ZeeWee™ I Bird DNA Sex Test

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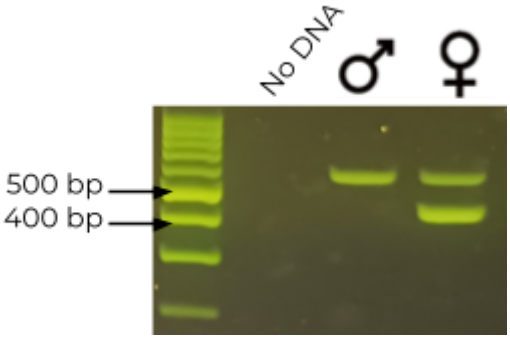
INTRODUCTION

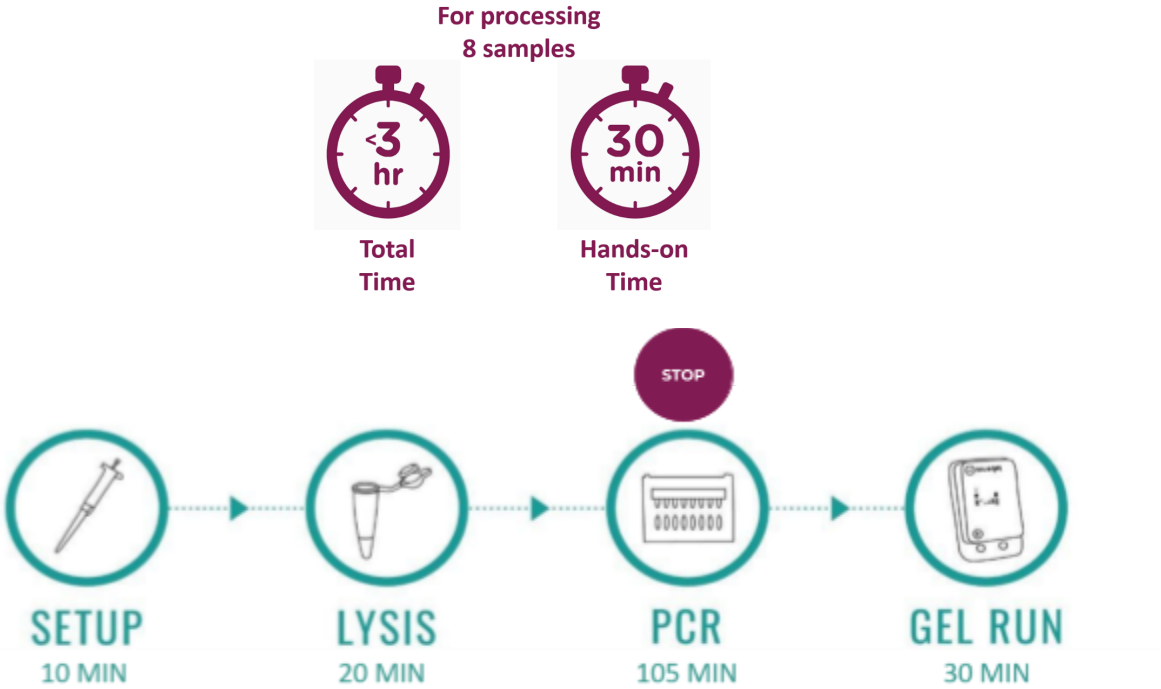
OVERVIEW

The ZeeWee™ I Bird DNA Sex Test is a DNA test to determine the sex of chickens (*Gallus gallus domesticus).* This test uses PCR and gel electrophoresis to determine if your chickens are male or female using either dried blood spots or feather follicle samples.



Start Your On-Farm DNA Lab

PLANNING YOUR TIME



KIT COMPONENTS

The ZeeWee™ I Bird DNA Sex Test has material for testing 32 birds and 6 controls (38 reactions).

SUPPLIED IN KIT

Reagents and Supplies	Quantity	Storage
5X EZ PCR Master Mix	1 tube	Freezer
ZeeWee™ I Primers	1 tube	Freezer
gelPCR™ Control 1	1 tube	Freezer
DEB™ Extraction Buffer	2 tubes	Freezer
100 bp DNA ladder, Load Ready™, 20 lanes	1 tube	Freezer
Strips of 8 PCR tubes	10 strips	Room temp.

SUPPLIED BY USER

Supplies available at dx.minipcr.com

Equipment
These items will be a one-time purchase.

For starting your lab, we recommend our gelPCR™ Bundles (QP-2510-30 and QP-2510-40), that contain the basic equipment and consumables to start testing.

Alternatively, here is the list of all the recommended equipment.

