NEXTflex™ mtDNA-Seq for cell samples

Bioo Scientific

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.)

Citation: Bioo Scientific NEXTflex™ mtDNA-Seq for cell samples. **protocols.io** dx.doi.org/10.17504/protocols.io.dnn5dd
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Guidelines

Contents, Storage and Shelf Life

The NEXTflexÃ¢Â□Â¢ mtDNA-Seq Kit contains enough material to isolate mtDNA and prepare 8 libraries for IlluminaÃ□Â® 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 -20Ã□Â°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

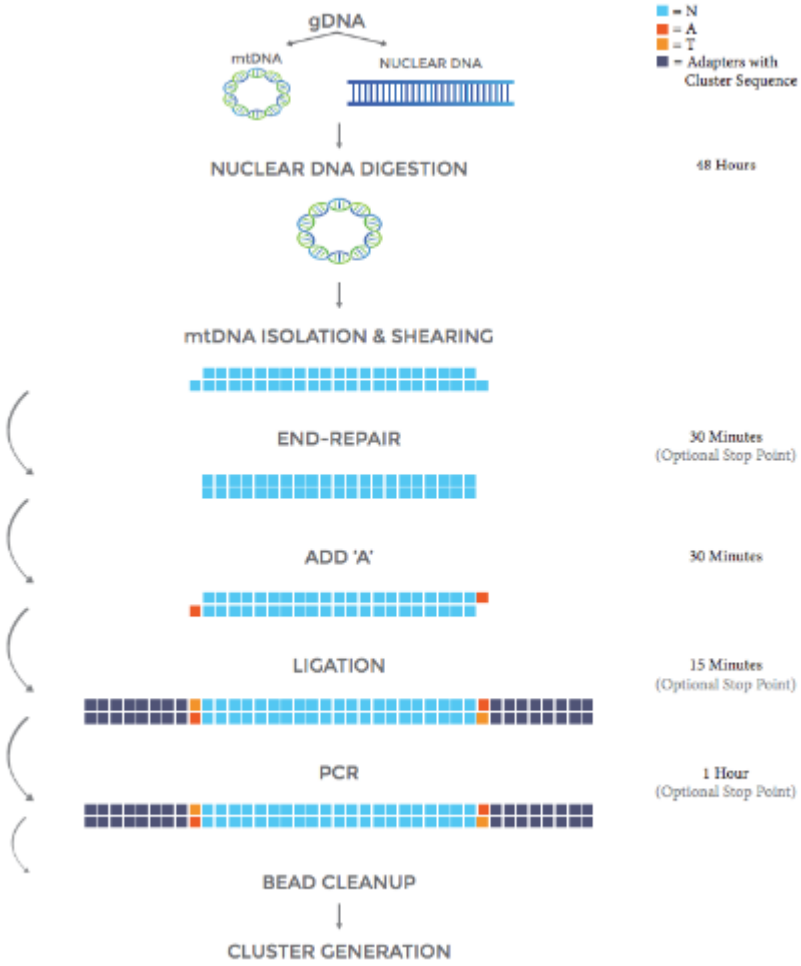
Required Materials Not Provided

Ã¢Â Â¢ 2 - 8 Ã Â¼g of genomic DNA in up to 35 Ã Â¼L nuclease-free water
Ã¢Â Â¢ (Optional)NEXTflexÃ¢Â Â¢ ChIP-Seq Barcodes Ã¢Â Â 6 , 12 , 24 , 48 or NEXTflex-96Ã¢Â Â¢ ChIP-Seq Barcodes (Cat # 514120, 514121, 514122, 514123, 514124)
Ã¢Â Â¢ Ethanol 100% (room temperature)
Ã¢Â Â¢ Covaris System (S2, E210) or other device for fragmenting DNA
Ã¢Â Â¢ 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
Ã¢Â Â¢ 96 well Library Storage and Pooling Plate (Fisher Scientific, Cat # AB-0765) or similar
Ã¢Â Â¢ Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
Ã¢Â Â¢ Agencourt AMPure XP 5 mL (Beckman Coulter Genomics, Cat # A63880)
Ã¢Â Â¢ Magnetic Stand-96 (Life Technologies, Cat # AM10027) or similar
Ã¢Â Â¢ Heat block at 37Ã Â°C and at 70Ã Â°C
Ã¢Â Â¢ Thermocycler
Ã¢Â Â¢ 2, 10, 20, 200 and 1000 Ã Â¼L pipettes, multichannel pipettes
Ã¢Â Â¢ Nuclease-free barrier pipette tips

