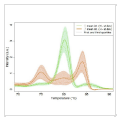

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Working

Real-Time Q-PCR Protocol for chicken sex identification.

PLOS One

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ABSTRACT

This is a Real-Time quantitative PCR based chicken gender determination protocol to identify the sex of fertilized egg at the early chicken embryo states. It is based on the differences between sexual chromosomes: ZZ for males and ZW for females. Two pairs of primers have been developped to give a robust differential signal between males and females: Xho-I and DMRT.

This protocol has been designed to sex chicken embryos in an invasive manner by openning the eggs.

EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

He L, Martins P, Huguenin J, Van T, Manso T, Galindo T, Gregoire F, Catherinot L, Molina F, Espeut J (2019) Simple, sensitive and robust chicken specific sexing assays, compliant with large scale analysis. PLoS ONE 14(3): e0213033. doi: 10.1371/journal.pone.0213033

PROTOCOL STATUS

Working

Lysis of chicken embryo's tissue and DNA extraction

1 **1.** Prepare the Lysis Buffer:

Composition for 100 ml Buffer	Quantity (Volume)	Final Concentration
Chelex Resin (Biorad Chelex 100)	10g	10%
SDS 20 %	1 ml	0.2%
Tris 1M pH8	1 ml	10 mM
Nuclease Free Water	qsp 100 ml	

2. Put 150 µl of lysis buffer in each 1.5 ml tube or well of 96-well plate for each sample to analyze.

3. Pipet 10~20 mg of embryo tissues from the embryo head (brain), membrane, chorion or other into the tube/well containing the lysis buffer.

4. Add 1.5 µl proteinase K at 20 mg/ml stock into each tube/well and vortex 3 seconds.

5. Incubate the tubes/plate in the thermocycler. **03:15:00**

Program:

· Heat lid at 60°C

· Incubate 3h at 55°C

· Incubate 15 min at 95°C

· Hold at 4°C

6. Centrifuge 5 minutes at 13000 rpm (or 15 mins at 8000 rpm) at room temperature. 00:05:00

7. Pipet 50 µl-100 µl of the supernatant. Put it in a new 96-well plate (this primary stock solution contains the template DNA at a concentration between 500 and 1000 ng/µl per tube) and store it:

- at 4°C if you are doing the PCR the same day.

- at -20°C if you are going to do the PCR another day (DNA is degraded with time in biological extracts).

8. Dilute the supernatant 4 times into a new 96-well plate (For example add 25 µl of the supernatant to 75 µl of nuclease free water). It will give a final concentration of prepared template DNA between 125 and 250 ng/µl.

Prepare the Primers Ddilutions

2 Two set of primers (Xho-I & DMRT) are used in this protocol.

Xho-I Primers

Xho-I Forward: CCCAAATATAACACGCTTCACT

Xho-I Reverse: GAAATGAATTATTTTCTGGCGAC

DMRT Primers

DMRT-57 Forward: CTTTCACAAATGTGTTCTGCTGT

DMRT-57 Reverse: AGCAGATACAACCTAAGAATGCC

Prepare a 10 µM primer working solution from 100 µM primer stock.

q-PCR setup

3 **1.** Assemble all reaction components **on ice in this order:**

Components	10 µl reaction	Final Concentration
Nuclease Free Water	qsp 10 µl	-
Sybr Green I Master Mix	5 µl	1x
10 µM Forward Primer Xho-I-F	0.5 µl	0.5 µM
10 µM Forward Primer DMRT-F	0.5 µl	0.5 µM
10 µM Reverse Primer Xho-I-R	0.5 µl	0.5 µM
10 µM Reverse Primer DMRT-R	0.5 µl	0.5 µM
DNA extract	1 µl	

2. Seal the plate, vortex and centrifuge briefly.

3. Transfer the Q-PCR plate into the LightCycler® 480 system.

In the lab we use the LightCycler® 480 System and the LightCycler® 480 SYBR GREEN I master reaction mix (Roche, Bâle, Swiss). It's advised to use the 384 q-PCR plate to perform the reaction with 10 µl per well.

Thermo-cycling conditions

4 The following table shows the PCR parameters set on a LightCycler ® 480 system PCR run using a 384 multiwell plate.

Lightcycler Setup					
Block type: 384	Reaction volume: 10 µl				
Detection: Sybr Green /HRM dye	Excitation filter: 465 nm	Emission filter: 510 nm			
Thermocycling program					
	Temperature	Acquisition Mode	Time	Ramp rate (°C/sec)	Acquisition per °C
Pre-incubation	95°C	None	5 min	4.4	-
Amplification 45 Cycles	95°C	None	10 sec	4.4	-
	53°C	None	20 sec	2.2	-
	72°C	Single	30 sec	4.4	-
Melting Curve	95°C	None	5 sec	4.4	-
	65°C	None	1 min	2.2	-
	97°C	Continuous	-	0.11	5
Cooling	40°C	None	10 sec	1.5	-

Results Analysis

5 2 parameters need to be analyzed for sex determination: Crossing Point (CP) and Melting Curve (MC). A script in R studio can be done for the routine analysis of the curves. In our hand this assay is able to discriminate 100% of chicken samples.

This Q-PCR method was evaluated in term of reproducibility and robustness with 176 sexed embryos without DNA quantification and purification. For more detail please look at our publication "Simple, sensitive and robust chicken specific sexing assays, compliant with large scale analysis" by Lyan He et al.

1. Crossing Point Analysis (Cp):

Average Cp was 8.6 for females, 21.1 for males and 32 for negative control. By defining a Cp value cut-off at 18, the assay is able to discriminate between males and females with 100% concordance. A Cp difference of more than 10 cycles between female and male samples containing between 1 ng and 1 µg DNA is observed. It makes it suitable for sex determination without DNA quantification.

As a second step, to reinforce the Cp discrimination, the melting temperature (tm) profiles need to be compared.

2. Melting Curve Analysis (Tm):

Female chicken exhibit one single peak around 80°C, whereas males show two peaks around 75°C and 84°C. To discriminate males and females chicken, use the ratio of the intensities between 84°C and 80°C.

The averages Tm ratios of 84°C/80°C intensities are 0.033 ±0.1 for females and 1.76 ±0.5 for males. A 84°C/80°C ratio cut-off at 0.33 lead to 100% of discrimination between males and females.

3. Results summary:

	Females	Males	Negative control
Cp	~ 8.6	~ 21.1	~ 32
Tm pics	80°	75°C and 84°C	-
Ratio Tm 84°C/80°C	0.033 ±0.1	1.76 ±0.5	-