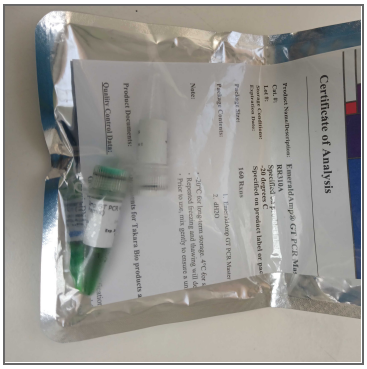

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OPEN ACCESS

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External link:
https://sites.google.com/view/lsleclercq/projects/phd-project

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MANUSCRIPT CITATION:
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).

PCR Amplification of Clock and Adcyap1 genes with EmeraldAmp® GT PCR Master Mix in Avian species for polymorphism elucidation

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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 PCR protocol is used to amplify *Clock* and *Adcyap1* gene regions in avian species which have previously shown polymorphisms, such as poly-Q runs, that correlated to migration phenology. It was tested and optimized in Woodlands kingfisher *(Halcyon senegalensis)* and Diederik cuckoos *(Chrysococcyx caprius).* The primers were designed based on those previously used by Johnson *et al.* (2007) and Steinmeyer *et al.* (2009) by comparing the relevant gene sequences for chickens *(Galus galus)* with several other available avian species to select primers that would account for the most common variations in primer regions, enabling more universal amplification. Individual clock gene sequences were retrieved from Genbank and aligned in BioEdit 7.2. Primers were then selected based on the annotated regions. The assay was designed using 25 µL (half) reactions of EmeraldAmp® GT PCR Master Mix, which is premixed with loading buffer for easy gel loading following PCR and does not require a long initial denaturation step (thereby shortening the run time). Gel electrophoresis was able to confirm successful amplification of a product ±280 bp long in both species. The same primers were subsequently used for sanger sequencing. A BLAST search of the resulting sequences confirmed the identity of the amplified regions.

ATTACHMENTS

Clock_anot_Primers+Product [BioEdit].bio

Adcyap anot Primers+Product [BioEdit].bio

NZG_LeClercq_PCR_template Clock and Adcyap1 genes.xlsx

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

Created: Jun 19, 2021

Last Modified: Nov 23, 2023

PROTOCOL integer ID: 50910

Keywords: Emerald Amp, Clock genes, Avian, PCR, Clock, Adcyap, Kingfisher, Cuckoo

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

IMAGE ATTRIBUTION

https://www.takara-bio.com/

GUIDELINES

- A PCR worksheet template is included for download to automatically calculate volumes.
- Check DNA template quantity and purity prior to use in PCR.
- EmeraldAmp® GT PCR Master Mix **Takara Bio Inc. Catalog #RR310A** allows for direct gel loading.
- Equipment used are interchangeable with industry equivalents.
- Experiments performed at Room temperature is always at 21 °C .
- Treated PCR products may be stored at -20 °C until required for sequencing.
- Briefly vortex reagents and mixes as needed.

MATERIALS

Reagents:

- EmeraldAmp® GT PCR Master Mix **Takara Bio Inc. Catalog #RR310A**
- **Primers**: (Inqaba Biotec. Industries; Annotated "BioEdit" alignment files are included)

	A	B	C	D	E	F
	Adcyap F	GATGTGAGTAACCAGCCACT	Adcyap1	Gene ID: 408251	20	61.3
	Adcyap R	ATAACACAGGAGCGGTGA	Adcyap1	Gene ID: 408251	18	59.7
	Clock F1	TGGAGCAGTAATGGTACCAAGTA	clock	Gene ID: 373991	23	62.9
	Clock F2	TGGAGCGGTAATGGTACCAAGTA	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

- UltraPure™ TBE Buffer 10X **Thermo Fisher Scientific Catalog #15581044**
- SeaKem® LE Agarose **Lonza Catalog #50004**
- SYBR SAFE DNA stain **Life Technologies Catalog #S33102**
- Quick-Load 100 bp DNA Ladder - 375 gel lanes **New England Biolabs Catalog #N0467L**
- ExoSAP-IT™ PCR Product Cleanup Reagent **Thermo Fisher Scientific Australia Catalog #78201.1.ML**

