A single simple procedure for dewaxing, hydration and heat-induced epitope retrieval (HIER) for immunohistochemistry in formalin-fixed paraffin-embedded tissue

I.M.S. Paulsen,[1] H. Dimke,[2] S. Frische[1]

[1]Department of Biomedicine, University of Aarhus

[2]Department of Cardiovascular and Renal Research, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark

Abstract

Heat-induced epitope retrieval (HIER) is widely used for immunohistochemistry on formalin-fixed paraffin-embedded tissue and includes temperatures well above the melting point of paraffin. We therefore tested whether traditional xylene-based removal of paraffin is required on sections from paraffin-embedded tissue, when HIER is performed by vigorous boiling in 10 mM Tris/0.5 mM EGTA-buffer (pH=9). Immunohistochemical results using HIER with or without prior dewaxing in xylene were evaluated using 7 primary antibodies targeting proteins located in the cytosol, intracellular vesicles and plasma membrane. No effect of omitting prior dewaxing was observed on staining pattern. Semiquantitative analysis did not show HIER to influence the intensity of labelling consistently. Consequently, quantification of immune labelling intensity using fluorescent secondary antibodies was performed at 5 dilutions of primary antibody with and without prior dewaxing in xylene. No effect of omitting prior dewaxing on signal intensity was detectable indicating similar immunoreactivity in dewaxed and non-dewaxed sections. The intensity of staining the nucleus with the DNA-stain ToPro3 was similarly unaffected by omission of dewaxing in xylene.

In conclusion, the HIER procedure described and tested can be used as a single procedure enabling dewaxing, hydration and epitope retrieval for immunohistochemistry in formalin-fixed paraffin-embedded tissue.

Introduction

Epitope retrieval procedures are pivotal for successful application of immunohistochemical techniques on formalin-fixed paraffin-embedded tissue. Previous studies have comprehensively shown, that the immunoreactivity of formalin-fixed paraffin embedded tissue can be restored by heating to almost boiling temperatures. Buffers adjusted to pH 9-10 appear to be the generally most effective choice.[1] Addition of Ca^{2+} chelating agents further improve the retrieval of antigenic epitopes.[2] However, since no general heat induced epitope retrieval (HIER) procedure has been established, a range of buffers, exposure times, temperatures and pH intervals is used in laboratories over the world.[3,4] In general the HIER procedures have been incorporated in classical staining protocols for paraffin sections, which are initiated by dewaxing and rehydration of paraffin sections through the use of xylene (or equivalent solvents) and a series of decreasing alcohol concentrations.

It has long been recognized, that the use of HIER could have made the time-consuming dewaxing and rehydration obsolete since paraffin melts at 60°C and thus would be anticipated to disappear from the sections when performing HIER at 97°C.[5,6] Initially this assumption was reported to hold true in a brief letter to the editor[6] and the possibility to exclude dewaxing from the protocol was later investigated in an extensive study from the Dako-Cytomation lab.[5] This study concluded that HIER did not remove paraffin sufficiently and therefore a 5 min incubation in the commercial dewaxing product Histoclear® (National Diagnostics, Atlanta, GA, USA) was recommended in the simplified immunohistochemistry protocol.[5] The HIER procedure employed involved immersion of slides in Dako cytomation Target Retrieval Solution pH 6.1 for 30 min at 97°C followed by a continuous wash in hot tap water for about 5 min. Although this was overall a promising simplified procedure, it was noted that an impractical gentle shaking during the washing step was necessary to remove the paraffin, although solubilised with Histoclear®. Moreover, the initial hope that dewaxing could be completely omitted as a separate procedure without the loss of immunohistochemical quality was not fulfilled.

A commercially available product Skipdewax® (Insitus Biotechnologies, Albuquerque, NM, USA) has been reported to be able to remove paraffin and perform HIER simultaneously.[7] In a direct comparison between traditional dewaxing in xylene followed by HIER in EDTA at pH 8 and the Skipdewax one-step protocol (both treatments performed for 2.5 min in a pressure-cooker), the Skipdewax protocol was found to perform better for 3 out of the 8 tested antibodies, and equivalent for the remaining 5 antibodies.[7] The exact composition of Skipdewax® is a proprietary of Insitus Biotechnologies. Trilogy® (Cell Marque, Rocklin, CA, USA) is another commercially available product with patented composition claimed to combine dewaxing, rehydration, and unmasking in a single step. However, no systematic comparison between traditional protocols and Trilogy® with respect to immunohistochemical results can be found in the scientific literature.

In the laboratories at the former Institute of Anatomy at Aarhus University (since 2011 part of Department of Biomedicine), we have used traditional xylene mediated dewaxing followed by HIER as the standard procedure for the last approximately 15 years. Although we have tried several HIER protocols, we have invariably seen the best results with boiling the sections for 16 min in Tris-buffer at pH 9 supple-

Correspondence: Sebastian Frische, University of Aarhus, Department of Biomedicine, Vilh. Meyers Allé 3, Universitetsparken, Bygn. 1233, 8000 Århus C, Denmark.
Tel. +45.87.168291 - Fax: + 45.87.167102.
E-mail: sfri@biomed.au.dk

Key words: Immunolabelling; antibodies; histology; protein localization; HIER.

