SOP Title:

KAPA Library Quantification (Illumina Platforms)

1.0 Purpose and scope

This standard operating procedure (SOP) is for the quantification of next generation sequencing (NGS) libraries for Illumina platforms on the Applied Biosystems (ABI) ViiA7 instrument using QuantStudio Real-Time PCR Software v1.2.

Related documents:

- Manufacturer supplied protocol for library quantification: KAPA Library Quantification Kit Illumina Platforms (KR0405-v8.17). Link to document.
- Manufacturer supplied reference material: KAPA Library Quantification Technical Guide-KAPA library Quantification Kits for Illumina Platforms V1.14. Link to document.
- Manufacturer supplied: QuantStudio Real-Time PCR Software v1.2 application, “Help” tab link to manual

Related TGL documents on R drive:

- YYYY_MM_DD_SAMPLE SUBMISSION FORM_PI_Lastname_Firstname_TGL.xls
- SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.doc
- 16_05_11_Kapa_Hyperprep_lot_tracking_sheet.xls
- 2016-04-22 095434_Dax_plate_cyling_template.eds
- SOP_RT-qPCR_Analysis_in_Excel_Template.xlsx

2.0 Materials

Workspace		
Post-PCR area		
Equipment and associated consumables		
ThermoFisher	ABI Applied Biosystems ViiA7	
	QuantStudio Real-Time PCR Software v1.2	
	MicroAmp® Optical 384-Well Reaction Plate with Barcode	4309849
	MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	4346906
	MicroAmp® Optical Adhesive Film	4311971
	MicroAmp Adhesive Film Applicator	4333183
BioRad	Sealing Roller	MSR0001
Roche (KAPA)	Kapa Library Quantification Kit-Illumina platforms ROX Low qPCR Master Mix (includes 6 standards)	7960336001 (KK4873)
	KAPA DNA Standards 1-6 (80ul each, 0.0002pM- 20pM)	7960387001 (KK4903)
Medstore	Nuclease-free water	W4502-1L
	Tween-20, Biotech grade, Nuclease free, 500ML	TWN510.500

3.0 General Guidance

- Do not mix and match reagents from multiple kits!
- When opening a new kit, record all kit lot numbers in TGL master lot tracking sheet (R:\Lot_tracking_forms\16_05_11_Kapa_Hyperprep_lot_tracking_sheet.docx, associate RT-qPCR excel file with lot letter key (“A”, “B”) for KAPA kit and KAPA standards
- Before beginning work every day, wipe down all pipetors and bench surfaces with Peroxide wipes, then wipe with 70% ethanol (made from bulk ethanol, 4L)
- Standards and QPCR mix may be briefly vortexed to ensure thorough mixing (exception to rule)
- Thawed standards and aliquoted master mix may be put in the fridge for short term storage or thawing prior to use
- When loading qPCR plate, all library dilutions and master mix may be kept at room temperature, as chilled reagents will affect pipetting volume
- PCR master mix should kept in the dark when not in use to prevent SYBR degradation
- Liquid in PCR plate may be electrostatic and “jump” to the adhesive covers. Handle carefully.

4.0 Procedure

POST PCR AREA

Preparation for Plate Loading:

1. When opening a new kit, add 1mL of 10X Primer Premix to 5mls of provided 2X KAPA SYBR FAST qPCR Master Mix. Aliquot into six separate 1.5ml microtubes, labeling with date and lot for storage in -20C and update lot tracking sheet. After each use, indicate freeze thaw with a "dot" on the lid of the tube to track freeze thaw cycles (avoid excessive freeze-thaws).
2. Make a 50ml mix of molecular grade H20 and 0.05% Tween-20 (25ul of tween-20 in 50mls H2O). Solution will be used for RT-qPCR 1 in 1000 dilutions. Solution may be saved for later use (stable for 3 weeks or so at room temp).
3. In a dark drawer, thaw 1ml aliquot of premixed RT-qPCR mix (containing primer and master mix, from step 1), standards, and libraries for quantification. Thawing may be done at room temperature if ready, or tubes may be placed in fridge until ready to begin. Briefly vortex samples, and RT-qPCR mix, spin down.

