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Le Clercq, L.S., 2023. *Biological clock measures: Assessing the association between the circadian and epigenetic clock as predictors of migration phenology and biological aging in wildlife* (Doctoral thesis, University of the Free State).

ABI Sanger Sequencing of Avian Clock genes to elucidate markers for Migration Phenology

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Biological clock measures the association between the circadian and epigenetic clock as predictors of migration and age

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ABSTRACT

This protocol follows up on "PCR Amplification of Clock genes with EmeraldAmp® GT PCR Master Mix in Avian species" and is intended to provide the next steps used in the sanger sequencing of the produced amplicons. This protocol uses ABI BigDye reagents (but suitable alternatives exist). The same primers used to produce the PCR products, of the respective clock genes, are used individually in a forward and reverse sequencing reaction. Cycling conditions mimic those used for PCR. Sequencing reactions are purified and subsequently resolved on an ABI Genetic Analyzer. The sequence read data was used in a BLAST search and confirmed to be the genes and regions of interest for all tested species.

ATTACHMENTS

NZG_Clock-genes_Big_Dye_sequencing_Le-Clercq.xlsx

GUIDELINES

- A sequencing worksheet template is included for download to automatically calculate volumes.
- Equipment used are interchangeable with industry equivalents.
- Experiments performed at Room temperature is always at 21 °C.
- Plates can be stored for up to 48:00:00 until analysis.
- Briefly vortex reagents and mixes as needed.

MATERIALS

Protocol status: Working
We use this protocol and it's working

Created: Jun 20, 2021

Last Modified: Nov 23, 2023

PROTOCOL integer ID: 50917

Keywords: BigDye, Sanger, DNA sequencing, clock, adcyap1, avian

Funders Acknowledgement:
National Research Foundation (RSA)
Grant ID: 112062

Reagents:

- BigDye™ Terminator v3.1 Cycle Sequencing Kit **Applied Biosystems**
- **Primers:** (Inqaba Biotech. Industries)

	A	B	C	D	E	F
	Adcyap F	GATGTGAGTAACCAGCCACT	Adcyap 1	Gene ID: 408251	20	61.3
	Adcyap R	ATAACACAGGAGCGGTGA	Adcyap 1	Gene ID: 408251	18	59.7
	Clock F1	TGGAGCAGTAATGGTACCAA GTA	clock	Gene ID: 373991	23	62.9
	Clock F2	TGGAGCGGTAATGGTACCAA GTA	clock	Gene ID: 373991	23	65.0
	Clock R1	TCAGCTGCGACTGAGCTGG	clock	Gene ID: 373991	19	66.0
	Clock R2	TCAGCTGTGGCTGAGCTGG	clock	Gene ID: 373991	19	66.1

Summary of primer details for the assay including the primer name, sequence, gene, gene ID, length and Tm

- BigDye XTerminator™ Purification Kit **Thermo Fisher Catalog #4376484**

Equipment:

Equipment

SimpliAmp Thermal Cycler

NAME

PCR

TYPE

Applied Biosystems

BRAND

A24811

SKU

https://www.thermofisher.com/order/catalog/product/A24811

LINK

Any standard PCR thermocycler will suffice

SPECIFICATIONS

Equipment

IKA MS 3 Digital Vortex Mixer

NAME

Vortex mixer

TYPE

IKA

BRAND

3319000

SKU

https://www.m2sci.com/ika-ms-3-digital-vortex-mixer/

LINK

Vortex mixing of plates

SPECIFICATIONS

Equipment	
3500 Genetic Analyzer	NAME
Sequence analyzer	TYPE
Applied Biosystems	BRAND
4440470	SKU
https://www.thermofisher.com/order/catalog/product/4440470?SID=srch-hj-4440470#/4440470?SID=srch-hj-4440470	LINK
DNA sequence fragment analysis	SPECIFICATIONS

DNA Amplicons:

- BioSample information information has been deposited to the BioProject (PRJNA737185) linked to this protocol.

