WP6 STANDARD OPERATING PROCEDURES

BARCODING OF FRESH SAMPLES AT UNIFI

CONTENTS

1. DNA Extraction

This protocol is based on a minimally destructive extraction process that allows recovery of the fresh sample for further subsequent analysis. It involves only a digestion phase without further purification. Listed here are materials that are commonly used in our laboratory. However you can use whichever ones you already own or are easiest for you to acquire.

Reagents and consumables

- Extraction buffer (100mM Tris-HCl, 5mM EDTA, 100mM NaCl, 0.5%SDS, pH 8)
- Proteinase K
- 15ml Falcon tubes
- 50ml Falcon tubes
- 96 well plates
- 1000ul tips
- 200ul tips
- 10ul tips
- Gloves

Equipment

- Single channel pipette (100-1,000 µL, 20-200 µL, 2-20 µL)
- Multichannel pipette (10-100 µL)
- Shaker (should be able to run a stable 56ºC overnight)
- Falcon tube racks
- Vortex
- Tabletop minifuge
- Plate centrifuge
- PCR strip cap tool
- 70-80% ethanol for cleaning surfaces

Procedure

1. Take the samples out of the freezer and leave to thaw at room temperature
2. Preheat the incubator to 40°C
3. Centrifuge the plate at maximum speed for 30 seconds
4. Remove as much ethanol from the plate using a multichannel pipettes avoiding causing any damage to the samples
   NOTE: change the tips for each plate row
5. Leave the open plate in the incubator at 40°C until all ethanol evaporates (typically takes about 15 minutes if there is < 10 µL of liquid remaining in each well).
   NOTE: Be careful not to let the sample dry out totally

6. Meanwhile prepare the extraction mix: add 5ul of proteinase K and 95ul of extraction buffer for each sample. In a 15ml vial, add 9.5ml of extraction buffer and 500ul of proteinase K (volumes for 100 samples)
   NOTE 1: Add Proteinase K just before use for maximum enzymatic activity.
   NOTE 2: A larger volume of extraction buffer without Proteinase K can be stored at room temperature for a prolonged time; just take an adequate aliquot and add Proteinase K to it once you are ready to perform the overnight extraction.
7. Mix the buffer by slowly inverting the vial. Pour the prepared buffer into a multichannel reservoir
8. Add 100ul of extraction mix to each well (using a multichannel pipette) once ethanol has fully evaporated, making sure the samples are submerged.
   NOTE: change the tips for each sample
9. Once all samples are submerged in appropriate buffer volumes, close the plate with strip caps making sure no caps are loose as that might cause evaporation.
10. Vortex the plate for approximately 15 seconds at 2000 RPM and then do a short spin to collect the drops
11. Incubate the plate **overnight** in a shaker at 56°C and 2000 RPM
12. After incubation is complete, transfer the DNA extract to a new 96-well plate using a multichannel pipette. Store the plate at 4°C for subsequent PCR or store at -20°C for subsequent use.

2. Amplification and indexing of COI fragment using a single-step PCR protocol

This SOP describes the procedure for high-throughput generation of mitochondrial cytochrome c oxidase subunit I (COI) DNA barcode amplicons using very small quantities of crude DNA extracted using non-destructive protocol (i.e., without grinding or disruption to the organism). The amplification consists in the use of a single-step PCR.

A cocktail of two barcoded primer pairs are used: Folmer primers (LCO1490, HC02198, Folmer et al., 1994) and Lep primers (LepF1, LepR1, Herbert et al., 2004). Forward (or reverse) primers have the same barcode in a single PCR mix.

Primer sequences:

LepF1: 5’ ATTCAACCAATCATAAAGATATTGG 3’
LepR1: 5’ TAAACTTCTGGATGTCCAAAAAATCA 3’
LCO1490: 5’ GGTCAACAAATCATAAAGATATTGG 3’
HC02198: 5’ TAAACTTCAGGGTGACCAAAAAATCA 3’

This protocol use 36 barcodes for forward primers (LCO1490 and LepF1) and 32 barcodes for reverse primers (HC02198 and LepR1) to multiplex up to **1152 samples**.

Barcode sequences used for forward primers:

Barcode Name	Barcode Sequence (5'-3')
BC1001	CACATATCAGAGTGCG
BC1002	ACACACAGACTGTGAG
BC1003	ACACATCTCGTGAGAG
BC1004	CACGCACACACGCGCG
BC1005	CACTCGACTCTCGCGT
BC1006	CATATATATCAGCTGT
BC1007	TCTGTATCTCTATGTG
BC1008	ACAGTCGAGCGCTGCG
BC1009	ACACACGCGAGACAGA
BC1010	ACGCGCTATCTCAGAG
BC1011	CTATACGTATATCTAT
BC1012	ACACTAGATCGCGTGT
BC1013	CTCTCGCATACGCGAG
BC1014	CTCACTACGCGCGCGT
BC1015	CGCATGACACGTGTGT
BC1016	CATAGAGAGATAGTAT
BC1017	CACACGCGCGCTATAT
BC1018	TCACGTGCTCACTGTG
BC1019	ACACACTCTATCAGAT
BC1020	CACGACACGACGATGT
BC1021	CTATACATAGTGATGT
BC1022	CACTCACGTGTGATAT
BC1023	CAGAGAGATATCTCTG
BC1024	CATGTAGAGCAGAGAG
BC1025	CGCGACACGCTCGCGC
BC1026	CACAGAGACACGCACA
BC1027	CTCACACTCTCTCACA
BC1028	CTCTGCTCTGACTCTC
BC1029	TATATATGTCTATAGA
BC1030	TCTCTCTATCGCGCTC
BC1031	GATGTCTGAGTGTGTG
BC1032	GAGACTAGAGATAGTG
BC1033	TCTCGTCGCAGTCTCT
BC1034	ATGTGTATATAGATAT
BC1035	GCGCGCGCACTCTCTG
BC1036	GAGACACGTCGCACAC

