Loxodonta Localizer: Guidelines for extracting DNA from samples, and for generating elephant mitochondrial sequences for the appropriate region using PCR and Sanger sequencing.

DNA extraction from elephant samples may use one of the many kits available for extraction of blood, tissue or fecal DNA. The primers below may be used to amplify and sequence DNA extracted from elephant blood, tissue or feces; or on DNA extracted from ivory. A separate protocol for extracting DNA from ivory is included as a link in the input page of the *Loxodonta* Localizer.

I. Step-by-step protocols: from PCR to sequencing reaction

Workspace - Pre PCR area

1. Custom order desalted primers (see below) from a commercial vendor or synthesize them by using an in-house oligonucleotide synthesizer.

   Primers
   CR-F1: TGGTCTTGTAAGCCATAAATGAAA
   CR-R1: GCTTTAATGTGCTATGTAAGACTATG
   CR-F2: TCGTGCATCACATTATTTACCC
   CR-R2: TGGTCCTGAAGAAAGAACCAG

   Amplicon size (excluding primer lengths)
   CR-F1/CR-R1: 318 bp
   CR-F2/CR-R2: 306 bp
   CR-F1/CR-R2: 522 bp

   For good quality DNA, use the primer set CR-F1/CR-R2. For poor quality DNA, use two separate amplification reactions, one using CR-F1/CR-R1 and the other CR-F2/CR-R2. The two reactions generate fragments that overlap for 102 bp.

2. Rehydrate primers with an appropriate volume of TLE (10 mM Tris-HCl, 0.1 mM EDTA), enough TLE to generate a 20 μM stock solution for each primer. Vortex thoroughly for 15 sec to dissolve primers, spin down and transfer an aliquot of the primer to a new tube to use as a working stock.

3. Prepare the PCR master mix by multiplying the amounts below by the number of samples to be amplified, plus the number of positive or negative controls, plus an additional amount of 10-15% to account for pipetting error. Prepare the mix on ice. Mix the components by pipetting or by tapping the tube with a finger, and centrifuge briefly to lower all components to a common mixture at the bottom of the tube.

Component	volume (µl) for 1 sample
Sterile mili-Q filtered water	14.8*
10X PCR Buffer II[+]	2.5
dNTP Mix (10mM) [#]	2.0
$MgCl_2$ (25mM)[^]	1.5
AmpliTaq Gold DNA Polymerase[+]	0.2
Forward primer (20 µM)	0.5[¥]
Reverse primer (20 µM)	0.5[¥]
BSA (10 mg/ml)[§]	2.5
TOTAL volume to aliquot	24.5*

*Depending on the amount of DNA used, these volumes need to be adjusted to produce a total reaction volume of 25 µl. If the DNA is dried in the plates, use 15.3 µl of water per sample, and aliquot 25 µl of the mix to each well.
[#]2.5mM of each dNTP (dATP, dCTP, dGTP and dTTP) blend (ABI, N8080260).
[^]AmpliTaq Gold DNA Polymerase with Buffer II and $MgCl_2$ solution (ABI, N8080249)
[§]60 mg Bovine Serum Albumin (100X) 10 mg/ml (New England BioLabs Inc., B9001S), optional for poor quality DNA

4. Place 0.5 µl of each DNA sample at the bottom of each PCR tube or well of a PCR plate (you can increase or decrease the amount of DNA depending on the quality of samples) and visually check that each well has received DNA. Cover each plate or tube and spin down briefly. If PCR doesn't work well, increase (or decrease) the amount of DNA used for the PCR reaction.

5. Pipette 24.5 µl or appropriate amount of mix into each well that contains DNA (being careful to avoid cross-contamination of wells). Cover the plate or tubes tightly and spin down briefly. In case of a dried DNA plate, pipette 25 µl of the mix, cover the plate tightly, briefly vortex to allow the DNA to rehydrate and then spin down (or spin down and let sit in the refrigerator to rehydrate).

