

# Immunodetection of 5-methylcytosine in preimplantation embryos

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## Introduction

This procedure for assessing DNA methylation in embryos was developed in our laboratory (Carambula et al., 2009). It is very simple and reliable procedure. Simplicity is enhanced by the use of the Zenon labeling system from Invitrogen to allow one-step labeling of cells with antibody. Modifications to this procedure can allow the procedure to be used for any cell type.

## Reagents

Triton X (Fisher BP151100)  
Paraformaldehyde (Electron Microscopy Sciences cat # 157-8)  
Tween 20 (Sigma cat # P9416)  
Bovine serum albumin (BSA)  
HCl (Fisher cat # A144SI-212)  
Tris base (Sigma cat # T-3253)  
Phosphate-buffered saline (PBS) – see below for recipe  
Polyvinylpyrrolidone (PVP) (Kodak cat # 15420)  
Propidium iodide (Sigma catalog number P4170)  
RNase A (heat treated) (Qiagen, cat.#1901)  
Zenon Alexa fluor 488 Mouse IgG1 Labeling Kit (Invitrogen cat # Z-25002)  
Anti-5-methylcytosine mouse MAAb (Calbichem cat # NA81)  
Hoescht 33342 (Sigma B2261)  
ProLong® Gold antifade reagent (Invitrogen cat # P36930)  
Slides - Superfrost (Fisher cat# 12-550-143)  
Cover slips (Gold Seal cat#3305)

## Solutions

### 1. 100 mM PO<sub>4</sub>, pH7.4 (Stock solution)

To prepare 100 mM PO<sub>4</sub> pH 7.4, add sodium phosphate monobasic (1a) to 300 ml sodium phosphate dibasic until the pH reaches 7.4. Approximately, 100 ml of monobasic solution will be added.

1a. 100 mM sodium phosphate monobasic (NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O)

13.8 g NaH<sub>2</sub>PO<sub>4</sub>  
1 L double-distilled (dd) ddH<sub>2</sub>O

1b. 100 mM sodium phosphate dibasic (Na<sub>2</sub>HPO<sub>4</sub>)

14.2g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O  
1 L ddH<sub>2</sub>O

### 2. 10 mM PBS/PVP (Work solution)

Dilute 100 mM PO<sub>4</sub> pH 7.4 with 800 ml ddH<sub>2</sub>O, add 9 g of NaCl and 1 g PVP, and bring volume to 1000 ml with water. Check pH.

