Biomek Automated 384-well qPCR Setup using KAPA Biosystems Library Quantification Kit for Illumina Sequencing Platforms

Introduction:

Library quantification is an essential step in the Illumina next-generation sequencing (NGS) workflow. We have automated a 384-well setup for the KAPA Biosystems Library Quantification Kit for Illumina Sequencing Platforms on the Biomek FX^P Workstation with Multichannel Pipettor and Span-8 Pipettors. This system provides the flexibility to quantify 1-96 NGS library samples in triplicate with low variability.

Experimental Design:

To determine the consistency of library quantification while also testing for the presence of cross-contamination, we analyzed 48 replicate samples of a single NGS library preparation in a checkerboard pattern (Figure 1).

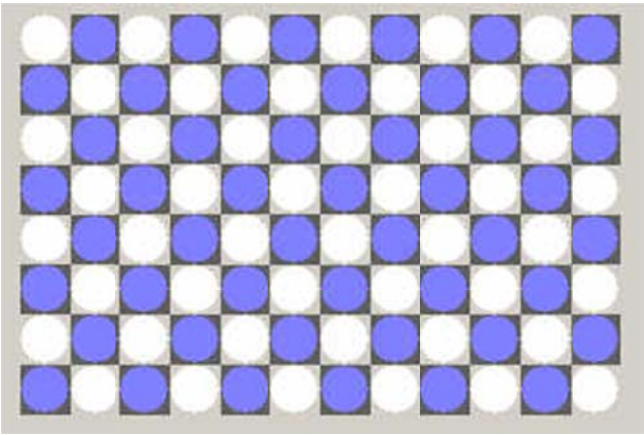

Figure 1. NGS Library Sample Pattern. Wells shaded blue contain replicate samples of an NGS Library, illustrating the cross-contamination test pattern. White wells contain nuclease-free water.

This library plate was created manually and placed on the deck of the Biomek FXP (Figure 2, "Clean Library"). The Span-8 Pod was used to add Library Dilution Buffer to three dilution plates. The NGS library samples were diluted 1:50 three times using the Multichannel Pod for a total dilution factor of 1:125,000. The KAPA mastermix and standards were transferred from 2ml tubes (mMix) to the 384-well plate using the Span-8 Pod. Finally, the diluted libraries/blanks were stamped into the first three quadrants of the qPCR plate using the Multichannel Pod. Figure 3 shows the final locations of the samples/blanks/standards in the 384-well qPCR plate.