Contributions: IMSP, experimental design, data acquisition (sectioning and labelling) and interpretation, manuscript revision and final approval; HD, experimental design, data acquisition (labelling) and interpretation, manuscript revision and final approval; SF, experimental design, data acquisition (microscopy) and interpretation, manuscript drafting and final approval.

Conflict of interest: the authors declare no conflict of interest.

Funding: the Water and Salt Research Center at the University of Aarhus established and supported by the Danish National Research Foundation (Danmarks Grundforskningsfond). H. Dimke is funded by the Novo Nordisk Foundation, the Carlsberg Foundation and the A.P. Møller Foundation for the Advancement of Medical Science. S. Frische is funded from the DFF:Medical Sciences and "MEMBRANES" at Aarhus University.

Acknowledgments: the authors thank the histologists JST, MB, JP, and HBM for inspecting the sections for semiquantitative analysis.

Received for publication: 5 May 2015.
Accepted for publication: 14 October 2015.

European Journal of Histochemistry 2015; 59:2532
doi:10.4081/ejh.2015.2532

mented with EGTA (Protocol A in this study). Results from this procedure have formed the basis for hundreds of scientific publications over these years employing hundreds of different antibodies.[8-11] As this procedure differs in temperature, time, heating method and pH from the HIER procedures investigated in previous studies of simultaneous dewaxing and HIER,[5-7] we decided to test if dewaxing in xylene could be excluded before HIER in our protocol without loss of quality of the immunohistochemical results.

Materials and Methods

Tissues

A range of archived formaldehyde-fixed paraffin-embedded tissues were used to determine if the omission of traditional dewaxing and rehydration procedures before HIER would affect immunohistochemical results. The tissues originate from our archive of animal tissues from a number of studies performed in accordance with Danish legislation on animal experiments. Accordingly, no animals were sacrificed for this study. The tissues were all embedded in a blend of microcrystalline paraffin wax and plastic polymers with a melting point between 54 and 57°C [Cellwax Plus (Polymer Added) from Cellpath Ltd., UK, local distributor: Hounisen, Aarhus, Denmark]. The formaldehyde-fixed tissues were dehydrated through incubation in aqueous solutions of increasing ethanol concentration (2 h in 70% ethanol, 2 h in 96% ethanol, 2 h in 99% ethanol) and thereafter left in xylene overnight (minimum 12 h). Tissues were thereafter transferred to liquid paraffin at 60ºC and allowed to infiltrate for 2 h at this temperature. While still at 60ºC, the tissues were embedded in paraffin using a paraffin dispenser and left to cool at a freezing plate.

The following tissues from rat were used:

Upper and lower jaw. Fixed by perfusion with cold 4% formaldehyde (from paraformaldehyde powder) in 0.1 M cacodylate buffer pH 7.4. Left in fixative overnight. Decalcified in 4.13% EDTA (pH 7.4) at 4C for 20 days. Archived for 5 years.

Kidney. Fixed by perfusion with room temperature 4% formaldehyde (from paraformaldehyde powder) in PBS pH 7.4. Archived for 1 year.

Spleen and heart. Fixed by perfusion with cold 4% formaldehyde (from paraformaldehyde powder) in 0.1 M cacodylate buffer pH 7.2. Archived for 10 years.

From the tissue-blocks, sections were cut at a thickness of 2 µm on a Leica 2165 rotary microtome a few days before the immunolabelling. The sections were collected on SuperFrost+ slides (Mentzel Gläser) and allowed to dry at room temperature for a minimum of 2 h and subsequently heated to 60ºC for 1 h.

Primary antibodies

To test the hypothesis most extensively, we chose a range of primary antibodies raised in different species, from different companies and against various types of proteins (globular, secretory and membrane-bound,) and known to be located in different cellular compartments (cytosol, intracellular vesicles and plasma membrane). We also included one antibody recognizing a phosphorylated epitope.

AE2a/b: a custom made rabbit polyclonal antibody recognizing the a and b isoform of the anion exchanger AE2.[12]

Cadherin: C1821 (Sigma-Aldrich, Brøndby, Denmark), mouse monoclonal antibody recognizing various cadherin isoforms.

Calbindin: 10R-C106A (Fitzgerald Industries Int., MA, USA), mouse monoclonal antibody recognizing the 28 kD isoform of the calcium-binding protein Calbindin.

CD68: MCA341R, (AbDSerotec, Kidlington, UK), mouse monoclonal antibody recognizing the macrophage marker CD68 from rat.[13]

Connexin 43: Ab11370, (Abcam, Cambridge, UK), rabbit polyclonal antibody to the gap junction protein connexin 43.

Pendrin: 2176, a custom made rabbit polyclonal antibody recognizing the anion-exchanger pendrin (SLC26A4).[14]

pNKCC2: H934, a custom made rabbit polyclonal antibody specifically recognizing a double phosphorylated form of the furosemide sensitive sodium, potassium, 2 chloride co-transporter, NKCC2.[9]

Renin: ISASRREN-GF, (Innovative Research, Novi, MI, USA), sheep polyclonal anti-body recognizing mouse and rat renin.

Secondary antibodies

P0447: horseradish peroxidase conjugated goat anti-mouse immunoglobulins from Dako (Glostrup, Denmark).

P0448: horseradish peroxidase conjugated goat anti-rabbit immunoglobulins from Dako.

P0163: horseradish peroxidase conjugated rabbit anti-sheep immunoglobulins from Dako.

