

dpcR a Swiss-army knife for the analysis of digital PCR experiments

Michał Burdukiewicz^{1,6}, Jim Huggett², Alexandra Whale², Bart K.M. Jacobs³, Lieven Clement³, Piotr Sobczyk¹, Andrej-Nikolai Spiess⁴, Valérie Taly⁵, Peter Schierack⁶, Stefan Rödiger^{6*}

¹Department of Genomics, Faculty of Biotechnology, University of Wrocław, Wrocław, Poland and ²Molecular and Cell Biology Team, LGC, Teddington, United Kingdom and ³Department of Applied Mathematics, Computer Science and Statistics, Ghent University, Belgium and ⁴University Medical Center Hamburg-Eppendorf, Hamburg, Germany and ⁵Université Paris Sorbonne Cité, Paris, France and ⁶Faculty of Natural Sciences, Brandenburg University of Technology Cottbus–Senftenberg, Großenhainer Str. 57, 01968, Senftenberg, Germany

Received January 1, 2009; Revised February 1, 2009; Accepted March 1, 2009

ABSTRACT

The digital Polymerase Chain Reaction (dPCR) enables an absolute quantification of nucleic acids. Different statistical analysis frameworks were proposed. However, most analysis is done in closed source software as provided by the vendors. This makes it harder to compare results, such as the confidence interval estimates. An unified open software framework for reproducible research is not available.

To perform dPCR analysis we implemented peer-review statistical methods and plots into the dpcR framework, based on the sophisticated statistical computing environment R. dpcR is versatile open source cross-platform software framework, which provides functions to process dPCR data independent of the hardware. Our software can be used for data analysis and presentation, as framework for novel technical developments and as reference for statistical methods in dPCR analysis. Features such as functions to estimate the underlying Poisson process, calculation of confidence intervals based on single samples as well as on replicates, a novel Generalized Linear Model-based procedure to compare dPCR experiments and a spatial randomness test for assessing plate effects have been integrated. We use a plug-in like architecture and abstraction layers to make the framework usable for droplets and (real-time) chamber based technologies.

dpcR is implemented with interfaces to the command-line, graphical user interfaces and interactive web application. Therefore, it can be used by novices in a graphical user interface or by experts via a command-line interface. The dpcR framework can be used to build a custom-made analyser according to the user requirements. dpcR is an open framework, which can be easily adapted to the growing knowledge in dPCR.

INTRODUCTION

The digital PCR (dPCR) is an important contender for precise nucleic acids quantifications. Application of the dPCR 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 the real-time quantitative qPCR (qPCR). Though, approaches based on isothermal amplification were also developed (20, 26, 30). 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 (33). In 1999 Vogelstein *et al.* (PNAS) described the first true dPCR (40). In contrast to qPCR, the amplification reaction does not take place in a single reaction chamber. Rather its a process of clonal amplification in small separate “partitions” (e.g., nl volume droplets of water oil emulsions, chambers on micro structured chips). The number of positive partition in relation to the number of total partitions. By applying Poisson statistics it is possible to determine the number of the starting material in given volume. Therefore, the dPCR does not require an external calibration (30, 35). Since approximately ten years the dPCR is gaining momentum in the mainstream user-base and will likely have the same impact as the qPCR methodology (14, 24, 30). 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.

The dPCR has some principle assumptions and fundamental properties. First of all the chemical reaction should be not affected by inhibitors. The distribution of the single molecule target regions follows a Poisson distribution. The Poisson distribution appears like a normal distribution but without negative values and being zero the lowest. First a large number (n) of amplifications reactions as required to have a high statistical power. Therefore, a high number of PCR

*To whom correspondence should be addressed. Tel: +49 357385936; Fax: +49 357385801; Email: stefan.roediger@b-tu.de

reactions is needed. For Poisson distributions an n of XY (get reference from table/text book from statistics/biostatistics?) is considered large. Second that the molecules required for the amplification reactions are randomly distributed in the compartments. Visual analysis, Ripley's K functions or ??? can be used to test for randomness of the reaction and thus to exclude the clustering of positive reactions. A clustering of positive wells might be due to sample loading or analysis process (systematical error). The outcome of an amplification can be no amplification at all (less than 1 copy per volume), an unsaturated reaction with a binary/“multinary” amplification (usable to calculate the “concentration”) or a saturated reaction where virtually all compartments are positive.

Chambers or emulsion based droplets are the dominant technical approaches to create partitions for dPCR reactions (24). However, other approaches based on beads were shown too (36). Chamber based dPCR systems have fixed geometries, including the volume of the reaction chambers. Despite the fact that dPCRs is an endpoint analysis the chamber based technologies allow generally the real-time monitoring of the amplification reaction and subsequent confirmation of the amplification reaction by melting curve analysis (22). Thus, such technologies enable easier trouble shooting and quality management of the data. However, the downside of these technologies is the fixed limited number of compartments and the price. The emulsion based dPCRs are easier to perform since the compartments are generated by microfluidic technologies and have practically no limitation regarding the number of compartments. This results in a higher statistical power to quantify small differences in sample quantities. The emulsion chambers are made of water-in-oil emulsions with similar sizes.

There is a need for an vendor independent data analysis. For example, others have written custom made scripts for data analysis in **Mathematica** (Wolfram Research), **MS EXCEL** (Microsoft) or **R** (8, 9, 38, 39). Recently, Mathew *et al.* published the open access bioinformatic pipeline, designated **definetherain** (17). The tool is coded in JavaScript and has been made available for free in a web browser. However, this is of limited use, since the solutions are tied to a specific dPCR platform (e.g., droplet dPCR by Bio-Rad), operating system platform for data analysis and only usable for a single task. Moreover, we found no software packages with GUIs and bindings to a sophisticated statistical computing environment for reproducible research.

