PCR

The Ubiquitous Molecular Biology Workhorse Reasserts its Continued Relevance

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Introduction

In this eBook, sponsored by Thermo Fisher Scientific, we examine the rising role of genetics in medical diagnostics, including polymerase chain reaction (PCR), genomic sequencing, and gene-based microbiome disease association. This collection of articles looks at the dramatic increase in prevalence of PCR testing since the onset of the COVID-19 pandemic, and explores the possibilities of still deeper, wider spread clinical applications for PCR in the not-so-distant future. As the prominence of PCR continues to climb, we analyze various methodologies to improve its efficiency, and offer insights on the most suitable laboratory equipment to ensure accuracy and precision when performing Real Time PCR (qPCR). The need for precision and accuracy when performing qPCR is paramount. It is an extremely sensitive assay and slight differences due to pipetting errors can be disastrous. We look at the impact that lab equipment has on performance when preparing samples, miniaturizing qPCR assays and simplifying standard curve preparations. Also, a discussion on the benefits of automating qPCR, such as saving time and avoiding repetitive motion injuries.

The increased role of genome sequencing in surveilling—and potentially combatting—the COVID-19 pandemic is also studied with an eye on growing sequencing programs across the United States, something emphasized by both the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO). Underscoring this critical need in a recent study, WHO stated, "The accelerated integration of genome sequencing into the practices of the global health community is a must if we want to be better prepared for the future threats." We attempt to understand how the US remains lagging far behind other countries in viral genome surveillance.

Meanwhile, a dive into the discovery that people with gut microbiota that contain the same collections of bacteria appear more likely to have certain health conditions. A 2021 study analyzed the genetic makeup of bacteria in the human gut, and linked groups of bacterial genes to disorders including atherosclerotic cardiovascular disease, cirrhosis of the liver, inflammatory bowel disease, colorectal cancer, and type 2 diabetes. "Overall, our work is not only a step towards gene-based, cross-disease microbiome diagnostic indicators, but it also illuminates the nuances of the genetic architecture of the human microbiome, including tension between gene- and species-level associations," wrote the research team. ■

SARS-CoV-2 Under Surveillance

To keep track of emerging SARS-CoV-2 variants, institutions tasked with viral surveillance need to devote more resources to genome sequencing

By Julianna LeMieux, PhD

Joao Paulo Burini / Getty Images

In this illustration of SARS-CoV-2, just half the virion is displayed to reveal coiled RNA that would otherwise remain hidden within the capsid. This RNA accumulates mutations, resulting in SARS-CoV-2 variants that threaten to outpace the development of vaccines and antivirals. To identify the variants as they arise, laboratories must step up their sequencing efforts.

Every pandemic is attended by expressions of fear and grief, the imposition of life-altering public health measures, and attempts to blame foreigners or other disfavored peoples. In all these respects, the current COVID-19 pandemic is like its predecessors. But the COVID-19 pandemic is playing out differently in terms of surveillance. COVID-19's causative agent—the SARS-CoV-2 virus—can be subjected to exceptionally close surveillance because we now possess sophisticated genomic sequencing technology. Sequencing information about the viral genome can be gathered so quickly—almost in real time—that it can power a dynamic public health response.

The information revealed through genomes requires analysis and interpretation on the part of genomic epidemiologists, professionals who rely on a steady flow of new sequences to do their research. Unfortunately, it has become increasingly clear that the United States—despite being home to the firm that generates more than 90% of the world's sequencing data—is not doing enough sequencing. Not even close.

Today, the United States sequences roughly

James Lu, Helix

0.2–0.3% of all positive cases, a fraction compared to the United Kingdom, which is leading the effort with 7%. Until now, the sequencing has been done primarily through state-led efforts, hospitals, and academic institutions. It has been "fairly disjointed" up until now, according to James Lu, MD, PhD, the co-founder and CSO of Helix.

How important is genomic surveillance? The World Health Organization (WHO) recently published a study, "Genomic sequencing of SARS-CoV-2: A guide to implementation for maximum impact on public health," asserting that sequencing is a top priority. It stated, "The accelerated integration of genome sequencing into the practices of the global health community is a must if we want to be better prepared for the future threats."

The Centers for Disease Control and Prevention (CDC) seems to agree and is boosting the United States' sequencing efforts through the National SARS-CoV-2 Strain Surveillance (NS3) program. Part of this program hopes to see state health departments and other public health agencies ramp up their sequencing. Indeed, the program states that its surveillance system has been scaled up to process 750 samples per state per week. In December 2020, the CDC contracted with three commercial diagnostic laboratories to conduct additional sequencing. The CDC has commitments from these laboratories to sequence 6,000 samples per week. Besides Helix, the laboratories include LabCorp and Quest Diagnostics.

Sequence Trackers

Some scientists, like Emma Hodcroft, PhD, at the University of Bern, have been analyzing the SARS-CoV-2 genome for the past year, since the first sequences of SARS-CoV-2 were available. In doing so, they have gained insight into different aspects of the pandemic. Hodcroft recently published research on a variant that expanded in Spain and then continued to move throughout Europe. Hodcroft says that this work was "incredibly eye-opening" because it uncovered the role that travel tends to play in the dispersion of a variant.

In addition to tracking variants through travel, Hodcroft says that genome information can yield information about which changes in the genome

At Helix, a population genomics company, efforts to sequence SARS-CoV-2 variants have shed light on the transmission of the B.1.1.7 variant. Together with other San Diego–based organizations, Helix published a medRxiv preprint in February reporting data on the prevalence and growth dynamics of this variant in the United States.

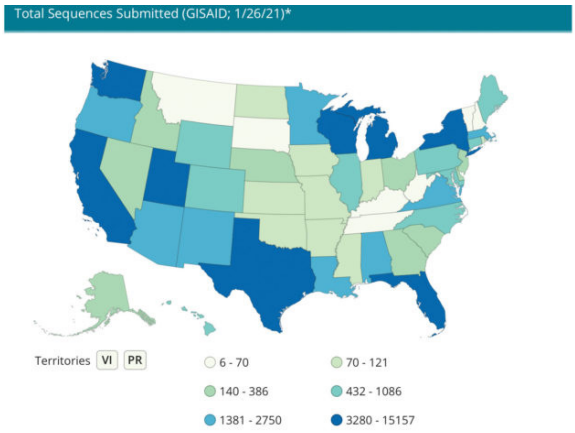
are important. "We see a lot of changes in the virus," she points out, "and it's not always obvious which ones are the ones we want to be paying attention to." She adds that when genomic changes can be associated with changes in transmissibility or antigen avoidance, "we can start to piece together a better picture of how the virus works [and] what changes we need to really be paying attention to."

Indeed, the world is watching the evolution of SARS-CoV-2 as it moves around the globe, with new variants becoming important factors in the state of the pandemic. The B.1.1.7 variant that originated in the United Kingdom has garnered much attention due to preliminary (and unconfirmed) reports of phenotypic changes such as increased transmissibility or virulence.

In South Africa, another variant of SARS-CoV-2 appeared independently of B.1.1.7 that shares some of the same mutations. In Brazil, a variant of SARS-CoV-2 known as P.1 emerged with 17 unique mutations, including several in the spike protein. Although these top the list of known variants at the moment, there is no doubt that many more will follow.

Helix Steps Up

Helix started working on SARS-CoV-2 sequencing the day before Christmas 2020. That started "a very interesting and busy month," recalls Lu. Helix



After a slow start, the CDC recently began moving quickly to bolster SARS-CoV-2 surveillance efforts in the United States. These efforts include sequencing and measures to improve the "infrastructure and workflow to ensure efficient sequence data submission to public repositories." Here, a map shows the cumulative number of SARS-CoV-2 sequences by state that have been published in public repositories from January 2020 to the present (as of February 2021). More such data can be found on the CDC's National Genomic Surveillance Dashboard.

had been COVID-19 testing, using the standard PCR method, since July. Known for its population genomics business, the company was a relative latecomer to the COVID-19 testing arena, according to Lu. But Helix quickly ramped up and currently runs half of the samples for the San Diego area.

Every day in its California facility, Helix runs tens of thousands of samples—from all 50 states. But those are PCR tests, which are designed for detection. Helix has been funded to reach 100,000 samples/day. Lu says that Helix expects to achieve this rate.

Out of those samples, roughly 15–20% are positive. From the positives, roughly 1,000 samples are chosen each week to be driven a mile down the road and run on the NovaSeq instruments at Illumina; the two companies formed a partnership in January. Which samples take the ride? Lu says the partners prioritize samples deemed most likely to give a good geographic representation of the United States. They also prefer to include B.1.1.7 variants in the mix.

How many tests, on a percentage basis, need to be sequenced to detect emerging strains? Lu says that if you sequence about 5% of positives in the United States today, which means sequencing roughly 10,000 samples/day, you can catch an emerging variant that is present in about 0.1% or 1% of samples. At that level of sequencing, an emerging variant that has a circulation figure of 0.1% or 1% would be detectable. This circulation figure is roughly the one currently estimated for the B.1.1.7 variant.

For Lu, this work goes beyond SARS-CoV-2, stretching into a future that will bring more viruses that need to be watched closely. Viral surveillance, he notes, will be part of the pandemic infrastructure over time. But it will take resources. And he wonders where the investment is going to be for readiness in the future.

How Much Sequencing is Best?

If every country in Europe could provide a couple hundred sequences every week, or even every month, Hodcroft says, that would "transform what we could say about different variants in Europe and how they're spreading." Hodcroft notes that if sequences were provided quickly—within a week

or so of sample collection—it would be possible to "track different variants in real time across Europe." Such a capability, she emphasizes, would be "game changing." She adds, "If we could expand this to the world, that would be even better."

A Platform to Detect Variants—or Sequence Them

Pathogen detection tests in food are challenging; the test has to be quick, cheap, and robust. Making a genomics-based test with those attributes is no small feat. Started in 2014, Clear Labs built such a test, hoping to modernize how food companies identify pathogens. When COVID-19 hit, the company adapted its platform, ClearDX, for SARS-CoV-2 detection. The ClearDX uses Oxford Nanopore's gridION sequencing platform to sequence the sample.

The platform can run sequencing-based modes for either detection or whole genome sequencing. The diagnostic mode runs in 11 hours and can process 192 samples at a time; whole genome sequencing takes 18 hours for 32 samples. Clear Labs' goal is to democratize such testing so that "anyone can have the capabilities to go from sample to answer without the need to go to a specialized lab," declares Sasan Amini, PhD, the company's co-founder and CEO.

Sequencing Wears Many Hats

Much emphasis is being placed on sequencing positive COVID-19 samples. More viral surveillance will lead to a deeper understanding of SARS-CoV-2 and may help inform public health decisions. Is it possible, though, for sequencing technology to play a second role in combatting the COVID-19 pandemic? Could it also help bolster the diagnostic testing effort?

Illumina came out early with its sequence-based clinical test. This test, called COVIDSeq, was the first next-generation sequencing (NGS) test granted EUA approval. An Illumina spokesperson declined to provide a detailed list of early adopters without prior permission but said global partners include the Institute of Genomics and Integrative Biology in New Delhi and the Communicable Disease Genomics Network in Australia.

But the merits of using NGS for COVID-19 clinical testing are questioned by Alex Dickinson, PhD, co-founder and executive chairman of ChromaCode. "NGS is great when you don't know what you're looking for," says Dickinson, who spent seven years as a senior vice president at Illumina. "It's fantastic when you're doing discovery." But PCR is amazingly efficient when you do know what you're looking for. When you know your target, he says, PCR is so simple and inexpensive. That is one of the reasons he thinks that most of the COVID-19 testing is happening, and will continue to happen, in PCR machines.

The biggest issues with NGS assays, according to Lu, concern turnaround time. A PCR run is 45–90 minutes, whereas a sequencer run is 10 hours. And

ChromaCode asserts that its High-Definition PCR (HDPCR) technology can assess as many as four disease targets for every target that is already part of an assay—and do so in a single reaction. Because the standard SARS-CoV-2 PCR assay includes 4 targets, ChromaCode's HDPCR SARS-CoV-2 assay could be used to assess 16 targets, facilitating the tracking of emerging SARS-CoV-2 variants.

although NGS has prodigious multiplexing ability, given that 25,000 samples can be run on a sequencer, the technique poses a serious operational challenge: loading all the samples at one time.