Library Dilutions and Standards:

Usually the top stock library will require dilution which depends on library type and sample input quality (i.e. fresh vs FFPE material), ask for assistance to determine appropriate dilution. Keep parental library dilutions as they will be used for sequencing. The 1 in 999ul dilutions may be discarded after analysis of RT results. See appendix A reference diagram.

1) Each sample library requires 3 separate dilutions, switch tips between dilutions to prevent contamination to top stock. Briefly vortex each stock library and spin down. Dilute each top stock library into 3 replicate dilutions, of 1ul library in 999ul of H20 +0.05% Tween-20 (25 ul of Tween-20 in 50 mls of H20). Vortex briefly. Each dilution will be loaded into 2 separate wells on the PCR plate. Therefore each library will use 6 wells of master mix.
2) Standards require 18 wells (3 wells per standard, 6 standards).

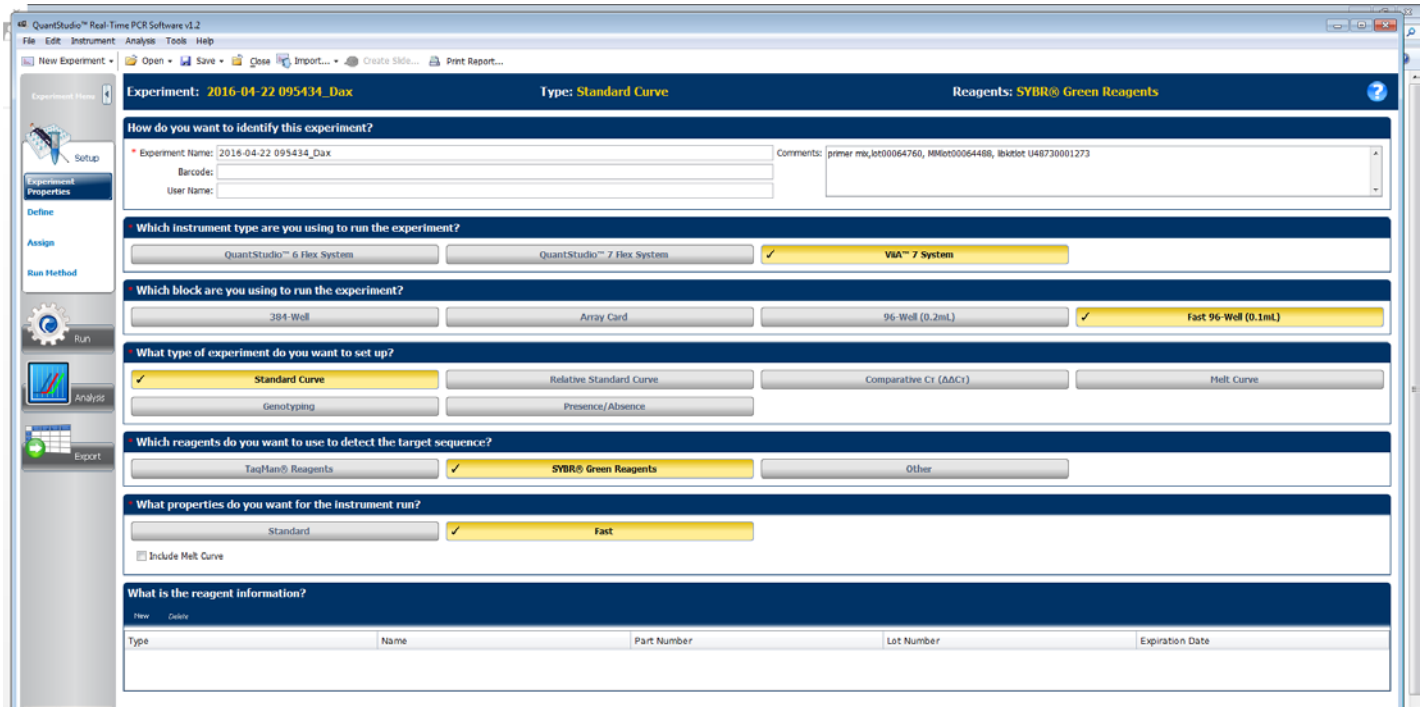
Plate Loading:

