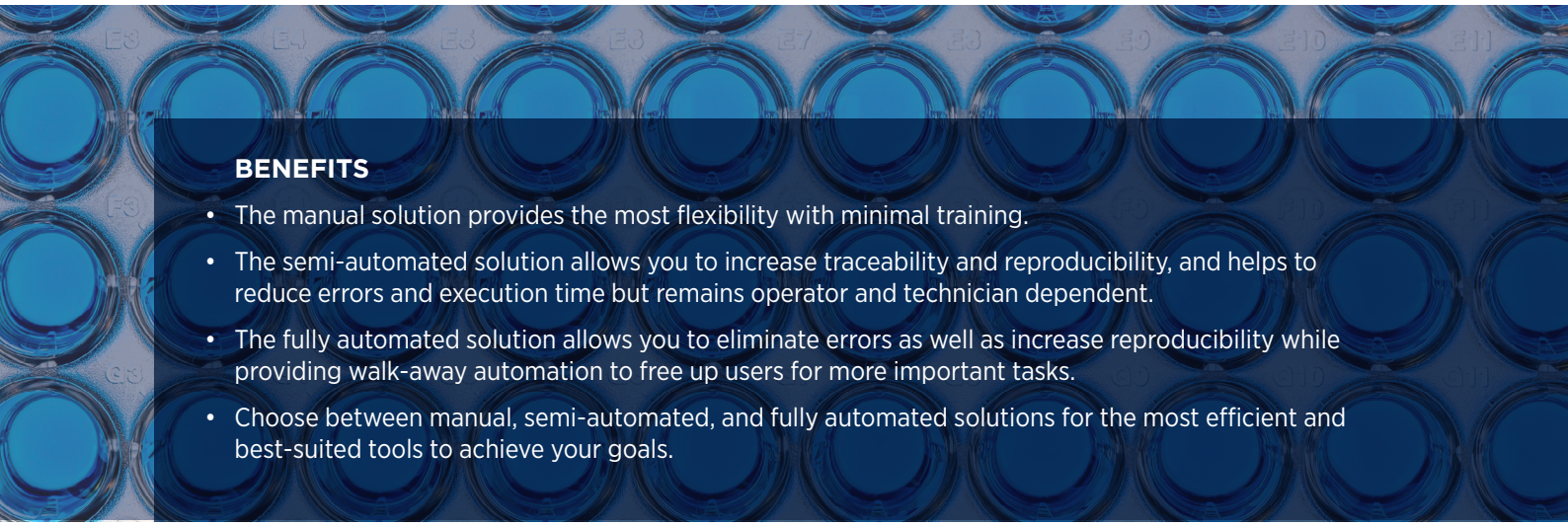
THREE APPROACHES TO qPCR REACTION SETUP

APPLICATION NOTE AN1053



BENEFITS

- The manual solution provides the most flexibility with minimal training.
- The semi-automated solution allows you to increase traceability and reproducibility, and helps to reduce errors and execution time but remains operator and technician dependent.
- The fully automated solution allows you to eliminate errors as well as increase reproducibility while providing walk-away automation to free up users for more important tasks.
- Choose between manual, semi-automated, and fully automated solutions for the most efficient and best-suited tools to achieve your goals.

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INTRODUCTION

Quantitative PCR (qPCR) is one, if not the most commonly used technique in the life science laboratory. It's used in many different applications, such as gene expression in response to a treatment or pathogen identification in diagnostics. qPCR is a versatile and sensitive method for DNA quantification. This very powerful method relies on efficient liquid handling to ensure quality results. However, in qPCR, plate preparation is tedious and time-consuming and relies on skilled technicians to ensure good reproducibility. One solution to answer these challenges is automation[1].

Efficiency and reproducibility have become the main challenges in every lab. To achieve good results in these areas, labs turn to automation, which offers many advantages. However, the number of different solutions makes it a challenge to choose the best one, depending on the lab's objectives and direction[2, 3].

In this study, seven housekeeping genes were quantified from genomic DNA from HeLa cells. We compared the qPCR results obtained using three different solutions: manual pipettes (Gilson PIPETMAN®), the semi-automated TRACKMAN® Connected pipetting solution, and the fully automated PIPETMAX® 268 automated liquid handler. Here, we'll show that all solutions give precise and similar results with different degrees of automation. Sample serial dilutions and master mix were all prepared manually, the different tools were then used for sample and master mix dispenses. Standard curves and efficiencies were determined for each of the different Gilson tools.

