Manual MPS Library Amplification of the Mitochondrial DNA Control Region with the PowerSeq® CRM Kit

1 Purpose

1.1 To increase the amount of mtDNA available for DNA sequencing by performing an *in vitro* replication of template DNA in ten amplicons. Adaptors are incorporated to facilitate sequencing-by-synthesis.

2 LIMS Processing

2.1 Refer to the LIMS Process Manual for the general worksheet processing protocols.

2.2 The index primer combinations used will be indicated by adding the index primer identifier as a suffix to each sample name (e.g., sample_A1, sample_B1).

2.2.1 Refer to Table 1 below to determine the sample suffix for the index primer combinations used.

Table 1: Suffixes for Index Primer Combinations

PRIMERS	D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D501	_A1	_A2	_A3	_A4	_A5	_A6	_A7	_A8	_A9	_A10	_A11	_A12
D502	_B1	_B2	_B3	_B4	_B5	_B6	_B7	_B8	_B9	_B10	_B11	_B12
D503	_C1	_C2	_C3	_C4	_C5	_C6	_C7	_C8	_C9	_C10	_C11	_C12
D504	_D1	_D2	_D3	_D4	_D5	_D6	_D7	_D8	_D9	_D10	_D11	_D12
D505	_E1	_E2	_E3	_E4	_E5	_E6	_E7	_E8	_E9	_E10	_E11	_E12
D506	_F1	_F2	_F3	_F4	_F5	_F6	_F7	_F8	_F9	_F10	_F11	_F12
D507	_G1	_G2	_G3	_G4	_G5	_G6	_G7	_G8	_G9	_G10	_G11	_G12
D508	_H1	_H2	_H3	_H4	_H5	_H6	_H7	_H8	_H9	_H10	_H11	_H12

3 Preparation

3.1 Retrieve the following reagents and thaw if necessary. After thawing, the components should be vortexed for 5 seconds.

PowerSeq® 5x Master Mix
PowerSeq® CRM Nested 10x Primer Pair Mix
10x Index D5 Primer (All relevant tubes)
10x Index D7 Primer (All relevant tubes)
2800M control DNA (10 ng/µL)
Laboratory-grade water
Promega amplification-grade water

3.2 Log all reagent lot numbers in LIMS.

3.3 Retrieve a 96-well Eppendorf PCR plate and label it with the run name and date, followed by "amplified libraries."

3.4 Locate the amplification sheet for your run in LIMS.

3.5 Preparing Sample Dilutions:

3.5.1 Referring to the amplification sheet, determine whether the samples for your run require dilution prior to amplification**.** The dilution factor is based on the nuclear DNA concentration for each sample.

3.5.1.1 A target amount of 500 pg of template for each sample will be added to each well.

3.5.2 If dilutions are needed, retrieve and label 1.5 mL microcentrifuge tubes.

3.5.3 Perform the sample dilutions by adding template DNA and laboratory-grade water in the volumes indicated in Table 2 below. Briefly vortex and centrifuge samples prior to aliquoting for dilution.

Table 2: Dilutions		
Dilution	**Amount of DNA Template (µL)**	**Amount of Laboratory Grade Water (µL)**
0.25	3 or (2)	9 or (6)
0.2	2	8
0.1	2	18
0.05	2	38
0.04	4 or (2)	96 or (48)
0.02	2 or (1)	98 or (49)
0.01	2	198
0.008	4 or (2)	496 or (248)

3.6 Preparing Positive Control:

3.6.1 Retrieve and label a 1.5 mL microcentrifuge tube.

3.6.2 Vortex and briefly centrifuge the stock 2800M Control DNA (10 ng/µL).

3.6.3 Dilute the control DNA to 0.1 ng/µL by adding the 2800M Control DNA and Promega amplification-grade water in the volumes indicated in Table 3 below.

Table 3: Preparing Positive Control	
Volume of 2800M Control DNA (µL)	**Volume of Water, Amplification-Grade (µL)**
2	198

Note: 2800M Control DNA should be stored at 4 °C after initial thaw.

