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

DNA extraction from lion samples may use one of the many kits available for extraction of blood, tissue, bone, or fecal DNA.

The primers and procedures below may be used to amplify and sequence DNA extracted from lion blood, tissue or feces; or on DNA extracted from bone. Alternative methods and/or primers can be used so long as the correct region of mitochondrial Cytochrome B is generated.

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
LEO_2F: GTGGGGCCAAATATCCTTTT (From Bertola *et al.* 2016)

LEO_835R: GGTTTGATATGGGGAGGGGTG (Unpublished)

Amplicon size (excluding primer lengths) LEO_2F/LEO_835R: 367 bp

2. Rehydrate primers with an appropriate volume of TLE (10 mM Tris-HCl, 0.1 mM EDTA), enough TLE to generate a 10 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)
Sterile mili-Q filtered water	11.8*
10X PCR Buffer	2.0
dNTP Mix (2.5mM)	1.6
MgCl2 (25mM)	0.8
5Uµ/Taq Polymerase	0.4
Forward primer (10 µM)	0.8
Reverse primer (10 µM)	0.8
BSA (10 mg/ml)	0.8
TOTAL volume to aliquot	19.0*

*Depending on the amount of DNA used, these volumes need to be adjusted to produce a total reaction volume of 20 µl. The volumes here assume 0.1 µl of template DNA, but the actual volume of DNA used will differ depending on concentration.

4. Place 1.0 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 20 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 20 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.

4 min 0 sec at 94˚C
40 cycles of 20 sec at 94˚C, 60 sec at 50-55˚C, 60 sec at 72˚C
10 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. This table assumes 0.5 µl of PCR product.
[#]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

Lion Localizer: trimming sequences to be used as queries.

TRIMMING SEQUENCES (quick guide)

Sequences must be trimmed to an exact 367 bp portion of the Cytochrome B region of mitochondrial DNA running in the 5'-3' direction. **Here are the steps involved:**

(1) You will need to use one of several programs that are useful for visualizing, checking, aligning and trimming DNA sequences. These include, but are not limited, to:
Geneious, https://www.geneious.com
Sequencher, https://www.genecodes.com
EMBL-EBI EMBOSS Matcher (free tool), https://www.ebi.ac.uk/Tools/psa/

(2) Once you have established that your sequences are of high quality, you can align your sequence(s) to a reference sequence from the Lion Localizer, and then use the reference sequence as a guide to trim your generated sequence(s) to the correct region and size. Here is a reference sequence (running in the 5'-3' direction) that can be copied and pasted into the software described above (or similar software), to use as a guide for trimming your sequences:
>LEO001
GAGGTGCAACTGTAATCACTAATCTCCTATCAGCAATCCCATACATCGGGGCCGACCTAGTAGAGTG
GATCTGAGGAGACTTCTCAGTAGACAAAGCCACCCTGACACGATTCTTTGCCTTCCACTTCATCCTTC
CATTTATCATCTCAGCCCTAGCAGCAGTCCACCTCCTGTTCCTCCATGAAACAGGATCTAATAACCCCT
CAGGAATGGTATCTGACTCAGATAAAATTCCATTCCATCCATACTATACAATCAAAGATATCCTAGGC
CTTCTAGTACTAATCTTAACACTCATACTACTCGTCCTATTCTCACCAGACCTATTAGGAGATCCCGAC
AACTATACCCCCGCCAATCCTCTAAG

(3) Using the reference sequence in (2) as a guide, trim your generated sequence.

(4) After trimming the sequences, it should be useful to first identify any identical mtDNA haplotype sequences across your trimmed sequences, and query each distinct haplotype only once. You can record information on all of the samples that share the same haplotype, and may choose to include this information in the first line of the FASTA format in the text line preceded by ">" as this information will be populated to the Lion Localizer output listing.

(5) Use each trimmed sequence as a query on the home page of the Lion Localizer. In FASTA format, any notes describing your query can be included in the first line, as text preceded by ">"

(6) Additional quality control step. If the Lion Localizer output listing does not show an exact match to your query, the output listing can be used for quality control on any novel (previously unreported) sequence. In the output listing, use the "show alignment" option to identify the position of the character state that makes your novel sequence unique. Then verify in the raw data that this character state is real and does not reflect a technical error or ambiguity.

ADDITIONAL INFORMATION (details not generally needed to conduct trimming)
Ambiguous character states (such as N or R) are not allowed in the Lion Localizer query sequence, which must only include the letters A, C, G, and T.

Description of sequences immediately upstream and downstream of the sequences to be input as a query:

If you are using the primers Leo_2F and Leo_835R, which are included in the Lion Localizer protocol for amplification and Sanger sequencing, then follow these guidelines: the query sequence will include all bases found between the primers Leo_2F and Leo_835R, excluding all bases that form a part of each primer. Below are shown only the primer sequences that immediately flank the appropriate 367 bp control sequence; these will be trimmed (along with any regions further upstream or downstream) in order to generate a sequence of the correct length.

5' side GTGGGGCCAAATATCCTTTT

3' side CACCCCTCCCCATATCAAACC

If using other primers or other methods to generate sequences, use the quick guide to trimming sequences at the top of this document.

Querying sequences that are identical across samples only once:

In confiscated seizures of lion parts, there may be samples from many different lions that carry the same mtDNA sequences in the 367 bp region of Cytochrome B used by the Lion Localizer. The user should, after trimming the sequences, compare all of the sequences to each other. For samples that carry the same 367 bp mtDNA sequence, these identical sequences should be queried against the Lion Localizer only once, to avoid redundancy in the workload and in the Lion Localizer output results.

When samples share identical sequences, text that identifies all of the identical samples can be included within a FASTA header line that can form part of the query against the Lion Localizer database. The header line beginning with “>” can be followed by information on all samples that carry the query sequence, such as a description, sample number, haplotype ID, or other identifying information. All of the information included in FASTA format on the input page will be reproduced on the output page, which also displays a map and a listing of matching haplotypes, and which includes a timestamp that lists the date and time that the output was generated (in Greenwich Mean Time).

If a distinct novel sequence is detected in only one sample, the single nucleotide polymorphism(s) (SNPs) unique to that sample should be identified. This may be done using the “show alignment” option for each haplotype on the Lion Localizer output page. As an extra quality control step, the sequence should be double-checked at the position(s) of the mismatching SNP(s), in order to ensure that any character state unique to the haplotype is not due to mis-calling of a base.