ChromaCode makes the reagents and computer services to execute PCR tests, including its own COVID-19 test, which has been available for the past eight months. To date, about 3 million COVID-19 tests have been shipped. Unlike traditional PCR tests that can detect 4 targets, ChromaCode's technology can detect 16 targets with a combination of reagents and software. The extra channels can be used to identify more respiratory diseases or to target other regions of the SARS-CoV-2 viral genome. In the latter case, SARS-CoV-2 variants could be identified.

In May 2020, Ginkgo Bioworks announced a $70 million investment, from Illumina and others, to build up its NGS COVID-19 testing capacity. Birgitte Simen, PhD, Ginkgo's senior director of genomics and computational biology, says that the company works closely with Illumina and talks to Illumina's personnel on a regular basis. But Ginkgo doesn't use Illumina's test.

Ginkgo has two tests—both are NGS based. One test, Concentric, is a PCR-NGS mashup: it's not a qPCR test, but it's not a full genome sequencing test either. It's a ratiometric test based on the Swab-seq test developed by Octant Bio, but with modifications, explains Simen. The test result comes from the ratio of two quantities: the amount of a "spike in" product (a synthetic RNA sequence that is similar to,

Alex Dickinson, PhD, ChromaCode (right, and Birgitte Simen, PhD, Ginkgo Bioworks (left).

but distinguishable from, the one for SARS-CoV-2) and the amount SARS-CoV-2 virus. The samples are barcoded, and the readout is done on an Illumina NovaSeq or NextSeq.

Concentric does not require the full time it takes for a normal sequencing run. The sequencer needs to read only the barcode and enough of the sample to differentiate between the "spike in" and the actual virus.

What is the benefit of Concentric over the standard PCR test or the Illumina COVID-seq? In Simen's view, the benefit is scale—or the potential to scale (Ginkgo did not reveal how many tests it runs at the moment.) Simen clarifies that when she cites scale, she means that you can run a lot of samples at the same time, driving down turnaround time with a few sequencers. How many samples can be run at one time using Concentric? Ginkgo didn't say, but it's safe to say that the number exceeds the capacity of qPCR.

What about surveillance? Ginkgo launched a whole-genome sequencing test in May 2020 that the company runs "at fairly low scale on a weekly basis for a number of partners," according to Simen. Ginkgo has done 4,000 of those tests total. She says that the company is "looking to scale this up."

Like too many aspects of the COVID-19 pandemic, the United States is lagging far behind other countries in viral genome surveillance. It's hard to understand how that can be. A year has passed, and some of the companies involved in surveillance are worth billions of dollars. Time and resource constraints are poor excuses.

Kári Stefánsson, MD, CEO of deCODE genetics, led an aggressive effort to stamp out COVID-19 in his home country of Iceland—which has current daily case counts in the single digits. In April 2020, he said that doing the same would be "even easier" in the United States due to the country's talent and resources. He added that it was "pretty sad" that although the methods used by Icelanders to quell the pandemic in their island nation originated in America, the Americans have not been using these methods themselves. According to Stefánsson, it all comes down to having "the will and desire." ■

The E1-ClipTip Equalizer Electronic Pipette and Multidrop Combi Reagent Dispensers Help Improve the Efficiency of Preparing Samples for RT-PCR

Abstract

This study demonstrates improved efficiency using the Thermo Scientific™ E1-ClipTip™ Equalizer expandable-spacing electronic pipette and the Thermo Scientific™ Multidrop™ Combi Reagent Dispenser in preparing samples for RT-PCR. The results show a 63% reduction in the amount of time required to prepare samples for nucleic acid extraction when using the E1-ClipTip Multichannel Equalizer pipette and the Multidrop Combi Reagent Dispenser. This time reduction also enables as much as a 24% increase in throughput by reducing instrument idle time between RT-PCR runs.

Background

One of the challenges in preparing samples for RT-PCR is the manually intensive, error-prone and inconsistent sample transfer step before nucleic acid isolation. In most cases, prior to nucleic extraction, samples must be transferred from one vessel, such as sample tubes, to a completely different configuration such as the more commonly used 96-well sample plate. This can be very cumbersome. The E1-ClipTip Multichannel Equalizer is an electronic pipette

with adjustable tip spacing that allows users to quickly and efficiently transfer samples between various vessels including tubes, racks, microplates, a horizontal gel box, etc. replacing the otherwise labor-intensive and time-consuming process using a single-channel pipette.

During nucleic acid extraction, there are multiple wash steps and an elution step that require 96-well plates to be filled with the appropriate solutions. Lastly, the isolated nucleic acid must then be transferred to a PCR plate and combined with the RT-PCR reagents. To help alleviate time and effort in these steps, the Multidrop Combi Reagent Dispensers can be used to swiftly and accurately fill the wash and elution plates required for the nucleic acid isolation process and the E1-ClipTip Multichannel Equalizer pipette can be used to combine the sample and necessary reagents for RT-PCR.

Generally, preparation for RT-PCR requires numerous manual pipetting steps that not only increase the risk of repetitive stress injuries but also significantly contribute to the inefficiencies and errors that impact the throughput and performance of the RT-PCR workflow. Used together, an E1-ClipTip Multichannel Equalizer pipette and Multidrop Combi Reagent Dispenser can save significant time during preparation of samples for RT-PCR.

Materials and Methods

In order to determine the amount of time saved during all the liquid transfer steps needed prior to RT-PCR, a technician was timed using manual pipetting versus using the E1-ClipTip Multichannel Equalizer electronic pipette and a Multidrop Combi Reagent Dispenser. To ensure that only liquid transfer steps were measured, all reagents and equipment were ready for use. The protocol for the Thermo Scientific™ MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Cat. No. A48310) was followed. First, the technician prepared the processing plates (Table 1) using either an 8-channel manual pipette or the Multidrop Combi Reagent Dispenser (one Multidrop Combi Reagent Dispenser per solution). Next, the technician prepared the 96-well sample plate by adding the reagents for nucleic acid isolation using an 8-channel E1-ClipTip Equalizer electronic pipette (Table 2). Lastly, the technician prepared the plates

Table 1: Preparation of the nucleic acid isolation processing plates.

Plate ID	Plate type	Volume per well	Device
Wash plate 1	96 deep-well plate	1,000 µL	8-channel pipette or Multidrop Combi
Wash plate 2		1,000 µL	
Wash plate 3		500 µL	
Elution plate		50 µL	

Table 2: Steps used to prepare plates for nucleic acid isolation.

Step	Volume	Device
1. Add Proteinase K (96 wells)	10 µL	8-channel pipette or 8-channel E1-ClipTip Equalizer pipette
2. Add negative control (1 well)	400 µL	Single-channel pipette
3. Add sample for extraction (94 wells)	400 µL	Single-channel pipette or 8-channel E1-ClipTip Equalizer pipette
4. Add bead binding mix (96 wells)	550 µL	8-channel pipette or Multidrop Combi
5. Add MS2 Phage control (96 wells)	10 µL	8-channel E1-ClipTip Equalizer pipette

Table 3: Steps used to prepare 96-well PCR plate for RT-PCR.

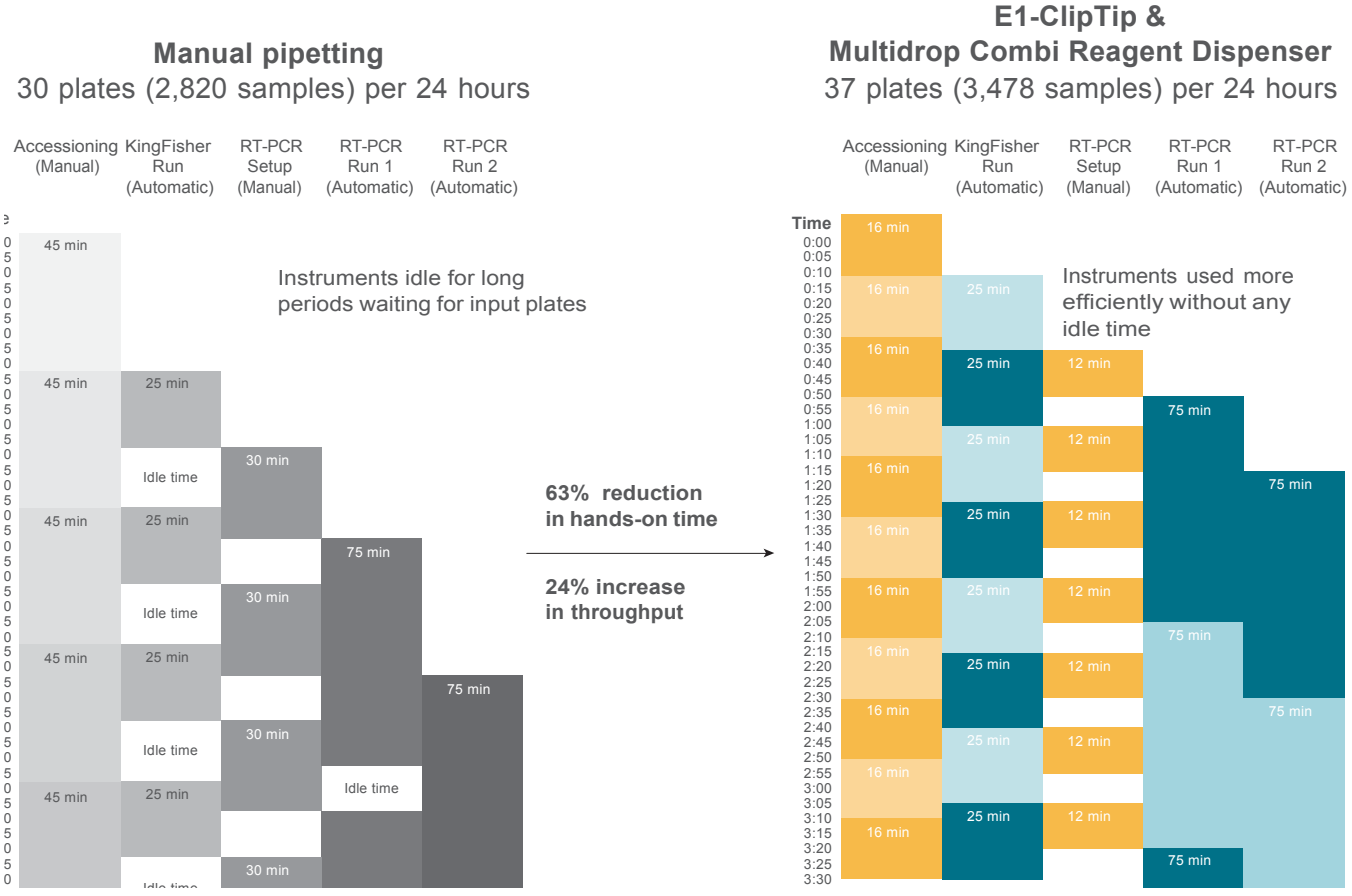
Step	Volume	Device
1. Preparation of positive control and reaction mix	Various	Single-channel pipette
2. Add reaction mix to PCR plate (96 wells)	20 µL	8-channel pipette or 8-channel E1-ClipTip Equalizer pipette
3. Add nucleic acid samples to PCR plate (94 wells)	5 µL	
4. Add positive control (1 well)	2 µL	Single-channel pipette
5. Dilute positive control with nuclease-free water (1 well)	3 µL	
6. Add negative control (1 well)	5 µL	

for RT- PCR (Table 3) and the time required was recorded.

Results and Conclusions

The results of this study show that there is a 63% reduction in hands-on time required for preparation of samples for RT-PCR using an 8-channel E1-ClipTip Equalizer pipette and Multidrop Combi Reagent Dispensers compared to performing these steps manually (Figure 1). The amount of time it took to prepare the samples and plates for nucleic acid extraction manually took 45 minutes compared to 16 minutes using the 8-channel E1-ClipTip Equalizer pipette and Multidrop Combi Reagent Dispensers. The preparation of the 96-well plate for RT-PCR went from 30 minutes to 12 minutes using the 8-channel E1-ClipTip Equalizer pipette. Taken together, the total time savings is 47 minutes (75 minutes – 28 minutes). This cuts the time it takes for manual pipetting by more than half and is also more ergonomic-friendly because of the reduced number of pipetting steps. A substantial number of these pipetting steps are eliminated by using the 8-channel E1-ClipTip Equalizer pipette to transfer the 94 samples from their collection tube to the 96-well plate by utilizing its adjustable tip spacing.

Figure 1: Increased RT-PCR throughput by 24% and 63% reduction in hands-on time using E1-ClipTip Equalizer and Multidrop Combi Reagent Dispenser.