1) Each well requires 6 ul of master mix. Briefly vortex master mix and spin down.
2) Add 6 ul of master mix to all required wells of PCR plate. You may touch the side of the well with the pipette tip. When all wells are loaded, tap the plate gently to collect pipetted master mix at the bottom of the wells. Seal the plate with MicroAmp adhesive film and plate sealer roller or applicator, and spin at 1000RPM for 1 minute.

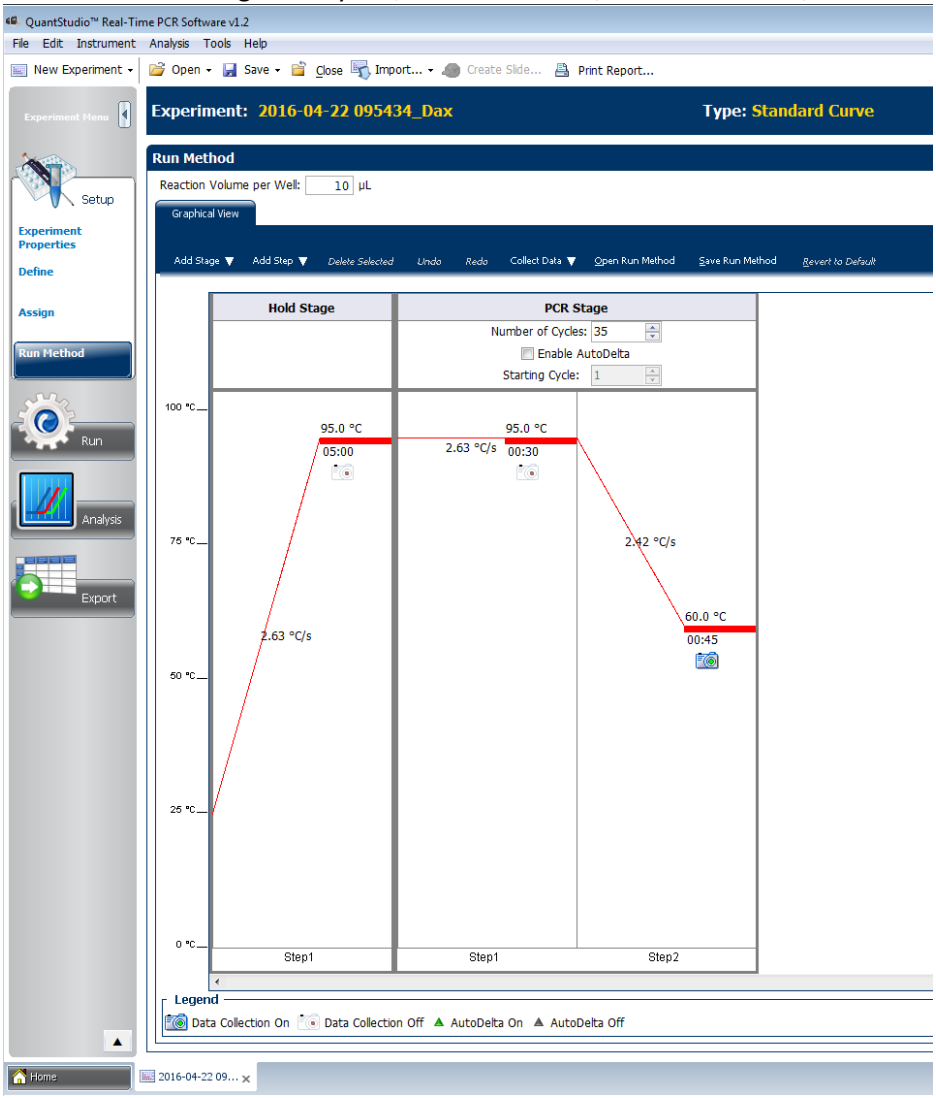
3) Remove adhesive film without disturbing solution at bottom of wells. If you do, spin the plate down again, and verify no mix was lost.
4) Aliquot 4 ul of diluted library (1 ul in 999ul of H20 + 0.05X Tween-20) or 4ul of standard into PCR well. You may touch the side of the well with the pipette tip. Each standard uses 3 wells, each library dilution requires 2 wells. Change pipette tips between individual library dilutions, and between standards. Seal plate with MicroAmp Adhesive film with plate sealer roller/wedge. Run around the plate margins with the end of the plastic wedge to seal the cover tightly (otherwise evaporation can occur).
5) Spin the plate at 1000RPM for 1 minute.
6) Wipe the optical surface of the plate with a Kimwipe. Plates may be kept in the fridge for approx. 4 hours before loading, but spin again before loading.

