Sexing Bovine Preimplantation Embryos by PCR

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This protocol uses primers directed against Y-specific and autosomal sequences to determine embryo sex. The primers are those designed by Park et al., Theriogenology 2001; 55: 1843-1853.

Sample Collection and Embryo Preparation

Materials

- 100 mM Sodium Phosphate Monobaisc (NaH_2PO_4•$2H_2O$)
  - 13.8 g NaH_2PO_4 (Sigma S9638-250G)
  - 1 L ddH_2O
- 100 mM Sodium Phosphate Dibasic (Na_2HPO_4)
  - 14.2 g Na_2HPO_4•$2H_2O$ (Sigma S0876-1KG)
  - 1 L ddH_2O
  - M Phosphate Buffer (pH 7.4)
  - 100 mL 100mM Sodium Phosphate Monobaisc
  - ~300 mL 100mM Sodium Phosphate Dibasic
    - Add only enough to reach a pH of 7.4
- PBS
  - 100 mL 0.1 M Phosphate buffer
  - 900 mL ddH_2O
  - 9 g NaCl (Sigma S7653-1KG)
- PBS-PVP (1 mg/mL)
  - 13 mL PBS
  - 13 mg polyvinylpyrrolidone (Kodak 15420)
- 0.1% DEPC H_2O
  - 1 mL Diethyl pyrocarbonate (DEPC) (Sigma D5758)
  - 1000 mL H_2O
    - Mix overnight
    - Autoclave 30 min 15 psi to remove residual DEPC
    - Store at room temp ≤ 2 months
- Pronase (0.1%)
  - 0.1 g Protease (P8811-1G) from Streptomyces griseus

- 100 mL PBS
  - Note filtering here will prevent particulate from being in aliqouts.
  - Make 1 mL aliquots and freeze at -20ºC
- 1.5 mL microcentrifuge tubes
- 8-strip 0.2 mL tubes (Fisher 951010022)
  - cross-linked with UV light (120 sec)

Preparation of Embryos

1. Remove Day 7 embryos from culture.
2. Wash once in PBS-PVP.
3. Place embryos in 0.1% pronase for 1-5 min to remove any cumulus cells or sperm that may be adherent to the zona.
4. Wash 3 times in 50 µl PBS-PVP.
5. While washing, mix the embryos well in the PBS-PVP drop to ensure a proper wash and less likelihood of transferring loose cumulus cells and sperm to clean drops.
6. Prepare 10 µl drops of DEPC water. Transfer a single embryo to each drop.
7. Using a new pipette tip for each embryo transfer a single embryo into 0.2 ml PCR tube in 5 µL DEPC H_2O.
8. Store at -20ºC until analysis.

Preparation of Somatic Cells as a Positive Control

1. Collect about 1 million cells, centrifuge at 600 g for 5 min, remove supernatant, and add 1 mL of DEPC H_2O.
2. Centrifuge at 600 g for 5 min.
3. Remove DEPC H_2O while being careful to leave the pellet and resuspend in 200 µL of DEPC H_2O.
4. Create 5 µL aliquots in 0.2 mL centrifuge tubes and freeze at -20ºC.

DNA PCR

Materials

- dNTPs 100mM (Invitrogen 10297-018)
  - 25 µL dATP
  - 25 µL dTTP
  - 25 µL dGTP
  - 25 µL dCTP
  - 900 µL DEPC H_2O
    - store at -20ºC

- 10xPCR Buffer and $MgCl_2$ (Invitrogen 18067-017)
- Taq DNA Polymerase (Invitrogen 18038-042)
- Primers: (we purchase from Integrated DNA Technology (IDT))
  - P1 SEX 5´-GAT CAC TAT ACA TAC ACC ACT-3´
  - P2 SEX 5´-GCT ATG CTA ACA CAA ATT CTG-3´
  - P1 AUT 5´-TGG AAG CAA AGA ACC CCG CT-3´
  - P2 AUT 5´-TCG TCA GAA ACC GCA CAC TG-3´
    - **Note:** Primers come as lyophilized powder. Pay attention to the 'Amount of Oligo' on the information sheet that comes with each primer. Reconstitute primers in 10x the equivalent DEPC water. (i.e. 26.30 nMoles – add 263 µl DEPC water)
    - Prepare working stock of each primer individually by adding 50 µl of reconstituted Primer to 450 µl DEPC water.
- 8-strip 0.2 mL tubes (Fisher 951010022) - cross-link with UV light (120 sec)

Sample Preparation

1. Wipe down the bench area and clean all pipettors with 10% bleach.
2. Remove embryo samples and positive control sample from freezer and thaw quickly at room temperature.
3. Repeat freeze-thaw of samples 2 more times.
   - This is to aid in rupturing the blastomeres and sperm cells to release the DNA into solution.
4. Place samples into the themral cycler.
   - Heat samples at 98ºC for 10 minutes.
   - While samples are in thermal cycler prepare Master Mix 1.

Master Mix 1

1. Take the PCR components (i.e. dNTP mix, 10x PCR Buffer, $MgCl_2$, Taq DNA Polymerase) out of the freezer and gently thaw on ice.
2. Once thawed, assemble PCR Master Mix 1 in a RNase free tube.
   - Prepare enough for (n+3), where n = number of samples.

