qPCR on the QuantStudio 6 Pro for Illumina Library Quantification

Adapted from KAPA's technical data sheet for KK4873.

Updated 29-Feb-2024 by Katie Murphy

BACKGROUND:

This quantification method will work for any Illumina-compatible libraries. We most often only run qPCR on final library pools prior to sequencing, but you may wish to quantify individual libraries or subpools pending your project needs.

HELPFUL HINTS:

- We recommend familiarizing yourself with the Technical Data Sheet for the KAPA library quantification kit prior to use.
- Before setting up your qPCR plate, make sure you have a template set up in the "Design and Analysis" software so that it will be ready to go. This software can be installed on your own computer if desired.
- If you have more than one sample to quantify, we recommend making serial dilutions in a regular PCR plate instead of individual tubes. You should dilute your positive control alongside of your libraries.
- Include a positive control, such as KAPA standard 0 or a previously quantified library.
- Include a no-template control (NTC) – this will be your dilution buffer.
- The master mix is light sensitive, so please make sure to keep it in a dark location except for during use.
- Choose the appropriate dilution(s) for your sample(s) ahead of time. You want your <u>diluted</u> sample to be **< 20 pM**.
    - Depending on your sample, this could require anywhere from a 1:100 to a 1:1,000,000 dilution.
    - Use Qubit to determine ng/ul and make a rough estimate of nM, then choose your dilution(s) accordingly.
- For the most accurate quantification of your final library pool, we recommend running three dilutions, with three replicates of each (9 rxns per pool).
    - If needed for space reasons, you could decrease this to two dilutions, but still maintain three replicates of each.

MATERIALS & REAGENTS:

- KAPA KK4873 – complete quant kit (*ROX low*) for Illumina libraries
- qPCR dilution buffer: 10mM Tris (pH 8.0-8.5) + 0.05% Tween-20
- 0.1mL MicroAmp PCR plate (these are the veriti plates – do NOT use our normal PCR plates!)
- Optically clear plate seals
- Filter tips
- P10 or P20 multichannel pipette
- Libraries to be quantified
- Positive control library (this could be KAPA standard 0, their positive control)

PROTOCOL:

Step 1 – Setting up your qPCR plate

1.1 Plan out your qPCR plate layout ahead of time. Bring a sketch or printout into the lab with you.
   a. Note that you should run the 6 KAPA standards and the no-template control (NTC) in triplicate, so these will use up 21 wells in addition to any samples or positive control.

1.2 Remove 2X KAPA master mix, standards 1-6, and Standard 0 from the -20C and thaw at **room temp.**
   a. ***The master mix must be protected from light,*** as it is light sensitive (use a covered box or other dark location).

1.3 While reagents are thawing, make the appropriate library dilution(s) in qPCR dilution buffer.
   a. Dilute your positive control alongside your libraries and run as an unknown.
   b. You <u>do not</u> dilute standards 1-6; they are run as-is.

1.4 Vortex the 2X KAPA master mix and standards 1-6, then spin briefly.

1.5 Add 6uL of master mix to each required well in your qPCR plate.
   a. We recommend aliquoting the master mix into strip tubes first, and then using a P10 or P20 multichannel pipette to add to your qPCR plate.

1.6 To the designated standard wells: add 4 uL of standards 1-6 to the appropriate well(s).

1.7 To the designated NTC wells: add 4 ul of qPCR dilution buffer.

1.8 To the designated sample/unknown wells: add 4ul of the appropriate dilution of sample or positive control.

1.9 Mix by pipetting up/down 5-10 times, ***MAKING SURE TO NOT MAKE BUBBLES.***
   a. Helpful tip: We recommend that you don't go to the second pipette stop completely, as this will cause bubbles.

1.10 Carefully seal the plate with optically-clear sealing film, pressing firmly to ensure it fully adheres.

1.11 Briefly spin the plate down in a centrifuge to collect contents to the bottom of the wells.

1.12 Inspect the wells. If there are any bubbles present in a well, repeat the spin.

1.13 Your plate is ready to run now.

1.14 Make sure to return all reagents to the -20C freezer.

Step 2 – Running the plate on the QuantStudio 6 Pro (QS6P) at NHB

2.1 Turn on the QS6P (switch is on the back-right) and computer if you haven't already done so.

2.2 Open the Design & Analysis software from the desktop computer, then click "Set up Plate".

2.3 Load your qPCR template if you've already made one, then skip to Step 2.16. If you don't already have a template, continue to Step 2.4.

2.4 Make sure you are in the System Templates screen. In the list of filter options on the left side, select "QuantStudio 6 Pro" under Instrument, "96-well 0.1 mL" under Block, and "Fast" under Run Mode.

2.5 From the template options that populate, select "Standard Curve with Melt".

2.6 Define the run method on the "Run Method" screen:

- a. Set the reaction volume to 10 µL.
- b. Heated cover should be set to 105°C.
- c. Set the run method as follows:
  - i. 95°C – 5 min
  - ii. 35 cycles of:
    - 95°C – 30 sec
    - 60°C – 45 sec (use 90 sec if library inserts are > 700 bp)
  - iii. Melt curve analysis: set range to 65 – 95°C.
- d. Ensure that the camera icon is turned on at the 60°C step.

2.7 Next, select "Plate Setup" from the options along the top of the screen.

2.8 Ensure that "Target" is highlighted in blue on the top left, and set the Passive Reference to "ROX".

2.9 In the "Target" panel on the lower right of the screen, click the "+" icon to add a target. Make sure to set the target's reporter to SYBR.

