

Massively Parallel Sequencing of Mitochondrial Control Region using the PowerSeq[®] CRM Nested System, Custom

Promega Corporation

Materials Required

- PowerSeq[®] CRM Nested System, Custom (Promega Cat.# AX5810)
- MicroAmp[®] Optical 96-well Reaction Plate and MicroAmp[®] Cap Strips (Applied Biosystems Cat.# N8010560, Cat.# N8010535)
- GeneAmp[®] PCR System 9700 with a gold-plated or silver-plated sample block (Applied Biosystems)
- PowerSeq[®] Quant MS System (Promega Cat.# PS5000)
- Qiagen GeneRead[™] Size Selection Kit (Qiagen Cat.# 180514) or Agencourt AMPure[®] XP System (Beckman Coulter Cat.# A63881)
- Magnetic separation stand: For individual tubes, we recommend the MagneSphere[®] Technology Magnetic Separation Stand (Promega Cat.# Z5342). For samples in 96-well plate format, we recommend Life Technologies DynaMag[™]-96 Side (Part# 12331D)
- Absolute ethanol (200 proof, molecular biology grade)
- Nuclease-free water
- 10mM Tris-HCl (pH 8.5)
- 2N NaOH (molecular biology grade)
- 10mM Tris-HCl + 0.1% Tween[®] 20 (pH 8.5)
- 50mM Tris-HCl (pH 8.0), 10mM CaCl₂
- PhiX Control (Illumina Cat.# FC-110-3001)
- Illumina MiSeq[®] Reagent Kit v3 (600 cycles, Illumina Cat.# MS-102-3003)
- Illumina MiSeq[®] or MiSeq FGx[™] Instrument

Description

Analysis of mitochondrial DNA (mtDNA) is sometimes the only way to identify unknown human remains in mass disaster and missing person cases. mtDNA analysis is traditionally performed using Sanger Sequencing on a capillary electrophoresis instrument. Benefits of using massively parallel sequencing for mtDNA analysis include better mixture deconvolution and higher throughput. The PowerSeq[®] CRM Nested System targets the human mtDNA control region, including HV1, HV2, and HV3—the most polymorphic regions in the mitochondrial genome. Due to smaller amplicon size, the PowerSeq[®] CRM Nested System generates more data from degraded DNA samples.

Increased mixture deconvolution and heteroplasmy resolution are achieved by deep sequencing coverage and digital read counts. Additionally, the use of small amplicons to sequence the mtDNA control region improves sequencing results from degraded samples. However, library preparation for many massively parallel sequencing workflows requires multiple enzymatic and purification steps that are time consuming and are often a source of variability and sample loss. The PowerSeq[®] CRM Nested System utilizes a nested amplification protocol that greatly reduces the number of steps and time required to produce libraries ready for sequencing. The protocol consists of a single PCR step to both amplify the target amplicons and incorporate indexed sequencing adapters. The system generates 10 small amplicons covering the control region of the mitochondrial genome in one multiplex. The targeted regions for amplification are designed to be in the range of 144–237bp to ensure optimal results from degraded samples.

This Application Note contains a protocol for use of the PowerSeq[®] CRM Nested System, Custom^(a) (Cat.# AX5810). Please contact your Promega representative to order the PowerSeq[®] CRM Nested System, Custom. Contact information available at: www.promega.com. E-mail: genetic@promega.com

Product Components and Storage Conditions

Product	Size
PowerSeq® CRM Nested System, Custom	100 reactions

Includes:

- 1 × 500µl PowerSeq® 5X Master Mix
- 1 × 250µl PowerSeq® CRM Nested 10X Primer Pair Mix
- 2 × 1,250µl Water, Amplification Grade
- 1 × 25µl 2800M Control DNA, 10ng/µl
- 1 × Proteinase K 10mg
- Indexing Primers
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D701
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D702
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D703
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D704
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D705
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D706
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D707
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D708
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D709
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D710
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D711
 - 1 × 75µl PowerSeq® Nested System Index Primer 1 D712
 - 1 × 75µl PowerSeq® Nested System Index Primer 2 D501
 - 1 × 75µl PowerSeq® Nested System Index Primer 2 D502
 - 1 × 75µl PowerSeq® Nested System Index Primer 2 D503
 - 1 × 75µl PowerSeq® Nested System Index Primer 2 D504
 - 1 × 75µl PowerSeq® Nested System Index Primer 2 D505
 - 1 × 75µl PowerSeq® Nested System Index Primer 2 D506
 - 1 × 75µl PowerSeq® Nested System Index Primer 2 D507
 - 1 × 75µl PowerSeq® Nested System Index Primer 2 D508

The PowerSeq® CRM Nested System includes sufficient reagents for 100 amplification reactions at 25µl per reaction. Components are shipped on dry ice.

