firefly® technical note

Automated KAPA Library Quantification

This technical note provides supporting information for automating the KAPA Library Quantification Kits on firefly. These protocols are available to download from the firefly community. Here we give an overview of the protocol run times, consumables usage and share data to demonstrate the performance of these protocols.

Overview

firefly protocols have been developed to automate the setup of qPCR reactions using KAPA Library Quantification Kits, enabling qPCR reactions to be easily set up for 1-12 columns of samples contained in a 96-well plate.

KAPA Library Quantification Kits contain all the reagents needed for the accurate, reliable, and reproducible qPCR-based quantification of next-generation sequencing (NGS) libraries prepared for sequencing on Illumina platforms. Kits include KAPA SYBR FAST qPCR Master Mix (formulated with different passive reference dyes for different qPCR instruments), a platform-specific library quantification primer premix, and a pre-diluted set of DNA standards.

firefly from SPT Labtech is an innovative all-in-one liquid handling platform that brings together air-displacement pipetting and non-contact positive-displacement dispensing in a single compact design. Underpinned by powerful, intuitive software, firefly unlocks the potential of automation for all, to accelerate genomic research.

Protocol highlights

- 10 µL qPCR reactions can be set up for 96 libraries, in triplicate, in 22 minutes.
- Only 2 µL of library DNA is required to prepare the library dilution for each sample.
- Variables enable a user-specified final library dilution in the range 1:2000 to 1:12000, suitable for quantifying libraries up to 240 nM without the need for any upfront dilution.

firefly protocols

Protocol name	firefly run time	Library dilution[1]	Library dilutions on reaction plate
1-12 columns - KAPA Library Quantification	22 mins	1:2000 - 1:12000	Final dilution only
1-6 columns - KAPA Library Quantification	25 mins	1:2000 - 1:12000	1:1000 dilution and final dilution

Table 1. Overview of KAPA Library Quantification protocols available in the firefly community

[1]Where a 1:2000 dilution is the convention used to describe a dilution consisting of 1 volume of stock in a final volume of 2000 - equivalent to a dilution factor of 2000.

Consumables

Pipetting head tips												
Protocol name	Pipetting head tip type	Columns of samples to quantify										
		1	2	3	4	5	6	7	8	9	10	11	12
1-12 columns - KAPA Library Quantification	125 µL 8-strip tips	3	6	9	12	15	18	21	24	27	30	33	36
	50 µL 8-strip tips	2	3	4	5	6	7	8	9	10	11	12	13
	50 µL 384-tip array	1	1	1	1	1	1	1	1	1	1	1	1
1-6 columns - KAPA Library Quantification	125 µL 8-strip tips	3	6	9	12	15	18	21	24	27	30	33	36
	50 µL 8-strip tips	3	5	7	9	11	13	15	17	19	21	23	25
	50 µL 384-tip array	1	1	1	1	1	1	1	1	1	1	1	1

Table 2. Pipetting head tips required to automate the KAPA Library Quantification Kits on firefly

Plates and dispense head syringes			
Protocol name	Plates required		Number of standard syringes required
	Number	Details	
1-12 column & 1-6 column – KAPA Library Quantification	6	1x 96-well plate with input DNA 1x 96-well plate with Standards in one column 3x empty 96-well plates for DNA dilutions 1x empty 384-well qPCR reaction plate	5

Table 3. Plates and dispense head syringes required to automate KAPA Library Quantification Kits on firefly

Input variables

Protocol name	Number of columns of library to quantify	Starting volume of library per well	Starting volume of standards per well	Final library dilution
1-12 columns - KAPA Library Quantification	1-12 columns	5 µL-200 µL	15 µL-200 µL	1:2000 -1:12000
1-6 columns - KAPA Library Quantification	1-6 columns	5 µL-200 µL	15 µL-200 µL	1:2000 -1:12000

Table 4. Variables and their working ranges to automate KAPA Library Quantification Kits on firefly

Workflow overview

Figure 1. Overview of the steps firefly performs to automate KAPA Library Quantification Kits. The output is a 384-well qPCR reaction plate that is ready to load onto a qPCR instrument.

Protocol details

Library dilution

Library dilutions (1:2000 to 1:12000) are achieved through three consecutive dilutions of the library DNA. The volumes used to prepare the first two dilutions are constants in the firefly protocols. The volume of diluent added to the third dilution plate – the final library dilution plate – is varied to achieve the final library dilution specified by the user, see Figure 2.

1. A 1:50 dilution of the library DNA is prepared by adding 2 µL library DNA to 98 µL of DNA dilution buffer and tip mixing.
2. A 1:1000 dilution of the library DNA is prepared by adding 5 µL of the 1:50 dilution to 95 µL DNA dilution buffer and tip mixing.
3. The final library dilution is prepared by adding 10 µL of the 1:1000 dilution to a variable volume of DNA dilution buffer (10 µL to 110 µL) and tip mixing. In this way a final library dilution of 1:2000 to 1:12000 can be achieved.