Master Mix 1

2x RT mix	Reaction Volume (µL)	Number of samples + 3	Total Reaction Volume (µL)
10x PCR Buffer	2	x	=
2.5 mM dNTPs	2	x	=
50 mM $MgCl_2$	2	x	=
Forward Primer (10 µM)	2	x	=
Reverse Primer (10 µM)	2	x	=
Taq DNA polymerase	0.2	x	=
Total	**10.2**	**x**	**=**

3. Mix by gently vortexing then pulse centrifuge. Keep mix on ice.
4. Add 10 µL of Master Mix 1 to each sample and to a positive and negative control.
   - Positive Control: genomic DNA from sperm (male).
   - Negative Control: a tube with no template added (i.e. 5 µL DEPC) to check for contamination.
5. Mix by gently vortexing then pulse centrifuge. Keep mix on ice.
6. Run samples on thermal cycler using the following program.

Sex.1 (run time ~40 min)						
Step	**Denaturation**	**PCR** Cycle (10 cycles)			**Final Extension**	**HOLD**
		Denature	Anneal	Extend		
	HOLD	Cycle			Hold	
Temperature (ºC)/ Time (min)	95 7	95 30 sec	57 30 sec	72 30 sec	72 7	4 ∞
Volume (µL)	15					

7. While the is running prepare Master Mix 2.

Master Mix 2

1. Assemble PCR Master Mix 2 in a RNase free tube.
2. Prepare enough for (n+3), where n = number of samples. Mix by gently vortexing then pulse centrifuge. Keep mix on ice.
3. Once Sex.1 is finished running add 4µL of Master Mix 2 to each sample and to the positive and negative control.
4. Mix by gently vortexing then pulse centrifuge.
5. Run samples on thermal cycler using the following program.

Master Mix 2

2x RT mix	**Reaction Volume (µL)**	**Number of samples x 3**	**Total Reaction Volume (µL)**
Forward Primer (10 µM)	2	X	=
Reverse Primer (10 µM)	2	X	=
Total	**4**	**X**	**=**

Sex.2 (run time ~1 h 30 min)						
Step	**Denaturation**	**PCR** Cycle (25 cycles)			**Final Extension**	**HOLD**
		Denature	Anneal	Extend		
	HOLD	Cycle			Hold	
Temperature (ºC)/ Time (min)	95 7	95 30 sec	57 30 sec	72 30 sec	72 7	4 ∞
Volume (µL)	19					

6. Once Sex.2 is finished you can either freeze samples at -20ºC to do electrophoresis later in the week, or continue immediately to gel electrophoresis.

Electrophoresis of PCR Amplification Products

Materials

- 1X TAE Running Buffer
  - 900 mL ddH_2O
  - 100 mL 10X TAE (Sigma T9650-1L)
    - Store at room temp for 1 month.
    - May be stored in PCR tank for up to one week, label and put your name on tank.
- Agarose (Fisher BP160-500)
- 6x Loading Dye (Promega G1881)
- 100 bp DNA Ladder (New England Biolabs N0467S)
- Ethidium Bromide (10 mg/mL)
  - 10 mg Ethidium Bromide (EtBr) (Fisher BP102-1)
  - 1 mL ddH_2O
    - store in dark container at 4ºC

Casting Gel

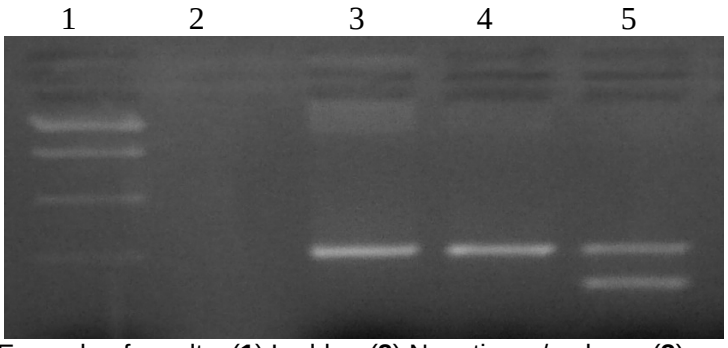
1. Prepare either a 3 or 4% agarose gel using the above formulation.
   - Combine the needed amount of agarose, TAE, and DEPC water in a **250 mL Erlenmeyer flask**.

	3%	4%
Agarose	3.0 g	4.0g
1X TAE	100 mL	100 mL
EtBr (10 mg/mL)	10 µL	10 µL
Total vol	**100 mL**	**100 mL**

2. Swirl to suspend agarose.
3. Place the gel solution into the microwave. Microwave for 2-3 min.
4. This will bring the solution to a boil.
5. Carefully swirl to mix and make sure all agarose is dissolved, if not completely dissolved microwave for 30 sec until dissolved.
6. Cool to 60ºC and then add 10 µL EtBr, swirl to mix.
7. Pour gel and allow to set (20-40 min).
8. Submerge the gel beneath 2 to 6 mm of 1x TAE buffer, then gently remove comb.
    - User a greater depth overlay when using higher voltages to avoid pH and heat effects.

Sample Preparation

1. Place a 2 µL drop of loading buffer for each lane that will have sample on the parafilm
2. Mix 10 µL of sample with 2 µL of loading buffer by repeat pipetting.
3. Place 10 µL of sample + loading buffer in each lane.
4. Place 6 µL of ladder in the first lane.
5. You may also want to add ladder to the last lane.
6. **Run gel Black to Red (70 V/160 mA) for about 1 h with constant voltage.**
7. After about 1 h check the progress of the gel by stopping running and looking at the gel under UV light. Do this until you have the separation you are wanting.
8. Stop electrophoresis when you have enough separation and photograph to record.



Example of results. (**1**) Ladder, (**2**) Negative w/embryo, (**3**) Female (216 bp), (**4**) Female, (**5**) Male (216 + 141 base pairs).

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