

# Xylene-free Tissue Processing – An Evaluation of Routine Use

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## Abstract

Xylene-free processing (XFP) was evaluated over several months using the Leica Peloris™ tissue processor. The criteria selected pre evaluation were processed block quality, microtomy properties, stained section quality, duration of processing, running costs, user-friendliness and reliability.

The Peloris was used to process tissue from the full range of specimen types received, including technically demanding blocks, such as surgical osteo-articular blocks measuring upto 60 x 55 x 4 mm, soft-tissue blocks of similar dimensions, as well as conventionally sized soft tissue blocks. In total several thousand pieces of tissue were processed xylene-free during the evaluation, alongside a similar range of tissues processed conventionally.

Whilst the evaluation had its limitations, the results were unequivocal, in that, with the possible exception of foetal CNS tissue, XFP exceeded expectations in every respect; it was demonstrably superior to conventional processing systems in key areas.

Three years on, users of XFP within this Department remain confident that it is the current “method of choice”, in that the incidence of any block being inadequately processed, or unsuitable for subsequent procedures in any way remains at zero; in our hands XFP is especially good for the processing of the high number of technically demanding tissues generated in this large histopathology department.

*NB The work upon which the paper was based was carried-out at the Freeman Hospital and it is being furthered at the Royal Victoria Infirmary where all of the histopathology is now centred. Both hospitals belong to Newcastle upon Tyne NHS Foundation Trust.*

## Introduction

The Leica Peloris xylene-free tissue processor was evaluated over several months during 2004 in the Osteo-articular Histology Laboratory at the Freeman Hospital, Newcastle upon Tyne. The aim was to determine whether Peloris xylene-free processing worked adequately and to discover any advantages it might have over conventional tissue processing. Given the challenging nature of much of the surgical work of the laboratory, the greatest pre-evaluation doubt was whether isopropanol would be as effective a de-fatting and clearing agent as xylene. The length of the protocols used in the evaluation reflects the difficult tissue being processed. While Peloris offer rapid protocols they are not required by the laboratory and were not tested.

## Conventional Tissue Processing

Conventional four-stage tissue processing encompasses:

- fixation (commonly formaldehyde based);
- alcohol dehydration (usually ethanol based);
- de-fatting/clearing (xylene, chloroform, toluene); and
- infiltration with paraffin wax.

Used by laboratories worldwide, this technique has stood the test of time. To be accepted, then, any variations to it must produce results that are both indistinguishable from those obtained conventionally, and confer significant advantages.

## Xylene-free Tissue Processing

Leica Microsystems' xylene-free tissue processing technology has five processing stages:

- formalin fixation;
- ethanol dehydration;
- continued dehydration and initial de-fatting/clearing with an 80/20 mixture of ethanol/isopropanol;
- full de-fatting/clearing with neat isopropanol; and
- paraffin wax infiltration.

## Workload

Tissues processed xylene-free as part of the evaluation were:

Surgical:

- Bone blocks up to 60 x 55 x 4 mm.
- Gastro-intestinal and hepato-biliary blocks up to 60 x 55 x 4 mm.
- Other large blocks, predominantly urological, up to 60 x 55 x 4 mm.

Post-mortem:

- Forensic and hospital specimens, including CNS, up to 60 x 55 x 4 mm.

The Freeman laboratory produces approximately 70,000 sections per year.

## Evaluation Method

1. Conventional and xylene-free systems were run in parallel; very similar ranges of specimen types were processed using each system.
2. For some large surgical cases tissue blocks were divided with some blocks processed conventionally, and others xylene-free.
3. As a safeguard, none of the smaller surgical specimens received were wholly processed xylene-free.
4. Sections from blocks processed xylene-free were identified to the reporting pathologist and to the Immunohistochemistry Laboratory.
5. During the initial phase of the evaluation Biomedical Scientists (BMSs) examined all sections microscopically in order to achieve a fair comparison. Thereafter microscopic quality assurance reverted to the norm, in which approximately one third of H&Es from each request were checked for section and stain quality.
6. BMSs ranked xylene-free block quality, microtomy and staining as good, fair or poor in each case.
7. Pathologists were asked to comment upon microscopic appearances.
8. Approximate quantities of reagents used were recorded.