We have developed the framework *dpcR* to perform analysis of dPCR experiments for **R** software that is widely used for statistical analysis of biomedical data and is freely available for the MacOS, Linux/Unix and Windows operating systems. *dpcR* may be used in conjunction with literate programming **R** packages (e.g., *knitr*) and tools for reproducible research (e.g., *rtrack*) (19, 30).

MATERIALS AND METHODS

Implementation

We have chosen **R** because it is cross-platform and the *lingua franca* in applied statistical bioinformatics. Since all software is open source it is possible to track numerical errors (30, 32).

Most **R** packages depend on other packages (25). The same holds true for *dpcR*. This results in a complex network of recursive dependencies (Figure 1A). Core packages include *qpcR* (28), *shiny* (7), *MBmca* (29), *chipPCR* (31). A basic design decision was to structure specific properties of dPCR systems (droplet vs. chamber) in auxiliary functions. Selected chamber dPCR systems rely on the proper preprocessing of qPCR data. This functionality is inherited from the *qpcR* **R** package.

The naming convention of *dpcR* is `underscore_sep` (1). The main *dpcR* functions (e.g., for analysis, simulations, plotting), several auxiliary functions (e.g., data import) and datasets of different dPCR systems are shown in the workflow of Figure 2. Figure 1B illustrates the implementation of the *dpcR* package. The function *read_dpcr*, *qpcr2pp* and *sim_dpcr*, sets the input for all objects in the `dpcr` class. Central calculation specific to Poisson statistics in are performed independently in main functions. This class manages details of the dPCR analysis that are subsequently processed by *dpcR* functions (e.g., reading/writing). Further details are explained in the supplementary information.

Figure 2 provides an overview of important functions available at each step of a dPCR analysis. The first step is to import sample data into the **R** session. *dpcR* accepts various data structures. This includes matrices of raw data and predefined structures provided by the different vendors (see Table 1). Moreover, *dpcR* accepts objects from the **R** workspace, which are converted via the *read_dpcr* function. Novel raw data structures are processed outside *dpcR* using tools as described elsewhere. The data input to *dpcR* should be raw data preferable, rather than gated summary data. The reasoning is to keep control over information loss for reproducible research.

Documentation

All functions of the *dpcR* package have its own documentation package, which specifies the input types, classes, parameters and output formats. The documentation is available as standard **R** package reference manual and as vignette.

According to the dMIQE guidelines (13) we used following notation:

- λ : average molecule numbers per partition,
- k : number of molecules per partition,
- m : total number of the molecules.

The following convention was kept, both in the documentation and in the package source code.

Import and export of results figures and data

Reading or importing data **R** has a rich set of tool to arrange data (reshape (43)) in order to prepare them for the analysis. This is important when it comes to the question how experiments should be treated.

Most *dpcR* import functions take a data matrix of annotated numeric (e.g. fluorescence amplitude) and integer values (e.g., counts) as input. The *raw_data* input was implemented make data input easier for the end user (see Supplement). Such input

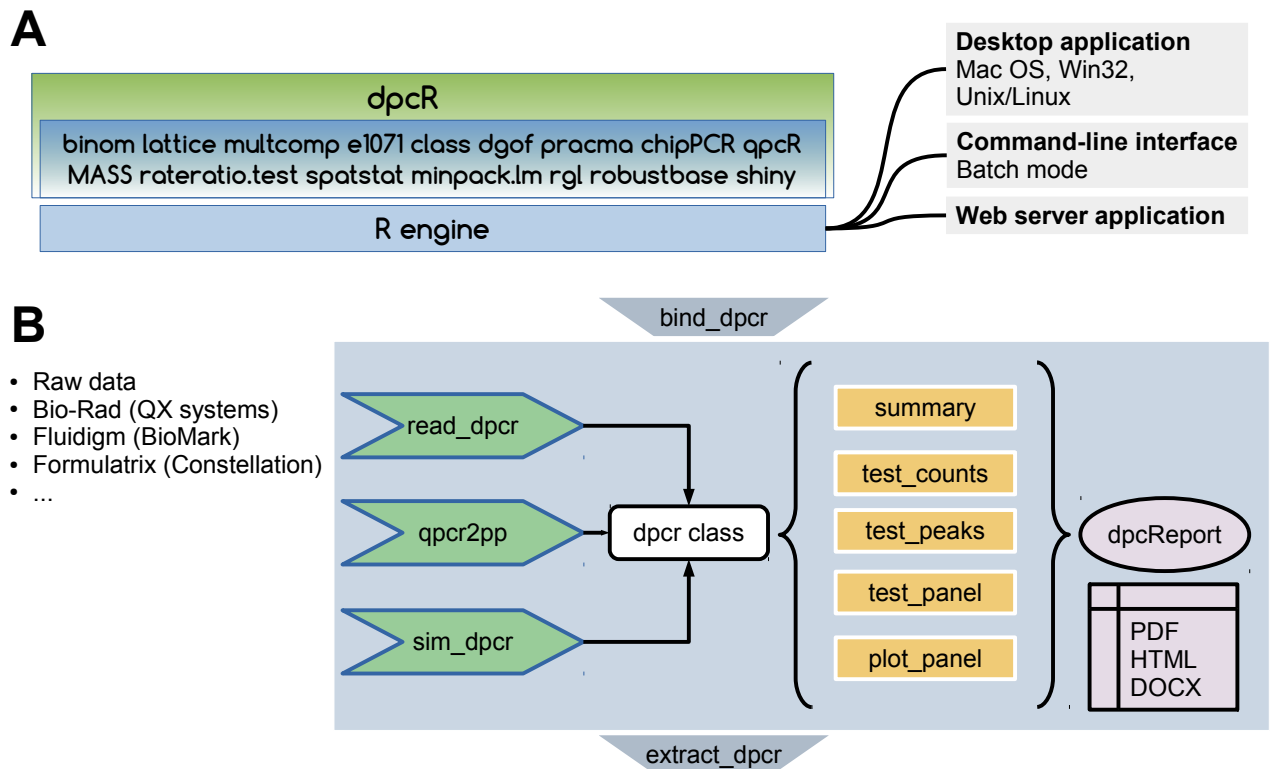


Figure 1. Implementation of the *dpcR* package. **(B)** Modular software framework structure. *dpcR* is typically run from a desktop computer or a server. The software can be operated by an GUI/IDE application such as **RStudio** or **RKward**. The *dpcR* package has dependencies to other **R** packages (middle layer). The functionality shared between the packages enables repaid addition and expansion of functionality.

