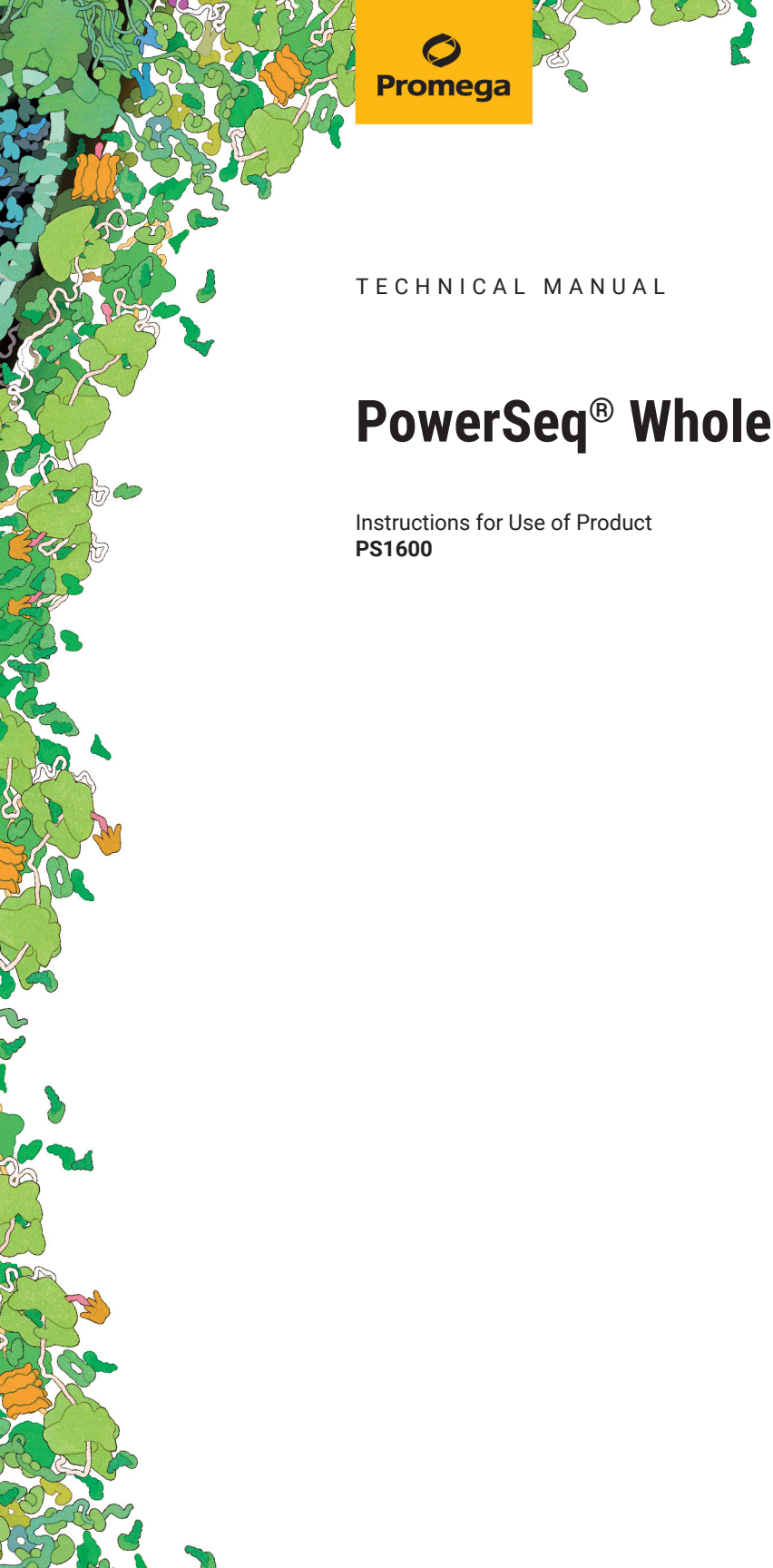

TECHNICAL MANUAL

PowerSeq® Whole Mito System

Instructions for Use of Product
PS1600

5/23
TM707

Promega

PowerSeq® Whole Mito System

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: genetic@promega.com

1. Description

In certain cases where forensic evidence was exposed to harsh conditions that can degrade DNA, mitochondrial DNA (mtDNA) sequencing may be essential for obtaining suitable DNA results (1,2). Massively parallel sequencing (MPS) has many advantages over traditional Sanger sequencing for mtDNA analysis, including increased resolution of mixtures and heteroplasmy, high-throughput sample processing and the ability to sequence the whole mitochondrial genome in fewer reactions (3–7). Whole mitochondrial sequencing can give added power of discrimination over control region alone (8–11).

The PowerSeq® Whole Mito System is designed to analyze the entire 16,569bp human mitochondrial genome using MPS. It includes reagents to amplify 161 small amplicons covering the entire mtDNA genome. The small amplicon format with a size range of 92–254 base pairs (167bp average) enables more efficient amplification from challenging degraded samples where nuclear DNA analysis may be difficult (12). Amplification instrumentation may vary. You may need to optimize protocols, including the amount of template DNA and cycle number for your laboratory instrumentation. In-house validation should be performed (13). The kit design includes a streamlined, integrated library prep workflow compatible with sequencing on the Illumina MiSeq® or Verogen MiSeq FGx™ instruments. A protocol to operate the massively parallel sequencing instrument should be obtained from the instrument manufacturer.

