MCCRD-SOP0007: Illumina Sequencing Library Quantitation using Droplet Digital PCR (ddPCR) Protocol

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APPROVALS

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VERSION INFORMATION

1. Change History

Revision	Description
	Internal SOP used by PDMR In Vitro Laboratory
8/27/2018	Standardize SOP for posting to PDMR internal site for use by designated NCI intramural laboratories
8/13/2019	Method of Deviation document added.

2. Reference Documentation

Document Number	Title
6407	Droplet Digital PCR Applications guide, BioRad bulletin http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf
10031906	QX200™ Droplet Reader and QuantaSoft™ Software Instruction Manual http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10031906.pdf
10023997	PX1™ PCR Plate Sealer Instruction Manual http://www.bio-rad.com/webroot/web/pdf/lsr/literature/bulletin_10023997.pdf
10021377	C1000 Touch™ Thermal Cycler Instruction Manual http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10021377.pdf
6402	ddPCR™ Library Quantification Kit for Illumina TruSeq Flier http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6402.pdf

Document Number	Title
10043138	Automated Droplet Generator Instruction Manual http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10043138.pdf
10031986	ddPCR™ Library Quantification Kit for Illumina TruSeq Product Insert http://www.biorad.com/webroot/web/pdf/lsr/literature/10031986.pdf

1.0 PURPOSE/SCOPE

This Standard Operating Procedure (SOP) describes the steps necessary to use digital droplet PCR (ddPCR) to quantitate libraries and/or pools of libraries for sequencing of Patient Derived Xenograft samples on the Illumina HiSeq 2500. This protocol begins with sample dilution, PCR plate set-up and processing, and concludes with data analysis. This SOP is intended for processing up to 24 samples per instrument run. **This SOP is for research purposes only and no clinical samples will be processed using this SOP.**

ddPCR is used to reproducibly determine the concentrations of library samples and library pools to achieve accurate cluster density for sequencing on the Illumina HiSeq 2500. The ddPCR Library Quantification Kit for Illumina TruSeq is used on the Bio-Rad QX200 AutoDG ddPCR systems and allows absolute quantification of both the P5 and P7 adapter sequences. The multiplexed, probe-based method also provides library quality information.

2.0 SAFETY

2.1 Lab coats, safety glasses, gloves must be worn at all times when handling hazardous or sensitive equipment, samples, reagents, and materials. These safety measures must also be followed when in close proximity to those who are working with these items.

3.0 REAGENTS & EQUIPMENT

3.1 Reagents

Description	Model#	Vendor
ddPCR Library Quantification Kit for Illumina TruSeq	1863040	Bio-Rad
PCR Plate Heat Seal, foil, pierceable	1814040	Bio-Rad
DG32™ Automated Droplet Generator Cartridges	1864108	Bio-Rad
Nuclease-free Molecular Biology Grade Water (not DEPC-Treated)	AM9937	Life Technologies
1.5mL Lo-Bind Tubes	022431021	Eppendorf
200-1000 µL Aerosol Barrier Pipette Tips	30389213	Rainin
20-200 µL Aerosol Barrier Pipette Tips	30389240	Rainin
0.2-20 µL Aerosol Barrier Pipette Tips	30389226	Rainin
ddPCR™ Droplet Reader Oil	1863004	Bio-Rad
Automated Droplet Generation Oil for Probes	1864110	Bio-Rad
Pipet Tip Waste Bins for the AutoDG™ System	1864125	Bio-Rad
Pipet Tips for the AutoDG™ System	1864121	Bio-Rad

Description	Model#	Vendor
Eppendorf™ 96-Well twin.tec™ PCR Plates	E951020346	Fisher Scientific
Buffer EB	19086	Qiagen
Disposable Pipetting Reservoirs	89094-662	VWR
96 well 0.8mL Storage Plates (MIDIPlates)	AB-0859	Fisher Scientific

