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Automated KAPA® Library Quantification Kit with the ep*Motion*® 5075t

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Abstract

Next-Generation Sequencing (NGS) is a high-throughput method that enables highly parallelized sequencing of multiple libraries, prepared from both DNA and RNA samples. Bolstered by growing applications, such as the whole genome, whole exome, and gene expression analyses, NGS is gaining importance and becoming a standard procedure in many laboratories.

The quantification of NGS libraries prior to sequencing is essential to obtain reliable results and optimal sequencing performance. This Application Note demonstrates the capability of the ep*Motion* 5075t liquid handling system to automate with high accuracy and high precision a complete qPCR assay setup using the KAPA Library Quantification Kit including library dilution steps.

Introduction

Accurate and precise quantification of the number of amplifiable molecules in a library is an essential step in sequencing workflows. It ensures consistent and optimal cluster densities, as well as equivalent representation of each library when sequencing in multiplex. Inaccurate quantification can result in decreased sequence quality metrics, inefficient flowcell use, and additional sequencing required for underrepresented samples – all of which translates to wasted time and money.

A quantitative PCR (qPCR) approach, such as the KAPA Library Quantification Kit, is the gold standard for NGS library quantification and is the only method that accurately measures the number of amplifiable molecules that have the ability to cluster. Additionally, this method has the sensitivity and broad dynamic range for accurate quantification of very dilute and PCR-free libraries.

Performing an accurate qPCR assay manually in a high-throughput setting becomes difficult and time consuming, and the use of an automated liquid handling platform is strongly recommended to reduce the risk of human error and increase the reliability of results. The Eppendorf ep*Motion*® family of automated pipetting systems is an essential tool for many laboratories looking to achieve consistent results. The ep*Motion* 5075t and other models in the Eppendorf family pipette volumes ranging from 0.2 µL to 1 mL with efficiency and accuracy.

The reliability of qPCR results is highly related to the accuracy and precision of liquid handling in both reaction setup and library dilution. Accuracy and precision are two terms that are often used interchangeably, but are actually differentiated. Accuracy refers to how close a measurement is to a true value, while precision refers to how close repeat measurements are to each other and is thus a measure of reproducibility. This Application Note presents experimental results aimed at verifying the accuracy and precision of the KAPA Library Quantification Kit processed on the ep*Motion* 5075t compared to manual handling.

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Materials and Methods

Materials

qPCR – Accessories

> LightCycler® 480 Sealing Foil (Roche, cat # 04 729 757 001)
> LightCycler® 480 Instrument, 96-well (Roche, cat # 05 015 278 001)
> Centrifuge MiniSpin®, non-refrigerated, with Rotor F-45-12-11, 230 V/50 – 60 Hz (Eppendorf, cat # 5452000010)
> Centrifuge 5810 R (IVD), refrigerated, without rotor, keypad, 230 V/50 – 60 Hz (Eppendorf, cat # 5811000015)

qPCR – Reagents

> KAPA Library Quantification Kit for Illumina® platforms (KAPA Biosystems, cat # 07960336001)
> KAPA Library Quantification Dilution Control Kit for Illumina® platforms (KAPA Biosystems, cat # 07960417001)
> UltraPure™ 1M Tris-HCl, pH 8.0 (Thermo Fisher®, cat # 15568025)
> Water, Sterile, Nuclease-free, Biotechnology Grade (VWR, cat # E476)

Methods

KAPA Library Quantification Kit contains all reagents required for quantification of NGS libraries for Illumina® sequencing, including KAPA SYBR® FAST qPCR Master Mix (2X), a Primer PreMix (10X) containing two primers based on Illumina P5 and P7 oligo sequences (Primer 1: 5'-AAT GAT ACG GCG ACC ACC GA-3' and Primer 2: 5'-CAA GCA GAA GAC GGC ATA CGA-3') and six ready-to-use DNA standards corresponding to a 10-fold dilution series. Standards consist of a linear, 452 bp dsDNA fragment flanked by qPCR primer binding sites. A KAPA Library Quantification Dilution Control, referred to as DNA Standard 0, is a 200 pM solution of the same linear, 452 bp dsDNA fragment. It is available for purchase separately and can be used to characterize impact of liquid handling on assay accuracy.