A11034: Alexa488, conjugated goat anti-rabbit IgG from Molecular probes (cat #A11034).

Dewaxing and HIER procedures

Protocol A. Sections were dewaxed in xylene overnight and subsequently passed through 3 x 10 min 99% ethanol and 2 x 10 min 96% ethanol. Endogenous peroxidase activity was then blocked with 0.35% H_2O_2 (35% H_2O_2 diluted 1:99 in methanol) for 30 min. Rehydration was completed by a rinse in 96% ethanol, 10 min in 70% ethanol and 3 rinses in ultrapure water. HIER was performed by placing the sections vertically in plastic slide holders (Tissue-Tek 4465, Sakura-Finetek, Værløse, Denmark) in plastic containers with lids (Tissue-Tek 4457, Sakura-Finetek) each containing 200 ml TEG-buffer (10 mM Tris (Sigma 7-9, cat#T1378, Sigma-Aldrich) pH 9 at RT with 0.5 mM Ethylene Glycol Tetraacetic Acid (EGTA) (Titriplex 6, Merck 1.08435), maintained at room temperature (RT). All 24 slots in each slide holder were filled with a slide. Three identically filled containers were placed symmetrically on the rotating plate in a microwave oven (Whirlpool MD101) and heated at 900 W for 6 min boiling vigorously followed by 10 min at 350W, resulting in gentle pulsatile boiling. Hereafter the sections were left cooling for 30 min in the TEG-buffer at RT.

Protocol B. This protocol was similar to protocol A, except that dewaxing and rehydration was omitted. The sections were thus directly exposed to the HIER procedure. Blockade of endogenous peroxidase activity was performed for 30 min using a 8:1:1 mixture of PBS, 35% H_2O_2 and methanol after HIER.

Protocol C. Sections were dewaxed in xylene overnight and subsequently passed through 3 x 10 min 99% ethanol and 2 x 10 min 96% ethanol. Endogenous peroxidase activity was then blocked with 0.35% H_2O_2 for 30 min. Rehydration was completed by a rinse in 96% ethanol, 10 min in 70% ethanol and 3 rinses in ultrapure water. Hereafter, the sections were placed in 10 mM TrisHCl pH 9 with 0.5 mM EGTA at 60°C overnight. Although this procedure is less efficient with respect to target retrieval than protocol A, we included it in this test because we have found it to be instrumental to prevent sections of bone from detaching the slide during HIER.

Protocol D. This protocol was similar to protocol C, except that dewaxing and rehydration was omitted. The sections were thus directly exposed to the 60°C overnight epitope retrieval procedure. Blockade of endogenous peroxidase activity was performed as in protocol B after the epitope retrieval procedure.

Immunolabelling procedure using HRP-conjugated secondary antibodies

The sections prepared through protocol A, B, C, and D were incubated for 30 min in 50 mM NH_4Cl to block free aldehyde groups. Subsequently the sections were washed 3 times in blocking solution (10 mM PBS containing 1% BSA, 0.2% gelatine, 0.05%

Saponin). Antibodies were diluted in 10 mM PBS containing 0.1% BSA, 0.3% Triton X-100 and the sections were incubated with the primary antibodies overnight for 1 h at RT and subsequently at 4°C overnight. The following day, the sections were allowed to reach RT before they were washed 3 times in PBS containing 0.1% BSA, 0.2% gelatine and 0.05% Saponin followed by incubation with secondary antibodies diluted in PBS with 0.1% BSA and 0.3% Triton X-100 for 1 h. After additional 3 washes in PBS containing 0.1% BSA, 0.2% gelatine and 0.05% Saponin, peroxidase activity was detected (10 min incubation) using diaminobenzidine (DAB) (DAB tablets pH 7, #4170, Kem-En-Tec Diagnostics, Taastrup, Denmark) in a final concentration of 1 mg/mL. DAB-tablets were freshly dissolved approximately 20 min before use and activated with 0.35% H_2O_2 just before use. Then sections were rinsed in PBS for 3 x 10 min, twice in ultrapure water and then counterstained in Mayers Hematoxylin for 2 minutes and placed in cold running tap water for 20 min. Dehydration was performed by 2x3 min incubation in 70%, 96% and 99% ethanol followed by 3 x 5 min in xylene before coverslips were finally mounted using DPX (Merck, 1.01979.0500).

Semiquantitative evaluation of the effects of omitting dewaxing in xylene

To semiquantitatively evaluate if the immunohistochemical results of protocol A and B (omission of dewaxing in xylene) differed consistently, 4 experienced histologists inspected 10 pairs of sections. The sections in each pair were from the same paraffin-block (rat kidney) and processed in parallel, except that one section followed protocol A and the other protocol B. Primary antibody against pendrin and HRP-conjugated secondary antibodies were used. The sections were anonymised and the pairs were made by randomly selecting sections prepared according to each protocol. Random pairs were prepared for each histologist. The histologists were asked if they recognized any difference between the immunohistochemical labelling intensity and specificity. If a difference was recognized, the histologist was asked to select the section in the pair, which showed superior immunohistochemical labelling with respect to intensity and specificity. If the assignment of superiority with respect to intensity and specificity is random, the number of superior sections from each protocol would follow a binomial distribution (0-hypothesis). Accordingly, we calculated the probability of assigning superiority to the number of sections from each protocol by each histologist according to the binomial distribution. A probability <0.05 was considered significant to reject the 0-hypothesis of randomness.