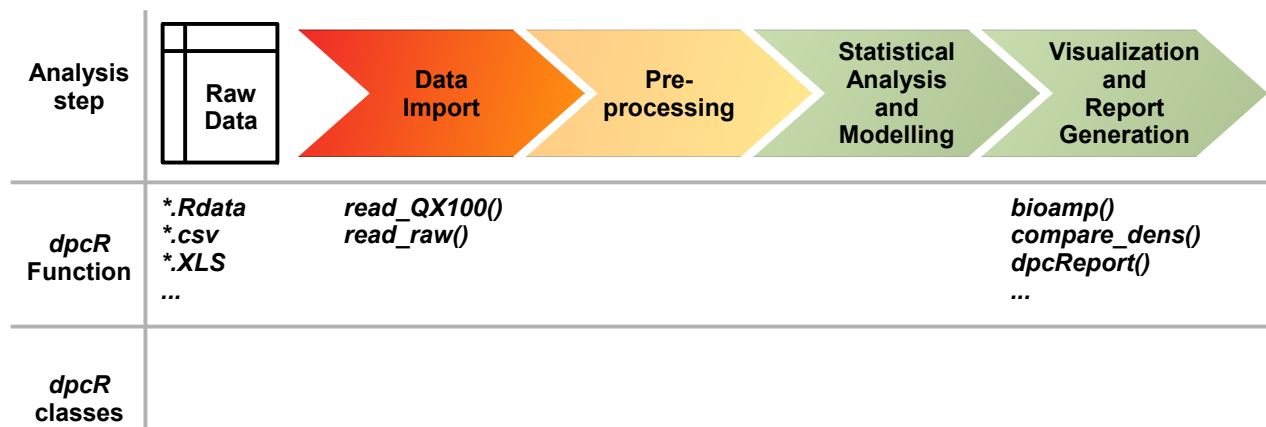


Figure 2. *dpcR* workflow. The diagram shows main functions available at each step of a dPCR data analysis.

files can be manually created in a spreadsheet program or text editor.

As described in (26, 27, 28, 30), there numerous methods to import amplification curve data into the **R** workspace. Prior processing the data for dPCR analysis it is recommended to use dedicated **R** packages, such as *chipPCR* (31), *MBmca* (29), *qpcR* (28), to pre-process (e.g., removal of missing values, smoothing (37)) the raw data.

(see Table 1).

De novo creation of dpcR objects from raw data In experimental setups user have the need to transform their raw data in a processable format. Since the **R** environment is cross-platform an ubiquitously used we aimed to ease this creation. In particular, this is relevant for reproducible research. The *dpcR* framework covers the types typically used in laboratories.

Public data sets *dpcR* includes data sets or refers to additional **R** packages for testing purposes. The data originate from

different dPCR and qPCR systems and were either published previously (30, 31, 41, 42) or *de novo* generated.

Export of analysis results Since *dpcR* is based on the **R** environment all facilities for a report generation are usable as described before (30).

(see Table 1).

A commonly available data exchange format is a prerequisite for reproducible research. So far no cross-platform and system-independent format has been introduced for dPCR experiments. Therefore, we decided to use **RData** as our default format. **RData** works across all **R** environments and saves the variable names along the content of multiple variables, which can be restored in any workspace.

Calculation of the uncertainty

To determine the uncertainty of the estimated λ we employ two previously published peer-reviewed methods (4, 10).

PUTDUBEANDBHATEQUATIONSHERE

Poisson distribution

Moments It is assumed that results from the dPCR reaction follow the Poisson distribution. We implemented several functions for computation of the four first moments (mean, variance, skewness, kurtosis) of the distribution from which the data is sampled. They can be calculated empirically from the sample data or as functions of the estimated λ .

Binomial approximation Since dPCR data contain binarized Poisson counts (all counts than zero are grouped together) it is possible to use methods appropriate for Binomial distribution. We employ several methods of computing confidence intervals (CI) for λ value (e.g., Wilson method, Agresti-Coull method). They especially advantageous when k is considerably smaller than m . They are beneficial for uncertainty calculation, because yielded CI's are not oscillating in such scenarios (6).

Simulation

The package covers two Simulation methods as described elsewhere in peer-reviewed publications. The method proposed by Dube *et al.* 2008 (10) is based on distributing template molecules over partitions. The procedure in Jacobs *et al.* 2014 (16) is more sophisticated and returns the fluorescence value obtains from droplet dPCR experiment including such phenomena as “rain”. We also added streamlined and high-performing simulation in assumptions similar to Dube *et al.* 2008.

Table 1. Structured vendor export data formats handled by *dpcR* v. 0.5 and later.

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* is growing. Numerous data formats can be processed with the functionality provided by the **R** environment (see (30)). CSV, comma separated values.

