

Automated qPCR Reaction Setup	
Document #: LIBPR.0125	Supersedes: Version 5
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## Automated qPCR Reaction Setup

### I. Purpose

This method is used for diluting constructed libraries and setting up qPCR reactions in plate format using the NIMBUS robot, and to measure concentrations of constructed Illumina libraries using the Bio-Rad CFX384 qPCR instrument.

**THIS SOP IS INTENDED FOR USE ON THE NIMBUS and JANUS G3.**

### II. Scope

All procedures are applicable to the Illumina Sequencing group and the Library Core group.

### III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a Quality Systems associate.

### IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Systems to audit this procedure for compliance and maintain control of this procedure.

### V. References

Document Title	Document Number
KAPA Library Quantification Kit, Technical Data Sheet KR0405	Version 6.14

### VI. Related Documents

Document Title	Document Number
CFX96 and CFX384 Real-Time PCR Detection Systems	IM.0274
96-well PCR Free Library Construction on NIMBUS for Illumina Sequencing	LIBPR.0133

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Document Title	Document Number
JANUS G3 Dispense qPCR Brew for NIMBUS	LIBPR.0147

## VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the safety data sheet (SDS) for additional information.

## VIII. Materials and Equipment

Name	Supplier	Number	Model or Catalogue #
Hard-Shell Thin-Wall 384-Well Skirted PCR Plates.	Bio-Rad	HSP3865	✓
Microseal 'B' Adhesive Seals (for qPCR)	Bio-Rad	MSB1001	✓
Edge Biosystems clear plastic sealers	Applied Biosystems	48461	✓
KAPA Library Quantification kit – Illumina/Universal	Roche Diagnostics	07960140001	✓
KAPA Library Quantification DNA Standards Only – Illumina: DNA Standards 1-6	Roche Diagnostics	07960387001	✓
E&K Reservoir Low Profile	E&K Scientific	EK-2036	✓
ABGene 1000 96-Well PCR Plate	Fisher	AB1000	✓
CFX384 TOUCH RT PCR SYSTEM	Bio-Rad	C1000 Touch	✓
RoboRack 25ul sterile Barrier Tips	Perkin Elmer	6000689	✓
RoboRack 175ul sterile Barrier Tips	Perkin Elmer	6000685	✓
Qiagen EB buffer	Qiagen		
Tween 20, 10%, for easy pipetting, 1L	Bio-Rad	161-0781	✓
Eppendorf Safe-Lock Tubes, 2mL, PCR clean	Eppendorf	022363344	✓
50mL Falcon Tubes	VWR	CATX21008-940	✓
Ice Bucket	VWR	35751-205	✓
Black ink permanent Marker pen	VWR	52877-310	✓

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Wet Ice	In-house	N/A	N/A	N/A
24-position tube rack for Biomek FX	Beckman Coulter	373661		✓
Beckman 1.5mL/2mL tube adapter	Beckman Coulter	373696		✓
Pipetman® Classic Starter Kit (P20, P200, P1000)	Mandel	GF-F167300		✓
Pipetman® Classic Micro Starter Kit (P2, P10, P100)	Mandel	GF-F167500		✓
Diamond Filter Tips 10 µL	Mandel	GF-F171203		✓
Diamond Filter Tips 30 µL	Mandel	GF-F171303		✓
Diamond Filter Tips 200 µL	Mandel	GF-F171503		✓
P200 Filter tips	Rainin	RT-200F		✓
Sorvall Legend Centrifuge	Mandel	RT	✓	
Sorvall RC3BP Centrifuge	Mandel	RC 3BP	✓	
Eppendorf Centrifuge	Eppendorf	5810R	✓	
Micro centrifuge	VWR	CABK368826	✓	

#### **KAPA qPCR Primer Sequences:**

Primer P1: 5'-AAT GAT ACG GCG ACC ACC GA-3'

Primer P2: 5'-CAA GCA GAA GAC GGC ATA CGA-3'

#### **Preparation of EB/0.05% Tween buffer**

Prepare a solution of 0.05% Tween 20 in EB buffer by adding 75µL of Tween 20 (10%) solution to 15mL of Qiagen EB buffer. Mix well. For larger volumes, use 250µL of Tween 20 (10%) to 49.75mL of Qiagen EB Buffer.

