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DI TRIESTE



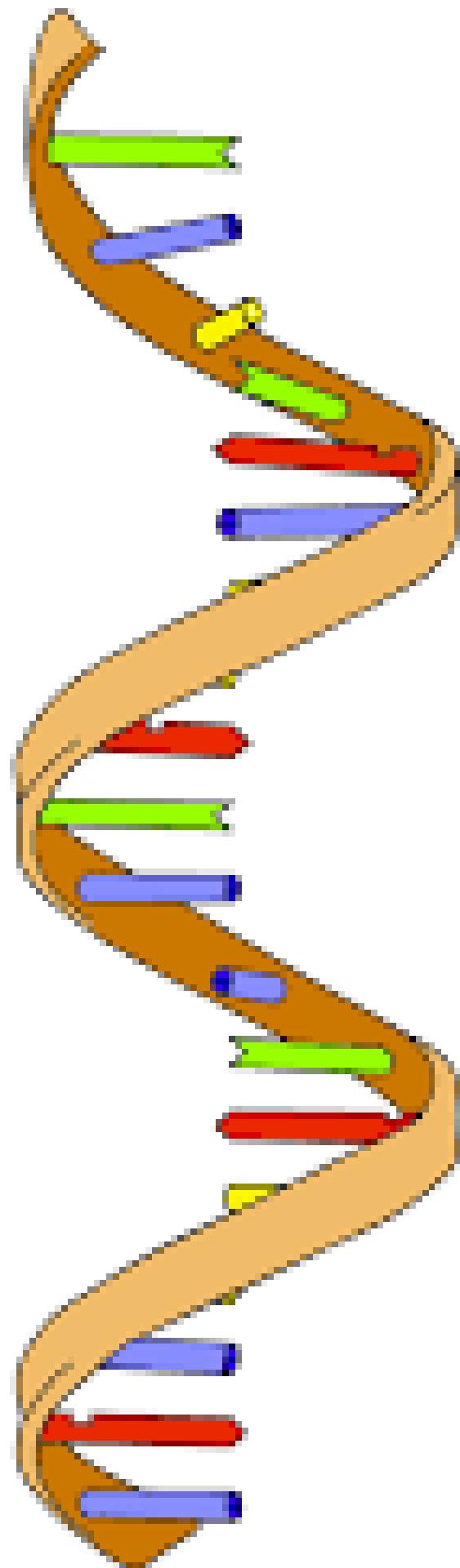
RNA PRESERVATION AND ANALYSIS STANDARDIZATION

OECL Oncology Days 2022

Serena Bonin

VALENCIA, 15TH JUNE 2022

RNA- A COMMON STRUCTURE FOR MULTIPLE FUNCTIONS



rRNA
t-RNA
m-RNA
nc-RNA (small nuclear, micro, interfering, long non coding, circular RNA)
y-RNA