Quantification of immunolabelling using fluorophore coupled secondary antibodies

Labelling procedure was similar to the one described above for HRP-conjugated secondary antibodies, except for the following modifications: i) The blockade of endogenous peroxidase activity was omitted. ii) ToPro3 (Molecular probes, T3605) diluted 1:1000 were added to the solution containing secondary antibodies for nuclear labelling. iii) Following the incubation with secondary antibodies only 3 x wash in PBS and a final rinse in ultrapure water were performed before coverslips were mounted using Glycergel (DAKO, C0563) which were supplemented with 2,5%w/v 1,4-Diazabicyclo[2.2.2]octane (Merck, 803456)

One section was labelled with each antibody dilution. The labelled sections were visualized using a Leica Konf SL confocal microscope with a PL APO CS 40.0x 1.25 OIL objective. Images using laser excitation at 488 and 633 were obtained separately and subsequently superimposed. ImageJ (http://imagej.nih.gov/ij/) was used for image analysis. Regions of interest were drawn by hand around cells which showed signal of immunolabelling following excitation at 488 nm and in which the ToPro3 nuclear staining were clearly visible following excitation at 633 nm. Between 8 and 26 Pendrin-positive cells were encircled in each section. Fluorescence signals from Alexa488 and Topro3 were integrated in each region of interest (ROI) and used for analysis (see Figure 3A for illustration of the ROI's).

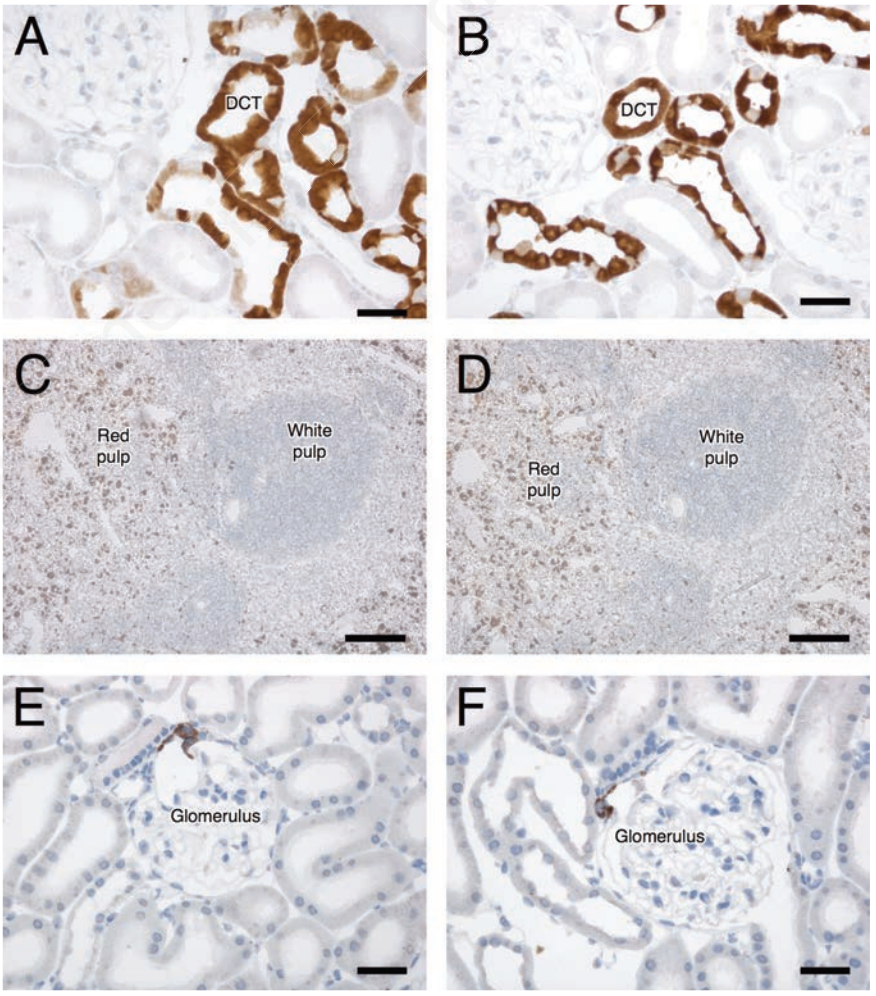

Figure 1. Comparison of immunolabelling of intracellular epitopes using protocol A (Xylene+HIER) (A,C,E,) and protocol B (HIER only) (B,D,F,). Immunolabelling for Calbindin in rat kidney (A *vs* B), CD68 in rat spleen (C *vs* D) and Renin in rat kidney (E *vs* F) are qualitatively similar in protocol A and B. DCT, distal convoluted tubule. Scale bars: A,B,E,F) 30 µm; C,D) 100 µm.