MATERIALS AND METHODS

Gilson materials used:

A complete set of PIPETMAN pipettes was used for the master mix preparation. These same pipettes were also used for manual pipetting qPCR experiments (PIPETMAN P20 single channel and PIPETMAN P10 single channel), and good pipetting practices were followed[4]. For the TRACKMAN Connected qPCR experiments, a TRACKMAN Connected tablet, a PIPETMAN M Connected P300M, and a PIPETMAN M Connected P10M single channel pipettes were used. The automated qPCR pipetting was performed using PIPETMAX 268 with the 8 x 20 pipetting head. All details and part numbers can be found in Table 1.

All experiments were performed using sterile filtered PIPETMAN® DIAMOND tips of the appropriate volume.

qPCR:

The qPCR experiments were performed on an Agilent AriaMX using a FAM filter. The qPCR Brilliant II Sybr master mix was used and prepared according to the manufacturer's instructions, except that the final reaction volumes were reduced to 20 µl (17 µl of master mix and 3 µl of DNA) instead of 25 µl. Primers were purchased from Merck Millipore Sigma already resuspended and desalted to a concentration of 100 µM. Sequences are given in Table 2. gDNA was obtained from HeLa cells using a QIAGEN blood and cell culture DNA kit. The initial concentration of DNA was quantified by UV-spectrophotometry at 260 nm. A standard curve was generated starting from 2 ng/L of DNA and serially diluted with a dilution factor of 4.

Table 1

Gilson material details with associated part number

TYPE OF INSTRUMENTS	MODEL	PART NUMBER
Manual Pipette	PIPETMAN P20	F144056M
	PIPETMAN P10	F144055M
Semi-Automated Pipetting Solution	TRACKMAN CONNECTED EU	FB1020
	PIPETMAN M CONNECTED P300M	F81044
	PIPETMAN M CONNECTED P10M	F81040
Automated Pipetting Solution	PIPETMAX 268 with Cover Cutouts	32100001
	MAX 8x20 Pipette Head	FC10022
	MAX 8x200 Pipette Head	FC10021
	PIPETMAX 268 Tray 384 well	32000091
	PIPETMAX 268 Tip Reload Block	32000175
	PIPETMAX 268 riser off-bed tip disposal	32000177
	Rack code 496 for 96 0.2 mL PCR Tubes	32000196

Table 2

Primer sequences

PRIMER SEQUENCES	
ActB_Ex4_1_FWD	AGCTTCTCCTTAATGTCACGCA
ActB_Ex4_1_REV	GGACCTGACTGACTACCTCATG
B2-Mic_Ex2_2_FWD	TGGGTTTCATCCATCCGACATT
B2-Mic_Ex2_2_REV	GACAAGTCTGAATGCTCCACTT
GAPDH_Ex8_1_FWD	ATCAAGAAGGTGGTGAAGCAGG
GAPDH_Ex8_1_REV	GTCAAAGGTGGAGGAGTGGG
Gusb_Ex10_1_FWD	CGCTCTGAATAATGGGCTTCTG
Gusb_Ex10_1_REV	GCTACTACTCTTGGTATCACGACT
Pgk1_Ex3_2_FWD	AGTCGGTAGTCCTTATGAGCC
Pgk1_Ex3_2_REV	GCAGAGATTTGAGTTCTACAGCA
TBP_Ex2_2_FWD	CACAGCTCTTCCACTCACAGA
TBP_Ex2_2_REV	AATCCCAGAACTCTCCGAAGC
TFRC_Ex17_1_FWD	TGTATTGGTTCAGATCCCTCACA
TFRC_Ex17_1_REV	TGAGAGGTACAACAGCCAACT

RESULTS AND DISCUSSION

To compare manual pipetting, semi-automation with TRACKMAN Connected, and personal automation with PIPETMAX 268, we performed qPCR on genomic DNA extracted from HeLa cells. Quantification was performed on four distinct concentration points, made by serial dilution and starting from the same initial sample. All concentrations were dispensed in triplicate (Figure 1). Primer sets were selected in the housekeeping gene exon. Each run included a non-template control to ensure no contamination could be observed.

