycerol), as well as various amounts of template DNA (0.01-1 ng/μL final concentration). Composition of buffer OPT (10x : 500 mM KCl, 100 mM Tris-HCl, pH 9.0, 0.1% Triton X) was designed experimentally taking into consideration optimal pH [13] of TsK1 enzyme.

Table 1. Compositions of amplification mixes for PCR optimization

	Concentration of stock	Volume per reaction, μL	Final concentration
MixA components			
Buffer OPT	10x	2.5	1x
Pr_F	10 μM	1.25	0.5 μM
Pr_R	10 μM	1.25	0.5 μM
dNTPs	10 mM	2	0.8 mM
Glycerol (DMSO)	50% (100%)	5 (1.25)	10% (5%)
$MgCl_2$	15 (30) (45) mM	2.5	1.5 (3.0) (4.5) mM
Template	100 ng/μL	0.25	1 ng/μL
TsK1 polymerase		1	
Reaction volume		25	

MixB components			
Buffer OPT	10x	2.5	1x
Pr_F	10 μM	1.25	0.5 μM
Pr_R	10 μM	1.25	0.5 μM
dNTPs	10 mM	2	0.8 mM
Glycerol (DMSO)	50% (100%)	5 (1.25)	10% (5%)
$MgCl_2$	5 (10) (15) (30) mM	2.5	0.5 (1.0) (1.5) (3.0) mM
Template	100 (50) ng/μL	0.25	1 (0.5) ng/μL
TsK1 polymerase		1	
Reaction volume		25	
MixC components			
Buffer OPT	10x	2.5	1x
Pr_F	10 μM	1.25	0.5 μM
Pr_R	10 μM	1.25	0.5 μM
dNTPs	10 mM	2	0.8 mM
Glycerol	50%	- Or 5	- Or 10%
$MgCl_2$	30 mM	2.5	3.0 mM
Template	1 (5) (50) ng/μL	0.25	0.01 (0.05) (0.5) ng/μL
TsK1 polymerase		1	
Reaction volume		25	

The 68^0C and 72^0C temperatures were tested for elongation, and elongation time was calculated based on expected amplicon length – 1kb/min. Temperature and time for annealing were applied according to primers' T_m and desired product size respectively. In control reactions, OneTaq (NEB) polymerase was used according to manufacturers' recommendations. PCR products were analyzed by 0.8% agarose gel electrophoresis.

Multiplex PCR

Human genomic DNA was used as template to amplify exons 3, 47, 50 and 52 (tab. 2) of human dystrophin gene using primer pairs suggested by [1] in optimized reaction mixture, with modifications. Cycling conditions for multiplex PCR were: 94^0C 5 min, [94^0C/30 sec, 65^0C/4 min] x 25, 65^0C/7 min. PCR products were analyzed on 3% agarose gel.

Table 2. Primers used in multiplex PCR according to [1]

Exon	**Forward primer (5′-3′)**	**Reverse primer (5′-3′)**	**Product size (bp)**
3	TCATCCATCATCTTCGGCAGATTAA	CAGGCGGTAGAGTATGCCAAATGAAAATCA	410
47	CGTTGTTGCATTTGTCTGTTTCAGTTAC	GTCTAACCTTTATCCACTGGAGATTTG	181
50	CACCAAATGGATTAAGATGTTCATGAAT	TCTCTCTCACCCAGTCATCACTTCATAG	271
52	AATGCAGGATTTGGAACAGAGGCGTCC	TTCGATCCGTAATGATTGTTCTAGCCTC	113

Results and Discussion. Initial experiments of PCR optimization were performed in commercial OneTaq Standard reaction buffer (NEB) (20 mM Tris-HCl pH 8.9, 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 22 mM KCl, 0.06% IGEPAL CA-630, 0.05% Tween 20) used in 25 μL reaction, containing dNTPs (0.2 mM), reverse and forward primers (2 μM each) TsK1 polymerase, without additional enhancers and $MgCl_2$. Cycling conditions were: 94^0C/1min, [94^0C/30 sec, 60^0C /30 sec, 68 ($72)^0$C/30 sec] x30, 68($72)^0$C/3 min. The same experiments were performed by increasing or decreasing annealing and extension temperatures as cycling parameters are dependent upon the sequence and length of the template DNA, the sequence and percent complementarity of the primers, and the ramp times of the thermal cycler [8]. The reactions resulted with none or non-specific amplicons using TsK1 (data are not shown), suggesting importance of appropriate composition of reaction components.

Enhancers were used to increase yield and specificity of PCR. Nonionic detergents (Triton X-100, Tween 20, or Nonidet P-40) neutralize charges of ionic detergents often used in template preparation, and should be used in the basic reaction mixture, rather than as optional enhancers at reaction concentrations of 0.1 to 1% in order to increase amplicon production. [8,10]. TsK1 polymerase shows its highest activity at pH 9.0 [13], thus, Mg^{2+} free buffer OPT (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X) was designed and used as basic reaction buffer in later experiments.

