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Avian DNA Sexing kit

REF **Cod MBK0059**

50 **50 REACTIONS /50 DNA samples**

INTENDED USE

The Avian DNA Sexing kit is intended for gender identification of monomorphic bird.

INTRODUCTION Gender identification of species is very important for veterinary, medical and ecological sciences. Nevertheless, sexing based on external morphology is often impossible, because more than the 50% of bird species are monomorphic (Vucicevic et al., 2012). Traditional methods for sexual identification as the observation of sex-specific behaviour, laparoscopy, laparotomy or cloacal examination are time consuming, expensive, and, in some case, invasive and dangerous. To avoid this limitation, molecular sexing-based methods using non-invasive means, such as feathers, were improved (Morinha et al., 2012).
The most common method for avian sexing is based on amplification and electrophoresis of the Chromo-helicase-DNA binding (CHD) gene on the W and Z chromosomes (Griffiths et al., 2008). The detection of these genes allows for sexing of unknown samples.

The Avian DNA Sexing kit is a PCR-based assay that allows the rapid avian molecular sexing using pre-assembled PCR mixes containing specific primers pair for amplification of CHD-W and CHD-Z genes.

PRINCIPLE OF THE ASSAY
The Avian DNA Sexing kit provides three ready reaction mixes for standard PCR, specific for distinct Avian species listed in Table I, II and III.

KIT CONTENTS

1X Avian Master Mix 1: 2 x 500 µl
1X Avian Master Mix 2: 2 x 500 µl
1X Avian Master Mix 3: 2 x 500 µl
Hot Rescue DNA Polymerase (5U/µl): 12 µl (60 U)
Male Control 1: lyophilized - 50 µl
Female Control 1: lyophilized - 50 µl
Male Control 2: lyophilized - 50 µl
Female Control 2: lyophilized - 50 µl
Male Control 3: lyophilized - 50 µl
Female Control 3: lyophilized - 50 µl
Dilution Buffer: 400 µl

OTHER SUPPLIES REQUIRED

- Disposable latex glove
- Precision pipettes
- Sterile pipette tips
- Sterile 1.5 ml vials and sterile 0.2 ml PCR vials
- Tabletop centrifuge

STORAGE

Store the kit at -20°C. Repeated freeze-thawing should be avoid. For discontinued use storage of mixes in working aliquots is recommended.

SHIPPING CONDITIONS

Shipping at room temperature has no detrimental effect on the performance of this kit.

GENERAL PRECAUTIONS FOR PCR

The operator should always pay attention to:

- MAINTAIN STRICTLY SEPARATE WORKING AREAS FOR DNA EXTRACTION AND PCR SET-UP
- use pipette tips with filter;
- store positive material (specimens and amplicons) separately from all other reagents and, if possible, add it to the reaction mix in a facility separated space;
- thaw all components and samples at room temperature before starting an assay;

PROTOCOL

1.1 SAMPLE PREPARATION AND DNA ISOLATION

Total DNA can be obtained by Genomic DNA Isolation Kit MBK0069 (Diatheva) (1).
Please follow the procedure described in Annex A.
Store the DNA at -20°C avoiding repeated freeze/thaw cycles.

(1) Other commercial DNA Purification systems can be used. Please refer to Diatheva technical support for additional information.

IMPORTANT NOTE: The Avian DNA Sexing kit has been formulated for testing several avian species. Be sure to use the right reagents and thermal protocol on the basis of species to be tested. Please refer to the Section 1.2, 1.3 or 1.4 Protocol for the specific specie to analyse.

1.2 Application of Avian DNA Sexing kit using the 1X Avian Master Mix 1

1.2.1 PCR SET UP

- Thaw the **1X Avian Master Mix 1**, vortex for 20 seconds and centrifuge briefly.
- Prepare the PCR mix 1 needed for each sample to be tested following the pipetting scheme below:

	For 1 sample *	**For 20 samples**
PCR mix 1	19.8 µl **1X Avian Master Mix 1** + 0.2 µl Hot Rescue DNA Polymerase	396 µl 1X Avian Master Mix 1 + 4 µl Hot Rescue DNA Polymerase

* For the analysis of more than one sample, simply multiply the volumes of mix and Hot rescue DNA Polymerase for the number of samples to be tested.

