

Standard Operating Procedure Approval Page:

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Name **Date**

Supersedes: **Name** **Date**

Annual Review and Approval

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Laboratory Director

Changes Made:
061620: New Protocol

Changes to previous procedures:
n/a

Note: *No laboratory personnel should use this piece of equipment without proper training by the laboratory supervisor or other designated person.*

Instrument Principles: The 7900HT Fast Real-Time PCR System is a real-time quantitative PCR system that combines 96- and 384-well plate compatibility and the TaqMan® Low Density Array with fully automated robotic loading-and now also offers optional Fast real-time PCR capability.

Specimen Types: KAPA Illumina Library Quantification Kits are ideally suited for the quantification of libraries constructed with Illumina adaptors qPCR primer sequences, with a broad range of concentrations, containing fragments with a wide range of GC contents, and containing fragment lengths ranging from 50 bp to 1000 bp.

Required Reagents and Consumables:

1. KAPA SYBR FAST qPCR Universal qPCR Kit (500 x 20 µL rxn) KAPA KK4824:
 - a. Illumina Primer Premix (10X, 1 ml)
 - b. ROX High Reference Dye (50X)
 - c. qPCR Master Mix (2X, 5 mL)
 - d. Illumina DNA Standards (6 x 80 µl)
2. Applied Biosystems™ MicroAmp™ Optical 384-Well Reaction Plate Applied Biosystems™ 4309849
3. Water, Molecular Grade, DNase-free, RNase-free
4. FisherBrand Super Friendly Air'It (catalog #23-002-523)

Procedural Steps:

Reagent and sample preparation:

1. **At least ten minutes prior to running a plate, power on the 7900 to allow it to warm up.**
2. **Thaw master mix, library pools, and library pool control on ice. Use an ice bucket with a lid to protect the master mix from light, as it is light sensitive.**
3. **Thaw standards at room temperature.**
4. **Verify the Illumina primer mix has been added to the KAPA qPCR master mix. This will be designated by the words “primers added” on the lid along with the date added. If using a new kit, thaw the Illumina primer mix and add 1 mL to a full, unopened bottle of the master mix and invert to mix. Write “primers added” on top of the lid and include the date.**
5. **The low and high ROX tubes in the kit are not used and can be discarded.**
6. **Determine the approximate concentration and fragment length of the library sample.**

7. If the investigator does not provide an estimated pool concentration or fragment length, email to obtain the values. If they do not have this information, measure 1 μL of the library on Nanodrop.
8. If below 1 $\text{ng}/\mu\text{L}$, use the high sensitivity Bioanalyzer assay to determine the estimated concentration (nM) and average fragment length (bp).
9. If above 1 $\text{ng}/\mu\text{L}$, use the DNA 1000 Bioanalyzer assay to determine the estimated concentration (nM) and average fragment length (bp).
10. If there is adequate pool volume, run the sample on the Bioanalyzer in triplicate and average the values obtained.

Library dilution:

11. Calculate the initial and serial dilutions for each pool based on the starting concentration. The 2 nM initial dilution value for most libraries should leave enough room to account for quant/Bioanalyzer error and allow for the values to fall within the standard curve. All dilutions in the protocol should be made with a fresh aliquot of molecular grade water.

If the starting concentration is:	≤ 1 nM	1 nM - 100nM	100 nM – 1000 nM
<u>Initial Dilution:</u>	N/A	Dilute to 2nM	1:500
<u>Serial dilutions</u> of the initial dilution for use in the qPCR assay:	1:100	1:1000	1:1000
	1:200	1:2000	1:2000
	1:400	1:4000	1:4000
	1:800	1:8000	1:8000

Example 1: 1 nM library pool \rightarrow no initial dilution \rightarrow one set of 1:100/200/400/800 serial dilutions to be run in duplicate

Example 2: 20 nM library pool \rightarrow 1:10 initial dilution (1 μL pool + 19 μL H_2O) in tube = \sim 2nM \rightarrow one set of 1:1000/2000/4000/8000 serial dilutions to be run in duplicate

Example 3: 200 nM library pool \rightarrow 1:500 initial dilution in tube \rightarrow one set of 1:1000/2000/4000/8000 serial dilutions to be run in duplicate

12. Perform the initial dilution of the library calculated in the previous step in a 1.5 mL tube. Dilute the libraries into a fresh aliquot of molecular grade water. Ensure that the volume of DNA was added successfully by checking the tip before and after adding the DNA to the water.
13. Perform the first serial dilution in a 1.5 mL tube. Ensure that the volume of DNA was added successfully.
14. The remaining serial dilutions will be performed in the intermediate plate.

Intermediate Plate:

11. Prepare a master mix of the KAPA qPCR master mix and water. Calculate the amount of master mix that you will need in order to run:

- a. Each sample or control in duplicate
- b. Each of the six standards in duplicate
- c. The NTC in quadruplicate
- d. An additional 15% added to the total number of reactions to account for error

Master Mix	
KAPA qPCR Master Mix (2x)	12 μ L
Water	4 μ L
DNA or NTC	4 μ L
Total volume	20 μL

12. Set up the Intermediate Plate. Follow the “Intermediate Plate” template found in the KAPA qPCR Template Excel file.

- a. After gentle vortexing and spinning down, pipette the master mix into column 1 of a 96 well plate, pipetting 1/8 of the total volume of master mix into each of the 8 wells.
- b. Vortex and spin down the six standards. Pipette 10 μ L of standard or water into column 2 of the plate, according to the template map.
- c. Pipette up to 200 μ L of the first serial dilution into Row A for each sample, according to the template map (column 3 and up).
- d. Pipette 100 μ L of water into each of the sample dilution wells for rows B-D.
- e. Transfer 100 μ L of the first serial dilution from row A to row B. Pipette up and down at least ten times to mix thoroughly.
 - i. A second multichannel pipet set to ~90 μ L can be used to pipette mix to avoid having to reset the primary multichannel.
- f. Transfer 100 μ L of the first serial dilution from row B to row C. Pipette up and down at least ten times to mix thoroughly.
- g. Transfer 100 μ L of the first serial dilution from row C to row D. Pipette up and down at least ten times to mix thoroughly.

