MCCRD-SOP0008: Denaturing and Diluting Sequencing Library Pools and Cluster Generation Protocol

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TABLE OF CONTENTS

APPROVALS

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VERSION INFORMATION

1. Change History

Revision	Description
	Internal SOP used by PDMR In Vitro Laboratory
12/1/2014	Standardize SOP for posting to PDMR internal site for use by designated NCI intramural laboratories
8/27/2018	Method of Deviation document added.

2. Reference Documentation

Document Number	Title
15050107 v02	HiSeq and GAIIx Systems: Denature and Dilute Libraries Guide http://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/hiseqkits/hiseq-ga-denaturing-diluting-libraries-reference-guide-15050107-02.pdf
15006165 v02	cBot System Guide http://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/cbot/cbot-system-guide-15006165-02.pdf
15050104	HiSeq Cluster Kit v4 Reference Guide

1.0 PURPOSE/SCOPE

This Standard Operating Procedure (SOP) describes the steps necessary to denature, dilute and cluster library samples on the cBot for sequencing of Patient Derived Xenograft samples on the Illumina HiSeq 2500. This protocol begins with sample denaturation and dilution and concludes with loading the cBot for cluster generation. This SOP is intended for processing up to 8 lanes of samples per instrument run. **This SOP is for research purposes only and no clinical samples will be processed using this SOP. Any deviation from this SOP will be noted but will not be formally documented.**

2.0 BACKGROUND

The cBot uses bridge amplification to create hundreds of millions of single-molecule DNA templates simultaneously on a sequencing flowcell. Clonal clusters are created from single DNA templates on a flowcell, preparing them for sequencing on the HiSeq 2500.

3.0 REAGENTS & EQUIPMENT

3.1 Reagents

Description	Model#	Vendor
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
Buffer EB	19086	Qiagen
0.2 mL PCR tube strips	14230210	Fisher Scientific
Sodium hydroxide, 10N volumetric solution	02004123	Fisher Scientific
HiSeq PE Cluster kit v4	401-4001	Illumina
Kimwipes™ Delicate Task Wipers, 1-Ply	06-666	Fisher Scientific
Phix Control kit v3	FC-110-3001	Illumina
Lens paper 6x8 inches	52845-009	VWR
Sterile isopropanol wipes prep pads, 70%	22-363-750	Fisher Scientific
Forceps	609T	Lerloy

3.2 Equipment

Description	Model #	Vendor
Single Channel Pipettes (p2, 20, 200, 1000)	Variable	Rainin
Vortex	58816-121	VWR
MiniFuge	93000-196	VWR
cBot	SY-312-2001	Illumina
Tabletop Centrifuge	5424	Eppendorf

4.0 AUTOMATION METHODS

The cBot is pre-programmed. The user must load the instrument with the necessary consumables and libraries and select the appropriate recipe to be used.

5.0 SAFETY

5.1 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.

6.0 ASSAY GUIDELINES

6.1 Thaw reagents in a water bath at room temperature and then store on ice during preparation.

6.2 Unless other noted, store library pool samples on ice during preparation.

6.3 Freshly prepare 0.1 N NaOH. Prepare at least 1mL of diluted NaOH to prevent small pipetting errors from affecting the final NaOH concentration.

6.4 Library pool samples are loaded for clustering at a concentration of 13.26pM with a 1% PhiX spike-in. This results in a cluster density of approximately 1050 K/mm^2 when sequenced on a HiSeq 2500.

7.0 PROCEDURE

7.1 Dilute pools

7.1.1 Following ddPCR pool quantitation, enter the calculated pool concentration values into the sheet called 'pool quant summary' in the library prep excel file.

7.1.2 Dilute each pool to a target concentration of 1.325 nM using the information in the table in the 'pool quant summary' sheet. Use EB to dilute the pools. Total volume for each diluted pool is 10 uL.

Note: RNA Access pools are sequenced 3 pools to a lane, with lanes run in duplicate. Dilute individual RNA Access pools to 1.325 nM as described above. After diluting individual pools, combine 5 uL of each of the 3 diluted pools together. Use 10 uL of this combined-pool mixture to continue with denaturing and loading for clustering.