Storage Conditions: For long-term storage, store all components except the 2800M Control DNA at –30°C to –10°C in a nonfrost-free freezer. Make sure that the 2800M Control DNA is stored at 2–10°C for at least 24 hours before use. For immediate use, components may be stored at 2–10°C for up to one year.

Protocol

Before You Begin

Determine the concentration of genomic DNA for your samples. Note that different quantification methods yield different values. We strongly recommend that you perform experiments to determine the optimal DNA template amount for the PowerSeq® CRM Nested System amplification reaction based on your DNA quantification method.

PCR Setup and Thermal Cycling

1. Thaw all PowerSeq® CRM Nested System components immediately before use.
2. Vortex the components thoroughly for 5 seconds.
3. Determine the number of reactions, including positive and negative controls. Add 1 or 2 reactions to this number.
4. The PCR amplification mix is prepared by combining the components shown below:

Component	Volume per Reaction	Number of Reactions	Final Volume
Water, Amplification Grade	To final reaction volume of 25µl		
PowerSeq® 5X Master Mix	5.0µl		
PowerSeq® CRM Nested 10X Primer Pair Mix	2.5µl		
10X Index Primer 1 (Added at Step 6)	2.5µl		
10X Index Primer 2 (Added at Step 7)	2.5µl		
Template DNA (0.5ng) (Added at Step 8)	Up to 12.5µl		
Total Volume	25.0µl		

5. Combine all PCR amplification mix components except for 10X Index Primer 1, 10X Index Primer 2 and Template DNA. Vortex for 5 seconds, and then add the required amount to each reaction well.
6. Add 2.5µl of the appropriate 10X Index Primer 1 to each reaction.
7. Add 2.5µl of the appropriate 10X Index Primer 2 to each reaction.

Note: Each sample in a sequencing pool will require a unique combination of Index Primer 1 and Index Primer 2.
8. Add template DNA. Mix by pipetting.
9. For the positive amplification control, vortex the 2800M

Control DNA and then dilute an aliquot to 0.1ng/μl. Add 5μl (0.5ng) of diluted DNA to a reaction well containing PCR amplification mix.

10. Seal the plate with strip caps. Briefly centrifuge the plate.
11. Program the thermal cycler using the conditions shown below. We recommend using 30 cycles. Be sure to select “Max” as the ramp speed and enter the reaction volume.

Thermal Cycling Protocol

96°C for 10 minutes, then:

30 cycles of:

96°C for 5 seconds,

60°C for 35 seconds,

72°C for 5 seconds, then:

60°C for 2 minutes

4°C soak

12. Place the plate in the thermal cycler, run the program and remove the plate from the thermal cycler.

Amplification Product Purification

Amplification reactions require size selection purification prior to sequencing. This can be performed using either column- or bead-based protocols described below.

Column Purification

Purify each amplification product using the Qiagen® GeneRead™ Size Selection Kit following the manufacturer’s protocol, “*GeneRead Size Selection of DNA Libraries Prepared with the GeneRead DNA Library Prep I Kit*”, eluting the purified DNA in 20μl of Buffer EB. Refer to the Qiagen® GeneRead™ Size Selection Handbook for details.

Bead Purification

To avoid bead clumping in downstream steps, the amplification reactions are treated with Proteinase K during the first bead purification step. Three rounds of bead purification are performed at a 1:1 sample to bead ratio to generate libraries ready for sequencing.

1. Proteinase K is supplied as a lyophilized powder. Reconstitute in 0.5ml of 50mM Tris-HCl (pH 8.0), 10mM CaCl₂ to make a 20mg/ml stock solution.

Note: The reconstituted protease should be dispensed into single-use aliquots and stored at –20°C, where it is stable for 2–3 months. Avoid multiple freeze thaws or exposure to frequent temperature changes as these can reduce product stability.