Ã¢Â□Â¢ Microcentrifuge
Ã¢Â□Â¢ 1.5 mL nuclease-free microcentrifuge tubes
Ã¢Â□Â¢ Low melt agarose such as Low Gelling Temperature Agarose with a melt point of 65Ã□ÂºC (Boston Bioproducts, Cat # P-730)
Ã¢Â□Â¢ 1X TAE buffer
Ã¢Â□Â¢ SYBR Gold (Invitrogen, Cat # S11494)
Ã¢Â□Â¢ UV transilluminator or gel documentation instrument
Ã¢Â□Â¢ Gel electrophoresis apparatus
Ã¢Â□Â¢ Electrophoresis power supply
Ã¢Â□Â¢ Vortex
Ã¢Â□Â¢ MiVac (SpeedVac)

NEXTflexÃ¢Â□Â¢ mtDNA-Seq Flow Chart

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



Starting Material

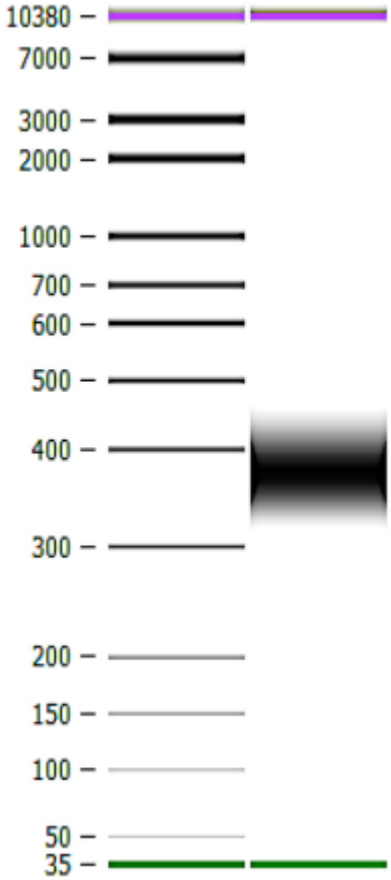
The NEXTflexÃ¢Â□Â¢ mtDNA-Seq Kit has been optimized and validated using 2 Ã¢Â□Â□ 8 Ã□Â¼g of genomic DNA isolated either from human blood or cell samples. This kit provides sufficient reagents for 8 nuclear DNA digestions and mtDNA library preparations.

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



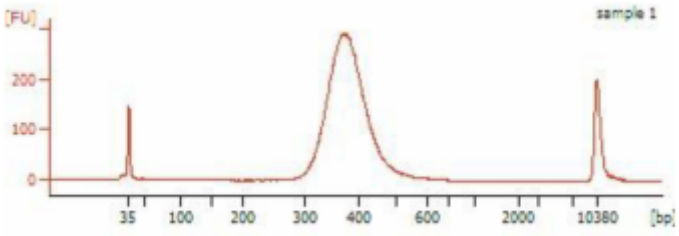


Figure 3 B: Bioanalyzer Electropherogram Image

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.

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.

Materials

96 well PCR Plate Non-skirted MPS-499 by Phenix Research

Adhesive PCR Plate Seal MSB1001 by Bio-rad Laboratories

Agencourt AMPure XP A63880 by Beckman Coulter

Magnetic Stand -96 AM10027 by Life Technologies

96 well Library Storage and Pooling Plate AB-0765 by Fisher Scientific

Low melt agarose such as Low Gelling Temperature Agarose with a melt point of 65ºC P-730 by Boston Bioproducts

SYBR Gold S-11494 by Thermo Scientific

Protocol

Reagent Preparation

Step 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.

Reagent Preparation

Step 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)

Step 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

NOTES

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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

Selective Digestion of Nuclear DNA (Cell Samples)

Step 4.
Mix well by pipetting.

Selective Digestion of Nuclear DNA (Cell Samples)

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

DURATION
48:00:00

Selective Digestion of Nuclear DNA (Cell Samples)

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

DURATION
00:30:00

Selective Digestion of Nuclear DNA (Cell Samples)

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

DURATION
00:00:10

Selective Digestion of Nuclear DNA (Cell Samples)

Step 8.
Add 50 µL of AMPure XP Beads to each sample and mix well by pipetting.

AMOUNT
50 µl Additional info:

REAGENTS
Agencourt AMPure XP A63880 by Beckman Coulter

Selective Digestion of Nuclear DNA (Cell Samples)

Step 9.
Incubate at room temperature for 5 minutes.