PROTOCOL MATERIALS

BigDye™ Terminator v3.1 Cycle Sequencing Kit **Applied Biosystems (ThermoFisher Scientific)**

Materials, Step 1

BigDye XTerminator™ Purification Kit **Thermo Fisher Catalog #4376484**

Materials, Step 3

SAFETY WARNINGS

- Set up master mixes in a "DNA-free" room and laminar flow cabinet.
- Add DNA to reaction tubes in a "DNA-loading" laminar flow cabinet.
- Always dispose of biohazardous waste appropriately in accordance to lab regulations.
- Always wear gloves and a lab coat.
- Never directly look at the UV lamps.

ETHICS STATEMENT

Protocol approval for the present study was obtained from the protocol committee of the Department of Genetics, University of the Free State (approval number: Res18/2020). Ethics approvals were obtained from the University of the Free State (approval number: UFS-AED2020/0015/1709) as well as the South African National Biodiversity Institute (approval number: SANBI/RES/P2020/30). Appropriate research permits were also obtained from South African regulatory authorities including the Department of Agriculture, Land Reform, and Rural Development (Section 20 permit: 12/11/1/1/18(1824JD)).

BEFORE START INSTRUCTIONS

- Thaw reagents On ice .
- Wipe workspace with [M] 10 % volume Bleach, followed by [M] 70 % volume Ethanol, and ddH_2O before (and after).
- UV the relevant laminar flow cabinets.

Big Dye Master Mix setup

1 Prepare BigDye™ Terminator v3.1 Cycle Sequencing Kit **Applied Biosystems** Master mix and Samples* for Sanger Sequencing.

*Sample information has been deposited to BioSample and associated to the BioProject (PRJNA737185) which used this protocol.

(An experiment template is included as an excel spreadsheet)

1.1 Prepare the following sequencing master mixes in duplicate, one for each primer (forward and reverse).

Master Mix:

	A	B	C	D
	BigDye™ 3.1 Ready MM	X2.5	X1	4
	Primer	3.2 µM	3.2 pM	1
	Nuclease free water	-	-	4

Components of sequencing reaction, indicating stock and final concentrations as well as the relative volume needed in microliters. (for a 20µL reaction you can double the volume of each component)

Plate set up for Sanger sequencing.

1.2 Add 9 µL Master Mix to 1 µL purified PCR product to the individual wells of a 96-well PCR plate or thin-walled PCR tubes.

Cycle sequencing

1m

2 Program and run the following cycle conditions on a thermal cycler, e.g. 3m 15s

Equipment	
SimpliAmp Thermal Cycler	NAME
PCR	TYPE
Applied Biosystems	BRAND
A24811	SKU
https://www.thermofisher.com/order/catalog/product/A24811	LINK
Any standard PCR thermocycler will suffice	SPECIFICATIONS

- Initial denaturation at 96 °C for 00:01:00
- 25 cycles of:

1. Denaturation at 96 °C for 00:00:10
2. Annealing at 50 °C for 00:00:05
3. Extension at 60 °C for 00:02:00

- Hold at 4 °C until next step.

Sequence reaction clean-up

3 Purify the sequencing products using
BigDye XTerminator™ Purification Kit **Thermo Fisher Catalog #4376484**

3.1 Vortex the bottle of BigDye XTerminator™ beads for 8 to 10 seconds before mixing with the SAM solution.

3.2 Prepare the SAM/BigDye XTerminator™ bead working solution:

Component	Volume per 10 µL reaction	Volume per 20 µL reaction
SAM solution	45 µL	90 µL
BigDye XTerminator™ bead solution	10 µL	20 µL
Total volume	**55 µL**	**110 µL**

Volumes of SAM solution and beads to add

3.3 Transfer the indicated volume of bead mix (BigDye XTerminator™ bead solution and SAM solution) to each.

3.4 Vortex the 96-well plate/tubes at 1800 rpm, 21°C, 00:20:00 on a shaker, e.g.

Equipment	
IKA MS 3 Digital Vortex Mixer	NAME
Vortex mixer	TYPE
IKA	BRAND
3319000	SKU
https://www.m2sci.com/ika-ms-3-digital-vortex-mixer/	LINK
Vortex mixing of plates	SPECIFICATIONS

