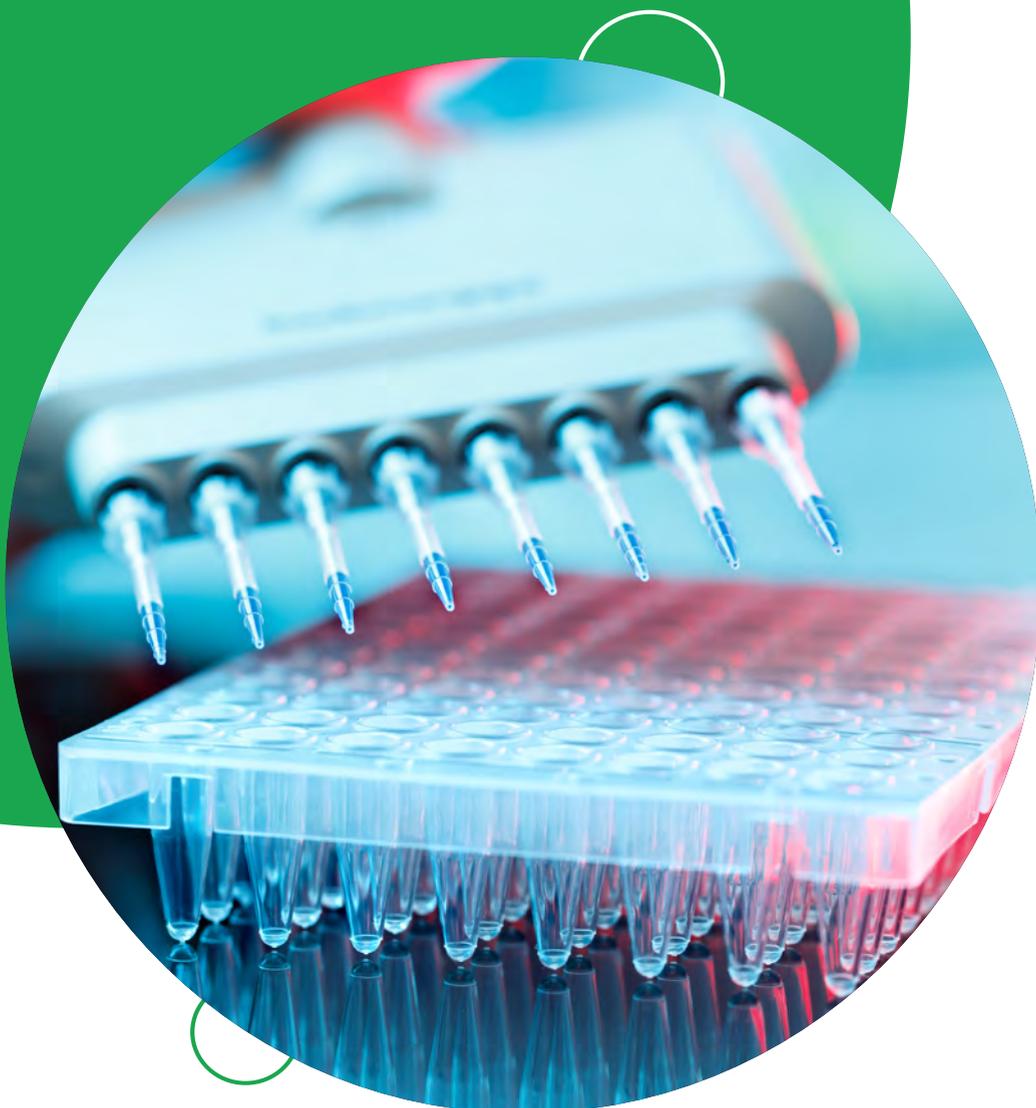


PUTTING THE PIECES TOGETHER: THE COMPONENTS OF EFFECTIVE qPCR FOR MOLECULAR DIAGNOSTICS AND INFECTIOUS DISEASE RESEARCH



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MODERN qPCR FOR APPLICATIONS IN MOLECULAR TESTING AND INFECTIOUS DISEASE RESEARCH