Comparison of dPCR experiments

Generalized Linear Models – GLM Generalized Linear Models (GLM) are linear models for data, where the response variables may have non-normal distributions (as binomially distributed positive partitions in dPCR experiments). We employ a simplistic model reflecting relationships in results of dPCR as given by:

$$\log Y = \beta^T X \quad (1)$$

where Y are counts, X are experiments names (categorical data) and β are coefficients for every run. Moreover, $\exp \beta = \lambda$. Estimated means copies per partitions obtained from the model are compared each other using multiple t-test (5).

Since the dPCR data is binomial, we advise to use binomial regression. It is also possible to choose the Poisson regression, but such usage should be limited to experimental settings where the concentration of template molecules in samples is small (positive partitions contain very rarely more than 1 template particle).

Multiple testing The dPCR experiments are compared pairwise using the uniformly most powerful (UMP) ratio test (11). Furthermore, computed p-values are adjusted using Benjamini-Hochberg correction (3) to control family-wise error rate. The UMP ratio test has following null-hypothesis:

$$H_0: \frac{\lambda_1}{\lambda_2} = 1 \quad (2)$$

The Wilson's confidence intervals (6) are calculated independently for every dPCR experiment and adjusted using Dunn – Šidák correction, where:

$$\alpha_{\text{adj}} = 1 - (1 - \alpha)^{\frac{1}{T}} \quad (3)$$

Such intervals are wider then usual, but ensure that confidence intervals simultaneously contain the true value of λ . For example, the 0.95 CI means that with 0.95 probability of λ is in the range of the confidence intervals.

Spatial distribution

Array based dPCR experiments provide information about spatial distribution of partitions. Procedures belonging to spatial statistics verify if the status (positive, negative) of partition depends on its location. To address such questions, we implemented a Complete Spatial Randomness test for dPCR arrays. Moreover, we provide functionalities to export array dPCR data to **R** packages specialized in spatial statistics as described in (2).

Converting qPCRs to dPCRs

High-Throughput qPCR is a well established and robust technology, which allows precise quantification of DNA material in high throughput fashion. However, the quantification by qPCR is challenging at very low and

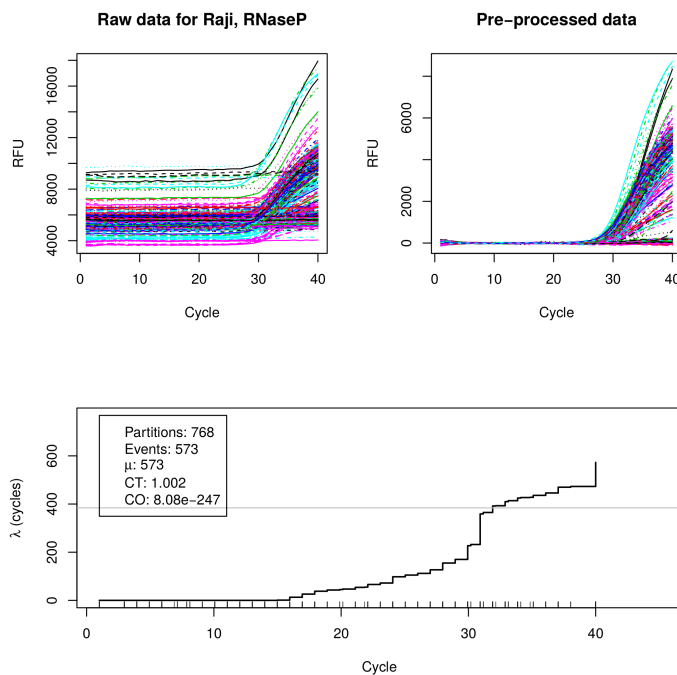


Figure 3. Uncover characteristics of dPCR data. Selected dPCR platforms are qPCR platforms at the same time. The function *qpcr2pp* uses the qPCR amplification curve data and interprets them as dPCR (Poisson process). A) Raw data of The function were B) preprocessed (baselined, smoothed) with functions from the *chipPCR* package and C) finally analysed (Cq calculation → binarize) with the *qpcr2pp* (qPCR to Poisson process) function from the *dpcR* package.

very high concentrations. In addition, pre-processing and data analysis is affected by numerous adverse effects (26, 34, 37). But the amplification curve real-time monitoring of the PCR product formation enables to determine quantification points (Cq), which can be binarized and analysed in the same way as dPCR experiments (22). This functionality is integral part of the *dpcR* package.

Figure3

RESULTS

In the following section we show applications of the *dpcR* package.

Evaluation of dPCR comparison methods

Two implemented approaches, GLM and multiple testing (MT) were compared over 15×10^5 simulated array dPCR experiments. Each simulation contained six reactions. Three of them had roughly the same amount of molecules per plate and other three had experiments with 10 to 50 molecules more. The GLM approach used binomial model and multiple testing framework employed ratio test.

A single GLM comparison took roughly 183 times longer than MT (on average 1.10 seconds versus 0.006 seconds on the Intel i7-2600 processor). The difference grows with the number of experiments and number of partitions (data not shown).

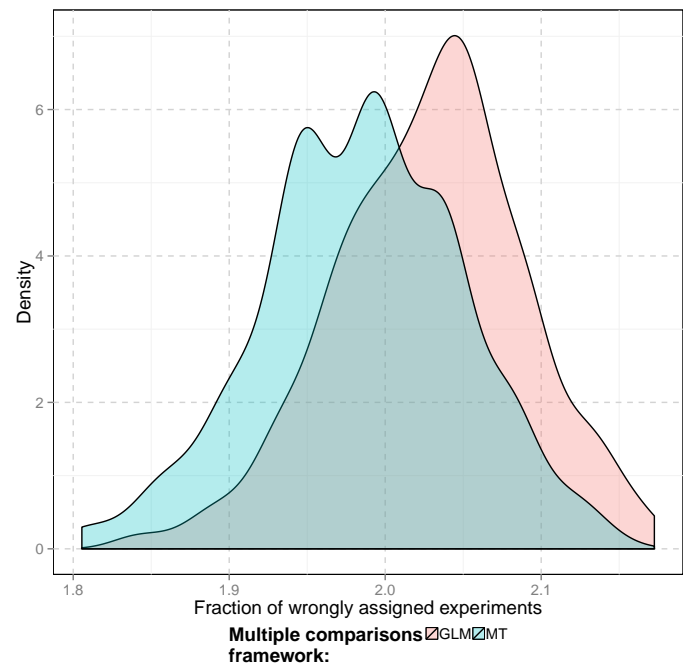


Figure 4. Results of simulation. Multiple testing (MT) and generalized linear model (GLM) were compared in simulation study.

