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NEXTflex™ mtDNA-Seq for cell samples

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Abstract

The NEXTflex™ mtDNA-Seq Kit is designed to prepare single or paired-end DNA libraries of mitochondrial DNA (mtDNA) for sequencing using Illumina® platforms. The procedure isolates mitochondrial DNA from genomic DNA (gDNA) by selective digestion of linear nuclear DNA, followed by fragmentation of mtDNA and library preparation.

Please see the **full manual** for additional details.

(Note: This is the procedure for Cell Samples; for Blood Samples, see **this** protocol.)

Guidelines

Contents, Storage and Shelf Life

The NEXTflexÃ¢ÂÂ¢ mtDNA-Seq Kit contains enough material to isolate mtDNA and prepare 8 libraries for IlluminaÃÂ® compatible sequencing. The shelf life of all reagents is 12 months when stored properly. 6X Loading Dye, Nuclease-free Water, and Resuspension Buffer can be stored at room temperature. All other components should be safely stored at -20ÃÂ°C.

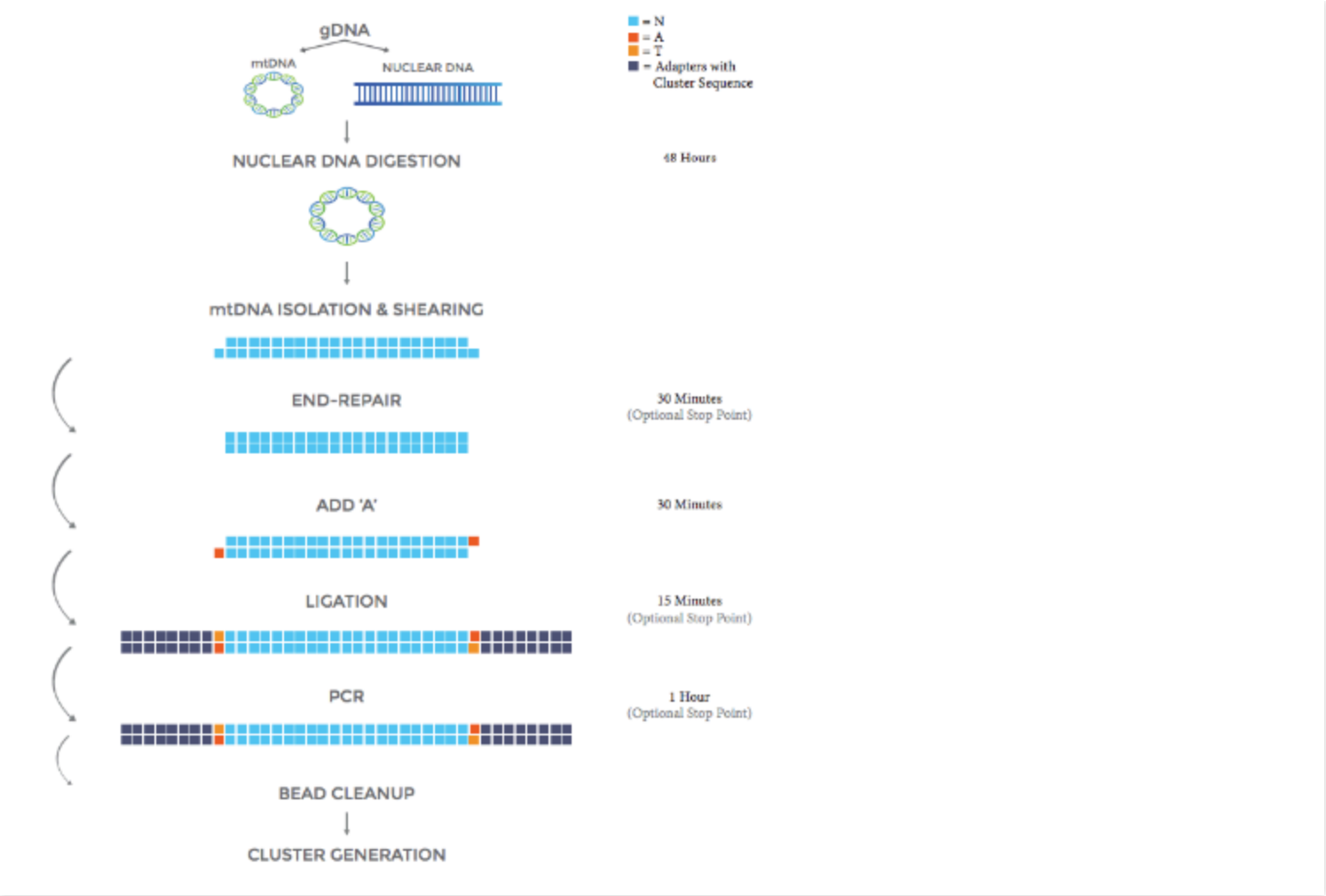
Kit Contents	Amount
BLUE CAP	
NEXTflex™ mtDNA Buffer Mix 1	80 µL
NEXTflex™ mtDNA Buffer Mix 2	80 µL
NEXTflex™ Nuclear DNA Digest Mix	80 µL
BROWN CAP	
NEXTflex™ mtDNA Primer Mix	13 µL
NEXTflex™ Nuclear DNA Primer Mix	13 µL
CLEAR CAP	
NEXTflex™ mtDNA End Repair Buffer Mix	56 µL
NEXTflex™ mtDNA End Repair Enzyme Mix	24 µL
RED CAP	
NEXTflex™ mtDNA Adenylation Mix	36 µL
PURPLE CAP	
NEXTflex™ mtDNA Ligation Mix	220 µL
NEXTflex™ ChIP Adapter	16 µL
GREEN CAP	
NEXTflex™ Primer Mix (12.5 µM)	16 µL
NEXTflex™ PCR Master Mix	176 µL
ORANGE CAP	
6X Loading Dye	500 µL
MW 100 bp Ready-to-Load Ladder	80 µL
WHITE CAP	
Nuclease-free Water	(2) 1.5 mL
Resuspension Buffer	(2) 1 mL