3.2 Equipment

Description	Model #	Vendor
Single Channel Pipettes (p2, 20, 200, 1000)	Variable	Rainin
Multi-Channel Pipettes (LTS 2, 20,200)	Variable	Rainin
Vortex	58816-121	VWR
MiniFuge	93000-196	VWR
PX1 PCR Plate Sealer	181-4000	Bio-Rad
QX200 AutoDG ddPCR system including laptop and droplet reader	186-4100	Bio-Rad
Plate Centrifuge	022628203	Eppendorf
C1000 Touch thermal cycler	1851197	Bio-Rad
PCR Workstation	AC648LFUVC	AirClean Systems

4.0 AUTOMATION METHODS

The AutoDG droplet generator is pre-programmed. The user must load the instrument with the necessary consumables, select the columns to be processed, and the type of probes that are to be used.

5.0 ASSAY GUIDELINES

5.1 Once thawed, the library quantification kit can be stored at 4oC for 2 weeks. Repeated freeze thawing is not recommended.

5.2 Thaw reagents at room temperature and then store on ice during use. After thawing, vortex and centrifuge reagents prior to pipetting.

5.3 For accurate quantitation, the ddPCR concentrations should be between 100-5000 copies/ul.

5.4 Assaying 2 dilution points in duplicate ensures that a reliable concentration measurement is obtained. In most cases 10^{-6} and 10^{-7} dilutions of libraries that were originally diluted to a target concentration of 5nM will yield data within the recommended range.

6.0 PROCEDURE

6.1 Program the C1000 Thermal Cycler

6.1.1 Program the C1000 thermal cycler with the cycler protocol outlined below.

Temperature	Time (mm:ss)	# Cycles
95ºC	HOLD	1
95ºC	10:00	1
94ºC	00:30	40
61ºC	02:00	2ºC/sec ramp rate
98	10:00	1
12ºC	HOLD	1

*Use a heated lid set to 105ºC and set the sample volume to 40 uL.

Note: Use the above thermocycler protocol instead of the protocol outlined in the assay documentation. This protocol has been optimized to maximize the fluorescent intensity between positive and negative droplets.

6.2 Preparation of ddPCR master mix

6.2.1 In a clean room or PCR box, prepare the ddPCR master mix as outlined in the table below.

Reagent	Volume/reaction, uL (including 5% overage)	Volume/96 well plate (including 5% overage)	Final concentration
2x ddPCR Supermix for Probes (no dUTP)	11.55	1108.8	1x
20x ddPCR library quantification assay	1.155	110.88	1x
Nuclease-free water	5.775	554.4	
Total volume to add per well	17.6 uL (+5% overage)	17.6 uL (+5% overage)	1x

Note: The AutoDG requires complete columns of samples. If necessary, fill remaining wells in the column with NTC samples (17.6 uL master mix + 4.4 uL nuclease-free water).

6.2.2 Add 17.6 uL master mix into each reaction well of a 96 well twin.tec plate. Seal the plate with adhesive film. Store the plate on ice in the dark until samples are added.

6.3 Create sample dilutions

6.3.1 Using the concentration information from the quality control TapeStation run, dilute the libraries or library pools with nuclease-free molecular biology grade water. Dilute samples to a final target concentration of about 5nM. Total dilution volumes should be at least 14uL. This ensures enough volume to use as a working stock for the remainder of the workflow.

6.4 Perform serial dilutions of samples

6.4.1 Create serial dilutions to be used for the ddPCR setup. Use EB buffer to dilute samples in a 96 well MIDI plate. Thoroughly mix samples between each step of the dilution series. Dilute each sample row-wise as outlined in the table below.