- Mix the PCR mix 1 prepared for 20 seconds and centrifuge briefly.
- Aliquot 20 µl of the PCR mix 1 into each of the PCR vials.
- In a separate area, add 5 µl of the DNA samples to be tested (prepared as indicated in section 1.1) into the corresponding PCR vial containing PCR mix, vortex and centrifuge briefly.
- In the same separate area thaw the Male Control 1 and Female Control 1 (lyophylized). Reconstitute each vial by adding 50µl of Dilution Buffer, vortex for 60 seconds and centrifuge briefly. Add 5µl of Male and Female control into the corresponding PCR vial containing amplification mix.

1.2.2 PCR RUN

- Program the PCR thermal cycler with the following parameters, specific for the PCR mix prepared in section 1.2.1:

  PCR mix 1

  Important note: Different species require different annealing temperature

1 cycle	95°C for 1min
30 cycles**	95°C for 30sec 52-58°C* for 15sec 72°C for 30sec
1 cycle	72°C for 5min
	cool down to 4°C

*****for appropriate annealing temperature (**See Table I**)
****** perform 35 cycles for species that require an annealing temperature of 52°C

- Set the reaction volume to **25 µl**
- Perform the PCR run.

When the run is completed, proceed immediately to the next step (section 1.5) or store the reaction at +4°C or at –20°C for a longer time.

1.3 Application of Avian DNA Sexing kit using the 1X Avian Master Mix 2

1.3.1 PCR SET UP

- Thaw the **1X Avian Master Mix 2**, vortex for 20 seconds and centrifuge briefly.
- Prepare the PCR mix 2 needed for each sample to be tested following the pipetting scheme below:

	For 1 sample *	**For 20 samples**
PCR mix 2	19.8 µl **1X Avian Master Mix 2** + 0.2 µl Hot Rescue DNA Polymerase	396 µl 1X Avian Master Mix 2+ 4 µl Hot Rescue DNA Polymerase

* For the analysis of more than one sample, simply multiply the volumes of mix and Hot rescue DNA Polymerase for the number of samples to be tested.

- Mix the PCR mix 2 prepared for 20 seconds and centrifuge briefly.
- Aliquot 20 µl of the PCR mix 2 into each of the PCR vials.
- In a separate area, add 5 µl of the DNA samples to be tested (prepared as indicated in section 1.1) into the corresponding PCR vial containing PCR mix, vortex and centrifuge briefly.
- In the same separate area thaw the Male Control 2 and Female Control 2 (lyophylized). Reconstitute each vial by adding 50µl of Dilution Buffer, vortex for 60 seconds and centrifuge briefly. Add 5µl of Male and Female control into the corresponding PCR vial containing amplification mix.

1.3.2 PCR RUN

- Program the PCR thermal cycler with the following parameters, specific for the PCR mix prepared in section 1.3.1:

PCR mix 2

1 cycle	95°C for 1min
30 cycles	95°C for 30sec 52°C for 15sec 72°C for 30sec
1 cycle	72°C for 5min
	cool down to 4°C

- Set the reaction volume to **25 µl**
- Perform the PCR run.
- When the run is completed, proceed immediately to the next step (section 1.5) or store the reaction at +4°C or at –20°C for a longer time.

1.4 Application of Avian DNA Sexing kit using the 1X Avian Master Mix 3

1.4.1 PCR SET UP

- Thaw the selected **1X Avian Master Mix 3**, vortex for 20 seconds and centrifuge briefly.
- Prepare the PCR mix 3 needed for each sample to be tested following the pipetting scheme below:

	For 1 sample *	**For 20 samples**
PCR mix 3	19.84 µl **1X Avian Master Mix 3** + 0.16 µl Hot Rescue DNA Polymerase	396.8 µl 1X Avian Master Mix 3 + 3.2 µl Hot Rescue DNA Polymerase

* For the analysis of more than one sample, simply multiply the volumes of mix and Hot rescue DNA Polymerase for the number of samples to be tested.