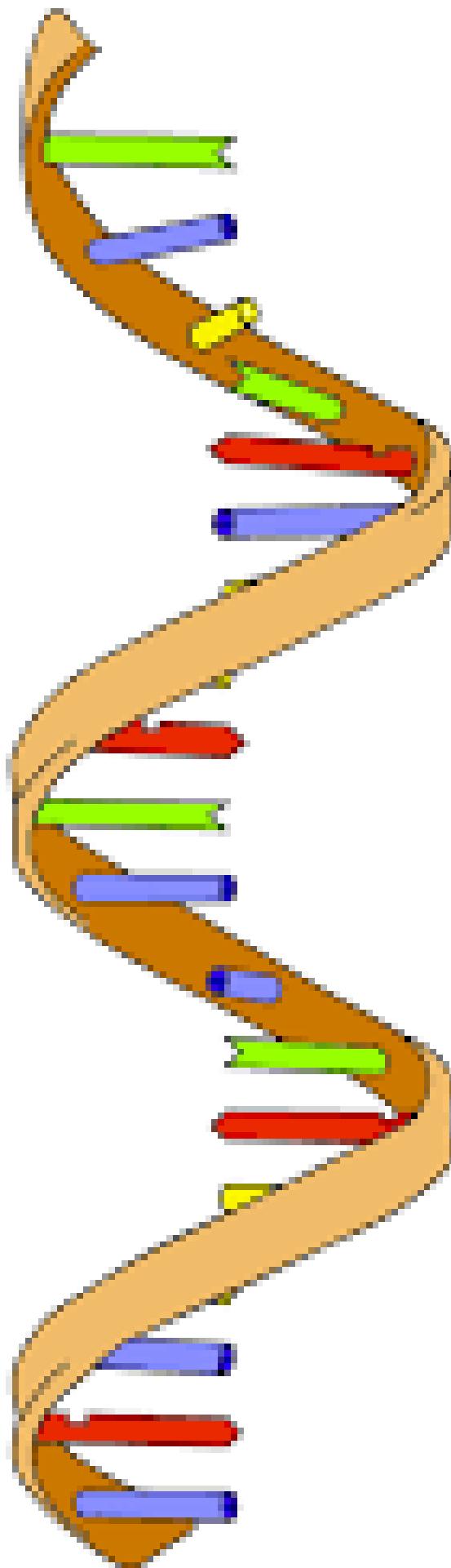
DIFFERENT FUNCTION



DIFFERENT STABILITY



RNA- A COMMON STRUCTURE FOR MULTIPLE FUNCTIONS



rRNA

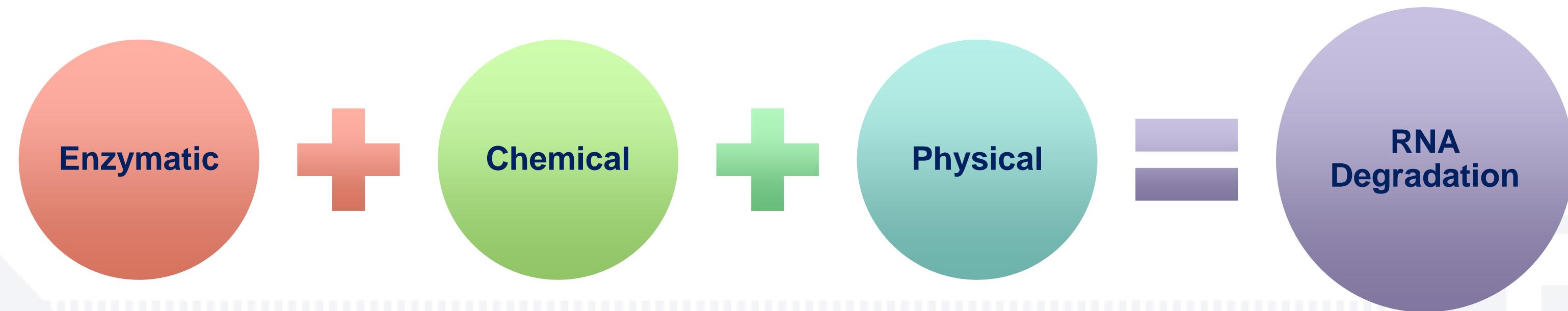
t-RNA

m-RNA

nc-RNA (small nuclear, **micro**, interfering, long non coding, circular RNA)

