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Comparison of histomorphology and DNA preservation produced by fixatives in the veterinary diagnostic laboratory setting

Abstract: Histopathology is the most useful tool for diagnosis of a number of diseases, especially cancer. To be effective, histopathology requires that tissues be fixed prior to processing. Formalin is currently the most common histologic fixative, offering many advantages: it is cheap, readily available, and pathologists are routinely trained to examine tissues fixed in formalin. However, formalin fixation substantially degrades tissue DNA, hindering subsequent use in diagnostics and research. We therefore evaluated three alternative fixatives, TissueTek® Xpress® Molecular Fixative, modified methacarn, and PAXgene®, all of which have been proposed as formalin alternatives, to determine their suitability for routine use in a veterinary diagnostic laboratory.

This was accomplished by examining the histomorphology of sections produced from fixed tissues as well as the ability to amplify fragments from extracted DNA. Tissues were sampled from two dogs and four cats, fixed for 24-48 hours, and processed routinely. While all fixatives produced acceptable histomorphology, formalin had significantly better morphologic characteristics than the other three fixatives. Alternative fixatives generally had better DNA amplification than formalin, although results varied somewhat depending on the tissue examined. While no fixative is yet ready to replace formalin, the alternative fixatives examined may be useful as adjuncts to formalin in diagnostic practices.

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16 **Introduction:**

17 Histopathology is the most useful tool for diagnosis of a number of diseases,
18 especially cancer. To be effective, histopathology requires that tissues be fixed prior to
19 processing. The ultimate tissue fixative for histopathology would create tissue
20 histomorphology identical to formalin, pose no hazard to human health, preserve nucleic
21 material for an extended period of time preferably at room temperatures, and be cost
22 effective. Formalin has been the most common histologic fixative for over 100 years. It
23 offers many advantages: it is cheap, readily available, and pathologists are routinely
24 trained to examine formalin-fixed tissues (Gugic et al. 2007; Srinivasan et al. 2002).

25 However, formalin has several disadvantages as well. It can cause respiratory
26 irritation and is classified as a carcinogen (Bolt et al. 2010; Bosetti et al. 2008; Buesa
27 2008; Gugic et al. 2007). It can also degrade nucleic acids and proteins, which make
28 formalin-fixed tissues less usable for downstream molecular diagnostics (Buesa 2008;
29 Gugic et al. 2007). Formalin is an aldehyde-based fixative that works by cross-linking
30 proteins, which irreversibly degrades proteins and nucleic acids (Srinivasan et al. 2002).

31 The most common method for preserving tissues for molecular diagnostics is
32 freezing at -80˚C, although this does not allow for histopathologic examination of tissues.
33 This method itself has a number of issues, including requiring special equipment,
34 difficulty in shipping samples to laboratories, and requiring duplicate samples to be taken
35 for histopathologic examination.

36 This is impractical for many private veterinary practices, as these generally lack
37 the facilities to freeze samples at -80˚C and many samples are too small to duplicate
38 samples. Therefore, alternative fixatives have been proposed to allow for both
39 histopathologic examination and molecular diagnostics {Cox, 2006 #2;Gugic, 2007
40 #3;Kap, 2011 #6;Vincek, 2003 #10}. These have been shown to preserve nucleic acids
41 with results similar to those obtained with fresh or frozen tissues, while still preserving
42 histomorphology. Some of the more successful alternatives include Tissue-Tek®
43 Xpress® Molecular Fixative (Gugic et al. 2007), PAXgene® (Kap et al. 2011), and
44 modified methacarn solution (Cox et al. 2006). All of these are alcohol-based and non-
45 cross-linking. Evaluation of histomorphology preservation has varied among the studies
46 evaluating these fixatives and generally involve research settings using techniques that
47 are not practical in most clinical situations. In addition, some of these fixatives are
48 currently cost prohibitive in the veterinary clinical setting. The majority of the studies
49 evaluating these fixatives have evaluated single organs from humans or rodents {Cox,
50 2006 #2} or multiple organs from humans {Kap, 2011 #6;Vincek, 2003 #10}. One study
51 evaluated Tissue-Tek® Xpress® Molecular Fixative and formalin comparing
52 histomorphology and RNA quality from a variety of animal tissues (small animals,
53 rodents, lagamorphs, birds, insects, and lizards) both at room temperature and high
54 ambient temperatures simulating field collection of samples {Gugic, 2007 #3}. They
55 concluded that Tissue-Tek® Xpress® Molecular Fixative protected RNA and provided
56 acceptable histomorphology that would not hinder histologic diagnosis in the species
57 studied. Some studies evaluating multiple animal species have included limited numbers
58 of fixatives for comparison {Gugic, 2007 #3;Vincek, 2003 #10}.