Item	Quantity	Recommended product	Cat. Number
Thermal cycler	1	miniPCR® mini8X thermal cycler	QP-1000-08
		miniPCR® mini16X thermal cycler	QP-1016-16
Gel electrophoresis and visualization system	1	blueGel™ electrophoresis with built-in transilluminator	QP-1500-01
		GELATO™ electrophoresis and visualization system	QP-1600-01
Micropipettes **• 1-10 µl** **• 2-20 µl** **• 20-200 µl** **• 100-1000 µl**	1 each	1-10 µl H-style	QP-1001-05
		2-20 µl H-style	QP-1001-01
		20-200 µl H-style	QP-1001-03
		100-1000 µl H-style	QP-1002-02
Microcentrifuge tube rack	1	Microcentrifuge tube rack, 60-well	CM-1003-10
PCR tube rack	2	miniRack	CM-1003-04
Microcentrifuge (optional)[1]	1	Gyro™ Microcentrifuge, fixed speed	QP-1800-01

[1] Microcentrifuging tubes will guarantee that the liquid collects at the bottom of the tubes, reducing contamination risk. Alternatively, flick tubes (see the "Liquid handling" section).

Consumables

DNA gel electrophoresis reagents and plastic consumables will need to be refilled.

We recommend the Learning Lab Companion Kit (KT-1510-01) to refill DNA gel electrophoresis reagents and microcentrifuge tubes. One Learning Lab Companion Kit will provide enough material for running 38 reactions of the ZeeWee I Bird DNA Sex Test.

Here is the complete list of all the necessary consumables.

Item	Quantity	Recommended product	Cat. Number
Micropipette filter tips	2 boxes each	1-10 µl	4AA75
		2-20 µl	4AA76
		20-200 µl	4AA77
		100-1000 µl	4AA78
TBE Buffer	Enough for making 600 ml – recommended if using blueGel™	TBE electrophoresis buffer, powder makes 600 ml	RG-1502-04
	Enough for making 3 L - recommended if using GELATO™	TBE electrophoresis buffer, powder makes 3 L	RG-1502-05
DNA gel electrophoresis reagents	Enough for preparing 8 small gels or 4 large gels.	SeeGreen™ All-in-One Agarose Tabs™, 8pcs	RG-1500-21
Microcentrifuge tubes	>10 tubes	Microtubes 1.5 ml	6AA02

Other laboratory supplies

- Disposable laboratory gloves
- Bottle and DI water for preparing TBE buffer
- Heat-resistant flask or beaker for preparing the gels
- Protective eyewear
- Lab coat
- Bottle or sprayer with 5% bleach (1 part bleach + 19 parts water)
- Permanent markers
- Disposable scalpels, blades, and/or tweezers

- Untreated disposable toothpicks or 2-200 µl tips (certain toothpick brands might be treated with chemicals that can impair DNA extraction and PCR)
- Containers with lid to dispose of tubes and tips
- Parchment paper or similar to place samples on
- Filter paper or printer/copy paper for collecting blood samples (e.g. unbleached coffee filters)
- Zip lock bags for collecting samples

BEFORE YOU START

LABORATORY GUIDELINES

PCR is an extremely sensitive technology that can detect minute amounts of DNA. Always follow the practices outlined below to minimize the risk of contamination.

How to Organize Your Lab

1. Set up your lab in an area that is removed from possible sources of bird DNA (e.g., away from the husbandry area).
2. Keep each step of the process in separate areas and avoid unnecessary trafficking between areas:
    - **Reagents area**: for handling and preparing the reagents only (except for the gelPCR control).
    - **Sampling area**: for working with samples and extracted DNA.
    - **PCR area**: for amplifying DNA and visualizing the results.

	Reagents area	**Sampling area**	**PCR area**
Purpose	Preparing extraction buffer (for gelPCR tests) Prepare PCR master mix	Preparing samples Transferring lysates to PCR mix (for gelPCR tests) Transferring control to PCR mix	Running PCR Test result visualization
Materials	*Reagents* DEB™ Extraction Buffer PCR Master Mix Primers *Equipment* 20-200 µl and 100-1000 µl micropipettes Marker miniRack Trash bin Bottle with bleach solution *Consumables* Tissue paper Gloves 200 µl and 1000 µl filter tips PCR tubes 1.5 ml tubes	*Reagents* gelPCR Control 1 *Equipment* Microcentrifuge (*optional*) 1-10 µl micropipette Marker miniRack Trash bin Bottle with bleach solution *Consumables* Tissue paper Gloves 10 µl filter tips Parchment paper Sample collection tools (razor blades, toothpicks, etc)	*Reagents* SeeGreen tabs TBE *Equipment* miniPCR Phone Gel-electrophoresis and visualization system (e.g. blue gel) 2-20 µl micropipette Trash bin *Consumables* 20 µl filter tips Gloves Trash bin

3. Dedicate a specific 2-20 µl micropipette and box of 20 µl filter tips for loading PCR samples into the gel. Never use this pipette and tips for preparing the PCR mix or for the DNA extraction step.
4. Maintain a clean work area. Spray 5% bleach on work surfaces before and after every use.
5. Prepare 5% bleach by 1 volume of household bleach to 19 volumes of distilled water.
6. Change gloves between samples, or spray them with 5% bleach.
7. Wear a clean lab coat when preparing the reagents.
8. Discard materials such as tips, tubes, and gloves in a container that can be closed. Empty it often and clean it with 5% bleach.
9. Keep all tubes closed except for the one that you are actively using.