Dilution step	Column 1	Column 2	Column 3	Column 4
Sample volume, uL	2 uL working stock	2 uL from column 1	2 uL from column 2	10 uL from column 3
EB buffer volume, uL	198 uL	198 uL	198 uL	90 uL
Dilution	100x	100x	100x	10x
Final dilution	10^{-2}	10^{-4}	10^{-6}	10^{-7}

6.5 Combine diluted samples and PCR master mix to create reaction plate

6.5.1 Add 4.4 uL diluted sample to each reaction well containing master mix according to the plate layout below. The total volume in each well should be 22 uL.

Sample #1 10^6	Sample #1 10^6	Sample #1 10^7	Sample #1 10^7	Sample #9 10^6	Sample #9 10^6	Sample #9 10^7	Sample #9 10^7	Sample #17 10^6	Sample #17 10^6	Sample #17 10^7	Sample #17 10^7
Sample #2 10^6	Sample #2 10^6	Sample #2 10^7	Sample #2 10^7	Sample #10 10^6	Sample #10 10^6	Sample #10 10^7	Sample #10 10^7	Sample #18 10^6	Sample #18 10^6	Sample #18 10^7	Sample #18 10^7
Sample #3 10^6	Sample #3 10^6	Sample #3 10^7	Sample #3 10^7	Sample #11 10^6	Sample #11 10^6	Sample #11 10^7	Sample #11 10^7	Sample #19 10^6	Sample #19 10^6	Sample #19 10^7	Sample #19 10^7
Sample #4 10^6	Sample #4 10^6	Sample #4 10^7	Sample #4 10^7	Sample #12 10^6	Sample #12 10^6	Sample #12 10^7	Sample #12 10^7	Sample #20 10^6	Sample #20 10^6	Sample #20 10^7	Sample #20 10^7
Sample #5 10^6	Sample #5 10^6	Sample #5 10^7	Sample #5 10^7	Sample #13 10^6	Sample #13 10^6	Sample #13 10^7	Sample #13 10^7	Sample #21 10^6	Sample #21 10^6	Sample #21 10^7	Sample #21 10^7
Sample #6 10^6	Sample #6 10^6	Sample #6 10^7	Sample #6 10^7	Sample #14 10^6	Sample #14 10^6	Sample #14 10^7	Sample #14 10^7	Sample #22 10^6	Sample #22 10^6	Sample #22 10^7	Sample #22 10^7
Sample #7 10^6	Sample #7 10^6	Sample #7 10^7	Sample #7 10^7	Sample #15 10^6	Sample #15 10^6	Sample #15 10^7	Sample #15 10^7	Sample #23 10^6	Sample #23 10^6	Sample #23 10^7	Sample #23 10^7
Sample #8 10^6	Sample #8 10^6	Sample #8 10^7	Sample #8 10^7	Sample #16 10^6	Sample #16 10^6	Sample #16 10^7	Sample #16 10^7	Sample #24 10^6	Sample #24 10^6	Sample #24 10^7	Sample #24 10^7

6.5.2 Seal plate with an optical seal and vortex thoroughly. Quick spin the plate. Inspect the plate to ensure there are no bubbles in the wells.

Note: Plate should be stored on ice away from light if it is not being immediately loaded onto the AutoDG for droplet generation.

6.6 Generate droplets

6.6.1 Load consumables on AutoDG instrument. For a run with 96 reactions:

6.6.1.1 Place 3 unused droplet generation cartridges on the back row

6.6.1.2 Remove lids and place 2 full boxes of droplet generation tips on the middle row

6.6.1.3 Place an empty waste bin on the middle row to the left

6.6.1.4 Place the 96 well cold chill block to the front right. Place an unused twin-tec 96 well plate in the chill block. Droplets will be deposited into this plate after they are formed.

6.6.1.5 Ensure that there is enough droplet generation oil to complete the run

6.6.2 Configure the AutoDG using the touchscreen on the instrument

6.6.2.1 ‘Probes’ should be selected as oil type

6.6.2.2 Configure the sample plate by selecting the columns containing samples

6.6.2.3 Place the reaction plate on the deck to the front left. Carefully remove the adhesive film.

6.6.2.4 Lower the lid of the AutoDG. Push start to generate droplets.

Note: Droplet generation will take approximately 40 minutes for a full 96 well plate of samples. Final volume in each reaction well is approximately 40 uL.

