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Abstract

Quantitation assays are a vital tool in various applications of molecular diagnostics. The demand for new DNA-, RNA- and immuno-diagnostic tests is constantly growing. They have found a number of applications in the diagnostics of genetic, parasite, and infectious diseases, as well as detection and monitoring of cancer, or the paternity testing. Such applications require tests that provide precise and specific quantitative assessment of the concentration of the analyte over a wide range of concentrations.

Quantitative Polymerase Chain Reaction (qPCR) is the central technique of molecular biology and an important solution in medical diagnostics. In was introduced in 1991 by Holland et al. in a format of Real-Time PCR [1]. This technique monitors the intensity of the physical signal (level of fluorescence, turbidity, etc.) connected with the formation of DNA product during PCR. The amount of this product (concentration C_p of the product) increases geometrically with the number n of PCR cycles: $C_p \propto q^n$. It is possible to assess the initial concentration of the analyte by specifying the number of PCR cycle after which the signal reached a given threshold, and comparing it with calibrated references. qPCR provides the assessment within a wide range of initial concentrations. However, the precision and accuracy of the assessment may be compromised by a number of factors, including the quality of samples and reagents, the presence of inhibitors, the quality of thermal cycling, and finally the reliability of signal detection in a qPCR device.

Despite the listed problems, real-time PCR is recognized as a *golden standard* qPCR technique, thanks to the relatively simple sample handling protocols (no partitioning of the sample is required), and well-established mathematical routines for calculating the final result (the comparison with a calibration curve).

However, there is an alternative PCR-based quantitative technique in a format of digital PCR (dPCR). Digital protocols were first introduced in 1915 by McCrady [2]. He described a the limiting-dilution quantitation assay for bacteria counting. Then, in 1992, digital protocols were

applied to quantitative PCR by Sykes et al. [3]. This idea was further developed by Vogelstein and Kinzler in 1999 [4]. They introduced the division of the sample into identical partitions which yielded either a positive ($s = 1$) or negative ($s = 0$) signal, depending on the presence of at least one molecule of the analyte in the inspected partition. Digital assays provide an absolute and highly reliable assessment of the initial concentration of the analyte, without any calibration of the experimental set-up. Moreover, the assessment by means of digital assays is usually very precise and sensitive [5, 6, 7].

Still, the popularity of digital techniques is increasing slowly. The main obstacle is the need for new apparata able to perform complicated partitiong and thermal treatment with the readout of tens or millions of partitions. This elevates the complexity and cost of devices.

In this work, we present the optimization of digital assay, and provide the systematic mathematical description. The algorithms we propose help to lower the laboratory requirements for running digital assessment by limiting the number of compartments.

We also describe an analog-digital method that combines the advantages of qPCR and dPCR and can be performed by means of standard qPCR devices. All the protocols presented in this work lower the number of compartments needed for the assessment and provide absolute quantitation. The protocols can be adapted to required parameters of a test: range of detected concentrations and precision of the assessment.

In the first chapter, we give a description of the project of optimisation of quantitation assays. The second chaper contains a short introduction to molecular diagnostics, with a brief description of the PCR-based methods. In the third chapter, we state the aim of our research, while the fourth chapter contains the description of the mathematical, numerical and experimental methods used to describe analytical assays, derive formulas for optimized assays and finally verify their performance. In chapters fifth, sixth, seven and eighth we describe the results of the analysis and the design of classic digital single- and multivolume assays, rationally designed digital assays and synergistic digital-analogue assays. We discuss and summarize the results and performance in the ninth chapter. Also, the instructions of how to prepare optimized assays and how to analyze the outcome from such assays are given in Appendix A, B, and C.

The findings of this work were published in the following research papers:

1. Debski, P.R., Gewartowski, K., Bajer, S., and Garstecki, P., *Calibration-free assays on*

standard real-time PCR devices., submitted

2. Debski, P.R., and Garstecki, P., *Designing and interpretation of digital assays: concentration of target in the sample and in the source of sample*, Biomolecular Detection, and Quantification, Available online 17 May 2016

3. Debski, P.R., Gewartowski, K., Sulima, M., Kaminski, T.S., and Garstecki, P., *Rational design of Digital assays*, Analytical Chemistry, 2015, 87 (16), 8203-8209

and the following patents and patent applications:

1. *Method for performing quantitation assays*, PCT/EP2013/000805 (patent granted in 2016; filed: 15 March 2013)

2. *Method for performing quantitation assays*, PCT/EP2012/004792 (patent granted in 2016; filed: 19 November 2012)

3. *Method for performing quantitation assays*, Polish Patent Application P-399908 (filed: 11 July 2012)

4. *Method for performing quantitation assays*, Polish Patent Application P-399673 (filed: 26 June 2012)

5. *Method for performing quantitation assays*, Polish Patent Application P-397026 (filed: 17 November 2011)

6. *Method for performing quantitation assays*, Polish Patent Application P-397027 (filed: 17 November 2011)

7. *Method for performing quantitation assays*, Polish Patent Application P-397028 (filed: 17 November 2011).

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