Failure to follow good laboratory practices can result in contamination that will invalidate results. A contaminated laboratory is difficult to clean up.

CONTROLS

Negative control (strongly recommended)

We strongly encourage you to run a negative control tube containing the PCR reagents but no DNA sample to check for possible contamination. We recommend running one negative control reaction per miniPCR® run. Presence of DNA bands in gel electrophoresis of the negative control is indicative of contamination and invalidates the results of the test batch.

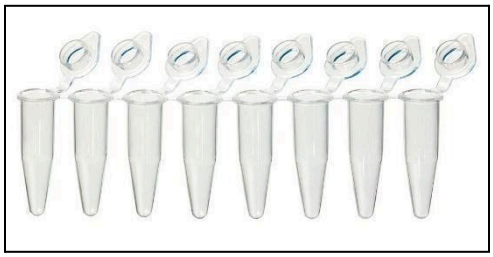
Positive control (recommended if available)

We encourage you to run a positive control reaction for every miniPCR® run. The ideal sample for the positive control is DNA extracted from a known female. This will help you recognize the two possible expected bands and clearly determine the genotype of the unknown samples. Check the section “Expected results'' for more information.

gelPCR Control (recommended to new users)

The gelPCR Control 1 reaction will generate a ~900 bp band. The presence of this band is indicative that the PCR master mix was prepared correctly and the PCR program was run as expected. We encourage you to run this reaction in parallel to your tests, especially when getting familiar with micropipetting technique and learning the PCR process.

TUBE TYPES

Tube type	Picture	Description
Screw-cap reagent tube		Reagents for DNA extraction, PCR, and DNA gel electrophoresis.
1.5 ml microcentrifuge tubes		Used to prepare PCR mix. They are not included in the kit. Available at dx.minipcr.com
PCR tubes		Used for the lysis and PCR steps. Included in the kit.

MICROPIPETTING

Micropipetting

Good micropipetting skills are essential to run the test successfully. Practice with water or colored liquids before using the reagents in this kit.

We recommend to dedicate
- one 100-1000 µl micropipette and one 20-200 µl micropipette to the "reagent setup area" for preparing the reagents (see section "STEP 1: SETUP")
- one 1-10 µl micropipette to the "PCR area" for transferring the lysate and gelPCR™ Control 1 into the PCR mix (see section "STEP 3: PCR")
- one 2-20 µl micropipette for the DNA gel electrophoresis area for loading gels only (see section "STEP 4: DNA GEL ELECTROPHORESIS AND VISUALIZATION").

Dedicating specific micropipettes and tip boxes to each area will reduce the contamination risk.

Always select the right micropipette and tip based on the volume that you need to transfer according to the table below.

Volume range	Micropipette	Tips
1-10 µl	 H10	
2-20 µl	 H20	
20-200 µl	 H200	
100-1000 µl	 H1000	

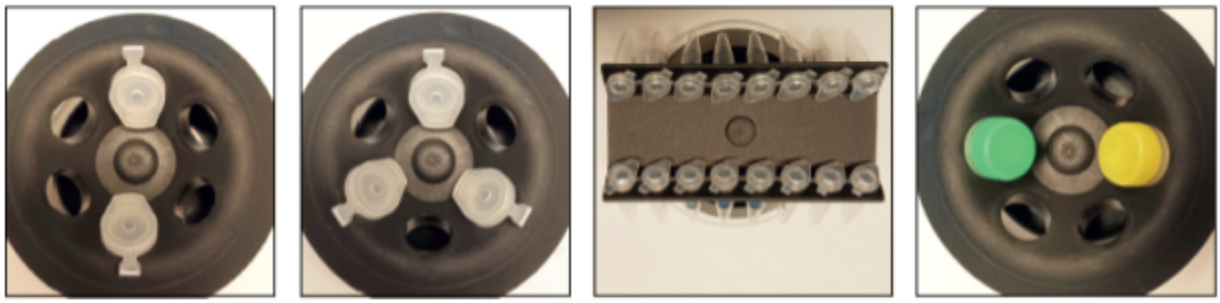
LIQUID HANDLING

Use a microcentrifuge to collect the liquid at the bottom of the tubes. Alternatively, use a strong and quick flick of the wrist to collect the liquid at the bottom of the tube.

To avoid contamination, make sure that all liquid is collected at the bottom of the tubes before opening them.

Follow these steps to use the microcentrifuge:

1. Ensure that the contents of the tubes are fully defrosted.
2. Place the tubes in the rotor, making sure they are balanced as shown in the pictures. The tubes should have approximately the same amount of liquid in them. If needed, prepare a tube with water to balance the rotor. Refer to the manufacturer's instructions for details.
3. Spin for 5 seconds at 10,000 RPM.



