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Pre-Sequencing V.2

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We use this protocol and it's working

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Abstract

This protocol details the information about pre-sequencing.

Materials

Mastermix for PCR 1:

	A	B	C	D	E	F	G	H	I
	Sample		**d5 NC**	**d5 R1**	**D5 R2**	**d5 R3**	**d13 R1**	**d13 R2**	**d13 R3**
	DNA Quants (ng/uL)		152	195	210	529	784	799	613
	Master Mix	x 1	x 8	x 8	x 8	x 8	x 16	x 16	x 16
	2X KAPA HiFi	25	200	200	200	200	400	400	400
	10um_F	1.5	12	12	12	12	24	24	24
	10um_R	1.5	12	12	12	12	24	24	24
	DNA	250 ng	13.2	10.3	9.5	3.8	5.1	5	6.5
	Water	to 25 uL	162.8	165.7	166.5	172.2	346.9	347	345.5
	Total	50	400	400	400	400	800	800	800

Mastermix for PCR 2:

	A	B
	Master Mix	**x 1**
	2X KAPA HiFi	12.5
	10um_F (TrueSeqR1)	0.75
	10um_R (NexteraR2)	0.75
	water	8.88
	100x Sybr Green	0.13
	Total	23
	AMPured Rxn_1	2
		25

Mastermix for PCR 3:

	A	B	C
	MasterMix	**x 1**	**x29**
	2X KAPA HiFi (RM)	12.5	362.5
	100x Sybr	0.13	3.6
	water	7.38	213.9
	Total	20	580
	10um_F (TrueSeq_P5)	1.5	
	10um_R (Nextera_P7)	1.5	
	AMPured Rxn_2	2	
		25	

DNA and RNA isolation:

1 For all cell pellets harvested at the first timepoint (referred to as Day 05, or D5), isolate both DNA and RNA using the a modified **QIAGEN AllPrep** kit (see manufacturer instructions for : https://www.qiagen.com/us/resources/resourcedetail?id=580866a6-56c6-4674-8566-2852164d8519&lang=en).

(1) **Determine amount of starting material** (either cell count or cell pellet size) and adjust protocol using the tables below (Table 1a, 1b).

Table 1a. Amount of starting material for AllPrep DNA/RNA Mini

	Cell pellet size	Number of cells
	Very small	< 0.1 × 10^6
	Small	0.1 × 10^6 – 5 × 10^6
	Medium	5 × 10^6 – 10 × 10^6
	Large	> 10 × 10^6

(2) **Prepare Buffer RLT Plus w/1% β-MercaptoEthanol (β-ME).** Determine volume of Buffer RLT Plus needed for the total number of samples and add 1% β-ME (Table 1b). Dispense β-ME in a fume hood.

Table 1b. Volumes of Buffer RLT Plus w/1%β-ME per starting material.

	Cell pellet size	Number of harvested cells	Volume ofBuffer RLT / sample	Volume of β-ME / sample
	Very small *	< 0.1 × 10^6	100ul	1ul
	Small	0.1 × 10^6 – 5 × 10^6	350ul	3.5ul
	Medium	5 × 10^6 – 1 × 10^7	600ul	6ul
	Large	> 1 × 10^7	1000ul	10ul

*For number of cells ≤0.1 × 10^6 (very small pellets), homogenization can be achieved by vortexing for 1 min without using a QIAshredder.

(3) Pipet up to 700µl of lysate directly into a **QIAshredder spin column** placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed. Save **homogenized cell lysate** in collection tube.

If processing >1×10^7 cells, apply volume to the same QIAshredder column. Collect homogenized cell lysate in a new collection tube, record lysate volumes, save **homogenized cell lysates**.
(Steps 1 thru 3 above replace STEP 1 of the AllPrep DNA/RNA Mini Kit Quick-Start Protocol, Part 1).

(4) Transfer the **homogenized lysate** to an **AllPrep DNA spin column** and centrifuge for 30s at ≥8000 x g (≥10,000 rpm). Place the **AllPrep DNA spin column** in a new 2 ml collection tube and keep column at RT for subsequent DNA purification. Proceed with the flow-through to RNA purification.
(^This is modified **STEP 2** of the **AllPrep DNA/RNA Mini Kit Quick-Start Protocol, Part 1)**.

(5) **Total RNA purification**. Add 1 volume of **70% ethanol** to the flow-through. Mix well by pipetting. Do not centrifuge. Immediately transfer up to 700µl of the sample, including any precipitate, to an **RNeasy spin column** placed in a 2 ml collection tube. Centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Save the collection tube for the following steps.

