

Extended kinetic model of real-time polymerase chain reaction process

A A Fedorov¹, D G Sochivko², D A Varlamov³ and
V E Kurochkin¹

¹ Institute for Analytical Instrumentation, Russian Academy of Sciences, St.
Petersburg, Russia

² Syntol JSC, Moscow, Russia

³ All-Russia Research Institute of Agricultural Biotechnology, Russian Academy of
Sciences, Moscow, Russia

E-mail: f_aa@mail.ru

Abstract. Real-time polymerase chain reaction (real-time PCR) is the main molecular genetic method used for qualitative and quantitative analysis of specific nucleic acid sequences in many areas of biomedical research. Theoretical study of PCR models allows to estimate the influence of various reaction components and parameters, and to determine the unknown parameter values by approximating the experimental real-time PCR curves. An extended kinetic model of real-time PCR is presented. The model takes into account the enzyme activity based on Michaelis-Menten kinetics, the hybridization of complementary DNA fragments, the presence of a fluorescent probe used for detection of the reaction products, and the temperature dependence of primers and probe hybridization.

1. Introduction

Currently, real-time polymerase chain reaction (real-time PCR) is the main molecular genetic method used for qualitative and quantitative analysis of specific nucleic acid sequences in many areas of biomedical research. The feature that distinguishes real-time PCR among other analytical methods is extremely high sensitivity and specificity, permitting detection and analysis of samples containing as few as single molecules of target sequence. Despite the fact that the overall molecular mechanism of the reaction is well known, new articles suggesting quantitative models of various complexity are published regularly [1-3]. The on-going research is stimulated by the fact that real-time PCR is a multistage reaction involving about dozen key molecules; therefore the analysis of its kinetics is a non-trivial task which requires a certain degree of simplification.

The main approach to model the real-time PCR process is based on the application of chemical kinetic equations. To do this, one has to create a chemical framework of PCR in the desired level of detailing, write down the reaction equations, derive from them the system of differential equations and then solve it, usually by numerical methods. This model study allows to estimate the influence of various reaction components and parameters, and to determine the unknown parameter values by approximating the experimental real-time PCR curves.

The review of simple kinetic models describing the major reaction components is given in [4]. The model suggested there includes the reversible primer hybridization, the hybridization of the



complementary DNA strands, and Michaelis-Menten kinetics of DNA fragment synthesis. However, most of the models don't take into account two aspects of the actual real-time PCR. The first aspect is the presence of the reporter probe that is used to detect reaction product in many real-time PCR realizations. The second one is the fact that hybridization is a temperature-dependent process. Indeed, the PCR primers and probes are designed to work at the specific temperature which is determined by their melting curve [5]. In the present paper, we introduce the model of real-time PCR which includes the reporter probe and hybridization temperature dependence.

2. The model description

We have developed an extended kinetic model of real-time PCR. The system takes into account enzyme activity based on Michaelis-Menten kinetics, the hybridization of complementary DNA fragments, the presence of the fluorescent probe used for detection of the reaction products, and temperature dependence of primers and probe hybridization. The model reactions are shown in Fig. 1.