Examples of balanced microcentrifuges. Tubes match in size and contain the same volumes, making their weights comparable.

ZeeWee™ I Bird DNA Sex Test

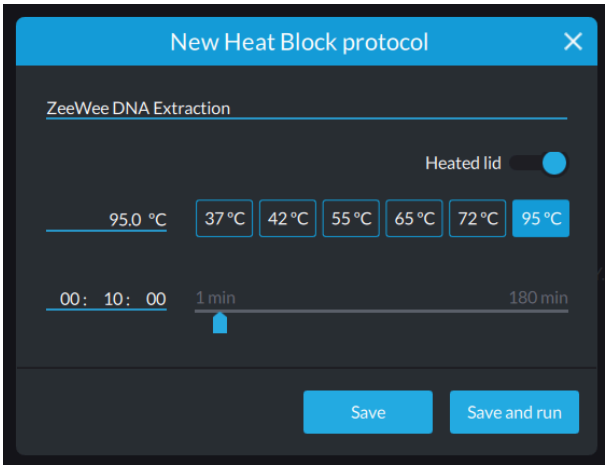
PROGRAM THE miniPCR® OR OTHER THERMAL CYCLER

This is a one-time setup. The program will be saved in the software library and can be reused.

Program the miniPCR®

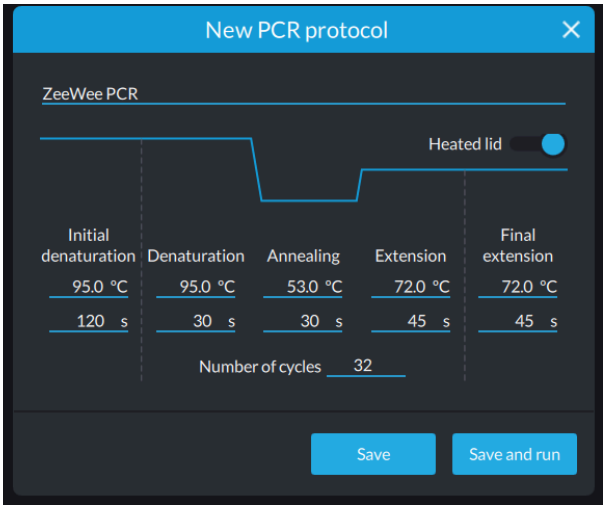
DNA extraction

1. Click or tap on the (+) symbol on the top right corner of the app.
2. Select “Heat Block”.
3. Enter information:
    a. Name: “ZeeWee DNA extraction” or name of your choice
    b. 95 °C
    c. 10 min
4. Click or tap “Save”.



ZeeWee PCR

1. Click or tap on the (+) symbol.
2. Select “PCR”.
3. Enter information:
    a. Name: “ZeeWee PCR” or name of your choice
    b. Initial denaturation 95 °C, 120 sec
    c. Denaturation 95 °C, 30 sec
    d. Annealing 53 °C, 30 sec
    e. Extension 72 °C, 45 sec
    f. Number of cycles 32
    g. Final extension 72 °C, 45 sec
5. Click or tap “Save”.



DRY BLOOD SPOT SAMPLE COLLECTION

Only properly trained individuals should collect blood for testing. Consult with a veterinarian for more information

10. For each bird you plan to test, prepare a 2 cm x 2 cm (approx. 0.4 inch x 0.4 inch) piece of filter paper.
11. Label the corner of the filter paper with a unique sample ID.
12. Spot a few drops of blood onto filter paper.
13. Let it dry at room temperature, then place the paper inside a zip-top plastic bag.
14. If collecting samples from more than one bird, repeat steps 3-4. Wear new gloves or clean them with bleach for each chicken and place each bird's sample in its own bag.
15. Dry blood spot samples can be stored at room temperature for up to a month before use.

FEATHER FOLLICLE SAMPLE COLLECTION

This kit requires freshly plucked feather follicles that include the lower tip of the feather shaft! This protocol cannot be used with molted feathers.

Only properly trained individuals should collect feathers for testing. Consult with a veterinarian for more information.

1. Wear lab gloves to collect feathers.
2. Place the feather from one bird into a zip-top plastic bag.
3. Label the bag with a unique identifier.
4. If collecting samples from more than one bird, repeat steps 1-3. Wear new gloves or clean them with bleach for each chicken and place each bird's feathers in their own bag.
5. Feathers can be stored in the freezer until you are ready to perform the DNA extraction.

PROTOCOL

STEP 1. SETUP (10 MIN)

REAGENTS AREA

In this step you will prepare the reagents needed for the lysis and PCR.

Reagents Preparation

Notes:

- *Complete mix* is the combination of 5X EZ PCR Master Mix and Primer.
- *Total number of reactions* refers to the sum of unknown samples that you are testing and the controls (e.g., negative control, positive control, and gelPCR control 1).