Results

Immunohistochemical results from protocol A and B revealed no qualitative differences

Cytoplasmic labelling was investigated by labelling for Calbindin, which is a cytosolic protein found (among other places) in the distal nephron. The expected labelling was evident in both protocol A and B (Figure 1 A,B). Further intracellular epitopes were investigated by labelling for CD68 in rat spleen, which localizes to the intracellular vesicular compartments associated with the endocytotic pathway (lysosomes) and by labelling for Renin, which is produced and released by juxtaglomerular cells in the afferent arterioles of renal glomeruli and thus is associated with the secretory pathway. The expected labelling for both proteins was evident in both protocols (Figure 1 C-F). Labelling of epitopes associated with the plasmamembrane was investigated by labelling for AE2, pNKCC2 and Connexin43. AE2 is a basolateral membrane protein found in several cell types, including osteoclasts.[15] Osteoclasts in rat upper jaw labelled for AE2 in both protocol A and B (Figure 2 A,B). An antibody recognizing a double phosphorylated epitope on NKCC2 in rat kidney, previously described to be localized only on the apical plasma membrane,[9] was used to evaluate qualitative differences in staining of apical plasma membrane proteins and to investigate the sensitivity of phospho-specific epitopes to the applied protocols. The expected labelling was found in both protocol A and B (Figure 2 C,D). Connexin43 is a widely distributed protein forming gap-junctions in many tissues including ameloblasts and papillary cells in the rat enamel organ.[11] The expected labelling was seen both in protocol A and B (Figure 2 E,F).

Semiquantitative evaluation did not reveal consistent effects of HIER

In 29 of the 40 comparisons of sections treated according to protocol A and B a difference in labelling intensity was noted (Table 1). In the sections in which a difference was recognized, 2 histologists found significantly better labelling intensity in protocol A, 1 histologist found significantly better labelling intensity in protocol B and 1 histologist did not find any significant difference (Binomial test $P<0.05$). In 9 of the 40 comparisons a difference in labelling specificity was noted (Table 1). In the sections in which a difference was

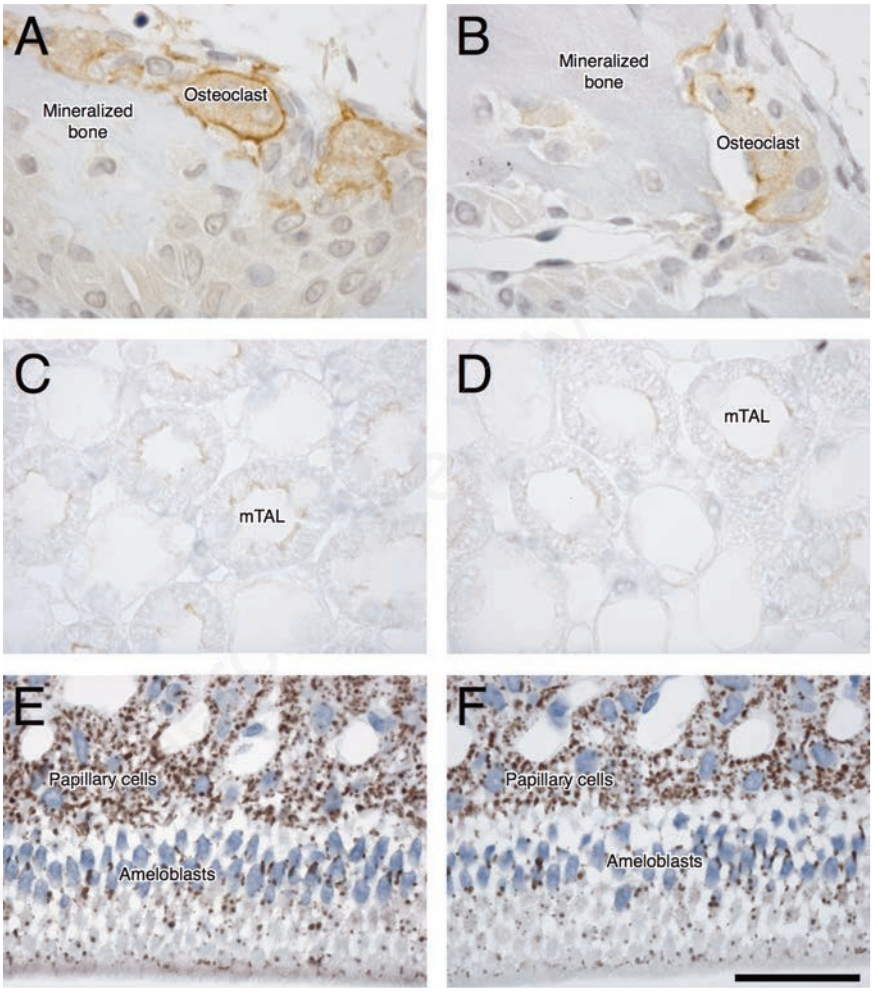

Figure 2. Comparison of immunolabelling of epitopes associated with the plasma membrane using protocol A (Xylene+HIER) (A,C,E,) and protocol B (HIER only) (B,D,F). Immunolabelling for AE2 in osteoclasts in rat jaw (A *vs* B), pNKCC2 in rat kidney (C *vs* D) and Connexin43 in rat enamel organ (E *vs* F) are qualitatively similar in protocol A and B. mTAL, medullary thick ascending limb. Scale bar: 30 µm.

Table 1. Results of the semiquantitative analysis of the results of protocol A and B. Each histologist inspected and classified 10 pairs of sections in which one section was treated according to protocol A and one section was treated according to protocol B.

