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Processing of qPCR signals obtained on microfluidic chips in the measurement sequence disorder event

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Abstract. The formation and movement of gas bubbles in microfluidic chips leads to the measurement sequence disorder in the form of a signal distortion. Two new methods for automatic processing of distorted qPCR signals were developed and implemented. Methods are based on the qPCR signals approximation by a sigmoid function and make it possible to successfully perform a microfluidic chips qPCR analysis in the event of bubbles, which is demonstrated on experimental and simulation curves.

Keywords: microfluidics, microfluidic chip, qPCR, bubbles, approximation

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Обработка полученных на микрофлюидных чипах сигналов ПЦР-РВ при разладке последовательности измерений

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Аннотация. Одним из основных недостатков микрофлюидных чипов является образование пузырей, приводящее к возникновению разладки в последовательности измерений в форме искажения сигналов ПЦР-РВ. Разработаны и реализованы две новые методики автоматической обработки искаженных сигналов, основанные на аппроксимации сигмоидальной функцией. Выполнена апробация методов на экспериментальных и имитационных сигналах. Данные методики позволяют получать удовлетворяющие значения погрешностей при определении порогового цикла и успешно выполнять количественный анализ ПЦР-РВ в микрофлюидных чипах при возникновении пузырей.

Ключевые слова: микрофлюидика, микрофлюидный чип, ПЦР-РВ, пузыри, аппроксимация

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Introduction

Recently, a large number of methods [1–4] and instruments [5–7] for diagnosing condensed matter have been developed in the world. The main disadvantages of such instruments are their large size, high consumption of reagents and samples, etc. [8, 9]. The use of microfluidic chips for the study of liquids and solutions in microsystems allows solving many of these problems. These chips provide reproducible control of the laminar flows of nano- and picoliter volumes of liquid in microsized channels, high sensitivity and low power, reagents and samples consumption [10–12]. So they are widely used in chemical engineering, pharmaceuticals, biotechnology, and medicine [13].

Quantitative polymerase chain reaction (qPCR) is a popular method of genetic analysis, which makes it possible to quantify the content of the target nucleotide sequence in a sample by determining quantification cycle values C_q [14]. Microfluidic qPCR chips are being actively developed and improved in the world [15-20]. A significant disadvantage of performing PCR in a chip chamber compared to test tubes is the formation of gas bubbles during thermal cycling. It has no noticeable effect on the reaction result, but prevents correct optical detection. Several methods have been used to inhibit the bubble generation: (i) the design of PCR chamber, (ii) the surface treatment, (iii) the sealing pressurization of the PCR chamber (iv) degasification of the PCR sample and (v) the addition of high boiling-point biocompatible reagents to the PCR sample [13]. Mathematical processing of qPCR signals makes it possible to evaluate the analysis result, despite the gas bubbles formation influence on signal. The PCR efficiency evaluation depending on the materials and design of the chips, the reagents used, and the reaction conditions is complex due to the occurrence of discord in the measurement sequence due to the formation and movement of bubbles. This results in a fluorescence intensity decrease over several cycles. Therefore, the development of methods for processing of qPCR signals distorted due to the formation of bubbles is an extremely urgent task.

Materials and Methods

Microchips for qPCR analysis were made of polycarbonate Novattro (SafPlast, Russia) and polypropylene PP 4445S (PJSC Nizhnekamskneftekhim, Russia) by thermal pressing in a MM-100 hydraulic press (MTDI, Korea) on a stainless steel master mold made by laser micro-processing. Microchip topology represents 3 chambers with supply channels [21]. Polymer film *P*-500 (LLC PKF Modern technologies, Russia) was used for sealing microchips.

The obtained microchips were used for qPCR of soybean DNA isolated manually using a set of M-sorb-OOM (LLC Sintol, Russia). The experiments were carried out for the initial concentration of isolated nucleic acids, for dilutions of 1, 2 and 3 orders, and for a positive control in three repeats for each variant. R6G and Cy5 dyes were used to detect soybean DNA samples and confirm a positive control respectively.

The measurements were carried out on a specially designed model that provides a thermal cycling mode for qPCR, dyes excitation at wavelengths of 530, 570 and 685 nm and signal registration at 580, 630 and 660 nm. The microchip chambers were filled with a reaction mixture with the addition of nucleic acids; the inputs/outputs were sealed with PCR film. The microchip was placed on the heating element with the film facing down. Overall, 114 experimental qPCR plots were obtained, about 70 % had distortion caused by bubbles.

Two signal processing methods, based on the qPCR plots approximation by a known sigmoid function [22] (formula 1) were developed and implemented in the MATLAB software to identify the correct C_q values:

$$F_c = \frac{F_m}{1 + e^{\frac{C_q - C}{k}}} + f_0, \quad (1)$$

where F_c is the fluorescence signal at cycle C, RFU; F_m is the maximal reaction fluorescence, RFU; C_q is the fractional cycle at which reaction fluorescence reaches half of F_m; k is related to the slope of the curve, f_0 is the fluorescence background, RFU.