Required Materials Not Provided

Ã¢ÂÂ¢ 2 - 8 ÃÂ¼g of genomic DNA in up to 35 ÃÂ¼L nuclease-free water
Ã¢ÂÂ¢ (Optional)NEXTflexÃ¢ÂÂ¢ ChIP-Seq Barcodes Ã¢ÂÂ 6 , 12 , 24 , 48 or NEXTflex-96Ã¢ÂÂ¢ ChIP-Seq Barcodes (Cat # 514120, 514121, 514122, 514123, 514124)
Ã¢ÂÂ¢ Ethanol 100% (room temperature)
Ã¢ÂÂ¢ Covaris System (S2, E210) or other device for fragmenting DNA
Ã¢ÂÂ¢ 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
Ã¢ÂÂ¢ 96 well Library Storage and Pooling Plate (Fisher Scientific, Cat # AB-0765) or similar
Ã¢ÂÂ¢ Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
Ã¢ÂÂ¢ Agencourt AMPure XP 5 mL (Beckman Coulter Genomics, Cat # A63880)
Ã¢ÂÂ¢ Magnetic Stand-96 (Life Technologies, Cat # AM10027) or similar
Ã¢ÂÂ¢ Heat block at 37ÃÂ°C and at 70ÃÂ°C
Ã¢ÂÂ¢ Thermocycler
Ã¢ÂÂ¢ 2, 10, 20, 200 and 1000 ÃÂ¼L pipettes, multichannel pipettes
Ã¢ÂÂ¢ Nuclease-free barrier pipette tips
Ã¢ÂÂ¢ Microcentrifuge
Ã¢ÂÂ¢ 1.5 mL nuclease-free microcentrifuge tubes
Ã¢ÂÂ¢ Low melt agarose such as Low Gelling Temperature Agarose with a melt point of 65ÃÂºC (Boston Bioproducts, Cat # P-730)
Ã¢ÂÂ¢ 1X TAE buffer
Ã¢ÂÂ¢ SYBR Gold (Invitrogen, Cat # S11494)
Ã¢ÂÂ¢ UV transilluminator or gel documentation instrument
Ã¢ÂÂ¢ Gel electrophoresis apparatus
Ã¢ÂÂ¢ Electrophoresis power supply
Ã¢ÂÂ¢ Vortex
Ã¢ÂÂ¢ MiVac (SpeedVac)

NEXTflexÃ¢ÂÂ¢ mtDNA-Seq Flow Chart

Figure 1: Sample flow chart with approximate times necessary for each step.



Starting Material
The NEXTflexÃ¢ÂÂ¢ mtDNA-Seq Kit has been optimized and validated using 2 Ã¢ÂÂ 8 ÃÂ¼g of genomic DNA isolated either from human blood or cell samples. This kit provides sufficient reagents for 8 nuclear DNA digestions and mtDNA library preparations.

It is recommended that the mtDNA isolation test (see "Optional Quality Control of Nuclear DNA Digest" section) is performed with each isolation. Primers and PCR master mix for this test are provided with the kit. Undigested gDNA may be used as a control (not provided).