Quantitative real-time PCR (qPCR¹) has the capacity to detect and measure nucleic acids at minute concentrations within a broad range of source materials. The advent of this technique has been nothing short of transformative for the life sciences, and qPCR is currently the most popular technique in molecular biology.^{1,2} From a research perspective, qPCR is used in every field that investigates gene expression. Additionally, it is used as a diagnostic tool for various clinical and industrial applications, including disease screening and risk assessment, biologics manufacturing, genetic engineering, and forensics.¹

Sensitivity, Specificity, and Flexibility

The popularity of qPCR largely stems from its high sensitivity and specificity. In theory, qPCR is so sensitive that it can detect as little as a single DNA fragment present within a sample,² although most practical situations use a lower limit threshold in the hundreds of copies for better consistency. Custom oligonucleotide primers offer exceptional targeting specificity against template sequences, and as qPCR primers are easy to customize and manufacture, the technique can be quickly and simply adapted for use against different targets of interest while maintaining as much

specificity as possible. Finally, primer design is largely dictated by the template and the target, rather than any need for compatibility with specific instruments or reagents. This flexibility and adaptability is critical for dealing with the rapidly changing pathogens and pathogen variants found in infectious disease research.

Understanding qPCR Performance

That is not to say that qPCR is completely “plug-and-play.” There are numerous factors that affect qPCR performance, including template quality and quantity, primer design, cycle conditions, polymerase selection, and master mix composition, which researchers must consider when comparing qPCR data gathered from runs against different targets, on different sample types, and in different laboratories. Understanding the factors that impact qPCR performance is critical for establishing qPCR data accuracy, reproducibility, and consistency. There is also the question of how to handle qPCR data, especially in high-throughput situations. Modern qPCR, while unable to reach the throughputs of microarrays, can still look at tens to hundreds of genes per run.³ A good data management workflow is critical to preventing missed findings of interest and analysis bottlenecks.

Rapid Results and Rapid Reports

Both effective performance and data management are necessary for qPCR to be viable as a diagnostic tool; rapid and accurate testing needs to be accompanied by rapid results acquisition and dissemination. Bio-Rad’s new CFX Opus Real-Time PCR Systems are designed with this in mind. These systems possess increased thermal uniformity across both 96- and 384-well formats, ensuring that a sample will yield the same result whether it is placed in the middle of the block or on the edges. Elevated thermal uniformity also boosts throughput by ensuring that every well can be used for every run, if needed.

The CFX Opus Systems also streamline data acquisition while promoting its integrity and security. CFX Maestro software facilitates automation, as well as protocol management and data analysis and visualization. Furthermore, CFX Opus Systems are WiFi-, Ethernet-, and USB-compatible and integrate within existing laboratory networking systems for data dissemination. Finally, both CFX Opus Systems and CFX Maestro software have “Dx” versions designed for in vitro diagnostic testing. In particular, CFX Maestro Dx SE software aids with regulatory compliance through its audit capabilities.

See references on page 7

EXPANDING THE qPCR TOOLBOX



qPCR is used for a wide range of applications across many fields, and as a result, scientists have devised an extensive array of reagents, kits, and adaptations geared at application-specific optimization. For infectious disease researchers, these advances have helped qPCR overcome the inherent challenges common to the field, including unique sample sources, sample scarcity, or hard-to-detect targets present in low concentrations. Because of this, qPCR has not only become a staple of infectious disease research, but also a key technology for infectious disease diagnostics—something that has been emphasized by qPCR’s widespread use during the COVID-19 pandemic.