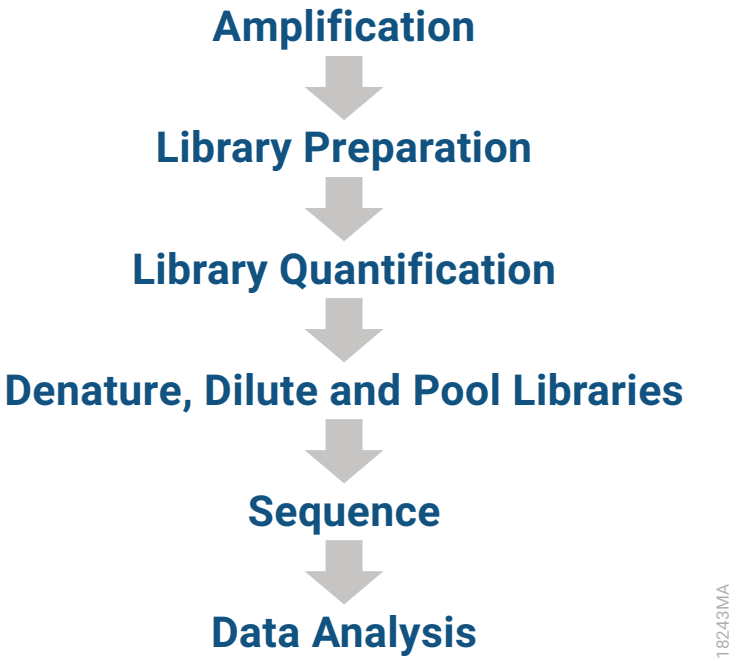

Figure 1. Overview of the PowerSeq® Whole Mito System workflow.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PowerSeq® Whole Mito System	**100 samples**	**PS1600**

Not for Medical Diagnostic Use. This system includes sufficient reagents for 100 reactions at 25µl per reaction.

The PowerSeq® Whole Mito System includes the PowerSeq® Whole Mito Amp & Prep Kit and PowerSeq® Whole Mito Purification Kit, including the following components:

PowerSeq® Whole Mito Amp & Prep Kit (components are shipped on dry ice):

Pre-Amplification Components Box:

- 1 × 500µl PowerSeq® Whole Mito 5X Master Mix
- 1 × 250µl PowerSeq® Whole Mito 10X Primer Pair Mix 1
- 1 × 250µl PowerSeq® Whole Mito 10X Primer Pair Mix 2
- 1 × 25µl 2800M Control DNA (10ng/µl)
- 2 × 1,250µl Water, Amplification Grade

Post-Amplification Components Box:

- 1 × 400µl Library Prep Reagent
- 6 × 500µl Library Prep Buffer

PowerSeq® Whole Mito Purification Kit (components are shipped at 4°C):

- 1 × 25ml ProNex® Size-Selective Chemistry
- 1 × 4ml ProNex® Size-Selective Binding Buffer
- 1 × 40ml Wash Buffer
- 1 × 15ml Elution Buffer
- 1 × 7ml Nuclease-Free Water
- 1 × 1ml Proteinase K

Storage Conditions: Upon receipt, store the PowerSeq® Whole Mito Amp & Prep Kit components at –30°C to –10°C in a nonfrost-free freezer. We strongly recommend that the pre-amplification and post-amplification reagents be stored and used separately with different pipettes, tube racks, etc. Store the 2800M Control DNA at +2°C to +10°C **for at least 24 hours before use**. After the first use, all other pre-amplification components can be stored at +2 to +10°C for up to 3 months. Store post-amplification kit components at –30°C to –10°C in a nonfrost-free freezer. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes for the Library Prep Reagent and Library Prep Buffer.

For the PowerSeq® Whole Mito Purification Kit, store the ProNex® Size-Selective Chemistry and Binding Buffer bottles at +2 to +10°C. Do not freeze. Store the remaining kit components at +15 to +30°C.

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3. Before You Begin

3.A. Precautions

The application of PCR-based typing for forensic and familial casework requires validation studies and quality-control measures that are not contained in this manual. Please refer to the appropriate guidelines (13–17).

The quality of purified DNA or direct-amplification samples, small changes in buffers, ionic strength, primer concentrations, reaction volume, choice of thermal cycler and thermal cycling conditions can affect PCR success. We advise strictly adhering to recommended procedures for amplification and library preparation. Additional research and validation are required if the recommended protocols are modified.

PCR-based mtDNA analysis is subject to contamination by very small amounts of human DNA. Take extreme care to avoid cross contamination when preparing template DNA, handling primer pairs, assembling amplification reactions and preparing sequencing libraries. Always include a negative-control reaction (i.e., no template) to detect reagent contamination. We highly recommend using gloves and aerosol-resistant pipette tips.