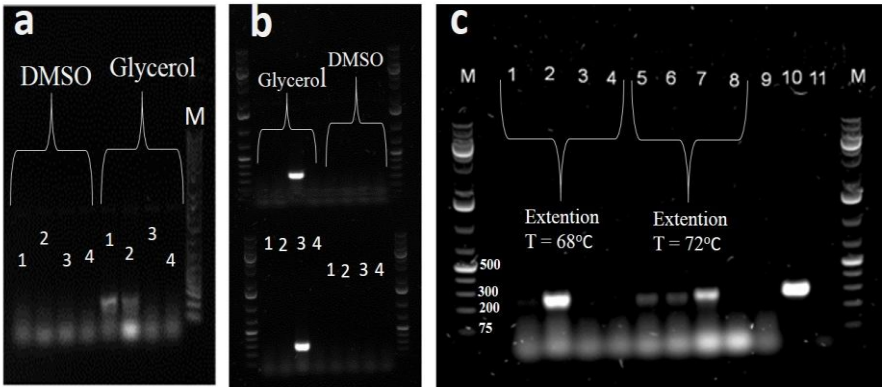

Fig. 1. a) Image of 265 bp amplicons using DMSO or glycerol as enhancers with different concentrations of $MgCl_2$: lines 1-4 – concentrations of $MgCl_2$ in reaction 3.0 mM, 1.5 mM, 4.5 mM, no $MgCl_2$, b) Image of amplicons with 25 ng (top) and 12.5 ng (bottom) of template, using DMSO or glycerol as enhancers with different concentrations of $MgCl_2$: lines 1-4 – concentrations of $MgCl_2$ in reaction 1.0, 1.5, 3.0, 0.5 mM respectively, c) Image of amplicons at 68^0C and 72^0C of extension temperature, using glycerol as enhancer with 3 mM $MgCl_2$, and different amounts of template: lines 1 and 5 – 0.25 ng template, lines 2 and 6 – 1.25 ng template, lines 3 and 7 – 12.5 ng template, 4 and 8-negative controls, line 9 – glycerol is absent, line 10 – amplicons with OneTaq enzyme (positive control), line 11 – negative control of PCR using OneTaq, M-molecular size marker (sizes are shown in bp)

The thermostable DNA polymerases require the presence of magnesium to act as a cofactor during the reaction process [2, 8, 10]. Optimum $MgCl_2$ concentration can vary even for different primers from the same region of a given template [8]. In general, the PCR product yield will increase with the addition of greater concentrations of Mg^{2+}. However, increased concentrations of Mg^{2+} will also decrease the specificity and fidelity of the DNA polymerase. Too much Mg^{2+} may prevent complete denaturation of the DNA template by stabilizing the duplex strand and also can stabilize spurious annealing of primers to incorrect template sites and decrease specificity resulting in undesired PCR products. When there is not enough Mg^{2+}, the reaction will not proceed, resulting in no PCR product [2, 10]. Therefore, to achieve maximal PCR yield the concentration needs to be optimized. Various concentrations of $MgCl_2$ (0, 0.5, 1.0, 1.5, 3.0, 4.5 mM) were tested with combination of glycerol or DMSO at 10% and 5%, respectively (see Mix A and Mix B in Table 1) under cycling conditions: 94^0C/1min, [94^0C/30 sec, 60^0C /20 sec, 68^0C /20 sec] x 30 cycles, 68^0C /3 min. Adding glycerol as enhancer in PCR mixture dramatically increased the yield (evaluated as intensity of bands on agarose gel images) of PCR with combination of $MgCl_2$ at 3 mM, and intensity of band is higher when using less amount of template DNA (fig.1b, line 3). By contrast, no amplification was occurred when adding DMSO instead of glycerol (fig.1. a,b). Glycerol is known to induce improvement of PCR, possibly explained by (i) enhancing hydrophobic interactions between protein domains, (ii) lowering the strand separation temperature, (iii) raising the

thermal transition temperature of proteins and (iv) enhancing association between enzyme and template [11, 14].

After determination of optimal $MgCl_2$ concentration and reaction components, the amplification efficiency of TsK1 depending on template amount (see MixC in tab. 2) and extension temperature were tested under cycling conditions: 94^0C/1 min, [94^0C/30 sec, 60^0C /20 sec, 68 (72)^{0}C /20 sec] x30, 68 (72)^{0}C /3 min. TsK1 shows ability to amplify DNA both at 68 and 72^0C. 1.25 ng of plasmid DNA is optimal for efficient amplification at extension temperature of 68^0C (fig. 1c, line 2), and at 72^0C of extension, intensity of amplicon bands increased along amount of used template (fig. 1c, lines 5-7). Interestingly, no amplification occurred in reaction mixture without adding glycerol (fig. 1c, line 9), suggesting importance of this agent for successful PCR using TsK1.

After optimization of optimal reaction conditions for TsK1, enzyme was tested for ability of amplification in multiplex PCR. Human genomic DNA was used as a template to amplify some exons of human dystrophin gene.

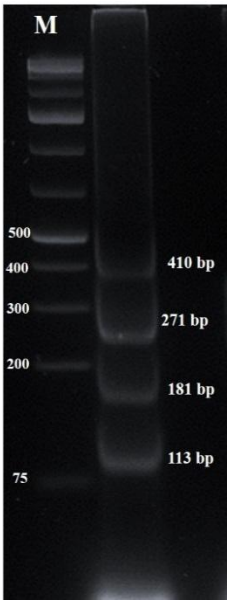

Fig. 2. Amplicons of multiplex PCR using TsK1. M-molecular mass marker GeneRuler 1kb Plus (NEB)

Deletions of various exons in human dystrophin gene are associated with Duchenne and Becker muscular dystrophies (DMD and BMD) and protocols for multiplex PCR assays allowing detection of different exons in single reaction were described [1] and are currently in use in diagnostics worldwide, including Armenia. Randomly chosen 4 primer pairs were used in equimolar concentrations for amplification of certain exons of human dystrophin gene in optimized basic reaction mixture containing 3 mM $MgCl_2$. Amplicons shown on fig. 2 prove the ability of TsK1 of multiplex PCR, suggesting that further investigations and improvement of TsK1 may make it very useful in molecular diagnostics as well.

The optimal conditions for PCR using TsK1 polymerase were determined. TsK1 polymerase exhibits PCR efficiency in both mono- and multiplex PCR, and further investigations to improve its activity and optimization may lead to make it promising tool for different molecular biological applications in routine research as well as in diagnostics.

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