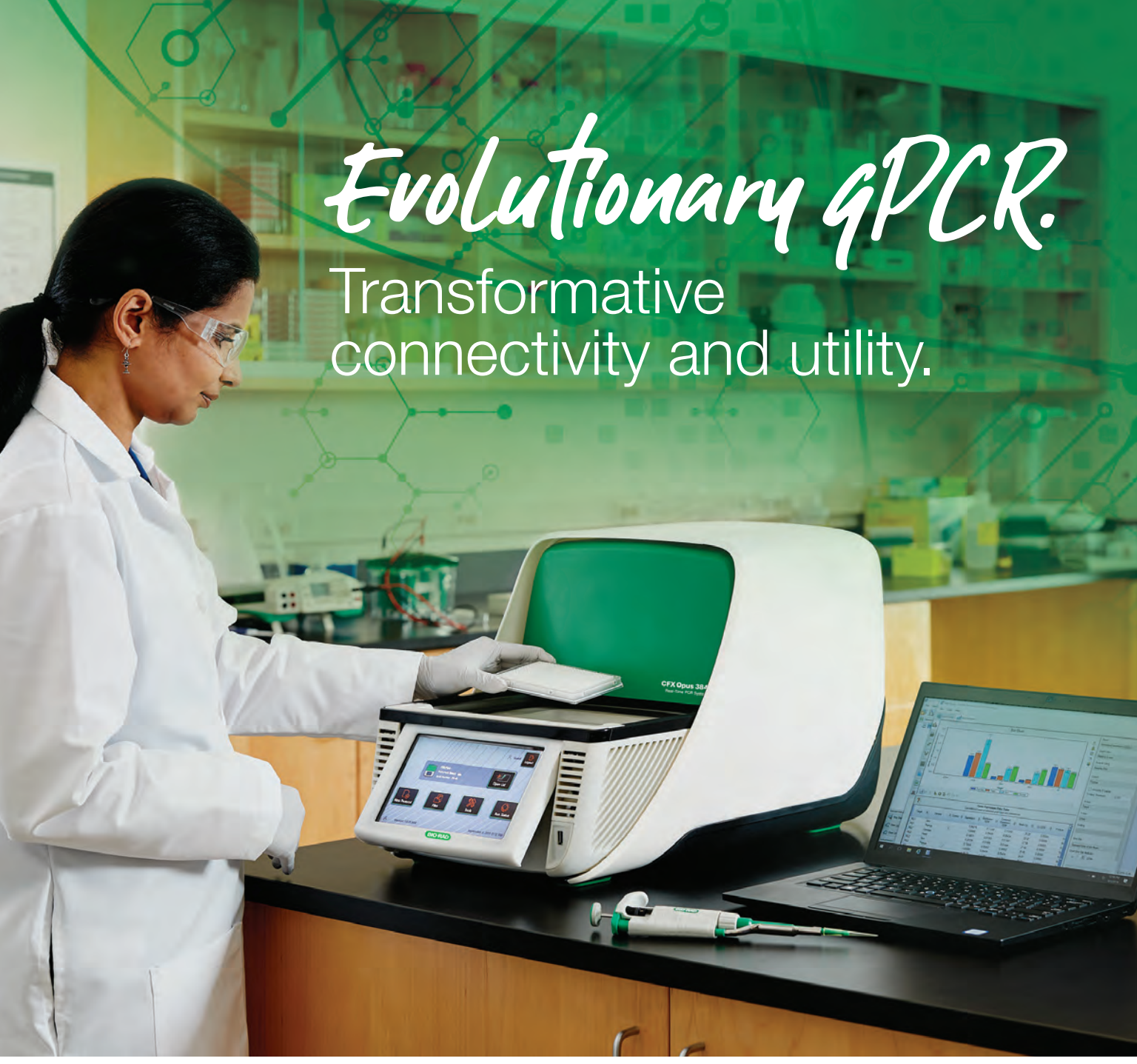
STREAMLINING qPCR FOR REPRODUCIBLE RESEARCH AND DIAGNOSTICS RESULTS

Custom Publishing From

TheScientist
EXPLORING LIFE, INSPIRING INNOVATION

Sponsored By

BIO-RAD



Introducing the *new* CFX Opus Real-Time PCR Systems

At Bio-Rad, we believe that if you can make something better, you should.

