Short scientific mission report
Standardization of digital PCR analysis workflow

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The report addresses four main milestones described in the workplan. During my short scientific mission, we have also worked on a scientific article describing our web server application for standardized and reproducible analysis of digital PCR (dPCR) data. The early draft of this publication is attached to the report.

Contents

1 Expand dpcR format of data exchange

REDF (Raw Exchange Digital PCR Format) has tabular structure. Each run of dPCR is described using following properties:

1. ***experiment***: name of the experiment to which run belongs.

2. ***replicate***: name or ID of the replicate of the experiment.

3. ***assay***: name of the assay to which experiment belongs. Often name of the channel used to detect positive partitions.

4. $\boldsymbol{k}$: number of positive partitions.

5. $\boldsymbol{n}$: total number of partitions.

6. $\boldsymbol{v}$: volume of the partition (nL).

7. $\boldsymbol{uv}$: uncertainty of partition's volume (nL).

Both our package and web server allows saving dPCR data in this format as .csv files (decimal sign: dot, column separator: comma). To standardize the output even more, the export function suggests .dpcR extension for these files.

2 Create more import functions standardizing data provided by vendors

During my short scientific mission I was able to add the import functions for QX200 (BioRad) and OpenArray (Thermo Fisher) systems. Additionally, we expanded our import functions to also cover raw amplitude data. Due to its unique structure and large size, I had to write more efficient import functions. The final version works with speed adequate for the web server application.

3 Expand documentation of the framework and complete it with examples

we document a web server as well as the **R** package underlying the web tool. The documentation is available online `http://michbur.github.io/dpcR_manual/`.

4 Add the R code generation to the web server

Although GUIs are convenient to use, they do not fulfill the requirement of reproducibility if they do not track all actions taken by the user. Our web server generates **R** script that can be used to fully reproduce the whole analysis including parameters manually adjusted.

The reproducibility is further enhanced by the preservation of the input data md5 checksum. Thanks to this, the script generated by our software can detect changes in data and ensures that the user knows that analysis is reproduced using exactly the same input.

5 Confirmation

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I hereby confirm that Michał Burdukiewicz completed the tasks described in this report during his Short Scientific Mission conducted in my workgroup.

24.10.2016

Stefan Rödiger

6 Draft of the publication

dpcReport: web server and software suite for unified analysis of digital PCRs and digital assays

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ABSTRACT

The digital Polymerase Chain Reaction (dPCR) enables an absolute quantification of nucleic acids. Results of the reaction, counts of partitions in which amplification occured, currently may be processed using several proposed frameworks. However, most studies are done in a closed source software provided by dPCR system vendors, which hinders standardized and reproducible statistical analysis of results.

To provide researchers with an unified open software framework for reproducible analysis of chamber and droplet dPCR data we implemented peer-reviewed statistical methods into the dpcReport web server. As dpcReport has a robust import functionality, it can process and compare dPCR data regardless of its source system. Additionally, abstraction layers make the framework usable both for droplet- and (real-time) chamber-based technologies. The main part of the framework includes functions to estimate parameters of the underlying Poisson distribution and compute confidence intervals based on single samples as well as on replicates. Experiments may be compared using methods based on Generalized Linear Model-based procedure and multiple ratio tests. The user may complete his analysis with a quality control tools as a spatial randomness test for assessing plate effects or test for deviations from assumed Poisson distribution. The statistical analysis is complemented with appropriate visualizations and an advanced report generation tool.

An analysis done with dpcReport is fully reproducible. Since our web server is based on the **R** package dpcR, all steps performed by the user may be exported as an **R** script and later exactly reproduced via a command-line interface. To the best of our knowledge is dpcReport the only dPCR web server software that follows the standardized notation of the Minimum Information for Publication of Quantitative Digital PCR Experiments guidelines.

INTRODUCTION

The digital PCR (dPCR) is an important contender for precise nucleic acids quantifications. Even though there are other digital assays (6, 7), dPCR may be considered as the most widely used. Applications include investigation of allele frequencies, single-cell analysis, gene expression analysis and absolute quantification of PCR products. The chemical basis (e.g., buffer, primer) of the dPCR and thermal cycling is similar to PCR. Approaches based on isothermal amplification were also developed (15).