The ep*Motion* was programmed to perform NGS library sample dilution and qPCR setup in one method. Reactions were set up according to Table 1 and thermocycled according to Table 2 using a LightCycler® 480 instrument (96 well). Template-free negative controls were included.

Table 1.: qPCR reaction setup

Reaction component	Volume per 20 µL reaction
2X KAPA SYBR® FAST qPCR Master Mix + 10X Primer Premix	12 µL
PCR grade water	4 µL
Template (DNA standard or library)	4 µL

Table 2: Thermocycling parameters

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	35
Annealing/Extension (data acquisition)	60°C	45 sec	
Melt curve analysis	65°C - 95°C		

Manual and automated standard curves were generated using triplicate reactions for each standard. ep*Motion* sample dilution accuracy was assessed using DNA Standard 0 diluted 1:10,000, as well as two NGS libraries diluted 1:10,000; 1:100,000; and 1:1,000,000. The same stock of reagents and samples were used in both manual and automated processes. The qPCR plate layout is illustrated on Figure 1.

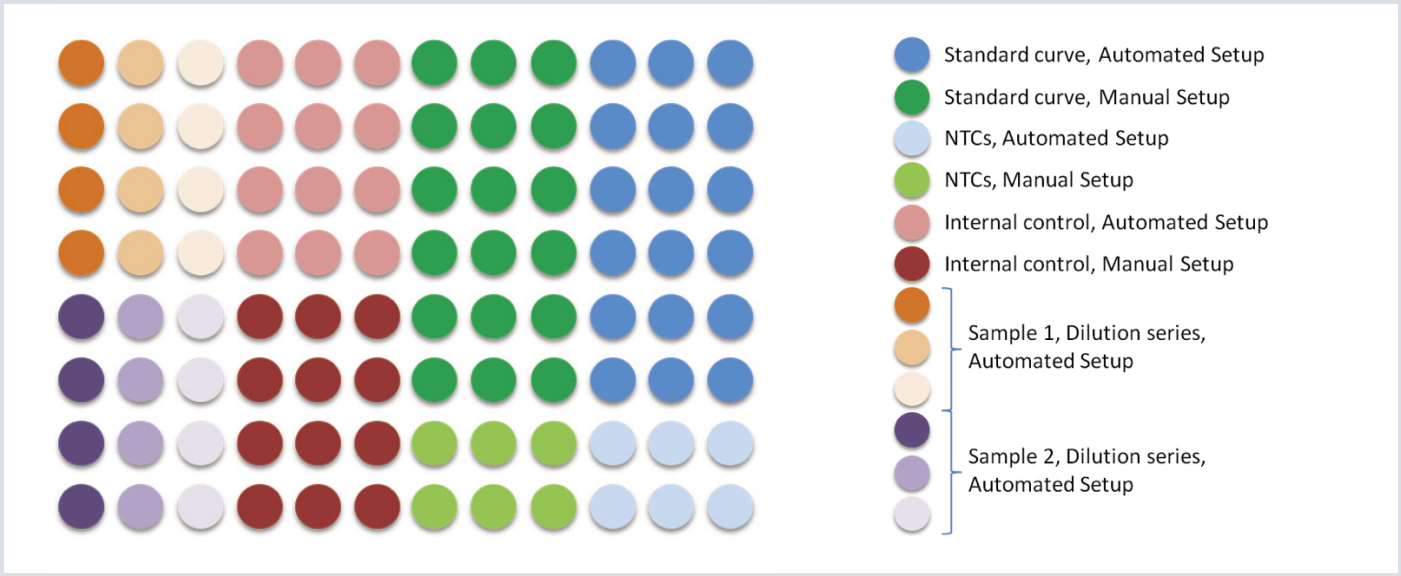


Figure 1: qPCR plate layout.

Before starting the program, the ep*Motion* surfaces and tools were cleaned using a DNA decontamination solution "DNA-Erase" and treated with UV-light for 15 minutes. The work-table of ep*Motion* 5075t instrument is equipped as in Figure 2.

