An Evaluation of Xylene-free Processing of Tissues From the Central Nervous System Using the Peloris™ Dual Retort Rapid Tissue Processor

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Abstract

This preliminary study demonstrated that xylene-free processing on the Peloris™ dual retort rapid tissue processor using isopropanol, can effectively prepare tissue from the central nervous system to a standard that is at least the equal of traditional xylene or chloroform schedules and in six hours instead of the more usual fourteen hours. The work described in this paper was carried out as an extension of a field trial conducted by Austin Health (Victoria Australia) together with Vision BioSystems (VBS) as part of the development of the Peloris tissue processor. Vision Biosystems has since formed part of the Biosystems Division of Leica Microsystems.

Tissues from the central nervous system, which are widely acknowledged as being difficult to section successfully, were processed using six, nine and twelve hour evaporative isopropanol schedules (xylene-free) using a Peloris processor. The results were compared to fourteen and twenty four hour schedules carried out using Tissue-Tek® VIP™ processors employing either xylene or chloroform. Sections were stained with H&E, Luxol Fast Blue/ Cresyl Violet, and Garvey Silver stain. Results were assessed separately by staff from Austin Health and Vision BioSystems.

Introduction

The Peloris dual retort rapid tissue processor has undergone extensive field trials as an essential part of its development. This processor has been designed to effectively process tissues using conventional schedules employing xylene, but its particular advantage is the ability to rapidly process tissues using xylene-free evaporative protocols with Isopropanol (IPA). Xylene is considered a relatively dangerous solvent and laboratories are being forced to seek less toxic alternatives for routine use(1-3). Isopropanol is considered less toxic (2, 4) and, providing it can be completely eliminated from specimens during wax infiltration, it can be used to both dehydrate and clear specimens during paraffin processing. The Peloris dual retort rapid tissue processor has been designed with this in mind.

As well as critically evaluating the performance of Peloris using a range of different tissues and schedules employing xylene, it was necessary to examine its xylene-free performance. The focus was to assess the overall quality of tissues processed in this way and to compare them to specimens processed on existing equipment using xylene and representing the accepted industry standard. As part of this overall evaluation it was felt that it would be useful to assess the performance of Peloris in processing a particularly challenging tissue such as brain. This paper presents the results of a preliminary study in this area.

It is widely accepted that tissue from the central nervous system (CNS) is difficult to process (5, 6). This is due to its unique histological structure. It consists of relatively large cells (neurones), a range of smaller supporting cells (glial cells) and their respective cytoplasmic processes which form a dense network around the cells. Apart from relatively small amounts around blood vessels, brain tissue contains little collagen or smooth muscle. However CNS tissue is rich in lipids particularly those associated with myelin that forms sheaths around nerve fibres (axons). Although CNS specimens may come in the form of small tumour samples or brain biopsies from neurosurgery, tissue from spinal cord or brain removed at operation or postmortem can be in the form of quite large slices. For these latter specimens most laboratories use long fixation times and extended processing schedules specifically designed for CNS tissues (5-7).

For our evaluation two independent processing trials were carried out in the Anatomical Pathology Laboratory at Austin Health (a large 840 bed teaching hospital). Senior histology scientists and a neuropathologist from Austin Health together with scientists from VBS were involved in assessing the processed blocks and the stained sections. These assessments were carried out independently using different scoring methods, with the assessors being unaware of the schedules used to process the specimens. The scoring system used by Vision BioSystems (VBS) staff has been extensively used throughout the development and testing of Peloris to evaluate the quality of tissue processing and as a mechanism for optimizing standard processing protocols. A score is calculated by assessing 23 parameters and is expressed as a percentage. The complete details are provided elsewhere (8).

Method

The dimensions of brain specimens processed ranged from 25x15x4 mm to 35x25x4 mm approximately. The specimens were processed on the Peloris using xylene-free isopropanol schedules, or a Tissue-Tek® VIP2000™ or Tissue-Tek® VIP5™, using conventional schedules incorporating either xylene or chloroform. These conventional schedules are routinely used to process CNS tissues at the Austin Health laboratories.