#### **Preparation of KAPA qPCR Master Mix / Primer Premix Brew aliquots:**

For each new KAPA qPCR Master Mix received, thaw the 5mL bottle of KAPA SYBR FAST qPCR Master Mix (2X) and add 1mL of Illumina GA Primer Premix (10X), received separately with the DNA Quantification Standards Kit. Mix well by inversions.

Prepare 500µL aliquots of primer/KAPA qPCR Master Mix and store frozen at -20°C or prepare larger 2 mL aliquots if necessary. Individual aliquots may be freeze-thawed for a number of qPCR assays.

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## **IX. Procedure**

**Note: The samples are diluted 1/2500X on a pre-PCR NIMBUS on the 5<sup>th</sup> floor or the Nimbus in the Library Construction Room on the 6<sup>th</sup> floor. All procedures related to Illumina library qPCR set-up are to be followed in a 6<sup>th</sup> floor (post-PCR product area) clean workspace, using the post-PCR NIMBUS.**

### **1. NIMBUS Set up of the 1/2500 dilution of the Illumina Library Sample Plate for qPCR**

Note: The user must choose from 3 radio buttons for this step on the NIMBUS.

1.1. QBC001 Addition as qPCR Control. Retrieve the QBC001 control and allow it equilibrate to room temperature. The average bp should be set to 450bp for the QBC control in LIMS. Vortex and quick spin. Replace designated well in the ligation tray with 10uL aliquot of QCB001 prior to the serial dilution.

1.2. Refer to LIBPR.0133 96-well PCR Free Library Construction on NIMBUS for Illumina Sequencing.

### **2. How to create the Bio-Rad qPCR Machine Sample Sheet in LIMS**

Note: See Appendix A if more than one TRA is submitted. APC will usually perform this.

2.1. Login onto LIMS using your username and password.

2.2. Scan the TRA of the submitted plate, and the barcode EQU2740 for the CFX384 qPCR machine.

2.3. Select "By Column-Nimbus" for qPCR set up as shown below.

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Printable Image  
Printable Page (extracts only this table)

		Select All Wells		Clear All Wells													
		1	2	3	4	5	6	7	8	9	10	11	12				
A												B58147 1477815	B58155 1477816				
B												B58148 1477817	B58156 1477818				
C												B58149 1477819	B58157 1477820				
D												B58150 1477821	B58158 1477822				
E												B58151 1477823	B58159 1477824				
F												B58152 1477825	B58160 1477826				
G												B58153 1477827	B58161 1477828				
H												B58154 1477829	B58162 1477830				

Create QPCR Run By Column - Nimbus

**Figure 1: Screenshot of dropdown menu for creating qPCR run**

- 2.4. Select “Create qPCR Run”, LIMS will generate a file name ending with “.plrn”; note the file name for step 7. The file is stored in:

Lab instruments on

‘isaac’\QPCR\_Run\sample\_sheets\run#.YearMonthDayTime(HrMinSec).plrn

Example: 73058.20150331074312.plrn

### 3. Dispense KAPA qPCR Master Mix Brew for the Samples and Standards/Water Control

- 3.1. Thaw an aliquot of KAPA SYBR FAST qPCR Master Mix (2X) brew (**containing primers**) for 30 minutes in the dark at room temperature. Quick spin and keep at room temperature until next step.
- 3.2. Use the JANUS G3 to dispense KAPA qPCR Master Mix into two temporary AB1000 plates. One for the samples and the other for standards/water control.
- 3.3. Keep any leftover qPCR brew mix in the 2mL Eppendorf tube.
- 3.4. Obtain the sample sheet from your APC or prepare one for the JANUS G3 to dispense brew mix using the same well map as per your 1/2500 diluted sample library plate “QPCR1”. Use the following spreadsheet as a template:

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R:\Library Core\JANUS G3 6th floor PPBC\Normalization, qPCR, Rearray, pooling templates\LibCore - qPCR Brew Dispensing Calculator.xls.