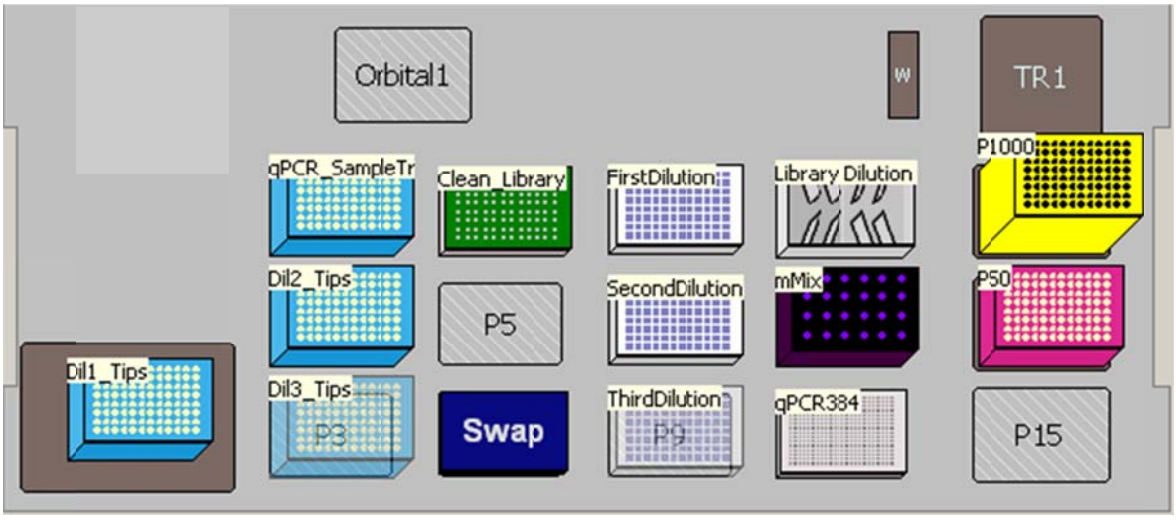

Figure 2. Sample Biomek FX^P deck layout for KAPA kit Automation.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Blank 1 Rep 1	Blank 1 Rep 2	Lib. 1 Rep 1	Lib. 1 Rep 2	Blank 2 Rep 1	Blank 2 Rep 2	Lib. 2 Rep 1	Lib. 2 Rep 2	Blank 3 Rep 1	Blank 3 Rep 2	Lib. 3 Rep 1	Lib. 3 Rep 2	Blank 4 Rep 1	Blank 4 Rep 2	Lib. 4 Rep 1	Lib. 4 Rep 2	Blank 5 Rep 1	Blank 5 Rep 2	Lib. 5 Rep 1	Lib.5 Rep 2	Blank 6 Rep 1	Blank 6 Rep 2	Lib. 6 Rep 1	Lib. 6 Rep 2
B	Blank 1 Rep 3	20 pM Rep 1	Lib. 1 Rep 3	20 pM Rep 2	Blank 2 Rep 3	20 pM Rep 3	Lib. 2 Rep 3		Blank 3 Rep 3		Lib. 3 Rep 3		Blank 4 Rep 3		Lib. 4 Rep 3		Blank 5 Rep 3		Lib. 5 Rep 3		Blank 6 Rep 3		Lib. 6 Rep 3	
C	Lib. 7 Rep 1	Lib. 7 Rep 2	Blank 7 Rep 1	Blank 7 Rep 2	Lib. 8 Rep 1	Lib. 8 Rep 2	Blank 8 Rep 1	Blank 8 Rep 2	Lib. 9 Rep 1	Lib. 9 Rep 2	Blank 9 Rep 1	Blank 9 Rep 2	Lib. 10 Rep 1	Lib. 10 Rep 2	Blank 10 Rep 1	Blank 10 Rep 2	Lib. 11 Rep 1	Lib. 11 Rep 2	Blank 11 Rep 1	Blank 11 Rep 2	Lib. 12 Rep 1	Lib. 12 Rep 2	Blank 12 Rep 1	Blank 12 Rep 2
D	Lib. 7 Rep 3	2 pM Rep 1	Blank 7 Rep 3	2 pM Rep 2	Lib. 8 Rep 3	2 pM Rep 3	Blank 8 Rep 3		Lib. 9 Rep 3		Blank 9 Rep 3		Lib. 10 Rep 3		Blank 10 Rep 3		Lib. 11 Rep 3		Blank 11 Rep 3		Lib. 12 Rep 3		Blank 12 Rep 3	
E	Blank 13 Rep 1	Blank 13 Rep 2	Lib. 13 Rep 1	Lib. 13 Rep 2	Blank 14 Rep 1	Blank 14 Rep 2	Lib. 14 Rep 1	Lib. 14 Rep 2	Blank 15 Rep 1	Blank 15 Rep 2	Lib. 15 Rep 1	Lib. 15 Rep 2	Blank 16 Rep 1	Blank 16 Rep 2	Lib. 16 Rep 1	Lib. 16 Rep 2	Blank 17 Rep 1	Blank 17 Rep 2	Lib. 17 Rep 1	Lib.17 Rep 2	Blank 18 Rep 1	Blank 18 Rep 2	Lib. 18 Rep 1	Lib. 18 Rep 2