On average, 2.03 and 1.98 reactions were assessed to a wrong group by respectively GLM and MT (Figure 4).

Simultaneous confidence intervals

Average coverage probability is the proportion of the time that the interval contains the true value of λ .

In Figure 5, we show the results of simulated 1×10^6 droplet dPCR experiments (2×10^4 droplets each) for each level of λ (1.2×10^7 experiments total). We computed average probability coverage of CI obtained by three methods: Dube's (10), Bhat's (4) and by our MT ($\alpha = 0.95$).

To assess simultaneous coverage probability, we randomly divided experiments into 2.000 groups (500 experiments each) for each possible value of λ . We counted frequency of groups in which all confidence intervals contain the true value of λ .

Graphical user interface

The critical functionalities of *dpcR* package are implemented in the *dpcReport* GUI. We aimed for a form factor (e.g., smart phone, tablet, desktop PCR) and operating system independent implementation of a graphical user interface. *dpcReport* is based on *shiny* technology and offers an intuitive user interface, which can be accessed by browsers (e.g., Google Chrome, Mozilla Firefox). The advanced plots are based on *ggplot2* (44).

The first panel “Input file” is responsible for loading the input data. Here part of data input structure (e.g., ID of experiments) may be accessed and modified during the analysis in the GUI. The second panel “Data summary” presents descriptive summary of the data in form of interactive tables and plots, offering filtering and selecting of individual

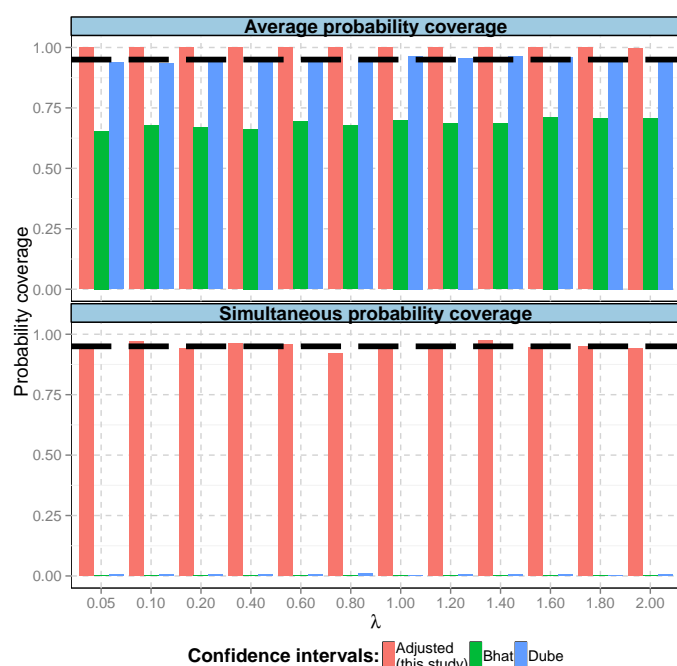


Figure 5. Evaluation of confidence intervals implemented in the *dpcR* package. Average coverage probability is the proportion of the time that the interval contains the true value of λ .

runs. The third panel “Comparison of runs” compares all runs of the experiment data. The fourth panel “Advanced analysis” consists of more specialized functionalities as testing individual arrays. All statistical methods used in the GUI are integral part of the *dpcR* package and described in the methods section. The last panel enables flexible report generation. The report can be customized by including various sections, which are equivalents of the GUI panels.

An interesting feature of the *shiny* technology is the automatic integration in environments, which support HTML5 and ECMAScript. The *dpcReport* integrates into Integration in third party software. This can be a modern web browser or an **R** IDE/GUI such as **RKward** (Figure 6) (32) or **RStudio**. *dpcReport* is a GUI tool for dPCR data mining and report generation. User can interact via a point-and-click interface on different tabs, which contain widgets such as sliders, input fields and check boxes. Other user input, such as parameters of test performed in GUI, are preserved and returned in the report to increase the reproducibility of the research study. The tabs cover relevant analysis steps for the report generation. An important option of *dpcReport* is an export of the **R** source code used for the report generation is provided to the user. This code can be used for recreating the analysis in the **R** environment or prototyping more complicated workflows.

Automatic report generation

In (30) we gave an example where we re-analyze droplet dPCR data from a Bio-Rad QX100 system with an early implementation of the *dpcR* package.

Availability

The *dpcR* framework is available as open source software package (GPL-3 or later) as part of the Bioconductor project (12). 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>.

DISCUSSION

Currently, there exist different dPCR analysis software solutions provided by the vendors. But most of the software packages are black boxes, which prevent deep insight into the data processing step. Other and we think that scientific software should be open (15, 23, 30). In addition, most of the software solutions are aimed to be used in very specific scenarios and a mutual exclusive to alternative platforms (e.g., droplet vs. chamber-based). We have chosen **R** because it is the *lingua franca* in biostatistics and broadly used in other disciplines (30). We developed the *dpcR* package, which is a software framework for analysis of dPCR. *dpcR* provides the scientific community a broadly applicable tool for teaching purposes, data analysis and theoretical research based on simulations. Our software framework can be used to accelerate the development of new approaches to dPCR.