So we're introducing the CFX Opus Systems. They have the reliable features you know and expect from Bio-Rad, including differentiated Bio-Rad optics and CFX Maestro Software, plus improvements in design, functionality, and connectivity. CFX Opus Systems are also fully integrated with the new BR.io cloud platform for experimental setup and data management. It's the perfect combination of everything you expect and everything you want for the modern lab.

Are the CFX Opus Systems right for you?
Visit bio-rad.com/CFXOpus to schedule a demo.

#ScienceForward

BIO-RAD

ESTABLISHING qPCR BEST PRACTICE GUIDELINES

Quantitative real-time PCR (qPCR)[1] lets scientists detect and measure small amounts of nucleic acids from a wide range of sources. qPCR is noted for its simplicity in concept and execution, its relatively low cost, and its speed, sensitivity, and specificity. As such, it has become a driving technology for nucleic acid quantification in research, clinical, and industry sectors around the world. A recent survey of the academic literature found that researchers from 184 different countries and territories have published at least one journal article using qPCR.[2]

qPCR's widespread popularity has also brought an inordinate array of different protocols, with diversity in reagents, cycle steps, analysis methods, and reporting formats. This lack of uniformity and consensus greatly hinders the accuracy, consistency, and cross-applicability of qPCR data.[1] Further complicating matters, many scientific articles gloss over information on key areas such as sample acquisition and handling, RNA quality and integrity, PCR efficiency, and analysis parameters, making reproducing results extremely difficult.[1]

MIQE: A Start for Standardization

This clear need for standardization led a group of scientists to devise and compile a set of recommended guidelines for qPCR. In 2009, Stephen Bustin and his colleagues published the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE).[1] The MIQE guidelines outline the minimum information that must be reported to ensure the relevance, accuracy, correct interpretation, and repeatability of a qPCR experiment. MIQE contains a detailed checklist of experimental parameters to monitor and report, but also offers recommendations for how to define and use terminology. It strives to improve both how qPCR experiments are conducted and how they are communicated.

MIQE covers a range of topics, but links everything together with one main underlying theme: thoroughness. Concerning samples, researchers should provide information on source, processing, storage, and composition, as well as nucleic acid quantity and quality assessment results. Minimum quality thresholds for qPCR should be clearly disclosed. Moreover, while the reverse transcription step is often overlooked as a means to convert RNA to cDNA, it can introduce substantial variation. MIQE guidelines therefore advocate that this step be fully documented and carried out in duplicate or triplicate.[1]

For the actual qPCR assay itself, researchers should provide sufficient information on both experimental parameters and reagents to allow for replication. This includes database accession numbers for each target and reference gene, specificity assessments for each primer or probe, and sequences and concentrations of each oligonucleotide used (including the identities, positions, and linkages of any dyes and/or modified bases). The MIQE guidelines also state that the PCR reaction mixture should be detailed, including the concentrations and identity of the polymerase used, the amount of template in each reaction, and the compositions, volumes, and concentrations for other reaction elements such as Mg^{2+}, salts, and any additives. Finally, investigators must identify the instrument that performed the cycling, as well as the properties and manufacturers of the consumables that they used, including tubes and plates.[1]

In terms of data rigor, analysis, and presentation, the MIQE guidelines provide detailed guidance on calibration, normalization, and controls. In particular, they highlight how researchers must compensate for potential differences in amplification efficiency between different primer sets and genes of interest.[1] Here, software programs and suites offer researchers a way to perform all of the necessary calibrations and standardizations to limit variability and promote consistency. Modern qPCR software packages are capable of mapping calibration and standardization results onto experimental data, allowing automatic adjustments to account for reaction efficiency and comparisons to multiple reference markers. They can even combine experimental data from multiple plates and collate them for larger gene studies.[3]