### 3. 4% Paraformaldehyde

2 ml of 8% paraformaldehyde + 2 ml of PBS/PVP

### 4. Wash buffer (0.05% Tween 20 in PBS)

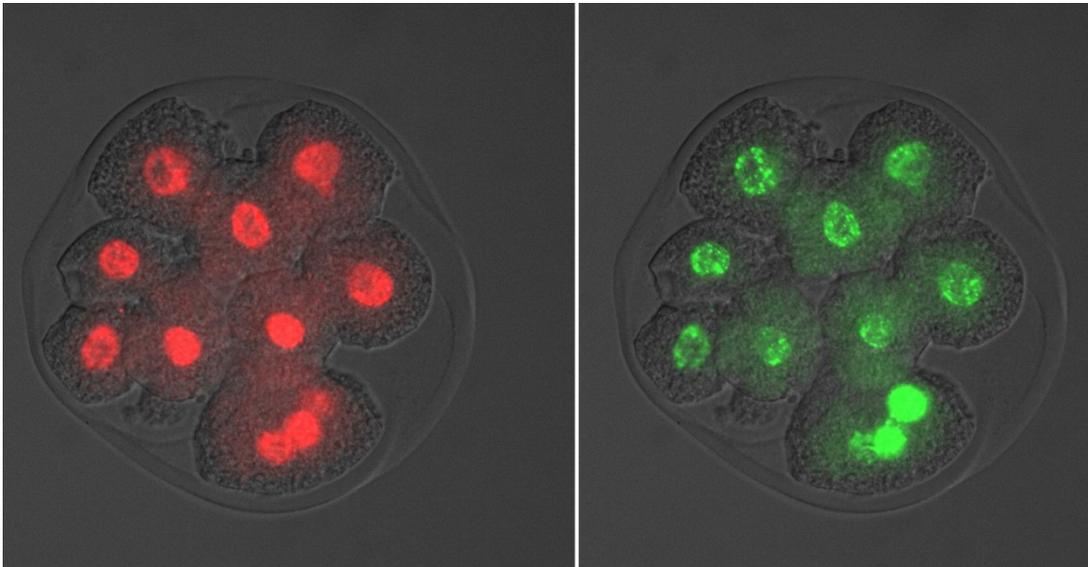
- 50  $\mu$ l Tween 20 in 100 ml PBS/PVP
5. **Permeabilization solution (0.2% Triton X)**  
200  $\mu$ l Triton X in 100 ml PBS/PVP
  6. **RNase (50  $\mu$ g/ml)**  
Use RNase A from Qiagen (100 mg/ml) as a stock. Dilute 1  $\mu$ l RNase A (stock) with 999  $\mu$ l PBS/PVP and then dilute this solution 1:1 with PBS/PVP to obtain a working solution (Use fresh).
  7. **3 M HCl/PVP**  
Mix 40 ml of 12 M HCl with 60 ml of ddH<sub>2</sub>O and add 0.1 g (0.1%) PVP.
  8. **100 mM Tris/PVP**  
1.211g of Tris base in 90 ml ddH<sub>2</sub>O, add 0.1 g (0.1%) PVP, correct the pH to 8.5 with 1 M HCl and bring volume to 100 ml with ddH<sub>2</sub>O.
  9. **Blocking Buffer (2% bovine serum albumin in 0.05% Tween PBS/PVP)**  
Add 2 g of BSA to 100 ml of 0.05% Tween PBS/PVP
  10. **Zenon / Antibody labeling**  
Dilute 1 volume of antibody with 5 volumes of the Zenon A reagent (labeled Fab fragments of anti-IgG1) and 5 volumes of the Zenon B reagent (blocking solution). For this application, 3  $\mu$ l of antibody is added to 15  $\mu$ l of Zenon A. After incubation for 5 minutes, add 15  $\mu$ l of Zenon B and incubate for 5 minutes. The procedure is done in the dark (no direct light). *Note that some embryos should be labeled with an isotype control labeled with the Zenon reagent. Also note that if the user chooses anti-methylcytosine antibodies or a different isotype, Zenon kits specific for that isotype should be used.*
  11. **Antibody dilution**  
The particular antibody used here is used at a final concentration of 0.7  $\mu$ g/ml. Labeled antibody is diluted 1:200 (v/v). In particular, a total of 3  $\mu$ l of antibody is added to 597  $\mu$ l of blocking solution. For other antibodies, the optimal concentration should be determined experimentally.
  12. **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  $\mu$ l PI to 900  $\mu$ l PBS/PVP and then add 50  $\mu$ l of this diluted solution to 200  $\mu$ l PBS/PVP to obtain the final working concentration of 50  $\mu$ g/ml.

## Procedures

1. Wash embryos in PBS/PVP 3 times in 50  $\mu$ l microdrops by transferring embryos drop to drop and allowing 2 min incubation in each drop.
2. Fix in 4% paraformaldehyde (50  $\mu$ l drop) for 15 min.
3. Wash the embryos in PBS/PVP 3 times in a 50  $\mu$ l drops (2 min per drop). At this point, embryos can be stored at 4°C for 1–7 days.
4. Wash the embryos in wash buffer 3 times in 50  $\mu$ l drops (2 min per drop).
5. Incubate the embryos in a 50  $\mu$ l drop of permeabilization solution for 30 min.
6. Wash the embryos as described in step 4.
7. Incubate embryos with RNase A (50  $\mu$ g/ml) for 1 h at 37°C in the dark.
8. Wash the embryos as described in step 4.
9. Incubate in 3M HCl/PVP for 30 minutes at 37°C to denature DNA .
10. Neutralize with 100 mM Tris for 10 minutes at RT.
11. Wash the embryos as described in step 4.
12. Block overnight at 4°C in blocking buffer.

13. Incubate in primary labeled antibody or labeled IgG control.
14. Wash the embryos 4 times in washing buffer.
15. Incubate the embryos with PI for 15 minutes in 25  $\mu$ l drops at room temperature in the dark.
16. Wash the embryos as described in step 4.
17. Add 5  $\mu$ l of Antifade to a slide.
18. Transfer up to 5 embryos in a volume of 20  $\mu$ l to the antifade drop and cover the sample with a cover slip.
19. Observe the embryos under epifluorescence microscopy. Green fluorescence indicates the methylated DNA and red fluorescence the total DNA.
20. For quantification, use an image analysis program like ImageJ to determine the amount of green and red fluorescence. The ratio of green to red fluorescence represents an estimate of degree of methylation.

### Representative Result



Shown here is a differential interference contrast image of a bovine embryo stained with anti 5-methylcytosine (green) and propidium iodide (red).

### Reference

Carambula, S., Oliveira, L.J., and Hansen, P.J. (2009) Repression of induced apoptosis in the two-cell bovine embryo involves DNA methylation and histone deacetylation. *Biochem. Biophys. Res. Commun.* **388**, 418-421.

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