2. Dilute the 20mg/ml Proteinase K solution in 50mM Tris-HCl (pH 8.0), 10mM CaCl₂ to make a 360μg/ml Proteinase K working solution.
3. Add 5μl of the 360μg/ml Proteinase K working solution to each 25μl amplification reaction.
4. Add 30μl of well mixed, room-temperature AMPure® XP beads to each amplification reaction to generate a 1:1 sample-to-bead ratio with a final Proteinase K concentration of 30μg/ml. Mix thoroughly by pipetting up and down at least 10 times. Optimal yield and balance are dependent on thorough mixing of beads and samples. Solution should appear homogenous after mixing. Incubate at room temperature for 5 minutes.
5. Place the samples on the magnetic stand at room temperature for 5 minutes. Remove and discard the supernatant. Take care not to disturb the beads during the wash steps. With the plate on the magnetic stand, add 200μl of freshly prepared 80% ethanol to each well and incubate the plate at room temperature for approximately 30 seconds. Remove and discard the supernatant from each well.
6. Repeat the 80% ethanol wash for a total of two washes.
7. With the plate on the magnetic stand, allow the beads to air-dry for 5 minutes.
8. To elute the DNA, remove the plate from the magnet and add 28μl of 10mM Tris-HCl (pH 8.5) to each well. Resuspend the samples by pipetting, then incubate at room temperature for 2 minutes. Place the plate on the magnet until the sample clears. Being careful not to disturb the bead pellet, transfer 25μl to a new well.
9. Perform two additional rounds of purification using a 1:1 sample-to-bead ratio and wash steps as described above. No additional Proteinase K treatment is required.

Quantify and Normalize Libraries

1. For optimal results from your sequencing run, we strongly recommend quantifying the libraries using a qPCR-based quantification kit for Illumina libraries. We recommend the PowerSeq® Quant MS System. Follow the qPCR instructions in the *PowerSeq® Quant MS System Technical Manual #TM511*.

Note: We recommend quantifying each library in triplicate. The reagents provided with the PowerSeq® Quant MS System are sufficient for testing 100 samples in triplicate.

2. If the PowerSeq® Quant MS System is used for quantification, dilute the PowerSeq® CRM Nested libraries 1:100,000.
 - a. Dilute the PowerSeq® System DNA libraries 1:100 by carefully adding 2µl of the undiluted library to 198µl of the PowerSeq® Quant MS 1X Dilution Buffer.
 - b. Mix the dilution thoroughly by gently pipetting 10 times using a 200µl single-channel or multi-channel pipette set to 198µl.
 - c. Dilute the PowerSeq® System DNA libraries to 1:10,000 by carefully adding 2µl of the 1:100 dilution (from Step 2.a) to 198µl of the PowerSeq® Quant MS 1X Dilution Buffer.
 - d. Mix the solution thoroughly by gently pipetting 10 times using a 200µl single-channel or multi-channel pipette set to 198µl.
 - e. Add 2µl of the 1:10,000 dilution (from Step 2.c) to 18µl of PowerSeq® Quant MS 1X Dilution Buffer.
 - f. Mix the dilution thoroughly by pipetting 10 times. This results in a 1:100,000 dilution of the initial library concentration.
3. Based on the DNA quantification results obtained, normalize the DNA library concentrations for pooling. In order to allow a final pool concentration of 4nM when accounting for low concentration samples or NTCs, we recommend using the following procedure to set the dilution target for libraries requiring normalization:
 - a. Count the total number of libraries, including negative controls, that you plan to sequence.
 - b. Count the number of libraries in the pool with concentration greater than 1nM, including positive controls.
 - c. Calculate the dilution target concentration using the following equation:

$$\text{Adjusted Dilution Target for Normalized Libraries} = \frac{\text{Total Number of Libraries in Pool}}{\text{Number of Libraries Greater than 1nM in Pool}} \times 4\text{nM}$$

- d. Dilute each library with Resuspension Buffer or 10mM Tris-HCl (pH 8.5) to the adjusted dilution target.
 - e. Libraries with concentrations below the dilution target are not diluted prior to addition to the pool. Dilute NTC reactions in the same volumes as the lowest concentration sample library, or pool undiluted if the lowest concentration sample library is below the adjusted dilution target.
4. Pool equal volumes of the diluted libraries prepared in Step 3. Vortex the pooled libraries for 10 seconds to mix.

