96-well PCR free Library Construction for Illumina Sequencing

I. Purpose

To provide specific guidelines for Plate based TruSeq Illumina PCR free genomic library construction (300-600bp) on Biomek FX.

II. Scope

All procedures are applicable to the BCGSC Library Core and Library TechD groups.

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 QA 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 Assurance Management to audit this procedure for compliance and maintain control of this procedure.

V. References

Reference Title	Reference Number
Sample Preparation for Paired-End Sample Prep Kit from Illumina	Version 1.1 (from Prep Kit)

VI. Related Documents

Document Title	Document Number
96-well DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR3V	LIBPR.0108
Operation of the Covaris LE220	LIBPR.0097
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples	LIBPR.0017
Operation and Maintenance of the Caliper Labchip GX for DNA samples using the High Sensitivity Assay	LIBPR.0051

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Document Title	Document Number
Quantifying DNA samples using the Qubit Fluorometer	LIBPR.0030
Span-8 Pooling of DNA Samples	LIBPR.0093
Normalization of Nucleic Acid Concentration using the JANUS Automated Workstation	LIBPR.0113
Automated PCR and qPCR reaction setup	LIBPR.0125

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 material safety data sheet (MSDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number: #	Model or Catalogue #	
NEB Paired-End Sample Prep Premix Kit – End Repair	NEB	E6875B-GSC		✓
NEB Paired-End Sample Prep Premix Kit – A Tail	NEB	E6876B-GSC		✓
NEB Paired-End Sample Prep Premix Kit – Ligation	NEB	E6877B-GSC		✓
Phusion Hotstart	Fisher	F540L		✓
Fisherbrand Textured Nitrile gloves – various sizes	Fisher	270-058-53		✓
Ice bucket	Fisher	11-676-36		✓
Covaris LE220 with WCS and Chiller	Covaris	LE220	✓	
DNA *AWAY*	Molecular BioProducts	21-236-28		✓
Gilson P2 pipetman	Mandel	GF-44801	✓	
Gilson P10 pipetman	Mandel	GF-44802	✓	
Gilson P20 pipetman	Mandel	GF23600	✓	
Gilson P200 pipetman	Mandel	GF-23601	✓	
Gilson P1000 pipetman	Mandel	GF-23602	✓	
Diamond Filter tips DFL10	Mandel Scientific	GF -F171203		✓
Diamond Filter tips DF30	Mandel Scientific	GF-F171303		✓
Diamond Filter tips DF200	Mandel Scientific	GF-F171503		✓
Diamond Filter tips DF1000	Mandel Scientific	GF-F171703		✓
Galaxy mini-centrifuge	VWR	37000-700	✓	
VX-100 Vortex Mixer	Rose Scientific	S-0100	✓	
Black ink permanent marker pen	VWR	52877-310		✓
Clear Tape Sealer	Qiagen	19570		✓
Aluminum Foils seals	VWR	60941-126		✓
Aluminum foil tape, 3"x 60 yds	Scotch/3M	34000740		✓
Eppendorf BenchTop Refrigerated Centrifuge 5810R	Eppendorf	5810 R	✓	
Bench Coat (Bench Protection Paper)	Fisher	12-007-186		✓
Small Autoclave waste bags 10"X15"	Fisher	01-826-4		✓
Anhydrous Ethyl Alcohol (100% Ethanol)	CommercialAlcohols	00023878		✓