For each tool, the seven primer sets were analyzed on the same 96-well plate in parallel. Analyses between tools were performed on different days. However, it is easy to see that regardless of the tool used, the primers gave very similar results with an efficiency between 90% and 110%, as advised in the MIQE guideline[5] (Table 3 & Figure 2). The GAPDH primer sets, after several freeze-thaw cycles, developed non-specific amplifications and were therefore excluded from the analyses. Depending on the primer analyzed, efficiencies were extremely consistent between runs, as seen for Actine-Beta, Beta2-MIC, GUSB, and PGK1. Small fluctuations could be observed for TBP and TFRC; however, all of them remained acceptable. Overall, for each primer set, R2 values are very consistent independently of the tool use.

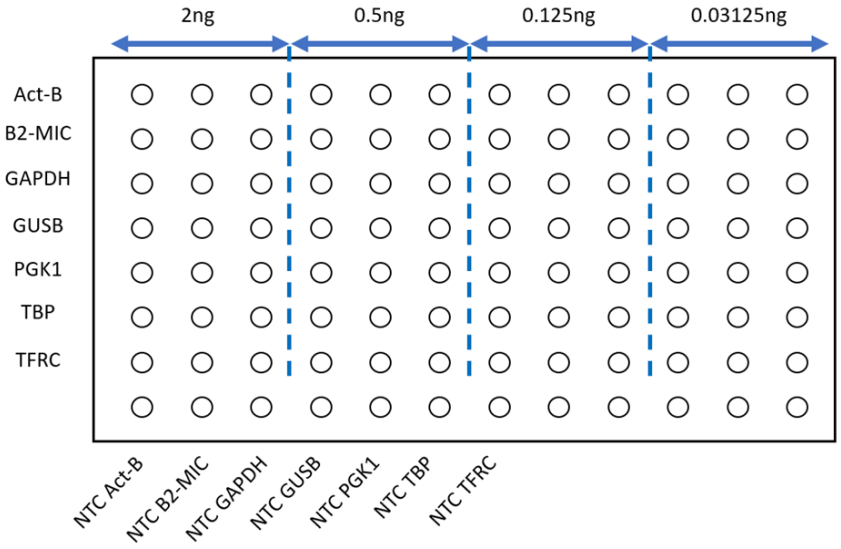

Figure 1

96 well plate qPCR scheme. qPCR dispense plan with different primer sets per row, as indicated. Dotted lines illustrate columns for concentration triplicate dispenses. Non-template controls (NTC) are dispensed on the last row with the primer set indicated.

Table 3

qPCR results and data per tool and primer sets. Table summarizing efficiency and R2 of each tool depending on the primer set. Efficiency is expressed in percentage.

PRIMER SET	ACT-B			B2-MIC			GUSB		
Tool	Manual	TRACKMAN Connected	PIPETMAX 268	Manual	TRACKMAN Connected	PIPETMAX 268	Manual	TRACKMAN Connected	PIPETMAX 268
Efficiency (%)	105.3	106.1	106.4	96.4	99.7	98.9	102.2	101.7	102.0
R2	0.9993	0.9987	0.9999	0.9996	0.9991	0.9969	0.9986	0.9966	0.9990

PRIMER SET	PGK1			TBP			TFRC		
Tool	Manual	TRACKMAN Connected	PIPETMAX 268	Manual	TRACKMAN Connected	PIPETMAX 268	Manual	TRACKMAN Connected	PIPETMAX 268
Efficiency (%)	105.4	101.0	101.6	97.6	91.1	99.9	90.2	102.9	90.6
R2	0.9971	0.9985	0.9996	0.9965	0.9988	0.9978	0.9905	0.9999	0.9944