See references on page 7

A UNIVERSAL qPCR DATA STANDARD

Researchers have generated vast volumes of information using qPCR. However, these data have been obtained using different instruments and analytical software programs—both supplied by instrument manufacturers and created by third parties looking to fill analysis gaps—leading to a large number of different data presentation and storage formats. The absence of a standardized or universal format for data dissemination and transmission hindered the interchangeability, exchangeability, and comparability of qPCR data, impacting data submission for publication, the establishment of public data repositories, and collaborations.[1]

The scientific community recognized the need for a standardized qPCR data format as early as 2005, with the first standardization initiative presented during that year. This initiative gathered momentum in the subsequent years, leading to the creation of the Real-Time PCR Data Markup Language (RDML) in 2009.[1] RDML is the product of a consortium driven by key developers but also actively taking feedback from the scientific community, instrumentation companies, reagent companies, software companies, organizations that offer instruction in qPCR use, and other key opinion leaders in the field, including a number of independent software development teams. RDML was created with the ultimate goal of facilitating easy cross-exchange of qPCR data between instrument software, third-party software, repository databases, and research collaborations.[1]

What is RDML?

RDML, first and foremost, offers a flexible and universal data file structure. The RDML standard is based on XML (eXtensible Markup Language), which was itself created to facilitate and streamline data sharing across different information systems and platforms. RDML files contain seven root element block types: ID, sample, target, experimenter, thermal cycling conditions, documentation, and experiment. The ID block holds identifying information such as database entry numbers or publisher information. Blocks for sample, target, experimenter, and thermal cycling conditions offer details on experimental parameters—what sample was used, what genes were targeted, who ran the experiment, and the nature of the qPCR program. Documentation contains other information such as MIQE checklists.[1,2] Finally, each experiment block contains data from a single experiment, which can be further subdivided on a run-by-run basis. The individual runs are themselves also subdivided into elements containing information on experimental setup, participating experimenters, reaction parameters, samples analyzed, and potentially both raw and post-analysis data. Multiple data element blocks can be created per reaction element in order to facilitate multiplexing. Here, rather than repeating information already present in other element blocks, IDs can be used to refer to specific experimenters, protocols, or other information. This has several benefits: it streamlines the overall file size, creates a more database-like structure, and facilitates easier transfer into standardized databases like repositories.[1]

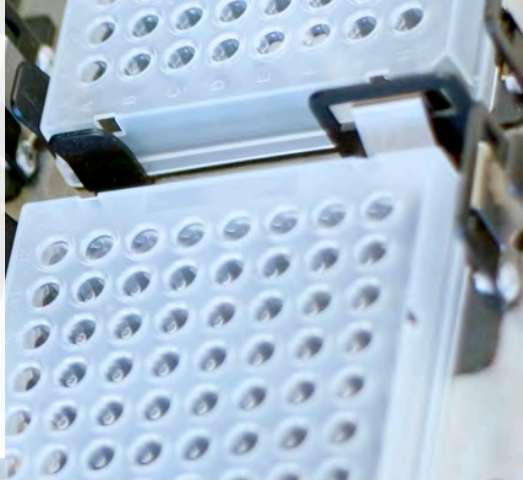
How to Use RDML

The RDML consortium is also active in the development and standardization of appropriate qPCR terminology, in alignment with the MIQE initiative.[1,2] Recognizing that RDML files may be populated both with richly annotated experimental data or just quantification cycle (Cq) values, the RDML consortium has proposed RDML guidelines as a subset of the MIQE guidelines to prevent the creation of "meaningless" RDML files.[1] Specifically, RDML files should contain, at minimum, Cq values for each reaction well in each experiment, results for each color being measured in multiplex experiments, and the settings used to obtain Cq values (amplification data can be provided in lieu of or in addition to the latter, but must be linked to samples and targets). Every sample and target gene should have a unique name or identifier and type designation (i.e. unknown, control, standard, target of interest, reference target). The meaning of target identifiers should also be easily accessible—they should be official gene symbols or references to primer databases, commercial assays, or publications. Barring any of those, annotation is required.[1]