The greatest impact on the RT-PCR workflow is the overall reduction in PCR instrument downtime as the devices wait for plates to be prepared. This time savings results in a 24% increase in sample throughput for a standard RT-PCR workflow (Figure 1). This also allows two PCR instruments to run continuously and increases the number of samples that can be processed in 24 hours from 30 plates (2,820 samples) to 37 plates (3,478 samples).

Furthermore, performing the liquid handling steps can be much faster and more consistent using an electronic pipette that includes step-by-step program creation versus having to change volumes with manual pipettes. E1-ClipTip has the ability to import protocols created in the My Pipette Creator App available on Connect. Protocols can also be shared between users and labs using the app. This reduces pipetting errors and maintains reproducibility across operators and labs.

Summary

- The E1-ClipTip Multichannel Equalizer pipette saves time by allowing users to perform multiple sample transfers simultaneously between virtually any tube, rack, microplate or horizontal gel box quickly and efficiently by simply sliding the scale to expand or contract the distance between tips to the desired labware.
- The Multidrop Combi Reagent Dispensers provides accurate automated dispensing allowing high-throughput filling of plates with any well size.
- A 63% reduction in the amount of time required to perform nucleic acid extraction preparation steps was determined with the use of the 8-channel E1-ClipTip Equalizer pipette and Multidrop Combi Reagent Dispenser. This time reduction enables as much as a 24% increase in throughput by reducing instrument idle time between RT-PCR runs. ■

PCR Gains New Powers and Broadens Its Clinical Remit

Already indispensable as a means of diagnosing COVID-19, PCR has the speed, sensitivity, and reliability to take on a wide range of clinical applications

By Vivienne Raper, PhD

Polymerase chain reaction (PCR) tests have become routine because of the COVID-19 pandemic, with more than a million PCR tests being carried out daily in the United States alone. What are now commonly known as RT-PCR tests have become key to the world's pandemic response.

Although "RT-PCR" is on everyone's lips, the term can be confusing. The "RT" can stand for "real time" or "reverse transcription." Also, RT-PCR tests may or may not incorporate a technique called quantitative PCR (qPCR). Finally, qPCR is sometimes taken to mean the same thing as real-time PCR.

For the sake of clarity, the gold standard diagnostic test for COVID-19 should be called "qRT-PCR" to emphasize that it incorporates reverse transcription PCR and quantitative PCR. Like other PCR techniques, qRT-PCR relies on PCR, a common method for making many copies of small DNA segments. In addition, qRT-PCR employs a reverse transcriptase, which creates complementary DNA copies of the RNA in a sample. Finally, qRT-PCR uses qPCR, which monitors the amplification of a targeted DNA molecule during the PCR by measuring fluorescent signals from the binding of fluorescent dyes or probes. (In qPCR, fluorescence is monitored in real time, that is, during amplification, not at the end of amplification, as in conventional PCR.)

Because qRT-PCR combines PCR amplification, reverse transcription, and fluorescence monitoring, it is able to measure the amount of RNA molecules that have been targeted for analysis, even RNA molecules that are present in quantities that would otherwise be too small to measure. Needless to say, these RNA molecules may correspond to viral RNA, such as the RNA from the SARS-CoV-2 virus.

Of course, PCR technologies have clinical applications beyond COVID-19 testing, as well as research applications. For example, reverse transcription can be used to monitor gene expression or mRNA synthesis. And if qPCR uses fluorescent DNA probes rather than dyes, it can measure multiple DNA targets—that is, it can realize multiplexing

unoL/Getty Images

applications. Yet another PCR technology is digital PCR. It involves partitioning PCR samples into thousands of nanodroplets, with a separate PCR reaction performed on each one. In digital PCR, the "digital" refers to the absolute quantification of target nucleic acids. Digital PCR does not rely on references or standards to derive absolute quantities from relative or "analog" measurements. Accordingly, it is capable of greater precision.

The latest advances in qPCR and digital PCR were discussed at the "8th qPCR & Digital PCR Congress," which was held December 6–7, 2021, in London. The event's presentations focused on the challenges of using qPCR and digital PCR in clinical settings—challenges such as accuracy, reproducibility, assay optimization, multiplexing, and standardization. Several of these challenges are discussed in this article, which shares insights from the event's most intriguing speakers.

Making PCR faster

The pandemic has highlighted some of the deficiencies in PCR testing. "It's become obvious testing is a crucial part of the response, but PCR testing is extremely infrastructure- and time-consuming," says Stephen Bustin, PhD, professor of molecular medicine, Anglia Ruskin University. If you're lucky, he continues, it might be six hours before you have a sample, but it's usually two or three days until you have an analyzable result.

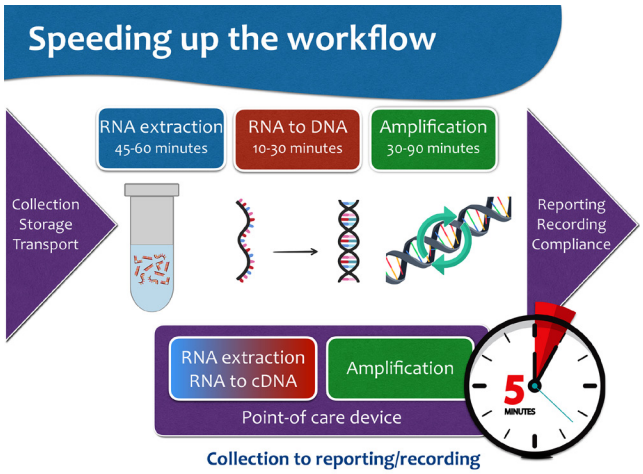


Figure 1: At Anglia Ruskin University, Stephen Bustin, PhD, has been working to make PCR analysis faster and more reliable. He believes that with the development of standardized workflows and optimized protocols, RT-qPCR systems could be devised that would provide five-minute reporting/recording of test results at the point of care.

Bustin has worked with PCR for decades. He achieved a measure of fame in 2007 as an expert witness for the U.S. Department of Justice. In this capacity, he presented a reanalysis of the RT-qPCR data underpinning Andrew Wakefield's infamous work linking the measles, mumps, and rubella (MMR) vaccine with autism.

Through his work, Bustin has learned that PCR is often performed poorly and applied inappropriately. He has also found that these problems have real-life consequences. In the case of the Wakefield reanalysis, he uncovered contamination issues in the original PCR analysis of intestinal samples taken from autistic children.

Bustin has been working to make PCR analysis faster and more reliable. He recalls how he reacted after a colleague reported that extreme PCR could perform 30 cycles of amplification in 20 seconds. Extreme PCR's speed, Bustin realized, could be valuable in COVID-19 diagnostics. He still feels that way: "We need a personalized point-of-care system where you can do PCR in as little time as possible."

Since 2019, Bustin has been working on a method to run 30 cycles of PCR amplification in 75 seconds. At the congress, he explained how to take a sample, enrich the RNA content, perform a reverse transcriptase reaction, and then run a rapid PCR on the DNA.

He explains that conventional PCR relies on a heating block to adjust temperature, whereas his method uses a robot to move samples rapidly between heated water baths. "I can't tell you a lot about it," he says. "The university is quite interested in it, and there are a lot of patents."

Although Bustin must be discrete, he does share that he's working with OptiSense, a small analytical instrument company based in Horsham, U.K. The collaborators have a two-year business plan to miniaturize the technology. They are currently developing a prototype instrument.

"If it works, it's going to revolutionize how we do PCR," Bustin asserts. "The aim is to do a PCR in five minutes. So, you might pop down to Boots [a U.K. pharmacist] and get a SARS-CoV-2 or flu test. You could test before you go into a waiting room or a hospital setting. Visitors could get tested before entering a care home, or people could get tested in an airport or on a cruise—the potential uses are quite limitless."

Amplifying the Applications

The U.K.'s Cell and Gene Therapy Catapult (CGTC) has been working with industry partners and academic collaborators to progress the use of PCR for gene therapy manufacturing. That's according to Lily Li, PhD, a viral vector analytical senior scientist at the CGTC. Li presented a study at the convention that compares qPCR with droplet digital PCR (ddPCR) for monitoring genome viral vector copy number in adeno-associated virus (AAV) manufacturing.

"When manufacturers make these AAV gene therapy products, they need to monitor the genome copy number of the AAV," Li says. "One of the most-used ways is by qPCR, and that's why adequate AAV characterization is critical for process development, manufacturing, clinical dosing, and ultimately product safety."

Li notes that the precision of qPCR can be poor for AAV characterization, and that qPCR can behave suboptimally when applied to complex gene therapy products.

"All this complexity may affect how qPCR, one of the most common [PCR] methods, works," she explains. Her research indicates that ddPCR performs better than qPCR. "It's less susceptible to complex factors and more robust," she details. "We know it's more capable of processing these variables." She acknowledges, however, that qPCR is cheaper and delivers higher throughput than ddPCR, and that many manufacturers still consider qPCR to be a gold standard technique.

Improving PCR Precision

A recent innovation in PCR that promises to advance precision diagnostics is two-tailed PCR, says Mikael Kubista, PhD, head of gene expression profiling, Institute of Biotechnology, Czech Academy of Sciences. "I think we first published on it three or four years ago," he recalls. "And that was the publication of an application for microRNA detection."

Regular qPCR uses two primers and, optimally, a probe. The primers are short sections of single-stranded DNA that flank the region of DNA to be copied (and amplified). The probe, meanwhile, is the fluorescently labeled DNA oligonucleotide that binds downstream of the primer and fluoresces when the DNA is cut during amplification. According to Kubista, the primers and probe tend to be 20–25 bases long and are thus unable to detect or amplify a DNA/RNA molecule shorter than 50 bases.

"Generally, this has been a major problem when analyzing short molecular targets like microRNAs," he says. "If you work with fragmented material, the standard method is to make the RNA longer to fit the two primers, but if you make the original RNA target longer, you have to include another reaction, the elongation."

Kubista explains that elongating the original RNA compromises the PCR yield by adding an additional process step. You also lose specificity, he explains, because the primer targets a small, specific sequence of bases.

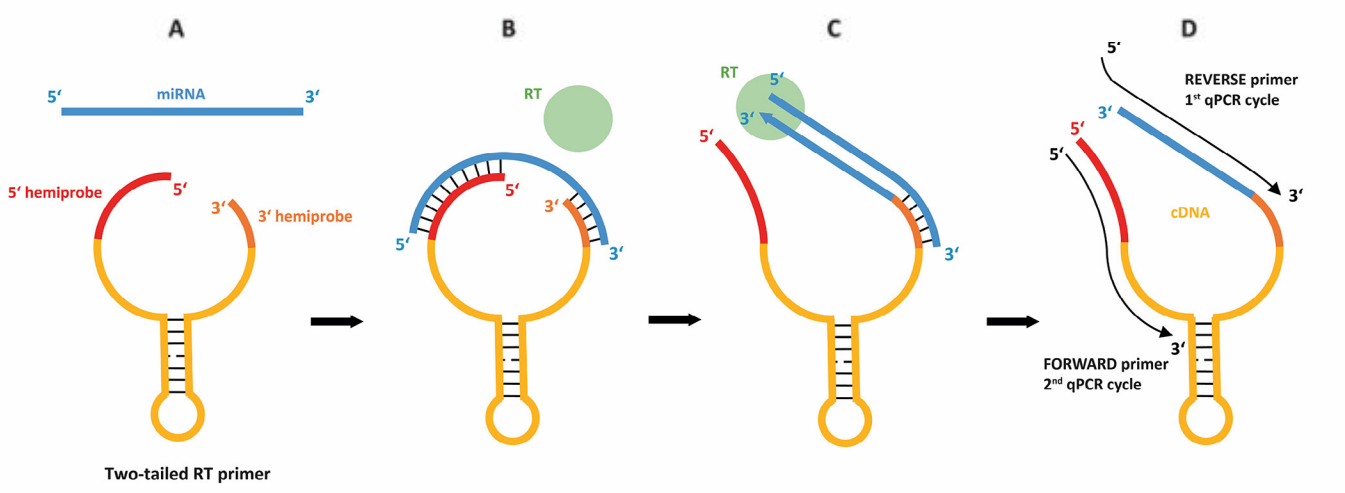


Figure 2: An advanced PCR technology called two-tailed RT-qPCR is being developed for microRNA detection by Mikael Kubista, PhD, head of gene expression profiling, Institute of Biotechnology, Czech Academy of Sciences. Instead of using a single probe, two-tailed PCR uses two hemiprobes that bind to different stretches of targeted microRNA and are connected by a tether. Using two probes ensures high sensitivity and enables discrimination of highly homologous microRNAs.