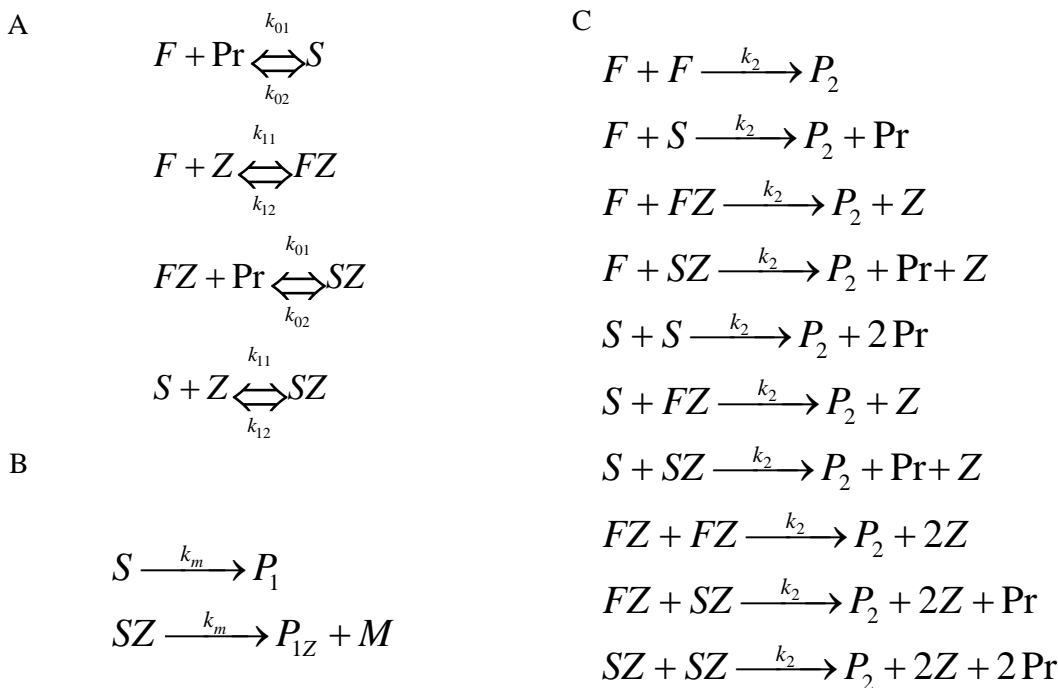


Figure 1. The model reactions. (A) reversible hybridization processes, (B) fragment synthesis by Taq polymerase, (C) irreversible complementary chains hybridization.

The model includes the following reactants:

- F – amplified DNA fragment,
- Pr – primer,
- Z – TaqMan fluorescent reporter probe,
- S – complex of fragment F and primer Pr (non-fluorogenic substrate for polymerase),
- FZ – complex of fragment F and probe Z ,
- SZ – complex of fragment F , primer Pr and probe Z (fluorogenic substrate for polymerase),
- P_1 – product of polymerase activity (double-strand fragment produced by synthesis),
- P_{1Z} – fluorescent product of polymerase activity,
- P_2 – duplex (double-strand fragment produced by hybridization of complementary chains),
- M – fluorescent reporter molecule (produced during synthesis of P_1 from SZ by polymerase).

Differential equation system corresponding to the reactions above was derived:

$$\begin{aligned}
 \frac{\partial[F]}{\partial t} &= k_{02}[S] + k_{12}[FZ] - k_{01}[F][Pr] - k_{11}[F][Z] - k_2[F]([F] + [S] + [FZ] + [SZ]) \\
 \frac{\partial[Pr]}{\partial t} &= k_{02}([S] + [SZ]) - k_{01}[Pr]([F] + [FZ]) + k_2[S]([F] + [S] + [FZ] + 2[SZ]) + k_2[SZ]([F] + [FZ] + [SZ]) \\
 \frac{\partial[Z]}{\partial t} &= k_{12}([FZ] + [SZ]) - k_{11}[Z]([F] + [S]) + k_2[SZ]([F] + [S] + [FZ] + [SZ]) + k_2[FZ]([F] + [S] + [FZ]) \\
 \frac{\partial[S]}{\partial t} &= k_{01}[F][Pr] - k_{11}[Z][S] - k_2[S]([F] + [S] + [FZ] + [SZ]) - k_m[S] \\
 \frac{\partial[FZ]}{\partial t} &= k_{11}[F][Z] - k_{01}[FZ][Pr] - k_2[FZ]([F] + [S] + [FZ] + [SZ]) \\
 \frac{\partial[SZ]}{\partial t} &= k_{01}[FZ][Pr] + k_{11}[S][Z] - [SZ](k_{02} + k_{12}) - k_2[SZ]([F] + [S] + [FZ] + [SZ]) - k_m[SZ] \\
 \frac{\partial[P_2]}{\partial t} &= k_2([F](0.5[F] + [S] + [FZ] + [SZ]) + [S](0.5[S] + [FZ] + [SZ]) + [FZ](0.5[FZ] + [SZ])) \\
 \frac{\partial[P_1]}{\partial t} &= k_m[S] \\
 \frac{\partial[P_{12}]}{\partial t} &= k_m[SZ] \\
 \frac{\partial[M]}{\partial t} &= k_m[SZ]
 \end{aligned} \tag{1}$$

The temperature dependence of primer and probe hybridization was introduced through the dissociation constant k_{02} and k_{12} . It is known that the association rate of oligonucleotides is weakly dependent on temperature, in contrast to the dissociation rate. To estimate the dissociation constant at the certain temperature, the melting curve of the primer or the probe was used.

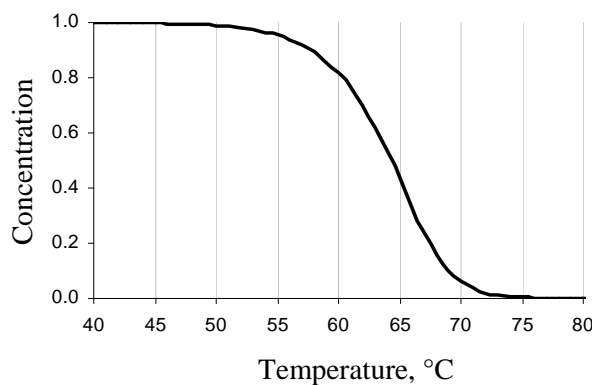


Figure 2. The melting curve of the model PCR primer. Normalized DNA hybrid concentration is plotted vs. temperature.

