

IMPACT OF STANDARDIZATION IN TISSUE PROCESSING: THE PERFORMANCE OF DIFFERENT FIXATIVES

Eleonora De Martino¹, Caterina Medeot¹, Lorenzo D'Amico^{2,3}, Giorgio Stanta¹ and Serena Bonin¹

Department of Medical Sciences, University of Trieste, Trieste, Italy

Department of Physics, University of Trieste, Trieste, Italy

Elettra-Sincrotrone Trieste S.C.p.A., Area Science Park, Trieste, Italy

Corresponding author:

Serena Bonin

DSM-Department of Medical Sciences

Cattinara Hospital

Strada di Fiume 447

34149 Trieste

Italy

Tel: +39 040 3996266

e-mail: sbonin@units.it

TABLE OF CONTENTS

Supporting results

- Nucleic acids yield and purity S3
- Real Time PCR efficiency S6
- Biomolecules analysis S7

Figure S1. Total RNA and DNA yield. Scatter dot plot showing mean of the yield (μg) of total RNA (a) and DNA (b). The p value refers to paired t-test for the comparison of the two years and Wilcoxon matched-pairs signed rank test for all sample comparison. FFPE means Formalin fixed and paraffin embedded, BFPE means Bouin's fixed and paraffin embedded, RFPE means RCL2[®] fixed and paraffin embedded and TFPE means TAG-1[™] fixed and paraffin embedded samples. S3

Figure S1: Total RNA and DNA purity. Scatter dot plot showing mean of the A260/280 values of RNA (a) and DNA (c), the A260/230 values of RNA (b) and DNA (d). The p value refers to Wilcoxon matched-pairs signed rank test and to pair t-test for the comparison of the two years absorbance values. FFPE means Formalin fixed and paraffin embedded, BFPE means Bouin's fixed and paraffin embedded, RFPE means RCL2[®] fixed and paraffin embedded and TFPE means TAG-1[™] fixed and paraffin embedded samples. The discontinuous horizontal line indicates the optimal cut-off (1.8 for RNA A260/280 and 2.0 for other ratios). S4

Figure S2: Nucleic acid integrity. Bar plot representing the percentage (mean and SD) of fragments in formalin fixed and paraffin embedded (FFPE) (a), Bouin's fixed and paraffin embedded (BFPE) (b), RCL2[®] fixed and paraffin embedded (RFPE) (c) and TAG-1[™] fixed and paraffin embedded (TFPE) (d) mouse livers. The p value refers to Wilcoxon matched-pairs signed rank test. S5

Table S1: Efficiency of Real Time PCR amplifications. The p value refers to Ancova test. S6

Table S2: Results of biomolecules amplification. It is reported the mean \pm SD of threshold cycle (Ct) of long (L), medium (M) and short (S) amplicon of HPRT gene for mRNA extracted from Bouin's fixed and paraffin embedded sample (BFPE). The p value refers to paired t-test. S7

SUPPORTING RESULTS

Nucleic acids yield and purity

NanoDrop™ ND-1000 spectrophotometer was used to check the nucleic acid yield and purity. Total RNA yield extracted from fixed and embedded samples was comparable between the two years. The average RNA yield extracted from fixed and embedded samples was 16.2 μg (5.3-47.5 μg), while from fresh frozen samples 128 μg (209-89 μg) were obtained. Total RNA yield from RCL2® extracted in 2021 (12 μg (10-13 μg)) was lower respect to RNA obtained in 2020 (19.6 μg (16- 22.5 μg); $p= 0.02$ (Figure S1a). Similar DNA amount was obtained from the two years. The mean DNA yield obtained from fixed and embedded specimens was 7 μg (0.5-21.5 μg) and the lowest DNA amount was obtained from BFPE samples (mean of 1.2 μg (0.5-2.2 μg)) (Figure S1b). On contrary, the average of 106.2 μg (74.4-302 μg) of DNA were extracted from fresh frozen tissues.