Some reagents used in mtDNA analysis are potentially hazardous and should be handled accordingly. Read the warning labels, take appropriate precautions when handling these substances and wear personal protective equipment, including eye protection, gloves and a lab coat. The reagents in the MiSeq® Reagent Cartridge contain formamide. Ensure that disposal of waste generated from the MiSeq® sequencing run complies with laboratory and governmental safety standards. Please refer to the MiSeq® System Guide #15027617 or the MiSeq FGx™ Instrument Reference Guide #VD2018006 for additional information on MiSeq® instrument waste.

Determine the genomic DNA concentration of your samples with a DNA quantitation method (e.g., PowerQuant® System). Note that different quantitation methods yield different quantitation values and that genomic DNA quantitation may not represent the amount of mtDNA present. We strongly recommend that you perform experiments to determine the optimal DNA template amount for the PowerSeq® Whole Mito System amplification reaction based on your DNA quantitation method.

The type and quantity of DNA samples to be sequenced should be considered when determining the number of samples to be multiplexed (i.e., pooled for simultaneous sequencing). Up to 24 reference samples can be sequenced simultaneously, with less multiplexing for more challenging samples (e.g., mixtures, degraded DNA samples). These are suggested guidelines; the number of samples that can be successfully multiplexed should be established through internal validation.

Deviations from the instructions provided in this technical manual are likely to impact system performance.

Please refer to the MiSeq® System Guide #15027617 or the MiSeq FGx™ Instrument Reference Guide #VD2018006 for troubleshooting instrument performance.

3.B. Materials to Be Supplied by the User

Instrumentation and Equipment

- ProFlex™ PCR System (Thermo Fisher Scientific), GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block, or Applied Biosystems Veriti® 96-well Thermal Cycler
- magnetic separation stand [For tubes, we recommend MagneSphere® Technology Magnetic Separation Stand (Cat.# Z5342). For 96-well plates, we recommend Thermo Fisher DynaMag™-96 Side (Part# 12331D).]
- centrifuge compatible with a 96-well plate
- Illumina MiSeq® or Illumina MiSeq FGx™ Instrument

Reagents

- IDT for Illumina - TruSeq® DNA UD Indexes v2 (Illumina Cat.# 20040870)
- MiSeq® Reagent Kit v3 (600 cycles, Illumina Cat.# MS-102-3003)
- PowerSeq® Quant MS System (Cat.# PS5000)
- absolute ethanol (200 proof, molecular biology-grade)
- Nuclease-Free Water (Cat.# P1193, P1195, or P1197)
- 2N NaOH (molecular biology-grade)
- PhiX Control v3 (Illumina Cat.# FC-110-3001)

Plastics

- aerosol-resistant pipette tips
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems, Cat.# N8010560)
- MicroAmp® Optical 8-Cap Strips or MicroAmp® Optical 12-Cap Strips (Applied Biosystems, Cat.# N8010535)
- adhesive seals for 96-well plates (Applied Biosystems Cat.# 4311971 or BioRad Cat.# MSB1001)

4. Protocols for DNA Amplification Using the PowerSeq® Whole Mito System

The PowerSeq® Whole Mito System is developed for amplifying extracted DNA. Slight protocol adjustments are recommended for optimal performance with each template source. The PowerSeq® Whole Mito System is compatible with the GeneAmp® PCR System 9700 thermal cycler with a gold-plated silver or silver sample block, the Veriti® 96-Well Thermal Cycler and the Applied Biosystems® ProFlex™ PCR System. This system has not been tested with the Veriti® 96-Well Fast Thermal Cycler or GeneAmp® PCR System 9700 with an aluminum block. We recommend using gloves and aerosol-resistant pipette tips to prevent cross contamination. Keep all pre-amplification and post-amplification reagents in separate rooms. Prepare amplification reactions in a room dedicated for reaction setup. Use dedicated equipment and supplies for amplification setup. Meticulous care must be taken to ensure successful amplification. A guide to amplification troubleshooting is provided in Section 7.

4.A. Amplification Setup

We recommend amplifying 100pg of total template DNA split into two separate 12.5µl reactions using the following protocol.

1. At the first use, thaw the PowerSeq® Whole Mito 5X Master Mix, PowerSeq® Whole Mito 10X Primer Pair Mixes 1 and 2, and Amplification Grade Water completely. After the first use, store the reagents at 2–10°C.
2. Ensure that the 2800M Control DNA has been stored at 2–10°C for at least 24 hours before use.

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mixes or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.
3. Determine the number of reactions to be set up (2 for each sample). This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that you will have enough amplification mix for all samples. It also ensures that each reaction contains the same amplification mix (see Tables 1 and 2).
4. Use a clean MicroAmp® plate for reaction assembly and label it appropriately. Alternatively, determine the number of new 0.2ml reaction tubes required and label them appropriately.
5. Add the final volume of each of the components in tables above to a separate, sterile tube. Prepare the amplification mix by combining volumes calculated in Step 3 of Amplification-Grade Water, PowerSeq® Whole Mito 5X Master Mix and PowerSeq® Whole Mito 10X Primer Pair Mix 1 or 2 to their respective reactions in the order listed in Tables 1 and 2. **Do not** add the template DNA until directed to do so in Step 8.
6. Vortex Amplification Mix 1 for 5–10 seconds, and then pipet amplification mix into each reaction well.

