Technical report

Protocol for ultrarapid immunostaining of frozen sections

Thomas Richter, Jörg Nährig, Paul Komminoth, Jürgen Kowolik, Martin Werner

Abstract

Rapid immunostaining of frozen sections within a tolerable time span would be very helpful for intraoperative diagnosis. A protocol was therefore established using the enhanced polymer one-step staining (EPOS) system (Dako) with antibodies against leucocyte common antigen (LCA), cytokeratin (CK), and anti-melanoma (MEL). Best results with reliable and specific immunostaining and a labelling intensity comparable to standard immunostaining protocols were achieved with fixation of samples in 100% acetone for 20 seconds (CK, LCA) or two minutes (MEL), followed by incubation of the primary antibody and development of the chromogen reaction with 3,3'-diaminobenzidine (DAB) for three and five minutes at 37°C, respectively. The total procedure takes only 12 minutes, thus enabling rapid immunostaining on intraoperative frozen sections. Apart from its use in tumour classification, this method is especially useful in detecting tumour cells in sentinel lymph nodes.

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**Institute of Pathology,
Technical University
Munich, Ismaninger
Str 22, 81675 München,
Germany**
T Richter
J Nährig
M Werner

**Department of
Gynaecology and
Obstetrics, Technical
University Munich**
J Kowolik

**Department of
Pathology, University
of Zürich, Zürich,
Switzerland**
P Komminoth

Correspondence to:
Dr Richter.

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In frozen sections the distinction between lymphoma, carcinoma, malignant melanoma, sarcoma, and chronic inflammation may be difficult on a morphological basis alone. Immunohistochemistry can often resolve this problem, but for intraoperative cryosection diagnosis, standard immunostaining methods require a time span which cannot be tolerated in the operating room. Another important setting in which rapid immunostaining would be helpful is to confirm or exclude tumour clearance in resection margins or to detect micrometastases in sentinel lymph nodes—for example, the axillary sentinel lymph nodes in breast cancer patients or regional lymph nodes in patients with malignant melanoma.^{1,2}

Although acceleration of immunostaining has been achieved by the use of high quality reagents,³ preformed antibody-avidin-biotin-peroxidase complexes,⁴ or microwave oven treatment during incubation of reagents,^{5,6} these methods have not proven suitable for

routine diagnosis and frozen sections. The enhanced polymer one-step staining (EPOS) system, where primary antibodies and horseradish peroxidase (HRP) are linked to a chemical inert polymer complex (dextran), offers a one step immunostaining procedure.⁷ The polymer complex has been shown to be resistant to microwave treatment, which can be used to accelerate the incubation time.^{8,9}

We have established a rapid and reliable immunostaining protocol with EPOS monoclonal antibodies. This protocol is easily accomplished, delivers fast and reproducible results, and is therefore applicable to intraoperative frozen sections.

Methods

Fresh tissues were obtained within 10 minutes after surgical removal. Specimens comprised six lymph nodes (three normal, two metastases of colon carcinomas, and one metastasis of a malignant melanoma), three carcinomas of the breast, stomach, and colon, as well as two malignant melanomas and two fibroadenomas of the breast. An additional 19 sentinel lymph nodes from 15 breast carcinoma patients were also investigated. Detection of the sentinel lymph nodes was done by technetium-99m labelling.² Frozen sections (7 µm) were mounted on silane coated glass slides.

We compared different fixatives (4% buffered formaldehyde (pH 7.0), 100% methanol, 4% formaldehyde/100% methanol 1:1, 100% acetone), and variable fixation times (20 seconds, two minutes, five minutes), with or without subsequent air drying of the sample. Slides were then rinsed for 15 seconds in Tris buffered saline (TBS, pH 7.4).

The prediluted EPOS reagents and antibodies against leucocyte common antigen (LCA), cytokeratin (CK), and anti-melanoma (MEL) (LCA/HRP (CD45, clone 2B11+PD7/26), anti-CK/HRP (clone MNF116), and anti-MEL/HRP (clone HMB45)) were kindly provided by Dako and incubated for one, three, or five minutes at room temperature or at 37°C in an incubation chamber. Incubation in a microwave oven (power output 500 W) was tested for 30 seconds, 2 × 30 seconds, one minute, or 2 × one minute.