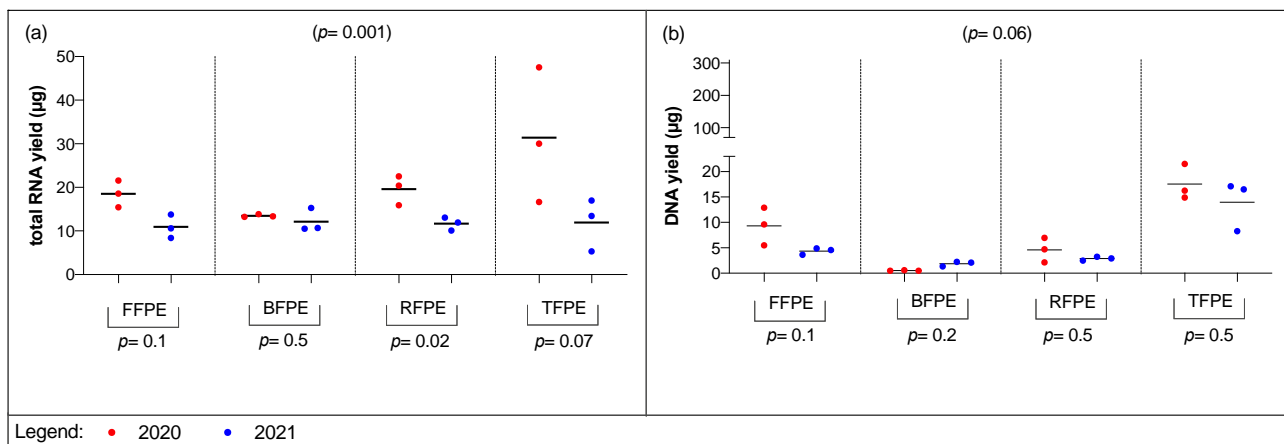


Figure S3. Total RNA and DNA yield. Scatter dot plot showing mean of the yield (μg) of total RNA (a) and DNA (b). The p value refers to paired t-test for the comparison of the two years and Wilcoxon matched-pairs signed rank test for all sample comparison. FFPE means Formalin fixed and paraffin embedded, BFPE means Bouin's fixed and paraffin embedded, RFPE means RCL2® fixed and paraffin embedded and TFPE means TAG-1™ fixed and paraffin embedded samples.