qPCR/7900 Plate:

13. Set up the 7900 Plate (384 well plate). Follow the “7900 Plate” template found in the KAPA qPCR Template Excel file. Note that the lettered wells are staggered in order to use a multichannel pipet. To increase the accuracy of the assay, change pipet tips between each pipetting step and avoid drips at the end of the pipet tips when drawing up liquid.

- a. Pipette 16 μ L of master mix per well from column 1 of the Intermediate Plate to the 7900 plate.
- b. Transfer 4 μ L of Standard DNA or NTC from column 2 of the Intermediate Plate to columns 1 and 2 of the 7900 Plate.
- c. Transfer 4 μ L of Sample DNA from column 3 and up of the Intermediate Plate to column 3 and up of the 7900 Plate, as shown in the template map.
- d. Seal the plate with a clear, **optical** film by repeatedly running the optical film applicator over the surface of the plate in multiple directions. Seal the edges of the plate with the edge of the applicator. Using a roller will not seal the optical film and will result in sample contamination when vortexed.

- e. Vortex the plate while covering the bottom of the wells with the optical film backing to avoid debris adherence to the well bottoms, which can affect instrument reads.
- f. Centrifuge the plate at 1000 rpm for 2 min to collect the sample in the bottom of the wells. Ensure no sample remains on the sides of the wells.
- g. Using an air duster, dust the bottom of the plate to remove any debris before loading into the 7900. Use only the FisherBrand Super Friendly Air'It (catalog #23-002-523).

qPCR run:

14. Set up the 7900 HT instrument.

- a. Open the SDS 2.4 software. The username to log in is “administrator.” Leave the password field blank.
- b. Open the SDS template file that is on the desktop called KAPA qPCR template or
- c. Set up a new experiment:
 - i. Select File > New.
 - ii. In the New Document window, select “standard curve (AQ)” as the assay and leave the other fields as the default.
 - iii. Define the detector for the assay: In the plate diagram, select all the wells on the plate by clicking the corner cell in the top left. Click the “add detector” button in the Setup tab. In the Find field, type “SYBR” and press enter. Select the SYBR detector in the list and select “copy to plate document”. Select only the wells that will contain a reaction and check the “use” box to the left of the detector name in the Setup tab.
 - iv. Set up sample names: Select the desired well or multiple wells, if they are duplicates of each other, and type in the sample name. Duplicate names will automatically trigger the software to calculate averages of the values during analysis.
 - v. Select the task for each well: For the samples and control, select “unknown”. For the NTCs, select “NTC”. For the standards, select “standard” and enter the standard concentration in the blank that appears to the right of the task. Use the following concentrations:

Standard 1	20 pM
Standard 2	2 pM
Standard 3	0.2 pM
Standard 4	0.02 pM
Standard 5	0.002 pM
Standard 6	0.0002 pM

- (i) Set up the Thermal Profile: Select the Instrument tab and navigate to the Thermal Profile section within the Thermal Cycler tab. Left click to select “stage 1” (will turn red when selected), right click, and select “delete step.” Click the “ok” button of the pop-up window regarding the hold time. Enter the following cycling conditions being sure to change the repeat number to “35”. Verify the reaction volume in the right-hand corner is set to “20”.

Step	Temp	Duration	Cycles
Initial Denaturation	95 °C	5 min	1
Denaturation	95 °C	30 s	35
Annealing/Extension	60 °C	90 s	

- d. Data collection: Under the Data Collection section, ensure the page icon appears under each temperature (indicates data will be collected for that step).
- e. Select File > Save As and save the SDS file to the desktop and not the PPMS drive to avoid data loss.
- f. Select the Instrument tab and click on “connect to instrument. Once connected, select the “open/close” button. Load the plate into the instrument with A1 in the top, left corner. Select the “start run” button to close the instrument and begin the run.

Data analysis:

15. When the run is complete, click the large, green “analyze” arrow in the toolbar to calculate the quantities.
16. Under the Results section that appears, verify the slope of the standard curve. This value should be above .99 and preferably above .995. Confirm that the samples (red X's) fall on the standard curve within the boundaries of the highest and lowest standards (black boxes).
17. Export the file.
 - a. Select only the wells that contained reactions. Select File > Export. Select “selected wells” as the from option and leave the other fields as the defaults. Name (include project ID, order number, “qPCR”, and date) and save the file to the PPMS order folder.
18. Copy the values from the exported file into the EIGC.014_Appendix A KAPA qPCR template to calculate the final concentration of the library pools. Use the “values only” paste option to retain the template formatting. Save only the first tab of the template into the customer order folder with the project, order, and date in the filename.
19. Adjusted value for the FAN control should be 45-50 nM. If this value is off, or if any of the NTCs appears to amplify, the entire qPCR will need to be repeated.
20. Zip and post the Excel file to the client data folder.

Troubleshooting:

For KAPA SYBR FAST qPCR Universal Kit troubleshooting, email: Support.SeqLS@roche.com or call: 800.262.4911. For instrument related troubleshooting, consult the Applied Biosystems 7900HT Fast Real-Time PCR System [Maintenance and Troubleshooting Guide](#).