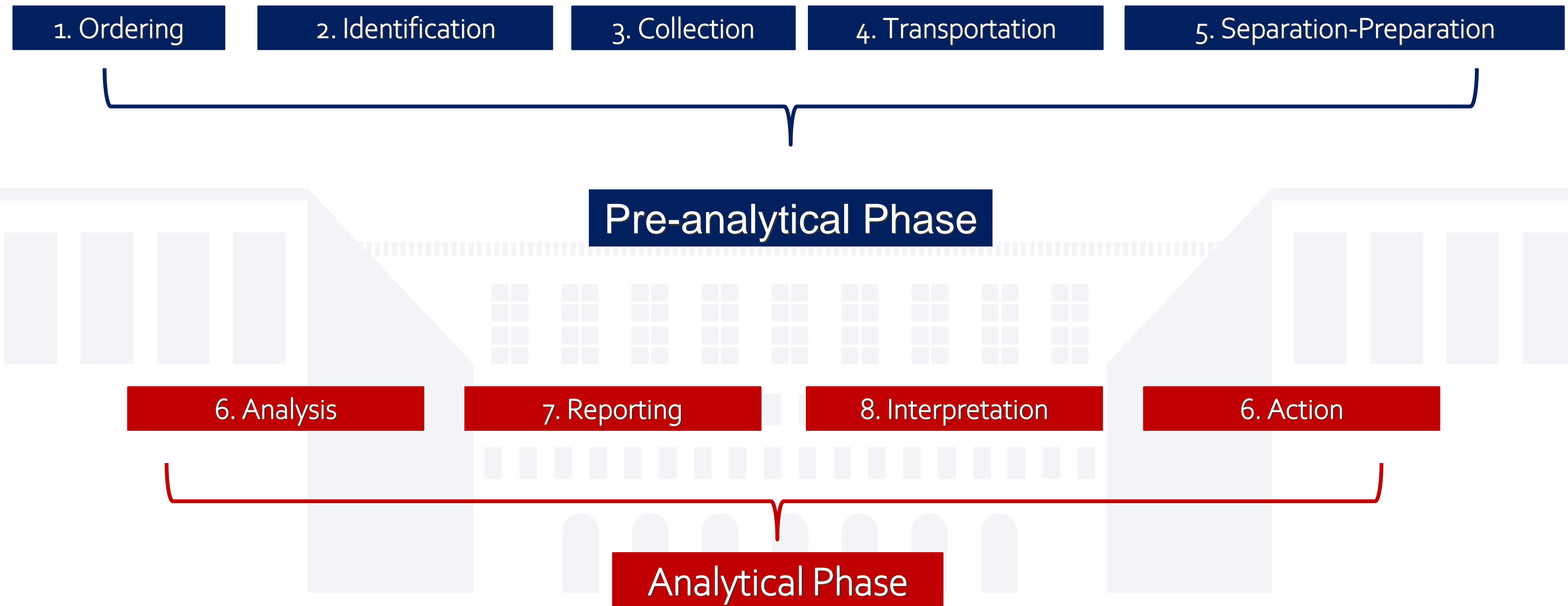
y-RNA





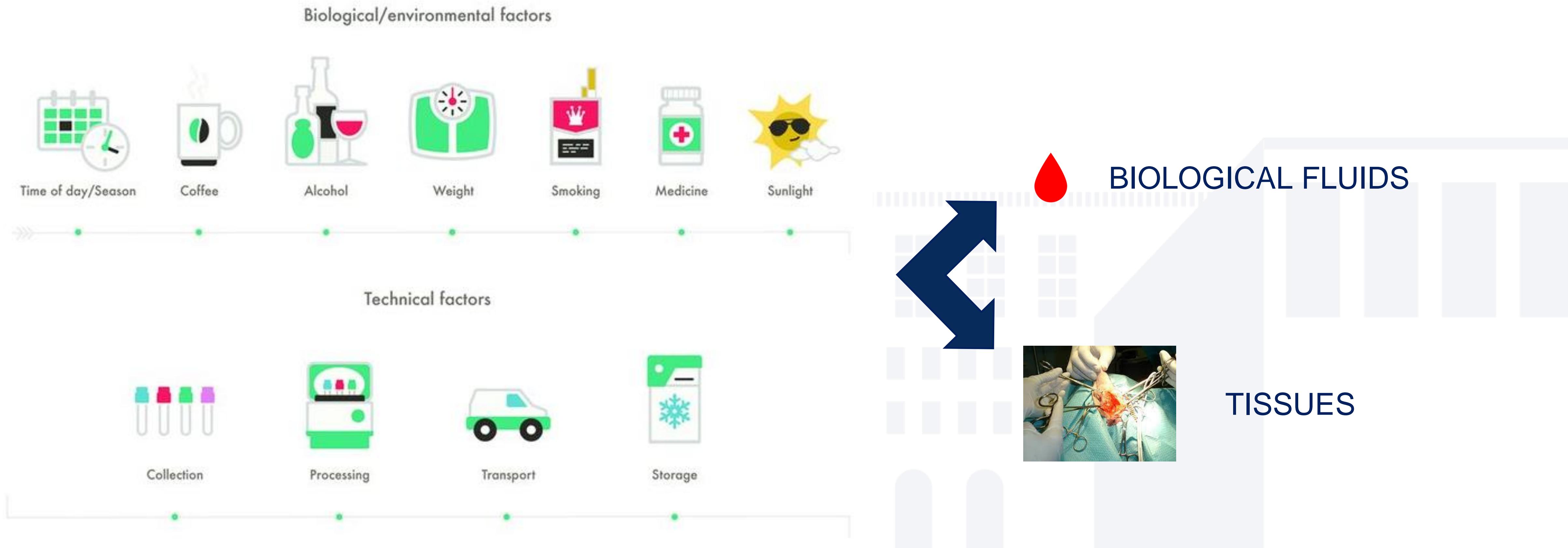
**STANDARDIZATION OF PRE-ANALYTICAL
PROCESSES AND ANALYTICAL SOPs**

TTP: TOTAL TESTING PROCESS



THE PRE-ANALYTICAL PHASE

Preanalytical phase



USE OF ISO STANDARDS AND CEN TECHNICAL DOCUMENTS

INTERNATIONAL
STANDARD

ISO
20166-1

First edition
2018-12

Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for formalin-fixed and paraffin-embedded (FFPE) tissue —

Part 1:
Isolated RNA

Analyses de diagnostic moléculaire in vitro — Spécifications relatives aux processus préanalytiques pour les tissus fixés au formol et inclus en paraffine (FFPE) —

Partie 1: ARN extrait

INTERNATIONAL
STANDARD

ISO
20184-1

First edition
2018-11

Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for frozen tissue —

Part 1:
Isolated RNA

Analyses de diagnostic moléculaire in vitro — Spécifications relatives aux processus préanalytiques pour les tissus congelés —