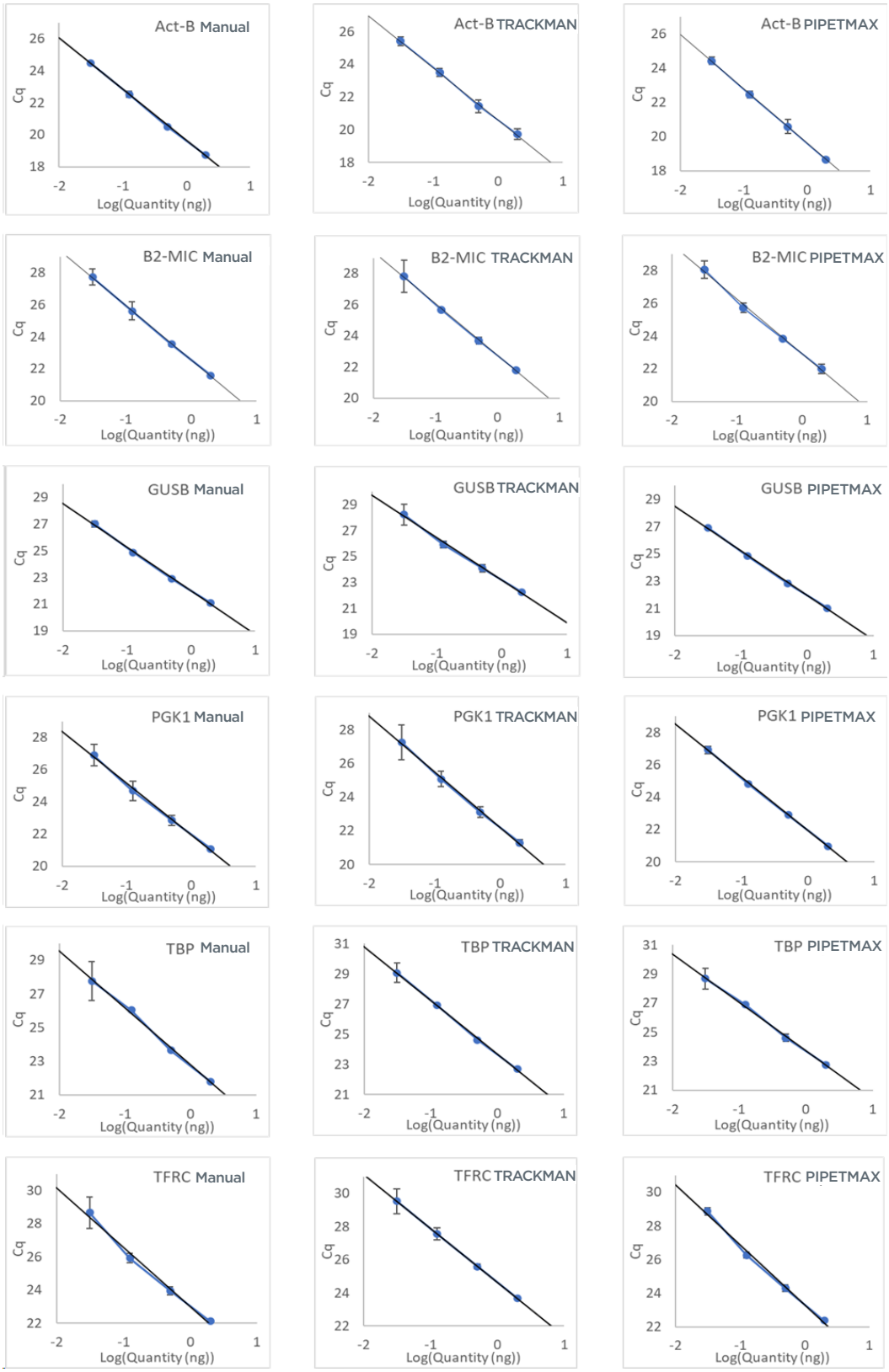

Figure 2

The efficiency curves of the different primer sets depend on the pipetting tools. Standard curve graphs for each primer set of qPCRs were analyzed using Cq values on the Y-axis and Log (Quantity (ng)) on the X-axis. The black line is the tendency curve. Error bars are 2 x SD (standard deviation), giving a confidence interval of 95%.

Another representation of the results was performed by plotting mean Cq values for each concentration depending on the different tools (Figure 3). For each histogram, error bars were added that were equal to double the standard deviation, giving a confidence interval equal to 95%. Error bars for each of the tools were quite narrow, demonstrating a high degree of precision in the obtained results. However, for the lowest concentration, regardless of which tool was used, the precision was lower. These results can be easily explained by the low concentration and distribution of DNA fragments in the quantification wells.