A first proposal for a dPCR-like approach and the use of the Poisson distribution to quantify the number of molecules on a "sample" was shown by Ruano *et al.* 1990 (PNAS) with the single molecule dilution (SMD) PCR (25). In 1999 Vogelstein *et al.* (PNAS) described the first true dPCR (29). Since approximately ten years the dPCR is rapidly gaining momentum in the mainstream user-base. There is an intensive research on dPCR platforms with the overall aim to make to technology broadly usable, cheap, robust and to enable high sample throughput (13, 16, 26).

A dPCR reaction consists of multiple amplifications occurring in numerous small "partitions" (e.g., nL volume droplets of water oil emulsions, chambers on micro structured chips). The result of dPCR is a binary data describing states of partitions (positive in case of detected amplification, negative otherwise). The amplification in positive partitions indicates the presence of one or more template molecules. It is assumed that distribution of template molecules over partitions is described appropriately by the Poisson distribution (16). This probability distribution is parametrized using only a single parameter, λ, which may be interpreted as the mean number of template molecules per partition. The relationship between

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λ, the number of positive partitions k and the total number of partitions n is as follows:

$$\lambda = -\log\left(1 - \frac{k}{n}\right) \tag{1}$$

Knowing the λ and the volume of the partition, v, the computation of the concentration of a template in the sample (c) seems to be straightforward ($c = \frac{\lambda}{V}$), yet methods of defining the uncertainty of λ or concentration are still emerging.
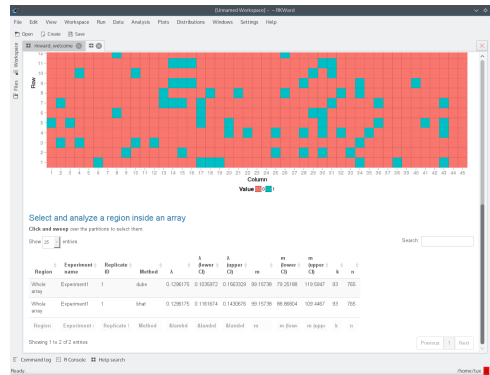
dPCR software landscape

The variety of existing procedures is disproportionate to the number of software packages dedicated to analysis of dPCR experiments. Most of the software consists of closed-source packages distributed by vendors of dPCR systems. The lack of open source code prohibits validation of the implementation of the assumed methodology and therefore has a limited usage in research. Moreover, vendors usually tightly tie software with their hardware, which prohibits comparisons of results from different systems in a uniform workflow.

The lack of a dedicated and open software for analysis of dPCR results leads to the rapid development of custom scripts in **Mathematica** (Wolfram Research) (27), **MS EXCEL** (Microsoft) (8) or **R** (9, 10, 28, 30). In addition, web-servers for very specific analysis of dPCR data, as the **definetherain** (14) and **ddpcr** (1), were developed. However, these tools have narrow use case scenarios, because they are tied to a specific task and in consequence cannot form the unified analysis framework.

The described situation is unfavorable for studies involving dPCR data or other digital assays. The absence of broader graphical user interfaces prohibits researchers not comfortable with the command line interfaces from using the custom-made frameworks. And those who are proficient in the command line interface heave to deal with a tedious task of integrating external code into their scripts. Others have to implement methods on their own, shifting their focus from an investigation of a scientific question to programming.

In 2013 we started the development of the *dpcR* framework to perform analysis of dPCR experiments (5). At this time *dpcR* was the first publically comprehensive package for the analysis and simulation dPCR data by Poisson statistics (17). We have chosen the **R** software environment (19), because it is already extensively used in bioinformatics and large parts of core, base and external **R** packages are peer-rewied. In particular, studies dealing with qPCR data (17, 23), both pre-processing (18, 22) and analysis (20, 21) have undergone this process. Furthermore, **R** is freely available for the MacOS, Linux/Unix and Windows operating systems.