(6) **Wash the RNA**

- Add 700µl **Buffer RW1** to the RNeasy spin column. Close the lid and centrifuge for 15s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Save the collection tube.
- Add 500µl **Buffer RPE** to the RNeasy spin column. Close the lid and centrifuge for 15s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Save the collection tube.
- Add 500µl **Buffer RPE** to the RNeasy spin column. Close the lid and centrifuge for 2min at ≥8000 x g (≥10,000 rpm).
- Place the RNeasy spin column in a new 2ml collection tube. Discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min to dry the spin column membrane.

(7) **Elute the RNA**
Place the RNeasy spin column in a new 1.5ml Eppendorf tube. Add 50µl **RNA Storage Solution** (AM7001) directly to the spin column membrane. Close the lid gently, let the column sit for 1 min and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA. Using the 50uL eluate, repeat step 7. Reuse the 1.5ml Eppendorf tube. Centrifuge for 1 min at ≥8000 x g (≥10,000 rpm).
(^These are modified **STEPS 1-6** of the **AllPrep DNA/RNA Mini Kit Quick-Start Protocol, Part 2, Total RNA purification)**

(8) **Genomic DNA purification**
Return to the **AllPrep DNA spin column** left at RT.

(9) **Wash the genomic DNA**

- Add 500 µl **Buffer AW1** to the AllPrep DNA spin column in 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube.
- Add 500 µl **Buffer AW2** to the AllPrep DNA spin column. Close the lid gently, and centrifuge for 2 min at full speed to wash the spin column membrane.
- Place the AllPrep DNA spin column in a new 2ml collection tube. Discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min to dry the spin column membrane.
- Incubate the AllPrep DNA spin column with lid open at room temperature (15–25°C) for 2 mins.

(10) **Elute the genomic DNA**
Place the AllPrep DNA spin column in a new 1.5ml Eppendorf tube. Add 50µl of Nuclease-treated water directly to the spin column membrane and close the lid. Incubate at room temperature (15–25°C) for 1 min. Centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the DNA. Using the 50uL eluate, repeat step 10. Reuse the 1.5ml Eppendorf tube. Centrifuge for 1 min at ≥8000 x g (≥10,000 rpm).
(^These are modified **STEPS 1-3** of the **AllPrep DNA/RNA Mini Kit Quick-Start Protocol, Part 2, Genomic DNA purification)**.

- Resuspend all DNA samples in 50 µL of Nuclease free water. Resuspend all RNA pellets in 50 µL of RNA storage solution (Invitrogen: https://www.thermofisher.com/order/catalog/product/AM7001).

- For subsequent timepoints (Generally Days 13, 17, and 21) isolate DNA only, using the **QIAGEN Dneasy** kit (see manufacturer instructions for 1d, Cultured Cells): https://www.qiagen.com/us/resources/resourcedetail?id=aa250d94-fc4b-4e27-bb74-d32391ff8a48&lang=en).
- We double Qiagen's recommendation of 20uL RNase A and 20uL of Proteinase K to 40uL each for cell pellets greater than 10 million cells.

- Resuspend all DNA samples in 50 µL of Nuclease free water.

PreSequencing Amplification:

2 Library prep from each DNA sample requires three consecutive PCRs:

- PCR1 or selection PCR leveraging genomic primers specifically designed to amplify the SNV library from DNA integrated in HAP1 genomes, and not from library plasmid DNA.
- PCR2, or target specific PCR, uses PCR1 amplicons as template to amplify a sequencing amplicon spanning the SNV library.
- PCR3, or index PCR, uses universal adapters in the sequencing amplicon as handles to link dual sequencing indices.

PreSequencing PCR1 or selection PCR:

3

Note

Gradient PCR: using unedited HAP-1 DNA as template, perform an 8-temperature gradient PCR (Tm range 58 °C - 68 °C) to determine the optimal PCR conditions for each PCR1 primer pair. Set up gradient PCRs in 10 µL reaction volume with 50 ng total DNA.

PreSequencing PCR1: Multiple technical replicates of each DNA sample are amplified in PCR1.

- For each Day 5 DNA sample, set up to amplify 8 replicates in independent PCR wells.
- For each Day 13, Day 17 and/or Day 21 samples set up to amplify 16 replicates in independent PCR wells.

Since cell populations are generally larger at later timepoints, an increased number of PCR reactions is used to minimize jackpotting and sufficiently sample the cell population.

3.1 Prepare one MasterMix per DNA to be amplified with enough reagents for the total number of replicates; include DNA in the matsermix and dispense equally in wells (8 for D5 samples, 16 for D13, D17 or D21 samples).

