Tunel Procedure in Bovine Embryos

Fabiola Paula-Lopes[1], F. Dean Jousan[1], Zvi Roth[1], P. Soto[1], and P. J. Hansen[1]

[1]*Dept. of Animal Sciences, University of Florida*

Modifications underway – we are adding details on use of Hoescht – the procedure works as written but we are trying to improve

Materials

8% (w/v) paraformaldehyde stock solution: Dissolve 8 g of powdered paraformaldehyde in 100 ml water. Heat and stir (55-60 C – do not go higher). Add a few drops of 2 N sodium hydroxide until the solution clears. Make fresh each day. Alternatively, 8% paraformaldehyde can be purchased from Electron Microscopy Sciences as a custom formulation in 4 ml aliquots (cat. no. 15710-SP). Throw away whatever is not used in one day.

4% (v/v) paraformaldehyde: 1:1 solution of 8% paraformaldehyde stock solution and 0.2 M PBS. Make up on the day of use.

Microscope slides: dip the slides in 1:10 poly-l-lysine solution (Sigma P8920) for 2 minutes. Allow the slides to dry.

DNase (50 U/ml) - The stock is RQ1-RNA free DNase, Promega cat. # 610A, concentration 1U/µl. Dilute 10 µl DNase with 190 µl PBS/PVP0µl. Use fresh.

RNase (50µg/ml) - Use RNase A (heat treated), QIAGEN, Germany, cat.#1901, concentration 100mg/ml as a stock. Dilute 1 µl RNase A (stock) with 999 µl PBS/PVP and then dilute this solution 1:1 with PBS/PVP to obtain a working solution (Use fresh).

Mounting medium and antifade: ProLong Antifade Kit (Molecular Probes P-7481)

TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein: Boehringer Mannheim; Cat. No. 1684795): Remove 100 µl Label Solution from bottle 2 for two negative controls. Add total volume of bottle 1 (50 µl) to the remaining 450 µl Label Solution in bottle 2 to obtain 500 µl TUNEL reaction mixture. Mix well to equilibrate components. Note: The TUNEL reaction mixture should be prepared immediately before use and should not be stored.

Hoescht 33342: Prepare Stock 1 by dissolving 25 mg Hoechst 33342 (Sigma B2261) in 2.5 ml of distilled water (10 mg/ml). Store at 4 C. On the day of use, prepare Stock 2 by diluting 5 µl Stock 1 in 10 ml PBS containing 1 mg/ml polyvinylpyrrolidone (PBS-PVP) to produce a 5 µg/ml solution. The working solution is prepared by diluting 200 µl Stock 2 with 800 µl PBS-PVP (final concentration = 1 µg/ml).

Propidium iodide (PI): Prepare a 2.5 mg/ml stock by dissolving PI (Sigma; catalog number P4170) in PBS. Store the stock at 4 C. Immediately before use, add 100 µl PI to 900 µl PBS/PVP and then add 50 µl of this diluted solution to 200 µl PBS/PVP to obtain the final working concentration of 50 µg/ml.

Note: Too much PI can obscure the TUNEL labeling and, if RNA is not completely removed, lead to excessive cytoplasmic staining. It may be necessary to use lower concentrations of PI, or shorter staining time to get good results. We have used concentrations as low as 0.5 µg/ml PI with good results. If possible we recommend use of Hoescht 33342 instead of PI.

Procedure for Performing TUNEL Reaction for Embryos in Solution (Preferred Method)

1. Remove embryos from embryo culture medium (KSOM) and wash 3 times in 50 µl drops of PBS-PVP (2 min for each wash) by transferring the embryos from drop to drop.
2. Fix embryos in 50 µl drops of paraformaldehyde solution [4% (w/v) in PBS, pH 7.4] for 1 h at room temperature. Drops may evaporate if incubation is continued for longer than 1 h.
3. Wash the embryos 3 times in a 50 µl drop PBS/ PVP by transferring the embryos from drop to drop (2 min for each wash).
4. Store the embryos at 4 °C in 4-well plates until the initiation of the TUNEL procedure (or proceed to Step 5 with TUNEL procedure). If embryos were stored, wash the embryos again as described in Step 3.
5. Incubate embryos in a 50 µl drop of permeabilization solution [0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate] for 30 min at room temperature in a humidified box (a plastic box with wet towels will do). Use of a PAP pen or other hydrophobic pen may aid in forming the permeabilization drop. Incubation in the permeabilization solution too long may cause embryos to lyse.
6. FOR PI: Prepare and label 4 dolphin-nosed tubes. One can add PBS/PVP immediately but don't add DNase, RNase, propidium iodide or TUNEL mixture until just before before use.
    a. DNase (190 µl PBS/PVP + 10 µl DNase)
    b. RNase A (999 µl PBS/PVP + 1 µl RNase)
    c. Prepare PI as described above.
    d. TUNEL (empty for now)
7. Wash the embryos as described in Step 3. For positive and negative controls go to Step 8. For samples, proceed to Step 9.
8. **For positive and negative control embryos only -** Incubate positive and negative control embryos with RNA free DNase (50 U/ml) at 37 °C for 1 h in the dark. Wash embryos as described in Step 3. (Continue to Step 9a).
9. Incubate embryos in 25 µl drops of the TUNEL reaction mixture for 1 hour at 37 °C in the dark (place embryos in box covered with aluminum foil).
    a. **For positive and negative control embryos only -** Incubate positive control embryos as described in Step 9. Incubate negative controls in the absence of the enzyme terminal transferase (bottle 1), only with label solution from bottle 2. Continue to Step 10.
10. Wash the slides as described in Step 3.
11. If using PI, incubate embryos with RNase A (50 µg/ml) for 1 h at room temperature in the dark. This step can be omitted if Hoescht 33342 is used to stain nuclei.

