

## SHORT REPORT

# Immunohistochemical assays in prostatic biopsies processed in Bouin's fixative

V Ananthanarayanan, M R Pins, R E Meyer, P H Gann

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**Aims:** To investigate the problems involved in undertaking immunohistochemistry (IHC) and nuclear morphometry using Bouin's fixed prostate biopsies.

**Methods:** Archival Bouin's fixed and formalin fixed, paraffin wax embedded prostatic biopsies were immunostained for three nuclear biomarkers (minichromosome maintenance protein 2 (MCM-2), p27, and Ki-67), one membrane localised biomarker (C-erb-B2), CD34, and  $\alpha$  methylacyl-CoA racemase (AMACR). The quality of IHC staining was compared between tissues prepared separately in both fixatives. Feulgen staining was also performed on Bouin's fixed tissues to check its suitability for nuclear morphometry.

**Results:** MCM-2 staining was completely negative in Bouin's fixed tissues, whereas p27 showed more background and excess cytoplasmic staining in Bouin's fixed versus formalin fixed tissues. C-erb-B2 showed non-specific, strong luminal cell staining in the Bouin's fixed tissue. Feulgen staining was also very weak in Bouin's fixed tissue. However, Ki-67, AMACR, and CD34 worked equally well in Bouin's and formalin fixed tissues.

**Conclusions:** Bouin's fixed tissues may be unsuitable when subsequent IHC and morphometry are contemplated. An awareness of which antibodies are suitable for use in Bouin's fixed biopsies is essential.

neoplasia. In this report, we present some of the problems encountered with the use of Bouin's fixative for prostatic biopsies.

## METHODS

In our study, we were interested in analysing the temporal and spatial variations in the expression of various biomarkers by IHC in the normal compartment of archival, Bouin's fixed (BF), paraffin wax embedded prostate biopsies. The markers used were: minichromosome maintenance protein 2 (MCM-2; clone-CRCT2.1; 1/40 dilution; Novocastra, Newcastle upon Tyne, UK), p27 (clone-G173-524; 1/800 dilution; Transduction Laboratories, Lexington, Kentucky, USA), Ki-67 (clone-MIB-1; 1/200 dilution; Dako, Carpinteria, California, USA), C-erb-B2 (clone-3B5; 1/100 dilution; Oncogene, San Diego, California, USA),  $\alpha$  methylacyl-CoA racemase (AMACR; clone-P504S; 1/100 dilution; Zeta Corp, Sierra Madre, California, USA), and CD34 (clone-QBEnd10; 1/50 dilution; Dako). Briefly, IHC was carried out as follows: 4  $\mu$ m thick sections were cut on to charged slides and dewaxed. After rehydration through descending grades of alcohol, heat induced epitope retrieval was carried out in a citrate bath (pH 6) using a steamer for 30 minutes. Primary antibodies were applied at the dilutions given above and a colour reaction was developed using the Dako EnVision+ system with diaminobenzidine as the chromogen. The BF specimens were also stained with Feulgen's stain to assess nuclear morphometry (nuclear size, shape, texture, and DNA content). NBF fixed specimens were stained in addition for comparison.