## Evaluation Criteria

1. Processed block quality:
  - adequacy of processing;
  - degree of shrinkage;
  - distortion.
2. Microtomy:
  - sectioning of blocks;
  - section quality.
3. Staining:
  - adequacy;
  - comparison with conventionally processed blocks from the same specimen.
4. Reagent volumes required in comparison with conventional processing.
5. Processor reliability and user friendliness.
6. Processing times.

## Evaluation Results

A spreadsheet was constructed in which BMSs recorded their processing, microtomy and staining rankings, with comments. After about three weeks, during which rankings (apart from foetal CNS tissue) failed to deviate from “good” and comments were few, it was decided to record only adverse findings for the remainder of the evaluation.

### Processed block quality

Block quality was graded “good” for all blocks except for a few samples of foetal CNS:

- the degree of shrinkage and distortion observed throughout the evaluation was unremarkable;
- large fatty slices processed well.

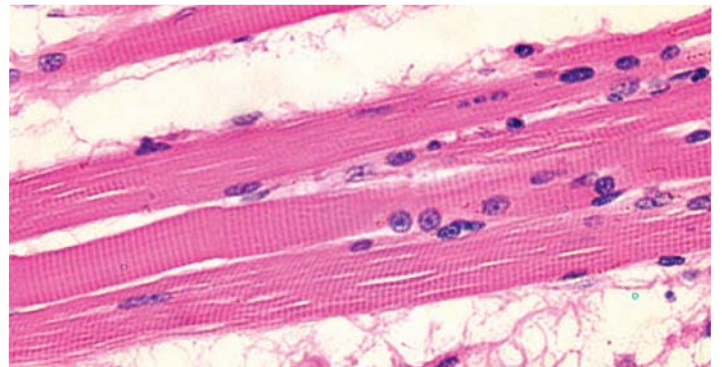
### Microtomy

Microtomy was graded “good” for all blocks:

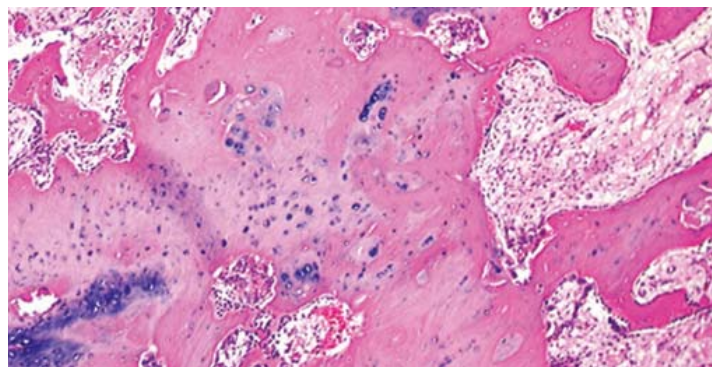
- blocks of haemorrhagic tissue were less friable and thus easier to section.

### Staining

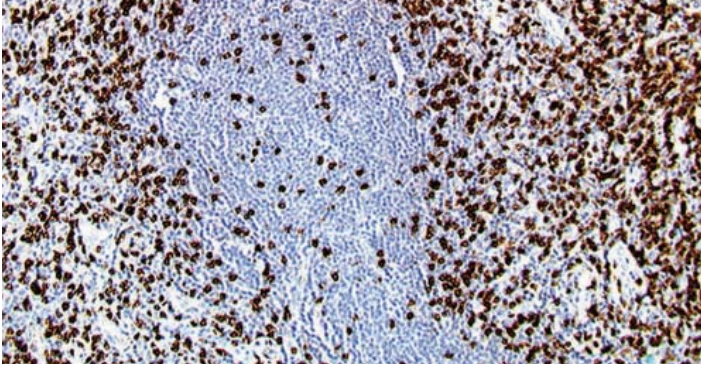
- H&Es and “specials”: BMS consensus was that staining was precise and vivid. No adverse observations throughout.
- Immunohistochemistry: results for xylene-free processed blocks were similar to conventionally processed blocks with no unusual problems or changes required to antigen-retrieval methods or reagent dilutions.
- ISH: staining of xylene-free processed tissue blocks was good.