Barcode sequences used for reverse primers:

Barcode Name	Barcode Sequence (5'-3')
BC1065	GCGCTCTCTCACATAC
BC1066	TATATGCTCTGTGTGA
BC1067	CTCTATATATCTCGTC
BC1068	AGAGAGCTCTCTCATC
BC1069	GCGAGAGTGAGACGCA
BC1070	TGCTCTCGTGTACTGT
BC1071	AGCGCTGCGACACGCG
BC1072	AGACGCGAGCGCGTAG
BC1073	GCGTGTGTCGAGTGTA
BC1074	TGTACGCTCTCTATAT
BC1075	TAGAGAGCGTCGCGTG
BC1076	GTGCACTCGCGCTCTC
BC1077	TATCTCTCGAGTCGCG
BC1078	CTCACACATACACGTC
BC1079	ATAGTACACTCTGTGT
BC1080	TATCTCTGTAGAGTCT
BC1081	GATATATATGTGTGTA
BC1082	GTGACACACAGAGCAC
BC1083	ATATGACATACACGCA
BC1084	CGTCTCTCGTCTGTGC
BC1085	ACACAGTAGAGCGAGC
BC1086	GTCGCGCATAGAGCGC
BC1087	CTATCTAGCACTCACA
BC1088	CGTGTCACTCTGCGTG
BC1089	CGCGCGAGTATCTCGT
BC1090	AGCACACATATAGCGC
BC1091	GTATATATATACGTCT
BC1092	TCTCACGAGAGCGCAC
BC1093	TAGATGCGAGAGTAGA
BC1094	ATAGCGACATCTCTCT
BC1095	GCACGATGTCAGCGCG
BC1096	TGTGCTCTCTACACAG

Reagents and consumables:

- Kapa3G Plant PCR Kit
- Primers
- 96 well plates

- PCR strip cap tool
- 1000 ul tips
- 200ul tips
- 10ul tips
- 1.5ml tube
- Gloves

Equipment:

- Single channel pipette (100-1,000 µL, 20-200 µL, 2-20 µL)
- Multichannel pipette (10-100 µL)
- Vortex
- Tabletop minifuge
- Plate centrifuge
- 70-80% ethanol for cleaning surfaces

Procedure:

1. Prepare a 1:10 dilution of the DNA extract with PCR grade water in a separate plate.
2. Prepare an adequate volume of Master Mix in a 1.5ml tube using the following table (make sure to adjust the initial conc. of stock components if they differ):

Component	Single mix	Master mix (100 samples)	Final concentration
DNA	1 µl	/	N/A
PCR-grade water	2,6 µl	260 µl	N/A
2X Kapa3G Plant PCR Buffer	5 µl	500 µl	1x
Primer Mix 10uM	0,5 µl	/	0.6µM
25mM $MgCl_2$	0,8 µl	80,0 µl	2mM
2,5U/ul Kapa3G Plant DNA polymerase	0,1 µl	10,0 µl	0,25U
TOT	10 µl	1000 µl	

NOTE: vortex each component (not polymerase) and do a short spin to collect the drops by a tabletop minifuge before prepare the mix

3. Vortex the mix and do a short spin to collect the drops by a Tabletop minifuge
4. Put a new 96 well plate in a cooling tube rack and add **8.5µl of Master Mix** to each well
5. Add **0.5 µl of primers mix** in each well in the right order
6. Add **1 µl of DNA** in each well in the right order
7. Vortex the plate for approximately **15 seconds at 2000 RPM** and then do a short spin to collect the drops
   NOTE: make sure there are no bubbles at the bottom of the well

8. Put the plate in a thermocycler with the following cycling protocol:

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	20 sec	35
Annealing	52°C	15 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1

9. Check DNA amplification on a 1.2 % agarose gel.

3. PacBio library preparation and sequencing

1. Pool amplicons (all remaining volume) in a 15 ml vial
2. Run the pool on a Fragment Analyzer and check concentration using Quibit
3. Proceed to PacBio library preparation following the protocol:
   Preparation of multiplexed amplicon libraries using SMRTbell prep kit 3.0 (Primer-barcoded samples workflow) - https://www.pacb.com/wp-content/uploads/Procedure-checklist-Preparing-whole-genome-and-metagenome-libraries-using-SMRTbell-prep-kit-3.0.pdf
4. Sequence library on a 8M ZMW SMRT cells (PacBio Sequel IIe platform)