Histologist	Intensity				Specificity			
	Pairs different	Dewaxed superior	Non-dewaxed superior	P Binomial test	Pairs different	Dewaxed superior	Non-dewaxed superior	P Binomial test
1	8	7	1	0.031*	5	0	5	0.031*
2	10	1	9	0.010*	0	0	0	n.a.
3	8	7	1	0.031*	2	2	0	0.250
4	3	0	3	0.125	2	0	2	0.250

*Statistically significant at $P<0.05$; n.a., not available.

recognized, 1 histologist found significantly better labelling specificity in protocol B and 3 histologists did not find significant differences (Binomial test $P<0.05$).

Quantification of immunolabelling intensity using fluorescent secondary antibodies and nuclear staining showed no differences between protocol A and B

To quantitatively measure the immunoreactivity of sections treated according to protocol A and B, labelling of a membrane protein in individual renal intercalated cells was quantified using laser confocal microscopy. We used a rabbit polyclonal antibody directed against Pendrin, which is a membrane protein expressed in intercalated B cells and so-called non-A, non-B intercalated cells.[14] An example of the images used for quantification is showed in Figure 3A. Quantification revealed a similar reduction in average labelling intensity per cell following dilution of the primary antibody while keeping the concentration of secondary antibody constant using protocol A and B (Figure 3B). Similarly, quantification using laser confocal microscopy revealed no difference between protocol A and B with respect to the average intensity of the nuclear stain ToPro3 per cell per section (Figure 3C).

Protocol C and D resulted in markedly weaker labelling intensity than protocol A and B and dewaxing was not complete in protocol D

Cadherin localizes to intercalated discs in rat heart.[16] As with the other epitopes investigated in this study, no qualitative differences were observed between the results obtained using protocol A and B (Figure 4 A,B). However, compared with protocol A and B (Figure 4 A,C), protocol C and D resulted in weaker immunolabelling (Figure 4 B,D). Similar results were obtained using other antibodies (*data not shown*). Further, dewaxing was not complete in protocol D, resulting in heterogeneous labelling intensity (Figure 4D).

Discussion

The overall conclusion from this study is that immunohistochemistry on paraffin-embedded tissue does not suffer significantly from the omission of dewaxing in xylene, when the HIER procedure of protocol A and B (boiling in alkaline buffer supplemented with EGTA) is used. This conclusion is based on successful immunodetection of epitopes located on a wide range of subcellular compartments in sections without dewaxing.