3.7 Preparing master mix:

3.7.1 Retrieve and label a 1.5 mL microcentrifuge tube.

3.7.2 Referring to the reagent tab in LIMS, create the master mix by adding PowerSeq® 5x Master Mix and PowerSeq® CRM Nested 10x Primer Pair Mix in the calculated volumes.

Reagent	Per Reaction
PowerSeq® 5x Master Mix	5.0 µL
PowerSeq® CRM Nested 10x Primer Pair Mix	2.5 µL

3.7.3 Vortex and centrifuge briefly.

3.8 Arrange Samples and Reagents:

3.8.1 Samples should be arranged to begin in well A2. Up to 32 samples can be batched to form an amplification set. Thus, samples should be loaded in wells A2-H5.

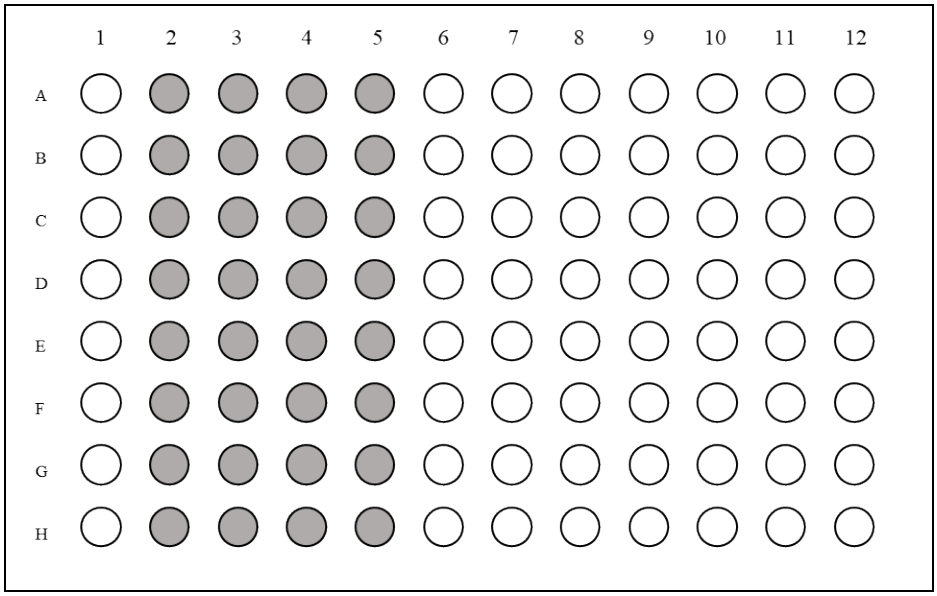

Figure 1: Samples will be loaded in wells A2-H5.

3.8.2 Vortex and centrifuge the samples briefly. Arrange your samples in the order indicated on the PowerSeq® Sample Sheet and/or LIMS.

3.8.3 Arrange the index primers for your run in the order reflected by the suffixes of the samples being tested. The arrangement of the index primers should result in a unique combination of index primers for each sample.

3.8.3.1 A different D5 index primer will be loaded in each row of the 96-well plate. The specific index primers due to be used for your run will be indicated in LIMS.

3.8.3.2 A different D7 index primer will be loaded in each column of the 96-well plate. The specific index primers due to be used for your run will be indicated in LIMS.