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IKA Works Vortexer	Agilent	MS2S9-5065-4428	✓	
22R Microfuge Centrifuge	Beckman	22R Centrifuge	✓	
Peltier Thermal Cycler	MJ Research	PTC-225	✓	
Power Supply, LVC2kW, 48VDCV	Tyco Electronics	RM200HA100	✓	
P165B Tips, sterile, 10 racks of 96/box	Ultident	24-FXF-180-LRS		✓
P50 (Universal) Tips, Presterile with Barrier, 50 µL, 96/rack, 10racks/case, CS960	Beckman	CABKA21586		✓
Plate, 96-Well reservoirs, diamond-bottom, Low-Profile	Seahorse	EK2036		✓
Plate, 96-Well reservoirs, diamond-bottom, Deep-Profile	Seahorse	S30014		✓
Plate, 96-Well reservoirs, 450uL EtOH & Waste	Axygen	P-96-450V-C		✓
AB1000 96-well 200µl PCR plate	Fisher	AB1000		✓
Biomek FX Liquid Handling System	Beckman	Biomek FX	✓	
Eppendorf Benchtop Centrifuge	Eppendorf	5810 R	✓	
70% Ethanol	In house	N/A	N/A	N/A
Qiagen Buffer EB – 250 mL	Qiagen	19086		✓
10 X TBE - 10 L	Invitrogen	15581-028		✓
1 X TBE	In House	N/A	N/A	N/A
UltraPure Distilled Water	Invitrogen	10977-023		✓
Nuclease Free 2.0 mL eppendorf tube	Ambion	12400		✓
5 mL Screw Cap tubes	Ultident	SCT-5ML-S		✓
TruSeq Indexed Adapters	IDT	NA		
TruSeq Universal Primers	IDT	NA		
Alpaqua 96S Super Magnet Plate	E & K Scientific	A001322	✓	
Ampure XP Beads, 450mL	Agencourt	A63882		✓
PCR Clean DX (ALINE beads)	ALINE Biosciences	C-1003-450		✓

These sequences are for internal use only:

TruSeq Indexed Adapters
GATCGGAAGAGCACACGTCTGAACTCCAGTCACNNNNNNATCACGATCTCGTATGCCGTCTTCTGCTT
G

TruSeq Universal Primer (For Rescue)
AATGATACGGCGACCACCGACACTC
CAAGCAGAAGACGGCATACGAGAT

IX. Introduction and Guidelines

1. General Guidelines

1.1. Ensure proper personal protective equipment is used when handling sample plates, reagents and equipment. Treat everything with clean PCR techniques.

1.2. Wipe down the assigned workstation, pipetman, tip boxes and small equipment with DNA *AWAY*. Ensure you have a clean working surface before you start.

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1.3. Pre-PCR and Post-PCR work should be performed on the 5th Floor and 6th floor respectively.
1.4. Acronyms: NA stands for Not Applicable. Pre-LC refers to Pre-Library Construction. Post-LC refers to Post-Library Construction. BC refers to Bead Clean.
1.5. Discuss with the APC/PC/designated trainer the results of every QC step. Report and record equipment failures and/or malfunctions and variations in reaction well volumes.

2. General Plate Guidelines

2.1 Up to 4 plates can be processed at a time by one technologist using Biomek FX.
2.2 To avoid cross-well contamination, reaction plates should never be vortexed and plate seals should never be re-used. Use Biomek FX for mixing.
2.3 Use only VWR foil seals for both short term storage and tetrad incubations and 3M aluminum foil seal for long term storage.
2.4 Quick spin the plate(s) at 4ºC for 1 minute at 2000 g after incubation.
2.5 Sample plates can be stored at -20ºC overnight after every step except post Adenylation. Adenylation and Ligation must be performed on the same day.

3. Positive and Negative Controls

3.1. The positive control template to be used for this protocol is HL60 genomic DNA. The yield of library products constructed from positive controls is expected to differ from those of collaborators' samples. However, the yield should not differ significantly from that of previously constructed positive controls.
3.2. The negative control template to be used for this protocol is Qiagen Elution Buffer. This control will measure background products that result from the library construction process.

4. General Brew Preparation Guidelines

4.1. Double check the QA release and expiry date of each reagent.
4.2. Thaw required reagents and premixed brews and place them on ice. Enzymes should be left in the freezer until ready to use. Each premix can be freeze thawed 3 times for library construction use.
4.3. Reagents and enzymes should be well mixed, the former by pulse-vortexing and the latter by gentle flicking. Treat premixed brews as enzymes. After mixing, quick spin down in a mini-centrifuge.
4.4. Once prepared, all brews should be well mixed by gentle, repeated pulse-vortexing to ensure equal distribution of all components and thus uniformity of enzymatic reactions across a plate. The Ligation brew is particularly viscous.

