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Original article

# Troubleshooting immunohistochemical labelling of proliferating cell nuclear antigen (PCNA) in cryocut tissue sections of mouse prostate

Jin Han Xie [a], Frédéric Hollande [b,c,d,e], John M. Haynes [a], Colin W. Pouton [a], Sabatino Ventura [a,*]

[a] Prostate Research Cooperative, Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia
[b] CNRS, UMR 5203, Institut de Génomique fonctionnelle, Montpellier, F-34094, France
[c] INSERM, U661, Montpellier, F-34094, France
[d] Université Montpellier 1, Montpellier, France
[e] Université Montpellier 2, Montpellier, France

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ABSTRACT

**Introduction:** The use of antibodies to proliferating cell nuclear antigen (PCNA) for the immunohistochemical detection of proliferating cells has been limited in frozen tissue sections because of its temperature dependence and instability in formaldehyde. In this study various protocols for the immunohistochemical staining of PCNA in frozen mouse prostate tissue sections were tested in order to identify optimal conditions. **Methods:** Fresh prostate tissues from 8 week old mice were frozen in liquid nitrogen or fixed with formaldehyde or paraformaldehyde before freezing with liquid nitrogen. Frozen tissues were then cut in a cryostat and unfixed sections were fixed by dipping slides with sections into fixative. Slide-mounted tissue sections were permeabilized with Triton X-100 before incubating overnight in primary antibody to PCNA diluted to different concentrations in different diluting media at room temperature or 4 °C. Secondary antibody was applied in the same medium as the primary. 19 different experimental protocols were examined. **Results:** Only one protocol showed strong positive immunostaining for PCNA. PCNA-immunopositive cells were observed in greater abundance in the stromal layer. **Discussion:** Paraformaldehyde fixation with Triton X-100 permeabilization without any blocking protocol produced the strongest nuclear PCNA immunolabeling probably when cells are in S-phase of mitosis which indicates the feasibility of PCNA immunoflourescence staining on frozen tissues.



## 1. Introduction

Most previous proliferating cell nuclear antigen (PCNA) immunohistochemical studies have been conducted on paraffin embedded tissues as it has been suggested that antibodies to PCNA do not recognize their target antigen in frozen tissue even when appropriate fixation methods are used (Tsuji et al., 1992). Although PCNA expression can be measured in wax-embedded tissue, the sensitivity of the antigen to variations in fixation and storage time, limit its practicability. Previous studies have indicated that PCNA is both heat and formaldehyde sensitive (Hall et al., 1990). The most commonly used protocol for PCNA immunostaining using frozen tissues is to employ the avidin–biotin–peroxidase complex (ABC) to amplify the positive signal counterstained with hematoxylin. Although this technique is suitable in several tissues, other tissues including prostate (especially rodent prostate) frequently require higher resolution ultrastructural counterstaining to accurately localize PCNA immunohistochemistry to stromal and epithelial subcellular structures. This technique will be especially valuable in comparing tissues from different cancers with variable proliferation capacities and finding a correlation. However, the use of normal prostate tissue in this study is justified by the testosterone dependent growth and development which occurs in early adulthood. Post-pubertal testosterone dependent cell proliferation within the prostate makes the tissue a suitable test tissue for the detection of proliferating cells.

PCNA is an acidic nuclear protein which is recognized as a histological marker for the G1/S phase in the cell cycle. These phases are when DNA synthesis occurs in mitotic cell division. PCNA was originally defined as a cyclin as it was found to be expressed at high levels in cycling cells (Almendral et al., 1987). PCNA expression has been used widely for identifying proliferating cells, especially in prostate cancer and benign prostatic hyperplasia (BPH) (Zhong et al., 2008). It was originally believed that there were different forms of PCNA (Bravo & Macdonald-Bravo, 1987), but it has since been suggested that PCNA exists in either a bound or a free state. Free PCNA can be easily extracted from the nucleus by incubating lysed cells in Triton X-100, whereas bound PCNA cannot (Sasaki et al., 1993). The free form is extractable by detergents and is present in significant amounts in proliferating cells but is almost undetectable in resting cells. Following extraction, PCNA

* Corresponding author. Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia. Tel.: +61 3 9903 9566; fax: +61 3 99039638.
*E-mail address:* Sab.Ventura@pharm.monash.edu.au (S. Ventura).