7.2 Dilute PhiX

7.2.1 Combine 2 uL of 10nM stock PhiX with 9 uL EB to yield 2nM PhiX.

7.3 Denture pools and PhiX

7.3.1 Combine 10 uL of each diluted pool or PhiX with 10 uL 0.1N NaOH.

7.3.2 Vortex to mix.

7.3.3 Centrifuge at 280 x g for 1 minute.

7.3.4 Incubate for 5 minutes at room temperature to denature the library into single strands.

7.4 Dilute denatured pools

7.4.1 Add 980 uL pre-chilled HT1 to the denatured pools and PhiX to result in 13.25pM pools and 20pM PhiX.

7.4.2 Invert the tube several times to mix and pulse centrifuge.

7.4.3 Place the denatured samples on ice until ready to proceed.

7.5 Spike in PhiX

7.5.1 Remove and discard 10 uL from each denatured 13.25pM pool sample.

7.5.2 Add 10 uL of denatured 20pM PhiX sample to each pool sample for a 1% PhiX spike-in.

7.5.3 Place samples on ice until ready to prepare the 8 tube strip.

7.6 Prepare an 8 tube strip

7.6.1 Label the tubes of an 8 tube strip1-8.

7.6.2 Add 75uL diluted/denatured pool sample into the specified tubes.

Note: Tube 1 of the strip corresponds to lane 1 of the flowcell, tube 2 corresponds to lane 2, etc.

7.6.3 Set the tube strip aside on ice until ready to load onto the cBot.

7.6.4 When prompted by the cBot software, load the 8 tube strip onto the cBot in the TEMPLATES tube strip holder.

7.7 Prepare the cBot

7.7.1 Turn on the cBot.

7.7.2 Ensure that the waste bottle is empty and there is sufficient coolant for the run.

7.7.3 Select User Name. Enter your user name and select Enter.

7.7.4 Select start to proceed to the pre-run wash.

7.7.5 Perform a pre-run wash.

7.7.5.1 Raise the cBot lid and fill the wash reservoir, located behind the thermal stage with approximately 12 mL deionized water.

7.7.5.2 Select the checkbox on the screen to indicate that water is present.

7.7.5.3 Select Wash to start the wash.

7.7.5.4 Repeat the Wash 1x for a total of 2 washes.

7.7.5.5 Blot out excess water remaining in the wash reservoir with a Kimwipe

7.7.5.6 Select the checkbox on the screen to indicate that the wash reservoir is dry.

7.7.5.7 Select Next.

7.8 Setting up and starting the cBot run

7.8.1 Select Experiment Name. Type the experiment name and select Enter.

7.8.2 Select protocol PE_HiSeq_Cluster_Kit_v4_cBot_recipe_v9.0 and select Next.

7.8.3 Load reagents

7.8.3.1 Remove the clear plastic lid from the thawed cBot reagent plate.

7.8.3.2 Gently press down on the tubes in the plate to make sure they are securely seated.

7.8.3.3 Select Scan Reagent ID to activate the barcode scanner.

7.8.3.4 With the barcode label facing the instrument, hold the reagent plate level and move the reagent plate into the light path of the barcode scanner.

7.8.3.4.1 You will hear a beep when the scanner has successfully read the barcode. The reagent ID appears on the screen.

7.8.3.5 Pull the spring-loaded reagent plate lever towards you to release the clamp.

7.8.3.6 Place the regent plate onto the reagent stage, positioned with row 1 facing towards the front of the stage. Make sure the beveled corner of the plate is positioned in the front right corner.

7.8.3.7 Release the reagent plate level to secure the reagent plate.

7.8.3.8 Select the checkbox to indicate the reagent plate is loaded

7.8.3.9 Select Next.

7.8.4 Load the flow cell

7.8.4.1 Lift the flow cell clamp.

7.8.4.2 Wipe the adapter plate on the thermal stage with an isopropanol wipe and allow to dry.

7.8.4.3 Select Scan Flow Cell ID to activate barcode scanner.

7.8.4.4 Hold the flow cell container close to the scanner tray with the barcode positioned toward the instrument.

7.8.4.5 Slowly slide the flow cell container into the light path of the barcode scanner so the entire barcode crosses the scan light at the same time.

7.8.4.5.1 You will hear a beep when the scanner has successfully read the barcode. The flow cell ID appears on the screen.

7.8.4.6 Remove the flow cell from the storage tube using forceps.

7.8.4.7 Holding the flow cell by the edges, rinse the flow cell gently with deionized water and dry thoroughly using a lens cleaning tissue.

7.8.4.8 Place the flow cell on the thermal stage with holes facing upward, lane 1 on the right side, and lane 8 and the barcode on the left side.