Reagent Preparation
1. Briefly spin down each component to ensure material has not lodged in the cap or side of tube. Keep on ice and vortex each tube prior to use.
2. Allow Agencourt AMPure XP Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.

Figure 3A:ÃÂ Bioanalyzer Gel Image

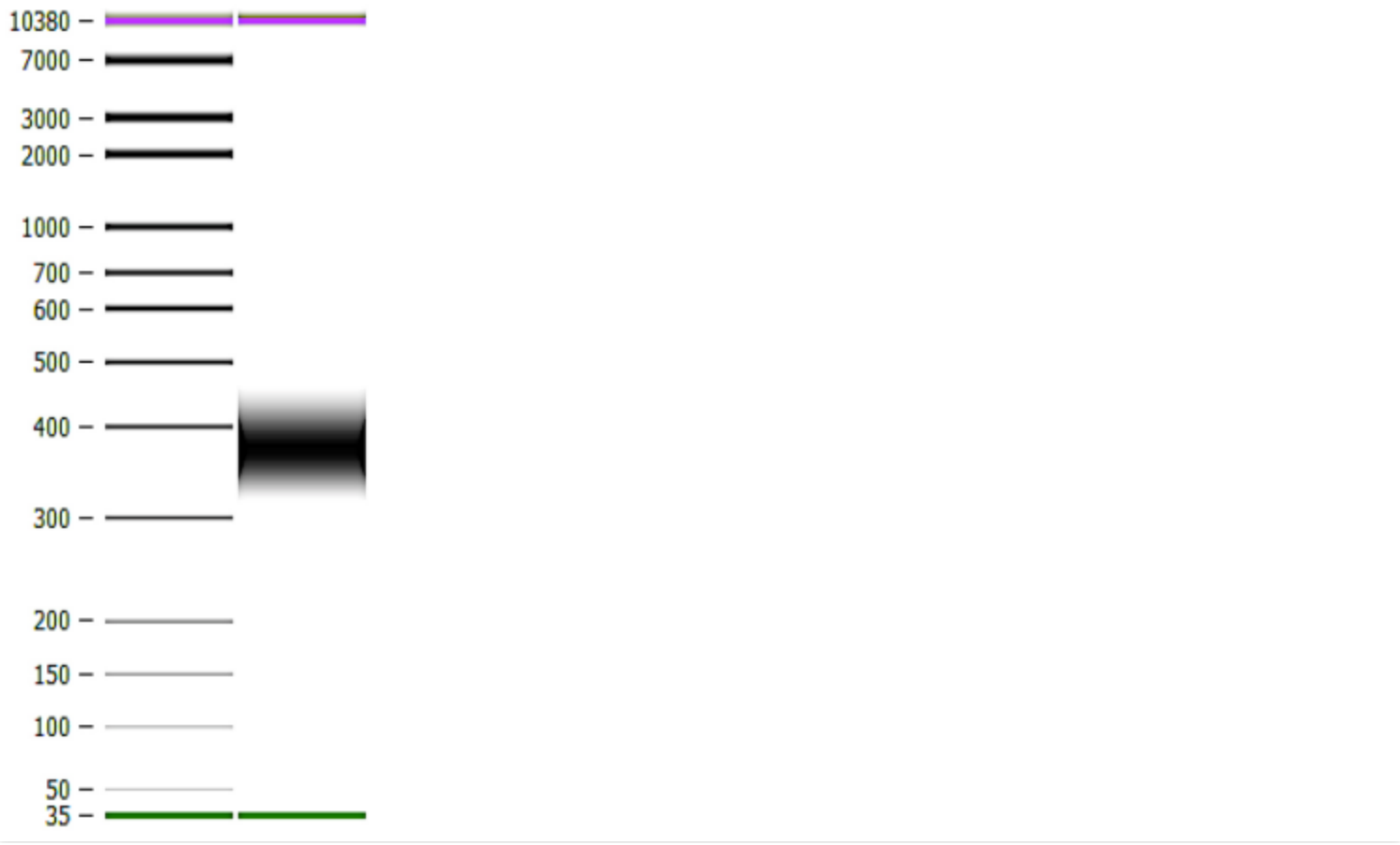


Figure 3 B: Bioanalyzer Electropherogram Image

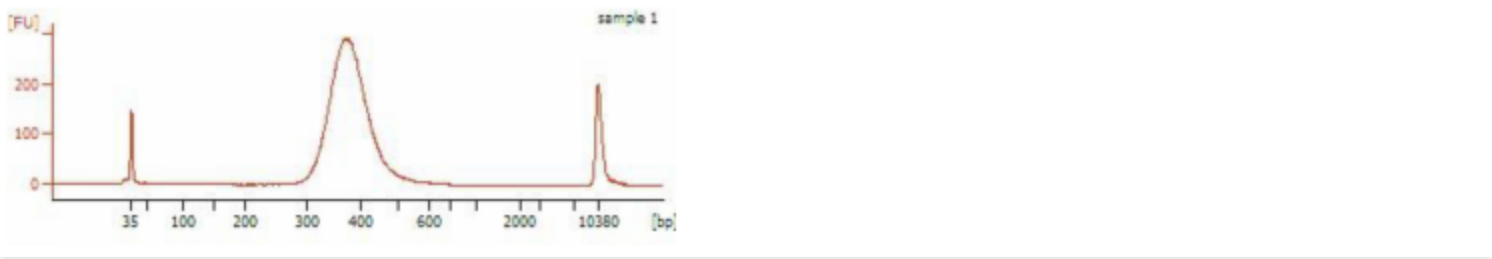


Figure 3A & 3B: Example of a mtDNA Library Size Distribution. Upon isolation the mtDNA was sheared to 150-200 bp, a total of 18 cycles of PCR were performed. 1 ÃÂ¼L of the resulting library was run on an Agilent High Sensitivity DNA chip to verify size. Using a QubitÃÂ® 2.0 Fluorometer & QubitÃÂ® dsDNA HS Assay Kit, the concentration of the library was determined to be > 10 nM.

Materials

MATERIALS

- 96 well PCR Plate Non-skirted **Phenix Research** **Catalog #**MPS-499
- Adhesive PCR Plate Seal **Bio-Rad Laboratories** **Catalog #**MSB1001
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- Magnetic Stand -96 **Life Technologies** **Catalog #**AM10027
- 96 well Library Storage and Pooling Plate **Fisher Scientific** **Catalog #**AB-0765
- Low melt agarose such as Low Gelling Temperature Agarose with a melt point of 65ºC **Boston Bioproducts** **Catalog #**P-730
- SYBR Gold **Thermo Scientific** **Catalog #**S-11494

STEP MATERIALS

- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- 96 well PCR Plate Non-skirted **Phenix Research** **Catalog #**MPS-499
- SYBR Gold **Thermo Scientific** **Catalog #**S-11494
- Adhesive PCR Plate Seal **Bio-Rad Laboratories** **Catalog #**MSB1001
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- Adhesive PCR Plate Seal **Bio-Rad Laboratories** **Catalog #**MSB1001
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
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- SYBR Gold **Thermo Scientific** **Catalog #**S-11494
- Adhesive PCR Plate Seal **Bio-Rad Laboratories** **Catalog #**MSB1001
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
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Protocol materials

- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- Magnetic Stand -96 **Life Technologies** **Catalog #**AM10027