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59 The main limitation of all of these previous studies is that they have evaluated
60 fixatives in a research setting. There has not been a systematic evaluation of these to
61 determine their utility in the veterinary diagnostic setting. Alternative fixatives would
62 have a number of benefits for veterinary diagnostic laboratories, including (depending on
63 the nature of the fixative) decreasing hazardous waste disposal costs, decreasing health
64 risks to laboratory workers, and enhancing the power of retrospective studies Therefore,
65 we conducted this study to determine how alternative fixatives would function in a
66 standard diagnostic laboratory setting by evaluating histomorphology of a variety of
67 tissues from dogs and cats, as well performing a quantitative evaluation of recoverable
68 DNA from tissues.

69

70 Materials and Methods:

71 All study protocols were approved by the University of Florida Institutional
72 Animal Use and Care Committee (approval #201105654), and all animals were
73 euthanized for reasons unrelated to this project. Necropsies were performed on four cats
74 and two dogs within four hours of euthanasia. Replicate 10 x 10 x 5 mm samples from
75 the liver, brain, lung, lymph node, kidney and spleen were collected. One sample of each
76 tissue was frozen at -80˚C. The remaining samples were placed into 10% neutral buffered
77 formalin, Tissue-Tek® Xpress® Molecular Fixative (TT-XMF), modified methacarn, and
78 PAXgene®, with a minimum of 1:10 tissue to fixative volume. Samples were allowed to
79 fix for 24-48 hours at room temperature with the exception of tissues in PAXgene®,
80 which were fixed and preserved according to the manufacturer's protocol.

81 All tissues were processed using a Tissue-Tek processor with a standard overnight
82 protocol (excluding formalin steps) followed by paraffin embedding and hematoxylin and
83 eosin staining. The 10% neutral buffered formalin, Tissue-Tek® Xpress® Molecular
84 Fixative, and PAXgene® fixatives were purchased commercially (ThermoFisher
85 Scientific, Waltham, MA). Modified methacarn was prepared as previously described,
86 using 8 parts methanol and 1 part glacial acetic acid (Cox et al. 2006).

87 Histomorphology was evaluated by two blinded board-certified veterinary
88 anatomic pathologists (MJD, JAC) and one blinded anatomic pathology resident (WFC).
89 Histomorphology of nuclear, cytoplasmic, and cellular membrane detail were evaluated
90 on a 1-4 scale (table 1). Sample scores were averaged between all three evaluators. For
91 one cat, the formalin-fixed lymph node sample was lost from the block; therefore,
92 formalin fixation histomorphometry scores for lymph node are based on the remaining
93 five samples. Both the individual components of the histomorphometry score as well as
94 the total score were evaluated using a Kruskal-Wallis test (Lowry 2012) to determine if
95 there was a difference between any of the four groups. If a significant difference was
96 found ($p < 0.05$), the Mann-Whitney test was used to compare each group to each other

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97 group, to determine significant differences between each individual fixative. Fixatives
98 were considered significantly different if the one-tailed Mann Whitney p value was less
99 than 0.05. The minimum, 25th quartile, median, 75th quartile, and maximum were
100 calculated for each tissue as well as for all tissues combined using Microsoft Excel
101 (v14.3.9, Microsoft Corp., Seattle, WA). Graphs were generated using GNUplot (v.4.6,
102 patchlevel 3).