Viral Challenges

Working with viral RNA and DNA creates challenges typically not present when working with cellular nucleic acids. Viral nucleic acids usually occur at much lower concentrations that can vary dramatically depending on the sample source (e.g., blood, saliva, or tissue) or when the samples are taken (e.g., early infection, late infection, or resolution). As such, qPCR must encompass a broad sensitivity range to detect viral DNA/RNA, especially for diagnostic purposes. Further, sample quality can be inconsistent. Patient samples, for example, whether for diagnostic or research purposes, can vary in terms of extraction protocol and abundance, as well as storage duration and conditions. These factors can considerably diminish DNA/RNA quality and integrity.

Special Tools for Special Situations

In the face of these challenges, scientists have developed reagents, kits, and protocols optimized for extracting and detecting nucleic acids. Chelex resin chelates polyvalent metal ions, preventing their direct ability to interfere with PCR and inactivating metallonucleases and other enzymes which may also interfere with PCR. Chelex is used in a number of fields, including forensics,¹ and forms the main component of products such as InstaGene matrix. These products not only increase DNA and RNA yields by preventing irreversible loss, but also streamline DNA and RNA purification by removing the need for time-consuming and labor-intensive deproteinization, organic extraction, dialysis, and alcohol precipitation protocols.

Sometimes, increasing sample nucleic acid yields is not feasible. In these circumstances, pre-amplification can help increase nucleic acid concentrations to a point where they can be detected following conventional qPCR. Pre-amplification-optimized reagents can help scientists obtain results from as little as 100 pg of starting material while avoiding amplification bias. Pre-amplification strategies have proven useful during the COVID-19 pandemic, with wastewater surveillance adopted by jurisdictions around the world as a means of evaluating the prevalence of COVID-19—especially novel variants—within a community.² Viral nucleic acid concentrations are diluted by the vast milieu of substances that make up wastewater, and pre-amplification helps researchers obtain measurable amounts of sample.

Scientists have also developed detection strategies pertaining

specifically to viruses and their properties. Bio-Rad’s Reliance One-Step Multiplex Supermix uses a novel chimeric reverse transcriptase derived from feline leukemia virus (FLV) and Moloney monkey leukemia virus (MMLV) reverse transcriptases in combination with a blend of DNA polymerases to facilitate the sensitive and accurate detection of up to five targets in a single reaction. This Supermix can also co-detect RNA and DNA within the same run across a wide range of concentrations using Droplet Digital PCR.

Pivoting for a Pandemic

Upon the onset of the COVID-19 pandemic, these existing technologies were quickly adapted to detect the SARS-CoV-2 virus, leading to the creation of kits like the Reliance SARS-CoV-2 RT-PCR Assay Kit from Bio-Rad. Approved for diagnostic use under emergency use authorization in the United States, and for in vitro diagnostic use in the European Union, this kit targets the SARS-CoV-2 nucleocapsid (N) gene instead of the spike protein (S) gene because the former has greater viral specificity and possibly lower mutation risk. The kit covers all major variants discovered up to February 2021, and in tandem with Bio-Rad’s one-step qPCR workflow, can deliver results in under two hours. In addition, Bio-Rad has also created the Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit for use in the European Union, which probes all three viruses simultaneously to distinguish COVID-19 infections from influenza cases.

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PLASTIC CONSUMABLES: MORE THAN “ONE AND DONE”

Consumables such as plates and tubes are necessary for qPCR workflows. These consumables are often “single-use” to limit cross-contamination. However, their “throw-away” nature leads them to become an afterthought for researchers. In actuality, plastic consumables can be integral to the success and reproducibility of a given qPCR experiment because they mediate heat transfer from the PCR instrument to the sample, directly impacting reaction efficiency. High-quality plastic consumables improve qPCR reaction efficiency and stability, thereby offering better data quality, consistency, and reproducibility.

Plastic Pitfalls

Plastic consumables tend to be regarded as interchangeable and generic. However, poor plastics can have a considerable deleterious impact on qPCR reactions. Given that they house the amplification reactions themselves, it is important that scientists recognize and understand the heat transfer properties of their consumables. Excessively thick well or tube walls, for example, can impede heat transfer and hinder amplification. Furthermore, it is important that plates or tubes fit properly within the thermal cycler. Gaps between the sample-holding vessel and the heat block can

create differences in temperature, leading to amplification variation.