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

Gel Doc XR+ Gel Documentation System	NAME
Gel Documentation System	TYPE
Bio-rad Laboratories	BRAND
1708195	SKU
https://www.bio-rad.com/en-us/product/gel-doc-xr-gel-documentation-system?ID=O494WJE8Z	LINK

Equipment

PowerPac Basic Power Supply	NAME
Power Supply	TYPE
Bio-Rad Scientific	BRAND
1645050	SKU
https://www.bio-rad.com/en-us/sku/1645050-powerpac-basic-power-supply?ID=1645050	LINK

Samples:

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

PROTOCOL MATERIALS

UltraPure™ TBE Buffer, 10X **Thermo Fisher Catalog #15581044** Step 3.1

EmeraldAmp® GT PCR Master Mix **Takara Bio Inc. Catalog #RR310A**

In Guidelines, Materials, Step 1.1

ExoSAP-IT™ PCR Product Cleanup Reagent **Thermo Fisher Scientific Australia Catalog #78201.1.ML**

In Materials and 2 steps

SeaKem® LE Agarose **Lonza Catalog #50004** Materials, Step 3.1

SYBR SAFE DNA stain **Invitrogen - Thermo Fisher Catalog #S33102**

In Materials and 2 steps

Quick-Load 100 bp DNA Ladder - 375 gel lanes**New England Biolabs Catalog #N0467L**

Materials, Step 3.2

UltraPure™ TBE Buffer 10X **Thermo Fisher Scientific Catalog #15581044**

Materials

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.

Master Mix set-up

1 Prepare Master Mix and Samples* for PCR.

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

(An experiment template is included in excel format.)

1.1 30m

Set up the following Master Mix with EmeraldAmp® GT PCR Master Mix **Takara Bio Inc. Catalog #RR310A** for a 25 µL reaction in a DNA-free lab and laminar flow cabinet.

	A	B	C	D
	EmeraldAmp® GT PCR Master Mix	X2	X1	12.5
	Forward primer	10 µM	0.2 µM	0.5
	Reverse primer	10 µM	0.2 µM	0.5
	Nuclease free water	-	-	9.5

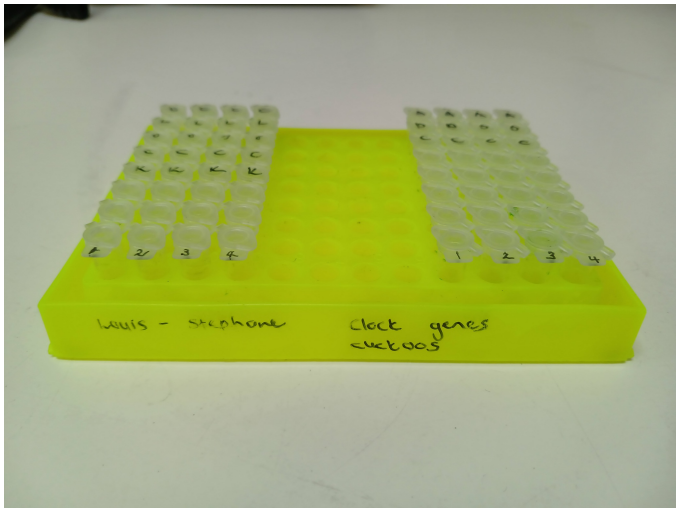
Summary of components to add to Master Mix with the original and final concentrations as well as the relative volume in µL

EmeraldAmp GT MM

Working solutions of forward (Clk F1) and reverse (Clk R1) primers.

1.2

- Add 23 µL Master Mix to 2 µL DNA template* in individual thin-walled PCR tubes in a DNA-loading laminar flow cabinet. 20m



PCR reactions prepared to run thermal cycles.

*DNA isolated from avian blood samples may be highly concentrated, ensure that it is still less that 500 ng of DNA per reaction.