- SYBR Gold **Thermo Scientific** **Catalog #**S-11494
- Adhesive PCR Plate Seal **Bio-Rad Laboratories** **Catalog #**MSB1001
- 96 well PCR Plate Non-skirted **Phenix Research** **Catalog #**MPS-499
- 96 well Library Storage and Pooling Plate **Fisher Scientific** **Catalog #**AB-0765
- Low melt agarose such as Low Gelling Temperature Agarose with a melt point of 65ºC **Boston Bioproducts** **Catalog #**P-730

Safety warnings

Bioo Scientific strongly recommends that you read the following warnings and precautions. Periodically, optimizations and revisions are made to the components and manual. Therefore, it is important to follow the protocol included with the kit. If you need further assistance, you may contact your local distributor or Bioo Scientific at nextgen@biooscientific.com.
• Do not use the kit past the expiration date.
• DTT in buffers may precipitate after freezing. If precipitate is seen, vortex buffer for 1-2 minutes or until the precipitate is in solution. The performance of the buffer is not affected once precipitate is in solution.
• Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.
• Do not heat the DNA Adapters above room temperature.
• Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).
• DNA sample quality may vary between preparations. It is the user's responsibility to utilize high quality DNA. DNA that is heavily nicked or damaged may cause library preparation failure. Absorbance measurements at 260 nm are commonly used to quantify DNA and 260 nm / 280 nm ratios of 1.8 - 2.0 usually indicate relatively pure DNA. Other quantification methods using fluorescent dyes may also be used. The user should be aware that contaminating RNA, nucleotides and single-stranded DNA may affect the amount of usable DNA in a sample preparation.
• Vortex the genomic DNA for 10 seconds before quantification. It is critical that the user quantifies the input gDNA correctly. The yield and purity of isolated mtDNA is dependent on the concentration of gDNA used.

Before start

Reagent Preparation
1. Briefly spin down each component to ensure material has not lodged in the cap or side of tube. Keep on ice and vortex each tube prior to use.
2. Allow Agencourt AMPure XP Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.