- a. Optional: If desired, enter a name for the target and/or set a color.

2.10 Define your samples and positive control in the "Samples" panel on the upper right of the screen. Click the "+" icon to list each dilution of each sample.

- a. Give each a descriptive name. For example, with two samples, a positive control, and three dilutions, you would have:
  - i. Sample 1 – Dilution 1
  - ii. Sample 1 – Dilution 2
  - iii. Sample 1 – Dilution 3
  - iv. Sample 2 – Dilution 1
  - v. Sample 2 – Dilution 2
  - vi. Sample 2 – Dilution 3
  - vii. Positive Control – Dilution 1
  - viii. Positive Control – Dilution 2
  - ix. Positive Control – Dilution 3
- b. Make sure that each sample and positive control dilution entry have "Unknown" selected as their type.

2.11 Define your six assay standards in the same "Samples" Panel, ensuring you select "Standard" as the type. Enter the standard's corresponding concentration in the "Quantity" column of this panel.

- a. Standard 1 – 20 pM
- b. Standard 2 – 2 pM
- c. Standard 3 – 0.2 pM
- d. Standard 4 – 0.02 pM
- e. Standard 5 – 0.002 pM
- f. Standard 6 – 0.0002 pM

2.12 Include a line for your No Template Control in the Samples panel; select "Negative Control" as the type.

2.13 If desired, select a color for each sample dilution and/or standard. This color does not impact your run.

2.14 Assign targets, samples, and standards to their specific wells on the plate. Highlight the wells of interest in the plate image, then click the corresponding check box in the Samples panel.
2.15 Make sure that your target (SYBR) from the Targets panel is also checked for all used wells.
2.16 Next, select "Run Summary" from the options along the top of the screen.
2.17 Review the run summary to ensure all settings and layout are correct.
2.18 Optional but recommended: If you will use this same plate layout in future and don't already have a template saved, click on the "Actions" button on the upper right. Selecting "Add to my Plates" from the dropdown menu will save this as a template for future use.
2.19 Recommended: Save your file as a .eds by clicking on the same "Actions" button. Make sure to navigate to your own folder when saving.
2.20 Scroll down on the "Run Summary" screen until you see the box of available instruments. Select our QS6P instrument, and then click "Send to Run Queue".
2.21 On the touchscreen of the QS6P instrument, log in using "1234" if needed.
2.22 On the touchscreen, select "Load Plate File". Then select Run Queue, and choose your run. You should see your plate properties now displayed on the screen.
2.23 Recommended: in the Plate Properties screen, edit the "Data file name" to your name of choice.
2.24 Select the location(s) to send the resulting data file; one or more can be selected. Options include on the QS6P instrument, sending to the desktop via the network drive, or saving directly to a USB drive. If saving to USB, connect a USB drive to the front of the QS6P now. If saving to the desktop, make sure you've navigated to your own folder.
2.25 Load your qPCR plate into the QS6P instrument:
    a. Touch the "eject" icon on the upper right of the screen to open the QS6P drawer. The drawer will extend out from the front of the instrument.
    b. Load your plate into the drawer. Well A1 should be on the upper left. Note that the plate will appear to be slightly raised when it's fully seated in the instrument.
    c. Touch the "eject" icon a second time to close the drawer.
2.26 Start your run. This will take approximately 1 hr 30 minutes, unless you are running long-insert libraries (>700 bp).
2.27 When the run is complete, remove your plate from the QS6P and dispose of it in the trash.
2.28 Open your data file in the Design & Analysis software. Review data as desired.
2.29 Export the results to an excel file in your own folder and/or to a USB drive by using the dropdown menu from the "Actions" button.

Step 3 – Analyzing your qPCR results

3.1 Open the KAPA qPCR analysis template excel file, and click 'Enable Macros" on the popup.
3.2 Re-save the file with your desired file name (specific to your current qPCR run).
3.3 Follow the directions given in the "Readme" tab to analyze your data.

ADDITIONAL INFORMATION/APPENDICES

- Example qPCR plate layout:

	columns 1, 2, & 3	columns 4, 5, & 6	columns 7, 8, & 9	columns 10, 11, & 12
A	standard 1 (20 pM)	sample 1 - 1st dil.	sample 1 - 2nd dil.	sample 1 - 3rd dil.
B	standard 2 (2 pM)	sample 2 - 1st dil.	sample 2 - 2nd dil.	sample 2 - 3rd dil.
C	standard 3(0.2 pM)	sample 3 - 1st dil.	sample 3 - 2nd dil.	sample 3 - 3rd dil.
D	standard 4 (0.02 pM)	sample 4 - 1st dil.	sample 4 - 2nd dil.	sample 4 - 3rd dil.
E	standard 5 (0.002 pM)	sample 5 - 1st dil.	sample 5 - 2nd dil.	sample 5 - 3rd dil.
F	standard 6 (0.0002 pM)	sample 6 - 1st dil.	sample 6 - 2nd dil.	sample 6 - 3rd dil.
G	NTC (0.0 pM)	sample 7 - 1st dil.	sample 7 - 2nd dil.	sample 7 - 3rd dil.
H		positive control - 1st dil.	positive control - 2nd dil.	positive control - 3rd dil.

REFERENCES

KAPA Biosystems. 2017. KAPA library quantification kit technical data sheet (KR0405 – v8.17).

Applied Biosystems. 2021. QuantStudio 6 Pro Real-Time PCR System and QuantStudio 7 Pro Real-Time PCR System: User Guide (Publication Number MAN0018045; Rev. H.0).