DURATION
00:05:00

Selective Digestion of Nuclear DNA (Cell Samples)

Step 10.

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

DURATION

00:05:00

Selective Digestion of Nuclear DNA (Cell Samples)

Step 11.

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

Selective Digestion of Nuclear DNA (Cell Samples)

Step 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.

DURATION

00:00:30

Selective Digestion of Nuclear DNA (Cell Samples)

Step 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.**

Selective Digestion of Nuclear DNA (Cell Samples)

Step 14.

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

DURATION

00:03:00

Selective Digestion of Nuclear DNA (Cell Samples)

Step 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

Selective Digestion of Nuclear DNA (Cell Samples)

Step 16.

Incubate resuspended beads at room temperature for 2 minutes.

DURATION

00:02:00

Selective Digestion of Nuclear DNA (Cell Samples)

Step 17.

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

DURATION

00:05:00

Selective Digestion of Nuclear DNA (Cell Samples)

Step 18.

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

Selective Digestion of Nuclear DNA (Cell Samples)

Step 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

Selective Digestion of Nuclear DNA (Cell Samples)

Step 20.

Incubate for 2 hours at 37°C.

DURATION

02:00:00

Selective Digestion of Nuclear DNA (Cell Samples)

Step 21.

Repeat steps 6-18.

NOTES

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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

Step 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)
5 µL	NEXTflex™ PCR Master Mix
1.6 µL	**NEXTflex™ mtDNA Primer Mix**
12.4 µL	Nuclease-free Water
20 µL	TOTAL

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

REAGENTS

96 well PCR Plate Non-skirted MPS-499 by Phenix Research

NOTES

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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

Optional Quality Control of Nuclear DNA Digest

Step 23.

Mix well by pipetting.

Optional Quality Control of Nuclear DNA Digest

Step 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	

Optional Quality Control of Nuclear DNA Digest

Step 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.

REAGENTS

SYBR Gold S-11494 by Thermo Scientific

Optional Quality Control of Nuclear DNA Digest

Step 26.

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

DURATION

01:00:00

Optional Quality Control of Nuclear DNA Digest

Step 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

Step 28.

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

NOTES
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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

Fragmentation of mtDNA
Step 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

Fragmentation of mtDNA
Step 30.
Transfer your sample from a Covaris tube to a 15 mL microcentrifuge tube.

Fragmentation of mtDNA
Step 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.

DURATION
02:00:00

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

PROTOCOL

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

CONTACT: Bioo Scientific
Step 32.1.
Add 260 µL of AMPure XP beads to each sample Mix thoroughly.

AMOUNT
250 µl Additional info:

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

Step 32.2.

Incubate sample at room temperature for 5 minutes.

DURATION

00:05:00

Step 32.3.

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

DURATION

00:05:00

Step 32.4.

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

Step 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.

DURATION

00:00:30

Step 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.**

Step 32.7.

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

DURATION

00:03:00

Step 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.

Step 32.9.

Incubate resuspended beads at room temperature for 2 minutes.

DURATION

00:02:00

Step 32.10.

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

DURATION

00:05:00

Step 32.11.

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

Fragmentation of mtDNA

Step 33.

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

NOTES

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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

Step 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

NOTES

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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

End Repair

Step 35.

Mix well by pipetting.

End Repair

Step 36.

Apply adhesive PCR plate seal and incubate on a thermocycler for 30 minutes at 22°C.

REAGENTS

Adhesive PCR Plate Seal MSB1001 by Bio-rad Laboratories

DURATION

00:30:00

Clean-Up

Step 37.

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

AMOUNT

80 µl Additional info:

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

NOTES

Bioo Scientific 18 Aug 2015
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

Clean-Up

Step 38.
Incubate sample at room temperature for 5 minutes.

DURATION
00:05:00

Clean-Up

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

DURATION
00:05:00

Clean-Up

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

Clean-Up

Step 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.

DURATION
00:00:30

Clean-Up

Step 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.**

Clean-Up

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

DURATION
00:03:00

Clean-Up

Step 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.

Clean-Up

Step 45.

Incubate resuspended beads at room temperature for 2 minutes.

DURATION

00:02:00

Clean-Up

Step 46.

Place plate on magnetic stand for 5 minutes or until the sample appears clear.

DURATION

00:05:00

Clean-Up

Step 47.

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

NOTES

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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

Step 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

NOTES

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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")

3’ Adenylation

Step 49.

Mix well by pipetting.

3’ Adenylation

Step 50.