Reagent Preparation

1 Briefly spin down each component to ensure material has not lodged in the cap or side of tube. Keep on ice and vortex each tube prior to use.

2 Allow Agencourt AMPure XP Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.

Selective Digestion of Nuclear DNA (Cell Samples)

3 For each sample, combine the following reagents on ice in nuclease-free microcentrifuge tubes:

	_ µL	Nuclease-free Water
	_ µL	gDNA (4 – 8 µg)
	5 µL	NEXTflex™ mtDNA Buffer Mix 1
	5 µL	NEXTflex™ mtDNA Buffer Mix 2
	5 µL	NEXTflex™ Nuclear DNA Digest Mix
	50 µL	TOTAL

Note

Materials

Bioo Scientific Supplied
BLUE CAP - NEXTflex™ mtDNA Buffer Mix 1, NEXTflex™ mtDNA Buffer Mix 2, NEXTflex™ Nuclear DNA Digest Mix
WHITE CAP - Nuclease-free Water

User Supplied
gDNA isolated from cells in up to 35 µL nuclease-free water
Heat block
1.5 mL Microcentrifuge tubes
Microcentrifuge
Ice
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic rack

4 Mix well by pipetting.

5 Incubate in a heat block for 48 hours at 37°C.
48:00:00

6 Incubate the sample at 70°C for 30 minutes.
00:30:00

7 Spin the tube for 10 seconds to collect contents of the tube.
00:00:10

8 Add 50 µL of AMPure XP Beads to each sample and mix well by pipetting.
50 µL
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

9 Incubate at room temperature for 5 minutes.
00:05:00

10 Place the tube on the magnetic rack at room temperature for 5 minutes or until the supernatant appears clear.
00:05:00

11 Remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in the tube.

12 Wash #1: With tube on rack, gently add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.
00:00:30

13 Wash #2: With tube on rack, gently add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette. **Ensure all ethanol has been removed.**

14 Remove the tube from the magnetic rack and let dry at room temperature for 3 minutes. Do not overdry the beads.
00:03:00

15 Resuspend dried beads with 36 µL Nuclease-free Water Mix well by pipetting Ensure beads are no longer attached to the side of the well

16 Incubate resuspended beads at room temperature for 2 minutes.
00:02:00

17 Place tube on magnetic rack for 5 minutes or until the sample appears clear.
00:05:00

18 Gently transfer 35 µL of clear sample to a fresh microcentrifuge tube.

19 To ensure complete removal of nuclear DNA contamination, repeat the digestion with 35 µL of eluted material from Step 18. Set up the reaction as follows:

	35 µL	Eluted DNA (Step 18)
	5 µL	NEXTflex™ mtDNA Buffer Mix 1
	5 µL	NEXTflex™ mtDNA Buffer Mix 2
	5 µL	NEXTflex™ nuclear DNA Digest Mix
	50 µL	TOTAL

20 Incubate for 2 hours at 37°C.
02:00:00

21 Repeat steps 6-18.

Note

If you wish to pause your experiment, the procedure may be safely stopped at this step and samples stored at -20 °C. To continue, thaw your frozen samples on ice before proceeding to section "Optional Quality Control of Nuclear DNA Digest".

Section "Optional Quality Control of Nuclear DNA Digest" is an optional quality control check of nuclear DNA digestion. Primers and PCR master mix for this test are provided with this kit. Performing this test with undigested gDNA as a control is highly recommended.

If not performing the section test, proceed to section "Fragmentation of mtDNA" for Fragmentation of mtDNA.

Optional Quality Control of Nuclear DNA Digest

22 For each sample prepare two separate reactions in adjacent wells of a 96-well PCR Plate on ice as described below:

1 µL	mtDNA (from STEP A1/A2)	1 µL	mtDNA (from STEP A1/A2)
5 µL	NEXTflex™ PCR Master Mix	5 µL	NEXTflex™ PCR Master Mix
1.6 µL	**NEXTflex™ mtDNA Primer Mix**	1.6 µL	**NEXTflex™ Nuclear DNA Primer Mix**
12.4 µL	Nuclease-free Water	12.4 µL	Nuclease-free Water
20 µL	TOTAL	20 µL	TOTAL

Note

Materials

Bioo Scientific Supplied
BROWN CAP - NEXTflex™ mtDNA Primer Mix, NEXTflex™ Nuclear DNA Primer Mix
GREEN CAP - NEXTflex™ PCR Master Mix WHITE CAP - 6X Loading Dye, MW 100 bp Ready-to-Load Ladder
WHITE CAP - Nuclease-free Water
User Supplied
2 µL of isolated mtDNA (from sections "Selective Digestion of Nuclear DNA (Cell Samples)" and " Selective Digestion of Nuclear DNA (Blood Samples)")
Thermocycler
2% Agarose gel
Electrophoresis system