103 Tissue scrolls were obtained from the paraffin blocks one week after processing
104 and DNA was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen Inc., Valencia,
105 CA). As a control, DNA was extracted from tissues frozen at -80˚C using the QIAamp
106 DNA Mini Kit (Qiagen Inc.). Primers were designed by aligning the sequences of the
107 retinol-binding protein 3, interstitial gene (IRBP) from dog, mouse, rat, and human, and
108 selecting regions that were relatively conserved, to generate 100, 200, 300, 500 and 750
109 base pair long amplicons (table 2). Extracted DNA was amplified via PCR on an Applied
110 Biosystems Veriti Thermal Cycler with the following conditions: 96ºC for 3 minutes,
111 followed by 35 cycles of 96˚C for 1 minute, 60˚C for 1 minute, then 72˚C for 1 minute.
112 This was followed by 7 minutes at 72˚C, with a final hold at 4˚C until the next morning.
113 Samples were examined on a 1.25% agarose gel via electrophoresis.

114 The presence or absence of bands for all sizes was noted. The Kruskal-Wallis test
115 was used to determine if there was a significant difference between the maximum band
116 size for any of the fixatives. If significant ($p<0.05$), the Mann-Whitney test was used to
117 compare each fixative against each other to determine which had significant differences
118 (one-tailed $p<0.05$). The minimum, 25th quartile, median, 75th quartile, and maximum
119 calculated for each fixative and for each tissue using Microsoft Excel. Graphs were
120 generated using GNUplot (v.4.6, patchlevel 3).

121 **Results:**

122 ***Histomorphology***

123 While the majority of the alternative fixatives produced adequate
124 histomorphology in the tissues examined, formalin fixed tissues consistently resulted in
125 superior histomorphology. There was no statistically significant difference between mean
126 histomorphology scores comparing dog and cat tissues, and these were combined for
127 subsequent analysis. Mean total, nuclear, cytoplasmic, and cellular membrane scores
128 (figs. 1A-D) for formalin fixed tissues were higher than for all other fixatives ($p<0.0001$),
129 although there is substantial variation with all fixatives (figs. 2A-D).

130 While a number of minor artifacts were noted, the primary difference noted
131 between formalin and the other fixatives was in erythrocytes. This is likely reflected in

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132 the significantly higher scores for formalin vs. other fixatives in the spleen (p=0.0026),
133 an organ made up in large part by erythrocytes.

134 ***DNA Preservation***

135 Formalin has significantly shorter total maximum DNA band sizes than TT-XMF
136 (p=0.0158), modified methacarn solution (p < 0.0001), and PAXgene (p=0.0004) (fig. 3).
137 In particular, the bands obtained from lymph nodes were significantly smaller with
138 formalin than with TT-XMF (p=0.0179) and modified methacarn (p=0.004) (fig. 4).
139 Overall, modified methacarn solution performed as well or better than the other fixatives
140 for all tissues, with the best score in brain (median amplicon length of 750bp).

141 **Discussion:**

142 While alternative fixatives have been found to work well in research settings (Cox
143 et al. 2006; Kap et al. 2011; Vincek et al. 2003), these are not ready to replace formalin
144 for routine tissue processing in the veterinary laboratory. All of the fixatives require
145 tissues be prevented from contacting formalin to benefit from their nucleic acid
146 preserving qualities, which would require laboratories to either maintain separate tissue
147 processors or bar submission of formalin-fixed tissues. Neither of these is practical in
148 veterinary practice. Several fixatives produce excellent histomorphology with alternative
149 processing techniques; this is also impracticable in most veterinary diagnostic
150 laboratories, as it would require separate processing runs.