Note*:* HiSeq flow cell lane orientation for loading onto the cBot is lane 8-1, left to right.

Note*:* HiSeq flow cells have a mechanically keyed corner, which provides visual orientation for loading the flow cell. Flow cells should be loaded so that the notched corner is in the far right corner of the thermal stage.

7.8.4.9 Select the checkbox to indicate that the flow cell has been loaded.

7.8.4.10 Select Next

7.8.5 Load the manifold

7.8.5.1 Remove the manifold from the packaging and inspect the sippers. Sippers should be straight. Ensure that the black rubber gaskets are evenly seated.

7.8.5.2 Position the manifold over the flow cell with the sipper comb pointing toward the front of the cBot.

7.8.5.3 Align the manifold with the guide pins on the thermal stage and set the manifold into place on top of the flow cell.

7.8.5.4 Wiggle the manifold to make sure it is evenly seated over the flow cell. The manifold must be evenly seated to form a tight seal.

7.8.5.5 Select the checkbox to indicate the manifold is seated.

7.8.5.6 Close the flow cell clamp to lock the manifold in position. Make sure the bracket snaps securely under the white clip.

7.8.5.7 Select the checkbox to indicate the flow cell clamp is closed

7.8.5.8 Connect the outlet end of the manifold to the outlet port in the wash reservoir and make sure it is evenly seated.

7.8.5.9 Snap the outlet clamp closed to secure the outlet end of the manifold.

7.8.5.10 Select the checkbox to indicate that you have connected the manifold to the outlet port and the rear clamp is secured.

7.8.5.11 Align the sipper comb with the two metal guide pins on the front edge of the thermal stage.

7.8.5.12 Snap the sipper comb into place using the plastic tabs on either side of the sipper comb. Make sure the sippers are straight and perpendicular to the reagent plate.

7.8.5.13 Select Next.

7.8.6 Load templates

7.8.6.1 Select Enter Template Name.

7.8.6.2 Type the template ID and select Enter.

7.8.6.3 Load the 8 tube strip containing the template into the TEMPLATE row of the tube strip holder such that the tube labeled ***#8 is on the left*** side and the tube labeled ***#1 is on the right side.***

7.8.6.4 Select the checkbox to indicate that templates have been loaded.

7.8.7 Perform a Pre-Run check and start the run

7.8.7.1 Select Next. The system will automatically perform a pre-run check.

7.8.7.2 After successful completion of the pre-run check, close the cBot lid and select Start. The Run Status screen opens and the run begins.

7.9 cBot post run procedures

7.9.1 When the run is complete, select Unload.

7.9.2 Raise the cBot lid.

7.9.3 Release the outlet clamp securing the outlet end of the manifold.

7.9.4 Disconnect the outlet end of the manifold form the outlet port in the wash reservoir.

7.9.5 Remove the sipper comb from the metal guide pins using the plastic tabs on either side of the sipper comb.

7.9.5.1 When the sipper comb is removed, the system selects the checkbox indicating that the manifold is removed.

7.9.6 Release the flow cell clamp.

7.9.7 Carefully remove the manifold from the cBot, making sure that the flow cell is still on the thermal stage.

7.9.8 Lift the flow cell from the thermal stage and place in the flow cell conical. Store on ice or at 4°C until loaded onto the HiSeq.

Note: The clustered flow cell is stable for 10 days if properly stored in storage buffer at 4°C.

7.9.9 Pull the reagent lever toward you to release the reagent plate.

7.9.10 Remove the reagent plate. Ensure that remaining reagent levels are equal across the rows.

7.9.11 Remove the 8 tube strip containing templates. Ensure that remaining template levels are equal.

7.9.12 Select the checkbox to indicate that reagents and templates have been unloaded. The Wash button becomes active when all components have been removed.

7.9.13 Perform a post-run wash

7.9.13.1 Fill the wash reservoir with approximately 12 mL deionized water. You must have sufficient volume to prevent air from entering the lines.

7.9.13.2 Select the checkbox on the screen to indicate water is present. The Wash button becomes active.

7.9.13.3 Select Wash.

7.9.13.4 Repeat the wash 1x for a total of 2 washes.

7.9.13.5 When the wash is complete use a Kimwipe to blot out any excess water remaining in the wash reservoir.

7.9.13.6 Select the checkbox on the screen to indicate the wash reservoir is dry. The Exit button becomes active.

7.9.13.7 Select Exit. The Start screen opens.