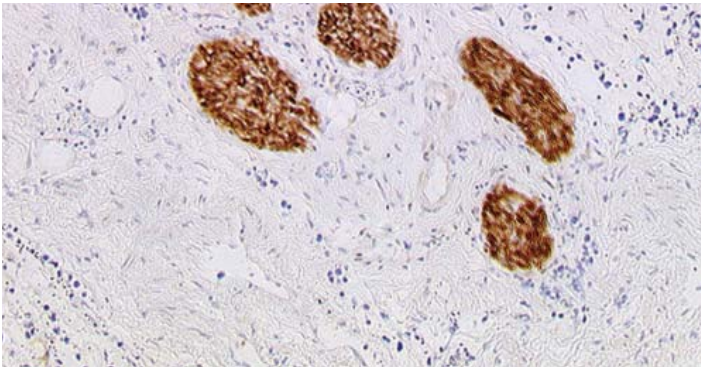
Striated muscle



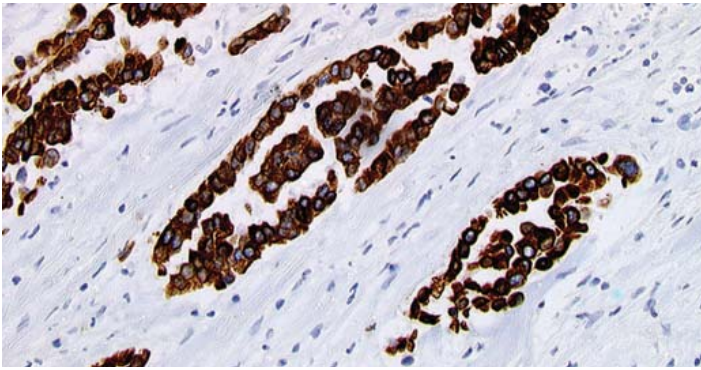
Decalcified bone, H&E



Soft tissue – osteo-articular specimen, CD3



Peripheral nerves, S100



Mesothelial cells post mortem block CK5/6

### Pathologist Comments

- “Minimal differences” were evident in sections of some xylene-free processed blocks, such as an increase in red blood cell lysis.
- With the exception of problems encountered in the processing of foetal CNS material, there were no adverse comments from over 20 pathologists during the evaluation.
- There were no requests for extra tissue blocks to be taken and processed conventionally.
- Reagent Use  
A typical snapshot of the reagent usage from the Peloris display reveals the number of cassettes processed per reagent since the last change.

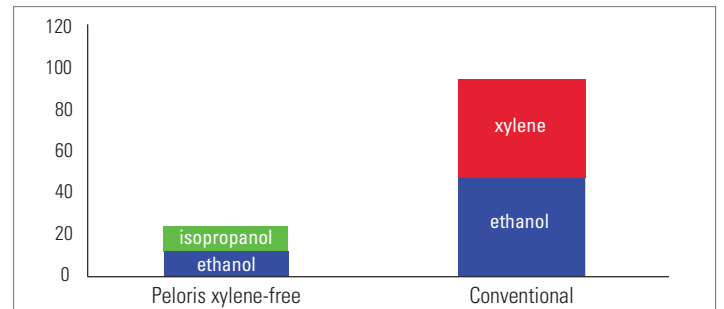
Bottle	Reagent	Cassettes
1	Formalin	916
2	Formalin	857
3	50% Ethanol	796
4	50% Ethanol	1513
5	50% Ethanol	828
6	80/20 Eth/IPA	2447
7	80/20 Eth/IPA	1038
8	80/20 Eth/IPA	1133
9	IPA	1133
10	IPA	2833
11	IPA	2424
12	IPA	1376
13/14	Clean	
15/16	Clean	
W1	Wax 1	1526
W2	Wax 2	943
W3	Wax 3	299
W4	Wax 4	2018