151 However, while no fixative is ideal from the standpoint of replacing formalin, all
152 fixatives produced interpretable slides. Therefore, using alternative fixatives may be
153 useful in specific circumstances where subsequent DNA isolation may be required. For
154 example, tissue samples from neoplasms may be saved separately to generate a tissue
155 bank for subsequent research projects. The specific alternative chosen should be based on
156 the tissue selected, as well as predicted needs for DNA amplification and preservation of
157 histomorphology. For example, while TT-XMF had better histomorphology scores in the
158 kidney than either modified methacarn or PAXgene, it had a lower median DNA
159 amplicon size.

160 One characteristic observed with alternative fixatives was that bloody or
161 congested tissues often had unfixed areas, which could result in missing lesions and
162 inaccurate diagnoses. This has not been found in previous studies (Cox et al. 2006), and
163 may be due to a number of factors. First, the size of sample taken will greatly influence
164 fixation. For most veterinary diagnostic laboratories, 1 cm thick samples are considered
165 standard for histopathologic examination. In many previous studies, samples taken for
166 fixation were substantially thinner; for example, the study by Cox et al. used 15 mm x 8
167 mm x 3 mm samples. Other possibilities include differences in processing; microwave

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168 fixation (Cox et al. 2006)} or rapid tissue processing (Vincek et al. 2003) techniques have
169 been used. Tuning the processing technique for the fixative selected would likely
170 improve fixation and the ultimate histomorphology.

171 Finally, our evaluation of macromolecule preservation of was limited to DNA.
172 Additional analysis would be required to determine whether these fixatives preserve
173 RNA equally well. Other variables require investigation to determine the best fixative for
174 a particular application. These include the effects of fixation time on nucleic acid quality,
175 as many samples will sit longer than 24 hours before processing, as well as the effect of
176 storage time after tissue processing but before sectioning for nucleic acid isolation, since
177 many blocks will be stored for a period of time between the evaluation of histopathology
178 and nucleic acid isolation. The latter is especially important if laboratories set up tissue
179 banks, as samples would be expected to be stored for prolonged periods.

180 Conclusions:

181 While no fixative is ideal to replace formalin, alternative fixatives have generally
182 acceptable histomorphologic characteristics in most tissues and are valuable adjuncts to
183 standard formalin fixation. Projects proposing to use an alternative fixative for a research
184 project should first evaluate the project requirements and ideally test the fixative with
185 samples of the target organ to determine the best fixative, processing characteristics, and
186 histomorphology compromises before actual sample collection begins.

187 Acknowledgements:

188 The authors would like to acknowledge Patrick Knisley for his assistance with samples,
189 the University of Florida College of Veterinary Medicine Histopathology Laboratory for
190 help with tissue processing, and Antoinette McIntosh for help with DNA processing.

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220

221 Figure Legends:

222 Figure 1. Histomorphology scores for all animals and tissues combined. The median is
223 represented by a red diamond, the box represents the 25th and 75th quartiles, and the
224 whiskers represent 1.5 x interquartile range.

225 Figure 2. Histomorphology scores for individual tissues. The median is represented by a
226 red diamond, the box represents the 25th and 75th quartiles, and the whiskers represent 1.5
227 x interquartile range.

228 Figure 3. DNA amplicon size ranges for all animals and tissues combined. The median is
229 represented by a red diamond, the box represents the 25th and 75th quartiles, and the
230 whiskers represent 1.5 x interquartile range.

231 Figure 4. DNA amplicon sizes for different tissue samples. The median is represented by
232 a red diamond, the box represents the 25th and 75th quartiles, and the whiskers represent
233 1.5 x interquartile range.

234 Figure 5. Representative fixative histomorphology. Samples are from the liver of a single
235 cat. A – formalin, B – TT-XMF®, C – modified methacarn, D – PAXgene®.