Two-tailed PCR overcomes this problem by priming with a single molecule that hybridizes to both ends of the microRNA, he explains. Although each hemiprobe is too small on its own to form a stable interaction with the microRNA, the hemiprobes hybridize with the same efficiency as a regular primer when they are put together on the same molecule.

"You gain sensitivity because you don't have to include an additional elongation reaction," Kubista explains. "You PCR the target directly, and you also have fantastic specificity." Two-tailed PCR, he says, can detect as little as one molecule of sequence variation among 100 to 1,000 sequences in a digital PCR droplet.

After the two-tailed PCR technology first appeared in published work in 2017, it began to be commercialized by BioVendor, which currently offers the technology in the form of off-the-shelf assays. The first panel to detect SARS-CoV-2 microRNAs was developed using two-tailed PCR. The technology also has applications for monitoring organ rejection. In these applications, the technology can monitor donor DNA—that is, DNA from a donor heart, lung, kidney, or liver—that enters the patient's bloodstream during rejection. The tiny fragments of donor DNA, Kubista asserts, are detectable with two-tailed PCR.

Turning to Advanced Sequencing Techniques

Another researcher working to improve the specificity of DNA detection in patient's blood is Viktor A. Adalsteinsson, PhD, associate director of the Gertsner Center for Cancer Diagnostics at the Broad Institute of MIT and Harvard. Adalsteinsson is focused on improving the sensitivity of techniques for detecting

minimal residual disease (MRD).

"There's been a lot of interest in tracking MRD—the cancer left after treatment," he says. "There are millions of cancer patients that undergo surgery for an early-stage cancer. But if there's no way to know if there's MRD left elsewhere in the body, it's difficult to assess the need for further treatment or the risk of a future recurrence.

"When there's very little tumor DNA in the blood, the likelihood that all mutations from a patient's tumor are drawn in any one tube of blood is slim. We think that looking for all mutations in a patient's tumor genome can improve detection."

He presented a study at the congress showing that tracking more mutations per patient improved the likelihood of detecting MRD. The study describes how Adalsteinsson and colleagues tracked mutations using an ultrasensitive blood test they developed for cell-free DNA. The test used exome sequencing for patient-specific single-nucleotide variants.

The researchers compared the results from their test to the results from a ddPCR test. Both tests were used to evaluate a cohort of breast cancer patients. The new test, the researchers found, had a thousand-fold lower error rate.

Adalsteinsson and colleagues have developed several other new methods. For example, they developed Concatenating Original Duplex for Error Correction (CODEC), a sequencing method that combines the massively parallel nature of next-generation sequencing with the single-molecule capability of third-generation sequencing. They developed Duplex-Repair, a method that can limit interior duplex base pair resynthesis, rescue the impact of induced DNA damage, and afford more accurate duplex sequencing. And they developed Minor Allele Enriched Sequencing through Recognition Oligonucleotides (MAESTRO), a method for mutation enrichment. According to Adalsteinsson, MAESTRO has made it possible to track genome-wide tumor mutations in blood. ■

Automation Tip Comparison

Performance Comparison of Automated Tips on an Automated TaqMan Assay

By Rosalinda M. Savoy, PhD., Product Applications Specialist II, LPD Divisional Marketing

The challenges of performing real time PCR are greatly reduced when using the most suitable equipment. Automating TaqMan® assays can greatly help reduce pipetting errors and improve precision and accuracy. In order to ensure the best tools are used for pipetting TaqMan assays, we plated several TaqMan assays and compared the results from using Thermo Scientific™ Automation Tips and a competitor's automation tips on the Tecan™ Freedom EVO™. Thermo Scientific Automation Tips showed less standard deviation between threshold cycle (Ct) values in the replicates, lower coefficient of variation (%CV), and demonstrated a higher precision than the competitor's automation tips. When the copy number variation (CVN) was compared between two different DNA sources, both sets of tips had the same copy number for the TaqMan assay, validating the accuracy of the Thermo Scientific Automation Tips.

Introduction

Real Time PCR (qPCR) is an extremely valuable and widespread tool in molecular biology, having applications in research, diagnostics, and forensics. It can be used for gene expression analysis, microRNA and noncoding RNA analysis, SNP genotyping, and to determine copy number variation.

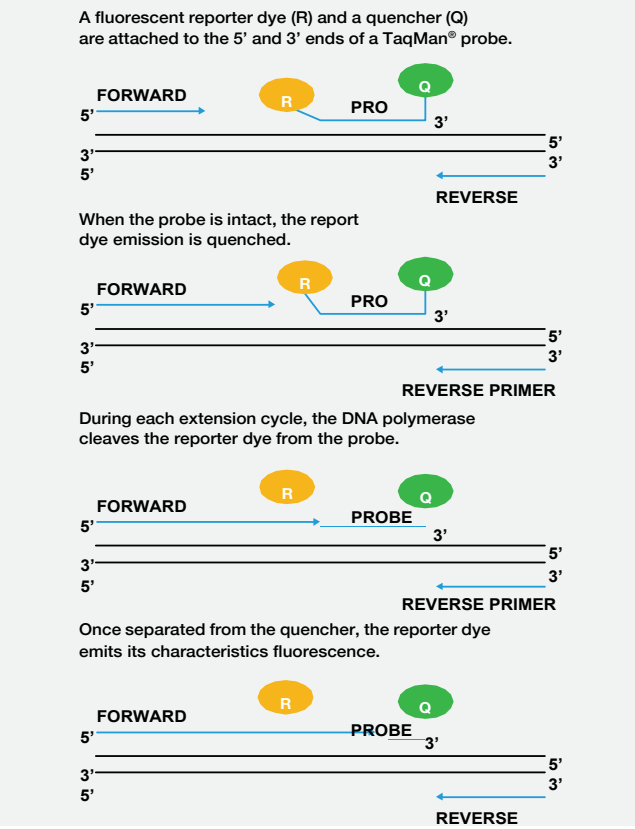


Figure 1. Overview of the TaqMan chemistry.

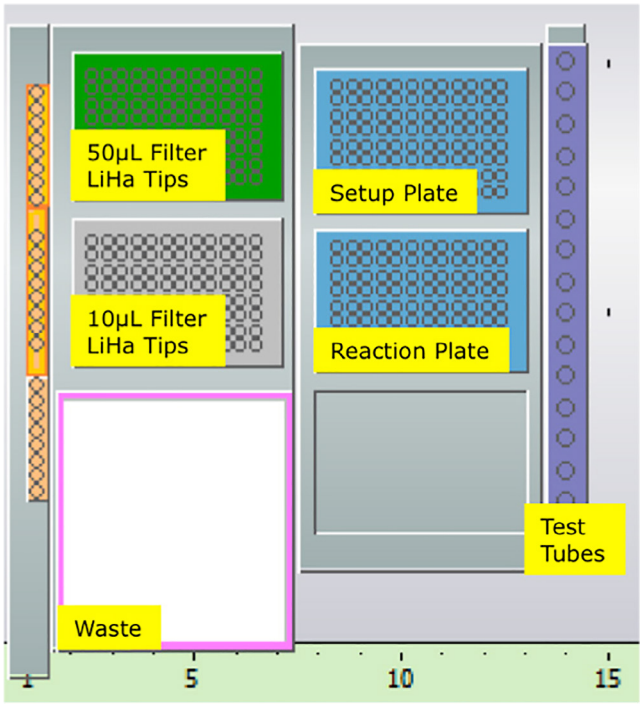
TaqMan probes are an extremely accurate method to employ when performing qPCR. They offer increased specificity when compared to primers alone. TaqMan probes are labeled with a fluorophore on the 5′ end and a quencher on the 3′ end. The polymerase extends from the primer and when it reaches the TaqMan probe, the 5′ to 3′ exonuclease activity cleaves the fluorophore releasing it from the quencher and

allowing its detection by the thermal cycler (Figure 1).

The need for precision and accuracy when performing qPCR is paramount. It is an extremely sensitive assay and slight differences due to pipetting errors can be disastrous. When combined with the large number of reactions that are often needed, this can become extremely time consuming and cause repetitive motion injuries. Automating qPCR has a huge advantage in preventing these issues and by using Thermo Scientific Automation Tips you can help ensure excellent repeatability, reproducibility, and accuracy, helping prevent pipetting errors that lead to incorrect data and costly reruns.

The purpose of this study is to highlight the precision and accuracy of Thermo Scientific Automation Tips for qPCR. To illustrate this we used the Tecan™ Freedom EVO™ system to automate the Applied Biosystems™ TaqPath™ ProAmp™ on two samples for CVN analysis (Figure 2). The sample preparation was automated using the Thermo Scientific 50 µL and 10 µL automation tips for Tecan™ LiHa and compared with equivalent competitor automation tips. The comparison demonstrates the precision of the Thermo Scientific Automation Tips and how effectively they can work on the Tecan™ Freedom EVO™ system in this and any application.

Figure 2. Deck layout using Thermo Scientific Automation Tips for TaqPath™ ProAmp™.



Materials and Methods

- Thermo Scientific Automation Tips
- 10 µL conductive, sterile, ART barrier tips for Tecan™ LiHa - P/N 901-011
- 50 µL conductive, sterile, ART barrier tips for Tecan™ LiHa - P/N 902-011
- Competitor's automation tips
- 10 µl tips for Tecan™ LiHa – conductive, sterile, filtered
- 50 µl tips for Tecan™ LiHa – conductive, sterile, filtered
- Tecan™ Freedom EVO™ Series with LiHa head was utilized for pipetting operations.
- Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 96-well, 0.1 mL (A28138)
- Applied Biosystems™ MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL (4346907)
- Applied Biosystems™ MicroAmp™ Optical Adhesive Film (4360954)

- Thermo Scientific™ Jurkat Genomic DNA (SD1111)
- Applied Biosystems™ TaqMan® Control Genomic DNA (4312660)
- Invitrogen™ RT-PCR Grade Water (AM9935)
- Applied Biosystems™ TaqMan® Copy Number Reference Assay, human, RNase P (4403328)
- Applied Biosystems™ TaqMan® Copy Number Assay, CYP2D6
- Applied Biosystems™ TaqPath® ProAmp™

Protocol

1. Make master mixes for samples according to user guide[1]. Combine the TaqPath™ ProAmp™ Master Mix, the RNase P TaqMan™ Copy Number Reference Assay, the CYP2D6 TaqMan™ Copy Number Assay, the RT-PCR Grade Water, and the gDNA for 5 replicas. Repeat for each gDNA sample and a negative for each of the assays.
2. Mix the samples on an orbital shaker and spin down in a centrifuge so there are no bubbles.
3. For each sample and negative, transfer four replicas to an empty MicroAmp™ Fast Optical 96-Well Reaction Plate.
4. Seal plate with the MicroAmp™ Optical Adhesive Film.
5. Spin down in a centrifuge so there are no bubbles.
6. Using the same conditions, repeat all steps for the competitor's automation tips.
7. Run on Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System[2].

Real Time PCR Protocol

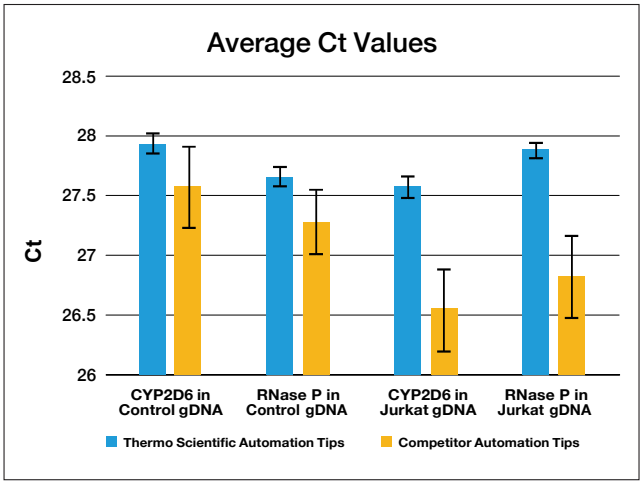
The samples were amplified using the following protocol:

1. Heated at 95°C for 5 min.
2. Performed 40 cycles of 95°C for 5 sec and 60°C for 30 sec.
3. Analyzed the data using the Design and Analysis Application and the Relative Quantification on the Thermo Fisher™ Connect™ Cloud.