Prepare the DEB™ Extraction Buffer and the PCR mix in an area isolated from where the sample collection and amplification are performed to prevent contamination with bird DNA.

1. Wear clean gloves and clean the surface of the reagent setup area with freshly prepared 5% bleach.
2. Prepare enough reagents to account for the number of samples that you are testing, the positive and negative controls, and the optional gelPCR control 1.

Example with gelPCR control 1

Unknown samples	Negative control	Positive control	gelPCR control	Total number of reactions
5	1	1	1	8

Example without gelPCR control 1

Unknown samples	Negative control	Positive control	Total number of reactions
6	1	1	8

3. Thaw the DEB™ DNA Extraction Buffer, the PCR Master Mix, and the Primers at room temperature.

4. Label a strip of PCR tubes with your sample's IDs.
5. Add **50 µl of DEB™ Extraction Buffer** to each tube. Close all the tubes. Tubes might be hard to close!

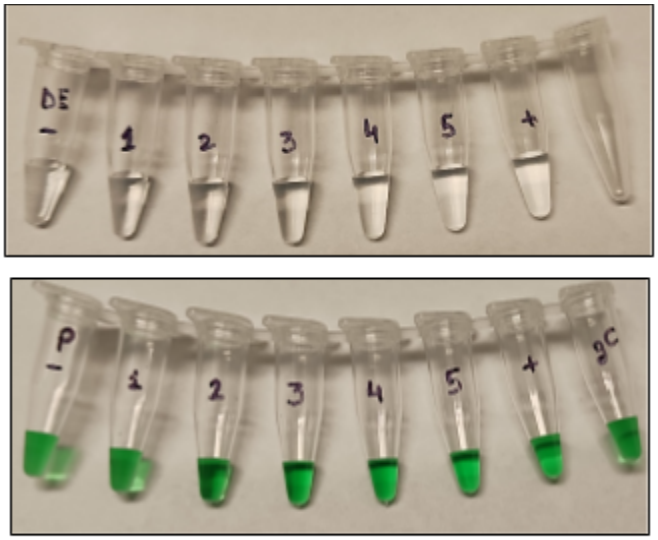
Example of a 20-200 µl micropipette set to 50 µl

6. Label a second strip of PCR tubes. Mark the strip with a "P" (as "PCR").
7. Prepare enough complete mix as indicated in the following table.

	Number of total reactions	
	1	**8**
5X EZ PCR Master Mix (µl)	5.5	44
ZeeWee I Primer (µl)	20	160
Total volume (µl)	**25.5**	**204**

8. Mix well but slowly by pipetting up and down at least six times, being careful not to introduce bubbles.
9. Add **23 µl of the PCR mix** to each PCR tube (use the strip designated for PCR labeled as "P") . Close all tubes.

Remember to bring the PCR Master Mix and the Primer back into the freezer. Once thawed, the DEB™ Extraction Buffer can be stored at room temperature.



Example of tubes containing the DEB™ Extraction Buffer (top, transparent liquid) and the complete mix (bottom, green liquid).

The negative control is indicated with "-", the positive with "+", and the gelPCR control with "gC".

STEP 2. LYSIS (20 MIN)

SAMPLING AREA

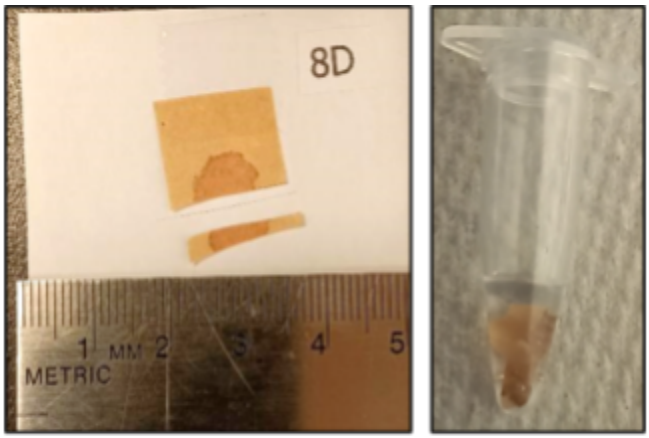
10 minutes hands-on followed by a 10 minutes incubation.

In this step you will extract DNA.

How to Extract DNA

If using dried blood spot samples

1. Wear gloves and clean the surface of the “sample collection area” with freshly prepared 5% bleach.
2. Take the strip labeled as DE (“DNA Extraction”, transparent liquid).
3. Always keep the first tube (negative control) closed.
4. Place the sample on a clean support (e.g., parchment paper) and take a clean disposable blade.
5. Cut a tiny piece of filtered paper with the dried blood spot (approximately 5 mm x 2 mm or 0.2 inch x 0.1 inch for thin filter paper. Use smaller samples if using thick filter paper).
6. Add the sample to the buffer. If needed, use a clean toothpick or a plastic 2-200 µl tip to push the paper into the buffer.
7. Dispose the parchment paper, clean the surface with 5% bleach, and clean the gloves with 5% bleach or change gloves.
8. Process the next samples following steps 4-8. Process the positive control as last.
9. When all the samples are ready, make sure that all the caps are safely closed.



