LIAI-SeqFac – SOLID DNA Fragment library preparation for Multiplex (8-96x) DNA Sequencing – Confidential - Version B

*Created based on previous experiments and Protocol written by Jeremy Day – LIAI – Edition 11MAR13: G.Seumois*

\* Samples are supposed to be quantified by picogreen and Biological QC passed: SOP tested from 20 to 500ng of DNA.

\* Samples supposed to be sonicated or not depending of the upstream application: >50% should be between 100 to 250 bp.

\* The entire procedure takes 7 hours including the Picogreen quantification, TS/Bioanalyzer QC, and Solid taqman PCR assay.

\* For consistency in analysis: use a spreadsheet associated to every experiment.

\*Reagents, Buffers and specific equipment:

- 5500 SOLiD™ Fragment Library Barcode Adaptors Modules, Life Technologies, 446440x (x=5-9), $1000.

- 5500 SOLiD™ Fragment Library Core Kit (48 samples), Life Technologies,

- SOLiD™ Library TaqMan® Quantitation Kit, Life Technologies, 4449639, $1066.

- Agencourt AMPure XP beads 60ml, Beckman Coulter, #A63881, $1050.

- Agilent High Sensitivity DNA Kit, Agilent, #5067-4626, $480.00.

- Black 96 well plate:

- 96 PCR plate: Biorad

- Plastic seals:

- Picogreen dye:

- Te Buffer: Tris-HCl pH8.0 10mM, EDTA 100µM (for 50ml Te: 500ul Tris 1M and 10ul EDTA 0.5M)

- Magnet

\* Before to start:

O Take out the Ampure XP beads out of the fridge, mix them, and prepare 80% ethanol solution – everything kept at RT!

O Thaw all reagents and samples on ICE.

O Check booking for common equipment.

START DATE: - END DATE: - Experimentalist: - Sample Owner:

O Work on 96-well PCR plate – for every condition, dilute your DNA up to **60ul** of Te.

O Prepare the End-repair and polishing MIX:



O Add the End polishing Mix – 40ul/ samples.

O Vortex for 5-10 sec, pulse-spin, incubate at RT for 20 minutes (sharp!!)

O Add **50ul** pre-warmed and mixed Ampure XP solution (=0.5volume).

O Pipet up and down to mix, at least 10 times, incubate at RT for 5 min.

O Place the plate on the magnet, give at least 2 min to capture beads.

O Collect **SUPERNATANT** (150ul) in a next set of plate PCR tube – Pellet will be used for QC>250s

O Add **30ul** of Ampure XP beads solution to the 150ul (=0.3v of initial volume).

O Pipet up and down to mix, at least 10 times, incubate at RT for 5 min.

O Place the plate on the magnet, give at least 2 min to capture beads.

O Discard supernatant and keep **PELLETS**.

O Wash all **PELLETS** (1st and second round of Ampure XP) 3x with 200ul of EthOH 80% : leave plate on the magnet during washes, do not mix.

O After last wash, make sure no liquid has left in the wells – QC with 20ul tips.

O Dry pellet 5 - 10 min at RT

O Elute size selected (SiSe) tubes with 2x 11 ul Water – store on ICE.

O Elute QC>250 with 25ul of Te.

O From the 22ul of SiSe condition, take out 17ul in new plate.

O Add 4ul of Te to the 4-5ul left of SiSe. Keep these conditions for QCs

O Prepare the dA-tailing MIX:



O Transfer 17ul of the SiSe product to a new set of well in a 96PCR plate.

O Add 8ul of dA-tailing mix, cover the plate, 5” of controlled Vortex and a 10” pulse-spin.

O Incubate at RT for 30 min.

O During the dA-tailing incubation, calculate primer volume:

Rules: < or = to 100ng as initial qty: dilute primer 1 in 20 and use 1.2ul per sample.

 > to 100ng as initial qty:

a. X=ug-to-pmol conversion factor= 1000000/(660 x average size of fragment)

b. Vol of adaptor needed= ((Qty post size selection (usually 30%) x X x 10)/50) x per the number of samples (but every BC is a different primer … !! this volume has to be < 4.3ul (if dilution needed)

O Prepare the Ligation MIX - !P1adapter is set for 500ng starting material!:



O Add Ligation mix 31ul directly to the dA-tailing product and add equivalent amount volume of specific Barcode-P2 adaptors: !=4ul of 1/20th diluted solution for 500ng starting material ligation!

O Cover the plate, 5” of controlled Vortex and a 10” pulse-spin.