After processing specimens were embedded and sections cut at 7µm, mounted on slides, dried at 37 °C overnight then at 60 °C for 2 hrs before staining. Three stains were carried out on slides from each block for each schedule:

1. H&E to provide general morphological information,
2. Luxol Fast Blue/Cresyl Violet (LFB/CV) which stains myelin and Nissl substance in neurones,
3. Garvey silver stain (GSS) which stains axons and is particularly good for identifying characteristic abnormalities associated with Alzheimer's disease.

The composite Tables below provide details of the various processing schedules used in both Trial 1 and Trial 2. Table 1 shows the xylene-free schedules used on Peloris and Table 2 shows the VIP schedules using xylene and chloroform.

	Schedule	**P1**	**P2**	**P3**				
		9 hour IPA	**12 hour IPA**	**6 hour IPA**				
Step No.	**Reagent**	**Time (minutes)**	**Time (minutes)**	**Time (minutes)**	**Drip Time (seconds)**	**Temp °C**	**P/V**	**Stir**
1	Formalin	30	40	20	10	60	Off	Med
2	50/50 ethanol/water	30	30	15	10	60	Off	Med
3	50/50 ethanol/water	30	30	20	10	60	Off	Med
4	80/20 ethanol/IPA	30	50	20	10	60	Off	Med
5	80/20 ethanol/IPA	60	80	30	10	60	Off	Med
6	IPA	60	80	30	10	60	Off	Med
7	IPA	60	80	50	10	60	Off	Med
8	IPA	60	80	50	10	60	Off	Med
9	Paraffin wax	60	60	50	10	85	Vac	Med
10	Paraffin wax	60	60	50	10	85	Vac	Med
11	Paraffin wax	40	70	30	10	65	Vac	Med
	Total step time	**520**	**660**	**365**				
	Total processing time	**547.5 (9.1 hours)**	**687.5 (11.5 hours)**	**385 (6.4 hours)**				

Table 1. Peloris processing schedules

	Schedule	V1	V2	V3		
		14 hour xylene	24 hour xylene	24 hour chloroform		
Step No.	**Reagent**	**Time (minutes)**	**Time (minutes)**	**Time (minutes)**	**Temp °C**	**P/V**
1	Formalin	120	-	-	45	Yes
2	70% Ethanol	30	120	120	40	Yes
3	90% Ethanol	30	120	120	40	Yes
4	100% Ethanol	60	120	120	40	Yes
5	100% Ethanol	60	120	120	40	Yes
6	50/50 Eth/Xyl	60	-	-	-	Yes
7	100% Ethanol	60	120	120	-	Yes
8	Xylene	30	90	Chloroform 90	-	Yes
9	Xylene	60	90	Chloroform 90	-	Yes
10	Xylene	60	90	Chloroform 90	-	Yes
11	Wax	30	60	60	58	Yes
12	Wax	30	60	60	58	Yes
13	Wax	60	120	120	58	Yes
14	Wax	60	240	240	58	Yes
	Total Step Time	**750**	**1350**	**1350**		
	Total processing time	**810 (13.5 hours)**	**1410 (23.5 hours)**	**1410 (23.5 hours)**		

Table 2. Tissue-Tek VIP processing schedules

Trial 1: The aim in this preliminary experiment was to compare a standard overnight Tissue-Tek VIP schedule (routinely used for surgical specimens) and an extended VIP schedule (specifically designed for CNS specimens) with equivalent Peloris Isopropanol schedules. Based on previous experience with other specimens we were confident that the Peloris schedules could be shorter in duration.

Tissues processed in this trial were human temporal lobectomy specimens which had been fixed in 20% NBF (neutral buffered formalin) for at least two months. A total of 12 blocks were processed using four different schedules as follows:

Schedule P1 Peloris 9 hr using Isopropanol

Schedule V1 VIP 14 hr using xylene (routinely used for surgical and autopsy specimens)

Schedule P2 Peloris 12 hr using Isopropanol

Schedule V2 VIP 24 hr using xylene (routinely used for brain)

Results of Trial 1:

Austin Health: The slides were carefully examined and scored. The consensus view of scientists and neuropathologist was that myelin and fibres were best demonstrated in slides prepared using Schedule P1 (Peloris 9 hour), closely followed by V1 (VIP 14 hour) and P2 (Peloris 12 hour). It was agreed that the 9 hour Isopropanol schedule on Peloris gave marginally better results than the other schedules.