236 Table Legends:

237 Table 1. Histomorphology scoring chacteristics.

238 Table 2. DNA primers used in this study.

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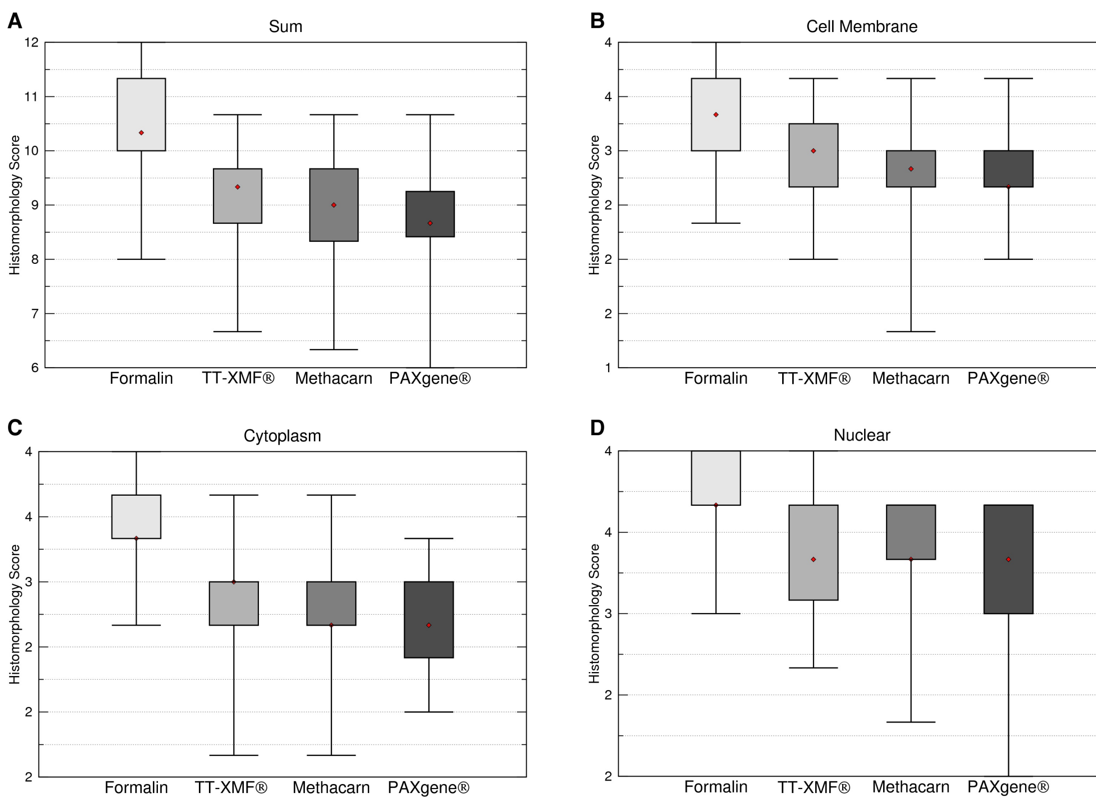

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241 Fig 1.