- Mix the PCR mix 3 prepared for 20 seconds and centrifuge briefly.
- Aliquot 20 µl of the PCR mix 3 into each of the PCR vials.
- In a separate area, add 5 µl of the DNA samples to be tested (prepared as indicated in section 1.1) into the corresponding PCR vial containing PCR mix, vortex and centrifuge briefly.
- In the same separate area thaw the Male Control 3 and Female Control 3 (lyophylized). Reconstitute each vial by adding 50µl of Dilution Buffer, vortex for

60 seconds and centrifuge briefly. Add 5μl of Male and Female control into the corresponding PCR vial containing amplification mix.

1.4.2 PCR RUN

- Program the PCR thermal cycler with the following parameters, specific for the PCR mix prepared in section 1.4.1:

  PCR mix 3

1 cycle	95°C for 1min
45 cycles	95°C for 30sec 55-58°C* for 15sec 72°C for 30sec
1 cycle	72°C for 5min
	cool down to 4°C

*for appropriate annealing temperature (**See Table III**)

- Set the reaction volume to 25 μl
- Perform the PCR run.
- When the run is completed, proceed immediately to the next step (section 1.5) or store the reaction at +4°C or at –20°C for a longer time.

1.5 AGAROSE GEL ELECTROPHORESIS

- Add 5 μl of DNA loading buffer directly to amplified samples.
- Load 25 μl of amplicons on a 2.5% agarose gel containing ethidium bromide or any other stain gel agent, in the presence of a DNA standard specific for the low range (100-1000 bp).
- To correctly identify all PCR products obtained, run the gel as long as amplicons are well distinguishable as shown below (**Fig.1**).

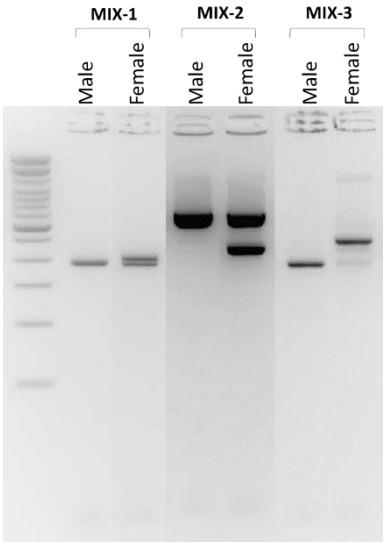

Fig.1: Representative agarose gel electrophoreses analysis of amplicons obtained through the amplification of Male and Female controls using Avian DNA Sexing kit.

1.6 INTERPRETATION OF RESULTS

PCR product loaded on 2.5% agarose gel will show two bands in female samples and only one band in male samples. For the interpretation of results please refer to:

- Table I for the samples amplified using **1X Avian Master Mix 1**
- Table II for the samples amplified using **1X Avian Master Mix 2**
- Table III for the samples amplified using **1X Avian Master Mix 3**

Table I - 1X Avian Master Mix 1: Optimal annealing temperature and sizing of PCR products.