Reagent table

### Reagent Usage

Estimated usage of ethanol, xylene and isopropanol (IPA) over a twenty working-day period showed that the xylene-free system used considerably less reagent (ethanol and isopropanol) than conventional processing (ethanol and xylene). This provides a significant cost saving.

### Reagent costs over a twenty working-day period



### Xylene-free

15 L ethanol @ approximately £4.00/5 L £12.00

15 L isopropanol @ approximately £4.00/5 L £12.00

**Total £24.00**

### Conventional

60 L ethanol @ approximately £4.00/5 L £48.00

60 L xylene @ approximately £4.00/5 L £48.00

**Total £96.00**

The far smaller volume of reagents used with xylene-free processing was attributed primarily to a more effective means of reducing reagent carryover from one reagent to the next than was achievable with the conventional processing system used in the laboratory at that time. Using conventional processing, some reagents were routinely changed after as few as three cycles in order to ensure thorough processing of large blocks.

## Processor Reliability and User Friendliness

All users found the processor easy to use. On-screen information was such that using Peloris rapidly became intuitive and reagent removal and replenishment was quick and clean throughout. Both retorts were frequently used simultaneously with different processing schedules, such as one overnight and one two-day cycle. The processor was 100% reliable through the evaluation period.

### Health and Safety

An important consideration in tissue processing relates to reagent handling; clearly the less toxic the reagent the lower the risk to health, especially over the longer term. A measure of toxicity (LD50) was compared for three well-known clearing agents (xylene, chloroform, toluene) and for isopropanol.

#### Xylene

LD50 oral 2,840 mg/kg

LD50 dermal 4,500 mg/kg

#### Chloroform

LD50 oral 908 mg/kg

#### Toluene

LD50 oral 636 mg/kg

LD50 dermal 12,124 mg/kg

#### Isopropanol

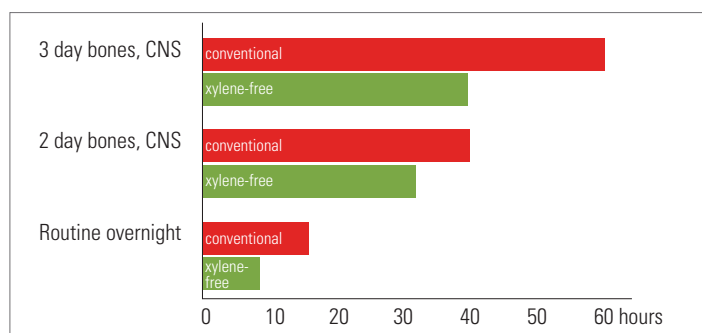
LD50 oral 5,045 mg/kg

LD50 dermal 12,800 mg/kg

Isopropanol has the highest LD50 figures, for both oral and dermal; it is considerably less toxic than the other reagents.

## Processing Times

Useful reductions in processing times were achieved using the xylene-free system:



Process	Conventional	Xylene-free	% Change
Routine overnight	16 hours	9 hours	-44%
2 day bones, large blocks, CNS	40 hours	32 hours	-20%
3 day bones, large blocks, CNS	60 hours	40 hours	-33%

## General Comments

### Foetal CNS

On very rare occasions conventionally processed blocks of fixed, intact foetal CNS tissue process poorly and disintegrate into small, disorganised fragments of pale staining tissue. Some xylene-free processed blocks also exhibited this effect. H&E microscopy revealed very pale cytoplasmic staining and pyknotic nuclei.