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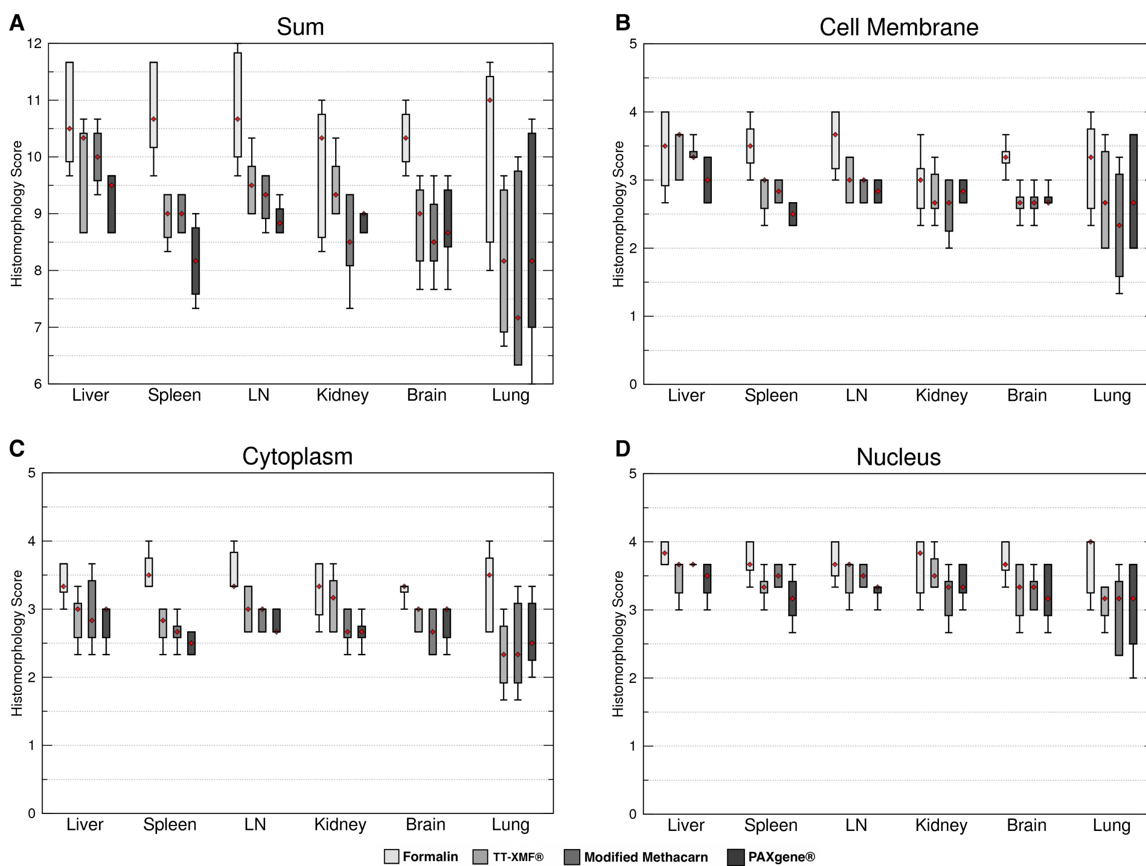

243 Fig 2.

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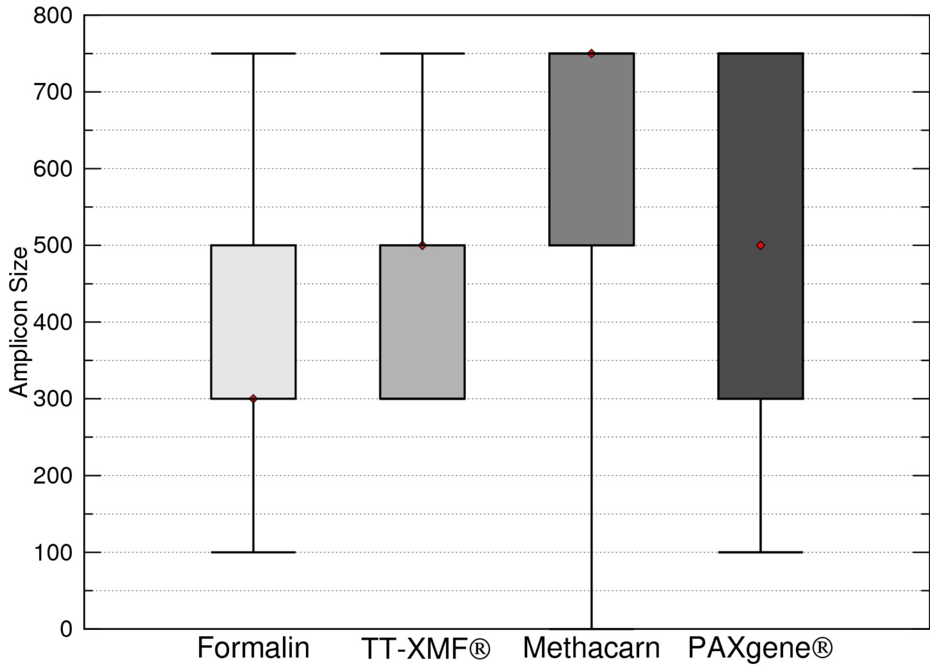