96 well PCR Plate Non-skirted **Phenix Research Catalog #**MPS-499

23 Mix well by pipetting.

24 Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

2 min	98°C	
30 sec	98°C	
30 sec	65°C	Repeat 25 cycles
45 sec	72°C	
4 min	72°C	

25 Prepare pre-stained SYBR Gold 2% or Ethidium Bromide TAE agarose gel. Mix and pour into gel tray. Load 3 µL of the PCR product mixed with 2 µL of 6X Loading Dye and 7 µL of Nuclease-free Water. Load the samples on the gel along with 6 µL of MW 100 bp Ready-to-Load Ladder.

SYBR Gold **Thermo Scientific Catalog #**S-11494

26 Run the gel with 1X TAE buffer at 100-120V for 60-120 minutes, or until bands have adequately resolved.
01:00:00

27 Visualize the gel on UV transilluminator or gel documentation instrument. Check for the presence of a mtDNA band and absence/significant reduction (compared to the control) of nuclear DNA bands to proceed with the subsequent steps (Fig. 2 in Guidelines).

Fragmentation of mtDNA

28 Adjust mtDNA sample volume to 130 µL with Nuclease-free Water. Set pipette to 130 µL and mix well by pipetting.

Note

Materials

Bioo Scientific Supplied
WHITE CAP - Nuclease-free Water

User Supplied
Up to 40 µL isolated mtDNA (from STEP A1/A2)
Covaris AFA microTUBE
Covaris Focused-Ultrasonicator System
Vacuum concentrator
Qubit® fluorometer

29 Transfer the sample to a Covaris tube. Follow manufacturer's instructions. For example, using the Covaris S2 system, the following parameters will produce fragments of 150-200 bp

Peak Intensity – 5
Duty cycle – 10%
Cycles per burst – 200
Time – 180 s

30 Transfer your sample from a Covaris tube to a 15 mL microcentrifuge tube.

31 Option 1: SpeedVac - following sonication, spin down sample in a SpeedVac for 2 hours at 45°C to reduce the volume of your sample to ≤ 40 µL. Do not let the sample dry down completely.
02:00:00

32 Option 2: Bead Cleanup – Alternatively a 2X AMPure XP bead clean up can be done as follows:

Protocol

NAME

Option 2: Bead Cleanup – Alternatively a 2X AMPure XP bead clean up for NEXTflex™ mtDNA-Seq Kit

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Preview

32.1 Add 260 µL of AMPure XP beads to each sample Mix thoroughly.

250 µL

Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

32.2 Incubate sample at room temperature for 5 minutes.

00:05:00

32.3 Place the tube on the magnetic rack at room temperature for 5 minutes or until the supernatant appears clear.

00:05:00

32.4 Remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in the tube.

32.5 Wash #1: With the tubes on the rack, gently add 500 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.

00:00:30

32.6 Wash #2: With the tubes on the rack, gently add 500 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate at room temperature for 30 seconds. Carefully, remove ethanol by pipette. **Ensure all ethanol has been removed.**

32.7 Remove the tube from the magnetic rack and let dry at room temperature for 3 minutes. Do not overdry the beads.

00:03:00

32.8 Resuspend the dried beads with 42 µL Nuclease-free Water. Mix well by pipetting. Ensure beads are no longer attached to the side of the well.

32.9 Incubate resuspended beads at room temperature for 2 minutes.

00:02:00

32.10 Place the tube on magnetic rack for 5 minutes or until the sample appears clear.

00:05:00

32.11 Gently transfer 40 µL of clear sample to a fresh microcentrifuge tube.

33 After concentrating the sample, Qubit® dsDNA reagents can be used to quantify the DNA concentration.

Note

Note that mtDNA accounts for 0.01% to 1% of total DNA and the concentration of isolated mtDNA can be very low. Concentrations typically vary between 0.1 to 1 ng/µL. It is recommended to use all of the fragmented mtDNA in library prep.