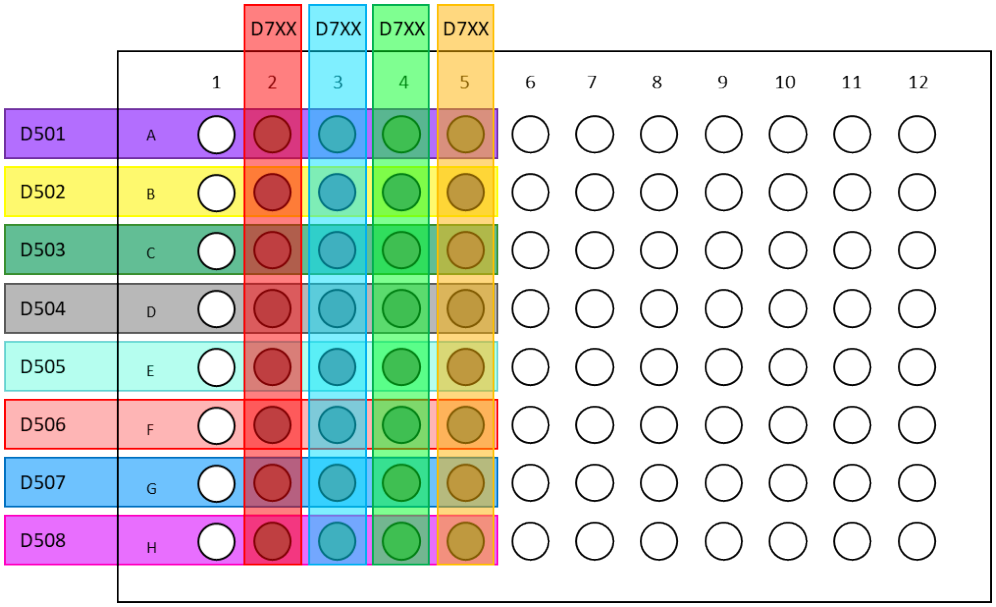

Figure 2: Generic Plate Layout for PowerSeq® CRM Library Amplification

3.9 Witness Step. Have another analyst witness the sample setup.

3.9.1 Read tube label to check sample order against the plate layout in LIMS.

4 Procedure

4.1 Load 7.5 µL of master mix into each well in use.

4.2 Load 2.5 µL of the index primers to each well in the appropriate arrangement.

4.2.1 A multichannel pipet may be used to load the index primers. If using a multichannel pipet, follow steps 4.2.1.1-4.2.1.5 below.

4.2.1.1 Retrieve an unused 96-well plate.

4.2.1.2 Into column 1 of the new plate, load 12.5 µL of each D5 primer in the appropriate order.

4.2.1.3 Beginning with well A7, load 22.5 µL of each D7 primer to the new plate horizontally (from well A7-A10). Make sure the index primers are loaded in the appropriate order.

4.2.1.4 Using a multichannel pipet, transfer 2.5 µL of D5 primer to each well of the amplified library plate that will be in use. Change pipet tips as each column is loaded.

4.2.1.5 Using a multichannel pipet, transfer 2.5 µL of D7 primer to each well of the amplified library plate that will be in use. Change pipet tips as each row is loaded.

4.3 Consult the amplification sheet in LIMS and load the indicated volume of each sample or control to the appropriate wells of the plate.

4.3.1 Positive Control

4.3.1.1 Aliquot 5 µL of diluted positive control.

4.3.2 Amplification Negative Controls

4.3.2.1 Aliquot 12.5 µL of amplification-grade water.

4.3.3 Samples and Extraction Negative Controls

4.3.3.1 Aliquot samples and extraction negative controls according to the amplification sheet.

NOTE: While extraction negative controls may be amplified at a dilution, the dilution factor must not exceed that of the associated samples.

4.4 Consult the amplification sheet in LIMS and load the indicated volume of Promega amplification-grade water to each well of the plate to bring the total amplification volume up to 25 µL.

4.5 Seal the plate using Bio-Rad film Microseal A.

4.6 Vortex the plate on a plate mixer at 1000 rpm for 1 minute. Centrifuge the plate at 1000 rpm for 1 minute.

4.7 For thermal cycler usage, see the Using the Mastercycler X50s manual.

4.8 The PowerSeq® CRM program is as follows:

Soak at 96 °C for 10 minutes		
	:	Denature at 96 °C for 5 seconds
30 Cycles	:	Anneal at 60 °C for 35 seconds
	:	Extend at 72 °C for 5 seconds
2-minute incubation at 60 °C		
Storage soak indefinitely at 4 °C		

4.9 After completion of the thermal cycling protocol, if not proceeding immediately with purification, centrifuge the plate and store the amplified product at -20 °C.