Results

The performance of Thermo Scientific Automation Tips on a Tecan™ Freedom EVO™ system using a LiHa head was evaluated as an ideal way to perform qPCR using Applied Biosystems™ TaqPath™ ProAmp™ Master Mix. All data points were averaged from quadruplicate

Figure 3. Quadruplicate average Ct and standard deviation for Thermo Scientific Automation Tips vs the competitor's.



	Thermo Fisher Scientific Automation Tips			Competitor Automation Tips		
Sample DNA	Average Ct	Standard Deviation	%CV	Average Ct	Standard Deviation	%CV
CYP2D6 in Control gDNA	27.937	0.085	0.304	27.574	0.354	1.283
RNase P in Control gDNA	27.662	0.087	0.316	27.287	0.271	0.995
CYP2D6 in Jurkat gDNA	27.558	0.100	0.361	26.557	0.327	1.231
RNase P in Jurket gDNA	27.888	0.062	0.221	26.845	0.330	1.228

Table 1. Quadruplicate average Ct, standard deviation, and %CV for Thermo Scientific Automation Tips vs the competitor's automation tips. All of the Thermo Scientific Automation Tips showed a standard deviation of less than 0.1, while the competitor's automation tips had a standard deviation closer to 0.3. In addition, %CV for the Thermo Scientific Automation Tips was almost 1% lower than the %CV for the competitor's automation tips.

replicas. The Thermo Scientific Automation Tips were compared with the competitor's automation tips and displayed highly consistent threshold cycle (Ct) values within the replicas of TaqMan assays compared to TaqMan assays pipetted with the competitor's automation tips (Figure 3).

The low coefficient of variation (%CV) of Thermo Scientific Automation Tips (between 0.221 and 0.361) showed a higher precision than the competitor's automation tips (between 0.995 and 1.283, Table 1).

Using the Thermo Fisher™ Connect™ Cloud, the copy number of CYP2D6 was determined in each of the gDNA samples. Using both the Thermo Scientific Automation Tips and the competitor's automation tips, the copy number of CYP2D6 was established to be 2 in the control gDNA and 3 in the Jurkat gDNA (Figure 4).

To ensure the quality of the data, negatives for each assay were performed. Negative samples are an extremely important part of qPCR to ensure no contamination happened during the pipetting process.

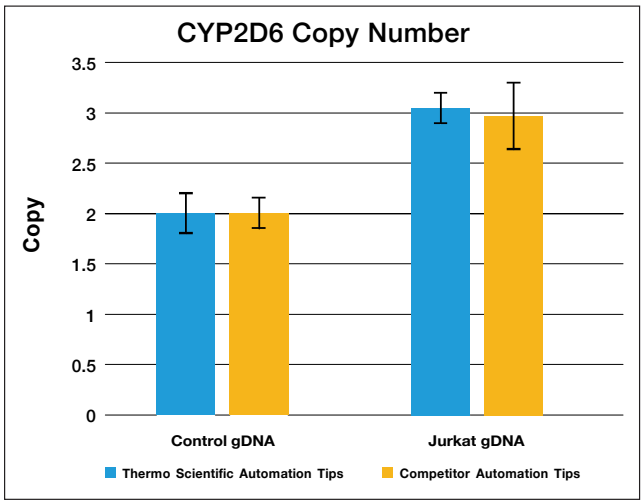


Figure 4. Copy Number difference of target gene between samples. The copy number of CYP2D6 in the control gDNA was 2, while it increased to 3 in the Jurkat gDNA. These results were proven with both Thermo Scientific Automation Tips and the competitor's automation tips, indicating a high level of accuracy.

Thermo Scientific Automation Tips have ART barriers that maintain the integrity of the samples and provide security against aerosols and liquid contamination. The negative samples had no amplification, highlighting the effectiveness of the ART barrier used in Thermo Scientific Automation Tips and the quality in the tips (Figure 5).

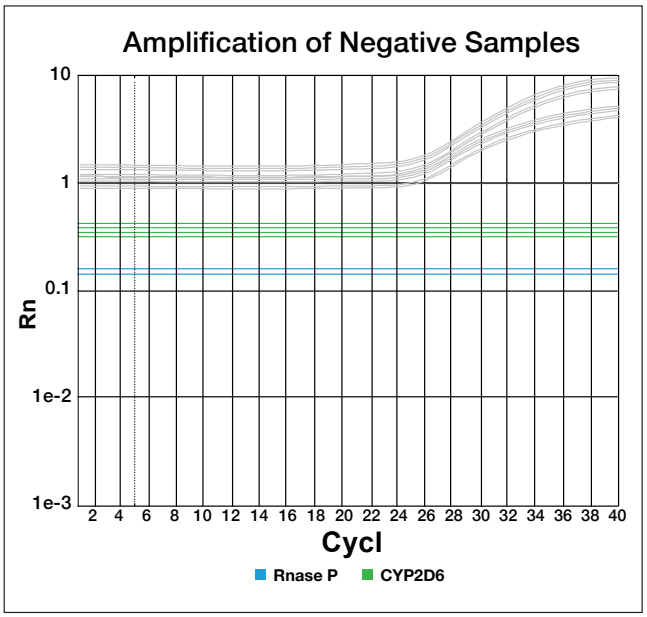


Figure 5. Negative samples for each assay using Thermo Scientific Automation Tips. The graph shows the Rn, or the magnitude of the fluorescent signal at each cycle, for the Thermo Scientific Automation Tips. The grey lines are the samples with gDNA and the highlighted green or blue lines are the negatives for each assay. The negative samples show that no leaking or spraying occurred during the pipetting process.

Conclusions

This study evaluated the suitability of using Thermo Scientific Automation Tips on a Tecan™ Freedom EVO™ with a LiHa head to perform qPCR with TaqMan probes. Both Thermo Scientific Automation Tips and the competitor's automation tips demonstrated the control gDNA had 2 copies of the CYP2D6 gene and the Jurkat gDNA had 3 copies of the CYP2D6 gene indicating a high level of accuracy. Furthermore, the Thermo Scientific Automation Tips had lower standard deviation and lower %CV between replicas than the competitor's automation tips, proving they provide better reproducibility. The ART barriers also helps ensure no contamination between samples or in the negatives, enabling the quality of the results. For over three decades Thermo Fisher Scientific has been manufacturing tips for automated liquid handling instruments. We adhere to a rigorous QC process, which includes lot-testing during production on the workstation from which the tips are created and helps ensure a low coefficient of variation. Thermo Fisher Scientific has tips available as sterile, non-sterile, filtered, non-filtered, liquid sensing, and non-liquid sensing. The sterile tips are certified free of RNase, DNase, DNA, endotoxin, bioburden, and pyrogen. Thermo Fisher Scientific has a wide range of pipette tips suitable for use in a 96 well or 384 well format, covering over 50 different liquid handlers. Our tips have equivalent performance to the robotic automation manufacturer's and other aftermarket manufacturer's tips with no need to reprogram the instrument. All of this allows for a seamless fit with identical formats to other manufacturers. ■

References:

1. TaqPath ProAmp User Manual https://tools.thermofisher.com/content/sfs/manuals/MAN0015758_TaqPathProAmpMMix_UG.pdf
2. QuantSudio 3/5 User Manual https://tools.thermofisher.com/content/sfs/manuals/MAN0010407_QuantStudio3_5_InstallUseMaint_UG.pdf

Signatures Comprising Groups of Gut Microbiome Genes Linked with Multiple Diseases

The human body is host to trillions of bacteria, fungi, viruses, and other microorganisms that make up the human microbiome. Researchers at Harvard Medical School (HMS) and Joslin Diabetes Center have now analyzed the genetic makeup of bacteria in the human gut, and linked groups of bacterial genes—genetic signatures—to disorders including atherosclerotic cardiovascular disease (ACVD), cirrhosis of the liver (CIRR), inflammatory bowel disease (IBD), colorectal cancer (CRC), and type 2 diabetes (T2D).

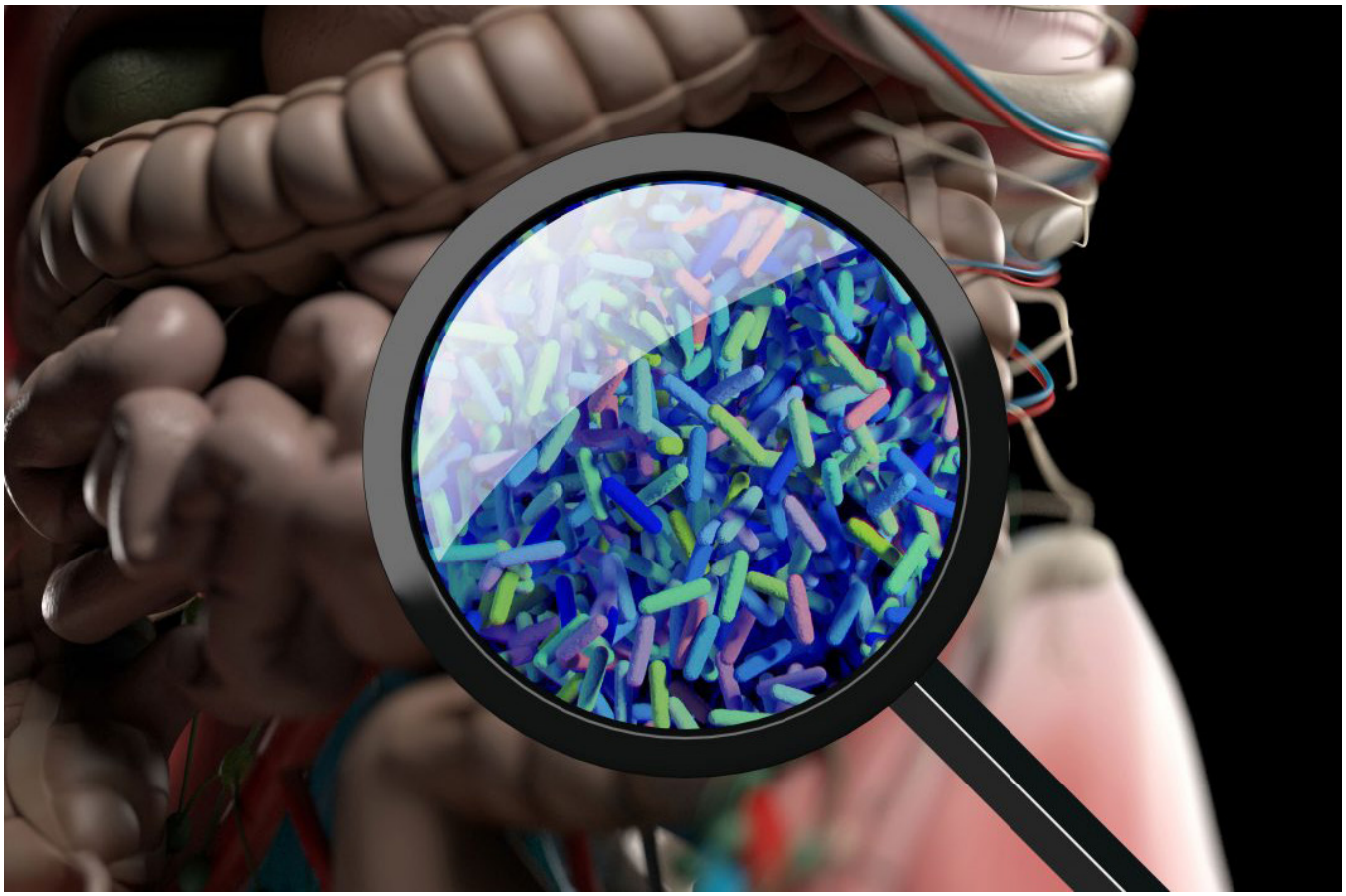
Data from the gene-level microbiome-disease association study indicated that coronary artery disease, inflammatory bowel disease, and liver cirrhosis share many of the same bacterial genes. In other words, people with gut microbiota that contain the same collections of bacterial appear more likely to have one or more of these three conditions.

The team says the work adds new understanding to what is already known about the relationship between the gut microbiome and specific diseases. If confirmed through further research, the results could inform the design of tools for gauging a person's risk for a range of conditions, based on analysis of a single fecal sample.