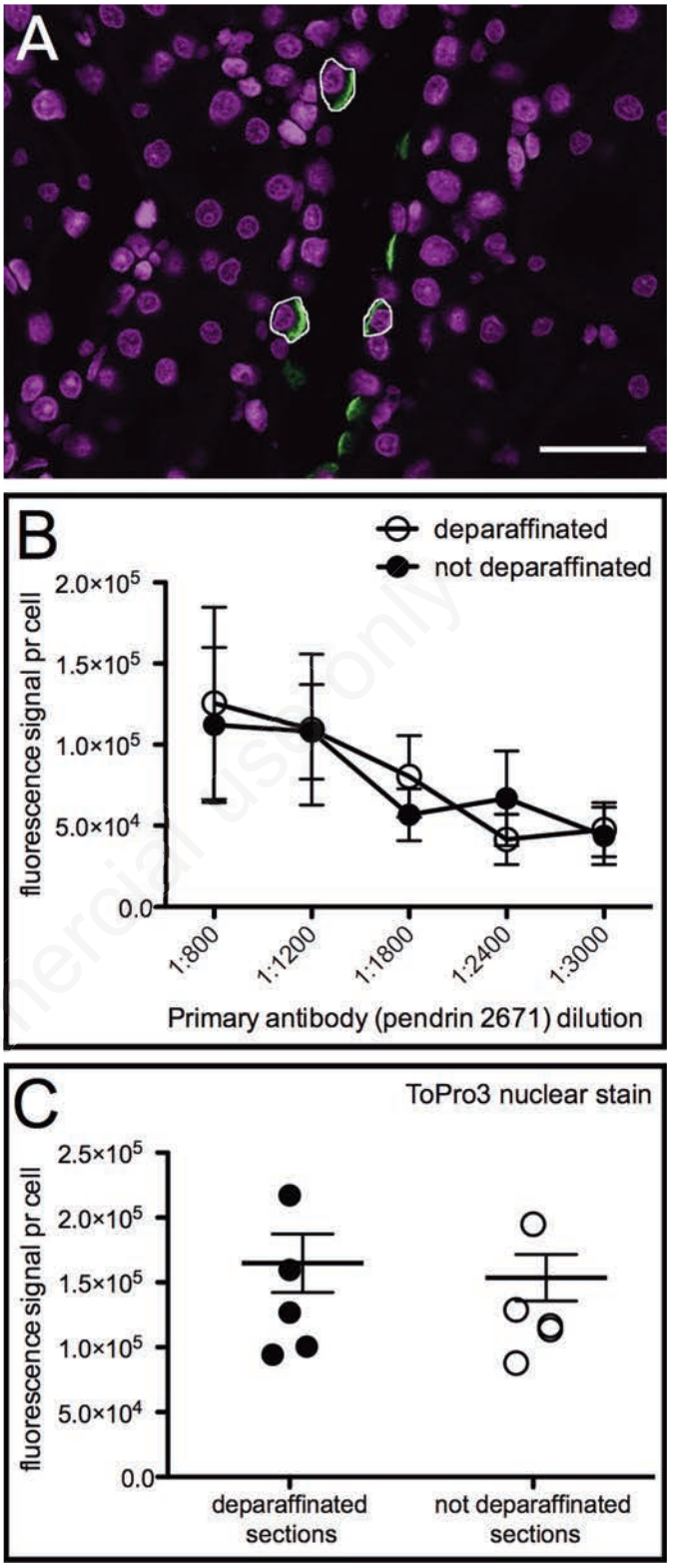

Figure 3. Quantitation of immunolabelling and nuclei staining after HIER with or without dewaxing in xylene. A) Rat kidney section immunolabelled for Pendrin using 1:1200 dilution of the antibody + Alexa488 GAR secondary antibodies and costained for nuclei using ToPro3; regions of interest were drawn by hand around cells, which labelled for pendrin and in which the nucleus was clearly visible (white encirclements); fluorescence signal from Alexa488 and Topro3 were integrated in each ROI; scale bar: 30 µm. B) The integrated Alexa488 fluorescence signal per cell decreased as a function of dilution of the primary antibody; no differences could be detected between the two labelling protocols. C) The average intensity per cell of ToPro3 nuclei staining did not differ between sections exposed to the protocol A and B.

Semiquantitative analysis showed that although 3 of 4 histologists noted significant differences between sections treated in protocol A and B with respect to intensity, they did not consistently find one protocol to produce better results than the other. It thus seems clear that experienced histologist are able to distinguish between sections treated according to protocol A and B, but this ability may depend on other aspects of the labelling, *e.g.*, counterstain intensity or colour tone of the DAB-precipitate, which is beyond the scope of this study to identify. In contrast to intensity, only 1 histologist was able to distinguish significantly between the protocols with respect to labelling specificity and found protocol B to produce the best results. Using fluorescent secondary antibodies and quantitation by confocal microscopy, no effect on specific signal intensity could be documented when omitting the dewaxing and rehydration steps. This confirms that the binding capability of the primary antibodies does not differ between dewaxed and non-dewaxed sections. Neither did the omission of xylene mediated dewaxing affect the staining intensity of nuclei using the DNA stain ToPro3.

In some of the previous studies of the possibility of omitting xylene mediated dewaxing, sections were heated for shorter time or to temperatures below the boiling point.[5,7] Our protocol involves vigorous boiling and we think it is important to adhere to this procedure to obtain reproducible results. Another parameter, which may be of importance, is section thickness. Sections cut to a thickness of 2 µm with a single tissue section on each slide were employed in this study, since this is our laboratory standard. In a previous study, Yörükoglu *et al.*[6] employed a wash in warm buffer to prevent the sections to be smeared with liquid paraffin after combined HIER and dewaxing. In our study we did not observe this phenomenon, but if thicker sections are used or several sections are placed on each slide we cannot exclude that the amount of paraffin to be removed may require additional procedures to avoid paraffin droplets to adhere to the sections. The most important parameter for paraffin removal in this procedure seems to be the heating to temperatures well above the melting point of paraffin, since in protocol D, the removal of paraffin was incomplete. This implies, that the exact type of paraffin embedding material could also influence the results, since various polymers have different melting temperatures and solubilities.

In this study we only applied our routine HIER solution and made no attempts to change this in order to improve its dewaxing effects by adding detergents or less polar solvents than water such as diethanolamine, which is a component in the Trilogy® solution. A prior study based on Raman-spectroscopy have showed hexane to be the most efficient dewaxing agent out of a range of solvents, including the commercially available dewaxing, hydration and HIER agents Trilogy® and Histoclear®.[17] However, the immunoreactivity after the dewaxing protocol was not systematically tested, so the practical benefit of hexane as dewaxing agent remains to be shown. The commercially available solutions Histoclear®, SkipDewax® and Trilogy® may possess unique beneficial properties based on their proprietary compositions, but this study shows that an aqueous solution, based on standard chemicals can remove paraffin in a HIER procedure without loss of immunoreactivity compared to sections dewaxed in xylene. This study thus supports a simplified procedure for immunohistochemistry, which may help to save time and reduce the exposure of laboratory personnel to organic solvents.