RDML Development Remains Ongoing

RDML is not intended to be a static entity, and development has continued post-initial release with RDML v.1.3 released on May 5, 2020. Additionally, the RDML consortium is committed to developing tools for creating, processing, and validating RDML files. As an example, two such tools, the open-source editor RDML-Ninja and the online repository RDMLdb, were released in 2015.[3] The RDML consortium is also working with manufacturers and developers to ensure that new instruments and software applications are RDML-compliant.[1] Currently, at least five major companies produce RMDL-compliant qPCR systems, with at least seven compliant third-party software suites available.[4,5]

See references on page 7



STANDARDIZING AUTOMATION

The main goal of qPCR standardization, both for experimental protocols and for data collection, analysis, storage, and dissemination, is to improve reproducibility. Even with best practice guidelines in place, there remain many points within a typical qPCR workflow where researchers can introduce variability despite their best efforts and preparations. For example, discrepancies in sampling and subsampling parameters, mechanical and technical pipetting variations, and environmental shifts between runs all impact qPCR reproducibility.[1] Implementing automation in tandem with adopting best practices can further improve qPCR data accuracy, consistency, and reproducibility by minimizing variability.

How qPCR is Automated

Automating qPCR workflows largely involves automating liquid and plate handling procedures. This greatly reduces volume and concentration variability from well to well and plate to plate. Automated liquid handling is also much faster than manual pipetting. Furthermore, programmable plate handler-thermocycler systems can handle plate loading, run initiation, and plate removal automatically, effectively allowing 24/7 workflow runtime.

Automation facilitates greatly increased qPCR throughput, which in turn allows for much higher volumes of data to be generated. This data needs to be processed and analyzed in a manner that minimizes user-introduced variation. Here, software packages designed for qPCR data analysis rapidly process, analyze, cluster/collate, and visualize generated results, ensuring that data analysis does not become a bottleneck. Modern software is also aligned with the data distribution needs of the modern laboratory, whether it is transferring data from reader instruments for analysis, or transferring analyzed information to laboratory information management systems (LIMS) for organization, storage, and future recall. The ability to integrate with LIMS is also critical for the rapid dissemination of qPCR data to necessary parties and agencies, and is pivotal to the viability of qPCR-based diagnostics for public health applications.[2]

The creation of best practices and standardization guidelines such as MIQE and RDML, paradoxically, creates another potential source of variation, as not all scientists may adhere to these standardization guidelines to the same degree.[3] This creates a need to "standardize standardization." Automation is useful in this area, as many of the instruments and software programs that drive automation are developed with MIQE and RDML-compliance in mind. By building in best practices, automation is further driving qPCR standardization.

Pushing Boundaries

The precision and throughput offered by automation-powered qPCR standardization is important for many scientific fields, but it has perhaps had the greatest impact on the scientific response to the COVID-19 pandemic. COVID-19 has reaffirmed how important qPCR is to the scientific and medical communities, with qPCR diagnostic testing the gold standard for detecting SARS-CoV-2 infection. At the same time, it has reinforced the need for qPCR to be fast, accurate, reproducible, and scalable.

In a showcase of what qPCR can accomplish in tandem with automation-guided workflows and data handling, Stephen Bustin and his colleagues created CoV2-ID,[4] a qPCR assay for detecting SARS-CoV-2 that was designed, developed, optimized, and validated based on MIQE guidelines. CoV2-ID is a multiplex assay that probes three viral genes and two controls simultaneously per run, increasing sensitivity by roughly three-fold by using the same fluorophore to detect all three targets. CoV2-ID can be performed in as little as 16 minutes on instruments capable of executing 1-second thermocycling step durations. As such, this assay represents not only a breakthrough for SARS-CoV-2 testing, but a demonstration of how qPCR might be incorporated into point-of-care therapeutics.[4]

In addition to improving SARS-CoV-2 testing, other researchers are pushing the sensitivity and throughput limits of qPCR for applications, such as identifying and validating novel biomarkers[5,6] and drug discovery and development.[7] Standardization, promoted through best practice guidelines like MIQE and put into practice by a combination of researcher efforts and technology, allows scientists to make the most of qPCR's current capabilities and push the technology further into the future.