VBS: The blocks and H&E stained slides were carefully examined and scores calculated. Schedules P1 (Peloris 9 hour) and P2 (Peloris 12 hour) scored better than either Schedule V1 or V2. It was agreed that both the Peloris Isopropanol schedules gave better results than the VIP xylene schedules based on an evaluation of the H&E stained sections. The results are shown in Table 3. Comparative micrographs are shown in Figures 1 and 2.

Schedule	**P1 9 hour IPA**	**V1 14 hour Xylene**	**P2 12 hour IPA**	**V2 24 hour Xylene**
Mean Score %	76	71	80	64

Table 3. Mean scores using the VBS scoring system for H&E slides from each Schedule used in Trial 1.

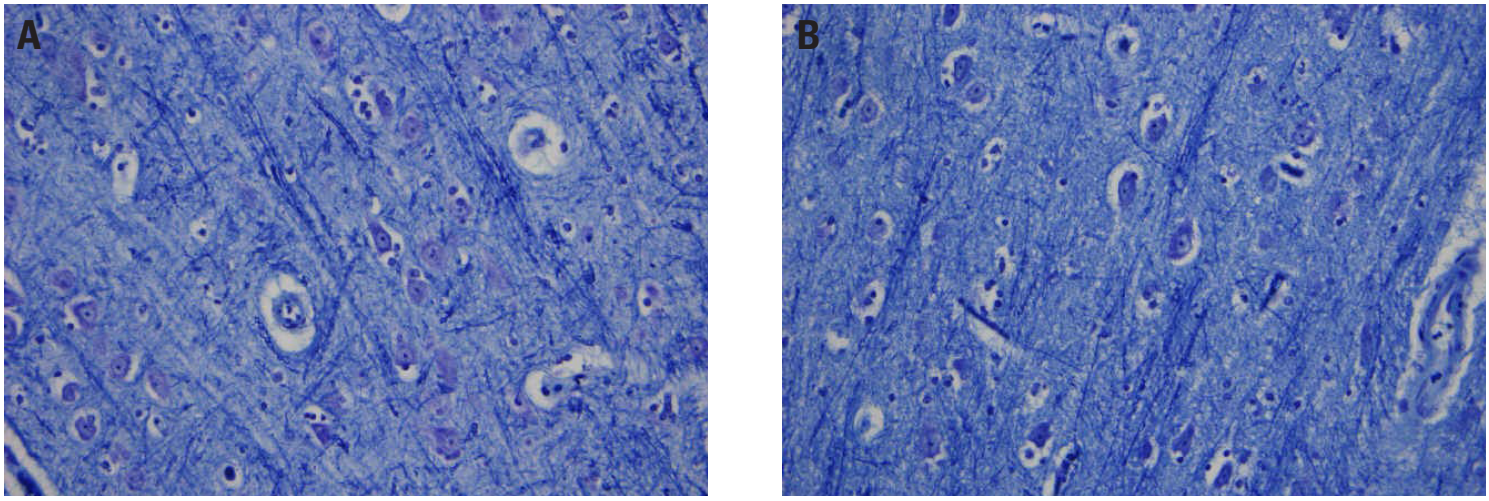

Figure 1. A comparison of LFB/CV stained sections from Trial 1. **A** processed by Schedule P1 (Peloris 9 hour using Isopropanol) and **B** processed by Schedule V1 (VIP 14 hour using xylene). Both sections show well-defined cell bodies. Myelinated fibres are very well demonstrated in **A**.