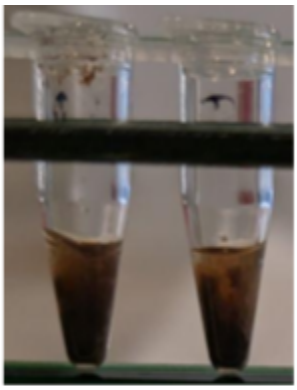
Example of dried blood spot sample

PCR AREA

10. Load the samples into the miniPCR® or another thermal cycler. Close the lid.
11. Run the “Avian DNA extraction” program until competition.

Following extraction, samples should appear clear or pale yellow/pink. A dark color suggests an excessive amount of starting material, which can inhibit amplification and yield inconclusive results.

Example of expected samples

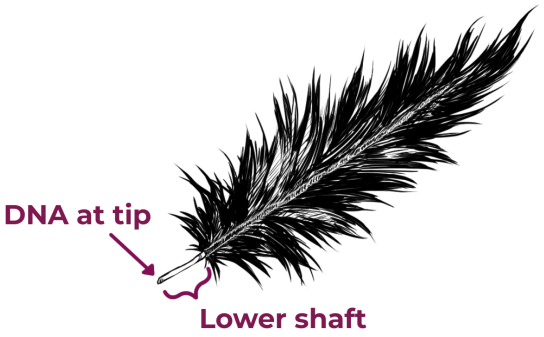


Example of too dark samples

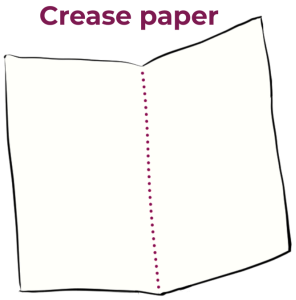
SAMPLING AREA

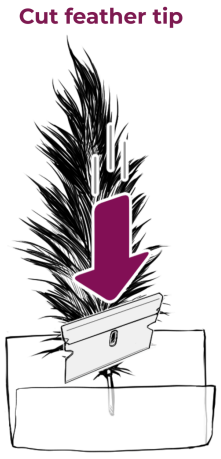
If using feather follicles samples

Make sure to use the very tip of a freshly plucked feather! DNA extraction might fail from molted feathers.



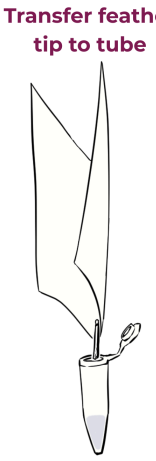
1. Wear gloves and clean the surface of the "sample collection area" with freshly prepared 5% bleach.
2. Take the strip labeled as DE ("DNA Extraction", transparent liquid).
3. Always keep the first tube (negative control) closed.
4. Fold a small square of paper in half. You will cut the tip of a feather off on this piece of paper and then use it like a chute to transfer the tip of the feather into a tube without touching it.
5. Place the base of a feather on the parchment paper, perpendicular to the fold. Having the top of the feather somewhat shielded by the paper is helpful if the tip of the feather "jumps" away when you cut it.
6. Use a clean disposable razor blade to cut off the lower shaft of the feather. This step varies slightly depending on the size of the feather shaft. Refer to the table below.

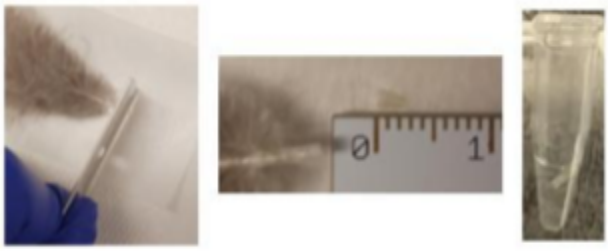




Lower shaft length	Collect
Longer than 0.3 cm	Only the tip of the lower shaft
Between 0.2-0.3 cm	Entire lower shaft
Shorter than 0.2 cm	Entire lower shaft from two feathers

7. Dispose of the top part of the feather.
8. Use the parchment paper like a chute to transfer the tip of the feather to the corresponding labeled tube containing DEB Extraction Buffer. Alternatively, you can lightly spear the feather shaft with the razor blade and use it to transfer the sample to the tube.
9. If needed, use a clean pipette tip or a toothpick to push the feather sample into the buffer. Dispose of the pipette tip or the toothpick after use.
10. Clean the surface with 5% bleach, and clean the gloves with 5% bleach or change gloves.
11. Process the next samples following steps 4-10. Process the positive control as last.
12. When all the samples are ready, make sure that all the caps are safely closed.