## RESULTS AND DISCUSSION

### MCM2 and p27

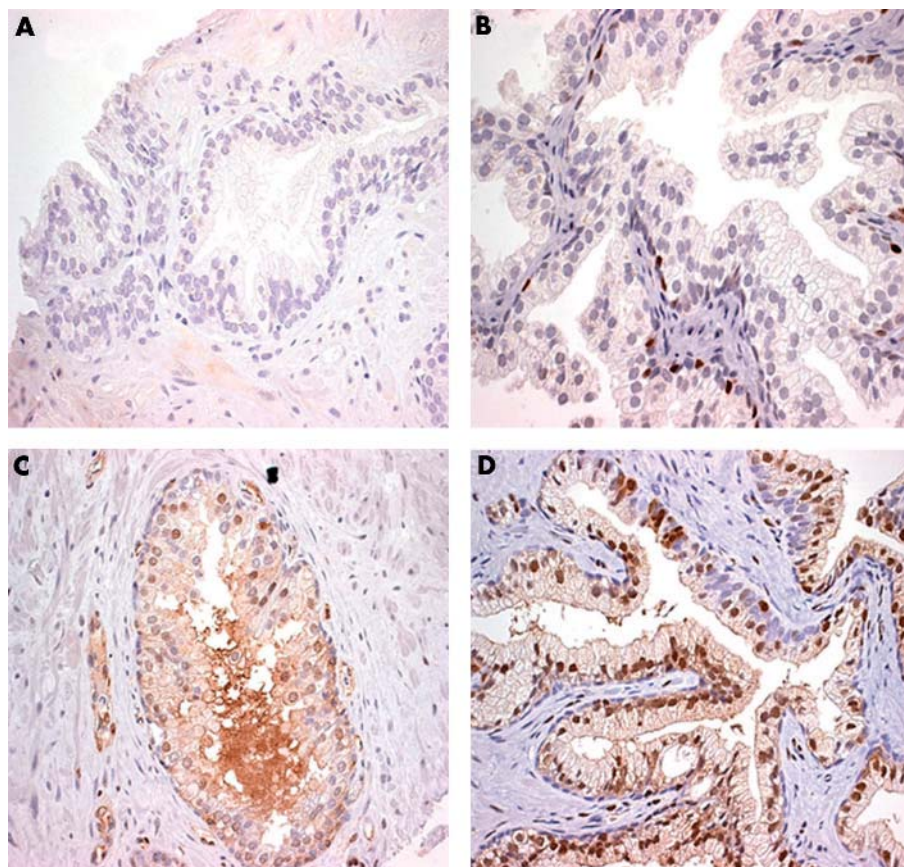
MCM-2, one of the six proteins of the MCM family involved in the initiation and regulation of DNA replication, showed no expression in the BF specimens (fig 1A), although the NBF fixed specimens showed strong expression of the MCM-2 protein in the basal and luminal cells of the normal glands (fig 1B), as reported previously.<sup>4</sup> The BF specimens continued to be immunonegative even under alternative experimental conditions, including prolonged incubation with the primary antibody, higher concentrations of primary antibody, overnight incubation with the primary antibody, prolonged antigen retrieval, different antigen retrieval techniques, and postfixation in methanol. To determine whether this difference was caused by the fixation time of the biopsy, we obtained multiple biopsy samples from the same subject and fixed them in either formalin or Bouin's solution for one, three, six, and 12 hours. All the BF biopsies were immunonegative for MCM-2, and altering the fixation time

The study of biomarker expression in archival paraffin wax blocks is often undertaken in the field of pathology. This may eventually translate into a clinical application, as in the case of Herceptin (Trastuzumab) and C-erb-B2 expression in breast carcinomas.

"Better nuclear detail can be particularly advantageous in prostate biopsies, because nuclear changes are a salient feature in defining prostatic intraepithelial neoplasia"

Biomarker detection by immunohistochemistry (IHC) in tissues is dependent on tissue fixation and processing methods. Traditionally, 10% neutral buffered formalin (NBF) has been the preservative of choice for most specimens; however, Bouin's fixative may be preferred over formalin in two situations. The first case is for small biopsies, because the yellow tinge imparted to the tissue facilitates visualisation during embedding, without an additional step of dipping the biopsies in ink. The second is when excellent nuclear detail and glycogen preservation properties are essential for an appropriate histopathological diagnosis.<sup>1,2</sup> For example, improved preservation of nuclear detail makes Bouin's fixative favourable for lymphoid lesions and testicular biopsies.<sup>3</sup> Better nuclear detail can be particularly advantageous in prostate biopsies, because nuclear changes are a salient feature in defining prostatic intraepithelial

**Abbreviations:** AMACR,  $\alpha$  methylacyl-CoA racemase; BF, Bouin's fixed; IHC, immunohistochemistry; MCM-2, minichromosome maintenance protein 2; NBF, neutral buffered formalin



**Figure 1** (A) Bouin's fixed (BF) prostate biopsy stained for MCM2 showing no reactivity. (B) The corresponding formalin fixed biopsy shows strong immunoreactivity, predominantly in the basal cell compartment. (C) BF prostate biopsy stained for p27 shows weak nuclear staining with abundant cytoplasmic positivity, whereas the formalin fixed biopsy (D) shows strong nuclear positivity with very weak cytoplasmic staining.

made no difference, whereas the corresponding formalin fixed specimens were immunoreactive under all conditions.

In the case of p27, a cell cycle inhibitor that has its highest expression in normal and quiescent epithelium, the mean percentage expression for p27 in the normal compartment in our BF specimens was around 66%. This is less than that expected from NBF fixed specimens (85–100%).<sup>5</sup> Added to this was the problem of persistent cytoplasmic staining at all dilutions in the BF specimens (fig 1C, D). In contrast, the formalin fixed specimens had a mean p27 index of 84%, with minimal cytoplasmic staining.

### C-erb-B2 and Feulgen

C-erb-B2 (HER2/neu) is known to exhibit a differential pattern of positivity in benign prostate glands, with basal cells showing strong staining and luminal cells showing negative to weak staining.<sup>6</sup> However, in BF biopsies, even the normal luminal cells were strongly positive for C-erb-B2 in

most samples, despite the use of the same clone of antibody previously reported in NBF.