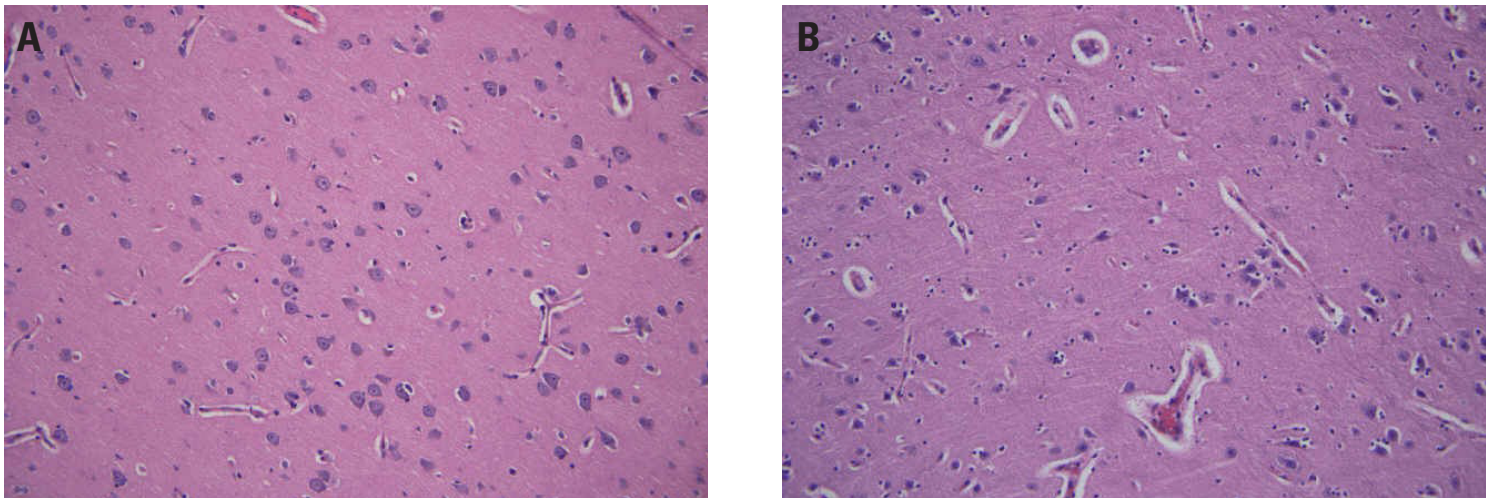

Figure 2. A comparison of H&E stained sections from Trial 1. **A** processed by Schedule P2 (Peloris 12 hour using Isopropanol) and **B** processed by Schedule V1 (VIP 14 hour using xylene). Both sections show well-preserved neural elements. Note the lack of shrinkage around neurons in **A**.

Trial 2: Based on the encouraging results of Trial 1 it was decided to try a shorter Isopropanol schedule (6 hours) and compare it with a 9 hour Isopropanol schedule and three VIP schedules. One of the VIP schedules included chloroform which Austin Health had previously used on occasions with good results. The slides produced from these schedules were also compared to the original diagnostic blocks and slides retrieved from Austin Health files.

Five schedules were run using human temporal lobectomy specimens from two different patients. In both cases specimens had been fixed in 20% NBF for at least two months. Sections were prepared and stained as in the previous trial.

A total of 10 blocks were processed as follows:

Schedule V1	VIP 14 hr using xylene (routinely used for surgical and autopsy specimens)
Schedule V2	VIP 24 hr using xylene (routinely used for brain)
Schedule V3	VIP 24 hr using chloroform (previously this schedule was routinely used for CNS by Austin Health – now replaced with xylene Schedule V2 to routinely process CNS tissue)
Schedule P1	Peloris 9 hr using Isopropanol
Schedule P3	Peloris 6 hr using Isopropanol

Results of Trial 2:

Austin Health: Compared with the original slides prepared (from Austin Health files) all slides were deemed to be of diagnostic quality by the consultant neuropathologist. Taking into account the small sample size and with particular reference to fibre demonstration in the CNS, the Austin Health team concluded that the tissues processed on Peloris using Isopropanol produced results equivalent to those using the Tissue-Tek VIP2000 and the VIP5 using xylene. Schedule V3 using chloroform was of the poorest quality.