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

DURATION

00:30:00

Adapter Ligation

Step 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

NOTES

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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)

Adapter Ligation

Step 52.
Mix well by pipetting.

Adapter Ligation

Step 53.
Apply adhesive PCR plate seal and incubate on a thermocycler for 15 minutes at 22°C.

REAGENTS

Adhesive PCR Plate Seal MSB1001 by Bio-rad Laboratories

DURATION

00:15:00

Clean-Up 2

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

AMOUNT

40 µl Additional info:

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

NOTES

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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

Clean-Up 2

Step 55.

Incubate at room temperature for 5 minutes.

DURATION

00:05:00

Clean-Up 2

Step 56.

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

DURATION

00:05:00

Clean-Up 2

Step 57.

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

Clean-Up 2

Step 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.

DURATION

00:00:30

Clean-Up 2

Step 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.**

DURATION

00:00:30

Clean-Up 2

Step 60.

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

DURATION

00:03:00

NOTES

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Do not overdry the beads.

Clean-Up 2

Step 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.

Clean-Up 2

Step 62.

Incubate resuspended beads at room temperature for 2 minutes.

DURATION

00:02:00

Clean-Up 2

Step 63.

Place plate on magnetic stand for 5 minutes or until the sample appears clear.

DURATION

00:05:00

Clean-Up 2

Step 64.

Gently transfer 50 µL of clear sample to new well.

Clean-Up 2

Step 65.

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

AMOUNT

40 µl Additional info:

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

Clean-Up 2

Step 66.

Incubate at room temperature for 5 minutes.

DURATION

00:05:00

Clean-Up 2

Step 67.

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

DURATION

00:05:00

Clean-Up 2

Step 68.

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

Clean-Up 2

Step 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.

DURATION

00:00:30

Clean-Up 2

Step 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.**

DURATION

00:00:30

Clean-Up 2

Step 71.

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

DURATION
00:03:00

NOTES
Bioo Scientific 02 Sep 2015
Do not overdry the beads.

Clean-Up 2

Step 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.

Clean-Up 2

Step 73.
Incubate resuspended beads at room temperature for 2 minutes.

DURATION
00:02:00

Clean-Up 2

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

DURATION
00:05:00

Clean-Up 2

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

NOTES
Bioo Scientific 02 Sep 2015
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

Step 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

NOTES
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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")

PCR Amplification

Step 77.
Mix well by pipetting.

PCR Amplification

Step 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	

NOTES
Bioo Scientific 03 Sep 2015
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.

PCR Amplification

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

AMOUNT
40 µl Additional info:

REAGENTS
Agencourt AMPure XP A63880 by Beckman Coulter

PCR Amplification

Step 80.
Incubate at room temperature for 5 minutes.

DURATION
00:05:00

PCR Amplification

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

DURATION
00:05:00

PCR Amplification

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

PCR Amplification

Step 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.

DURATION
00:00:30

PCR Amplification

Step 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.**

DURATION
00:00:30

PCR Amplification

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

DURATION
00:03:00

NOTES
Bioo Scientific 18 Aug 2015
Note: Do not overdry the beads.

PCR Amplification

Step 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.

PCR Amplification

Step 87.
Incubate resuspended beads at room temperature for 2 minutes.

DURATION
00:02:00

PCR Amplification

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

DURATION
00:05:00

PCR Amplification

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

PCR Amplification

Step 90.
Add 40 µL of AMPure XP Beads to each sample and mix well by pipetting.

AMOUNT
40 µl Additional info:

REAGENTS
Agencourt AMPure XP A63880 by Beckman Coulter

PCR Amplification

Step 91.
Incubate at room temperature for 5 minutes.

DURATION
00:05:00

PCR Amplification

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

DURATION
00:05:00

PCR Amplification

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

PCR Amplification

Step 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.

DURATION
00:00:30

PCR Amplification

Step 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.**

DURATION
00:00:30

PCR Amplification

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

DURATION
00:03:00

NOTES
Bioo Scientific 18 Aug 2015
Note: Do not overdry the beads.
Bioo Scientific 02 Sep 2015
Do not overdry the beads.

PCR Amplification

Step 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.

PCR Amplification

Step 98.
Incubate resuspended beads at room temperature for 2 minutes.

DURATION
00:02:00

PCR Amplification

Step 99.

Place plate on magnetic stand for 5 minutes or until the sample appears clear.

DURATION

00:05:00

PCR Amplification

Step 100.

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

PCR Amplification

Step 101.

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

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.