6.7 Seal the reaction plate

6.7.1 After droplet generation is complete, place a pierceable foil seal on the 96 well plate containing the droplets.

6.7.2 Remove the reaction plate from the AutoDG and seal the plate on the plate sealer.

6.7.2.1 Seal at 180 ºC for 5 seconds

6.8 Amplification

6.8.1 Place the reaction plate on the thermal cycler and start the program outlined in step 1.

Note: The amplification protocol takes about 2.5 hours.

Note: If droplets are not immediately read plate can be stored for up to 2 days at 4-12 ºC away from light.

6.9 Configure the plate template

6.9.1 Open the Quantasoft software and load the associated template file.

6.9.1.1 ABS should be selected as Experiment Type in the well editor

6.9.1.2 ddPCR Supermix for Probes (no dUTP) should be selected as Supermix Type in the well editor

6.9.1.3 Ch1 Unknown in Target 1 and Ch2 Unknown in Target 2 should be selected in the well editor

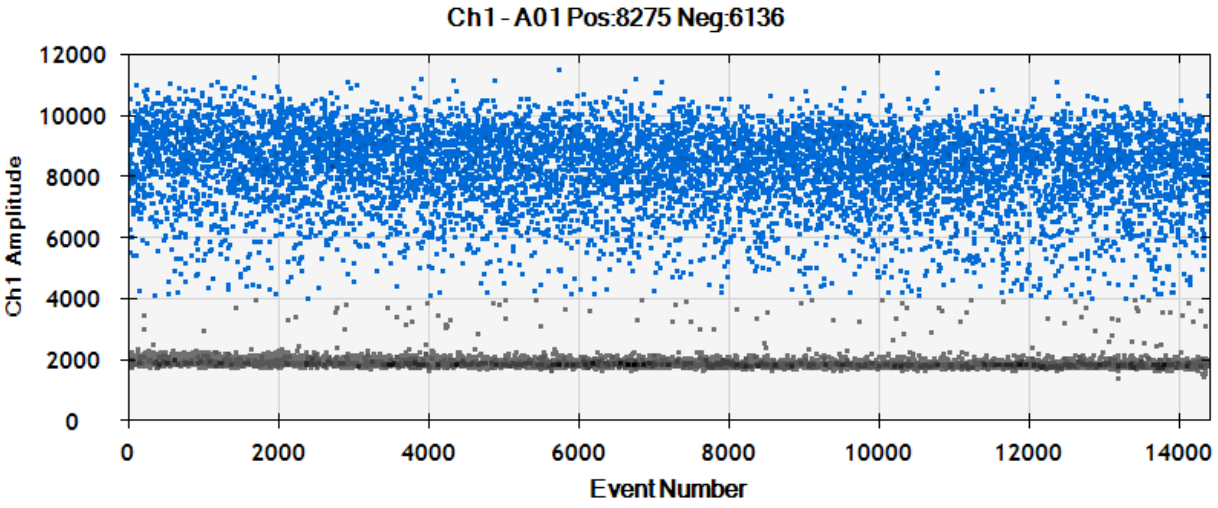
6.9.1.4 Load the reaction plate onto the droplet reader.

6.9.1.5 In the software, click Run and select the FAM/HEX dye set.

Note: A complete plate of 96 reactions will take approximately 2 hours to read.

6.10 Data analysis

6.10.1 Click Analyze in the Quantasoft software. Select the dilutions corresponding to a sample in the well selector under analyze. Examine the automatic thresholding applied to the amplitude data. It may be necessary to manually set the threshold between the positive and negative droplets using the amplitude and/or histogram plots. See below for an example of thresholding. Repeat for each dilution of each sample.