VBS: The blocks and H&E stained slides were carefully examined and scores calculated. Schedule P1 Peloris 9 hour gave the best results while the shorter Schedule P3 Peloris 6 hour produced results similar to the longer xylene and chloroform protocols. Results are shown in Table 4. Comparative micrographs are shown in Figures 3 and 4.

Taking the results from both trials 1 and 2 into account the best results were achieved using Schedule P1 Peloris 9 hour using Isopropanol.

Schedule	V1 14 hour Xylene	V2 24 hour Xylene	V3 24 hour Chloroform	P1 9 hour IPA	P3 6 hour IPA
Mean score %	72.5	72	74	81.5	74.5

Table 4. Mean scores using the VBS scoring system for H&E slides from each Schedule used in Trial 2

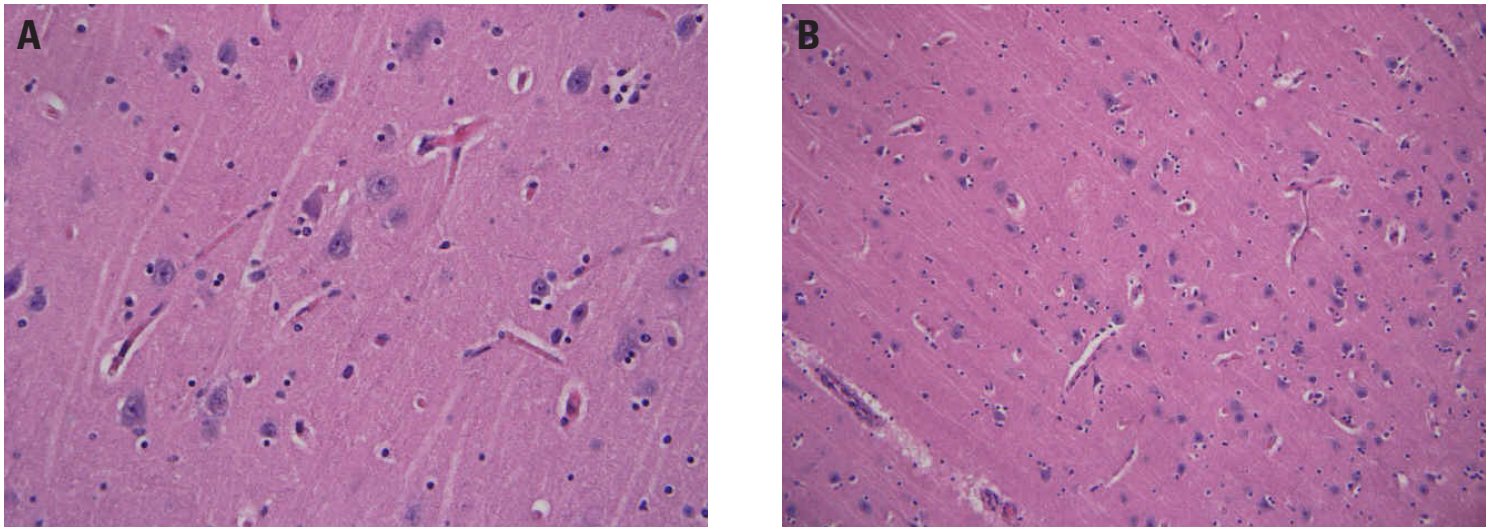

Figure 3. A comparison of H&E stained sections from Trial 2. **A** and **B** processed by Schedule P1 (Peloris 9 hour using Isopropanol) Both sections show well-preserved cellular and fibrous elements with minimal shrinkage.