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becomes a stable complex which participates in DNA replication during the S-phase of the cell cycle (Landberg & Roos, 1991).

In this study, a number of fixing and antibody incubation protocols were tested to optimize the immunohistochemical protocol designed for PCNA detection in frozen prostate tissue. Prostate tissue sections were also counterstained with α-actin and 4′,6-diamidin-2-phenyindole, dilactate (DAPI) to enable easy and accurate localization of PCNA immunostaining.

## 2. Methods

### 2.1. Animals

Seventy-six 8-week old male Swiss mice (4 per protocol) were obtained from Monash Animal Services and housed in a controlled environment at 22 °C with a photoperiod of 12 h light and 12 h dark. Ethical approval for these experiments was obtained from the Monash University Standing Committee on Animal Ethics in Animal Experimentation (Ethics number: VCPA 2006/9). Mice were killed by cervical dislocation and the ventral prostate was removed. Prostates from four mice were used for each protocol.

### 2.2. Fixation protocols

#### 2.2.1. Protocol 1

Freshly dissected prostate tissue was fixed in 4% formaldehyde in phosphate buffered saline (PBS: mM; NaCl 137, KCl 3, $KH_2PO_4$ 2, $Na_2HPO_4$ 8; pH = 7.4) for 2 h. Tissue was then washed in sucrose solution (PBS + 7% w/v sucrose + 0.01% w/v sodium azide) four times for 10 min each time. After washing, the tissue was cryoprotected in the same solution for 48 h at 4 °C. Tissue was then embedded in Tissue-Tek® optimal cutting temperature (OCT) embedding compound (product code: IA018; Sakura Finetek) in Cryomold® intermediate size specimen moulds (product code: 4566; Sakura Finetek) and snap frozen in liquid nitrogen for storage at −80 °C in an ultrafreezer. 8 μm sections were cut using a Leica CM 1850 cryostat at −19 °C and thawed on to positively charged glass microscope slides (product code: J1800AMNZ; Menzel-Glaser) and air-dried for 1 h at room temperature.

#### 2.2.2. Protocol 2

Freshly dissected prostate tissue was fixed in 0.5% paraformaldehyde in PBS for 5 min then washed in PBS three times for 5 min each time. Tissue was then embedded in Tissue-Tek® OCT embedding compound in Cryomold® intermediate size specimen moulds and snap frozen in liquid nitrogen for storage at −80 °C in an ultrafreezer. 8 μm sections were cut using a Leica CM 1850 cryostat at −19 °C and thawed on to positively charged glass microscope slides and air-dried for 1 h at room temperature.

#### 2.2.3. Protocol 3

Freshly dissected prostate tissue was directly embedded in Tissue-Tek® OCT embedding compound in Cryomold® intermediate size specimen moulds and snap frozen in liquid nitrogen for storage at −80 °C in an ultrafreezer. 8 μm sections were cut using a Leica CM 1850 cryostat at −19 °C and thawed on to positively charged glass microscope slides and air-dried for 1 h at room temperature. Slide-mounted sections were then fixed in 0.5% paraformaldehyde in PBS for 5 min before washing in PBS three times for 5 min each time.

### 2.3. Permeabilization

A subset of slide-mounted sections from each of the three fixing protocols were permeabilized in PBS + 0.5% (v/v) Triton X-100 (product code: X100; Sigma) for 15 min before washing in PBS three times for 5 min each time.