Functions included may be used to simulate dPCRs, perform statistical data analysis, plotting of the results and simple report generation.

CONCLUSION

In conclusion, *dpcR* provides means to understand how dPCR works, to design, simulate and analyze experiments, and to verify their results (e.g., confidence interval estimation), which should ultimately improve reproducibility. We have built what we believe to be the first unified, cross-platform, dMIQE compliant, open source software framework for analyzing dPCR experiments. Our *dpcR* framework is targeted at a broad user base including end users in clinics, academics, developers, and educators. We implemented existing statistical methods for dPCR and suggest the introduction of a standardized dPCR nomenclature. Our framework is suitable for teaching and includes references for an elaborated set of methods for dPCR statistics. Our software can be used for (I) data analysis and visualization in research, (II) as software framework for novel technical developments, (III) as platform for teaching this new technology and (IV) as reference for statistical methods with a standardized nomenclature for dPCR experiments. The framework enables the simulations and predictions of Poisson distribution for dPCR scenarios, the analysis of previously run dPCRs. Due to the plug-in structure of the software it is possible to build custom-made analyzers.

We decided not to implement algorithms for clustering and “rain” (positive droplets) definition of droplet dPCR data. This is because, there are several **R** packages from flow-cytometer research. Implementations range from manual to automatic clustering (18, 21, 39). Moreover, discussion with our peers and the literature suggest that a consensus of an appropriate method for dPCR is not available.

Our open framework includes to invitation to the scientific community to join and support the development of *dpcR*.

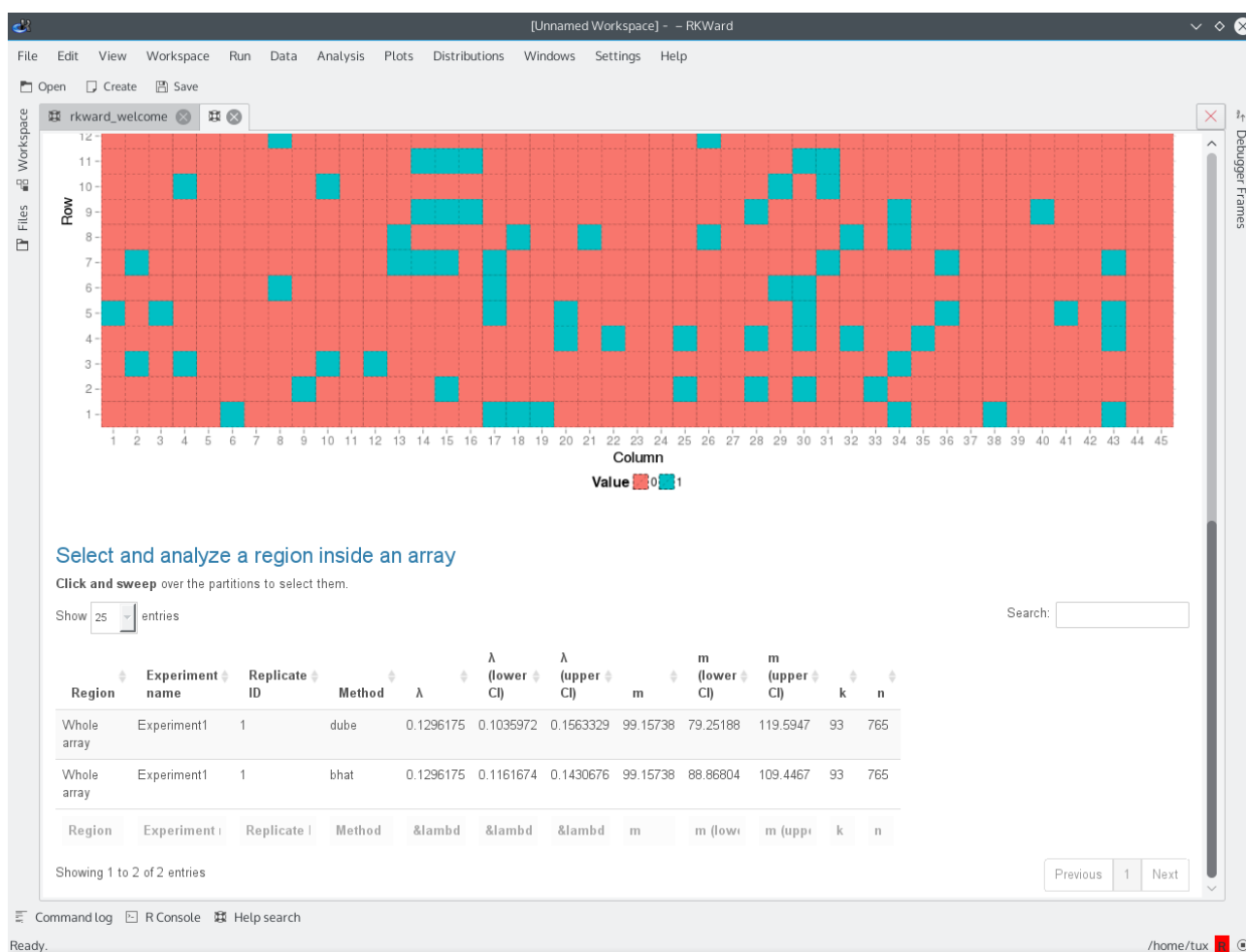


Figure 6. *dpcrReport()* function running in the graphical user interface and integrated development environment RKward.

ACKNOWLEDGEMENTS

Grateful thanks belong to the **R** community and the **RStudio** developers.