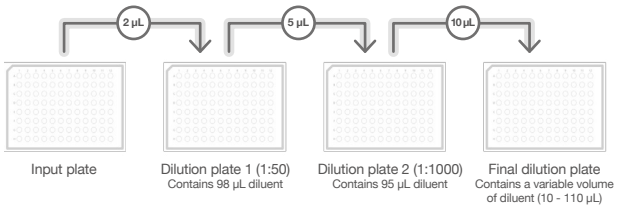

Figure 2. Illustration of the library dilution steps used to automate KAPA Library Quantification Kits on firefly.

Transfer of diluted libraries and standards to a 384-well reaction plate

1. 4 µL of the final library dilution is added in triplicate to a 384-well qPCR reaction plate. See Figure 4 for an example of the reaction plate layout.
2. 4 µL of standards are transferred in triplicate from a single column of a 96 well plate to the qPCR reaction plate. We recommend transferring standards from the kitted tubes into a single use Lo-bind plate that is discard after use. See Figure 3.
3. 6 µL of KAPA SYBR FAST qPCR Master Mix (2X) - with Primer Premix (10X) added - is dispensed from reservoir R1 to all wells of the qPCR reaction plate that contain the diluted library or a standard. Each reaction is then tip-mixed. See Figure 3.
4. The qPCR reaction plate can then be removed from firefly, sealed, spun down and loaded into a qPCR machine.

The *1-6 columns - KAPA Library Quantification* protocol follows the same steps as above, the only difference being that it adds both the final library dilution and the 1:1000 dilution to the qPCR reaction plate prior to the addition of the standards and KAPA SYBR FAST qPCR Master Mix. For this reason, a maximum of 6 columns of library samples can be prepared with this protocol when using a single 384-well reaction plate.

Deck layout

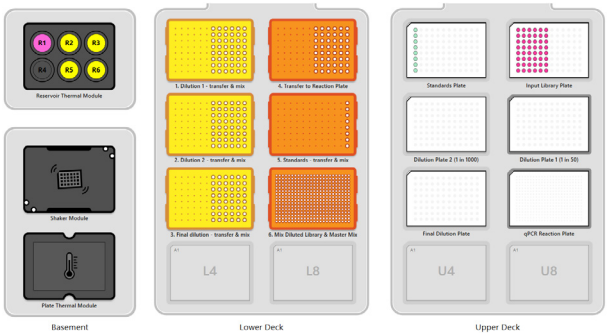

Figure 3. Starting deck layout for the "1-12 columns - KAPA Library Quantification" protocol – this example is for the quantification of six columns of libraries. Reservoir 1 (R1) contains the KAPA SYBR FAST qPCR Master Mix, reservoirs 2, 3, 5 & 6 (R2, R3, R5 & R6) contain the DNA dilution buffer. Lower deck contains 125 µL tips (yellow) and 50 µL tips (orange).

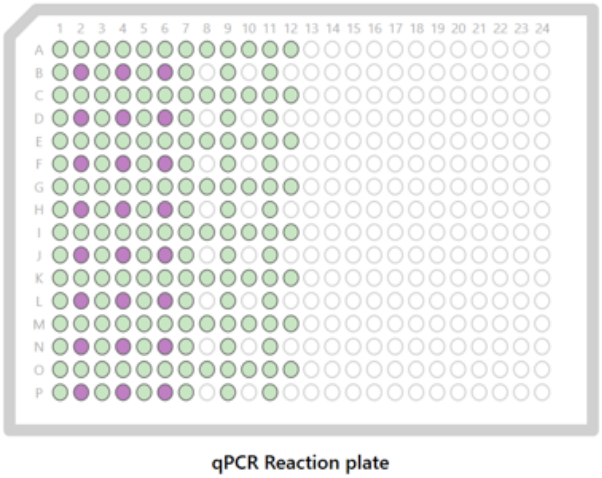

Figure 4. qPCR reaction plate layout for diluted libraries (green) and standards (purple). This example is for the quantification of six columns of libraries using the "1-12 columns - KAPA Library Quantification" protocol. Samples are loaded in triplicate into quadrants 1,2 and 3. Standards are loaded in triplicate into quadrant 4.

Protocol performance

The data presented here was generated using KAPA Library Quantification Kits (Complete kit, Universal, Cat. # 07960140001) and run on a LightCycler 480 System.