   Note: Failure to vortex Amplification Mix 1 sufficiently can result in poor amplification or locus-to-locus imbalance.
7. Vortex Amplification Mix 2 for 5–10 seconds, then pipet amplification mix into separate empty reaction wells.

   Note: Failure to vortex Amplification Mix 2 sufficiently can result in poor amplification or locus-to-locus imbalance.

Table 1. Amplification Mix 1. Final reaction volume = volume per reaction × number of reactions to be run.

Component	Volume Per Reaction	Number of Reactions	Final Volume
Water, Amplification-Grade[1]	To a final volume of 12.5µl		
PowerSeq® Whole Mito 5X Master Mix[1]	2.5µl		
PowerSeq® Whole Mito 10X Primer Pair Mix 1	1.25µl		
template DNA (50pg)[1–3]	(up to 8.75µl)		
total reaction volume	**12.5µl**		

Table 2. Amplification Mix 2. Final reaction volume = volume per reaction × number of reactions to be run.

Component	Volume Per Reaction	Number of Reactions	Final Volume
Water, Amplification-Grade[1]	To a final volume of 12.5µl		
PowerSeq® Whole Mito 5X Master Mix[1]	2.5µl		
PowerSeq® Whole Mito 10X Primer Pair Mix 2	1.25µl		
template DNA (50pg)[1–3]	(up to 8.75µl)		
total reaction volume	**12.5µl**		

[1]Add Water, Amplification Grade, to the tube first, then add PowerSeq® Whole Mito 5X Master Mix and PowerSeq® Whole Mito 10X Primer Pair Mix. The template DNA will be added at Step 8.

[2]Store DNA templates in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE^{-4} buffer with 20µg/ml glycogen. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the volume of DNA added should not exceed 20% of the final reaction volume. PCR efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl), available magnesium concentration (due to chelation by EDTA) or other PCR inhibitors, which may be present at low concentrations, depending on the source of the template DNA and the extraction procedure used.

[3]Apparent DNA concentrations can differ, depending on the DNA quantitation method used, and genomic DNA quantitation may not accurately reflect the amount of mitochondrial DNA present (18,19). We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method.

8. Split 100pg of template DNA of each sample into two reaction wells (50pg each), one containing Amplification Mix 1 and another containing Amplification Mix 2.

   Note: The PowerSeq® Whole Mito System was optimized and balanced using 100pg of total 2800M DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

4.A. Amplification Setup (continued)

9. For the positive amplification control, vortex the 2800M Control DNA and then dilute an aliquot to 20pg/µl by adding 2µl of 2800M Control DNA to 998µl of TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE^{-4} buffer with 20µg/ml glycogen. Add 2.5µl (50pg) of diluted DNA to separate corresponding reaction wells containing Amplification Mix 1 and Amplification Mix 2.

   Note: The 20pg/µl 2800M Control DNA dilution can be stored at 4°C for 24 hours.

10. For the negative amplification control, pipet Water, Amplification Grade, or TE^{-4} buffer instead of template DNA into reaction wells containing Amplification Mix 1 and Amplification Mix 2.

11. Seal or cap the plate, or close the tubes. **Optional:** Briefly centrifuge the plate or tubes to bring contents to the bottom of the wells, and remove any air bubbles.

4.B. Thermal Cycling

Amplification instrumentation may vary. You may need to optimize protocols including the amount of template DNA and cycle number for your laboratory instrumentation. Our recommended starting condition based on internal testing is 30 cycles for 50pg of purified DNA template per reaction.

After completing the thermal cycling protocol, prepare amplification products for sequencing or store amplified samples at –20°C. For short-term storage, amplified samples can be stored at 4°C.

1. Place the MicroAmp® plate or reaction tubes in the thermal cycler.

2. Select and run the recommended protocol provided in the table below. The total cycling time is less than 1 hour.

Thermal Cycling Protocol	
96°C for 1 minute, then:	
30 cycles of:	94°C for 10 seconds
	59°C for 1 minute,
	72°C for 30 seconds, then:
60°C for 10 minutes	
4°C soak	

Notes:

a. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.

b. When using the GeneAmp® PCR System 9700, the program must be run with 'Max Mode' as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.

3. After completion of the thermal cycling protocol, proceed to amplification product purification or store amplified samples at –20°C. For short-term storage, amplified samples may be stored at 4°C.

5. Preparing Amplification Products for Sequencing

5.A. Purifying Amplification Products

1. Let the ProNex® Size-Selective Chemistry stand for 30 minutes to 1 hour to equilibrate to room temperature.
2. Reconstitute the Wash Buffer by adding 200ml of 95–100% ethanol to the wash buffer provided in the kit. Alternatively, aliquots of the Wash Buffer may be removed and reconstituted in smaller containers if desired. Add 5 volumes of ethanol for every 1 volume of wash buffer.