- 3.4.1. Brew for maximum 96 samples can be dispensed, not counting standards and water control. Copy and then paste the well layout from your 1/2500 QPCR tray into the 'Setup' sheet. Do not include the standards or water control in the spreadsheet. Save the 'CSV' sheet as a CSV file and export it to folder:

geneexplab on "\\phage.bcgsc.ca\home\geneexplab\Library Core\JANUS G3 6<sup>th</sup> floor PPBC\Production csv files JANUS G3

- 3.5. Determine how much qPCR brew is needed:

- a) Calculating based on the following equation:

**Total volume of qPCR Brew needed:**

$(22.86\mu\text{L} \times \# \text{ of samples}) + (20\mu\text{L} \times 7 \text{ standards and water control}) + 100\mu\text{L}$   
dead volume

$(22.86\mu\text{L} \times \# \text{ of samples}) + 240\mu\text{L}$

i.e.  $22.86\mu\text{L} \times 92 \text{ samples} + 240\mu\text{L} = 2343.12\mu\text{L}$

- b) Alternatively, proceed to step 3.6 and the JANUS program will calculate the volume of qPCR brew required based on your .CSV file.

- 3.6. JANUS G3 setup the qPCR Brew Dispensing.

- Check there is enough ddH<sub>2</sub>O in water carboy.
- Log onto the JANUS G3 and open **JANUS Application Assistant**. Select **LibCore – G3 qPCR Brew Dispensing**. Click **4.Run** then **Start**.
- Flush/prime the fluid system by checking the *check box* if bubbles are visible in the liquid lines. Then click **Continue**. This takes approximately 3.5 minutes.
- Specify first full tip column of 25uL tip box. Then click **Continue**.
- Click **Browse** to load the dispense brew file. Here, log into PHAGE.bcgsc.ca\geneexplab\. Select and open the appropriate qPCR Brew

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Dispense csv file in \Library Core\JANUS G3 6<sup>th</sup> floor PPBC\Production csv files JANUS G3\. Click **Continue**.

- f) Confirm the number of samples at software prompt.
- g) Fill 2mL Eppendorf tube(s) with appropriate brew volume and load the tube(s) into rack position starting at **A1**.
- h) Load the required labware as indicated in pop-up picture and click **OK**.
- i) Click **OK** after ensuring Varispan waste tip box is empty.
- j) Click **OK** to begin.
- k) After brew dispenses, cover the plates and quick spin at room temperature. Keep in the dark until ready for next setup on the NIMBUS

#### 4. Prepare 384-well qPCR plate on NIMBUS

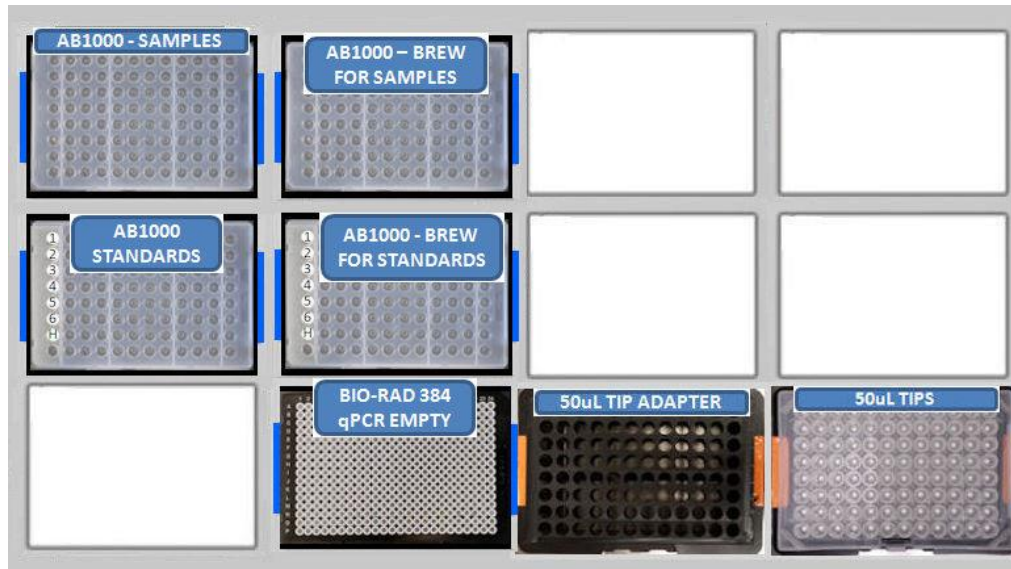
- 4.1. This step will combine the 1/2500 diluted samples and the standards plate with the two qPCR brew plates. You will be prompted to quick spin plates (at max speed) before the NIMBUS transfers the brew plus template into the 384 well qPCR plate.
- 4.2. **Please note that 80µL of standards 1-6 are dispensed in column 1 (wells A1- F1, respectively) for NIMBUS qPCR set up.**
- 4.3. Add 80µL of fresh Ultrapure Water to well G1 on the standards plate prior to each run. Each pre-aliquoted standards plate can be used up to 5 times.
- 4.4. Log into the following program. Keep the plates covered until the program prompts you to remove the covers.