Poor-quality consumables can also melt or warp during the qPCR process. Changes to the physical integrity of the storage vessel can result in contamination, either across different samples or between samples and the thermocycler environment. Melting or warping can also affect the integrity of plate and tube seals, which is a further source of potential contamination and can dramatically alter sample volumes and nucleic acid concentrations through evaporation. Finally, poor-quality consumables are prone to chemical leaching, with or without the presence of visible melting or warping. Chemical leaching during qPCR can be deleterious in many ways, including directly impeding enzyme function, inhibiting receptor-ligand binding, and absorbing light at key wavelengths.¹

In addition to material quality, the color and transparency of plastic consumables is also a potential factor for qPCR. First, opaque wells reduce light refraction compared to transparent wells, therefore limiting any potential signal cross-over between adjacent wells. This helps improve well-to-well consistency. Second, plastic color impacts how much light is reflected versus absorbed. White-colored well walls reflect the most light, allowing more signal to reach the detectors and thereby increasing sensitivity. Conversely, it is important that

tops, including sealing films and lids for plates or caps for tubes, are optically clear to minimize signal absorbance or distortion.

Optimized Plasticware for Optimized Results

High-quality plasticware can help researchers optimize their qPCR experiments for better data reproducibility and accuracy. Hard-Shell PCR plates from Bio-Rad, for example, are specifically designed to withstand the mechanical forces present in qPCR workflows. They exist in 96- and 384-well formats, as well as semi-skirted versions to accommodate different thermal cycler heat block configurations. The skirt and deck are molded from a thermostable polymer, preventing distortion and shrinkage when subjected to high temperatures. This material is also rigid, preventing any damage when handled by robotic arms. In contrast, the wells are V-shaped with thin walls to optimize heat transfer efficiency and recovery of low-volume samples. They are also molded from polypropylene that has a low DNA binding affinity. Finally, raised well rims allow for tight sealing using pressure-, adhesive-, and heat-sealing methods.

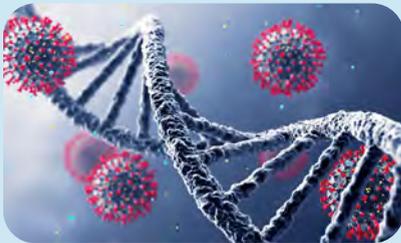
See references on page 7

qPCR in Action: Genomic Surveillance of SARS-CoV-2 Variants



Mutation Detection

Researchers use qPCR to confirm the presence or absence of specific SARS-CoV-2 genomic mutations. This helps characterize existing or novel viral strains and variants, potentially offering clues regarding virulence and transmissibility. Mutation detection also distinguishes between known SARS-CoV-2 viral variants by examining the presence or absence of specific mutations at key sites.¹



Variant Tracking

Mutation detection paves the way for variant tracking, and multiplexed mutation detection allows researchers to screen a sample for multiple variants simultaneously.^{2,3} Variant identification allows researchers to characterize variants relative to one another, and public health officials to track the prevalence and progression of a variant throughout a population.¹⁻³



Wastewater Surveillance

Wastewater surveillance can deliver information on broader virus trends within a community without the need for self-reporting. Researchers have used qPCR and Droplet Digital PCR (ddPCR) for this purpose. The former provides an extensive dynamic range, while the latter offers greater precision and more resistance to potential PCR inhibitors present in wastewater samples.^{4,5}



Consumables

High-quality plastic consumables like Hard-Shell qPCR plates decrease contamination risk, increase consistency, and lead to more reliable results and higher throughputs.



Instruments

Modern thermal cyclers emphasize even heat distribution and transfer for more accurate and reproducible results. The ability to integrate with existing networking infrastructure streamlines data handling workflows and promotes security.



Reagents

Robust reagents are necessary for robust qPCR assays. Novel polymerase combinations and chimeric reverse transcriptases optimized for working with viral nucleic acids improve sensitivity and accuracy.