Partie 1: ARN extrait

INTERNATIONAL
STANDARD

ISO
20186-1

First edition
2019-02

Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood —

Part 1:
Isolated cellular RNA

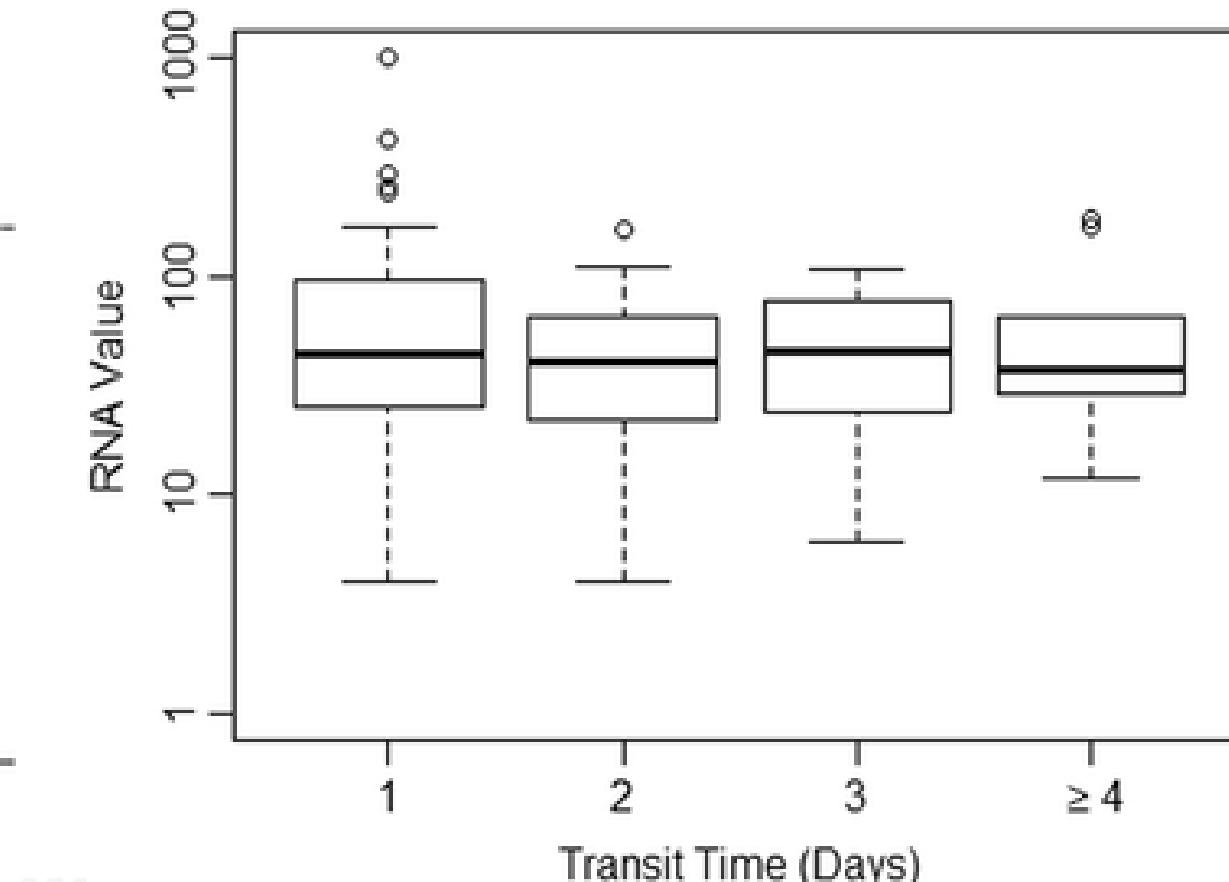
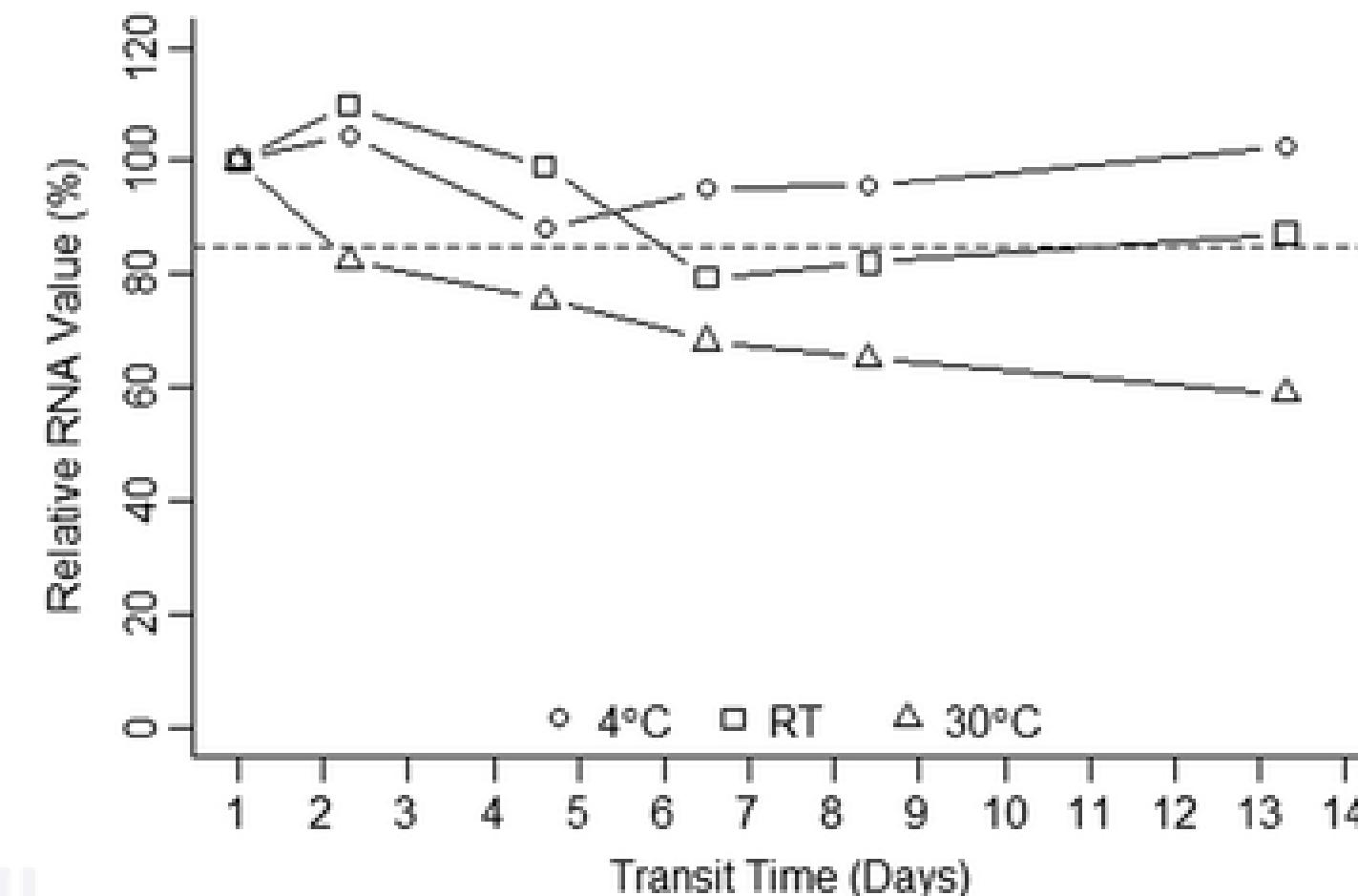
Analyses de diagnostic moléculaire in vitro — Spécifications relatives aux processus préanalytiques pour le sang total veineux —