Hamilton Run Control>File>Open>Production> LIBPR qPCR Reaction Setup>LIBPR qPCR Reaction Setup.med

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**Figure 2: Initial deck layout on Hamilton NIMBUS**

- 4.5. After the program has been completed, cover the standards and 1/2500 dilution plate with foil seal and store at -20°C.
- 4.6. Cover the Bio-Rad 384-well plate with the BioRad tape cover and then quick spin the plate at max speed in an Eppendorf 5810R centrifuge at room temperature for 1 minute.

## 5. Enter Sample Information and Start Run

- 5.1. Login onto the CFX384 qPCR workstation computer using your username and password.
- 5.2. Turn on the CFX384 qPCR machine.
- 5.3. Start the qPCR software, “Bio-Rad CFX Manager” after the instrument is ready for use.
- 5.4. Select “File” and “Open”, and select “LIMS File”, which defaults to the “sample sheets” folder. Select the appropriate file based on your created LIMS sample sheet.
- 5.5. Toggle to the “Plate” tab, to check the layout of the Samples, Standards and No-Template Controls.



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- 5.6. Ensure that the correct LIMS File is selected and opened.
- 5.7. Once all sample information is confirmed, go the **Start Run** tab.
- 5.8. Select “Open Lid” and place 384-well optical plate inside the qPCR machine, with well A1 at the top left.
- 5.9. Ensure that the lid is unobstructed, and select “Close Lid”.
- 5.10. Click on the **Start Run** button. You will be prompted for the run file name and destination. Confirm the appropriate file name and final destination is present then save the run.
- 5.11. The qPCR run will take approximately 1.5 hours to complete.

## 6. LIMS Protocol

- 6.1. Log on to LIMS using your username and password.
- 6.2. Scan the TRA of the submitted plate into the scan field, and select “Scan”.
- 6.3. Under the Lab Protocol Tracking field, select “qPCR Quant” from the protocol list and select “Continue with Lab Protocol”.
- 6.4. Enter the qPCR equipment number in the “Scan qPCR equipment” field and select “Completed Step”.
- 6.5. Scan Nimbus used for sample dilution in the Equ field and select “Completed Step”
- 6.6. Scan in the solution number (SOL) of the qPCR Kit and select “Completed Step”
- 6.7. Scan in the Equ number of the JANUS G3 which is necessary to indicate automated brew dispense.