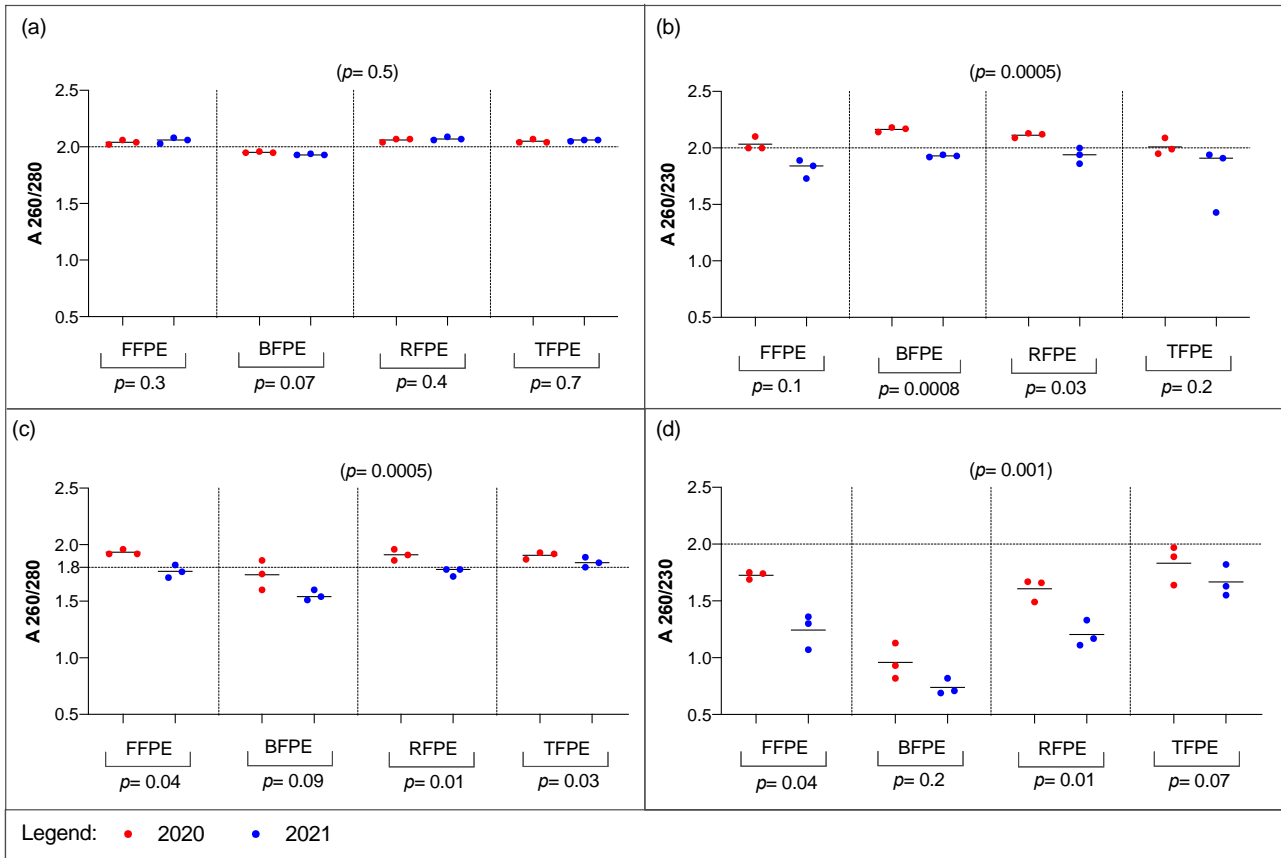


Figure S2. Total RNA and DNA purity. Scatter dot plot showing mean of the A260/280 values of RNA (a) and DNA (c), the A260/230 values of RNA (b) and DNA (d). The p value refers to Wilcoxon matched-pairs signed rank test and to pair t-test for the comparison of the two years absorbance values. FFPE means Formalin fixed and paraffin embedded, BFPE means Bouin's fixed and paraffin embedded, RFPE means RCL2[®] fixed and paraffin embedded and TFPE means TAG-1[™] fixed and paraffin embedded samples. The discontinuous horizontal line indicates the optimal cut-off (1.8 for RNA A260/280 and 2.0 for other ratios).

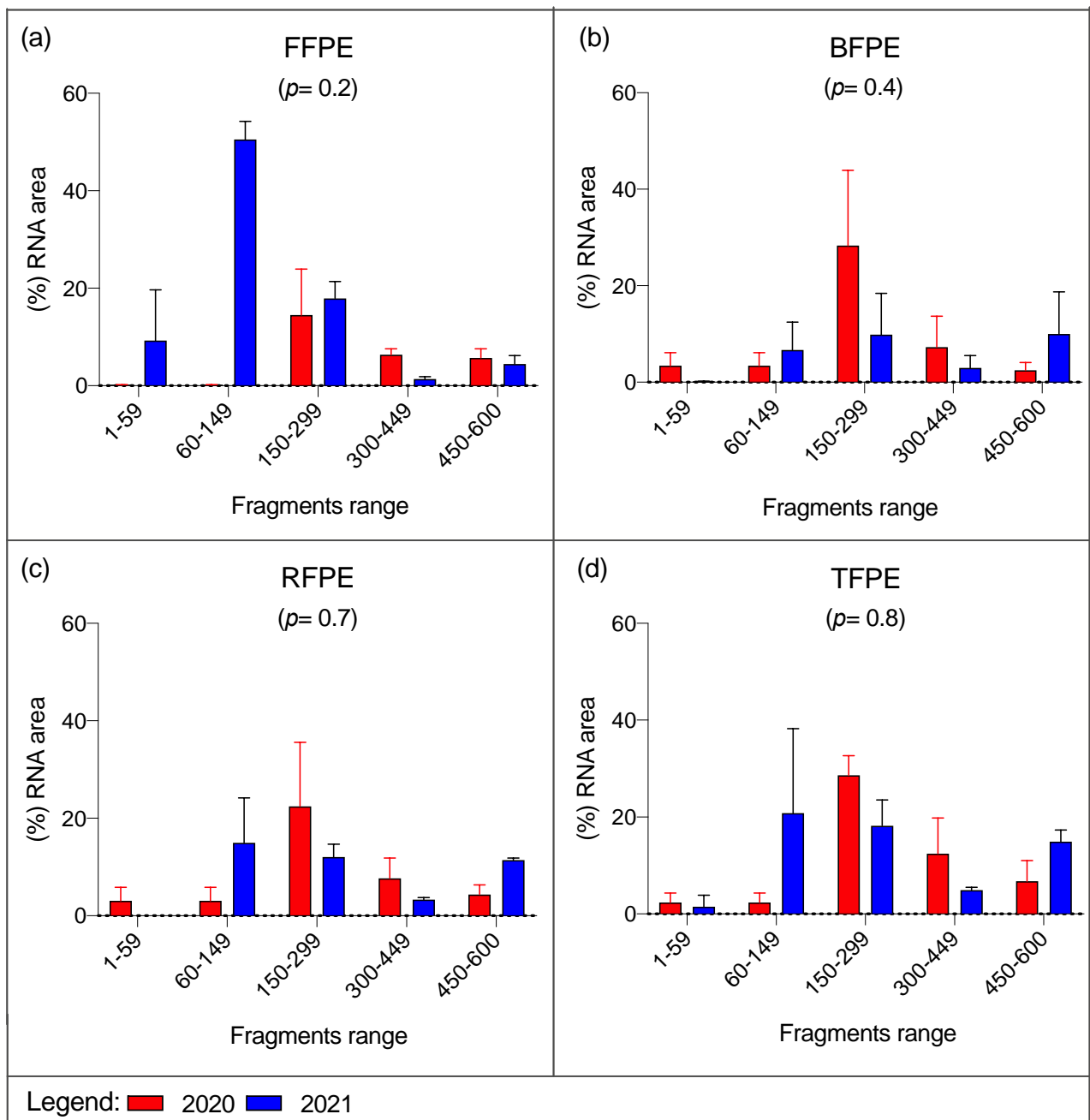


Figure S4: Nucleic acid integrity. Bar plot representing the percentage (mean and SD) of fragments in formalin fixed and paraffin embedded (FFPE) (a), Bouin's fixed and paraffin embedded (BFPE) (b), RCL2[®] fixed and paraffin embedded (RFPE) (c) and TAG-1[™] fixed and paraffin embedded (TFPE) (d) mouse livers. The p value refers to Wilcoxon matched-pairs signed rank test.