The researchers do caution that their study was not designed to determine how and why these microbial genes may be linked to different diseases. Thus far, they said, it remains unclear whether these bacteria are involved in disease development or are mere bystanders in this process. Nevertheless, as Braden Tierney, a graduate student in the Biological and Biomedical Sciences program at HMS, stated, "This opens a window for the development of tests using cross-disease, gene-based indicators of patient health … We've identified genetic markers that we think could eventually lead to tests, or just one test, to identify associations with a number of medical conditions."

Tierney is first author of the team's published paper in *Nature Communications*, which is titled "Gene-level metagenomic architecture across diseases yield high-resolution microbiome diagnostic indicators."

The ecology of the human microbiome is known to be associated with both phenotype and environment, the authors wrote. Previous studies have



ChrisChrisW / Getty Images

linked the mix of resident bacteria, and the presence of specific bacterial species, with conditions ranging from obesity to multiple sclerosis. The goal of the newly reported study was to determine whether groups of bacterial genes, rather than the species themselves, could reliably indicate the presence of different diseases.

The researchers started out by collecting microbiome data from 13 groups of patients totaling more than 2,500 samples. Next, they analyzed the data to pinpoint linkages between seven diseases and millions of microbial species, microbial metabolic pathways, and microbial genes. By trying out a variety of modeling approaches—computing a total of 67 million different statistical models—they were able to observe those microbiome features that consistently emerged as the strongest disease-associated candidates.

The team found that of all the various microbial characteristics—species, pathways, and genes—microbial genes had the greatest predictive power. In other words, the researchers said, groups of bacterial genes, or genetic signatures, rather than merely the presence of certain bacterial families, or individual bacterial genes were linked most closely to the presence of a given condition.

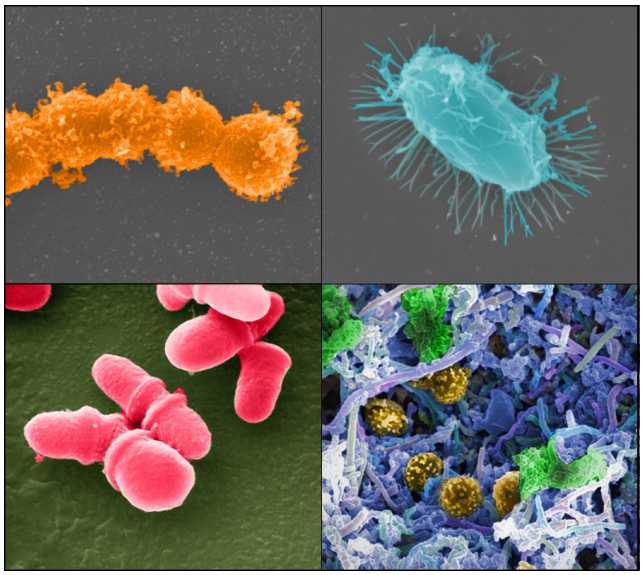
The results indicated that while coronary artery disease, inflammatory bowel disease, and liver

cirrhosis had similar gut microbiome genetic signatures, type 2 diabetes, was associated with a microbiome signature unlike any other phenotype tested. "Overall, we found striking and previously unrecognized high resolution genetic and taxonomic signatures associated with ACVD, IBD, CRC, and cirrhosis …" the authors noted.

Interestingly, the analysis did not find a consistent link between presence of the bacterial species Solobacterium moorei and colon cancer—an association that has previously reported in numerous studies. However, the researchers did identify particular genes from a S. moorei subspecies associated with colorectal cancer. This finding indicates that gene-level analysis can yield biomarkers of disease with greater precision and more specificity compared with current approaches.

Co-senior study author Chirag Patel, PhD, associate professor of biomedical informatics in the Blavatnik Institute at HMS, suggested that this result underscores the notion that it is not merely the presence of a given bacterial family that may indicate risk, but rather it is the strains and gene signatures of the microbes that matter.

The ability to identify interconnections with such precision will be critical for designing tests that can measure risk reliably, he added. So, for example, a test intended to measure colon-cancer risk by merely detecting the presence of S. moorei in the gut may not be as reliable as a more refined test that measures bacterial genes to detect the presence of specific strains of S. moorei that are associated with colon cancer.



Jonathan Bailey, National Human Genome Research Institute, NIH

Figure 1: An example of the diversity of the human microbiome. Clockwise from top left: Streptococcus (Tom Schmidt); microbial biofilm of mixed species, from human body (A. Earl, Broad Institute/MIT); Bacillus (Tom Schmidt); Malassezia lopophilis (J.H. Carr, CDC).

"Our gene-level architecture analysis captured a previously undocumented strain-level exploration of pan-disease-associated microbes," Tierney and colleagues reported. "We additionally find that at the species-level, the prior-reported connection between Solobacterium moorei and colorectal cancer is not consistently identified across models—however, our gene-level analysis unveils a group of robust, strain-specific gene associations." Patel added, "Our study underscores the value of data science to tease out complex interplay between microbes and humans."

In contrast with highlighting positive associations

between gut microbiome gene signatures and some diseases, the study data also indicated that two conditions, ear inflammation (otitis) and adenomas—benign soft-tissue tumors—showed only weak associations with the gut microbiome, suggesting that microorganisms residing in the human gut are not likely to play a role in the development of these conditions, nor are they likely to be reliable indicators that these conditions are present. "We specifically chose to examine otitis as a form of negative biological control, as, to our knowledge, it has limited reported association with the gut microbiome, and we expected it to have a negligible metagenomic architecture," the investigators noted.

In a previous study, the HMS team used massive amounts of publicly available DNA-sequencing data from human oral and gut microbiomes to estimate the size of the universe of microbial genes in the human body. The analysis revealed that there may be more genes in the collective human microbiome than stars in the observable universe. Given the sheer number of microbial genes that reside within the human body, the new findings represent a major step forward in understanding the complexity of the interplay between human diseases and the human microbiome, the researchers said.

The newly identified microbial genetic signatures could be studied further to determine what role, if any, the organisms play in disease development. "Overall, our work is not only a step towards gene-based, cross-disease microbiome diagnostic indicators, but it also illuminates the nuances of the genetic architecture of the human microbiome, including tension between gene- and species-level associations," the team wrote in their paper. "Focusing on the gene level may have an additional practical advantage over analysis at the species- or pathway level in the clinic: it allows for high-throughput, multiplexed, PCR-based, and specific diagnostics."

"The ultimate goal of computational science is to generate hypotheses from a huge swath of data," said Tierney. "Our work shows that this can be done and opens up so many new avenues for research and inquiry that we are only limited by the time, people, and resources needed to run those tests."

The authors concluded, "Overall, this work depicts a path for researchers for moving microbiome associations from the abstract to the robust. In short, fitting and reporting a single model is simply not sufficient. However, if we are able to identify robust-to-specification associations that reproduce across cohorts, we will increase the efficiency of biomedical experiments." ■

Miniaturizing qPCR Assays and Simplifying Standard Curve Preparations

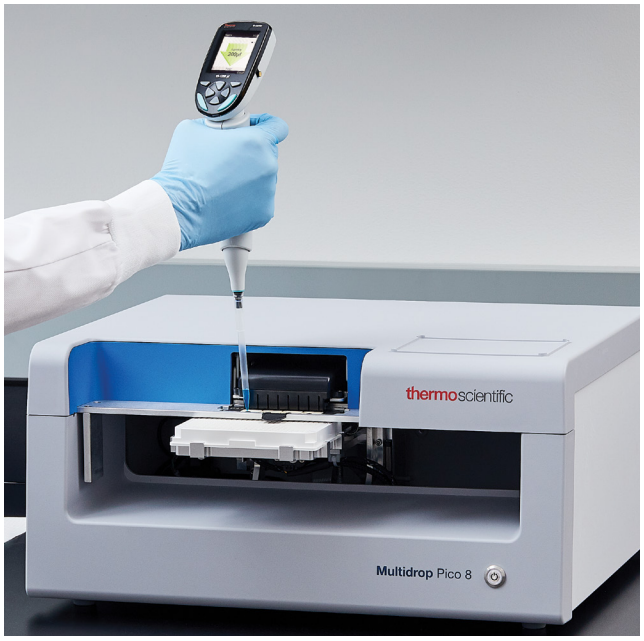
Abstract

This study demonstrates and executes a successfully miniaturized qPCR assay with the Thermo Scientific™ Multidrop™ Pico 8 Digital Dispenser and PicoIT 8 software. In three simple steps, the PicoIT 8 software enables the setup of a qPCR assay plate including titrations of reagents needed for a standard curve. Then, with the Multidrop Pico 8 Digital Dispenser, the reagents for a miniaturized volume (5 µL reaction) in a 384-well qPCR assay plate are dispensed, mixed, and ready to be placed on the Applied Biosystems™ QuantStudio™ Real-Time PCR System for amplification. The results show how the Multidrop Pico 8 Digital Dispenser and PicoIT 8 software allows users to easily set up a miniaturized TaqMan qPCR assay plate with successful amplification of the input cDNA.

Introduction

The Multidrop Pico 8 Digital Dispenser is a non-contact liquid dispenser that enables assay miniaturization and liquid dispensing down to the picoliter range. The Multidrop Pico 8 Digital Dispenser precisely delivers fluids with %CV values of ≤ 8% for volumes as low as 11 pL into any well of a microplate including 96, 384, and 1,536 well-plate formats. The non-contact dispense technology removes the risk of contamination of the reagents. Additionally, the Multidrop Pico 8 Digital Dispenser is capable of direct mixing of the dispensed reagents to ensure a homogenous reaction.

The PicoIT 8 software included with the Multidrop Pico 8 Digital Dispenser provides an easy to use user interface. PicoIT 8 software includes pre-set protocol templates made for qPCR layouts or the user



can simply create a custom protocol using the PCR function. With the pre-set protocol qPCR templates, the software guides you through 3 steps: opening a pre-set template, customizing the protocol template to your specific assay, and finally running your newly developed protocol with the Multidrop Pico 8 Digital

Dispenser. In addition, the software automatically generates associated reports in the appropriate format (.DA.csv) to be imported into the QuantStudio Real-Time PCR System. The direct importing of the plate layout with the sample information eliminates the additional steps of recreating the plate set-up in the QuantStudio allowing the user to start the qPCR run instantly. A common hurdle in setting up TaqMan qPCR assays is ensuring pipetting precision at small reaction volumes. In many cases of qPCR assay development, the scientists settle for a larger reaction volume such as 20 µL because of pipetting errors. Decreasing the qPCR reaction volume while maintaining precision improves cost-effectiveness as well as increases throughput.

In this study, we highlight the use of the Multidrop Pico 8 Digital Dispenser and PicoIT 8 software to set up a 5 µL reaction qPCR assay in a 384-well plate. The %CV of Ct values from each technical replicate in this study provides evidence of the Pico 8 dispenser precision when creating low volume TaqMan reactions. The plate layout including sample information is transferred directly to a QuantStudio 5 for thermal cycling steps. The purpose of this study is to demonstrate the ability to miniaturize and successfully execute a qPCR standard curve using the Multidrop Pico 8 Digital Dispenser and PicoIT 8 software. It is important to note that this is an example of a qPCR workflow and can be used as a guide. However, the sample type, reagents, and conditions will differ depending on experimental design.

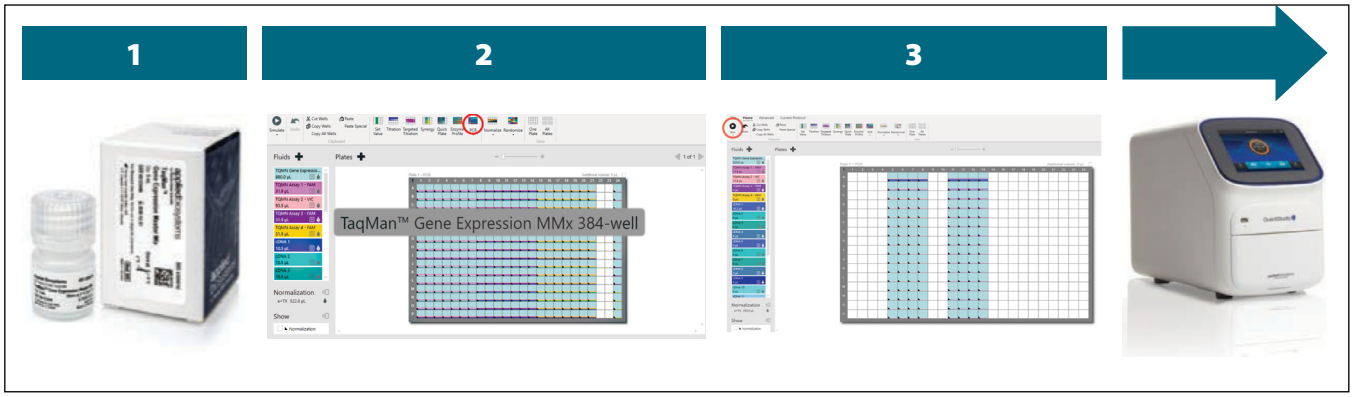
Materials

Table 1: Equipment, reagents, and consumables used in this example qPCR workflow.