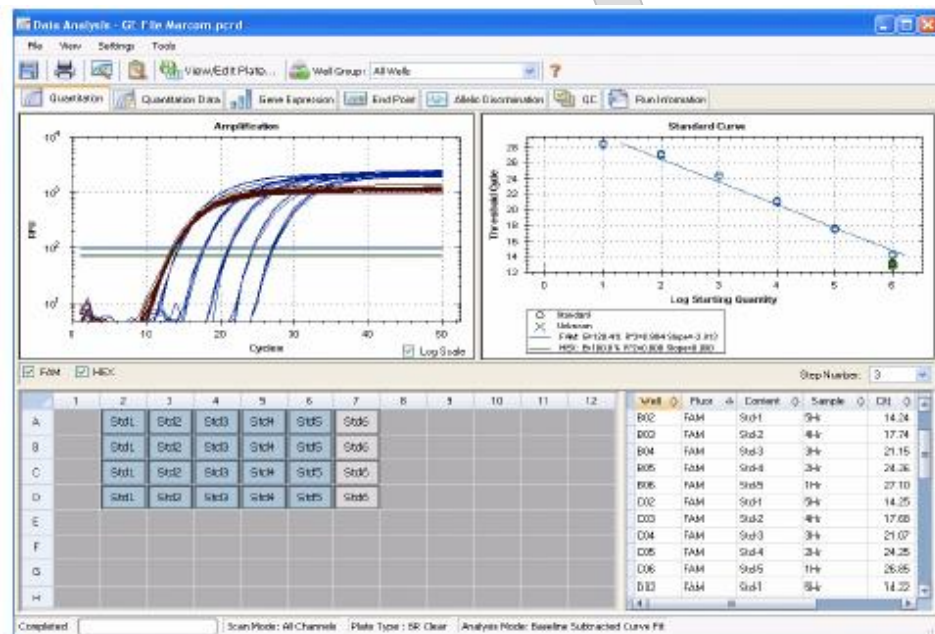
## 7. Review Analyzed Data

- 7.1. Select the **Quantitation** tab in the Data Analysis window for an overview of the qPCR run results (Figure 9). There are 4 windows displayed under this tab: Amplification, Standard Curve, Plate Layout, and Quantitation Data. To view the amplification plots of

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select wells, highlight the specific wells in the Plate Layout window, and the corresponding amplification curves will appear darker in the Amplification window. The Standard Curve shows the plot of Threshold cycle vs. Log Starting Quantity. The plot of the DNA standards (represented by open circles) should be linear. Samples are represented by cross-hatch lines.



**Figure 3: Quantitation Tab in the Data Analysis Window**

- 7.2. To view the analyzed data, open the **Quantification Data** tab. The spreadsheet contains the qPCR data for each Library sample (Unknown) including the individual well data (Cq and Starting Quantity) as well as the data for the triplicate set (Cq Mean, Cq Standard Deviation, and Starting Quantity [SQ] Mean).
- 7.3. Log onto LIMS using your user name and password.
- 7.4. Go to the Summaries drop down menu and select QPCR Run Summary. Select the correct TRA and click on “Display QPCR Run Results”. Select “Replicate” to organize the data. The Cq Std Mean for each triplicate is displayed. If the Cq Std Mean is greater than 0.2, look for outliers in the triplicate quants and select them for removal from the calculation. In the event where the Cq values of a triplicate are evenly distributed, the values may remain for calculation. If Cq Std Mean values are >0.3 and there isn’t an

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obvious outlier, the APC may decide to remove that sample entirely and rerun it as such a large range could cause cluster density issues if the incorrect outlier is removed. Remove outlier data points from the data set with the APC, and review before selecting “Remove QPCR well”.

- 7.5. After the outliers have been removed, toggle to select all the wells.
- 7.6. After the APC has reviewed the results, select “Confirm QPCR Results”.

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## Appendix A

In order to combine multiple TRAs, if more than one Library plate is being submitted, the following method should be applied:

1. In the “Scan” field, scan in the TRAs of the submitted library plates.
2. In the “Actions” tab, enter in the amount to be aliquoted (2uL), the container type should be entered as a “96-well ABGene”, scroll down to “Include/Exclude” and select "Include", Select “Aliquot” and “Go”.
3. The next screen in LIMS will allow you to reassign the libraries to different wells, if needed, otherwise select the “TRAY” tab and choose “Aliquot to 96-well ABGene”.

current plates: 773500,773501,785005,785006,785007,785008

Aliquot to 96-well ABGene

Exclude:

Source Tube	Well	By Row	By Column	By Thunderstorm Row Layout	By Thunderstorm Column Layout
Pla773500: P00030-1.	A03	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Pla773501: P00029-1.	B03	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Pla785005: P00037-1.	A04	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Pla785006: P00036-1.	B04	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Pla785007: P00040-1.	C04	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Pla785008: P00039-1.	D04	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>

ReArray

Tray

This will track samples individually upon the target plate

Adding to existing tray:

Aliquot to 96-well ABGene

4. The next screen will show a 96 well plate lay out, select “Confirm Aliquot to 96-well ABGene as Tray”.
5. A new TRA will be created, use this new TRA when creating your sample sheet in step 4 in LIMS to get the “plrn.” run file.