See references on page 7

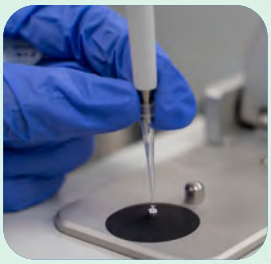
An Automated qPCR Workflow

Reverse Transcription (RT)

Variations in key parameters, such as amount of sample loaded, enzyme used, priming conditions, and cycling conditions, during the RT step can introduce substantial variation into downstream qPCR.

Automation

Liquid handlers maintain better RT master mix volume and concentration consistency. Plate loading instruments run multiple RT assays in parallel or sequence without manual intervention.



Sample Preparation

Accurate concentration measurements help prevent unbalanced amplification during RT and qPCR steps. Quality assessment is vital for identifying degradation and contamination.

Automation

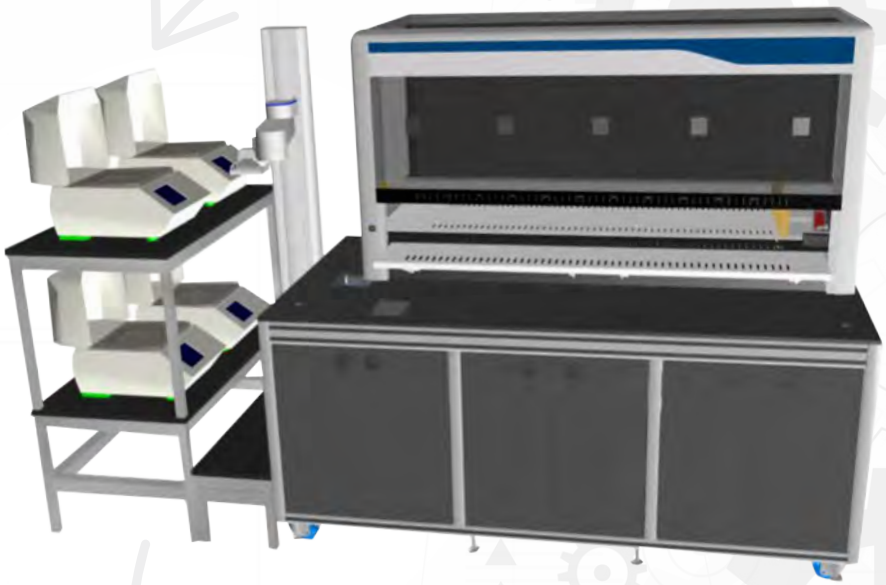
Liquid handlers accelerate sample extraction and QC processes. More consistent volume control yields more accurate concentration measurements and quality assessments.

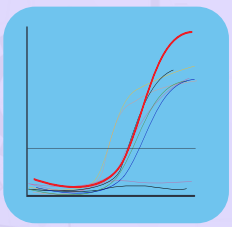
qPCR Reaction

Primer sequences and binding positions need to be carefully considered, especially in the context of secondary nucleic acid structures. All information necessary to replicate the qPCR run should be recorded. No-reverse transcription controls identify unintended amplification products, while positive controls monitor assay variation.

Automation

Automating liquid handling reduces variation in master mix composition and sample loading, helping control amplification disparities. Programmable thermocycler instruments, in tandem with plate loaders, schedule and run multiple qPCR assays without user intervention.



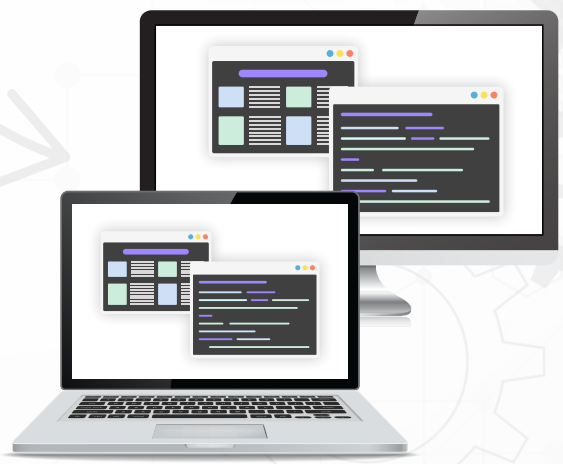


Data Processing and Analysis

Researchers must select and validate reference genes based on the parameters of each experiment. Enough samples and replicates must be run to obtain sufficient statistical power.