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246 Fig. 3

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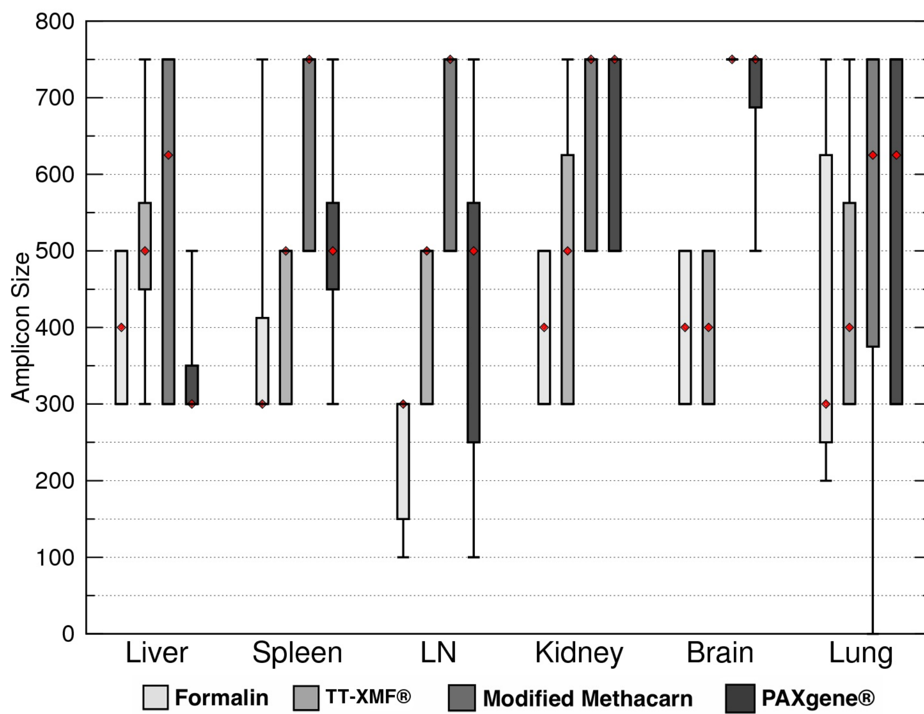