Loading the QPCR Plate in the RT-qPCR Machine:

1) Turn on the Applied Biosystems ABI ViiA7 RT-qPCR machine.
2) Ensure that the correct thermal block "Fast 96-Well (0.1mL)" or 384 well block, and PCR plate rack is installed. Blocks are stored in the drawers below the instrument.
3) Open the QuantStudio Real-time PCR software which must connect to the instrument.
4) Load the plate in the ViiA7 RT-qPCR machine.
5) Open TGL's template file (2016-04-22 095434_Dax_plate_cyling_template.eds) which contains pre-set run conditions for cycling; verify settings below are loaded.



6. Under the run method tab, verify the following settings (see image below)
    a. Reaction volume =10 ul
    b. Hold stage= 95C for 5 minutes, data collection off
    c. PCR Stage= 35 cycles, 95C-30 seconds, 60C- 45 seconds, data collection on

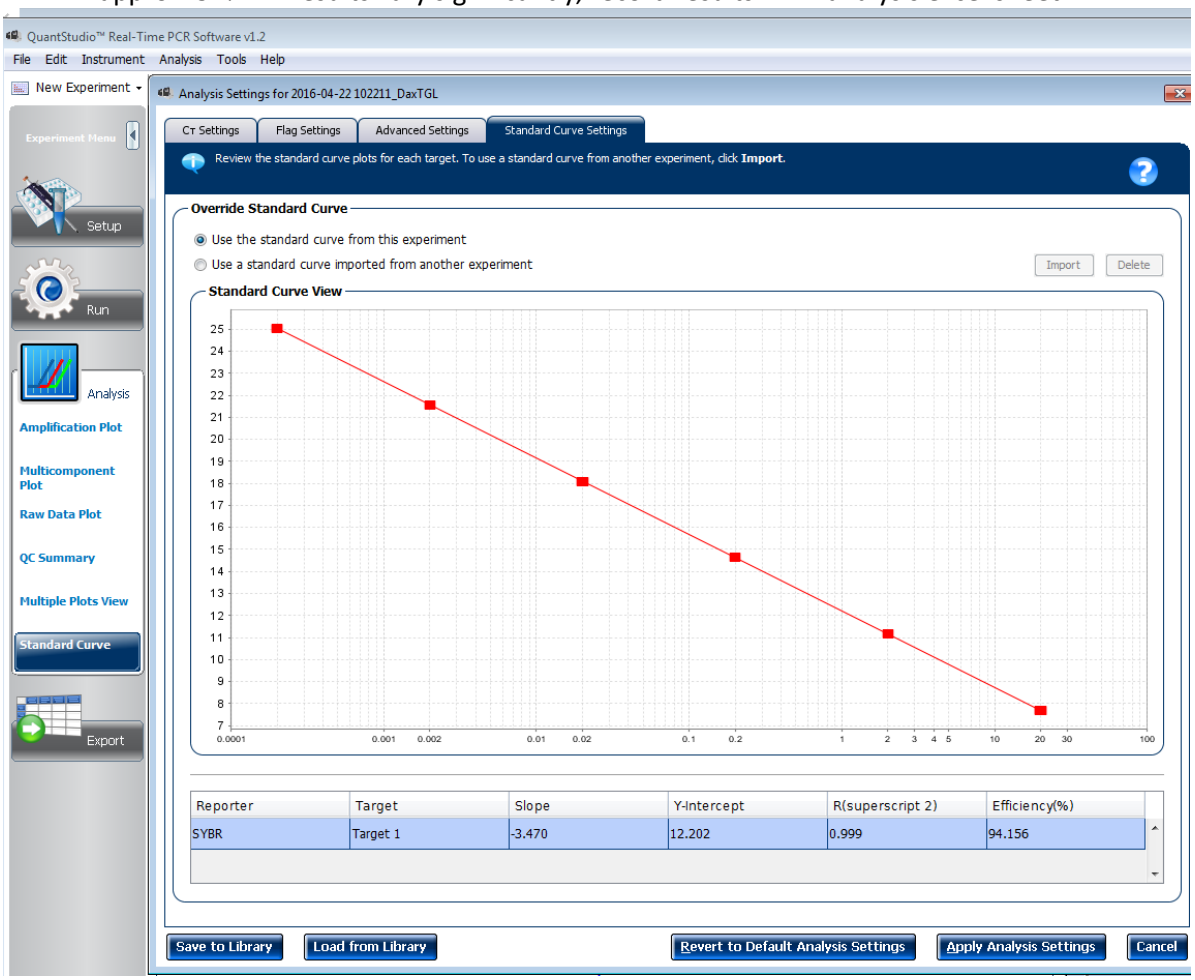


7. Targets may be defined under the “define tab” on the left margin of the window. Individual samples may be indicated here and will export when the run is complete. Ensure all used wells and indicated “targets” use “SYBR” reporter, if SYBR is not indicated sample will not be detected.

8. Assign samples and standards to PCR plate wells. The instrument will not detect/measure a well if it is not indicated with template or standard. Save a new file on the desktop in TGL folder.