Each cycle of the program, when implementing the developed methods, the graph is approximated. In method No. 1, the signal value with the largest absolute deviation from the approximating function (1) ΔF is assigned the value corresponding to the approximating dependence point. In method No. 2, the value corresponding to the deviation ΔF is excluded from the experimental set. The program end is determined automatically.

The use of the program on experimental data does not allow estimating the errors in determining the threshold cycle values since there is no a priori information about the true C_q values. The experimental curves errors study was carried out, which revealed the following:

- the mathematical expectation of errors close to zero;
- the distribution of errors is more consistent with the normal law: information discrepancy of the Kullback–Leibler histogram of the real distribution of interference over 6 intervals with a uniform law of 0.0710 nits, with a normal one – 0.0422 nits, a coefficient of kurtosis – 3.07;
- the error is close to multiplicative, its standard deviation is possibly proportional to the value of the average signal.

Based on the results of the error study, Set A was created from 10000 simulation curves (Fig. 1,*a*) with parameters $F_m = 1000$; $C_q = 30$; $k = 1$, $f_0 = 600$, the dependence law of errors normally distributed standard deviations (RMS) on the signal value $\sigma = 0.0024F_c + 1.1368$, uniform distributions of the duration of signal distortion from 3 to 7 cycles, and the intensity of the signal drop when a bubble occurs $(0.22–0.24)\cdot F_c$. The beginning of the bubble is also random, its distribution is even.

We separately created five sets of 10000 curves: with signal distortion caused by bubbles that occur in the ranges: Set B from 1 to 24 cycles, Set C from 25 to 35 cycle and Set D from 36 to 50 cycle; as well as with signal distortions of duration: Set E 3 cycles and Set F 7 cycles.

Results and Discussion

As a result of applying the method No. 1 (Fig. 1,*b*) on Set A errors 76.41 % of values C_q lie in the range ±0.15 cycle from the true value, as a result of applying method No. 2, 77.39 %. The mean C_q scores for the methods were 30.36 ± 0.84 and 30.52 ± 1.30, respectively. The estimate bias is caused by large positive errors, the proportion of which is about 20 %. The acceptable value of 0.15 cycles corresponds to a quantification error of less than 10 % and was chosen as the deviation in the determination of C_q, which is acceptable for most tasks of qPCR analysis. According to this criterion, method No. 2 has advantages.

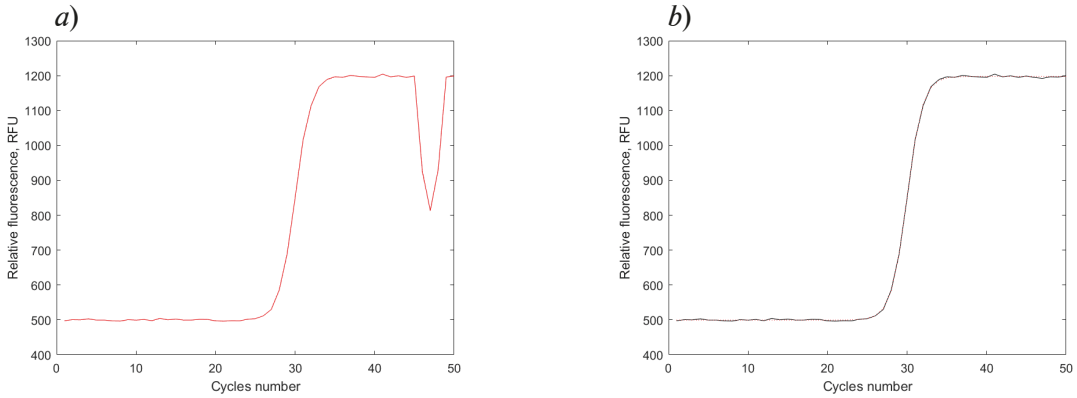

Fig. 1. Graphs for qPCR: model graph (*a*); model graph with eliminated distortion according to Method No. 1 (*b*)