As **R**'s command-line interface might not meet the needs of all users, hereby we present a web server *dpcReport*. It grants access to the most of *dpcR* functionalities through the Graphical User Interface (GUI) as a web server. *dpcReport* addresses both the chamber dPCR and the droplet dPCR in contrast to other implementations. To broaden even more the range of our software, *dpcReport* can be also installed as the stand-alone software in situations when using web servers is impossible or inadvisable.

We aimed for a form factor (e.g., smart phone, tablet, desktop PC) and operating system independent implementation of a graphical user interface. *dpcReport* integrates with all environments that support `HTML5` and `ECMAScript`, including not only into modern web browsers, but also **R** Integrated development environments (IDEs) such as **RKWard** (Figure 1) (24) or **RStudio**.

Figure 1. *dpcReport* running in the graphical user interface and integrated development environment **RKWard**.

STRUCTURE OF THE WEB SERVER

The workflow of *dpcReport* encloses complete data analysis, starting from data import and ending with report generation (Figure 2). We separate different tasks by panels: utility input/output functions are in "Input file" and "Save report" panels, basic descriptive statistics in the "Data summary" panel and multiple comparisons of experiments in the "Comparisons of runs" panel. More specific functions tied mostly to the quality control are in "Advanced analysis" panel. All panels are independent from each other, so the user interested only in computation of confidence intervals is not enforced to dive into other parts of the web server aside from the necessary data import. The modularity extends also to report generation, where user selects parts of analysis that should be saved. Although we strongly advise users to use the report generation functionality for sake of research reproducibility, all tables and figures may be also saved separately.

To standardize the notation used in our web server we followed dMIQE (Minimum Information for Publication of Quantitative Digital PCR Experiments) guidelines (12):

- λ: average molecule numbers per partition,

- k: number of molecules per partition,

- m: total number of the molecules.

The same notation is also consequently used in report and, whenever it is possible, in the **R** script generated during the analysis.

Data import

The first panel of GUI, "Input file", is responsible for loading the input data. As any processing of dpcR data is inevitably tied to the information loss, we suggest to start analysis with raw data rather than summaries exported by vendor-provided software. If it is not possible, part of *dcpReport* functionalities will be not available, but most of the analysis can be still successfully conducted.

Our GUI supports majority of common dPCR systems. It is extremely important that user specifies precisely which system generated the data, because it guarantees a proper import along with addition of necessary metadata as partition volume. To cover experimental or not yet included systems, we created a "raw data" format (described in GUI and Supplementary Files). The user can manually arrange his data in this format and import it to the *dpcR* package. Such input files can be created in a text editor, a spreadsheet program or using *dpcR* functions.

Imported datasets, regardless of their origin (array- or droplet-based dPCRs) and type (total number of positive partitions, states of partitions, CQs of partitions), are stored in the standardized class. Subclasses further define the more specific features of objects as for example the spatial organization of chambers in case of the array-based dPCR. This abstract representation allows creation of unified workflows that do not depend on a data source. Currently only one module (array spatial distribution analysis) is source-specific from obvious reasons.

It is possible to edit the input dataset using the mini-spreadsheet included in the "Input file" panel. All important features, starting with experiment names and ending with results, can be freely modified allowing manual correction of the input. A modified dataset may be downloaded from the application, but otherwise currently cannot be incorporated in the final report. However, the note about the manual modification of the input data is automatically added to the report.

Table 1. Structured vendor export data formats handled by *dpcReport*.

Vendor	System	Format	Type
Bio-Rad	QX100 & QX200	CSV	Summary export
Fluidigm	BioMark	CSV	Summary export
Formulatrix	Constellation Digital PCR	CSV	Summary export

The number of structured export data formats handled by *dpcR*. CSV, comma separated values.

Data summary

The descriptive summary of the data in form of interactive tables and plots is included in the panel "Data summary". Box-and-whisker diagrams cumulatively describe relationships between experiments and their λ value or concentration of the template, while scatter charts allow individual representation of runs.

To determine the uncertainty of the estimated λ we employ two previously published peer-reviewed methods The first

(11) uses the normal approximation to compute the confidence intervals for binomially distributed $\frac{k}{n}$. Next, the confidence intervals are altered using Equation **1**.