Equipment	Cat. No.
Multidrop Pico 8 Digital Dispenser	Thermo Scientific, 5840600
QuantStudio 5, 384-well heat-block	Applied Biosystems, A28575
E1-ClipTip Electronic Single Channel Pipettes	Thermo Scientific, 4670000BT, 4670020BT, 4670040BT
Reagents and consumables	
TaqMan Gene Expression Master Mix (2x)	Applied Biosystems, 4370048
RT-PCR grade, Nuclease-free water	Invitrogen, AM9935
Triton X100, 1%	Invitrogen, HFH10
TaqMan Assay A (20x, custom) FAM-MGB	Applied Biosystems, custom
TaqMan Assay (20x) RNaseP, VIC-TAMRA	Applied Biosystems, custom
cDNA (34 ng/µL)	Generated from Total RNA Control (human), Applied Biosystems, 4307281
384-well MicroAmp EnduraPlate with barcode	Applied Biosystems, 4483285
MicroAmp Optical Adhesive Film	Applied Biosystems, 4360954
ClipTip filter-barrier tips (various sizes)	Thermo Scientific, 94420043, 94420318, 94420813
200 µL 4-channel Cartridges for Pico 8	Thermo Scientific, LTR0003
20 µL 8-channel Cartridges for Pico 8	Thermo Scientific, LTR0004

Table 2: PCR reaction components in each well (5 µL total volume).

TaqMan Gene Expression Master Mix (2x)	2.5 µL
TaqMan Assay (20x) + Triton X100 (1%)	0.275 µL (0.1% Triton X100)
cDNA Template + Nuclease free water + Triton X100 (1%)	2.225 µL (0.1% Triton X100)
Total Volume	**5 µL**



3-steps used to prepare the plate for this qPCR assay
Step 1: Select a pre- programmed protocol template; Step 2: Customize and save as a protocol (adjust variables including standards); Step 3: Run the dispense protocol and follow the software prompts. Plate ready for qPCR.

Methods

First, the PCR reaction components were prepared. Using Thermo Scientific™ E1-ClipTip™ pipettes, the TaqMan Assay reagent was prepared and cDNA template was diluted in nuclease-free water. Triton X100 was added to both mixtures at a final concentration of 0.1% to facilitate dispensing by Multidrop Pico 8 Dispenser (1). Table 2 shows the volume of each reaction component for a 5 µL reaction volume of a TaqMan qPCR assay.

Step 1:

Open a protocol template from the PicoIT 8 software Click File > Open Template > TaqMan Gene Expression MMx 384-well (Figures 1 and 2).

Figure 1: Screenshot of Multidrop PicoIT 8 software, the protocol template option for TaqMan Gene Expression Master Mix, 384-well 5 µL reaction volume, and Multiplex highlighted in red.

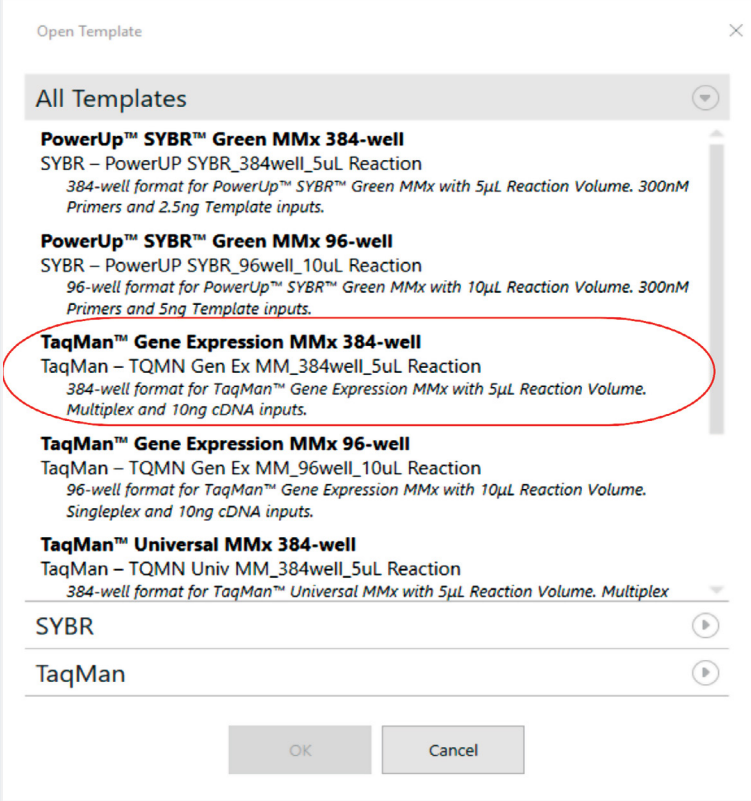


Table 3: Volume dispensed by Pico 8 for each titration.

Level	cDNA volume
1	1.9 µL
2	0.95 µL
3	0.475 µL
4	0.238 µL
5	0.119 µL
6	59.4 nL
7	29.7 nL
8	14.8 nL
9	7.42 nL
10	3.71 nL
11	1.86 nL
12	0.928 nL
13	464 pL
14	232 pL
15	116 pL

Step 2:

Customize the protocol template with the PCR function within the PicoIT software. First, select PCR function from the ribbon (red circle Figure 2), then proceed according to the PCR function: Clicked Plate > Fluids > Unknown Groups > Standards/NTCs > Layout > Summary to set up the parameters specific to the experimental design (Figures 2, 3, 4, and 5).

Figure 2. Screenshot of Multidrop PicoIT 8 software showing the TaqMan Gene Expression MMx 384-well protocol template. Red circle indicates the PCR function icon.

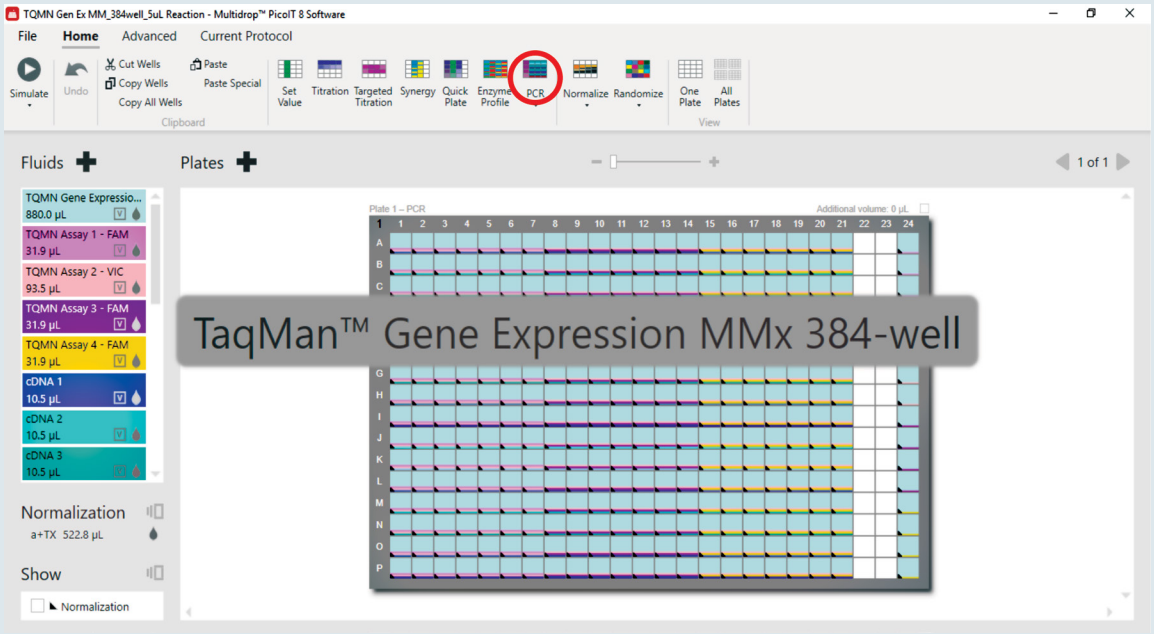
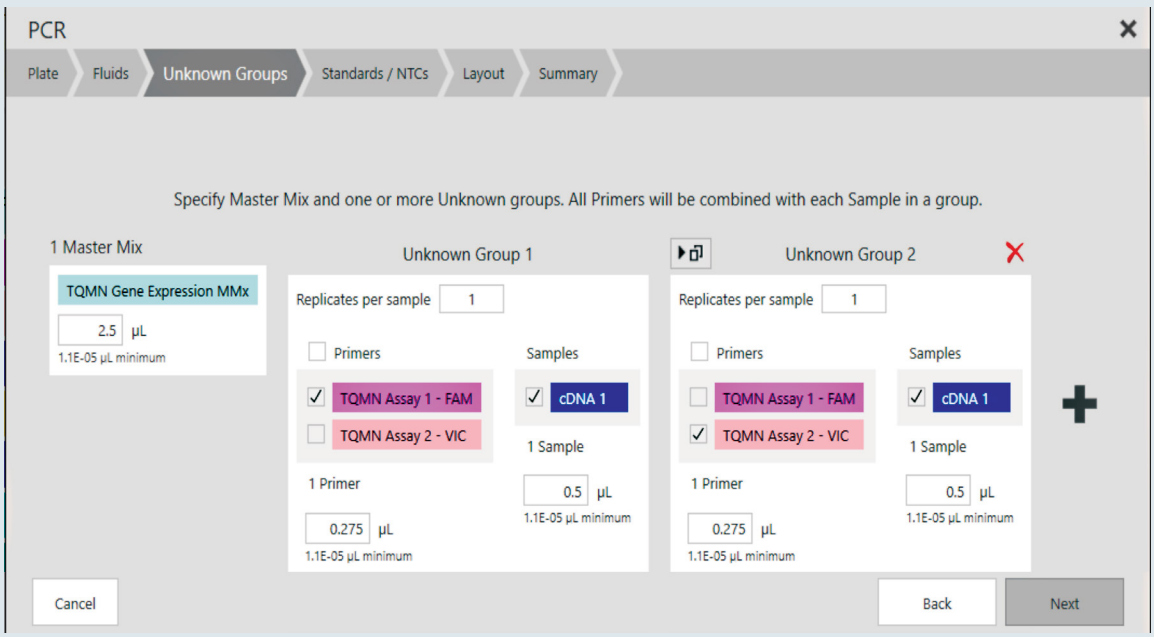


Figure 3. Screenshot of PCR function window and the parameters set for this study in the Unknown Groups tab.



Customization, such as sample numbers or replicates, can also be done manually. In this study, the PCR function was used to create a set of titration curves (15 points, linear titration of 1:2 starting from cDNA 1.9 µL, 4 technical replicates) with 4 NTC (Table 3). All reactions are normalized to 5 µL.

Figure 4. Screenshot of PCR function window with the parameters set for this study in the Standards/NTCs tab.