The developmental step of the chromogen reaction (Dako HRP kit) was carried out again at room temperature, at 37°C in a incubation

Table 1 Treatments tested for fixation, incubation, and development of frozen sections

Fixation		Incubation		Development	
Fixative	Time	Temperature	Time	Temperature	Time
Acetone	20 s, 1, 2 min	RT	1, 2, 5 min	RT	1, 2, 5 min
Formaldehyde	20 s, 1, 2 min	37°C	1, 2, 5 min	37°C	1, 2, 5 min
Methanol/ formaldehyde	20 s, 1, 2 min	MW	30 s, 2×30 s	MW	1, 2 min
Methanol	20 s, 1, 2 min		1, 2×1 min		

MW, microwave; RT, room temperature.

Table 2 Protocol for rapid immunostaining in frozen sections

1. Mount frozen sections (7 µm) on silane coated slides.
2. Fix tissues with 100% acetone for 20 seconds (CK, LCA) or with 100% acetone (MEL) for two minutes.
3. Air dry when fixed in acetone; rinse in TBS when fixed in 4% buffered formaldehyde.
4. Incubate with EPOS antibody for three minutes at 37°C.
5. Rinse twice in TBS for 15 seconds.
6. Develop with Dako peroxidase/DAB detection kit (code No K 5001) at 37°C for five minutes.
7. Rinse in tap water.
8. Nuclear counterstain with haematoxylin for 10 seconds.
9. Rinse in tap water and coverslip (Kaisers glycerine-gelatine, Merck).

CK, EPOS antibody anti-cytokeratin/horseradish peroxidase (HRP) (clone MNF116); LCA, EPOS antibody anti-leucocyte common antigen/HRP (CD45, clone 2B11+PD7/26); MEL, EPOS antibody anti-melanoma/HRP (clone HMB45).

chamber, or in a microwave oven (power output 500 W) for one, three, or five minutes, respectively. During microwaving the slides were cooled by a cold water bath.⁶ All treatments that were tested are summarised in table 1. During incubation at 37°C in the incubation chamber, drying of the samples was prevented by covering the slides with a small coverslip.

All reactions were compared with standard immunostaining on serial sections of the material fixed in formaldehyde and paraffin embedded, using the same monoclonal antibodies and a HRP/DAB detection kit (Dako peroxidase/DAB detection kit; code No K 5001). One serial section from sentinel lymph nodes was stained with haematoxylin and eosin (H&E) for routine diagnosis.

Results

For immunostaining with CK and LCA, fixation of the frozen sections was optimal with

Figure 1 Rapid immunostaining with CK-EPOS antibodies on a lymph node with a metastases of a colon carcinoma (A) and with MEL-EPOS on a lymph node metastasis of a malignant melanoma (C), each compared with standard immunostaining (B) (D). (E) An example of LCA-EPOS on a normal lymph node. (F) Sensitivity of the rapid immunostaining technique with a strong immunoreaction of a single tumour cell in an axillary lymph node in a breast cancer patient with CK-EPOS antibodies. Note the faint staining of sinus endothelial cells in the lymph node (arrowhead).

two minutes of 4% formaldehyde fixation or 20 seconds of 100% acetone fixation. Preservation of morphology was slightly better with formaldehyde fixation. Fixation in ethanol and the formalin/ethanol mixture led to faint staining with CK and LCA.

Incubation of the EPOS reagents at room temperature for one, three, or five minutes showed specific staining of the target cells, but signal intensity was always reduced compared to standard immunostaining. Incubation at 37°C for three minutes, in combination with DAB development at 37°C for five minutes, showed excellent preservation of morphology with strong and specific immunostaining, slightly reduced compared with standard immunostaining in serial sections. Microwave heating during incubation of the EPOS reagents resulted in specific antibody binding by 30 seconds. Staining intensity reached an optimum at 60 seconds, and heating for periods longer than 90 seconds produced non-specific binding and tissue shrinkage. Overall, with microwave accelerated incubation, morphology was less well preserved than with the other treatments, but still delivered consistent results. Application of our protocol (table 2) to sentinel lymph nodes of breast cancer patients detected metastases in five of 15 patients positive by both rapid standard immunohistochemistry and immunohistochemistry, while only four patients were positive with H&E stains. Figure 1 shows the results of rapid immunostaining with CK, MEL, and LCA and an example of the application to sentinel lymph nodes of breast cancer patients.