F	Blank 13 Rep 3	0.2 pM Rep 1	Lib. 13 Rep 3	0.2 pM Rep 2	Blank 14 Rep 3	0.2 pM Rep 3	Lib. 14 Rep 3		Blank 15 Rep 3		Lib. 15 Rep 3		Blank 16 Rep 3		Lib. 16 Rep 3		Blank 17 Rep 3		Lib. 17 Rep 3		Blank 18 Rep 3		Lib. 18 Rep 3	
G	Lib. 19 Rep 1	Lib. 19 Rep 2	Blank 19 Rep 1	Blank 19 Rep 2	Lib. 20 Rep 1	Lib. 20 Rep 2	Blank 20 Rep 1	Blank 20 Rep 2	Lib. 21 Rep 1	Lib. 21 Rep 2	Blank 21 Rep 1	Blank 21 Rep 2	Lib. 22 Rep 1	Lib. 22 Rep 2	Blank 22 Rep 1	Blank 22 Rep 2	Lib. 23 Rep 1	Lib. 23 Rep 2	Blank 23 Rep 1	Blank 23 Rep 2	Lib. 24 Rep 1	Lib. 24 Rep 2	Blank 24 Rep 1	Blank 24 Rep 2
H	Lib. 19 Rep 3	0.02 pM Rep 1	Blank 19 Rep 3	0.02 pM Rep 2	Lib. 20 Rep 3	0.02 pM Rep 3	Blank 20 Rep 3		Lib. 21 Rep 3		Blank 21 Rep 3		Lib. 22 Rep 3		Blank 22 Rep 3		Lib. 23 Rep 3		Blank 23 Rep 3		Lib. 24 Rep 3		Blank 24 Rep 3	
I	Blank 25 Rep 1	Blank 25 Rep 2	Lib. 25 Rep 1	Lib. 25 Rep 2	Blank 26 Rep 1	Blank 26 Rep 2	Lib. 26 Rep 1	Lib. 26 Rep 2	Blank 27 Rep 1	Blank 27 Rep 2	Lib. 27 Rep 1	Lib. 27 Rep 2	Blank 28 Rep 1	Blank 28 Rep 2	Lib. 28 Rep 1	Lib. 28 Rep 2	Blank 29 Rep 1	Blank 29 Rep 2	Lib. 29 Rep 1	Lib.29 Rep 2	Blank 30 Rep 1	Blank 30 Rep 2	Lib. 30 Rep 1	Lib. 30 Rep 2
J	Blank 25 Rep 3	0.002 pM Rep 1	Lib. 25 Rep 3	0.002 pM Rep 2	Blank 26 Rep 3	0.002 pM Rep 3	Lib. 26 Rep 3		Blank 27 Rep 3		Lib. 27 Rep 3		Blank 28 Rep 3		Lib. 28 Rep 3		Blank 29 Rep 3		Lib. 29 Rep 3		Blank 30 Rep 3		Lib. 30 Rep 3	