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4.5. All reactions require the preparation of a Brew Source Plate. The Biomek FX will be used to aliquot the brew from the Brew Source Plate into the reaction plates.
4.6. All brew calculators include excess volume to account for dead volume required by the Biomek FX in the Brew Source Plate and to account for pipetting loss.
4.7. Follow instructions in this SOP to determine the volume of premixed brew per well.

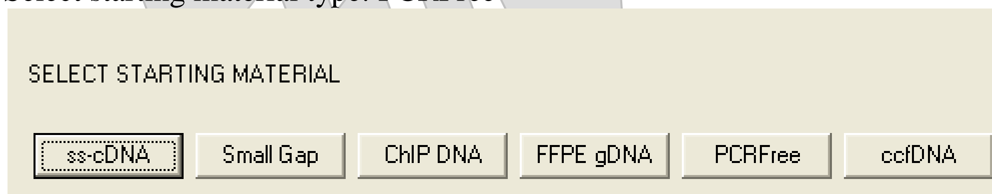
5. Biomek FX Handling Guidelines

5.1. Home all axes before starting each Biomek run.
5.2. Reaction brews vary in viscosity, selecting the correct Biomek technique is therefore essential to ensure accurate volume transfer.
5.3. The default dead volume required by the Biomek FX in each well of a Brew Source Plate is 5µL/well regardless of the number of plates being processed.
5.4. The dead volume required by the Biomek FX in the 96-well reservoir is 25mL.
5.5. Confirm the plate and tip box locations on the Biomek deck matches the software deck layout on the computer screen.
5.6. Ensure plate seals and tip box covers are removed before starting the Biomek program.

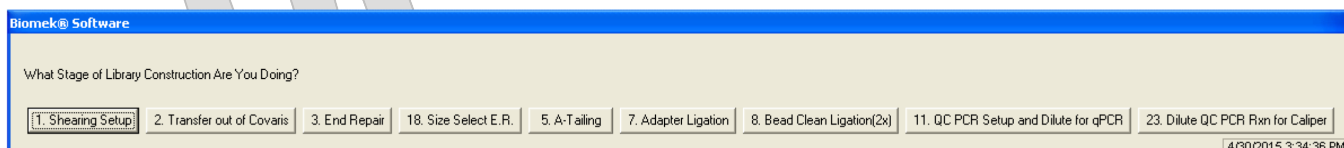
6. General note on Biomek programs

The following steps are generally followed:

A. Open project: LIBPR
B. Select and Run: LibraryConstruction
C. Select starting material type: PCRFree



D. Select Library Construction step. An example is shown below:



The Biomek bead cleanup modules employed in this SOP are based on the following conditions:

Bead Binding Time (mins)	1st Magnet Clearing Time (mins)	2 X 70% EtOH Wash Vol (µL)	Ethanol Air-dry Time (mins)	Elution Volume (µL)	Elution time (mins)	2nd Magnet Clearing time (mins)
15	**7**	**150**	**5**	**20-52**	**3**	**2**

Note: Bead to reaction ratios are defined at each step

X. Procedure

Note: ALINE beads (PCR Clean DX) and Ampure XP beads can be used interchangeably in the magnetic bead clean up steps.

1. Initial QC

1.1 For each gDNA 96 well stock plate, quantify according to the following SOP:

LIBPR_WORKINST.0108 96-well DNA Quantification using Quant-iT and VICTOR3

2. Sample Normalization on the JANUS Automated Workstation

2.1 Samples must be diluted in 62.5 µL of Qiagen elution buffer prior to shearing. The minimum requirement is 250 ng or 4.17 ng/µL in 62.5µL, the maximum input is 1000ng or 16ng/µL in 62.5µL.