Discussion

The EPOS system is a synthetic, high polymeric glucopolysaccharide (dextran) with chemically bound primary antibody and enzyme (HRP) molecules. In spite of the high molecular weight, even a few minutes of incubation at room temperature results in sufficient immunostaining, as shown here for CK, LCA, and MEL, and also for proliferating cell nuclear antigen and Ki-67 antigen by Tsutsumi *et al.*¹⁰ The reaction may even be accelerated with heating during incubation. Microwave heating, as proposed by Chilosi *et al.*,⁸ was less optimal in our hands because of decreased preservation of the morphology. Moreover, incubation in the microwave oven was very sensitive to incubation time and power setting. We suggest incubation of both

the EPOS reagents and the developmental step at 37°C, which results in stronger staining compared with incubation at room temperature and produces fewer artefacts than occur with incubation in a microwave oven.

Fixation with acetone for two minutes, as proposed by Chilosi *et al.*,⁸ did not improve the staining intensity except in the case of MEL. Blocking of endogenous peroxidase (2% H₂O₂, one minute) was not necessary in our specimens (data not shown), but may be needed when tissue samples from the gastrointestinal tract, tissues with haemorrhage, or tissues with infiltrates of neutrophil granulocytes are investigated.¹⁰

Table 2 summarises the optimal protocol for rapid immunostaining using the EPOS system. This protocol is easily accomplished within a few minutes and since all reagents are prediluted and ready for use no special experience in immunohistochemistry is necessary. The antibodies applied—for example, CK, LCA, and MEL—are helpful for the many differential diagnostic problems that may be encountered in intraoperative frozen sections, as well as for ascertaining tumour clearance at resection margins. Our first results on axillary sentinel lymph nodes in breast cancer patients show that rapid intraoperative immunostaining of frozen sections with CK increases the detection rates of micrometastases (paper in preparation).

- 1 Morton DL, Wen DR, Wong JH, *et al.* Technical details of intraoperative lymphatic mapping for early stage melanoma. *Arch Surg* 1992;127:392-9.
- 2 Veronesi U, Paganelli G, Galimberti V, *et al.* Sentinel-node biopsy to avoid axillary dissection in breast cancer with clinically negative lymph-nodes. *Lancet* 1997;349:1864-7.
- 3 Heyderman E, Neville M. A shorter immunoperoxidase technique for the demonstration of carcinoembryonic antigen and other cell products. *J Clin Pathol* 1977;30:138-40.
- 4 Lloyd RV, Schmidt K, Blaivas L, *et al.* A rapid immunostaining method utilizing preformed antibody-avidin-biotin-peroxidase complexes. *Am J Clin Pathol* 1985;83:636-9.
- 5 Leong ASY, Milios J. Accelerated immunohistochemical staining by microwaves. *J Pathol* 1990;161:327-34.
- 6 Werner M, von Wasielewski R, Georgii A. Immunodetection of a tumor associated antigen (TAG-12): comparison of microwave accelerated and conventional method. *Biotech Histochem* 1991;1:79-81.
- 7 Bisgaard K, Linne A, Rolstad H, *et al.* Polymeric conjugates for enhanced signal generation in enzyme linked immunoassays [abstract]. Aarhus: XXIVth Annual Meeting of the Scandinavian Society for Immunology, 1993.
- 8 Chilosi M, Lestani M, Pedron S, *et al.* A rapid immunostaining method for frozen sections. *Biotech Histochem* 1994;69:235-9.
- 9 Vitarelli E, Sipelli G, Tuccari G, *et al.* The use of microwave irradiation for immunohistochemistry: a new methodological proposal. *Histol Histopathol* 1995;10:35-8.
- 10 Tsutsumi Y, Serizawa A, Kawai K. Enhanced polymer one-step staining (EPOS) for proliferating cell nuclear antigen (PCNA) and Ki-67 antigen: application to intraoperative frozen diagnosis. *Pathol Int* 1995;45:108-11.