K	Lib. 31 Rep 1	Lib. 31 Rep 2	Blank 31 Rep 1	Blank 31 Rep 2	Lib. 32 Rep 1	Lib. 32 Rep 2	Blank 32 Rep 1	Blank 32 Rep 2	Lib. 33 Rep 1	Lib. 33 Rep 2	Blank 33 Rep 1	Blank 33 Rep 2	Lib. 34 Rep 1	Lib. 34 Rep 2	Blank 34 Rep 1	Blank 34 Rep 2	Lib. 35 Rep 1	Lib. 35 Rep 2	Blank 35 Rep 1	Blank 35 Rep 2	Lib. 36 Rep 1	Lib. 36 Rep 2	Blank 36 Rep 1	Blank 36 Rep 2
L	Lib. 31 Rep 3	0.0002 pM Rep 1	Blank 31 Rep 3	0.0002 pM Rep 2	Lib. 32 Rep 3	0.0002 pM Rep 3	Blank 32 Rep 3		Lib. 33 Rep 3		Blank 33 Rep 3		Lib. 34 Rep 3		Blank 34 Rep 3		Lib. 35 Rep 3		Blank 35 Rep 3		Lib. 36 Rep 3		Blank 36 Rep 3	
M	Blank 37 Rep 1	Blank 37 Rep 2	Lib. 37 Rep 1	Lib. 37 Rep 2	Blank 38 Rep 1	Blank 38 Rep 2	Lib. 38 Rep 1	Lib. 38 Rep 2	Blank 39 Rep 1	Blank 39 Rep 2	Lib. 39 Rep 1	Lib. 39 Rep 2	Blank 40 Rep 1	Blank 40 Rep 2	Lib. 40 Rep 1	Lib. 40 Rep 2	Blank 41 Rep 1	Blank 41 Rep 2	Lib. 41 Rep 1	Lib.41 Rep 2	Blank 42 Rep 1	Blank 42 Rep 2	Lib. 42 Rep 1	Lib. 42 Rep 2
N	Blank 37 Rep 3	Water Rep 1	Lib. 37 Rep 3	Water Rep 2	Blank 38 Rep 3	Water Rep 3	Lib. 38 Rep 3		Blank 39 Rep 3		Lib. 39 Rep 3		Blank 40 Rep 3		Lib. 40 Rep 3		Blank 41 Rep 3		Lib. 41 Rep 3		Blank 42 Rep 3		Lib. 42 Rep 3	
O	Lib. 43 Rep 1	Lib. 43 Rep 2	Blank 43 Rep 1	Blank 43 Rep 2	Lib. 44 Rep 1	Lib. 44 Rep 2	Blank 44 Rep 1	Blank 44 Rep 2	Lib. 45 Rep 1	Lib. 45 Rep 2	Blank 45 Rep 1	Blank 45 Rep 2	Lib. 46 Rep 1	Lib. 46 Rep 2	Blank 46 Rep 1	Blank 46 Rep 2	Lib. 47 Rep 1	Lib. 47 Rep 2	Blank 47 Rep 1	Blank 47 Rep 2	Lib. 48 Rep 1	Lib. 48 Rep 2	Blank 48 Rep 1	Blank 48 Rep 2
P	Lib. 43 Rep 3	Water Rep 1	Blank 43 Rep 3	Water Rep 2	Lib. 44 Rep 3	Water Rep 3	Blank 4 Rep 3		Lib. 45 Rep 3		Blank 45 Rep 3		Lib. 46 Rep 3		Blank 46 Rep 3		Lib. 47 Rep 3		Blank 47 Rep 3		Lib. 48 Rep 3		Blank 48 Rep 3	