6. Place the plate or tubes in a thermocycler and use the following cycling algorithm.

9 min 45 sec at 95˚C
3 cycles of 20 sec at 94˚C, 30 sec at 60˚C, 30 sec at 72˚C
5 cycles of 20 sec at 94˚C, 30 sec at 58˚C, 30 sec at 72˚C
5 cycles of 20 sec at 94˚C, 30 sec at 56˚C, 30 sec at 72˚C
5 cycles of 20 sec at 94˚C, 30 sec at 54˚C, 30 sec at 72˚C
5 cycles of 20 sec at 94˚C, 30 sec at 52˚C, 30 sec at 72˚C
22 cycles of 20 sec at 94˚C, 30 sec at 50˚C, 30 sec at 72˚C
7 min final extension at 72 ˚C
Hold at 4˚C

7. Remove the plate or tubes from the thermocycler and take them to the post PCR working area. Plates and tubes should be refrigerated when not in use.

Workspace - Post PCR area

8. Spin down the PCR plate or tubes briefly. Mix 5 µl of PCR products and 1 µl of loading dye on a plate or on a section of parafilm. Apply samples with dye or a DNA ladder into each well of a 1.5 to 2 % agarose gel with ethidium bromide (or alternative) in 1x TAE buffer and run at 120 V for 30 to 40 min. Using a face shield, briefly examine the gel under UV light and take photos. Keep the samples in a -20˚C freezer dedicated to post PCR materials until the next step.

Workspace - Pre PCR area

9. Exonuclease I and shrimp alkaline phosphatase (SAP) are used to enzymatically destroy primers and unincorporated dNTPs. Calculate the amount of mix needed given the number of samples that successfully amplified, plus 10-15% pipetting error. Prepare the mix on ice, mix by pipetting or tapping with finger, and spin down briefly.

Component	volume (µl) for a 20 µl of product
Sterile mili-Q filtered water	3.88
Exonuclease I (10 units/µl)*	0.38
Shrimp alkaline phosphatase (1 unit/µl)[#]	0.74
TOTAL volume to aliquot	5.0

*Exonuclease I (USB Corporation, 70073X)
[#]Shrimp alkaline phosphatase (USB Corporation, 70092Z)

Workspace - Post PCR area

10. Bring the mix to the post PCR workspace. Spin down the PCR plate or tubes briefly. Add 5 µl of the mix to each 20 µl of PCR product and cover the plate or tubes tightly. Spin down briefly and place in a thermocycler, using the following algorithm: 75 min at 37˚C (to promote enzyme activity), followed by 5 min at 95˚C to inactivate the enzymes.

Workspace - Pre PCR area

11. Prepare sequencing reactions based on the table below, depending on the number of samples plus an additional 10-15% for pipetting error. Add reagents on ice, mix by pipetting or tapping with a finger, and spin down briefly.

Component	volume (µl) for a reaction
Sterile mili-Q filtered water	6.775*
5X Sequencing buffer [#§]	1.875
BigDye Terminator v3.1[#]	0.25
2 µM primer (M13F for forward or M13R for reverse)	0.6
TOTAL volume to aliquot	9.5*

*Depending on the PCR product volume, these volumes may need to be adjusted to produce a 10 µl total volume.
[#]BigDye Terminator v3.1 Cycle sequencing Kits (ABI, 4337454 – 4337457)
[§]Use proprietary 5X buffer, or a mix of 400 mM Tris-HCl PH 9.0, 10 mM $MgCl_2$

12. Apply 9.5 µl of the mix to each well of the PCR plate or PCR tubes. Cover and spin down briefly.

Workspace - Post PCR area

13. Take the prepared plates or tubes to the post PCR working area. Spin down the enzyme-treated PCR products briefly.

14. Add 0.5 µl of PCR products into each well of the plates or tubes prepared for sequencing reaction. Cover the plates or tubes tightly, spin down briefly and set in a thermocycler using the following algorithm.

1 min at 95˚C
34 cycles of 15 sec at 95˚C, 5 sec at 45˚C, 4 min at 60˚C
Hold at 4˚C and refrigerate until transfer to core sequencing facility