FUNDING

This work was funded by the Federal Ministry of Education and Research (BMBF) InnoProfile–Transfer–Projekt 03 IPT 611X.

Conflict of interest statement. None declared.

REFERENCES

- Rasmus Bååth. The State of Naming Conventions in R. *The R Journal*, 4(2):74–75, December 2012.
- Adrian Baddeley and Rolf Turner. spatstat: An R package for analyzing spatial point patterns. *Journal of Statistical Software*, 12(6):1–42, 1 2005.
- Yoav Benjamini and Yosef Hochberg. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, 57(1):289–300, 1995.
- Somanath Bhat, Jan Herrmann, Paul Armishaw, Philippe Corbisier, and Kerry R Emslie. Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number. *Analytical and Bioanalytical Chemistry*, 394(2):457–467, May 2009.
- Frank Bretz, Torsten Hothorn, and Peter Westfall. *Multiple Comparisons Using R*. Chapman & Hall/CRC Press, Boca Raton, Florida, USA, 2010.
- Lawrence D. Brown, T. Tony Cai, and Anirban DasGupta. Interval estimation for a binomial proportion. *Statist. Sci.*, 16(2):101–133, May 2001.
- Winston Chang, Joe Cheng, JJ Allaire, Yihui Xie, and Jonathan McPherson. *shiny: Web Application Framework for R*, 2015. R package version 0.12.2.
- David Dobnik, Björn Spilsberg, Alexandra Bogoalec Koir, Arne Holst-Jensen, and Jana el. Multiplex Quantification of 12 European Union Authorized Genetically Modified Maize Lines with Droplet Digital Polymerase Chain Reaction. *Analytical Chemistry*, 87(16):8218–8226, August 2015.
- Tanja Dreö, Manca Pirc, Iva Ramak, Jernej Pavi, Mojca Milavec, Jana el, and Kristina Gruden. Optimising droplet digital PCR analysis approaches for detection and quantification of bacteria: a case study of fire blight and potato brown rot. *Analytical and Bioanalytical Chemistry*, 406(26):6513–6528, August 2014.
- Simant Dube, Jian Qin, and Ramesh Ramakrishnan. Mathematical analysis of copy number variation in a DNA sample using digital PCR on a nanofluidic device. *PloS one*, 3(8):e2876, 2008.
- Michael Fay. Two-sided exact tests and matching confidence intervals for discrete data. *Proceedings of the National Academy of Sciences of the United States of America*, 2(1):53–58, June 2010.
- Robert C Gentleman, Vincent J Carey, Douglas M Bates, Ben Bolstad, Marcel Dettling, Sandrine Dudoit, Byron Ellis, Laurent Gautier,