The other method (3) is based on the uncertainty of the measurement and includes also the uncertainty caused by the variation of volume. The exact formula for the uncertainty of the λ value, u_λ, is:

$$u_\lambda = \sqrt{\frac{k}{n^2(1 - \frac{k}{n})}} \tag{2}$$

and for the uncertainty of the concentration, u_c, of the template molecules:

$$u_c = C\sqrt{\left(\frac{1}{\log 1 - \frac{k}{n}}\sqrt{\frac{k}{n(n-k)}}\right)^2 + \left(\frac{u_V}{V}\right)^2} \tag{3}$$

where u_V is uncertainty of the volume.

Comparison of dPCR experiments

The third panel "Comparison of runs" facilitates comparison of multiple runs contained by the input dataset. In contrast to the previous panel which provides only descriptive statistics, here the can test against each other different values of λ. The proposed groups of runs are presented in tabular format and in charts.

The *dpcReport* workflow covers peer-reviewed methods of comparing results of dPCR experiments. Here, by the comparison we understand a procedure, where all data points from all runs are considered and affect the final outcome.

Two methods, GLM and MRT, conduct such analysis on the run level by comparing individual runs against each other (4). The GLM method is named after Generalized Linear Model, which is used to estimate the λ values for all runs. Next, results are compared against each other using the max-t test. The obtained p-values of comparison does not require further comparisons.

The MRT method estimates dissimilarities between runs using the uniformly most powerful ratio test to compare the value of λ between two runs. The uniformly most powerful test has the highest probability of rejecting the false null hypothesis among all tests with the same confidence level.

Since confidence intervals computed in this panel are adjusted for multiple comparison, they simultaneously contain the mean number of template molecules per partition of several experiments at a given significance level. This property makes them suitable for testing.

Advanced statistical analysis

The "Advanced analysis" panel consists of two more specialized functionalities related mostly to the quality control of the dPCR data.

Spatial distribution of positive droplets

Here an user may test the array data for randomness of spatial distribution of positive droplets over the panel using using
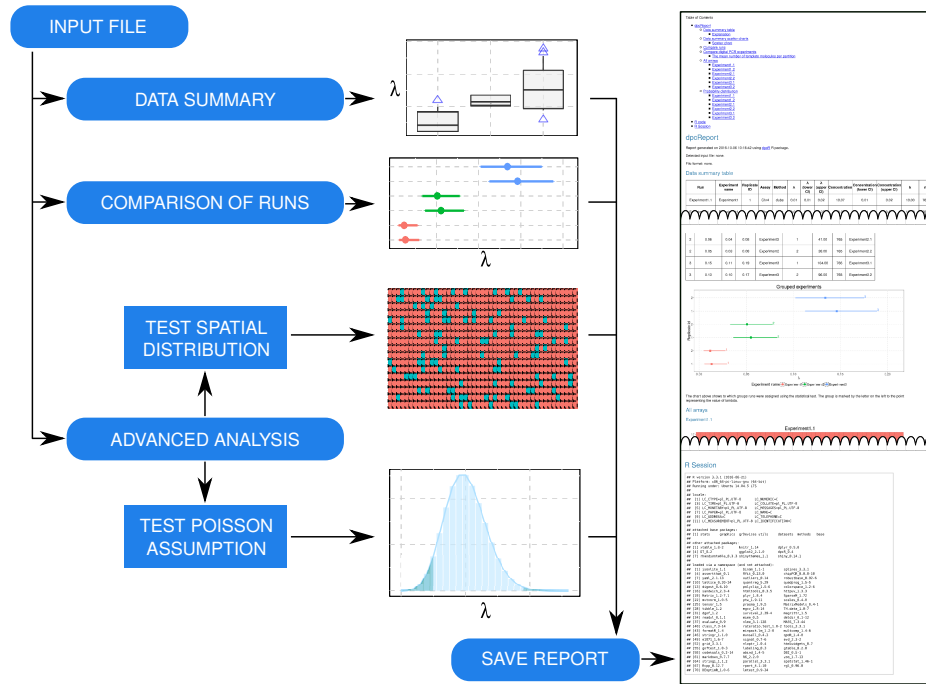
Figure 2. The diagram shows main parts of the modular *dpcReport* workflow (data summary, comparison of runs and advanced analysis). The analysis is concluded with report generation.