   Notes:

   a. The ProNex® Size-Selective Chemistry contains 0.02% sodium azide. The reconstituted Wash Buffer contains 80% ethanol, which is flammable. Please safely handle and dispose of the liquid and dry wastes generated by this product in accordance with your institutional policies. Wear safety glasses, lab coat and gloves while handling samples and kit components.

   b. All purification steps should be performed at room temperature (15–30°C).

   c. This protocol and the sections following refer specifically to 96-well plates, but reactions may be performed in tubes with appropriate adjustments.
3. For each sample, pipette 10µl of the Amplification Mix 1 reaction and transfer to a new well of a 96-well plate.
4. Pipette 10µl of the Amplification Mix 2 reaction and combine it with the 10µl of Amplification Mix 1 reaction of the corresponding sample from Step 3.
5. Dilute the 20mg/ml Proteinase K by adding 50µl of 20mg/ml Proteinase K to 950µl of Nuclease-Free Water to make a 1mg/ml solution.
6. Add 5µl of the 1mg/ml Proteinase K to the 20µl of combined Amplification Mix 1 and 2 for each sample.

   Note: If the volume of the amplification reaction to be purified is not 20µl, then the final concentration of the Proteinase K must be adjusted so that, in a final reagent volume that includes the amplification reactions, ProNex® Size-Selective Chemistry and Proteinase K, the final concentration of Proteinase K is 20µg/ml.
7. Make sure the ProNex® Size-Selective Chemistry bottle cap is tightened securely and vortex vigorously 10 seconds or longer until well dispersed.
8. Add 75µl of the ProNex® Size-Selective Chemistry to each well (3:1 ratio to the amplification reaction volume and Proteinase K). Vortex the bottle of ProNex® Size-Selective Chemistry frequently to make sure that it is evenly distributed to the samples.
9. Mix thoroughly by gently pipetting 10 times using a 200µl single-channel or multichannel pipette set to 80µl.

   Note: The amplification reaction and ProNex® Size-Selective Chemistry are both viscous solutions that do not mix easily. Optimal yield and balanced representation of all whole mito amplicons is dependent on thorough mixing of the Size-Selective Chemistry and samples. The solution in the well should become an even light brown color after mixing. *For best practice*, draw from the bottom and release liquid near the top of the mixture. Continue mixing if the color of the solution is lighter or clear near the top. For more information, refer to the bead purification video on the PowerSeq® 46GY product page: **www.promega.com/products/forensic-dna-analysis-mps/target-amplification-and-library-prep/powerseq-46gy-system/**

5.A. Purifying Amplification Products (continued)

10. Incubate the plate at room temperature for 10 minutes.
11. Place the plate on a magnetic stand at room temperature for approximately 5 minutes or until liquid is clear.
12. Remove and discard the supernatant from each well, avoiding the resin.
13. With the plate on the magnetic stand, wash 2 times with 80% ethanol as follows:
    a. Add 200µl of Wash Buffer to each well.
    b. Incubate on the magnetic stand for 30 seconds.
    c. Remove and discard the supernatant from each well, taking care not to disturb the bead pellet.
    d. Repeat Steps 13.a–c for the second wash.
14. Use a 20µl pipette to remove residual ethanol from each well.
15. Air-dry the samples on the magnetic stand for 5 minutes.
16. Add 23µl of the Elution Buffer to each well.
17. Remove the plate from the magnetic stand.
18. Mix thoroughly by gently pipetting 10 times. For best mixing practices follow the technique noted in Step 9.
19. Incubate at room temperature for 2 minutes.
20. Place the plate on the magnetic stand and wait until the liquid is clear (~1 minute).
21. Being careful not to disturb the bead pellet, transfer 21µl of the supernatant to a new 96-well reaction plate.

Note: This is a safe stopping point. Seal the plate and store at –25°C to –15°C for up to 7 days.

5.B. Preparing the DNA Libraries

1. Leave the Sample Purification Beads out for 30 minutes to 1 hour to equilibrate to room temperature.
2. Thaw the Library Prep Buffer at room temperature. If precipitate is present, vortex the buffer until it is resuspended in solution.
3. Place the Library Prep Buffer and Library Prep Reagent on ice until use. Be sure to return Library Prep Buffer and Library Prep Reagent to –20°C after use.
4. Thaw the DNA Adapters in the IDT for Illumina TruSeq® DNA UD Index v2 Plate at room temperature for 10 minutes.
5. Briefly centrifuge the IDT for Illumina TruSeq® DNA UD Index v2 Plate to collect the contents at the bottom of the wells.
6. Pierce the foil seal on the IDT for Illumina TruSeq® DNA UD Index v2 Plate with the pipette tips to assemble the adapter ligation reactions described in the following step. (Return IDT for Illumina TruSeq® DNA UD Index v2 Plate to –20°C after use for up to four freeze-thaw cycles.) Cover the used wells with an adhesive seal.
7. Assemble the library prep reactions in the same plate and wells containing the purified amplification product as shown in the table below:

Component	Volume Per Sample
Purified Amplification Product (from Section 5.A)	21µl
Library Prep Buffer	30µl
Library Prep Reagent	4µl
DNA Adapter (from the IDT for Illumina TruSeq® DNA UD Index v2 Plate)	5µl

Note: If fewer than 16 libraries will be sequenced simultaneously, please refer to the Illumina Index Adapters Pooling Guide Document# 1000000041074 for strategies to select compatible adapters.