End Repair

34 For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR Plate:

	40 µL	mtDNA (from section "Fragmentation of mtDNA")
	7 µL	NEXTflex™ mtDNA End Repair Buffer Mix
	3 µL	NEXTflex™ mtDNA End Repair Enzyme Mix
	50 µL	TOTAL

Note

Materials

Bioo Scientific Supplied
CLEAR CAP - NEXTflex™ mtDNA End Repair Buffer Mix, NEXTflex™ mtDNA End Repair Enzyme Mix
WHITE CAP - Nuclease-free Water

User Supplied
mtDNA in 40 µL Nuclease-free Water (from section "Fragmentation of mtDNA")
96 well PCR Plate
Adhesive PCR Plate Seal
Microcentrifuge
Ice

35 Mix well by pipetting.

36 Apply adhesive PCR plate seal and incubate on a thermocycler for 30 minutes at 22°C.
00:30:00
Adhesive PCR Plate Seal **Bio-Rad Laboratories Catalog #**MSB1001

Clean-Up

37 Add 80 µL of AMPure XP Beads to each sample and mix well by pipetting.
80 µL

Note

Materials

Bioo Scientific Supplied
WHITE CAP - Resuspension Buffer

User Supplied
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand

Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

38 Incubate sample at room temperature for 5 minutes.
00:05:00

39 Place the 96 well PCR Plate on the magnetic stand at room temperature for 5 minutes or until the supernatant appears clear.
00:05:00

40 Remove and discard clear supernatant taking care not to disturb beads. Some liquid may remain in wells.

41 Wash #1: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.
00:00:30

42 Wash #2: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette. **Ensure all ethanol has been removed.**

43 Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.
Note: Do not overdry the beads.
00:03:00

44 Resuspend dried beads with 17 µL Resuspension Buffer. Mix well by pipetting. Ensure beads are no longer attached to the side of the well.

45 Incubate resuspended beads at room temperature for 2 minutes.
00:02:00

46 Place plate on magnetic stand for 5 minutes or until the sample appears clear.
00:05:00

47 Gently transfer 16 µL of clear sample to a new well.

Note

If you wish to pause your experiment, the procedure may be safely stopped at this step and samples stored at -20°C. To restart, always thaw your frozen samples on ice before proceeding to section "3' Adenylation".

3' Adenylation

48 Combine the following in the 96 well PCR Plate:

	16 µL	End-Repaired DNA (from section "Clean-Up")
	4.5 µL	NEXTflex™ mtDNA Adenylation Mix
	20.5 µL	TOTAL

Note

Materials

Bioo Scientific Supplied
RED CAP - NEXTflex™ mtDNA-Seq Adenylation Mix

User Supplied
Thermocycler (set to 37°C)
16 µL of End Repaired DNA (from section "Clean-Up")

49 Mix well by pipetting.

50 Apply adhesive PCR plate seal and incubate on a thermocycler for 30 minutes at 37°C.
00:30:00

Adapter Ligation

51 For each sample, combine the following reagents (in this order) in the 96-well PCR Plate:

	20.5 µL	3’ Adenylated DNA (from the above section)
	27.5 µL	NEXTflex™ mtDNA Ligation Mix
	2.0 µL	NEXTflex™ mtDNA Adapter or NEXTflex™ ChIP Barcode
	50 µL	TOTAL

Note

Materials

Bioo Scientific Supplied
PURPLE CAP - NEXTflex™ mtDNA Ligation Mix (Thaw right before use and store immediately after use at -20°C), NEXTflex™ ChIP Adapter

User Supplied
20.5 µL 3’ Adenylated DNA (from section "3’ Adenylation")
(Optional) NEXTflex™ ChIP Barcodes – 6 / 12 / 24 / 48 / 96 (Cat # 514120, 514121, 514122, 514123, 514124)

52 Mix well by pipetting.

53 Apply adhesive PCR plate seal and incubate on a thermocycler for 15 minutes at 22°C.
00:15:00
Adhesive PCR Plate Seal **Bio-Rad Laboratories Catalog #**MSB1001

Clean-Up 2

54 Add 40 µL of AMPure XP Beads to each sample and mix well by pipetting.
40 µL

Note

Materials

Bioo Scientific Supplied
WHITE CAP - Resuspension Buffer

User Supplied
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand

Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

55 Incubate at room temperature for 5 minutes.
00:05:00

56 Place the 96 well PCR Plate on the magnetic stand at room temperature for 5 minutes or until the supernatant appears clear.
00:05:00

57 Set pipette to 88 µL and gently remove clear supernatant taking care not to disturb beads. Some liquid may remain in wells.

58 Wash #1: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each sample and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.
00:00:30

59 Wash #2: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each sample and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette. **Ensure all ethanol has been removed.**
00:00:30

60 Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.
00:03:00

Note

Do not overdry the beads.

61 Resuspend dried beads with 52 µL Resuspension Buffer. Mix well by pipetting and ensuring beads are no longer attached to the side of the well.