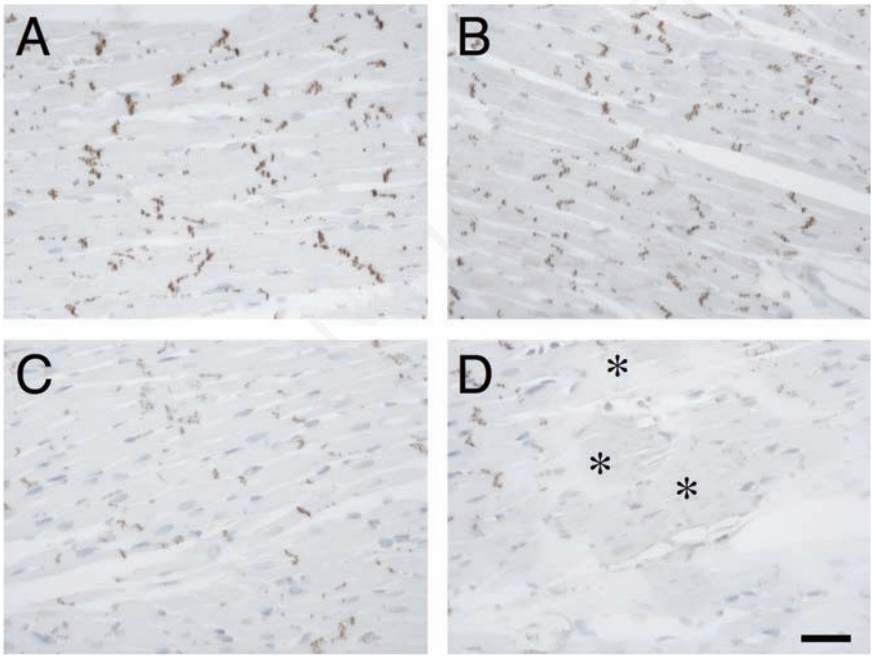

Figure 4. Comparison of immunolabelling of cadherin in rat heart tissue using protocol A (Xylene+HIER) (A); protocol B (HIER only) (B); protocol C (Xylene+HIER at 60°C) (C); and protocol D (HIER at 60°C only) (D). No differences were detected between protocol A and B. HIER at only 60°C resulted in weaker immunolabelling of Cadherin irrespective if the sections were dewaxed in xylene (A *vs* C) or not (B *vs* D). HIER at 60°C without dewaxing in xylene gave rise to heterogeneous staining intensity due to incomplete removal of paraffin (C *vs* D). Asterisks indicate areas with incomplete dewaxing in D. Scale bar: 30 µm.

References

1. Shi SR, Imam SA, Young L, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. J Histochem Cytochem 1995;43:193-201.
2. Shi SR, Cote RJ, Hawes D, Thu S, Shi Y, Young LL, et al. Calcium-induced modification of protein conformation demonstrated by immunohistochemistry: what is the signal? J Histochem Cytochem 1999;47:463-70.
3. D'Amico F, Skarmoutsou E, Stivala F. State of the art in antigen retrieval for immunohistochemistry. Immunol Methods 2009; 341:1-18.
4. Shi SR, Shi Y, Taylor CR. Antigen retrieval immunohistochemistry: review and future prospects in research and diagnosis over two decades. J Histochem Cytochem 2011;59:13-32.
5. Boenisch T. Pretreatment for immunohistochemical staining simplified. Appl Immunohistochem Mol Morphol 2007;15: 208-12.
6. Yorukoglu K, Cingoz S. Epitope retrieval technique: a simple modification that reduces staining time. Applied Immunohistochem 1997;5:71.
7. Wong SCC, Chan JKC, Lo ESF, Chan AKC, Wong MCK, Chan CML, et al. The contribu-

tion of bifunctional SkipDewax pretreatment solution, rabbit monoclonal antibodies, and polymer detection systems in immunohistochemistry. Arch Pathol Lab Med 2007;131:1047-55.
8. de Seigneux S, Malte H, Dimke H, Frøkiaer J, Nielsen S, Frische S. Renal compensation to chronic hypoxic hypercapnia: downregulation of pendrin and adaptation of the proximal tubule. Am J Physiol Renal Physiol 2007;292:F1256-66.
9. Dimke H, Flyvbjerg A, Bourgeois S, Thomsen K, Frøkiaer J, Houillier P, et al. Acute Growth hormone administration induces antidiuretic and antinatriuretic effects and increases phosphorylation of NKCC2. Am J Physiol Renal Physiol 2007;292:F723-35.
10. Praetorius J, Kim YH, Bouzinova EV, Frische S, Rojek A, Aalkjaer C, et al. NBCn1 is a basolateral Na+-HCO3- cotransporter in rat kidney inner medullary collecting ducts. Am J Physiol Renal Physiol 2004;286:F903-12.
11. Josephsen K, Takano Y, Frische S, Praetorius J, Nielsen S, Aoba T, et al. Ion transporters in secretory and cyclically modulating ameloblasts: a new hypothesis for cellular control of preeruptive enamel maturation. Am J Physiol Cell Physiol 2010;299:C1299-307.
12. Frische S, Zolotarev AS, Kim YH, Praetorius J, Alper S, Nielsen S, et al. AE2 isoforms in rat kidney: immunohistochemical localization and regulation in response to chronic NH4Cl loading. Am J Physiol Renal Physiol 2004;286:F1163-70.
13. Dijkstra CD, Döpp EA, Joling P, Kraal G. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. Immunology 1985;54:589-99.
14. Kim YH, Kwon TH, Frische S, Kim J, Tisher CC, Madsen KM, et al. Immunocytochemical localization of pendrin in intercalated cell subtypes in rat and mouse kidney. Am J Physiol Renal Physiol 2002;283:F744-54.
15. Josephsen K, Praetorius J, Frische S, Gawenis LR, Kwon TH, Agre P, et al. Targeted disruption of the Cl-/HCO3- exchanger Ae2 results in osteopetrosis in mice. Proc Natl Acad Sci USA 2009; 106:1638-41.
16. Brüel A, Nyengaard JR. Design-based stereological estimation of the total number of cardiac myocytes in histological sections. Basic Res Cardiol 2005;100:311-9.
17. Faolain EO, Hunter MB, Byrne JM, Kelehan P, Lambkin HA, et al. Raman spectroscopic evaluation of efficacy of current paraffin wax section dewaxing agents. J Histochem Cytochem 2005;53:121-9.