Trend lines displayed on the histograms (Figure 3) directly reflect the standard curves obtained and shown in Figure 2. Besides having very similar slopes, for most of the primer sets, PIPETMAX 268 and manual standard curves could be superposed except for the TBP primer sets.

Regarding TRACKMAN Connected, the standard curves were also similar to manual pipettes and PIPETMAX 268 for the primer set of PGK1 and Beta2-MIC. However, for other primer sets, TRACKMAN Connected gave parallel standard curves, but slightly shifted, with higher Cq values for each.

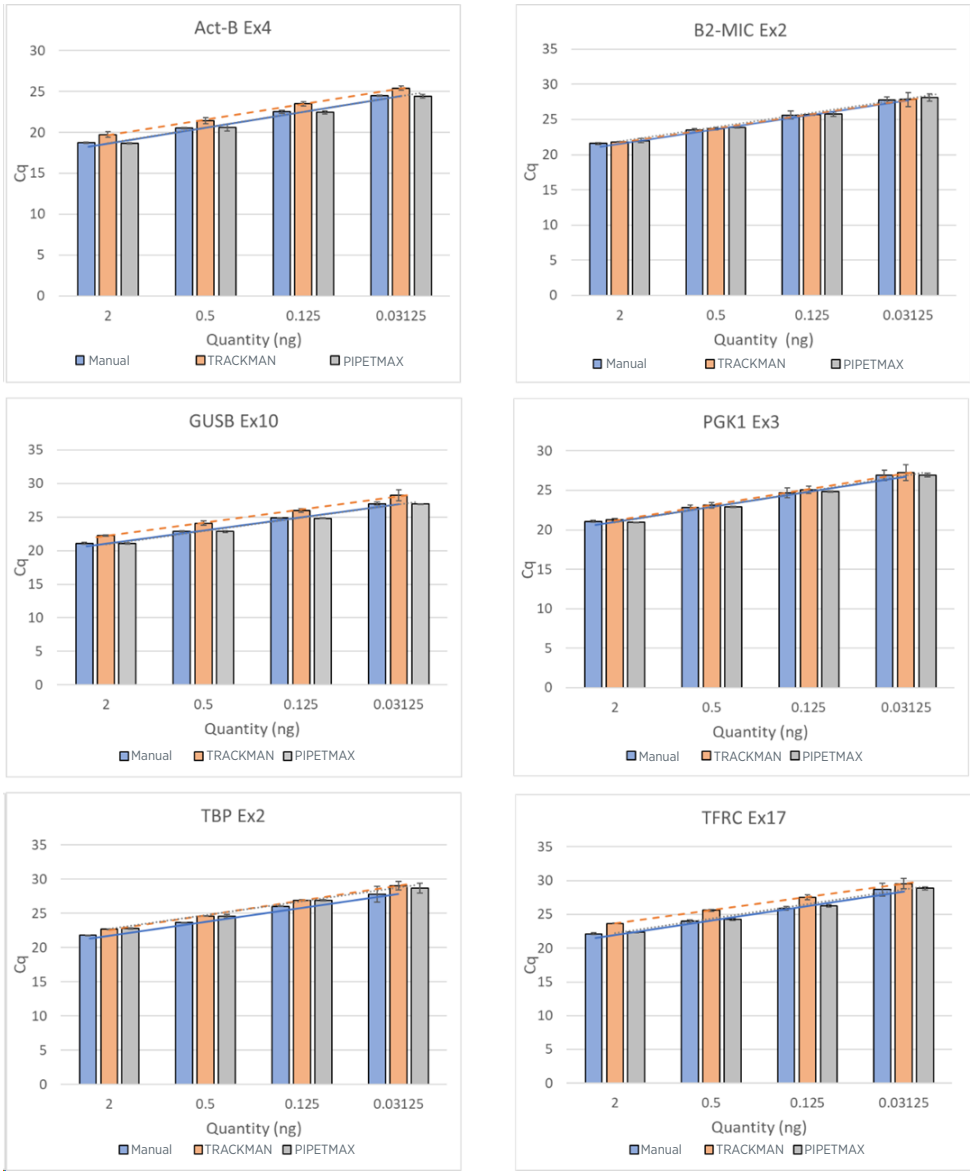

Figure 3

Comparison of qPCR standard curves between each pipetting tool. Histograms for each primer sets of the different Cq value obtained with each tool, tendency curve are displayed on the same graph. Manual pipetting results in blue, TRACKMAN Connected results in orange and PIPETMAX 268 results in grey. X-axis is the quantity in ng and Y-axis Cq values. Errors bars are 2 x SD (standard deviation) which correspond to 95% confidence interval.