Order	Family	Latin name	Annealing temperature	PCR product sizes (bp)
Psittaciformes	Psittacidae	*Agapornis roseicollis*	58°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Agapornis fischeri*	58°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Agapornis personatus*	58°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Poicephalus senegalus*	58°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Agapornis nigrigenis*	58°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Forpus coelestis*	58°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Ara ararauna*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Aratinga solstitialis*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Aratinga pertinax*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Amazona ochrocephala*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Psittacus erithacus*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Pionites heine*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Pionus maximiliani*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Amazona aestiva xanthopteryx*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Cacatuidae	*Nymphicus hollandicus*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittaculidae	*Platycercus eximius*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittaculidae	*Trichoglossus rubritorquis*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Trichoglossus haematodus*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Myiopsitta monachus*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Psittacula krameri*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Amazona amazonica*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittaculidae	*Chalcopsitta atra*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittaculidae	*Chalcopsitta duivenbodei*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittaculidae	*Polytelis anthopeplus*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittaculidae	*Pseudeos fuscata*	55°C	M (380 bp); F(380 & 400 bp)
Psittaciformes	Psittacidae	*Ara chloropterus*	55°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Fringillidae	*Carduelis carduelis*	52°C	M (310 bp); F(380 & 310 bp)
Passeriformes	Fringillidae	*Carduelis major*	52°C	M (310 bp); F(380 & 310 bp)
Passeriformes	Fringillidae	*Chritagra mozambica*	52°C	M (310 bp); F(380 & 310 bp)
Passeriformes	Fringillidae	*Eophona personata*	52°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Fringillidae	*Loxia leucoptera*	52°C	M (350 bp); F(350 & 400 bp)
Passeriformes	Oriolidae	*Oriolus chinensis*	52°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Pycnonotidae	*Spizixos semitorques*	52°C	M (350 bp); F(350 & 400 bp)
Passeriformes	Sylviidae	*Paradoxornis gularis o Psittiparus gularis*	52°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Thraupidae	*Dacnis cayana*	52°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Thraupidae	*Tangara arthus*	52°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Thraupidae	*Tangara Cyanocephala*	52°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Thraupidae	*Tangara guttata*	52°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Thraupidae	*Tangara nigroviridis*	50°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Turdidae	*Turdus merula*	55°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Turdidae	*Turdus iliacus*	55°C	M (380 bp); F(380 & 400 bp)

Passeriformes	Turdidae	*Turdus philomelos*	55°C	M (380 bp); F(380 & 400 bp)
Passeriformes	Turdidae	*Turdus pilaris*	55°C	M (380 bp); F(380 & 400 bp)
Columbiformes	Columbidae	*Columba livia (pigeon)*	48°C	M (360 bp); F(360 & 350 bp)
Galliformes	Phasianidae	*Chrysolophus pictus*	55°C	M (360 bp); F(360 & 380 bp)
Galliformes	Phasianidae	*Chrysolophus pictus luteus*	55°C	M (360 bp); F(360 & 380 bp)
Galliformes	Phasianidae	*Phasianus colchicus mongolicus*	55°C	M (380 bp); F(380 & 400 bp)
Galliformes	Phasianidae	*Syrmaticus reevesii*	55°C	M (380 bp); F(380 & 400 bp)
Galliformes	Phasianidae	*Lophura nycthemera*	55°C	M (360 bp); F(360 & 380 bp)
Galliformes	Phasianidae	*Chrysolophus amherstiae*	55°C	M (360 bp); F(360 & 380 bp)
Galliformes	Phasianidae	*Gallus domesticus*	55°C	M (360 bp); F(360 & 380 bp)

NOTE:

- Sometimes male DNA from *Agapornis sp.* can also generate a weak band at 400 bp, anyway, there is no ambiguity in results interpretation, because in female samples the band at 400 bp is stronger than the one at 380 bp and not fainter as happens in that particular male samples
- Sometimes species amplified using an annealing temperature of 52°C can also generate a band at 250 bp, anyway, there is no ambiguity in results interpretation.

If Male and Female control have been included check the validity of results according to the following scheme:
Male control 1: presence of one band with size corresponding to 380 bp (indicating a successfully performed PCR)
Female control 1: presence of two bands with size corresponding to 380 & 400 bp (indicating a successfully performed PCR)

Table II - 1X Avian Master Mix 2: Optimal annealing temperature and sizing of PCR products.