8 *Nucleic Acids Research*, 2009, Vol. 37, No. 12

- Yongchao Ge, Jeff Gentry, Kurt Hornik, Torsten Hothorn, Wolfgang Huber, Stefano Iacus, Rafael Irizarry, Friedrich Leisch, Cheng Li, Martin Maechler, Anthony J Rossini, Gunther Sawitzki, Colin Smith, Gordon Smyth, Luke Tierney, Jean Y H Yang, and Jianhua Zhang. Bioconductor: open software development for computational biology and bioinformatics. *Genome biology*, 5(10):R80, 2004.
13. Jim F Huggett, Carole A Foy, Vladimir Benes, Kerry Emslie, Jeremy A Garson, Ross Haynes, Jan Hellemans, Mikael Kubista, Reinhold D Mueller, Tania Nolan, Michael W Pfaffl, Gregory L Shipley, Jo Vandesompele, Carl T Wittwer, and Stephen A Bustin. The digital MIQE guidelines: Minimum information for publication of quantitative digital PCR experiments. *Clinical Chemistry*, 59(6):892–902, June 2013.
14. Jim F. Huggett, Justin OGrady, and Stephen Bustin. qPCR, dPCR, NGS A journey. *Biomolecular Detection and Quantification*, 3:A1–A5, March 2015.
15. Darrel C. Ince, Leslie Hatton, and John Graham-Cumming. The case for open computer programs. *Nature*, 482(7386):485–488, February 2012.
16. Bart KM Jacobs, Els Goetghebeur, and Lieven Clement. Impact of variance components on reliability of absolute quantification using digital pcr. *BMC Bioinformatics*, 15(1):283, 2014.
17. Mathew Jones, James Williams, Kathleen Gärtner, Rodney Phillips, Jacob Hurst, and John Frater. Low copy target detection by Droplet Digital PCR through application of a novel open access bioinformatic pipeline, definetherain. *Journal of Virological Methods*, 202(100):46–53, June 2014.
18. Nolwenn Le Meur. Computational methods for evaluation of cell-based data assessmentBioconductor. *Current Opinion in Biotechnology*, 24(1):105–111, February 2013.
19. Zhifa Liu, Stan Pounds, Zhifa Liu, and Stan Pounds. An R package that automatically collects and archives details for reproducible computing. *BMC Bioinformatics*, 15(1):138, May 2014.
20. Andrew T. Ludlow, Jerome D. Robin, Mohammed Sayed, Claudia M. Litterst, Dawne N. Shelton, Jerry W. Shay, and Woodring E. Wright. Quantitative telomerase enzyme activity determination using droplet digital PCR with single cell resolution. *Nucleic Acids Research*, 42(13):e104–e104, July 2014.
21. Mehroush Malek, Mohammad Jafar Taghiyar, Lauren Chong, Greg Finak, Raphael Gottardo, and Ryan R. Brinkman. flowdensity: reproducing manual gating of flow cytometry data by automated density-based cell population identification. *Bioinformatics*, 31(4):606–607, 2015.
22. Mitra Mojtahedi, Aymeric Fouquierd’Hroul, and Sui Huang. Direct elicitation of template concentration from quantification cycle (Cq) distributions in digital PCR. *Nucleic Acids Research*, 42(16):e126–e126, September 2014.
23. A. Morin, J. Urban, P. D. Adams, I. Foster, A. Sali, D. Baker, and P. Sliz. Shining Light into Black Boxes. *Science*, 336(6078):159–160, April 2012.
24. Alexander A. Morley. Digital PCR: A brief history. *Biomolecular Detection and Quantification*, 1(1):1–2, 2014.
25. Jeroen Ooms. Directions for improved dependency versioning in R. *The R Journal*, 5(1):197–207, 2013.
26. Stephan Pabinger, Stefan Rödiger, Albert Kriegner, Klemens Vierlinger, and Andreas Weinhäusel. A survey of tools for the analysis of quantitative PCR (qPCR) data. *Biomolecular Detection and Quantification*, 1(1):23–33, 2014.
27. James R. Perkins, John M. Dawes, Steve B. McMahon, David LH Bennett, Christine Orenge, and Matthias Kohl. ReadqPCR and NormqPCR: R packages for the reading, quality checking and normalisation of RT-qPCR quantification cycle (Cq) data. *BMC Genomics*, 13(1):296, July 2012.
28. Christian Ritz and Andrej-Nikolai Spiess. qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. *Bioinformatics*, 24(13):1549–1551, January 2008.
29. Stefan Rödiger, Alexander Böhm, and Ingolf Schimke. Surface Melting Curve Analysis with R. *The R Journal*, 5(2):37–53, December 2013.
30. Stefan Rödiger, Michał Burdukiewicz, Konstantin A. Blagodatskikh, and Peter Schierack. R as an Environment for the Reproducible Analysis of DNA Amplification Experiments. *The R Journal*, 7(2):127–150, 2015.
31. Stefan Rödiger, Michał Burdukiewicz, and Peter Schierack. chipPCR: an R package to pre-process raw data of amplification curves. *Bioinformatics*, 31(17):2900–2902, 2015.
32. Stefan Rödiger, Thomas Friedrichsmeier, Prasenjit Kapat, and Meik Michalke. RKWard: A Comprehensive Graphical User Interface and Integrated Development Environment for Statistical Analysis with R. *Journal of Statistical Software*, 49(9):1–34, 2012.
33. G. Ruano, K. K. Kidd, and J. C. Stephens. Haplotype of multiple polymorphisms resolved by enzymatic amplification of single DNA molecules. *Proceedings of the National Academy of Sciences*, 87(16):6296–6300, January 1990.
34. Jan M Ruijter, Michael W Pfaffl, Sheng Zhao, Andrej N Spiess, Gregory Boggy, Jochen Blom, Robert G Rutledge, Davide Sisti, Antoon Lievens, Katleen De Preter, Stefaan Derveaux, Jan Hellemans, and Jo Vandesompele. Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications. *Methods (San Diego, Calif.)*, 59(1):32–46, 2013.
35. David A. Selck, Mikhail A. Karymov, Bing Sun, and Rustem F. Ismagilov. Increased Robustness of Single-Molecule Counting with Microfluidics, Digital Isothermal Amplification, and a Mobile Phone versus Real-Time Kinetic Measurements. *Analytical Chemistry*, 85(22):11129–11136, November 2013.
36. Joe Shuga, Yong Zeng, Richard Novak, Qing Lan, Xiaojiang Tang, Nathaniel Rothman, Roel Vermeulen, Laiyu Li, Alan Hubbard, Luoping Zhang, Richard A. Mathies, and Martyn T. Smith. Single molecule quantitation and sequencing of rare translocations using microfluidic nested digital PCR. *Nucleic Acids Research*, 41(16):e159–e159, January 2013.
37. Andrej-Nikolai Spiess, Claudia Deutschmann, Michał Burdukiewicz, Ralf Himmelreich, Katharina Klat, Peter Schierack, and Stefan Rödiger. Impact of Smoothing on Parameter Estimation in Quantitative DNA Amplification Experiments. *Clinical Chemistry*, 61(2):379–388, January 2015.
38. Matthew C Strain, Steven M Lada, Tiffany Luong, Steffney E Rought, Sara Gianella, Valeri H Terry, Celsa A Spina, Christopher H Woelk, and Douglas D Richman. Highly precise measurement of HIV DNA by droplet digital PCR. *PloS one*, 8(4):e55943, 2013.
39. Wim Trypsteen, Matthijs Vynck, Jan De Neve, Pawel Bonczkowski, Maja Kiselina, Eva Malatinkova, Karen Vervisch, Olivier Thas, Linos Vandekerckhove, and Ward De Spiegelaere. ddpcRquant: threshold determination for single channel droplet digital PCR experiments. *Analytical and Bioanalytical Chemistry*, 407(19):5827–5834, July 2015.
40. Bert Vogelstein and Kenneth W Kinzler. Digital PCR. *Proceedings of the National Academy of Sciences of the United States of America*, 96(16):9236–9241, August 1999.
41. Alexandra S Whale, Jim F Huggett, Simon Cowen, Valerie Speirs, Jacqui Shaw, Stephen Ellison, Carole A Foy, and Daniel J Scott. Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. *Nucleic Acids Research*, 40(11):e82, June 2012.
42. Richard A White, 3rd, Paul C Blainey, H Christina Fan, and Stephen R Quake. Digital PCR provides sensitive and absolute calibration for high throughput sequencing. *BMC genomics*, 10:116, 2009.
43. Wickham and Hadley. Reshaping data with the reshape package. *Journal of Statistical Software*, 21(12), 2007.
44. Hadley Wickham. *ggplot2: elegant graphics for data analysis*. Springer New York, 2009.