Thermal cycling

10s

2 Program and run the following thermal cycling profile on a thermal cycler, e.g. 2h

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

- 40 Cycles of:

1. Denaturation at 98 °C for 00:00:10
2. Annealing at 60 °C for 00:00:30
3. Elongation at 72 °C for 00:01:00

- Infinite hold at 4 °C until ready for next steps.

Example of PCR run lasting approximately 1h30.

Electrophoresis

3 Confirm success of amplification by TAE/TBE electrophoresis.

3.1 Prepare a [M] 2 % (v/v) gel with SeaKem® LE Agarose **Lonza Catalog #50004** and 45m

UltraPure™ TBE Buffer, 10X **Thermo Fisher Catalog #15581044**, pre-stained with SYBR SAFE DNA stain **Life Technologies Catalog #S33102** using a casting tray and comb with sufficient wells.

Resolution capacity of different concentrations of gels.

	A	B
	0.3	5 to 60 kbp
	0.6	1 to 20 kbp
	0.7	0.8 to 10 kbp
	0.9	0.5 to 7 kbp
	1.2	0.4 to 6 kbp
	1.5	0.2 to 3 kbp
	2.0	0.1 to 2 kbp

Concentration (%) of agarose gels and their efficient range of separation in kilo base pairs.

Amount of agarose required for a small (50 mL) and large (100 mL) gel.

	0.3	150mg	300mg
	0.6	300mg	600mg
	0.7	350mg	700mg
	0.9	450mg	900mg
	1.2	600mg	1.2g
	1.5	750mg	1.5g
	2.0	1g	2g

Concentration (%) of gels and their required amount of agarose.

*Note: SYBR SAFE DNA stain **Life Technologies Catalog #S33102** is usually added at a concentration of 1 µL Stock per 10 mL TBE

SYBR Safe

3.2 Load 4 µL of PCR product to the gel alongside a molecular weight marker, e.g. 40m

Quick-Load 100 bp DNA Ladder - 375 gel lanes **New England Biolabs Catalog #N0467L**, and run at 60 Volt for 00:30:00 . Possible settings for the

Equipment

PowerPac Basic Power Supply NAME

Power Supply TYPE

Bio-Rad Scientific BRAND

1645050 SKU

https://www.bio-rad.com/en-us/sku/1645050-powerpac-basic-power-supply?ID=1645050 LINK

are:

	A	B
	< 1kbp	5 V/cm
	1-12 kbp	4-10 V/cm
	>12 kbp	1-2 V/cm

Ideal voltages for resolving different size fragments.