62 Incubate resuspended beads at room temperature for 2 minutes.
00:02:00

63 Place plate on magnetic stand for 5 minutes or until the sample appears clear.
00:05:00

64 Gently transfer 50 µL of clear sample to new well.

65 Add 40 µL of AMPure XP Beads to each sample and mix well by pipetting.
40 µL
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

66 Incubate at room temperature for 5 minutes.
00:05:00

67 Place the 96 well PCR Plate on the magnetic stand at room temperature for 5 minutes oruntil the supernatant appears clear.
00:05:00

68 Set pipette to 88 µL and gently remove clear supernatant taking care not to disturb beads. Some liquid may remain in wells.

69 Wash #1: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each sample and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.
00:00:30

70 Wash #2: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each sample and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette. **Ensure all ethanol has been removed.**
00:00:30

71 Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.
00:03:00

Note

Do not overdry the beads.

72 Resuspend dried beads with 38 µL Resuspension Buffer Mix well by pipetting Ensure beads are no longer attached to the side of the well.

73 Incubate resuspended beads at room temperature for 2 minutes.
00:02:00

74 Place plate on magnetic stand for 5 minutes or until the sample appears clear.
00:05:00

75 Gently transfer 36 µL of clear sample to new well.

Note

If you wish to pause your experiment, the procedure may be safely stopped at this step and samples stored at -20°C. To restart, always thaw your frozen samples on ice before proceeding to section 'PCR Amplification'.

PCR Amplification

76 For each sample, combine the following reagents on ice in the 96 well PCR plate:

	36 µL	Purified Ligation Product (from section "Clean-Up 2")
	12 µL	NEXTflex™ PCR Master Mix
	2 µL	NEXTflex™ Primer Mix
	50 µL	TOTAL

Note

Materials

Bioo Scientific Supplied
GREEN CAP - NEXTflex™ Primer Mix , NEXTflex™ PCR Master Mix
WHITE CAP - Resuspension Buffer

User Supplied
Thermocycler
96 Well PCR Plate
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand
*36 µL Ligation Product (from section "Clean-Up 2")

77 Mix well by pipetting.

78 PCR Cycles:

2 min	98°C	
30 sec	98°C	
30 sec	65°C	Repeat 16-18 cycles*
60 sec	72°C	
4 min	72°C	

Note

PCR cycles will vary depending on the amount of starting material and quality of your sample. Further optimization may be necessary. Always use the least number of cycles possible.

79 Add 40 µL of AMPure XP Beads to each sample and mix well by pipetting.

40 µL

Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

80 Incubate at room temperature for 5 minutes.

00:05:00

81 Place the 96 well PCR Plate on the magnetic stand at room temperature for 5 minutes or until the supernatant appears clear.

00:05:00

82 Set pipette to 88 µL and gently remove clear supernatant taking care not to disturb beads. Some liquid may remain in wells.

83 Wash #1: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each sample and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.

00:00:30

84 Wash #2: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each sample and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette. **Ensure all ethanol has been removed.**

00:00:30

85 Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.

00:03:00

Note

Note: Do not overdry the beads.

86 Resuspend dried beads with 52 µL Resuspension Buffer. Mix well by pipetting and ensuring beads are no longer attached to the side of the well.

87 Incubate resuspended beads at room temperature for 2 minutes.
00:02:00

88 Place plate on magnetic stand for 5 minutes or until the sample appears clear.
00:05:00

89 Gently transfer 50 µL of clear sample to new well.

90 Add 40 µL of AMPure XP Beads to each sample and mix well by pipetting.
40 µL
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

91 Incubate at room temperature for 5 minutes.
00:05:00

92 Place the 96 well PCR Plate on the magnetic stand at room temperature for 5 minutes or until the supernatant appears clear.
00:05:00

93 Set pipette to 90 µL and gently remove clear supernatant taking care not to disturb beads. Some liquid may remain in wells.

94 Wash #1: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each sample and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.
00:00:30

95 Wash #2: With plate on stand, gently add 200 µL of freshly prepared 80% ethanol to each sample and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette. **Ensure all ethanol has been removed.**
00:00:30

96 Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.
00:03:00

Note

Note: Do not overdry the beads.

Note

Do not overdry the beads.

97 Resuspend dried beads with 16 µL Resuspension Buffer. Mix well by pipetting. Ensure beads are no longer attached to the side of the well.

98 Incubate resuspended beads at room temperature for 2 minutes.
00:02:00

99 Place plate on magnetic stand for 5 minutes or until the sample appears clear.
00:05:00

100 Gently transfer 15 µL of clear sample to a well of a new 96 well PCR Plate.

101 qPCR is recommended to quantitate DNA library templates for optimal cluster density.