Globally, Cq values between tools were very similar, with only a few primer sets exhibiting higher Cq values for the TRACKMAN Connected tool with a maximum difference of 1.6 cycles. The difference in cycles not being reproducible from one primer to the other and the standard curves being very similar between all of the pipetting tools indicates that the difference in cycles is probably due to qPCR bias rather than the pipetting tools themselves.

Table 4 illustrates the tip consumption and execution time for each tool. The highest tip consumption, with 249 tips used and an execution time of 45 minutes, was observed with manual PIPETMAN pipettes. Our semi-automated TRACKMAN Connected exhibited the lowest tip consumption, with 108 tips and 15 minutes of execution time. This can be explained by the fact that electronic pipettes can do multi-dispensing, which saves time, and also tips by multi-dispensing samples without touching the master mix thanks to operator precision. PIPETMAX 268 is the fastest solution, saving operator time with only 5 minutes to start the system followed by the ability to allow unattended operation while performing the qPCR plate filling. However, it shows an intermediate tip consumption with 191 tips used, which is linked to the dispensing of the sample directly into the master mix to ensure the best performance of the method. Depending on the laboratory's objectives, PIPETMAX 268 protocols can be adapted either to ensure the best efficiency or the best tip savings. In this case, the PIPETMAX 268 protocol was developed to maximize accuracy and precision.

Table 4

Tip consumption and execution time

	MANUAL	TRACKMAN CONNECTED	PIPETMAX 268
Tips consumption (Overall)	249	108	191
Operator execution time (min)	45	15	5

The data illustrates that all three solutions (manual pipettes, TRACKMAN Connected, and PIPETMAX 268) give similar results and do not lead to any data quality loss.

CONCLUSION

All solutions give comparable results, but what tool is right for certain lab configurations and objectives is dependent on their requirements. PIPETMAN manual pipettes offer the best flexibility and control over experiment completion. The fully automated solution, PIPETMAX 268, offers the best throughput thanks to walk-away time resulting from unattended operation. Between these two solutions stands the semi-automated TRACKMAN Connected, taking advantage of electronic Bluetooth®-connected pipettes controlled by a tablet. Such a tool brings traceability to manual pipetting as well as efficiency and precision thanks to manually operated electronic pipettes.

In conclusion, we compared the qPCR results obtained using different levels of automation from Gilson. It appears clear that independently of the tool chosen (PIPETMAN manual pipettes, TRACKMAN Connected, or PIPETMAX 268) qPCR data was comparable, and the output produced very similar results. Such a conclusion indicates that manual, semi-automated, or fully automated solutions can all reach the same pipetting quality and precision, but PIPETMAN manual pipettes offer flexibility, PIPETMAX 268 offers reproducibility and unattended operation, and TRACKMAN Connected offers traceability and efficiency. This conclusion means that tools and solutions must then be chosen depending on the objectives, budget, and needs of each lab.

REFERENCES

1. Holland, I. & Davies, J. A. Automation in the LifeScience Research Laboratory. Front. Bioeng. Biotechnol. 8, (2020).
2. Schuster, J., Kamuju, V., Zhou, J. & Mathaes, R. Piston-driven automated liquid handlers. SLAS Technol. 29, 100128 (2024).
3. Rupp, N., Ries, R., Wienbruch, R. & Zuchner, T. Can I benefit from laboratory automation? A decision aid for the successful introduction of laboratory automation. Anal. Bioanal. Chem. 416, 5–19 (2024).
4. Gilson, INC. Guide to pipetting, Third edition, LT800550/H. (2024).
5. Bustin, S. A. et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622 (2009).