Feulgen staining involves a primary acid hydrolysis step that results in the removal of purine bases from the DNA molecule. The free aldehyde groups that are thus exposed subsequently react with pararosaniline in the Schiff's reagent to produce a magenta colour.<sup>7</sup> As noted previously, Feulgen staining of the nuclei was very weak in our BF specimens.<sup>8</sup>

However, some immunomarkers worked equally well with both fixatives, including AMACR, Ki-67, and CD34 (data not shown). Thus, the success of staining in BF specimens was unpredictable. Table 1 summarises the markers that gave discrepant results in the two fixatives.

Immunohistochemistry is, and will remain for the foreseeable future, a fundamental tool in diagnostic pathology. Bouin's fixative is known to provide excellent nuclear detail,<sup>1</sup> which is achieved by the addition of picric acid to formalin, but at the cost of the denaturation of DNA.<sup>3</sup> We speculate

**Table 1** Immunoreactivity of markers showing discrepant staining according to the fixative used

Antigen	Location	Bouin's	Results	Formalin	Results
		Number of slides (cases)		Number of slides (cases)	
MCM2	Nuclear	6 (5)	Negative	22 (11)	Good
p27	Nuclear	109 (23)	Decreased	10 (5)	Good
C-erb-B2	Membrane	23 (4)	Aberrant	15 (7)	Good

Good: the staining pattern was consistent with the expectation based on previous reports; decreased: for p27, a lower percentage of staining was seen, with persistent cytoplasmic staining; aberrant: for C-erb-B2, an altered pattern of spatial expression was noted—although the staining was crisp, the location of the staining was aberrant.

### Take home messages

- Although Bouin's fixative provides excellent nuclear detail its use may preclude tissues from subsequent immunohistochemical analysis and morphometry
- Some antibodies worked in Bouin's fixed biopsies (such as Ki-67, AMACR, and CD34), whereas others (such as C-erb-B2, MCM-2, and p27) showed aberrant or suboptimal staining
- Therefore, Bouin's should not be used as a fixative for prostatic biopsies when immunohistochemical analysis or Feulgen staining for morphometry are planned

that this could affect the immunoreactivity of some of the nuclear antigens and cause suboptimal IHC staining for nuclear antigens in BF prostatic biopsies compared with cytoplasmic antigens. Gala *et al* reported that the B cell antibody L26/CD20 gave unsatisfactory results on BF bone marrow trephine biopsies.<sup>9</sup> The authors mentioned in a subsequent reply to O'Briain *et al* that the antigen retrieval technique is also a prime deciding factor in IHC staining outcome,<sup>10</sup> and they also provided a list of antibodies that work well in BF trephine biopsies. Lehmann *et al* detected no staining for the peroxisomal proteins in BF rat liver sections, although they were all positive in Carnoy's fixed sections.<sup>11</sup> Hayat states that there are limitations to the use of formalin substitutes—such as Bouin's, Carnoy's, F13, and others—for tissue fixation because they not only cause poor cellular preservation, shrinkage, and brittleness, but also cause an artefactual shift in immunoreactivity. Finally, Hayat concludes that formalin substitutes cannot be recommended for IHC.<sup>12</sup> Bouin's fluid is also unsuitable for ultrastructural studies and the reverse transcription polymerase chain reaction.<sup>13</sup>

The various diagnostic modalities used in pathology can vary depending on the nature of the tissue and the pertinent clinical diagnosis. This in turn demands the use of a fixative best suited for its purpose, such as glutaraldehyde for electron microscopy. Most IHC biomarkers are used primarily in formalin fixed tissues, with very limited data available regarding their applicability to specimens processed in alternative fixatives. Thus, it seems that the better nuclear detail achieved with Bouin's fixative in prostate biopsies involves a serious trade off in terms of reduced capability for semiquantitative staining. Our experience suggests the need for exercising caution in using Bouin's as a fixative for prostatic biopsies when IHC analysis or Feulgen staining for morphometry are planned.

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### Authors' affiliations

**V Ananthanarayanan, P H Gann**, The Department of Preventive Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA

**M R Pins**, The Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

**R E Meyer**, The Pathology Core Facility, Northwestern University Feinberg School of Medicine

**P H Gann**, Department of Preventive Medicine, Robert H Lurie Comprehensive Cancer Center, Chicago, IL 60611, USA

Correspondence to: Dr V Ananthanarayanan, Department of Preventive Medicine, Northwestern University Feinberg School of Medicine, 680 North Lake Shore Drive, Suite 1102, Chicago, IL 60611, USA; [viju@northwestern.edu](mailto:viju@northwestern.edu)

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