3.3 Visualize and capture gel on an appropriate imager and paired software, e.g. 15m

Equipment

Gel Doc XR+ Gel Documentation System NAME

Gel Documentation System TYPE

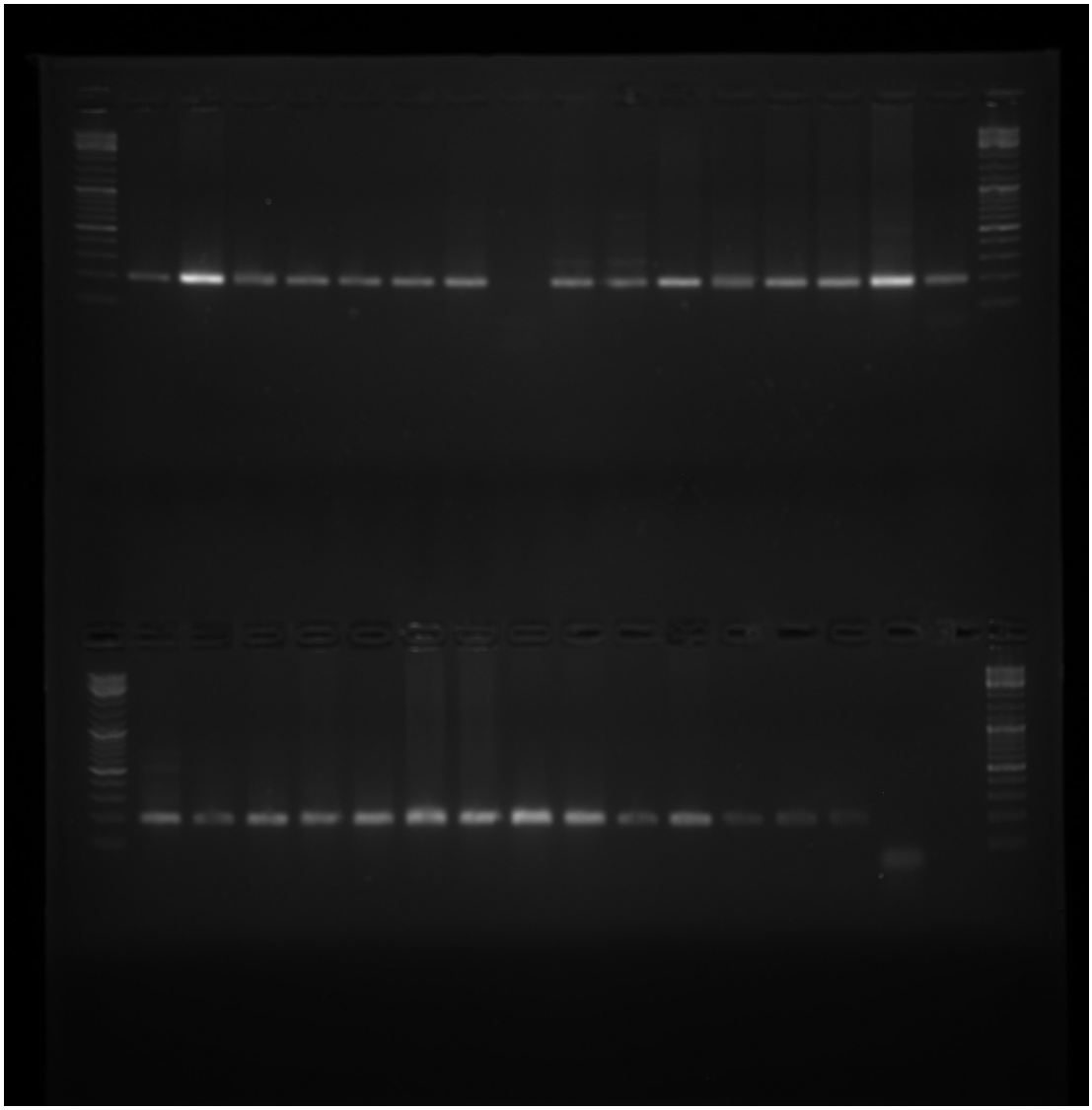
Bio-rad Laboratories BRAND

1708195 SKU

https://www.bio-rad.com/en-us/product/gel-doc-xr-gel-documentation-system?ID=O494WJE8Z LINK

Expected result

Gel image of molecular marker and clock gene amplicons for Diederik cuckoo:



Positive amplification of clock genes viewed on a 1.5% TAE-Agarose gel.

Amplicon purification

30m

4 Purify the positive amplicons with 1h

ExoSAP-IT™ PCR Product Cleanup Reagent **Thermo Fisher Scientific Australia Catalog #78201.1.ML**

prior to sequencing.

4.1 Mix 5 µL PCR product with 2 µL Exo-SAP IT reagent for a total volume of 7 µL . 30m

4.2 Incubate at 37 °C for 00:15:00 to degrade PCR primers and short products. 15m

4.3 Incubate at 80 °C for 00:15:00 to inactivate the 15m

ExoSAP-IT™ PCR Product Cleanup Reagent **Thermo Fisher Scientific Australia Catalog #78201.1.ML**

.

Exo-SAP digestion and inactivation cycles.

4.4 The PCR product is now ready for use in DNA sequencing*, SNP analyses, or other primer-extension applications.

*See Clock genes sequencing protocol (https://protocols.io/view/abi-sanger-sequencing-of-avian-clock-genes-to-eluc-bvydn7s6)