### 2.4. Primary antibody incubation

Slides were incubated with polyclonal rabbit anti-human PCNA (product code: 19166; Abcam) diluted in one of the three diluting media listed below. In order to distinguish association of PCNA-immunopositive staining with stromal and epithelial structures in the mouse prostate, sections were simultaneously incubated with a mouse monoclonal antibody for smooth muscle actin (product code; IR611; DAKO). Incubations were carried out in a humid perspex chamber at 4 °C or room temperature for 18–20 h.

Primary polyclonal antibody to PCNA and monoclonal antibody to smooth muscle actin were diluted 1 in 50 – 200 and 1 in 250 respectively, with: antibody diluting medium A (PBS + 0.1% w/v Na azide + 0.01% w/v BSA + 0.1% w/v lysine + 0.1% v/v Triton X-100); antibody diluting medium B (PBS + 0.1% w/v Na azide + 0.01% w/v BSA + 0.1% w/v lysine + 0.1% v/v Triton X-100 + 2% v/v mouse serum) or antibody diluting medium C (PBS + 0.1% v/v Triton X-100).

### 2.5. Secondary antibody incubation

Slide-mounted sections were washed in PBS three times for 10 min each time prior to incubation with the secondary antibodies: fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG(H+L) (product code: FI-1000; Vector; 1:250) and Texas Red conjugated horse anti mouse IgG(H+L) (product code: TI-2000; Vector; 1:200) diluted in the same medium as the primary antibodies at room temperature for 1 h. After washing in PBS three times for 10 min each time, cell nuclei were counterstained with DAPI (50 ng/ml) for 5 min. Sections were then washed with PBS three times for 5 min each time again and mounted in Vectashield® mounting medium (product code: H-1000; Vector) and sealed with a glass coverslip.

### 2.6. Microscopy

Immunoreactivity was visualized using an Olympus BX60 fluorescence microscope fitted with an Olympus U-MNUA2 filter cube consisting of a DM400 dichroic beam splitter, BP360–370 exciter filter and BA420–460 barrier filter to view DAPI staining. FITC was viewed with the same microscope fitted with an Olympus U-MNIBA3 filter cube consisting of a DM505 dichromatic mirror, BP470–495 exciter filter and BA510–550 barrier filter. Texas Red was viewed with the same microscope fitted with an Olympus U-MWIY filter cube

**Table 1**

| Protocol number | Fixation protocol | Permeabilization | Incubating temperature | Diluting medium | Results |
|---|---|---|---|---|---|
| 1 | Protocol 1 | Yes | RT | Medium A | Negative |
| 2 | Protocol 1 | Yes | RT | Medium B | Non-specific |
| 3 | Protocol 1 | Yes | RT | Medium C | Negative |
| 4 | Protocol 1 | No | RT | Medium A | Negative |
| 5 | Protocol 1 | No | RT | Medium B | Non-specific |
| 6 | Protocol 1 | No | RT | Medium C | Negative |
| 7 | Protocol 2 | Yes | RT | Medium A | Negative |
| 8 | Protocol 2 | Yes | RT | Medium B | Non-specific |
| 9 | Protocol 2 | Yes | RT | Medium C | Negative |
| 10 | Protocol 2 | No | RT | Medium A | Negative |
| 11 | Protocol 2 | No | RT | Medium B | Non-specific |
| 12 | Protocol 2 | No | RT | Medium C | Negative |
| 13 | Protocol 3 | Yes | 4 °C | Medium A | Negative |
| 14 | Protocol 3 | Yes | 4 °C | Medium B | Non-specific |
| 15 | Protocol 3 | Yes | 4 °C | Medium C | Positive |
| 16 | Protocol 3 | No | 4 °C | Medium A | Negative |
| 17 | Protocol 3 | No | 4 °C | Medium B | Non-specific |
| 18 | Protocol 3 | No | 4 °C | Medium C | Negative |
| 19 | Protocol 3 | Yes | RT | Medium C | Negative |

PCNA labelling of cryostat sections of mouse prostate with different fixation, permeabilization and primary antibody incubation protocols.
RT = room temperature.