8. Mix each adapter ligation reaction thoroughly by gently pipetting 10 times.
9. Incubate the library prep reactions for 30 minutes at room temperature.

5.C. Purifying the DNA Libraries

1. If the Sample Purification Beads are not already at room temperature, equilibrate for 30–60 minutes at room temperature.
2. Using the provided Binding Buffer, make a binding buffer mixture for each sample, adding at least two samples to account for pipetting error, as shown in Table 3.

Table 3. Preparing Binding Buffer Mixture. Final volume = volume per reaction × number of libraries.

Component	Volume Per Reaction	Number of Libraries	Final Volume
Binding Buffer	30µl		
Nuclease-Free Water	15µl		
total reaction volume	**45µl**		

3. Tighten the ProNex® Size-Selective Chemistry bottle cap securely and vortex vigorously 10 seconds or longer until well dispersed.
4. Add 120µl of the Sample Purification beads to each well of the plate containing ligation products (2:1 ratio to the library prep reaction volume). Vortex the beads frequently to make sure that the beads are evenly distributed to the samples.
5. Mix thoroughly by gently pipetting 10 times using a 200µl single-channel or multichannel pipette set to 160µl.

   Note: The library prep reaction and purification beads are both viscous solutions that do not mix easily. Optimal yield and balanced representation of all whole mito amplicons is dependent on thorough mixing of the beads and samples. The solution in the well should become an even light brown color after mixing. For best practice, draw from the bottom and release liquid near the top of the mixture. Continue mixing if the color of the solution is lighter or clear near the top.
6. Incubate at room temperature for 10 minutes.
7. Place the plate on a magnetic stand at room temperature for approximately 2–3 minutes until clear.
8. Remove and discard the supernatant from each well, avoiding the resin.
9. Add 45µl of the binding buffer mixture (from Step 2) to each sample.
10. Remove the plate from the magnetic stand.
11. Cover the plate with an adhesive seal or strip caps and mix by vortexing.
12. Briefly spin the plate to remove liquid from the adhesive seal or strip caps.
13. Incubate at room temperature for 10 minutes.

14. Place the plate on a magnetic stand at room temperature for approximately 2–3 minutes until clear.
15. Remove and discard the supernatant from each well, avoiding the resin.
16. With the plate on the magnetic stand, wash 2 times with Wash Buffer as follows:
    a. Add 200µl of the Wash Buffer to each well.
    b. Incubate on the magnetic stand for 30 seconds.
    c. Remove and discard the supernatant from each well, taking care not to disturb the bead pellet.
    d. Repeat Steps 16 a.–c. for the second wash.
17. Use a 20µl pipette to remove residual ethanol from each well.
18. Air-dry the samples on the magnetic stand for 5 minutes.
19. Add 23µl of the Elution Buffer to each well.
20. Remove from the magnetic stand.
21. Mix thoroughly by gently pipetting 10 times. Alternatively, cover the plate with adhesive seal and mix by vortexing.
22. Incubate at room temperature for 5 minutes.
23. Place the plate on the magnetic stand, and wait until the liquid is clear.
24. Being careful not to disturb the bead pellet, transfer 20µl of the supernatant to a new 96-well plate.

Note: This is a safe stopping point. Seal the plate and store at –25°C to –15°C for up to 7 days.

5.D. Quantify and Normalize Libraries

1. To get optimal data from your sequencing run we strongly recommend quantifying the libraries using a qPCR-based quantitation kit for Illumina libraries. We recommend the PowerSeq® Quant MS System. Follow the qPCR instructions in the *PowerSeq® Quant MS System Technical Manual*, #TM511 at: **www.promega.com/protocols**

   Note: We recommend testing each library in triplicate. The reagents provided in the kit are sufficient for testing 100 samples in triplicate.

2. If the PowerSeq® Quant MS System is used for quantification, dilute the PowerSeq® Whole Mito 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 multichannel pipette set to 198µl.
   c. Dilute the PowerSeq® System DNA libraries 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 dilution thoroughly by pipetting 10 times.
   e. Dilute the PowerSeq® System DNA libraries 1:100,000 by carefully adding 2µl of the 1:10,000 dilution (from Step 2.c) to 18µl of the PowerSeq® Quant MS 1X Dilution Buffer.
   f. Mix the dilution thoroughly by pipetting 10 times.

3. Based on the DNA quantitation results obtained, normalize the DNA library concentrations for pooling. For 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 Elution Buffer to the adjusted dilution target.
   e. Libraries with concentrations below the dilution target are not diluted prior to addition to the pool.
   f. 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.

5.E. Denature and Dilute Libraries for Sequencing

Denature and dilute libraries following the protocol provided. For additional information, refer to the MiSeq® System Denature and Dilute Libraries Guide Document #15039740, v10.