The melting curve represents the steady-state fraction of DNA hybrid assessed in the given temperature range. The melting curve can be obtained using fluorescent probes of different kinds. For example, two complementary oligonucleotide fragments can be mixed with an intercalating fluorescent dye and heated in a PCR machine. At lower temperatures, the fluorescent signal will be maximal as all the chains will be in the hybridized state. At higher temperatures, fluorescence will reach minimum as all the double-stranded complexes will dissociate. The normalized curve obtained is presented in Fig. 2. This curve can be well approximated by generalized logistic function with parameters a_1 , a_2 , T_M (a_1 – slope parameter, a_2 – asymmetry parameter, T_M – midpoint parameter) which allows calculating the hybridized fragment fraction $M(T)$ at any temperature T (Eq. 1). Next, the

dissociation rate constant can be calculated from equilibrium condition reached during melting curve analysis (a_3 is equal to the concentration of melted oligonucleotides) assuming that association constant k_1 does not depend on temperature (Eq. 2):

$$M(T) = \frac{1}{(1 + e^{-a_1(T-T_M)})^{a_2}}, \quad (2)$$

$$k_{-1} = k_1 a_3 \frac{(1 - M(T))^2}{M(T)}. \quad (3)$$

Enzymatic synthesis of products by Taq polymerase was described by Michaelis-Menten kinetics of k_m rate constant (V_{max} – maximum rate, K_M – Michaelis constant, $[S]$ – substrate S or SZ concentration):

$$k_m = \frac{V_{max}}{K_M + [S]}. \quad (4)$$

The resulting system of kinetic equations was solved and analyzed by numerical simulation using the C++ language. The selection of parameters of the system was carried out on the basis of literature data and own experimental results: initial DNA concentration – 10^{-11} M, primer and probe concentration $2 \cdot 10^{-7}$ M (unless otherwise specified), $k_{01} = 10^6 \text{M}^{-1} \text{s}^{-1}$, $k_2 = 3 \cdot 10^6 \text{M}^{-1} \text{s}^{-1}$, $k_{11} = 10^6 \text{M}^{-1} \text{s}^{-1}$, $a_1 = -0.31$, $a_2 = 3.4$, $a_3 = 2 \cdot 10^{-7} \text{M}$, $V_{max} = 1 \cdot 10^{-8} \text{M} \cdot \text{s}^{-1}$, $K_M = 2 \cdot 10^{-9} \text{M}$.

3. Results

In contrast to the most existing models of real-time PCR counting DNA fragments synthesized during reaction, product registration in our model is carried out indirectly by the number of fluorescent reporter molecules M generated in the reaction. This allows us to assess the influence of probe kinetics on the PCR curve shape and amplitude. We analyzed the PCR curves for product P_1 and reporter M at several probe Z concentrations (Fig. 3A). The concentration of the reporter at the PCR plateau is lower than the concentration of the product when they are added to the reaction in the same amount. When we look at the curves normalized to the plateau level we can see that the reporter curves saturate slightly earlier than the product curve (Fig. 3B).

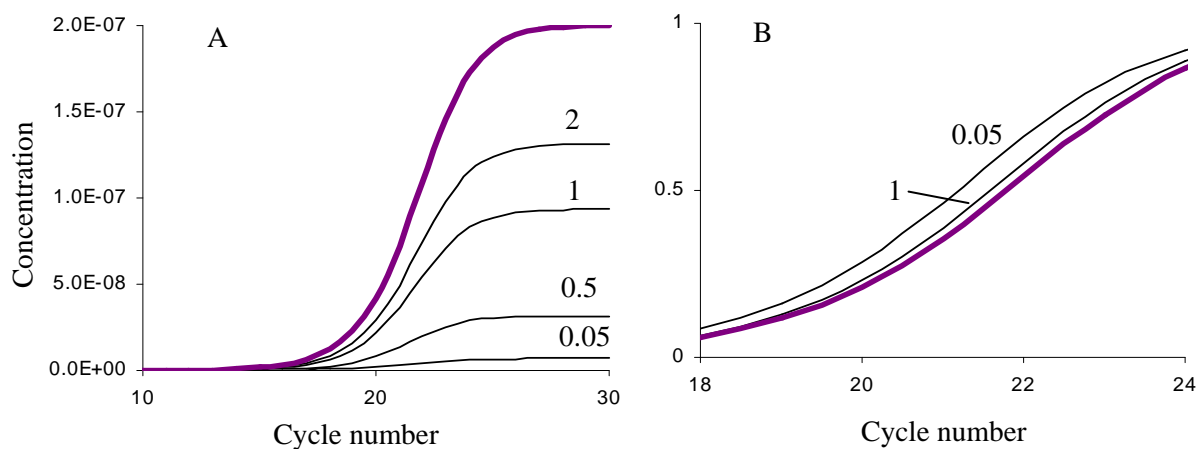


Figure 3. The PCR curves for product (thick line) and reporter (thin lines). (A) absolute concentrations, (B) normalized curves. The initial probe concentration relative to the primer concentration is denoted.