Partie 1: ARN cellulaire extrait

CEN/TS 17688-1:2021-Molecular in vitro diagnostic examinations - Specifications for pre-examination processes for Fine Needle Aspirates (FNAs) - Part 1: Isolated cellular RNA

CEN/TS 17390-1:2020-05-Molecular in vitro diagnostic examinations - Specifications for pre-examination processes for circulating tumor cells (CTCs) in venous whole blood - Part 1: Isolated RNA

USE OF NEW TECHNOLOGIES FOR SECURING HIGH QUALITY SAMPLES

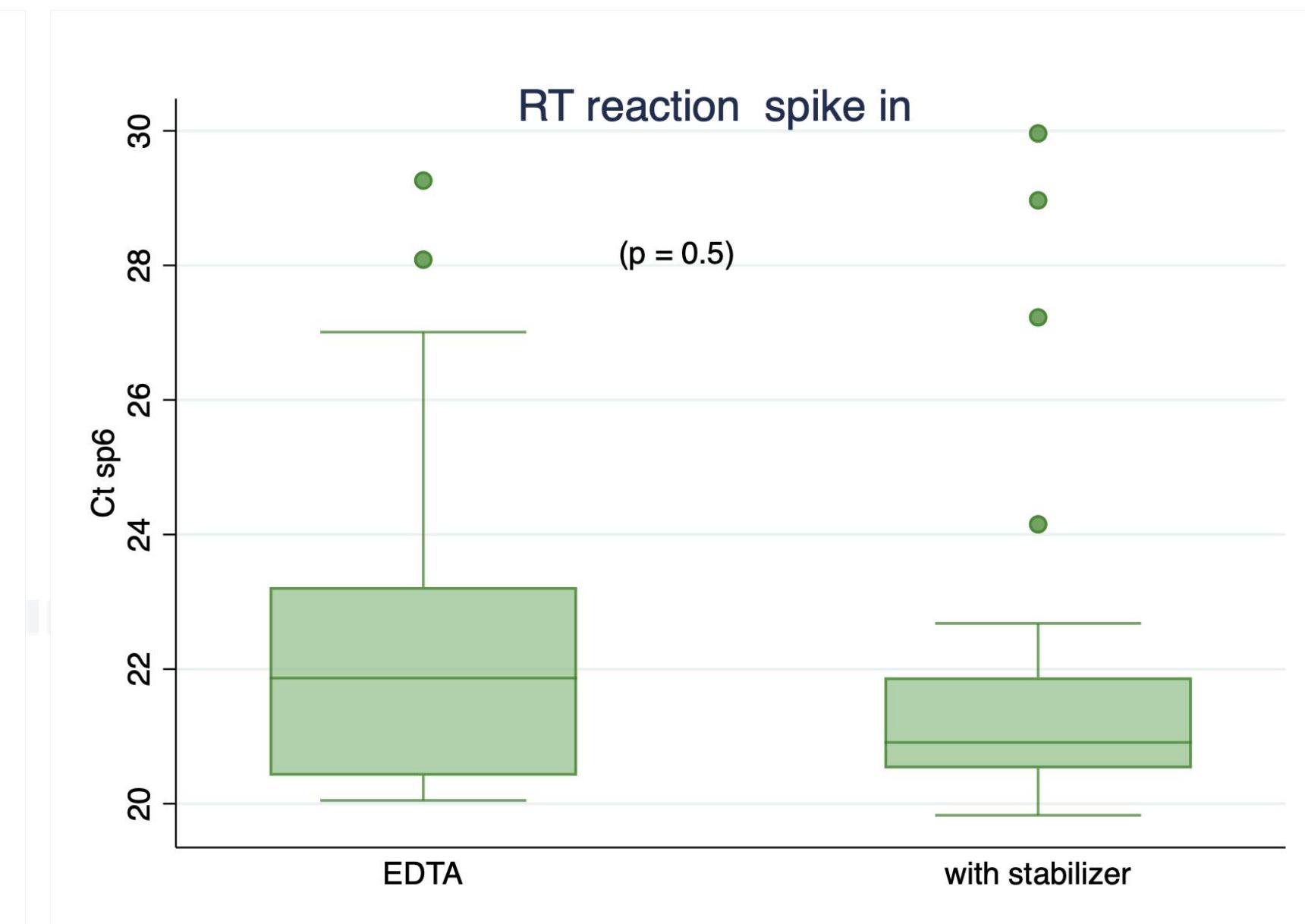