	A	B	C	D	E	F	G	H	I
	Sample		**d5 NC**	**d5 R1**	**D5 R2**	**d5 R3**	**d13 R1**	**d13 R2**	**d13 R3**
	DNA Quants (ng/uL)		152	195	210	529	784	799	613
	Master Mix	x 1	x 8	x 8	x 8	x 8	x 16	x 16	x 16
	2X KAPA HiFi	25	200	200	200	200	400	400	400
	10um_F	1.5	12	12	12	12	24	24	24
	10um_R	1.5	12	12	12	12	24	24	24
	DNA	250 ng	13.2	10.3	9.5	3.8	5.1	5	6.5
	Water	to 25 uL	162.8	165.7	166.5	172.2	346.9	347	345.5
	Total	50	400	400	400	400	800	800	800

Example table depicting mastermix volumes of Negative Control, 3 biological replicates of Day 05 harvests, and 3 biological replicates of Day 13 harvests.

	A	B	C
	STEP	**TEMP**	**TIME**
	Initial Denaturation	95°C	4 minutes
	10-20 Cycles*	98°C	30 seconds
		(tbd)°C	15 seconds
		72°C	40 seconds
		72°C	4 minutes
	Hold	4°C	o/n

*Do not over-amplify, end PCR at inflection point before reaching plateau.

3.2 Carry out PCR at optimal Tm and cycle number (based on gradient PCR parameters), do not over-amplify.

3.3 Pool 1/5 volume from each PCR sample (e.g. 10 µL of 50 µL reaction) and merge in an Eppendorf tube.

3.4 Clean with 1.2x AMPure beads followed by two 80% EtOH washes and resuspend in initial volume of water (resuspend in 80 µL water if pooling 10 µL from each of the 8 PCR replicates and 160 µL water if 16 PCR replicates).

3.5 Verify amplicon size on a 2% agarose gel in TAE buffer (120V, 00:00:00).

4 **PreSequencing PCR2:** amplify the specific target use sequencing primers to link universal adapters to the sequencing amplicon.

4.1 Set up a MasterMix for the total number of samples (PCR1 replicates have been merged) in 25 µL reaction volume, adding DNA (AMPured PCR1) independently to each PCR reaction.

	A	B
	Master Mix	**x 1**
	2X KAPA HiFi	12.5
	10um_F (TrueSeqR1)	0.75
	10um_R (NexteraR2)	0.75
	water	8.88
	100x Sybr Green	0.13
	Total	23
	AMPured Rxn_1	2
		25

	A	B	C
	STEP	TEMP	TIME
	Initial Denaturation	98°C	3 minutes

	A	B	C
	10-20 Cycles (TBD)*	98°C	20 seconds
		60°C	15 seconds
		72°C	15 seconds
	Hold	4°C	o/n

*Do not over-amplify, end PCR at inflection point before reaching plateau.

4.2 Perform PCR2 following standard parameters (Tm 60 °C); abort reaction at amplification inflection before reaching amplification plateau.

4.3 Clean with 1.2 x AMPure beads and resuspend in 100 µL volume.

4.4 Use 2 µL as template for PCR3.

5 **PreSequencing PCR3:** amplify the sequencing amplicon from PCR2 to add sequencing indices.

5.1 Prepare a MasterMix for the total number of samples to be amplified; add primers and DNA (AMPured PCR2) independently to each well.

	A	B	C
	MasterMix	**x 1**	**x29**
	2X KAPA HiFi (RM)	12.5	362.5
	100x Sybr	0.13	3.6
	water	7.38	213.9
	Total	20	580
	10um_F (TrueSeq_P5)	1.5	

	A	B	C
	10um_R (Nextera_P7)	1.5	
	AMPured Rxn_2	2	
		25	

	A	B	C
	STEP	TEMP	TIME
	Initial Denaturation	98°C	30 seconds
	10-20 Cycles (TBD)**	98°C	10 seconds
		66°C	15 seconds
		72°C	15 seconds
	Hold	4°C	o/n

*Do not over-amplify, though make sure to obtain enough library for pooling.

5.2 Clean with 1.2 x AMPure beads and resuspend in 25 µL volume.

5.3 Check PCR2 and PCR3 on a 2% agarose gel; if loaded next to each other, it should be possible to observe the larger size of the PCR3 amplicon due to the sequencing indices.

5.4 Prepare a 1:10 dilution of the AMPured PCR3.

5.5 Using a Qubit High Sensitivity DNA kit, or similar, determine the concentration of each sample and dilute each library to 2 µL.

5.6 In an Eppendorf tube, pool equal volumes (e.g., 5 µL) of each 2 µL library for sequencing.

5.7 Check concentration using a Qubit High Sensitivity DNA kit.