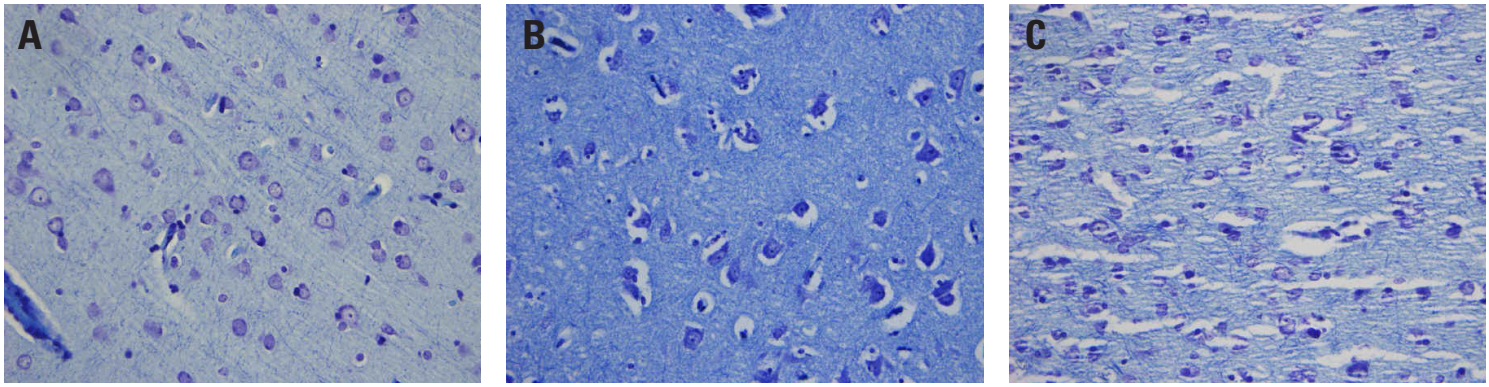

Figure 4. A comparison of LFB/CV stained sections from Trial 2. **A** processed by Schedule P1 (Peloris 9 hour using Isopropanol), **B** processed by Schedule V2 (VIP 24 hour using xylene) and **C** processed by Schedule V3 (VIP 24 hour using chloroform). Greater shrinkage is visible in both **B** and **C**. **C** showed extensive microscopic and macroscopic cracking due to the brittleness of the block processed using chloroform.

Conclusion

Two strong indications can be drawn from our results. Namely, evaporative processing on Peloris using Isopropanol can effectively prepare tissue from the central nervous system to a standard which is at least the equal of both traditional chloroform and xylene processing, and tissue can be processed in a much shorter time.

The replacement of the more toxic xylene with isopropanol as a processing reagent has obvious Occupational Health and Safety (OH&S) advantages.

This preliminary study has produced some encouraging results which will require further investigation with larger sample sizes. All of the blocks, sections and stains were assessed and found to be of a satisfactory standard.

Because of the quality of the results achieved we encourage Peloris users to try for themselves the xylene-free protocols presented here.

Acknowledgements

The Biosystems Division of Leica Microsystems would like to thank Mr Terry Cass and the staff of the Anatomical Pathology Department, Division of Laboratory Medicine, Austin Health, Victoria, Australia for their valuable assistance in conducting the trials described in this paper and in the preparation of this manuscript. Thanks are also due to Mr David Roche and Mr Neville Farmer (Biosystems Division) for coordinating the project and collecting and collating the data.

References

1. Richard-Allan Scientific. MSDS xylene [WWW document]. January 1995. http://www.rallansci.com/msds/xylene.pdf [9/08/05].
2. Carson FL. Histotechnology. 2nd ed. Chicago: ASCP Press; 1997.
3. Langman JM. d-Limonene: Is it a safe alternative to xylene? Journal of Histotechnology 1995;18(2):131-137.
4. Richard-Allan Scientific. MSDS isopropanol [WWW document]. December 2001. http://www.rallansci.com/msds/Isopropyl_Alcohol.pdf [9/05/05].
5. Wainwright H, Blumbergs PC. Central Nervous System. In: Woods AE, Ellis RC, editors. Laboratory Histopathology. New York: Churchill Livingstone; 1994.
6. Lowe J. Techniques in neuropathology. In: Bancroft JD, Stevens A, Turner DR, editors. Theory and practice of histological techniques. 4th ed. New York: Churchill Livingstone; 1996.
7. Lillie RD, Fullmer HM. Histopathologic technic and practical histochemistry. 4th ed. New York: McGraw-Hill; 1976.
8. Vision BioSystems. Assessing the quality of tissue processing and the performance of Peloris using the VBS scoring system. Melbourne: Vision BioSystems Ltd May 2005.