Standard curve

firefly was used to prepare qPCR reactions for the DNA standards provided in the KAPA Library Quantification Kits. The Cq scores (Table 5) and standard curve (Figure 5) demonstrate consistency between the technical replicates and show that the standards from the KAPA Library Quantification protocol exhibit the expected ΔCq and reaction efficiency when firefly is used to prepare the qPCR reactions.

DNA Standard	Concentration (pM)	Cq score			Average Cq	ΔCq*	Standard deviation	CV %
		1	2	3				
Standard 1	20	7.48	7.59	7.41	7.49	-	0.09	1.21
Standard 2	2	10.89	11.01	10.83	10.91	3.42	0.09	0.84
Standard 3	0.2	14.41	14.51	14.22	14.38	3.47	0.15	1.02
Standard 4	0.02	17.78	17.92	17.71	17.80	3.42	0.11	0.60
Standard 5	0.002	21.25	21.44	21.00	21.23	3.43	0.22	1.04
Standard 6	0.0002	24.61	24.78	24.35	24.58	3.35	0.22	0.88

Table 5. Example of the Cq scores for the six DNA standards to demonstrate the repeatability of technical replicates.

*Expected ΔCq is 3.1-3.6 for a 10-fold dilution.

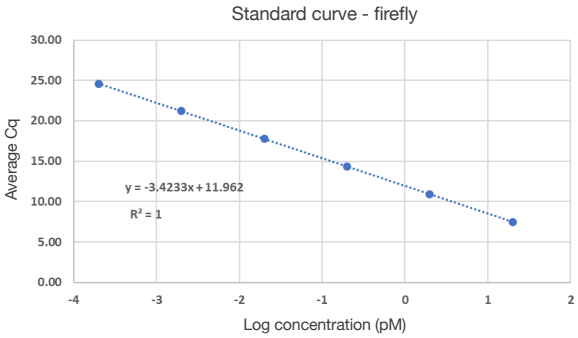

Figure 5. Standard curve generated with the KAPA Library Quantification protocol on firefly. Reaction efficiency = 95.9%.

Accuracy of library dilutions

The firefly KAPA Library Quantification protocol prepares three consecutive dilutions using 2 µL of the library DNA being quantified. The user specifies the total dilution of library DNA required and calculations within the protocol vary the volume of diluent in the final dilution plate to achieve this dilution.

To assess the accuracy of the library dilutions, a 1:2000 final library dilution was prepared using KAPA Standard 0, which has a known concentration of 0.2 nM, see Table 6 for details of the dilution volumes. The 1:50 and 1:1000 dilutions are fixed in the firefly protocol and the diluent volume is varied to achieve desired final dilution of 1:2000.

4 µL was transferred from each dilution plate (Dilution plate 1, Dilution plate 2, and the Final Dilution Plate) in triplicate, into a 384-well qPCR reaction plate and 6µL of KAPA SYBR FAST qPCR Master Mix was added to each reaction well to prepare 10 µL qPCR reactions for each sample.

Plate name	Input	Input volume	Diluent volume	Total dilution of input DNA
Dilution plate 1	Standard 0	2 µL	98 µL	1:50
Dilution plate 2	Dilution plate 1	5 µL	95 µL	1:1000
Final dilution plate	Dilution plate 2	10 µL	10 µL	1:2000

Table 6. Worked example: Details of the three consecutive dilutions used to dilute the Standard 0 (0.2 nM) in the KAPA Library Quantification protocol on firefly to assess the dilution accuracy. The diluent volume used to create the "final dilution plate" is varied to achieve the desired final dilution of 1:2000.

	Total dilution	Cq 1	Cq 2	Cq 3	Mean Cq	Dilution factor	ΔCq	Expected ΔCq	Diluted Standard 0		Undiluted Standard 0	
									Concentration (pM)	Expected concentration (pM)	Concentration (nM)	Expected concentration (nM)
Rep 1 - Standard 0 (0.2nM)	1:50	9.61	9.63	9.65	9.63	20	4.29	4.32	3.905	4.000	0.20	0.20
	1:1000	13.85	14.03	13.87	13.92				0.224	0.200	0.22	0.20
	1:2000	15.00	14.94	15.16	15.03	2	1.12	1.00	0.106	0.100	0.21	0.20
Rep 2 - Standard 0 (0.2nM)	1:50	9.50	9.67	9.59	9.59	20	4.32	4.32	4.019	4.000	0.20	0.20
	1:1000	13.85	13.98	13.88	13.90				0.226	0.200	0.23	0.20
	1:2000	14.88	14.89	14.94	14.90	2	1.00	1.00	0.116	0.100	0.23	0.20
Rep 3 - Standard 0 (0.2nM)	1:50	9.48	9.66	9.51	9.55	20	4.31	4.32	4.119	4.000	0.21	0.20
	1:1000	13.83	13.85	13.90	13.86				0.232	0.200	0.23	0.20
	1:2000	14.86	14.86	14.93	14.88	2	1.02	1.00	0.117	0.100	0.23	0.20
Rep 4 - Standard 0 (0.2nM)	1:50	9.46	9.53	9.49	9.49	20	4.35	4.32	4.278	4.000	0.21	0.20
	1:1000	13.78	13.91	13.84	13.84				0.235	0.200	0.23	0.20
	1:2000	14.85	14.95	14.91	14.90	2	1.06	1.00	0.116	0.100	0.23	0.20