We analyzed the effect of temperature on the product synthesis. The primer melting curve is shown in Fig. 2, the corresponding approximated T_M from Eq. 1 was 69°C. Reaction temperature T was varied, and the resulting PCR curves were depicted in Fig. 4. Reaction kinetics started to change as the temperature reached 70°C when the melting curve shows just 10% of the primer is hybridized. At higher temperatures the reaction declined dramatically.

Next, we studied how the reaction curve amplitude is affected by the relative melting temperatures of the primers and the probe. Reaction temperature T was 60°C. Primer melting temperature T_M was fixed at 69°C, whereas T_M of the probe was varied from 60 to 80°C. The reporter concentration curves are presented at Fig. 5. Surprisingly, the curve amplitude differed just slightly for all melting temperatures equal or higher than primer T_M . This is in contrast to the general belief that the probe melting temperature should be about 10 degrees higher than the one for the primer. Normalized curves were identical for all the temperatures studied (data not shown).

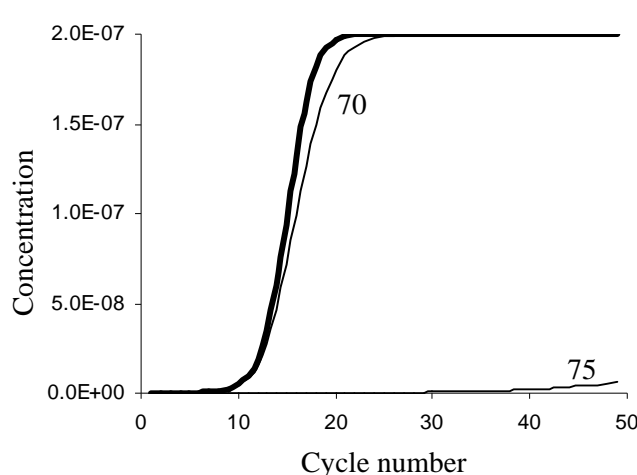


Figure 4. The effect of reaction temperature T on the product synthesis. Thick line – $T = 65^\circ\text{C}$ and lower. Thin lines – T is denoted in the panel.

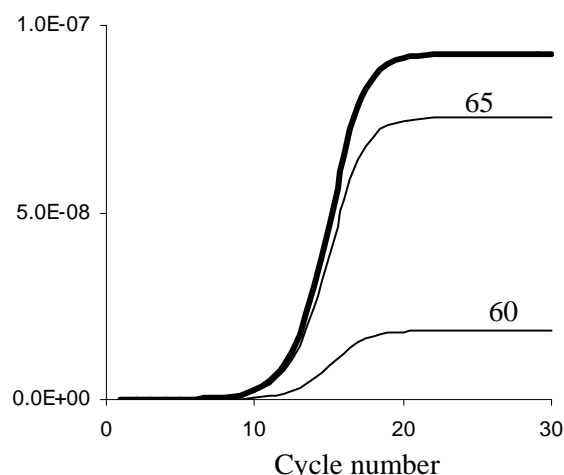


Figure 5. The effect of probe melting temperature T_M on the PCR curve amplitude. Thick line – T_M is 70°C and higher. Thin lines – T_M is denoted in the panel.

4. Conclusion

The extended kinetic model of real-time PCR presented here was designed to study how reaction results depend on various reagents and reaction conditions. The model parameters have clear meaning and allow experimental or theoretical evaluation. The results of modeling demonstrate realistic behavior characteristic for the actual real-time PCR curves.

Acknowledgements

The work was supported by the Ministry of education and science of the Russian Federation, agreement 14.579.21.0012 (ID # RFMEFI57914X0012).

References

- [1] Mehra S and Hu W S 2005 *Biotechnol Bioeng* **91** 848-860
- [2] Cobbs G 2012 *BMC bioinformatics* **13** 203
- [3] Marimuthu K, Jing C and Chakrabarti R 2014 *Biophys J* **107** 1731-1743
- [4] Sochivko D G, Fedorov A A, Varlamov D A, Kurochkin V E and Petrov R V 2016 *Dokl Biochem Biophys.* **466** (in press)
- [5] SantaLucia Jr J 2007 *PCR Primer Design* (New York: Humana Press) chapter 1 pp 3-33