O Incubate 30min @ 20C or at RT. Total reaction volume should be 60ul

O Add to the 36ul of Ampure XP beads (0.6 volume).

O Pipet up and down to mix, at least 10 times, incubate at RT for 5 min.

O Place the plate on the magnet, give at least 2 min to capture beads.

O Discard supernatant and keep **PELLETS**.

O Wash the **PELLETS** 1x with 200ul of EthOH 80%: Leave tubes in the magnet during washes, do not invert/mix.

O After last wash make sure no liquid has left in the wells – QC with 20ul tips.

O Dry pellet 3-5 min at RT (dried pellet start to crack – that is a nice QC)

O Add 40ul of Te buffer, pipet up and down to mix, at least 10 times, incubate at RT for 5 min.

O Take the plate out of the magnet and add 44 ul of Ampure XP beads (1.1 volume)

O Pipet up and down to mix, at least 10 times, incubate at RT for 5 min.

O Place back the plate on the magnet, give at least 2 min to capture beads.

O Discard supernatant and keep **PELLETS**.

O Wash the **PELLETS** 3x with 200ul of EthOH 80%: Leave the plate on the magnet during washes, do not invert/mix.

O After the last wash, make sure no liquid has left in the wells – QC with 20ul tips.

O Dry pellet 3-5 min at RT**.**

**O Elute DNA with 2x 12ul Te – KEEP -20C and on ICE**

O For QC purposes, in a new plate, dilute freshly ligated samples in Te buffer.

 - Dilution1: Take 3ul + 57 ul of Te (20x) -> Picogreen Qty

 - Dilution2: Take 3ul of dilution1 + 147 ul of Te (1000x) -> Solid PCR quantification

O QC – Bioanalyzer: Randomly pick 2 samples from QC>250bp, 3 samples from the QC-SiSe, and the rest from the freshly ligated samples. With 1ul for every sample run a High sensitivity Bioanalyzer chip. If required, samples could be run on Tape Station also.

Pass QC if:

1. >250bp fractions: <15% should be <250bp
2. Sise fraction (before ligation):
	1. >80% of Sise samples are 100bp> x <250bp.
	2. Mode peak size and median fragment size should be ranging between 170 and 190bp.
	3. ∆ size (median – mode) < 20nt.
3. Ligated samples:
	1. >70% of ligated samples are >200bp and <350bp.
	2. Fraction < 200 or >350 has to be <10%.
	3. Mode peak size and median fragment size should be ranging between 270 and 290bp.
	4. ∆ size (median – mode) < 20nt.
	5. ∆ Size (Lig Median – SiSe median) > 80 nt.
4. If we started with 500ng, Qty (for 1 ul) of the entire peak should be > to 1000pg.
5. Adaptor contamination should be <5% (50 bp).

O QC – Picogreen.

O Prepare fresh Pico stock solution (keep dark): N x 200ul of Te

Add 2ul of Picogreen dye (aliquots kept in -20C) per ml of TE

 O Prepare a Sonicated Standard Lambda DNA serial dilution: Stock Conc=40ng/ul:

 In a 8 tube PCR strip:



 O Use a Black, flat Bottom 96 well plate

 O Dispense 190ul of Pico solution. Be sure to add prior to DNA, since DNA may be absorbed by the plastic in the plate!

 O Dispense in triplicate 10ul of the standards

 O Dispense in du/tri-plicates at least 10ul of the Ligated samples Dilution1.

O Read on the spectramax without a lid – don’t address reference on this machine, there is a excel sheet to do the analyses. You may use an adhesive foil in order to vortex, spin, and keep covered until reading.

Pass QC if = or > than 15% of the starting material.

O QC – Solid Taqman PCR assay (required to estimate the ligation efficiency % and will inform about the number of P1/P2 amplification cycle to perform).

O Prepare PCR mix:



O Prepare 10x serial dilution from the Standard DNA (100pM) – use 2ul/well in duplicates.

O Dispense Template : use Dilution2 samples – 2ul / well in duplicates.

O Run on Step one ABI machine:

 - 20 min @ 72˚C

 - 2 min @ 95˚C

 - 40x ( 15” @ 95˚C – 30” @ 60˚C)

O Analysis: set Threshold at 0.25, adjust baseline – export Ct value and analyze on Excel spreadsheet.

Pass QC if ligation efficiency = or > than 5%.

O Determination of the P1/P2 amplification: follow Excel spreadsheet – Less then 8 cycles.