2.2 Normalize input as directed by your APC and according to the following SOP:

LIBPR.0113 Normalization of Nucleic Acid Concentration using the JANUS automated workstation

3. Shearing

3.1 To transfer normalized DNA into the Covaris plate, log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction > RUN> PCRFree >**Shearing Setup**

3.2 Refer to the following SOP for shearing conditions:

LIBPR.0097 Operation of the Covaris LE220 Program: **Plate_90sec_PCR_Free_Genome_Large_Gap.e1proc**

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Make sure that you have performed the shearing twice with a spin in between according to the SOP above.

4. Agilent HS DNA QC after shearing – Spot Check

4.1 For each 96 well plate of sheared samples, use 1 µL from 11 random samples (ensure that at least one of these samples is a positive control) to spot check on a High Sensitivity DNA Agilent Assay. To prevent overloading on the HSDNA chip, dilute 6X by transferring 1µL of sheared gDNA to 5µL of EB. Mix dilution well before running the QC. For lower input starting amounts, confirm the dilution with the APC.Refer to the following SOP:

LIBPR.0017 Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples

4.2 The profile for sonicated DNA should have the peak close to 425bp. Consult with your APC to confirm the sonicated DNA profiles. Repeat shearing may be necessary in some cases, and if so ask your APC for the additional required sonication time.

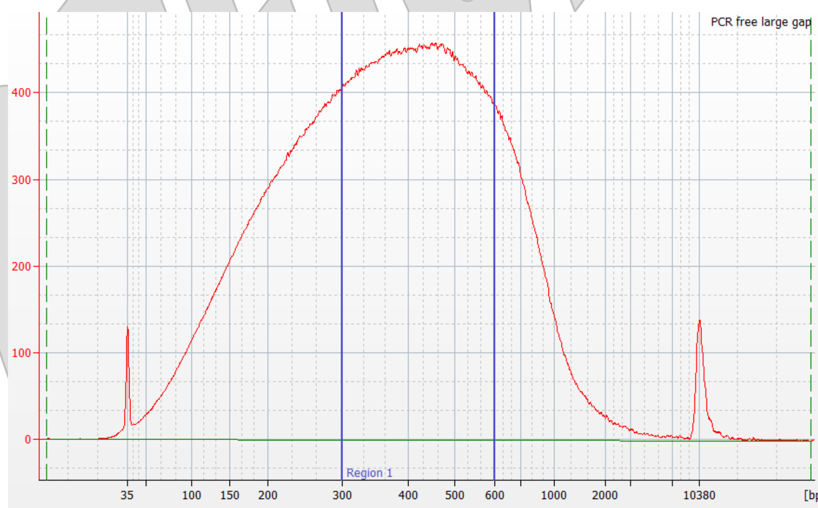

Figure 1: High Sensitivity DNA chip showing ideal shearing in the 300:600 bp range

5. Transferring DNA out of Covaris Plate

5.1 Log into the following Biomek program to transfer sheared gDNA to an AB1000 plate.

Biomek: Project > LIBPR> LibraryConstruction > RUN> PCRFree > **Transfer out of Covaris**

6. End-Repair and Phosphorylation Reaction

6.1. End Repair Premix must be thawed on ice, and then mixed with gently flicking prior to dispensing brew into the brew plate using volumes described below. Note that aliquot volumes include 5µL of dead volume.

	End Repair Premix (µL/well)
Aliquot: 1 plate	45
Aliquot: 2 plates	85
Aliquot : 3 plates	125
Aliquot : 4 plates	165
Volume per reaction	40
Total Reaction Volume	100.00

6.2. Log into Biomek FX Program as follows:

6.3. Biomek: Project > LIBPR> LibraryConstruction > RUN> PCRFree >**End Repair** The brew plate is the "Source" and the DNA plate(s) is the "Dest." After Biomek program completion, seal the plates and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

6.4. Incubate End-Repair reaction plate(s) at 20ºC for 30 minutes. Total reaction volume is 100ul.

Tetrad Program: Run > LIBCOR > ER

6.5. After the 30 minute incubation, store plate at -20ºC or proceed to Size Selected End Repaired DNA.

7. Size Selected End Repaired DNA

7.1 Log into the Biomek FX and select the pipeline-specific bead clean up method:

Biomek: Project > LIBPR> LibraryConstruction > RUN>PCRFree>**Size Select E.R.**

7.2 Note that end-repaired product can be stored at -20 °C after size selection.

8. Addition of an 'A' Base (A-Tailing) Reaction

8.1.A-Tailing Premix must be thawed on ice, and then mixed with gently flicking prior to dispensing brew into the brew plate using volumes described below. Note that aliquot volumes include 5µL of dead volume.