i. **NOTE: DO NOT WASH EMBRYOS AFTER THIS STEP!!!**

12. Incubate embryos in a 25-50 µl drop of Hoescht 33342 or PI for 15 min at room temperature in the dark.
13. Wash the embryos 7-8 times in PBS/PVP as described in Step 3.
14. Add 2-3 µl of mounting medium (w/ antifade) to a poly-L-lysine coated slide.
15. Transfer the embryos (5 to 20 embryos per drop) and cover the sample with a cover slip.
16. Determine the number of fluorescent nuclei. Blue (Hoescht) or red (PI) fluorescence indicates non-apoptotic cells and greenish-blue or teal (Hoescht) or green/yellow (PI) fluorescence indicates apoptotic cells.

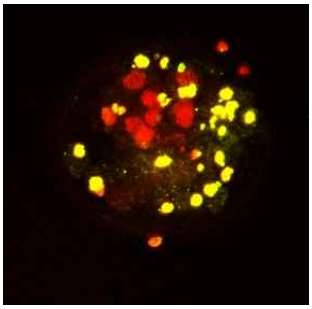
HINT: If background fluorescence is a problem, wash the embryos for a longer duration in Step 13. It is crucial that the non-specific PI is washed away from embryos prior to mounting them.

Procedure for Performing TUNEL Reaction for Embryos Affixed to Microscope Slides

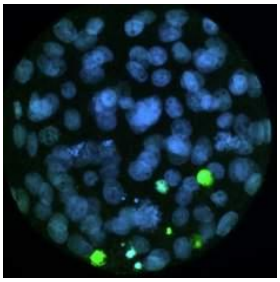
NOTE: The procedure is written for PI but can also be used with Hoescht instead

1) Remove embryos from embryo culture medium (KSOM) and wash 4 times in 100 µl drop of PBS + 1 mg/ml polyvinyl-pyrrolidone (PVP) by transfering the embryos from drop to drop.
2) Fix embryos in 100 µl drop of paraformaldehyde solution [4% (w/v) in PBS, pH 7.4] for 1 h at room temperature. Wash the embryos 3 times in 100 µl drop PBS/ PVP by transfering the embryos from drop to drop. Transfer the embryos to a poly-l-lysine coated slide and allow embryos to dry for 24 hours at room temperature.
3) Wash the slides twice by dipping in a Coplin jar containing PBS/PVP (2 minutes each).
4) Incubate in permeabilisation solution [0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate] for 30 min at room temperature. IF you go too long, the embryo may lyse.
5) Wash the slides as described in step 3.
6) Incubate positive and negative control with DNase (50 U/ml) at 37C for 1 h.
7) Wash the slides as described in step 3.
8) Dry the area around the sample and add 50 µl of TUNEL reaction mixture. Incubate for 1 hour at 37 C in the dark. Incubate negative controls in the absence of the enzyme terminal transferase (label solution from bottle 2).
9) Wash the slides as described in step 3.
10) Incubate the slides with RNase A (50 µg/ml) for 1 h at room temperature.

11) Blot the slides, dry the area around the sample and add propidium iodide (0.5 µg/ml) for 1 h at room temperature.
12) Wash the slides 4 times in PBS/PVP (2 minutes each).
13) Dry the area around the sample, add 16 µl of mounting medium (w/ antifade) to the slide and cover the sample with coverslip.
14) Determine the number of fluorescent nuclei. Red fluorescence indicates nonapoptotic cells and green/yellow (PI) or (Hoescht)fluorescence indicates apoptotic cells.



Confocal image of a bovine embryo heat shocked at 41 C for 9 h and subjected to the TUNEL procedure using PI.



Epifluorescent image of a bovine embryo heat shocked at 41 C for 9 h and subjected to the TUNEL procedure using Hoescht 33342.

modified 6-8-03