More frequent disintegration was observed in blocks that had undergone xylene-free processing. This may have been due to, or exacerbated by, the processing method; or it may have been due to an unusual "cluster" of autolyzed CNS tissue. However, to err on the side of caution, it was decided that tissue of this type would be processed conventionally.

The unsatisfactory outcome may have been reflective of the degree of autolysis present, though not evident macroscopically, in these lipid rich, but myelin poor tissues, irrespective of the processing system used.

## External Quality Assurance (EQA)

Anonymised H&E stained sections from xylene-free processed blocks were submitted for external quality assurance on a regular basis in the usual manner; the unconventional processing system used was not declared. Processing, microtomy and staining qualities were assessed on each occasion. A summary of results over the period April 2004 to April 2005 indicated that:

- The scoring range was 7/10 to 10/10
- The average score over the period was 8.80, against a participant background of 7.47
- The average score over the previous year (during which conventional processing was used) was 8.70, against a participant background of 7.46.

These results suggested that, whilst technically dissimilar, tissue processed xylene-free was essentially the same in quality to tissue processed using conventional regimes in a broad range of laboratories involved in the EQA scheme.

## Limitations of the Evaluation

Whilst at least 50% of the laboratory workload (amounting to several thousand individual pieces of tissue) was processed xylene-free during the trial period, the evaluation had some limitations:

- Workload in this specialised laboratory generated relatively few requests for IHC, ISH and special stains.
- The laboratory processed very few small (eg core or endoscopic) biopsies, and there was no demand for rapid (1-2 hour) processing over the evaluation period. While it is unlikely that xylene-free processing would be inadequate in these cases, no assessment could be carried out.
- The majority of IHC and special stain requests were performed on either surgical bone specimens, with potentially adverse effects of decalcification, or autopsy material, in which the various post-mortem effects also had to be considered.

The limited number of IHC, ISH and special stain sections that were processed xylene-free clearly met, or exceeded, quality standards. There were no requests for extra sections of this type for repeat staining.

## Post-script: Two years on...

The laboratory continues to use xylene-free processing on a daily basis, for all specimen types, except foetal CNS material.

- After approximately two years use, no problems associated with xylene-free processing have been identified by laboratory staff.
- Similarly, no problems have been brought to the attention of laboratory staff by medical colleagues ranging in experience/seniority from Specialist Registrar to Professor.
- The quantity of reagent required for xylene-free use remains far lower than quantities used for conventional processing, representing a considerable saving in consumable costs.
- Hands-on time spent using the xylene-free processor and changing reagents remains usefully lower than that spent on a conventional processor.
- The protocol time for very dense bones (3 day bones) has been further reduced to 32 hours (from 60 hours with conventional processing).

## Summary

This paper describes the main findings of the evaluation:

- Block quality was graded "good"; large fatty slices processed well.
- Microtomy was graded "good" for all blocks; in particular, blocks of haemorrhagic tissue were easier to section.
- H&E's and "specials" staining was precise and vivid.
- Immunohistochemistry staining of xylene-free processed blocks was similar to conventionally processed blocks with no changes required to antigen-retrieval methods or reagent dilutions.
- ISH staining of xylene-free processed tissue blocks was good.
- Foetal CNS tissue is known to process poorly on rare occasions; this occurred during the trial for both conventional and xylene-free processing .
- Reagent costs on Peloris xylene-free processing were £24.00, compared with £96.00 on the conventional processor over a 20-day period.
- Processing times were dramatically reduced:
  - the conventional 16 hour protocol was replaced by a 9 hour xylene-free protocol, and
  - the long 60 hour protocol was replaced by a 40 hour xylene-free one.
- All users found the processor easy to use.
- The processor was 100% reliable.

## Acknowledgements

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