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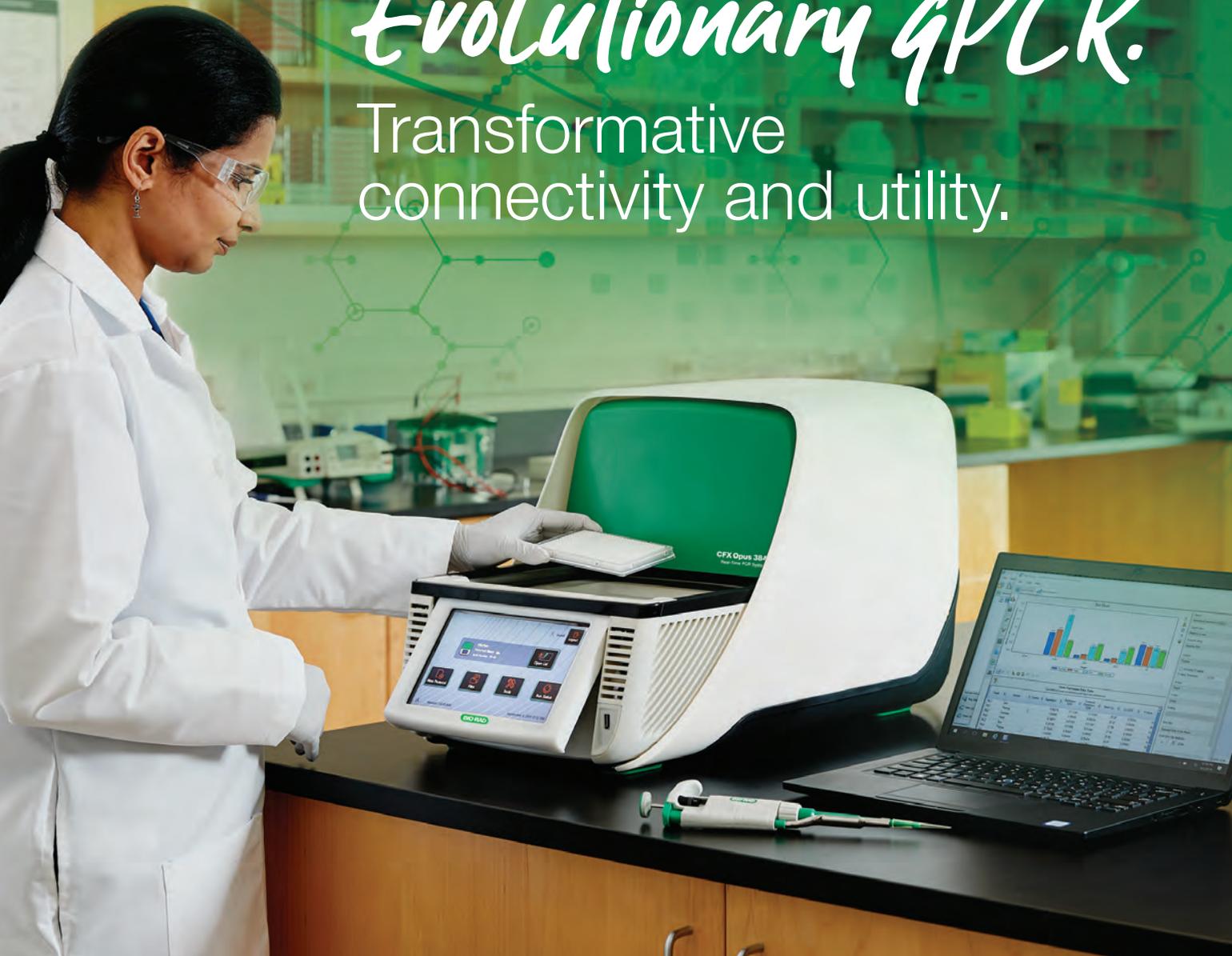
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