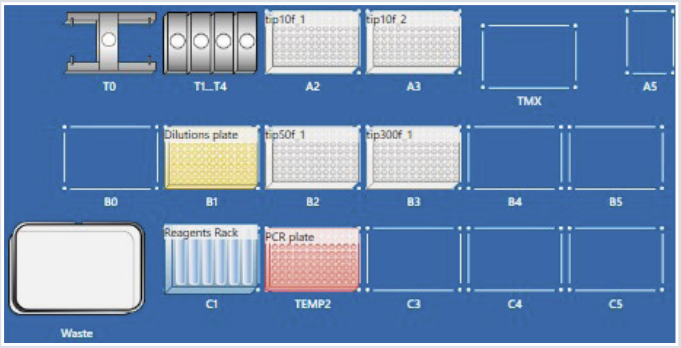


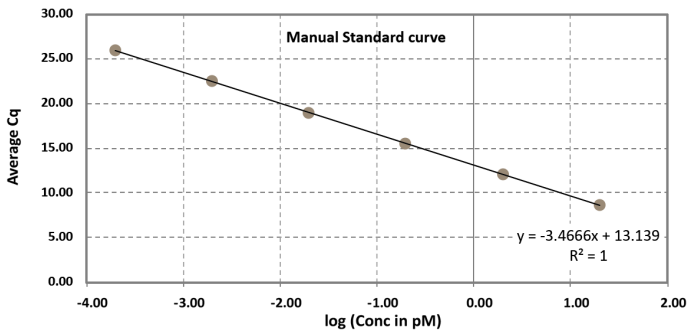
Figure 2: ep*Motion* 5075t worktable for sample dilution and qPCR setup with the KAPA Library Quantification Kit for Illumina® platforms.

Results and Discussion

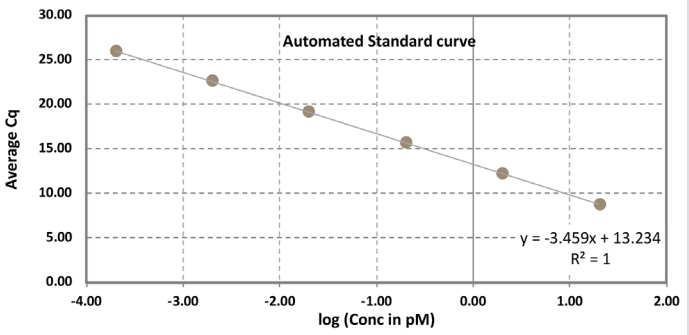
Automated qPCR Setup Accuracy and Precision

The accuracy and precision of the qPCR setup was evaluated based on standard curve analysis (Figure 3). Amplification efficiency is equivalent between automated (95%) and manual (94%) setup, and both values are within the acceptable range of 90 – 110%. The coefficient of variation (R^2) obtained for the two liquid handling methods is similar with a value of 1.00, meeting the minimum requirement of 0.99. For standards on the automated standard curve, the standard deviation of Cq values between triplicate data points was less than 0.1, translating to a coefficient of variation less than 0.4%. This indicates that any quantification data obtained is accurate, assuming accurate and precise library dilution.

A



B



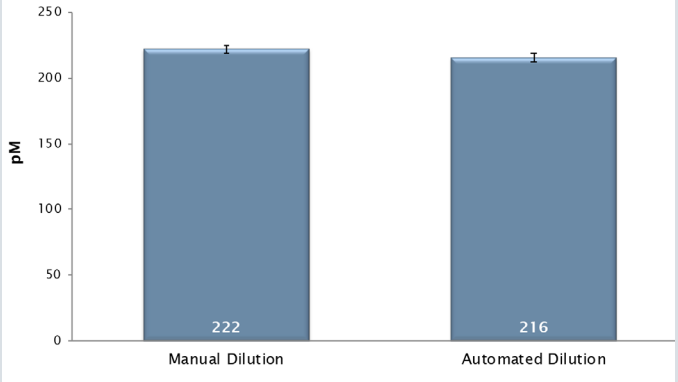
C

	R^2	Slope	Y-intercept	Efficiency
Manual	1	-3.4666	13.139	94%
Automated	1	-3.459	13.234	95%

Figure 3: Standard curves of manual (A) and automated (B) qPCR setups, as well as a summary of associated data (C).