Order	Family	Latin name	Annealing temperature	PCR product sizes (bp)
Falconiformes	Falconidae	*Falco peregrinus*	52°C	M (680 bp); F (680 & 460 bp)
Falconiformes	Falconidae	*Falco tinnunculus*	52°C	M (680 bp); F (680 & 460 bp)
Falconiformes	Falconidae	*Falco cherrug*	52°C	M (680 bp); F (680 & 460 bp)
Falconiformes	Falconidae	*Falco biarmicus feldeggii*	52°C	M (680 bp); F (680 & 460 bp)
Accipitriformes	Accipitridae	*Parabuteo unicinctus*	52°C	M (700 bp); F (700 & 460 bp)
Accipitriformes	Accipitridae	*Aquila nipalensis*	52°C	M (680 bp); F (680 & 460 bp)
Galliformes	Phasianidae	*Pavo cristatus*	52°C	M (660 bp); F (660 & 460 bp)
Anseriformes	Anatidae	*Anser cygnoides*	48°C	M (660 bp); F (660 & 460 bp)
Anseriformes	Anatidae	*Anser anser domesticus*	48°C	M (660 bp); F (660 & 460 bp)
Coraciiformes	Coraciidae	*Coracias garrulus*	52°C	M (700 bp); F (700 & 460 bp)

Table III - 1X Avian Master Mix 3: Optimal annealing temperature and sizing of PCR products.

Order	Family	Latin name	Annealing temperature	PCR product sizes (bp)
Strigiformes	Strigidae	*Bubo bubo*	55°C	M (380 bp); F (380 & 480 bp)
Strigiformes	Strigidae	*Bubo scandiacus*	55°C	M (380 bp); F (380 & 480 bp)
Strigiformes	Strigidae	*Bubo africanus*	55°C	M (380 bp); F (380 & 480 bp)
Strigiformes	Strigidae	*Bubo bengalensis*	55°C	M (380 bp); F (380 & 480 bp)
Strigiformes	Tytonidae	*Tyto alba*	58°C	M (380 bp); F (380 & 480 bp)
Strigiformes	Strigidae	*Ninox novaeseelandiae*	58°C	M (380 bp); F (380 & 480 bp)
Strigiformes	Strigidae	*Ninox boobook*	58°C	M (380 bp); F (380 & 480 bp)
Strigiformes	Strigidae	*Surnia ulula*	58°C	M (380 bp); F (380 & 480 bp)
Anseriformes	Anatidae	*Aix sponsa*	58°C	M (380 bp); F (380 & 480 bp)
Anseriformes	Anatidae	*Tadorna ferruginea*	58°C	M (380 bp); F (380 & 480 bp)
Anseriformes	Anatidae	*Cairina moschata*	58°C	M (380 bp); F (380 & 480 bp)
Anseriformes	Anatidae	*Anas platyrhynchos*	55°C	M (380 bp); F (380 & 480 bp)
Galliformes	Numididae	*Numida meleagris*	50°C	M (380 bp); F (380 & 480 bp)

TROUBLESHOOTING

Observation	**Possible cause**	**Suggested solutions**
No PCR product	Missing components (e.g. template or DNA Polymerase)	Check the assembly of the reaction
	Missing or incorrect essential step in the cycler protocol	Check the cycler protocol
	Poor quality template	DNA extraction should be carried out according to manufacturer suggestions of recommended extraction kit (as indicated above)
	Insufficient starting template	Increase starting template
		Perform PCR reaction in a final volume of 50 µl using 10 µl of DNA starting template.
	Degraded reagents	Store reagents at -20°C. Avoid multiple freeze thaw cycles
	Pipetting mistake	Check pipetting and repeat the test
	Presence of inhibitory substances	Dilute DNA starting template
Extra bands on gel	Too much starting template	Dilute DNA starting template
	High performance of your PCR platform	Reduce to 20-25 the number of PCR cycles
	Cross-reaction with non-target sequences	Increase gel concentration from 2.5 to 3% agarose and run amplicons for a longer time in order to distinguish possible non-specific amplicon from target amplicons

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