START DATE: - END DATE: - Experimentalist: - Sample Owner:

 **P1/P2 Amplification for EZ beads system**

O Determine the number of cycle of amplification.

O USE 1/2th or the ligation product per amplification! take this in account to determine your # of amplification cycles.

O So take 12ul of ligated product for amplification and add 8 ul of Water in a PCT tube/or plate.

O Prepare the P1/P2 amplification MIX:



O Add 105ul of Amplification MIX.

O On PCR thermoblock proceed with this PCR-based amplification protocol:

- nick translation: 20 min @ 72˚C

- 5min @ 95C

- X cycles of (15” @ 95˚C, 15”@ 62˚C, 2’ @ 70˚C)

- Last cycles add 20 min @ 70˚C

- Hold 4˚C.

O Add 150ul of Ampure XP beads.

O Pipet up and down to mix, at least 10 times, incubate at RT for 5 min.

O Place the plate on the magnet, give at least 2 min to capture beads.

O Discard supernatant and keep **PELLETS**.

O Wash the **PELLETS** 1x with 200ul of EthOH 80%: Leave tubes in the magnet during washes, do not invert/mix.

O After last wash make sure no liquid has left in the wells – QC with 20ul tips.

O Dry pellet 3-5 min at RT (dried pellet start to crack – that is a nice QC)

O Add 40ul of Te buffer, pipet up and down to mix, at least 10 times, incubate at RT for 5 min.

O Clear sample on magnet for 5 mins

O Take the plate out of the magnet and add 44 ul of Ampure XP beads (1.2 volume)

O Pipet up and down to mix, at least 10 times, incubate at RT for 5 min.

O Place back the plate on the magnet, give at least 2 min to capture beads.

O Discard supernatant and keep **PELLETS**.

O Wash the **PELLETS** 3x with 200ul of EthOH 80%: Leave the plate on the magnet during washes, do not invert/mix.

O After the last wash, make sure no liquid has left in the wells – QC with 20ul tips.

O Dry pellet 3-5 min at RT**.**

**O Elute DNA with 2x 12ul Te – KEEP -20C and on ICE**

O For QC purposes, in a new plate, dilute freshly ligated samples in Te buffer.

 - Dilution1: Take 3ul + 147 ul of Te (50x)

 - Dilution2: Take 3ul of dilution1 + 147 ul of Te (2500x)

O QC – Tape Station (High sensitivity): Using 2ul of each undiluted sample run a High sensitivity Tape Station.

Pass QC if:

1. >70% of Amplified samples are 200bp> x <350bp.
2. Median size ranging between 270-290bp.
3. Quantity should be >2000pg/ul
4. Molarity should be >10000pM
5. Adaptor contamination should be <5%.

O QC – Picogreen (optional).

O Prepare fresh Pico solution (keep dark): N x 200ul of Te

Add 2ul of Picogreen dye (aliquots kept in -20C) per ml of TE

 O Prepare a Sonicated Standard Lambda DNA serial dilution: Stock Conc=40ng/ul:

 In a 8 tube PCR strip:



 O Use a Black, flat Bottom 96 well plate

 O Dispense 190ul of Pico solution. Be sure to add prior to DNA, since DNA may be absorbed by the plastic in the plate!

 O Dispense in triplicate 10ul of the standards

 O Dispense in du/tri-plicates at least 10ul of the Ligated samples Dilution1.

O Read on the spectramax without a lid – don’t address reference on this machine, there is a excel sheet to do the analyses. You may use an adhesive foil in order to vortex, spin, and keep covered until reading.

Pass QC if > Qty = (Ligated QTy (ng)/2) \* (power(# of P1/P2 cycles,2) / 2) (-> Taken in account the fact we take only 12ul of ligated samples, the fact the the Ampure XP could take away some DNA and the fact that the PCR efficiency could be < 2).

O QC – Solid Taqman PCR assay – required to normalization and pooling.

O Prepare PCR mix:



O Prepare 10x serial dilution from the Standard DNA (100pM) – use 2ul/well in duplicates.

O Dispense Template : use Dilution2 samples – 2ul / well in duplicates.

O Run on Step one ABI machine:

 - 5 min @ 95˚C

 - 40x ( 15” @ 95˚C – 30” @ 60˚C)

O Analysis: set Threshold at 0.25, adjust baseline – export Ct value and analyze on Excel spreadsheet.

Pass QC = all samples should be >10000 pM.

O If QC passed, proceed to sample normalization and pooling strategy.