1. Thaw the MiSeq® v3 reagent cartridge following the instructions provided in the MiSeq® System Guide #15027617.
2. Prepare a fresh dilution of 0.2N NaOH by adding 10µl of 2.0N NaOH to 90µl of Nuclease-Free Water. Dilution should be used within 12 hours. Vortex the dilution to mix thoroughly.

   Note: The NaOH stock may concentrate over time. Higher concentrations of NaOH can have deleterious effects on sequencing.
3. Thaw the HT1 hybridization (Hyb) buffer at room temperature. Store the HT1 buffer at 2–8°C until needed to dilute the denatured libraries.
4. Denature the libraries by combining components in a microcentrifuge tube as indicated below:

Component	Volume Per Sample
Pooled 4nM libraries (from Step 4, Section 5.C)	5µl
0.2N NaOH	5µl
total volume	**10µl**

5. Briefly vortex and incubate for 5 minutes at room temperature.
6. Add 990µl of chilled HT1 buffer to the tube containing the denatured library. Mix by inverting the tube or pipetting.

   Note: Library concentration is now 20pM.

7. Dilute and denature the PhiX Control v3 by combining components in a microcentrifuge tube as indicated below:

Component	Volume Per Sample
PhiX Control v3 (10nM)	2µl
Elution Buffer	3µl
0.2N NaOH	5µl
total volume	**10µl**

8. Briefly vortex and incubate for 5 minutes at room temperature.
9. Add 990µl of chilled HT1 buffer to the tube containing the denatured PhiX control. Mix by inverting the tube or pipetting.

   Note: PhiX Control concentration is now 20pM.
10. Prepare the sequencing dilution by combining components in a microcentrifuge tube as indicated below:

Component	Volume Per Sample
HT1 Buffer	195µl
20pM pooled and denatured libraries (from Step 6, Section 5.D)	365µl
20pM denatured PhiX Control (from Step 9, Section 5.D)	40µl
total volume	**600µl**

11. Mix the sequencing dilution by inverting the tube or pipetting.

    Note: If the sequencing dilution will not be immediately added to the cartridge for sequencing, place the dilution on ice until ready to add to the cartridge.

5.F. Instructions for Loading Sample Libraries onto the MiSeq® v3 Reagent Cartridge

1. Mix the thawed reagent cartridge by inverting 10 times.
2. Use a new 1ml pipette tip to pierce the foil seal over the well labeled “Load Samples”.

   Note: Do not pierce any other well positions.
3. Pipet all of the 600µl of prepared sample libraries into the “Load Samples” well. Avoid touching the seal while dispensing.

6. Operating the Illumina MiSeq

6.A. Setting up a Run in Local Run Manager

Create a run using the protocol provided. For additional information, refer to the Illumina Local Run Manager v3 Software Guide (Document# 1000000111492 v00) and Local Run Manager Generate FASTQ Analysis Module Workflow Guide (Document# 1000000003344 v03).

1. Open the Local Run Manager Software in Chromium.
2. Select **Create Run**.
3. Select **Generate FastQ**.
4. Enter a run name.
5. From the 'Library Prep Kit' drop-down library prep workflow, select **TruSeq DNA PCR-Free**.
6. Select **IDT-ILMN TruSeq DNA UD Indexes v2 (96 Indexes)** from the 'Index Kit' drop-down.
7. Select either **Single Read** or **Paired Read** (we recommend **Single Read** because of accumulated sequencing errors in read 2 and reduced time on the sequencer).
8. For Read Length enter **275** for Read 1. If **Paired Read** is selected enter **275** for Read 2.
9. Make sure Adapter Trimming is set to **ON**.
10. Enter a unique **Sample ID** for each sample.
11. Under Index Well select the appropriate well from the TruSeq® DNA UD Index v2 Plate that is associated with each sample.
12. Select **Save Run**.

6.B. Starting a MiSeq Sequencing Run

1. Perform DNA sequencing using the MiSeq® Reagent Kit v3 and MiSeq® Reagent Kit v3 Cartridge as directed by the manufacturer:

   For the MiSeq® instruments, refer to the MiSeq® System Guide, Document# 15027617 v05, Material# 20000262, Cat.# SY-411-9001DOC, August 2019.

   For the MiSeq FGx™, refer to the MiSeq FGx™ Instrument Reference Guide, Part# VD2018006 Rev. A, Cat.# SY-411-9001DOC, June 2018.

7. Sequencing Analysis

Analyze with commercial software such as SoftGenetics GeneMarker® HTS software.

7.A. Instructions for Analysis with SoftGenetics GeneMarker® HTS Software v2.6

The following instructions are for use with SoftGenetics GeneMarker® HTS software, **version 2.6 or later**. These instructions are **not** compatible with earlier software versions.