procedures belonging to spatial statistics. The dispersion test for spatial point pattern is based on quadrat counts and verifies if the status (positive, negative) of partition depends on its location (2). Any spatial dependency in data is contrary to commonly assumed uniformly random distribution of positive partitions over the plate and suggests that experiment The interactive visualization of the array allows analysis of whole plate or manually selected region

Distribution analysis

Table 2. Empirical and theoretical moments of the Poisson distribution. $\mu = \frac{1}{n}\sum_{i=1}^{n} x_i$, $m_j = \frac{1}{n}\sum_{i=1}^{n}(x_i - \mu)^j$, $s = \sqrt{m_2}$.

Moment	Empirical	Theoretical
Mean	μ	λ
Variance	s^2	λ
Skewness	$\frac{m_3}{s^3}$	$\frac{1}{\sqrt{\lambda}}$
Kurtosis	$\frac{m_4}{s^4}$	$\frac{1}{\lambda}$

The second part of the "Advanced analysis" panel is devoted to the investigation of distribution of the positive droplets in individual runs via computation of its confidence intervals and

its moments. The highly customizable functionalities allows choice between few implemented methods of confidence interval estimation for binomial variable.

We enable computation of four first moments (mean, variance, skewness, kurtosis) of the distribution from which the data is sampled. It is also possible to compare these empirical moments with theoretical moments of the Poisson distribution computed using the estimated λ (Table 2).

Save report

The last panel enables flexible report generation. The report can be customized by including abovementioned modules, which are equivalents of the GUI panels. The user input, including manually adjusted parameters of the test performed in the GUI, are preserved and returned in the report to increase the reproducibility of the research study. To limit the size of final report, we excluded from it tha data imported by the user. Instead, every report is enriched with the MD5 checksum of the input data allowing precise identification of the input file.

An important option of *dpcReport* is an export of the **R** script used for the report generation. Since *dpcReport* utilizes function from the *dpcR* package, whole analysis can be recreated in **R** environment. Therefore, by exporting **R** script the user virtually overcomes low reproducibility and challenging inter-exchange of analysis made in GUI. The

automatically generated code, aside from recreation of the analysis, may be also used by **R** proficient users to modify or improve the workflow, allowing for customization such details as figure colors and so on.

Since the constant evolution of *dpcR* and **R** may potentially affect results, the report is always complemented with the basic information about versions packages used by *dpcReport*.

DISCUSSION AND CONCLUDING REMARKS

The main focus of our package was to provide users of dPCR technology with a flexible workflow that incorporate most fundamental needs: estimation of the λ value, comparison of template concentration among several runs and quality control. The *dpcReport* web server provides researchers with means to analyze experiments and to verify their results. Being independent of the dPCR vendor, our web server helps in comparison of data coming from different systems.

dpcReport is dMIQE compliant, therefore it helps researchers in facing the challenge of standardization. All charts and tables produced by our workflow already follow the suggested convention, lessening the additional effort required to follow guidelines advised by the dPCR community.

The unique ability to generate **R** code increases reproducibility of analysis performed using *dpcReport* and let researchers share their own frameworks or easily make valuable supplemental materials. Moreover, every step of the analysis may be scrutinized and validated, because the source code is open and publicly available.

Considering all factors mentioned above, we have built what we believe to be the first unified, cross-platform and reproducible open source software framework for analyzing dPCR experiments.

Availability

dpcReport is avaible as a web server under http://smorfland.uni.wroc.pl/shiny/dpcReport. It is also available as the *dpcReport()* function in the *dpcR* **R** package. *dpcR* is available as an open source software package (GPL-3 or later). The stable version is hosted at http://cran.r-project.org/web/packages/dpcR and the source code is available from https://github.com/michbur/dpcR.

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