Real Time PCR efficiency

The efficiency of all amplification systems was studied by two-fold serial dilution of DNA or cDNA pools of fixed and embedded samples. Data obtained in 2020 and 2021 from linear regression lines were reported in Table S1. Significant differences were found only between the slopes obtained in 2020 and 2021 of short HPRT sequence both in mRNA and DNA analysis ($p= 0.03$ and $p= 0.003$, in order). Anyway, if the greatest dilution is not considered in the regression line, the 2020 and 2021 slopes are equal; thereby, analyzing highly diluted system error deduction could be made.

Table S3: Efficiency of Real Time PCR amplifications. The p value refers to Ancova test.

<i>Biomolecule</i>	<i>Gene name</i>	<i>Amplicon length</i>	<i>Slope 2020</i>	<i>Slope 2021</i>	<i>Y-intercept 2020</i>	<i>Y-intercept 2021</i>	<i>R2 2020</i>	<i>R2 2021</i>	<i>p value</i> ¹
mRNA	<i>Mus-musculus mt-COI</i>	60 bp (S short)	-3.2	-3.2	23.6	23.6	0.99	0.99	0.82
		179 bp (M medium)	-3.4	-3.3	25.3	25.3	0.99	0.99	0.75
		302 bp (L long)	-3.2	-3.6	32.3	33.5	0.99	0.99	0.09
	<i>Mus-musculus GAPDH</i>	90 bp (S short)	-3.4	-3.3	29.1	29.3	0.99	0.99	0.25
		185 bp (M medium)	-3.4	-3.4	31.8	32.5	0.99	0.99	0.85
		267 bp (L long)	-3.6	-3.6	38.3	38.3	0.98	0.98	0.98
	<i>Mus-musculus HPRT</i>	60 bp (S short)	-3.5	-3.4	33.2	34.0	0.99	0.99	0.03
		223 bp (M medium)	-3.5	-4.2	38.0	40.2	0.99	0.98	0.08
		395 bp (L long)	-2.2	-2.3	42.1	41.9	0.97	0.76	0.98
DNA	<i>Mus-musculus HPRT</i>	60 bp (S short)	-3.6	-3.5	32.8	32.2	0.99	0.99	0.003
		223 bp (M medium)	-3.8	-3.6	34.5	34.0	0.99	0.99	0.06
		1062 bp (L long)	-3.9	-3.9	40.3	41.6	0.99	0.98	0.85

¹ p value refers to Ancova test

Biomolecules analysis

HPRT gene expression was investigated by Real-Time PCR to analyze the effect of storage on mRNA and DNA molecules. For mRNA analysis, the 60 bp (short, S), 140 bp (medium, M) and 184 bp (long, L) amplicons of HPRT gene were amplified. 184 bp amplicon of HPRT gene was detected in both years by Real-Time PCR assay (Table S2). After one year of storage the threshold cycles had increased in all amplicon length. Namely 5 Ct of difference was calculated between the 2020 and 2021 analysis of long stretch (184 bp) ($p= 0.04$), while the discrepancy decreases in medium and short sequences (respectively three and one Ct of difference; $p= 0.004$ and 0.005) (Table S2).

Table S4: Results of biomolecules amplification. It is reported the mean \pm SD of threshold cycle (Ct) of long (L), medium (M) and short (S) amplicon of HPRT gene for mRNA extracted from Bouin's fixed and paraffin embedded sample (BFPE). The p value refers to paired t-test.

<i>Biomolecule</i>	<i>Gene name</i>	<i>Fixative</i>	<i>Ct L 2020 (184 bp)</i>	<i>Ct L 2021 (184 bp)</i>	<i>p value¹</i>	<i>Ct M 2020 (140 bp)</i>	<i>Ct M 2021 (140 bp)</i>	<i>p value¹</i>	<i>Ct S 2020 (60 bp)</i>	<i>Ct S 2021 (60 bp)</i>	<i>p value¹</i>
mRNA	<i>Mus-musculus HPRT</i>	BFPE	37.3 \pm 3.2	42.4 \pm 1.3	0.04	35.4 \pm 1.9	38.8 \pm 1.6	0.004	30.6 \pm 0.7	31.6 \pm 0.6	0.005

¹ p value refers to paired t test