Figure 3. qPCR Plate layout of triplicate samples, blanks and standards (green).

Analysis and Results:

The qPCR plate was processed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using the qPCR cycling settings recommended by the KAPA kit (35 cycles). Figure 4 shows raw Ct data for all triplicate samples. Blank samples that had a Ct lower than 35 are highlighted in orange. Of these 4 wells, the lowest Ct is 33, which corresponds to 0.002% of the library sample concentrations, indicating that even if these readings correspond to cross-contamination rather than primer dimers or other background signals, the effects on sample quantification would be negligible.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Undet.	Undet.	16.53	16.46	Undet.	Undet.	16.56	16.55	Undet.	33.32	16.65	16.62	Undet.	Undet.	16.56	16.57	Undet.	Undet.	16.58	16.55	Undet.	Undet.	16.44	16.48
B	Undet.	8.10	16.51	8.21	Undet.	8.19	16.60		Undet.		16.74		Undet.		16.59		Undet.		16.62		Undet.		16.51	
C	16.50	16.48	33.87	Undet.	16.54	16.40	Undet.	Undet.	16.64	16.63	Undet.	Undet.	16.48	16.51	Undet.	Undet.	16.59	16.57	Undet.	Undet.	16.56	16.51	Undet.	Undet.
D	16.59	11.64	Undet.	11.88	16.57	11.65	Undet.		16.69		Undet.		16.59		Undet.		16.63		Undet.		16.65		Undet.	
E	34.06	Undet.	16.50	16.40	Undet.	Undet.	16.57	16.56	Undet.	Undet.	16.68	16.66	Undet.	Undet.	16.42	16.62	Undet.	Undet.	16.67	16.61	Undet.	Undet.	16.63	16.60
F	Undet.	15.01	16.57	15.14	Undet.	15.20	16.62		Undet.		16.72		Undet.		16.64		Undet.		16.72		Undet.		16.70	
G	16.47	16.53	Undet.	Undet.	16.50	16.50	Undet.	Undet.	16.61	16.51	Undet.	Undet.	16.50	16.50	Undet.	Undet.	16.63	16.54	Undet.	Undet.	16.50	16.51	Undet.	Undet.
H	16.54	18.49	Undet.	18.54	16.59	18.65	Undet.		16.63		Undet.		16.60		Undet.		16.64		Undet.		16.61		Undet.	
I	Undet.	Undet.	16.51	16.53	Undet.	Undet.	16.57	16.51	Undet.	Undet.	16.60	16.56	Undet.	Undet.	16.65	16.63	Undet.	Undet.	16.56	16.57	Undet.	Undet.	16.52	16.53
J	Undet.	22.02	16.59	21.88	Undet.	21.94	16.61		Undet.		16.66		Undet.		16.64		Undet.		16.64		Undet.		16.59	
K	16.45	16.60	Undet.	Undet.	16.42	16.45	Undet.	Undet.	16.47	16.44	Undet.	Undet.	16.55	16.53	Undet.	Undet.	16.56	16.57	Undet.	Undet.	16.55	16.54	Undet.	Undet.
L	16.52	25.44	Undet.	25.16	16.49	25.18	Undet.		16.54		Undet.		16.61		Undet.		16.60		Undet.		16.61		Undet.	
M	Undet.	Undet.	16.58	16.67	Undet.	Undet.	16.68	16.58	Undet.	Undet.	16.63	16.56	Undet.	Undet.	16.36	16.41	33.00	Undet.	16.53	16.57	Undet.	Undet.	16.56	16.62
N	Undet.	Undet.	16.67	Undet.	Undet.	Undet.	16.69		Undet.		16.64		Undet.		16.44		Undet.		16.61		Undet.		16.63	
O	16.55	16.49	Undet.	Undet.	16.57	16.54	Undet.	Undet.	16.62	16.53	Undet.	Undet.	16.55	16.62	Undet.	Undet.	16.54	16.53	Undet.	Undet.	16.49	16.35	Undet.	Undet.
P	16.46	Undet.	Undet.	Undet.	16.51	Undet.	Undet.		16.62		Undet.		16.59		Undet.		16.52		Undet.		16.54		Undet.	

Figure 4. Cycle threshold (Ct) values from KAPA Library Quantification qPCR analysis.

Figure 5 shows the low CVs of the triplicate Ct values of the KAPA standards, illustrating the precision that can be achieved through automation of this process.

Standard Concentration Curve (pM)

	Known Conc.	Log of known conc.	Ct rep 1	Ct rep 2	Ct rep 3	Ct Average	Ct StDev	Ct %CV
A	20.0000	1.3010	8.1047	8.2067	8.1921	8.17	0.06	0.7%
B	2.0000	0.3010	11.6380	11.8835	11.6483	11.72	0.14	1.2%
C	0.2000	-0.6990	15.0124	15.1380	15.2011	15.12	0.10	0.6%
D	0.0200	-1.6990	18.4869	18.5439	18.6503	18.56	0.08	0.4%
E	0.0020	-2.6990	22.0240	21.8761	21.9442	21.95	0.07	0.3%
F	0.0002	-3.6990	25.4403	25.1565	25.1771	25.26	0.16	0.6%

Figure 5. KAPA standard Ct values and analysis.

To determine the consistency of the automated method we analyzed CVs for individual triplicates of the 48 samples. All triplicates gave a CV at or below 8.3%, with all but 4 samples falling below 5% CVs (data not shown). For the entire plate, we determined the average library concentration (calculated as recommended in the KAPA kit, using 315bp as the average fragment length) and variability between the 48 samples. Figure 6 shows that the starting library was at an average concentration of 13.29nM. In addition, we see low variability across the plate, illustrated by a CV of 4.1%.

Average	13.29 nM
Stdev	0.55 nM
CV	4.1%

Figure 6. Average concentration and variability of the 48 wells of the starting NGS library.

Conclusion:

These data illustrate how the Biomek FX^P can be utilized to reliably automate the preparation of the KAPA Biosystems Library Quantification Kit for Illumina Sequencing Platforms in a 384-well format. This rapid method provides a walk-away means of quantifying 96 samples in triplicate within 45 minutes.