9. Ensure instrument is connected (Run status), and open PCR door by pressing open on ViiA7 touch screen to extend loading rack. Place spun, Kimwiped PCR plate on rack; align plate to the A1 rack placeholder, and close door.
10. Start run (approximately 1.5 hours).

Post Run Analysis and Data Export to Excel:

6. Click on "Analysis" menu at top of screen and a new window will open. Click on "Standard curve settings" and verify slope of the line (approx. -3.470, and R2 value approx. 0.999, and efficiency approx. 94%. If results vary significantly, record results in RT analysis excel sheet.



7. Email your results (ex: 2016-04-22 102211_DaxTGL.eds) from the instrument PC and store on TGL's R drive R:\TGL\DNA_QC\RT-qPCR. Do not use USB keys!
8. At your desk, open the run "file_name.eds". Click on the "Export" tab in the left margin. Verify export file location) and export "Results".
9. Use the "SOP_RT-qPCR_Analysis_in_Excel_Template.xlsx" as an example on how to format your excel sheet.
    - Keep a tab "0" with the original export data
    - In the run information tab, under notes, record the master mix, standards
    - In the calculations tab, take the average of 6 sample wells. Cq values should be no more than 0.5 Cq apart. Up to two data points may be removed from the average if they fall outside of 0.5 Cq from the average.
    - Summarize the analysis in the "Summary tab". Include the BA (tapestation) file name, RT-qPCR file name, and tech signatures. You will need results from the TapeStation to size correct your library using the equation:

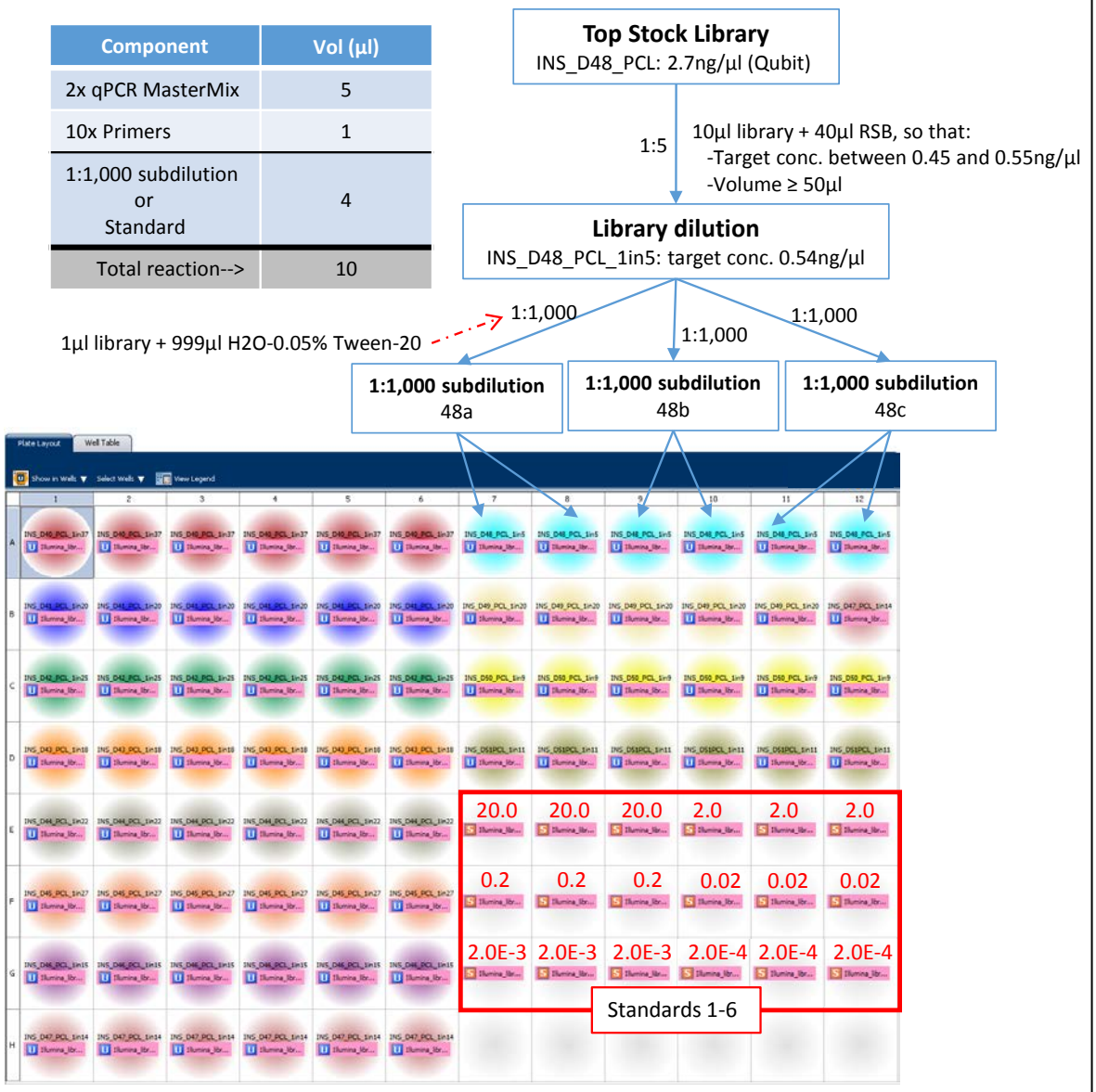
      Final size corrected library concentration (nM)
      =pM*(452/(average library size from 100-1000 region))

    - Record bad results in the summary and note if variability was found! This will be useful if an issue arises with the instrument or reagents that may not currently be apparent.

10. Transfer the summary results to respective sample sheets.

5.0 Appendix A: RT-qPCR Dilution Diagram

Component	Vol (µl)
2x qPCR MasterMix	5
10x Primers	1
1:1,000 subdilution or Standard	4
Total reaction-->	10



6.0 Revision History

Version Number	Date *(yyyy-mm-dd)*	History of change
1.0	2017-05-24	First draft by Dax Torti
1.0.1	2017-05-29	Edited by Alberto Leon
1.0.2	2017-05-29	Kayla Marsh reviewed, in production