Automation

The latest data analysis software suites can process, compile, and visualize data per researcher instructions. These suites can create and populate RDML-compliant files for storage and dissemination.



References

Article 1: Establishing qPCR Best Practice Guidelines

1) S.A. Bustin et al., "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments," *Clin Chem*, 55(4):611-22, 2009.
2) A. Nour et al., "The MIQE Guidelines' tenth anniversary: The good and bad students," *Gene Rep*, 2020:19, 100630.
3) S. Taylor et al., "A practical approach to RT-qPCR—publishing data that conform to MIQE guidelines," Bulletin 5859 Ver. E, Bio-Rad Laboratories, Inc., Hercules, CA, USA. https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5859.pdf

Article 2: A Universal qPCR Data Standard

1) S. Lefever et al., "RDML: structured language and reporting guidelines for real-time quantitative PCR data," *Nucleic Acids Res*, 37(7): 2065-69, 2009.
2) S.A. Bustin et al., "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments," *Clin Chem*, 55(4):611-22, 2009.
3) J.M. Ruijter et al., "RDML-Ninja and RDMLdb for standardized exchange of qPCR data," *BMC Bioinformatics*, 16(1):197, 2015.
4) "RDML – RDML compliant instruments," RDML, 2021, https://rdml.org/instruments.html. Accessed October 28, 2021.
5) "RDML – RDML compliant qPCR 3rd party software," RDML, 2021, https://rdml.org/software.html. Accessed October 28, 2021.

Article 3: Standardizing Automation

1) S.C. Taylor et al., "The ultimate qPCR experiment: producing publication quality, reproducible data the first time," *Trends Biotechnol*, 37(7):761-74, 2019.
2) T. Chang et al., "A method for campus-wide SARS-CoV-2 surveillance at a large public university," *PLoS One*, 16(12):e0261230, 2021.
3) S. Lefever et al., "RDML: structured language and reporting guidelines for real-time quantitative PCR data," *Nucleic Acids Res*, 37(7): 2065-69, 2009.
4) S. Bustin et al., "CoV2-ID, a MIQE-compliant sub-20-min 5-plex RT-PCR assay targeting SARS-CoV-2 for the diagnosis of COVID-19," *Sci Rep*, 10:22214, 2020.
5) K.S. Sharov et al., "8806 Russian patients demonstrate T cell count as better marker of COVID-19 clinical course severity than SARS-CoV-2 viral load," *Sci Rep*, 11:9440, 2021.
6) L.Z. Hong et al., "Systematic evaluation of multiple qPCR platforms, NanoString and miRNA-Seq for microRNA biomarker discovery in human biofluids," *Sci Rep*, 11:4435, 2021.
7) J. Chen et al., "Leveraging automation toward development of a high-throughput gene expression profiling platform," *SLAS Discov*, 26(3):410-19, 2021.

Low background.
High reproducibility.
Hard-Shell Plates.

Try quality PCR consumables from Bio-Rad

Bio-Rad offers a large selection of PCR tubes and plates, seals, and accessories for a variety of applications. Our PCR consumables are precisely manufactured to ensure efficiency, reproducibility, and reliability at each step of discovery, development, and manufacturing. The quality of PCR plastics matters — plate uniformity minimizes data variability, and white well options maximize qPCR sensitivity. Our plastic consumables are certified to be free of DNase, RNase, and human genomic DNA. Be confident in your PCR, qPCR, and next-generation sequencing research with our high-quality plastics.

To learn more, visit bio-rad.com/QualityPCRPlates

#ScienceForward

BIO-RAD

Bulletin 3326 Ver A