Example of feather follicle sample

PCR AREA

13. Load the samples into the miniPCR® or another thermal cycler. Close the lid.
14. Run the “Avian DNA extraction” program until competition.

STEP 3. PCR (105 MIN)

SAMPLING AREA

5 minutes hands-on followed by 100 minutes PCR run.

Setting Up PCR

In this step, you will add the extracted DNA (transparent liquid) to the PCR mix (green liquid) and run the PCR. Open only one tube at a time to avoid possible contamination.

1. Open the first tube on both strips (negative control).
2. Transfer 2 µl from the lysis tube to the PCR mix tube, then close the tubes.

Example of a 1-10 µl micropipette set to 2 µl

3. Repeat step 2 for all tests, making sure that you process one sample at the time.
4. If using gelPCR Control 1:
    a. Make sure that gelPCR Control 1 has thawed.
    b. Transfer 2 µl of gelPCR Control 1 into the assigned tube.
    c. Discard the tip.
    d. Close the gelPCR Control 1 and PCR tubes.
5. Make sure that all tubes are closed.

PCR AREA

6. Load the samples into the miniPCR® or another thermal cycler.
7. Run the “ZeeWee PCR” program till competition.

Remember to bring the gelPCR Control 1 back to the freezer.

Note:
Once the PCR program has finished, samples can stay at room temperature overnight before running the DNA gel electrophoresis.

STEP 4. DNA GEL ELECTROPHORESIS AND VISUALIZATION (45 MIN)

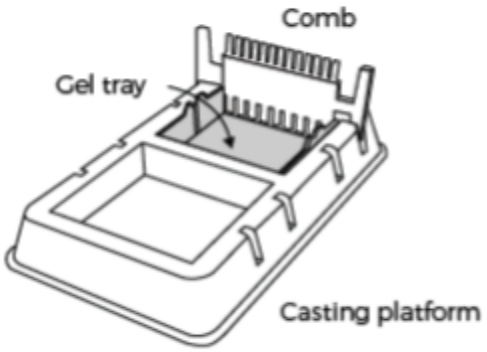
PCR AREA

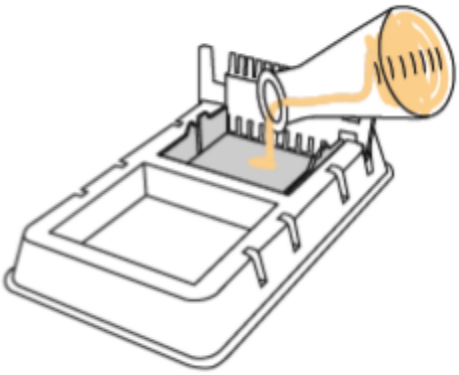
In this step you will prepare the setup for the DNA gel electrophoresis. You can cast the agarose gel and prepare the 1X TBE buffer while the PCR program is running.

1. Prepare the gel casting platform. Make sure that the comb is inserted.
2. If using SeeGreen™ All-in-One Agarose Tabs™ and blueGel™, follow the instructions for preparing a 2% gel.
    a. For one blue gel, soak one tab in 20 ml of water and swirl for about 3 min, till the tab is fully dissolved.
    b. Then, heat the solution until it is clear and all particles are dissolved (typically 30 - 60 seconds in a high-power microwave).
    c. Pour the liquid into the casting platform.
    d. Allow gel to solidify completely.
        - Gels will typically be ready in about 10 minutes.
        - Gel is ready when cool and firm to the touch.

Alternatively, prepare a 2% Agarose gel in 1X TBE buffer with the appropriate amount of DNA staining dye.

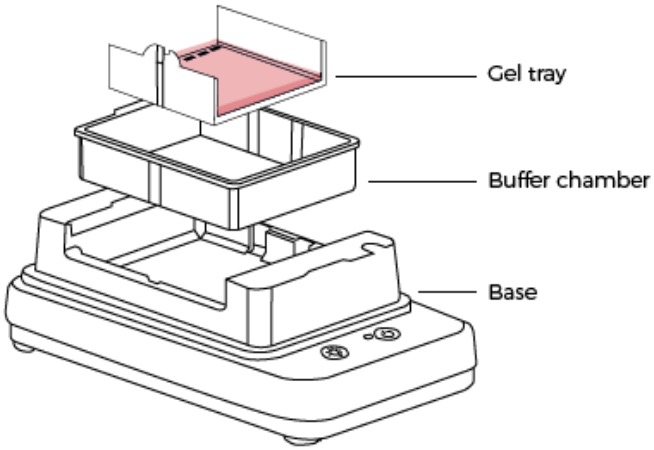
Prepare the Gel and 1X TBE





3. Once the gel is solid and the PCR program is concluded, it is time to run the DNA gel electrophoresis.
4. Remove the comb by pulling firmly upwards and place the gel tray containing your gel in the buffer chamber. If using a blueGel™:
    a. Ensure that the clear buffer chamber is inside the blueGel™ electrophoresis system.
    b. The wells of the gel should be on the same side as the negative electrode, away from the power button.