Prepare Libraries for Sequencing

- Denature and dilute the libraries prior to DNA sequencing as follows. For additional information, refer to the Denature and Dilute Libraries Guide (Illumina Part# 15039740 v03).
 - Dilute 2N NaOH to 0.2N with nuclease-free water.
 - Denature the libraries by combining 5µl of the pooled 4nM libraries and 5µl of 0.2N NaOH.
 - Incubate for 5 minutes at room temperature.
 - Add 990µl of chilled HT1 buffer (a component of the MiSeq® Reagent Kit v3). This results in a 20pM denatured pooled library.
- Prepare the PhiX Control as follows:

Component	Volume per Reaction
PhiX Control (10nM)	2.0µl
10mM Tris-HCl + 0.1% Tween® 20 (pH 8.5)	3.0µl
0.2N NaOH	5.0µl
Total Volume	10.0µl

- Incubate for 5 minutes at room temperature.
- Add 990µl of chilled HT1 buffer. The concentration of the PhiX Control is 20pM.

Note: The 20pM PhiX Control can be stored at –20°C for up to 2 weeks.
- Prepare the sequencing dilution as follows:

Component	Volume per Reaction
HT1 buffer	225µl
Pooled 20pM denatured libraries prepared in Step 2e	337.5µl
20pM PhiX Control working stock prepared in Step 5	37.5µl
Total Volume	600µl

- Note:** This dilution results in a 10% PhiX spike, which is required for low-complexity libraries.
- Perform DNA sequencing using the MiSeq® v3 Reagent Kit and MiSeq® v3 Reagent Cartridge as directed by the manufacturer. Perform 1 × 300 cycles run or 2 × 300 cycles paired-end run.

Please refer to section 5E, 5F and 5G in the *PowerSeq® 46GY System Technical Manual #TM522* for instructions to load samples, create a sample sheet and start a sequencing run on the MiSeq or MiSeq FGx instrument.

Instructions for Analysis with GeneMarker®HTS Software from SoftGenetics

GeneMarker®HTS Software is recommended for analysis of the sequencing data.

- Open the SoftGenetics GeneMarker®HTS software.
 - Under “Project Folder:” navigate to the folder where the analysis results are to be exported.
 - Under “Reference Path:” and “Motif Path:” default boxes can be checked.
 - Under the “Panel:” pulldown menu select **Promega_PowerSeq_CRM_v1**.
 - At the bottom of the screen select **Add** and navigate to the folder where the FASTQ files to be analyzed are located. Select all of the FASTQ files to be analyzed.
 - On the right hand side under “Sequencer:” make sure “Illumina” is marked.
 - Under “Filter Settings” go to the “Mito Alignment” tab and select “Input Region”. In the first row, enter 16013 in the Start column and 16569 in the End column. In the second row, enter 1 in the Start column and 592 in the End column. Make sure the “Template Length” box is not checked. This will set the region to be analyzed. Default settings in the “Mito Variant” tab are acceptable. Internal validation should be done to establish thresholds.
 - Click **OK** to begin analysis.
 - When the analysis is complete for each file, the file name font color will change from gray to black.
 - Select each file in the ‘Project Viewer’ one at a time. Results can be viewed in the “Mitochondrial Alignment” viewer panel.
 - Filter settings may be adjusted for each sample separately under “Filter Settings” in the Mito Variant and Mito Alignment tabs.
 - Major and minor variant reports, consensus sequences and other coverage information from each library can also be found as text files in the export folder specified in Step 2.

For more information about GeneMarker®HTS software, visit <https://softgenetics.com/GeneMarkerHTS.php> or contact SoftGenetics.
- Website: www.softgenetics.com
Technical Service: tech_support@softgenetics.com
Product Sales and Marketing: info@softgenetics.com

Ordering Information

Please contact your Promega representative to order the PowerSeq® CRM Nested System, Cat.# AX5810. Contact information available at www.promega.com
Email: genetic@promega.com

^(a)U.S. Pat. No. 6,242,235, Australian Pat. No. 761757, Canadian Pat. No. 2,335,153, Chinese Pat. No. ZL99808861.7, Hong Kong Pat. No. HK 1040262, Japanese Pat. No. 3673175, European Pat. No. 1088060 and other patents pending.

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