1. Open the SoftGenetics GeneMarker®HTS software.
2. Under Project Folder: navigate to the folder where the analysis results are to be exported.
3. Under Reference Path: and Motif Path: default boxes can be checked.
4. Under the 'Panel: pulldown' menu select **Promega_PowerSeq_WholeMito_v1**.
5. Under Panel Options: select **Allow Primer Mismatches.**
6. 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.
7. On the right hand side under Alignment Options: make sure **Illumina** is marked as the Sequencer, **Consensus and Motifs** options are selected, and Clip mismatched ends is not selected.
8. Under Filter Settings, we recommend a minimum 5% minor variant frequency percentage. Other default settings are acceptable. Internal validation should be done to establish thresholds.
9. Select **OK** to begin analysis.
10. When the analysis is complete for each file, the file name font color will change from gray to black.
11. Select each file in the Project Viewer one at a time. The file will open in the STR Results window. Select the **Mitochondrial Alignment** option in the top left side of the window to view mitochondrial results.
12. Filter settings may be adjusted for each sample separately.

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9. Troubleshooting

9.A. Amplification and Library Detection

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com** E-mail: **genetic@promega.com**

Symptoms	Causes and Comments
Low coverage	The PowerSeq® 5X Master Mix was not mixed well before use. Mix the 5X Master Mix using a vortex mixer for 15 seconds before dispensing into the PCR amplification mix.
	An air bubble formed at the bottom of the reaction tube or plate. Use a pipette to remove the air bubble or centrifuge the reactions briefly before thermal cycling.
	Thermal cycler, plate or tube problems. Review the thermal cycling protocol in Section 4. We have not tested other reaction tubes, plates or cyclers. Calibrate the thermal cycler heating block if necessary.
	Primer concentration was too low. Use the recommended primer concentration. Vortex the PowerSeq® Whole Mito 10X Primer Pair Mixes for 15 seconds before use.
	Samples were not denatured completely. Denature samples with 0.2N NaOH and incubate for 5 minutes at room temperature.
	Do not vortex the libraries once they are single-stranded.
Low or no coverage for the positive control reaction	Improper storage of the 2800M Control DNA. Ensure that the Preparing and Plating protocol is strictly followed.
High number of minor variants detected	Contamination with another template DNA or previously amplified DNA. Cross-contamination can be a problem. Use aerosol-resistant pipette tips, and change gloves regularly.
Imbalanced amplicon coverage	Miscellaneous balance problems. At first use, thaw the 10X Primer Pair Mixes and 5X Master Mix completely. Vortex the 10X Primer Pair Mix and 5X Master Mix for 15 seconds before use; do not centrifuge the 5X Primer Pair Mix and 5X Master Mix after mixing. Calibrate thermal cyclers and pipettes routinely.
	PCR amplification mix prepared in Section 4 was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.

9.A. Amplification and Library Detection (continued)

Symptoms	Causes and Comments
Imbalanced amplicon coverage (continued)	Instructions for library preparation were not followed. Ensure that each step of the workflow was performed as described.
	Variants that occur under primer amplification sites can cause loss of amplification efficiency at those locations.
	Purification steps with the Sample Purification Beads were not adequately mixed, or the ratio of beads was incorrect.

9.B. Amplification of Extracted DNA

The following information is specific to amplification of extracted DNA. For information about general amplification and detection, see Section 9.A.

Symptoms	Causes and Comments
Low coverage	Impure template DNA. Because a small amount of template is used, this is rarely a problem. Depending on the DNA extraction procedure used and sample source, inhibitors might be present in the DNA sample.
	Insufficient template. Use the recommended amount of template DNA if available.
	High salt concentration or altered pH. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the DNA volume should not exceed 20% of the total reaction volume. Carryover of K^+, Na^+, Mg^{2+} or EDTA from the DNA sample can negatively affect PCR. A change in pH also can affect PCR. Store DNA in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA), TE^{-4} buffer with 20µg/ml glycogen or Nuclease-Free Water. Faint or absent peaks may be seen more often when using the maximum template volume or reduced amplification reaction volume.
	The reaction volume was too low. This system is optimized for a final reaction volume of 12.5µl. Decreasing the reaction volume can result in suboptimal performance.

10. Related Products

Product	Size	Cat.#
PowerQuant® System	200 reactions	PQ5002
	800 reactions	PQ5008
Plexor® HY System	200 reactions	DC1001
	800 reactions	DC1000
PowerSeq® CRM Nested System, Custom	100 reactions	AX5810
PowerSeq® 46GY	100 reactions	PS4600

Not for Medical Diagnostic Use.

Accessory Products and Reagents

Product	Size	Cat.#
2800M Control DNA (10ng/µl)	25µl	DD7101
2800M Control DNA (0.25ng/µl)	500µl	DD7251
Water, Amplification Grade	6,250µl	DW0991

Not for Medical Diagnostic Use.

Product	Size	Cat.#
PowerSeq® Quant MS System	500 reactions	PS5000
CXR Reference Dye	100µl	C5411
Tris-HCl, Molecular Biology Grade (Tris-Hydrochloride)	100g	H5121
	500g	H5123
	2,500g	H5125
Proteinase K (Lyophilized)	100mg	V3021
Proteinase K (PK) Solution	4ml	MC5005
Nuclease-Free Water*	50ml	P1193
	150ml	P1195
	500ml	P1197

For Research Use Only. Not for Use in Diagnostic Procedures.
*For Laboratory Use.

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