5. Add 1X TBE electrophoresis buffer to the chamber. If using a blueGel™, use 30 ml of 1X TBE buffer.
   a. The buffer should just cover the gel and fill the wells.
   b. Ensure that there are no air bubbles in the wells (shake the gel gently if bubbles need to be dislodged).



blueGel™ User's Guide

Once the PCR program is completed:

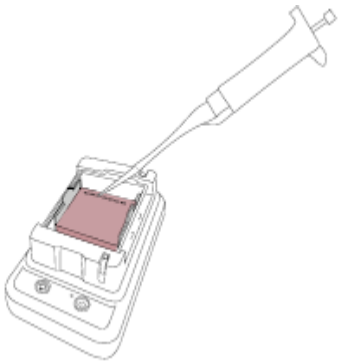
• Tap down or briefly centrifuge the tubes to make sure that the liquid (including possible condensation drops on the top of the tube) collects at the bottom before opening the tubes.
• Open the tubes only in the dedicated PCR area and use the dedicated micropipette and filter tips.
• Never open the tubes in the area where you prepare the PCR mix or collect the DNA samples.
• Never use the micropipettes and the filter tips that you use for the pre-PCR steps for handling the post-PCR samples.

These practices will reduce the risk of contamination.

6. Using the dedicated 2-20 µl micropipette and 20 µl filter tips, load the 100 bp DNA ladder, Load Ready™ in the first lane of the gel.
   a. If using the 8-wells comb, load 10 µl
   b. If using the 13-wells comb, load 7 µl

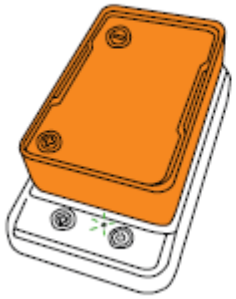
Once thawed, the DNA ladder can be stored at room temperature.

7. Load the PCR samples:
    a. If using the 8-wells comb, load 10 µl of each PCR test in each well. Keep track of the order and change tips between samples.
    b. If using the 13-wells comb, load 7 µl of each PCR test. Keep track of the order and change tips between samples.



Load Gels

8. Run the DNA gel electrophoresis. Specifically, if using a blueGel™:
    a. Place the orange cover on the blueGel™ electrophoresis system.
    b. To prevent fogging, make sure that ClearView™ spray has been evenly applied to the inside of the orange cover.
    c. Match the positive and negative electrode signs on the orange lid with the corresponding positive and negative signs on the blue base.
    d. The orange lid should sit flush with the blue base using little force.
    e. Press the “Run” button. Check that the green light beside the power button remains illuminated.
    f. Conduct electrophoresis for 15-45 minutes. Check gel and take a picture every 15 minutes to monitor sample migration (follow instructions on step 11 and 12).

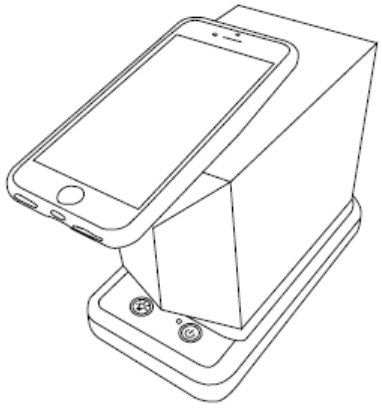


Notes

a. Longer electrophoresis times will result in better size resolution.
b. However, if run too long, small DNA fragments can run off the end of the gel or lose fluorescence.
c. If using GELATO™ electrophoresis and visualization system, run DNA gel electrophoresis at 135 V and take a picture at 10 minutes and at 20 minutes. Please, refer to GELATO™ User's Guide to operate the instrument.

GELATO™ User's Guide

9. Visualize the result using a transilluminator. If using a blueGel™
    a. Press the "light bulb" button to turn on the blueGel™ transilluminator.
    b. For best viewing, dim lights or use the Fold-a-View™ photo documentation hood with a smartphone camera.
    c. If the image appears hazy, wipe off the inside of the orange cover and reapply ClearView™ spray.
    d. Ensure that there is sufficient DNA band resolution in the 400-500 bp range of the 100 bp DNA ladder, Load Ready™. Run the gel longer if needed to increase resolution.
10. Take a picture and document your result.
    a. Place Fold-a-View™ photo documentation hood on the blueGel™ electrophoresis system to take a picture with a smartphone or other digital camera.
    b. Compare the bands from the DNA samples to the ladder to obtain size estimates and interpret the result (check "Expected results" section).



EXPECTED RESULTS

Sample	Expected bands (~ bp)
Negative control	No bands. The presence of DNA bands in the negative control lane indicates contamination; in this case, the test results are invalid and must be discarded.
Male	550
Female	550 bp + 450 or only 450
gelPCR Control 1	900

Results Interpretation