Automated Sample Dilution Accuracy and Precision

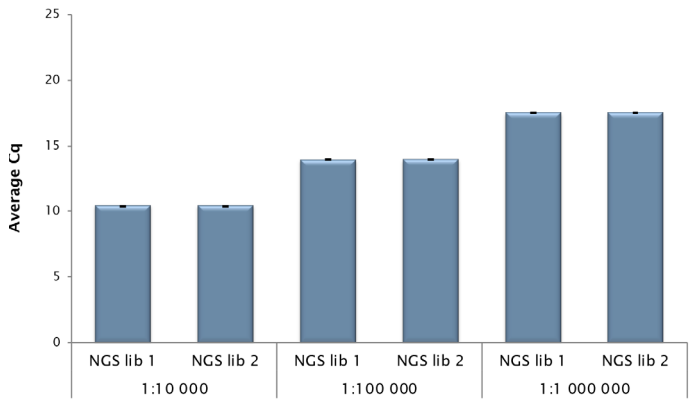
Standard 0 was diluted to 1:10,000 by two consecutive 1:100 dilutions either manually or with an automated process. The average pM value of the automated Standard 0 reactions (216 pM) is similar to the average pM value of the manual reactions (222 pM) showing equivalency of the automated sample dilution process on the ep*Motion* (Figure 4). Additionally, the automated process resulted in an estimated Standard 0 concentration of 216 pM, which deviates from the expected value of 200 pM by less than 10%. The low standard deviation (CV of 1.4%) among the twelve replicates shows high precision and highlights the reliability of the automated sample dilution process.



Process	SD for pM	CV (%) for pM
Manual	2.90	1.30
Automated	3.02	1.40

Figure 4: Comparison between manual and automated serial dilutions using Standard 0.

Performance was further evaluated using two Illumina NGS libraries diluted in series (1:10K, 1:100K, 1:1M) with four replicates per data point. As expected for a 10-fold dilution series, the Delta Cq values between the different dilution factors were within the acceptable range of 3.1 to 3.6. The Cq standard deviation of both NGS libraries does not exceed 0.2% showing high reproducibility (Figure 5).



NGS Libraries	Dilution	SD for Cq	CV for Cq
NGS Lib 1	1:10 000	0.019	0.18%
NGS Lib 2	1:10 000	0.008	0.08%
NGS Lib 1	1:1 00 000	0.018	0.13%
NGS Lib 2	1:1 00 000	0.006	0.04%
NGS Lib 1	1:1 000 000	0.030	0.17%
NGS Lib 2	1:1 000 000	0.017	0.10%

Figure 5: Comparison of automated serial dilutions for two NGS libraries.

Conclusions

The automation of the KAPA Library Quantification Kit on the Eppendorf ep*Motion* 5075t offers a solution for high-throughput quantification, reducing hands-on time and human error with a total runtime of only 28 minutes for the here presented setup. The Eppendorf ep*Motion* liquid handling systems have the advantage of having a user friendly interface, with flexible options for modifying the number of libraries, the number of qPCR replicates, and the dilution factor to desired experimental design with a maximum of 72 reactions per plate. Reliable, optimized and ready-to-use ep*Motion* methods are available for small ep*Motion* models such as the ep*Motion* 5073 and larger sizes such as the ep*Motion* 5075. The presence of a thermo-module on the worktable keeps the qPCR plate refrigerated during processing, and detection of tip and consumable positions before run start allows reliable processing without incident.

Ordering information

Description	Order no. International
ep*Motion*® 5075t	5075 000.302
Thermal module on position C2	5075 757.001
TS 10 single channel dispensing tool, 0.2 – 10 µL	5280 000.100
TS 50 single channel dispensing tool, 1 – 50 µL	5280 000.010
TS 300 single channel dispensing tool, 20 – 300 µL	5280 000.037
Thermoblock for PCR	5075 766.000
Reservoir Rack	5075 754.002
Reservoir Rack Module TC for Safe-Lock Tubes	5075 799.081
epT.I.P.S.® Motion, 10 µL, filtered	0030 015.193
epT.I.P.S.® Motion, 50 µL, filtered	0030 014.413
epT.I.P.S.® Motion, 300 µL, filtered	0030 014.456
ep*Motion*® Reservoir, 30 mL	0030 126.505
Eppendorf twin.tec® PCR Plate 96, skirted	0030 128.648
Eppendorf Deepwell Plate 96/500 µL, PCR clean	0030 501.101
Eppendorf DNA LoBind Tubes, 1.5 mL	0030 108.051

For more information about Roche sequencing solutions, please visit: **sequencing.roche.com**

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