	A-Tailing Premix (µL/well)
Aliquot: 1 plate	25
Aliquot: 2 plates	45
Aliquot : 3 plates	65
Aliquot : 4 plates	85
Volume per reaction	20
Total Reaction Volume	50

8.2.Log into Biomek FX Program as follows:

Biomek: Project > LIBPR> LibraryConstruction > RUN>PCRFree>**A-Tailing**

8.3.The brew plate is the "Source" and the DNA plate(s) is the "Dest." After Biomek program completion, seal the plates and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

8.4.Incubate A-Tailing reaction plate(s) at 37ºC for 30 minutes; 70ºC for 5 minutes; 4ºC for 5 minutes; 4ºC hold. Total reaction volume is 100ul.
Tetrad Program: Run > LIBCOR > ATAIL

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8.5. During the incubation prepare ligation brew for in-tandem ligation. **Note: do not bead clean adenylated library.**

8.6. After the incubation, immediately proceed to Adapter Ligation.

9. Adapter Ligation

9.1. Premix Ligation Brew must be mixed with gently flicking prior to dispensing brew into the brew plate using volumes described below. Note that aliquot volumes include 5µL of dead volume.

	Ligation Premix (µL/well)
Aliquot: 1 plate	26
Aliquot: 2 plates	47
Aliquot : 3 plates	68
Aliquot : 4 plates	89
Volume per reaction	21
Total Reaction Volume	75

9.2. Log into Biomek FX Program as follows:

Biomek: Project > LIBPR> LibraryConstruction > RUN> PCRFree>**Adapter Ligation**

9.3. The Biomek will transfer brew to the template, mix and then transfer everything to the TruSeq indexed adapter plate.

9.4. Seal the plates and quick spin at 4ºC for 1 minute after the Biomek process has completed. Inspect the reaction plates for any variations in volume.

9.5. Incubate Ligation reaction plate(s) at 20ºC for 15 minutes. The total reaction volume is 75 µL.

Tetrad Program: Run > LIBCOR > LIGATION

9.6. While the ligation incubation is running, set up the Biomek for Aline Magnetic bead clean up. Bead clean up must occur immediately after the ligation reaction is completed.

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9.7. After the 15 minute incubation, quick spin the plate and then proceed immediately to Aline Magnetic Bead Clean Up after ligation. Store plate on ice during set up.

10. AMPure XP Magnetic Bead Clean Up after Ligation

Note: Prior to the second bead clean elution, remove the tip corresponding to the PCR brew control well from the tip box to avoid carrying over elution buffer to the PCR brew control well.

10.1. The input volume for this step is 75 µL per well.

10.2. Log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction >RUN> PCRFree > **Bead clean Ligation (2x)**

10.3. Post-ligation bead cleanup is performed twice and a safe stopping point is after the first bead clean. A prompt will appear asking "Do you want to skip the first bead clean? Yes, No or Quit". If you want to proceed to the first bead clean and pause, select "No". If you have already finished one round of bead clean and are continuing, select "Yes" (see Figure below).

Do you want to skip the first bead clean step?

YES NO QUIT

10.4. Pour beads back to falcon tube and invert 10 times to mix the beads before the 2nd bead cleanup

10.5. Samples can be stored at -20°C or you may proceed immediately to prepare samples for qPCR and 10X PCR QCs. Refer to LIBPR.0125 for library QC setup including PCR setup, Caliper and qPCR setup.