6.10.2 Assess data quality

6.10.2.1 Concentration data for 10^6 dilutions should be about 10 times concentration data for 10^7 dilutions.

6.10.2.2 Replicate data should be similar.

6.10.2.3 Total number of droplets per reaction should be 12000-20000.

6.10.2.3.1 Concentration data for Ch1 and Ch2 should be similar. If these numbers are not similar it may indicate an issue with library quality. Properly formed library molecules will be positive for both the Ch1 and Ch2 targets.

6.10.2.4 When looking at the 2D plot, ddPCR data can also be used to assess library quality.

6.10.2.4.1 Inserts of varying sizes can be observed along a diagonal path. Larger fragments are nearer the negative droplets.

6.10.2.4.2 Adaptor/adaptor ligations will appear as a population of droplets in the upper right-hand corner.

6.10.2.4.3 See below for an illustration of library quality information that can be determined from a 2D plot.

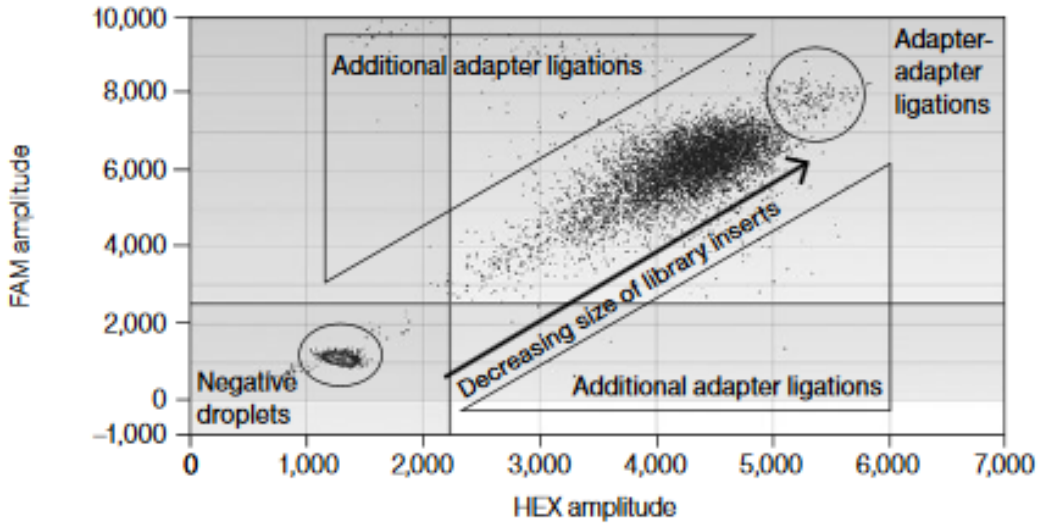


Fig. 1. Illumina TruSeq library quantified using the ddPCR Library Quantification Kit for Illumina TruSeq.

6.10.3 Save the post-analysis plate information in Setup, Plate, Save As.

6.10.4 Export both the merged and single well information and save as a csv file.

6.11 Concentration calculations

6.11.1 For each dilution for each sample, compare the merged Ch1 and Ch2 data and copy and paste the lower concentration into the lowest conc, copies/uL column. Once this concentration information is entered, the nM concentration of the library will be automatically calculated.

6.11.1.1 Calculation example: A library at the 10^6 dilution yielded 2000 copies/uL in the ddPCR mixture. Multiply 2000 by 10^6 and by 5 to account for the dilution in the reaction mixture (2000 x 10^6 x 5 = 10^{10} copies/uL of original stock library). To obtain nM: (10^{10} copies/uL x 10^6 uL)/(6.023 x 10^{23} copies/mole) = 1.66 x 10^{-8} M or 16.6 nM.

6.11.1.2 Use the ddPCR data from the least diluted reactions that yielded concentration information between 100 and 5000 copies per reaction. For most samples this means the 10^6 data will be used. Use these concentrations to further dilute the samples for pooling or clustering.