Table 7. qPCR run data for 4 replicates of KAPA qPCR Standard 0 (0.2 nM, 452bp) serially diluted to 1:50, 1:1000 then 1:2000 then quantified in triplicate. The standard curve for this run showed a reaction efficiency of 94.9% and r^2= 0.9999.

Table 7 shows that the ΔCq measured for the dilutions of Standard 0 prepared on firefly are in line with the ΔCq expected for each dilution. This suggests that firefly can accurately prepare library dilutions of the library DNA.

Consistency of library dilutions

Consistency of the library dilutions prepared on firefly was assessed using 16 replicates of the same library and measuring the concentration of the library in each of the three dilution plates generated in the KAPA Library Quantification protocol on firefly. A final library dilution of 1:12000 was prepared using a library with an average fragment length of 351bp and using the firefly protocol to prepare three serial dilutions, see Table 8 for details of the volumes used.

Plate name	Input	Input volume	Diluent volume	Total dilution of input DNA
Dilution plate 1	351bp library	2 µL	98 µL	1:50
Dilution plate 2	Dilution plate 1	5 µL	95 µL	1:1000
Final dilution plate	Dilution plate 2	10 µL	110 µL	1:12000

Table 8. Details of the three consecutive dilutions used to dilute a 351bp library using the KAPA Library Quantification protocol on firefly. The diluent volume used to create the "final dilution plate" is varied to achieve the desired final dilution of 1:12000.

Table 9 shows the %CV to be ≤3% for the variation in the concentration across 16 samples of the same library. This demonstrates that the KAPA Library Quantification protocol on firefly gives a consistent measure of sample concentration across multiple samples. Table 9 also demonstrates the accuracy of the library dilutions in that the concentration of the undiluted library varies by only 0.08 nM across the dilution range 1:50 to 1:12000.

Library dilution	Number of samples	Diluted library				Undiluted library	%CV
		Mean concentration (pM) size adjusted	Dilution factor	Expected concentration (pM)	% deviation from expected concentration	Mean concentration (nM) size adjusted	
1:50	16	19.74	n/a	n/a	n/a	0.99	2.2
1:1000	16	1.06	20	0.99	7.0	1.06	3.2
1:12000	16	0.09	12	0.09	1.2	1.07	2.6

Table 9. Dilution accuracy and variation in concentration across 16-replicates of the same sample. The expected concentration of the diluted library is based on the input concentration and the dilution factor.

Well-to-well contamination

The KAPA Library Quantification protocol was run using an input plate containing 48 library samples and 48 no template controls (NTCs), arranged in a checkerboard pattern. Figure 6 shows the amplification curves of the standards and the NTCs from this run and demonstrates that there was no detectable well-to-well contamination between the library samples and the NTCs.

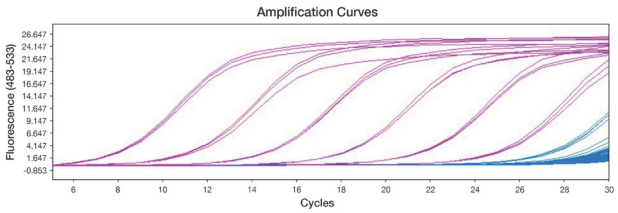

Figure 6. Amplification curves for KAPA qPCR standards 1 to 6 (Pink) and NTCs (Blue).

Conclusions

The KAPA Library Quantification Kits protocol has been successfully automated on firefly, enabling a 384-well qPCR reaction plate to be prepared with standards and up to 96 diluted samples, in just over 20 minutes. The qPCR reaction efficiency is as expected for KAPA Library Quantification Kits, sample dilutions are both accurate and consistent, and no well-to-well contamination was detected.

firefly provides accurate liquid handling and user-friendly software to remove the inconvenience and inconsistency of manual pipetting, enabling the fast, simple, high-throughput setup of qPCR reactions.

*Data on file. Project name: KAPA Library Quant on firefly. KAPA is a trademark of Roche. All other product names and trademarks are the property of their respective owners. LightCycler 480 System is for life science research only. All reagents are for Research Use Only. Not for use in diagnostic procedures.
For more information about Roche KAPA Library Quantification Kits, please visit: go.roche.com/GetKLQKits

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