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249 Fig. 4

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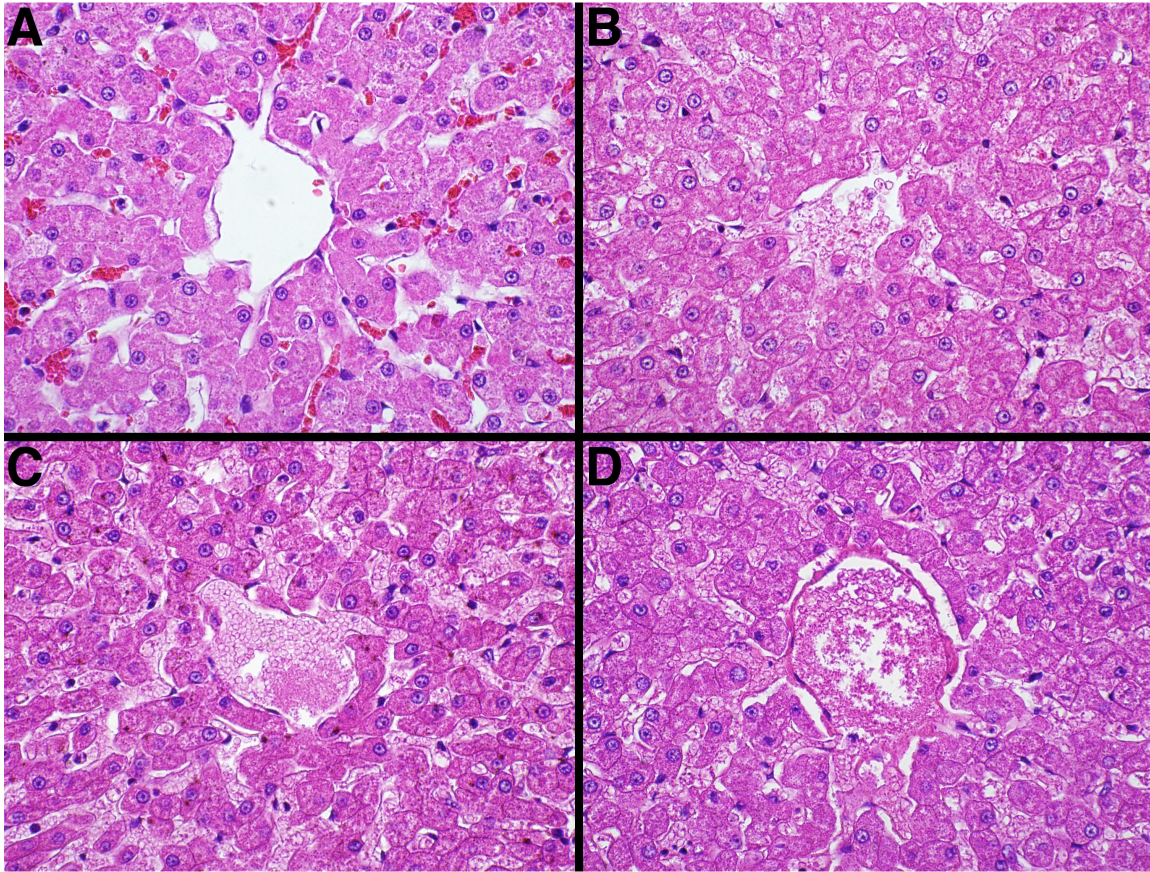

252 Fig 5.

253

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Characteristic	Score	Criteria
Nuclear	4	Sharp nuclear membrane;chromatin pattern and nucleolus, when present, are distinct
	3	Slight degradation in chromatin pattern Nucleolus, when present, less distinct but discernable, sharp nuclear membrane
	2	Less distinct nuclear membrane, fuzzy chromatin pattern Nucleolus, when present, is difficult to discern
	1	Fuzzy nuclear membrane Chromatin pattern difficult to determine Nucleoli indetectable
	0	Nucleus not able to be differentiated from cytoplasm
Cytoplasm	4	Normal cellular morphology easily determined
	3	Intracytoplasmic details fuzzy
	2	Only rare evidence of normal intracellular structures
	1	Increased cytoplasmic pallor, increased cytoplasmic eosinophilia
	0	Cytoplasm homogenously pale eosinophilic with no evidence of organelles
Cell Membranes	4	Cells have distinct intracellular borders Normal substructures, if present, are easily distinguished
	3	Loss of substructures in some cells Slight loss of intracellular details
	2	Loss of substructures in most cells Obvious fuzzing of many cellular borders
	1	No substructures detected Significant fuzzing of most cellular borders
	0	Cells unable to be distinguished from adjacent cells

255 Table 1

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257

Primer Name	Sequence
IRBP_F	CCT KGT RCT GGA NAT GGC
IRBP_R1_100bp	CTC TTG ATG GCC TGC TC
IRBP_R2_200bp	GGC TCA TAG GAG ATG ACC AG
IRBP_R3_300bp	CAG GTA GCC CAC RTT NCC CTC
IRBP_R4_400bp	CGG AGR TCY AGC ACC AAG G
IRBP_R5_500bp	GAT CTC WGT GGT NGT GTT GG
IRBP_R6_750bp	CTC AGC TTC TGG AGG TCC

258 Table 2

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