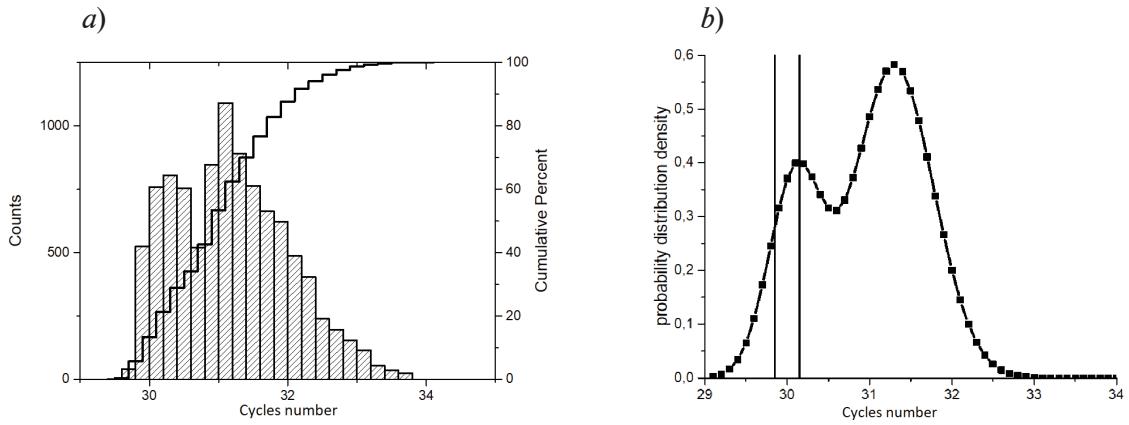

Fig. 2. Results of Method No. 1 application in Set C: threshold cycle position estimates histogram (*a*); possible probabilistic model for the distribution of scores (*b*), which is the sum of two Gaussian distributions

In case of distortion in the initial (Set B) or final (Set D) section of the kinetic curve, the average errors in estimating the position of the inflection point are at an acceptable level: 0.013 for the initial section and 0.022 for the final section when choosing any of the two methods. Permissible error in determining C_q obtained in 100 % of cases.

The main errors occur when the signal is distorted in the central section of the qPCR graph. 11% of the curves fall within the allowable range of ±0.15 cycles (Fig. 2).

The mathematical expectation (M) of the threshold cycle position in method No. 1 was 31.18 cycles. As follows from the histogram, the distribution is close to bell-shaped. According to the Novickij-Zograf rule, at least 90 % of the sample must fall within the interval $M \pm m \cdot \sigma$ standard deviation at $m = (1.65 \pm 0.05)$ [23], in our case in the interval {29.81; 32.55} with $m = 1.65$.

Set analysis shows that 9329 out of 10000 results fell into this interval (i.e., 93.3 %), which correlates well with the Novickij-Zograf rule. Depending on the method for constructing an approximating dependence and estimating the inflection point of the kinetic curve in the presence of bubbles in the section 25–35 cycles, the estimation error are given in Table 1. The average error according to method No. 1 is 1.18 cycles, according to method No. 2 0.99 cycles.

With method No. 2, the proportion of small (up to 0.3 cycles) errors is much larger (29.5 versus 18.5 %). However, the proportion of super-large estimation errors is almost the same: 20 % of the largest errors with method No. 1 - 1.89 cycles or more, with method No. 2 - 1.80 cycles or more. Approximately every 10th quantitative qPCR analysis, when distortion is on the central part, we will mistakenly exclude a reasonable and correct result.

Another stage of the simulation involved introducing a discord corresponding to a 3 or 7 distortion duration (Sets E and F).

Table 1

Comparison of the errors distribution in determining C_q by two methods when processing the Set C

Method No. 1		Method No. 2	
Level, cycle	Probability, %	Level, cycle	Probability, %
0.30	18.5	0.30	29.5
0.50	25.2	0.50	38.0
1.00	42.6	0.85	50.0
1.12	50.0	1.00	56.8
1.89	80.0	1.80	80.0

According to method No. 1 with the replacement of values with a distortion duration of 3 cycles, the average error in threshold cycle determining was 0.135; with an alternative method of exclusion, it was 0.065 cycles. That is, for a "small" bubble, it is more reasonable to exclude the corresponding 3 measurements from the signal.

When simulating a bubble with distortion duration of 7 cycles, the average errors in estimating the position of the threshold cycle are practically the same with an accuracy of several thousandths and are approximately equal to 0.254 cycles.

Conclusion

Two automated methods have been developed that allow to obtain a satisfying RMS value when determining the threshold cycle and successfully perform a quantitative analysis of qPCR in microfluidic chips when bubbles occur. Approbation on a set of 10000 created simulation curves showed that the acceptable error in determining C_q values, method No. 1 provides in 76.41 % of cases, method No. 2 - in 77.39 %. The main errors occur when the distortion is in the central section of the curve, which is characterized by the most intense fluorescence intensity change. When using the developed methods, 11 % of the processed curves fall within the allowable range of C_q value errors. Methods allow identifying such cases and recommending the operator to discard the values as false.

As a result, developed methods approbation on sets of simulation curves with different signal distortion lengths revealed that for short distortion durations, it is more reasonable to exclude the corresponding measurements from the signal, for large ones, the choice of the method is not fundamental.

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