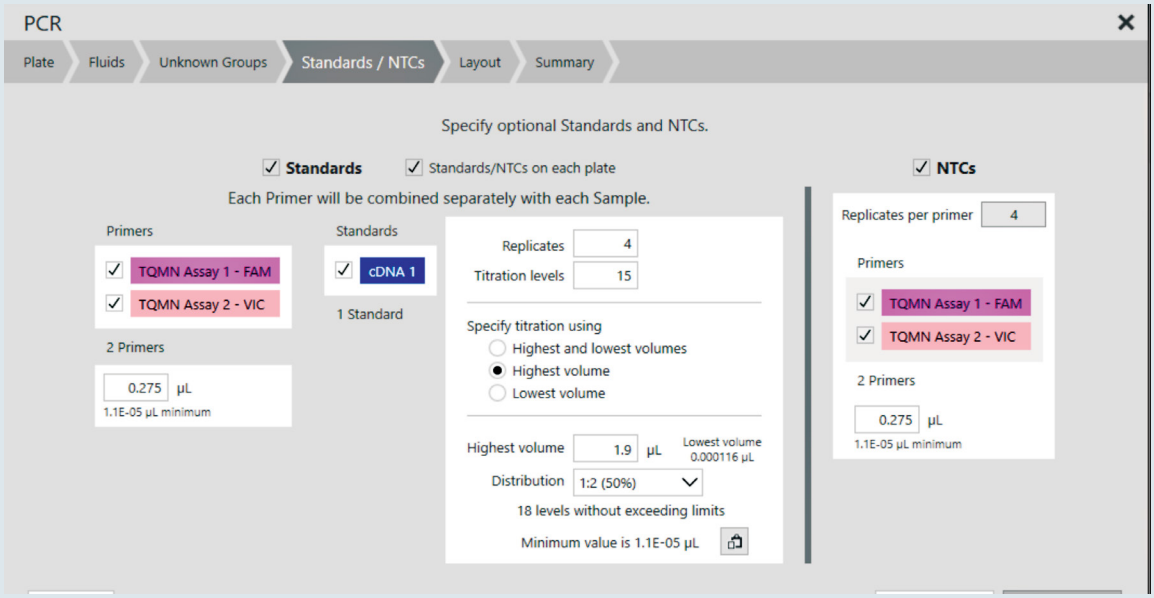
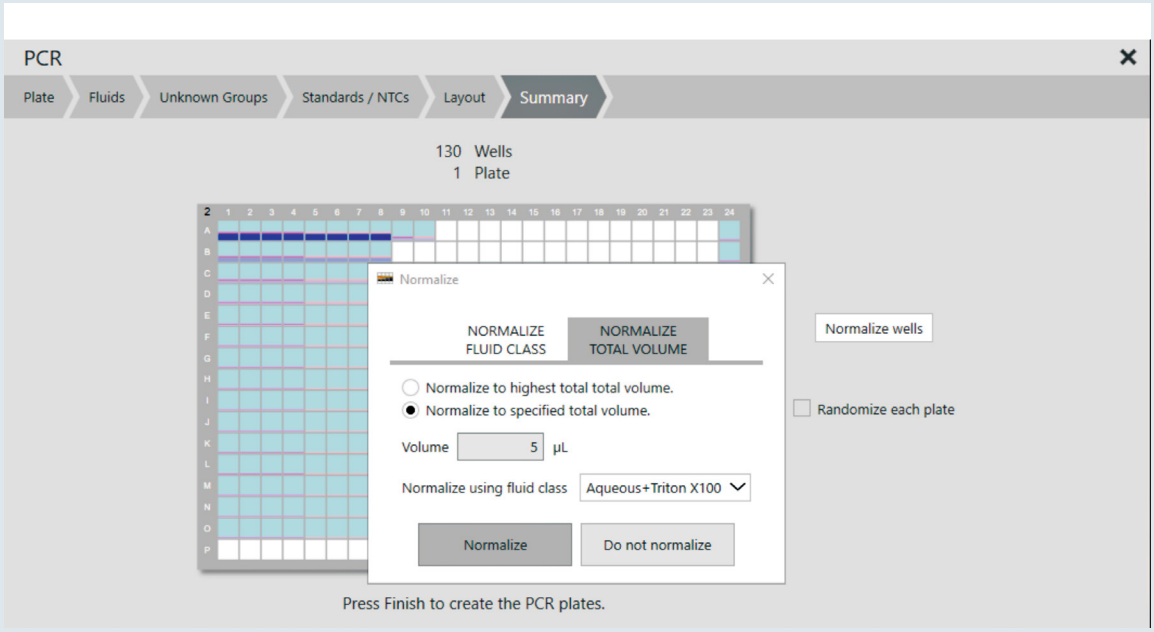


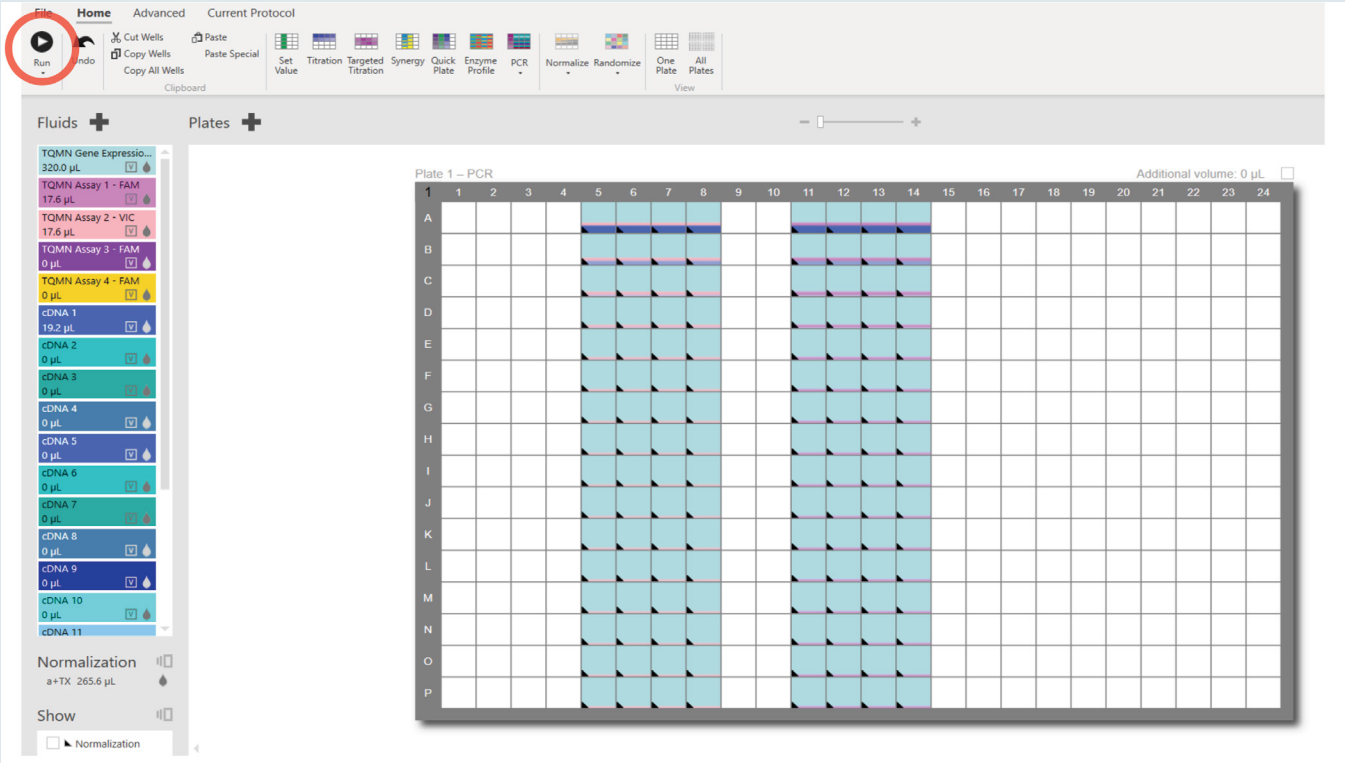
Figure 5. Screenshot of PCR function Summary window with the normalization criteria set for this study.



Step 3:

Click Run to start the dispense protocol. Using E1-ClipTip, load reagents and sample, and dispense into a 384-well microplate with the Multidrop Pico 8 Digital Dispenser (red circle Figure 6). See Table 2 for specific volumes dispensed for each well. After the run completes, the PicoIT 8 software automatically generates the report that includes a .DA.csv file. This file was then imported into QuantStudio Design and Analysis software v1.5.1 for the qPCR run.

Figure 6. Screenshot of the customized protocol and the parameters set for this study. Red circle indicates the Run command.



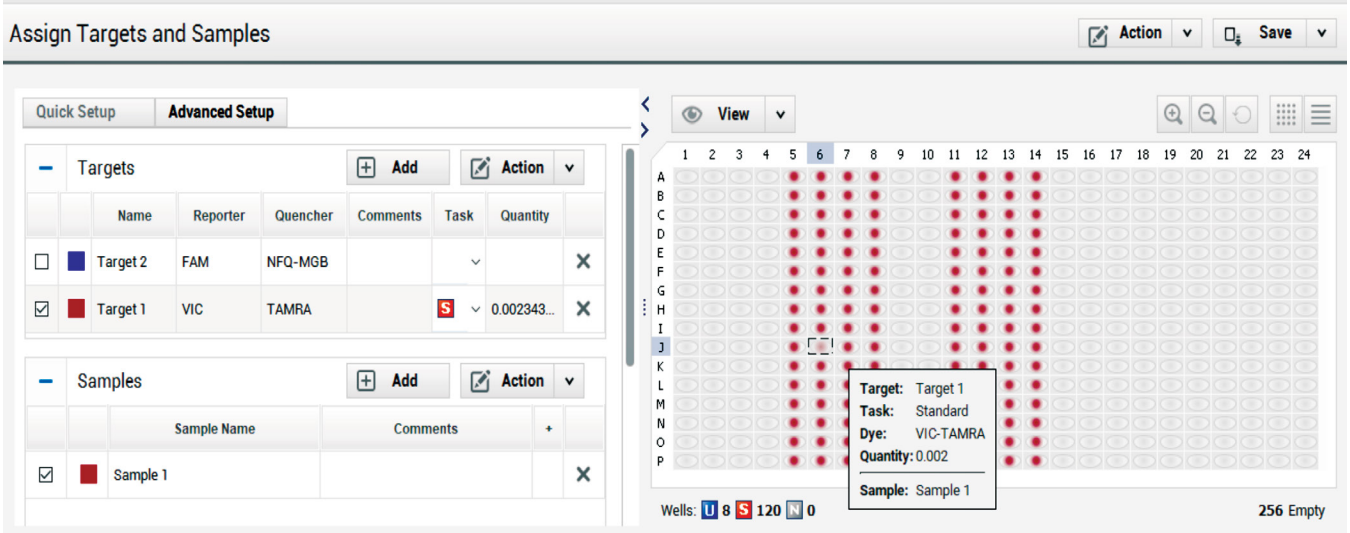


Figure 7. Screenshot of the plate setup in QuantStudio Design and Analysis software v1.5.1 and the parameters set for this study.

After the qPCR assay plate was prepared with the Multidrop Pico 8 Digital Dispenser and PicoIT 8 software, the plate was run on standard mode for qPCR with the QuantStudio 5 system. To import the .DA.csv file, click File > Import Plate Setup in the QuantStudio Design and Analysis software v1.5.1 (Figure 7).

Results

The Multidrop Pico 8 Digital Dispenser and PicoIT 8 software were successfully used to set up a standard curve qPCR assay plate as seen in the amplification of the cDNA (Figure 8). The run report indicated that the Pico 8 dispensed cDNA for each titration level (Table 3). The analyzed data in QuantStudio Design and Analysis software showed the standard curve (VIC) with a slope of -3.325 and primer efficiency of 99.9%. More specifically, R2 value was 0.996 indicating the accurate dispensing of reagents. The %CV of the Ct values from technical replicates (4) of cDNA at each dilution point ranged from 0.11 – 0.61% and the average of Ct %CV for all points was 0.27%, revealing outstanding precision.

Conclusions

This study demonstrates that the Multidrop Pico 8 Digital Dispenser and PicoIT 8 software is an excellent solution to prepare a miniaturized qPCR assay. Setting up a qPCR assay plate can be done in three easy steps when using the PicoIT 8 pre-programed protocol template and PCR function features. The results show that with the Multidrop Pico 8 Digital Dispenser, it is possible to miniaturize the TaqMan qPCR assay reaction total volume to 5 µL without compromising assay results. The Multidrop Pico 8 Digital Dispenser and PicoIT 8 software also titrates the reagents needed for a standard curve and

eliminates manual pipetting for serial dilution. The titration is beneficial to accelerating the standard curve preparation process in relative quantitative PCR assays, gene expression assays, primer efficiency validations, or PCR efficiency validation.

Furthermore, each dilution is generated directly in an individual well with non-contact dispensing technology. This feature reduces the risk of contamination and technical errors that are typically introduced in manual pipetting and serial dilution.

Taken together, the Multidrop Pico 8 Digital Dispenser and PicoIT 8 software provide increased productivity in assay development. They facilitate an accelerated and miniaturized qPCR assay with decreased manual pipetting, reduced reagent amounts, and precise results. ■

References

1. Weyent RS, Edmonds P, Swaminathan B. (1990) Effect of ionic and nonionic detergents on the Taq polymerase. Biotechniques Volume 9. Issue 3. Pages 308-309.

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