10.6. If the PCR-free genome samples are to be pooled, use the average bp from either the Caliper or Agilent and concentration from Qubit or Quant-iT to calculate the nM for the sub-libraries. For POG and specified HiSeqX genomes, pool tumour and normal in a 2:1 nM ratio and send the data to the APC for the pooled IX# generation and the instructions for qPCR setup on the pooled libraries. For non-tumour and normal paired pooling, follow the APC's instructions regarding pooling and subsequent qPCR.

10.7. If the PCR-free genomes are not pooled, proceed to step 11 following the qPCR setup using LIBPR.0125.

LIBPR.0125 Automated PCR and qPCR reactions setup

11. Re-array into 1.5mL Tubes for Submission

11.1. Discuss with supervisor to see if libraries pass the acceptance criteria. If libraries pass the acceptance criteria, libraries can be re-arrayed into 1.5mL tubes for submission.

12. Rescue PCR for failed PCR-Free library

12.1. Your Supervisor will review the data and determine if there are any candidates for PCR rescue.

12.2. Libraries that do not meet acceptance criteria can be rescued by doing 4 cycles PCR or more as specified by supervisor.

12.3. The volume requirement for 1 reaction set up is as follows:

Solution	1 rxn (µL)	
Undiluted ligated template	8	
5X Phusion HF Buffer	5	PCR Brew (17µL)
TruSeq Primer 1 & 2 Cocktail, 10 µM	2	
10mM dNTP	0.5	
100% DMSO	0.75	
Hot Start Phusion Enzyme, 2 U/µL	0.25	
Nuclease free water	8.5	
Reaction total	**25**	

12.4. Generate the PCR QC calculator using LIMS:

LIMS: Mix Standard Solution > **PCRFree_Rescue_PCR**> follow the prompts>Save Standard Solution

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12.5. Obtain the 1D large Solution/Box/Kit Label and Chemistry Label. Prepare the brew in an appropriate sized tube according to the chemistry calculator.

12.6. Carefully aliquot 17µL of brew to wells of a new AB1000 plate using a Distriman pipette.

12.7. Log into the following Biomek program:

Biomek: Project >LIBPR> Run> Transfer to AB1000

12.8. Follow the prompts. Program the Biomek to transfer 8µL of undiluted PCRfree template to 17µL of pre-dispensed brew.

12.9. Run 4X QC PCR program using the following tetrad program:

Tetrad Program: LIBCOR>PF'PCR4

PF'PCR4:

- 98°C 30 sec
- 98°C 10 sec
- 60°C 30 sec } total of 4 Cycles of PCR
- 72°C 30 sec
- 72°C 5 min
- 4°C ∞

12.10. When the PCR program is completed remove reaction plates from the tetrad and quick spin plates.

12.11. Proceed to 1X Bead Clean PCR rescued samples.

13. 1X Bead Clean PCR rescued samples

13.1. Log into the following Biomek program:

Biomek: Project > LIBPR> RUN > **PCR Rescue**

13.2. The Biomek will run one round of 1:1 bead:sample bead clean to remove unwanted small products and adapter artifacts. The final elution volume is 20µL.

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13.3. When the process is complete, cover the plate(s) with a VWR foil plate and then QC 4X-enriched samples by Caliper and Quant-iT as described above.

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Appendix A: LIMS Protocol

1. Start of Plate Library Construction– IDX pipeline

2. Bioanalyzer Run-QC Category: sonication QC

3. A-PCR Free Library Construction - IDX pipeline

4. Bioanalyzer Run or Caliper Run- QC Category: Post library construction size selection

   Enter the following attributes:
      1) Library_size_distribution_bp
      2) Avg_DNA_bp_size

5. qPCR Run generated in LIMS
6. qPCR quant

7. PCR_Rescue – only run this protocol when PCR rescue is required

   1. If Pooling: Action: Aliquot pooling volume into a new TRA

   2. If Pooling: Pooling and/or Manual Rearray into tubes – ITP pipeline

8. Final_Submission – TPE (no pooling); ITP (pooled)

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