3.5 In a swinging bucket centrifuge, centrifuge the plate at 1000 x g, 21°C, 00:02:00 . 2m

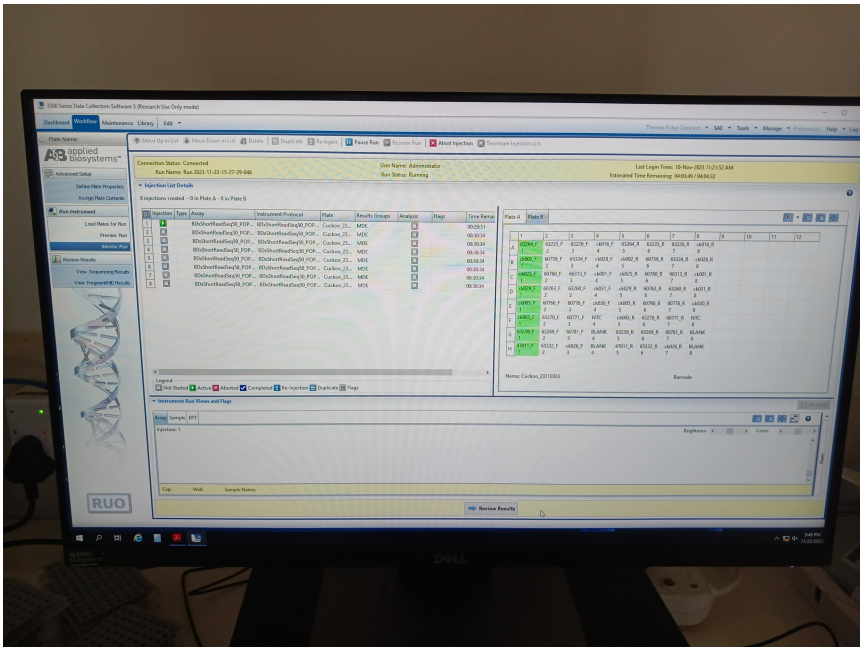
Capillary electrophoresis & Data capture

4 Load sequencing reactions to sequencing plate and set up a run on the genetic analyzer, e.g.

Genetic analyser with plates loaded for capillary electrophoresis.

Equipment

3500 Genetic Analyzer	NAME
Sequence analyzer	TYPE
Applied Biosystems	BRAND
4440470	SKU
https://www.thermofisher.com/order/catalog/product/4440470?SID=srch-hj-4440470#/4440470?SID=srch-hj-4440470	LINK
DNA sequence fragment analysis	SPECIFICATIONS



Set up plate layout for fragment analysis on genetic analyser.

- Export sequence read trace files once done!
- Files can be opened with

Software	
BioEdit	NAME
Windows 10 32-bit	OS
Tom Hall	DEVELOPER
Informer	SOURCE LINK

or

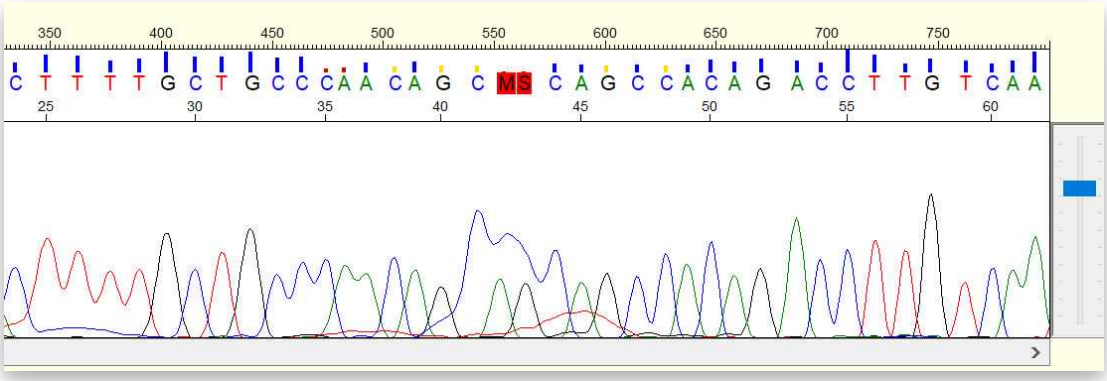
Software	
MEGA	NAME

or

Software

Sequence Scanner	NAME
Windows 10 32-bit	OS
Life Technologies	DEVELOPER
Informer	SOURCE LINK

Expected result



View of trace file in Seq Scanner 2