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Processing samples following intracellular FACS screen

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We use this protocol and it's working

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Abstract

This protocols describes methods for processing samples following intracellular FACS screens.

Guidelines

Use fresh everything- Libraries are easy to contaminate. Preserve coverage at every step until after PCRs

Materials

Arcturus PicoPure DNA Extraction Kit

3M sodium acetate pH 5.5

20 mg/mL glycogen

100% ethanol

ice cold, 70% ethanol

1% agarose gel

Q5 2X Master Mix (New England Biolabs)

SPRI beads in PEG

80% ethanol

Qubit and reagents

PCRs primers to amplify the hU6-sgRNA-tracrRNA region from the genome and add adapters for sequencing. List of i7 primer options, barcode is **bolded**.

	i7_BC1_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GACTCGGTGCCACTTTTTCAA
	i7_BC2_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GACTCGGTGCCACTTTTTCAA
	i7_BC3_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**CACTGT**GACTCGGTGCCACTTTTTCAA
	i7_BC4_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GACTCGGTGCCACTTTTTCAA
	i7_BC5_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**GATCTG**GACTCGGTGCCACTTTTTCAA
	i7_BC6_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**TACAAG**GACTCGGTGCCACTTTTTCAA

	i7_BC7_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GACTCGGTGCCACTTTTTCAA
	i7_BC8_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GACTCGGTGCCACTTTTTCAA
	i7_BC9_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**TCAAGT**GACTCGGTGCCACTTTTTCAA
	i7_BC10_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**AGCTAG**GACTCGGTGCCACTTTTTCAA
	i7_BC11_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**GTCGTC**GACTCGGTGCCACTTTTTCAA
	i7_BC12_Miseq_gRNAlib_rev	CAAGCAGAAGACGGCATACGAGAT**CGATTA**GACTCGGTGCCACTTTTTCAA

Troubleshooting

Reverse Crosslinking

1 1. We use the Arcturus PicoPure DNA Extraction Kit. This protocol is for 10^5 – 10^6 cells. Scale up/down appropriately. **We follow their protocol except we reverse crosslink overnight instead of their recommended 3 hours.**

2 Add 155ul of Reconstitution Buffer into one vial of Proteinase K.

2.1 Gently vortex and immediately place on ice, use ASAP

3 To your pelleted cells, add 150ul reconstituted Proteinase K

3.1 Vortex gently to mix

4 Incubate samples at 65 C overnight

5 Incubate 95 C for 10 mins to inactivate Proteinase K

Ethanol Precipitation

6 1. Add 1/10 volume 3M sodium acetate pH 5.5, 2.2X volume 100% ethanol, and 1/100 volume of 20 mg/mL glycogen to tubes (i.e. for a 100 uL gibson reaction, add 1 uL glycogen, 10 uL sodium acetate, and 220 uL ethanol)

7 Freeze overnight at -80 C

8 Allow tube to thaw on ice (should only take a few minutes)

9 Centrifuge for 15 minutes at 15,000 g, 4C, precipitate should be a visible at bottom of tube

10 Gently remove supernatant by pouring

11 Do two consecutive washes with 1 mL of ice cold, 70% ethanol. Hold tube at 180 degree angle and slowly dispense volume of ethanol, gently tilt back and forth, and then pour off ethanol.

11.1 Use pipette to remove residual ethanol, but do not interfere with pellet

12 Allow DNA to dry by opening lid and placing tube on 37C heat block with kimwipe covering tube for ~5 minutes.

13 Resuspend DNA in small volume (~25 uL) of water, quantify using Nanodrop

PCRs

14 Do a test PCR to ensure the rxn conditions are correct. If these look good, then proceed to full PCRs. Generally I do 1, 25ul PCR for each sample, then proceed to full if you see a clean band with each, but this can be modified depending on your coverage / volume / etc. These PCRs will amplify the hU6-sgRNA-tracrRNA region from the genome and add adapters for sequencing. See Materials for list of 12 barcode options for the i7 primer, barcode is **bolded.**

15 Set up test PCRs with i5 fwd primer, AATGATACGGCGACCACCGAGATCTACACAATTTCTTGGGTAGTTTGCAGTT, and one of the i7 barcoded primers (see Materials).

	A	B	C
	Reagent	**Single Rxn (25 ul)**	**Single Rxn (50 ul)**
	Q5 2X MM	12.5	25
	Fw Primer i5_miseq_gRNAlib_fw 10uM	1.25	2.5
	Rev Primer: (i7 BC##) 10uM	1.25	2.5
	Template	250 ng	500 ng
	Water	To 25 ul	To 50 ul

	A	B	C
	Initial denaturation	98 C	30 sec
	25 cycles	98 C	10 sec
		60 C	30 sec
		72 C	20 sec
	Final Extension	72 C	2 min
	Hold	4 C	inf

16 Run 5 ul PCR product on a 1% agarose gel, check band (product should be ~270bp)

17 If bands look good, proceed to full PCRs. Match conditions exactly except you can scale up volume if needed to a max of 50ul

18 If you split sample across multiple tubes, mix product together so you have 1 tube for each sample. After this point, you can move forward with an aliquot of PCR product

Double-sided selection with SPRI beads

19 Vortex SPRI bead bottle and aliquot required volume

20 Let SPRI beads in PEG solution equilibrate to RT

21 Add 0.6x SPRI beads to DNA based (i.e if 100 uL of DNA sample, add 60 uL of SPRI beads)

22 Incubate for 5 minutes

23 Place on magnet for 2 minutes

24 Transfer supernatant containing desired amplicon product and primer dimers into a new tube, beads contain unwanted plasmid DNA

25 Add 1.8x SPRI beads (since the PEG solution still remains from the 0.6x step, we only need to add 1.2x SPRI beads of the original volume, i.e if 100 uL of starting DNA sample, add 120 uL of SPRI beads)

25.1 Mix thoroughly by pipetting up and down 15+ times

26 Incubate at RT for 15 minutes

27 Place on magnet for 10 minutes

28 Discard supernatant containing primer dimer

29 Wash 2x with 200 uL freshly prepared 80% EtOH

30 Dry beads for 5 minutes on 37 C heat block with lid open and covered with KimWipe

30.1 Discard residual ethanol at bottom of tube

31 Elute with ~22 uL of H20

31.1 Mix thoroughly by pipetting up and down for 20+ times

32 Incubate at 37C heat block for 5 minutes, helps displace bound DNA from beads

33 Return to magnet for 2 minutes

34 Take ~20 uL of supernatant and transfer to a new tube

Measure DNA concentration with Qubit

35 Collect reagents and bring to RT

36 Prepare working solution: 1 uL of 200x fluorescent reagent + 199 uL uL of buffer (make enough for half another reaction, i.e if measuring 6 samples, prepare 1300 uL of working solution (6.5 * 200 uL)

37 Add 190 uL of working solution to standard tubes and 198 uL of working solution to samples

38 Add 10 uL of standards and 2 uL of DNA samples to corresponding tubes

39 Vortex to mix and incubate at RT for 2 minutes

40 Measure concentrations and change home screen to calculate ng/uL for subsequent calculations

41 Calculate the volume of each sample to constitute a 2 nM concentration based on amplicon length

42 Prepare 2 nM working stocks of each library