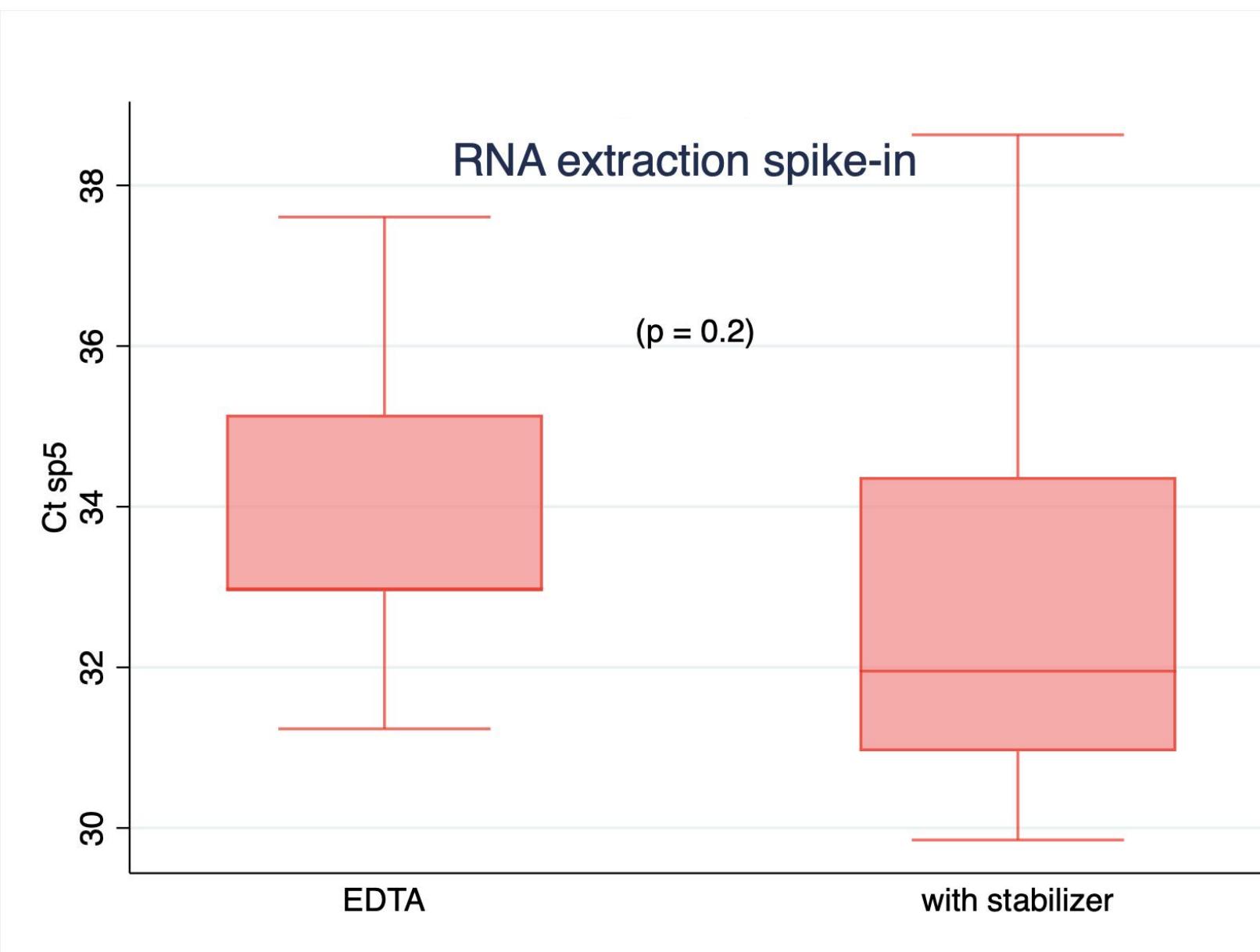
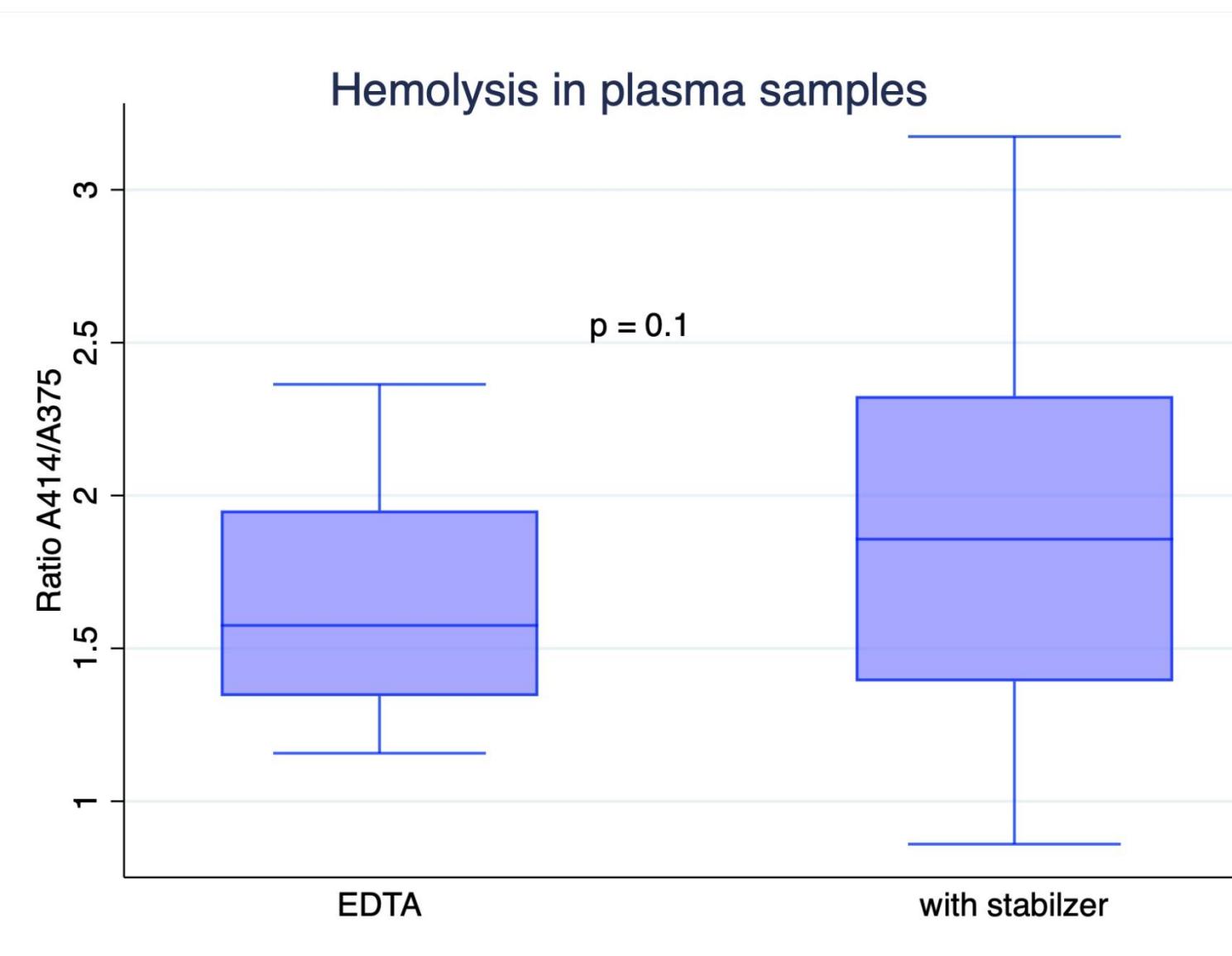


Hessels, D et al. *Transl med commun* 2, 5 (2017).

Advancement in technology allows the collection of body fluids (blood, urine...) in tube with stabilizer for:

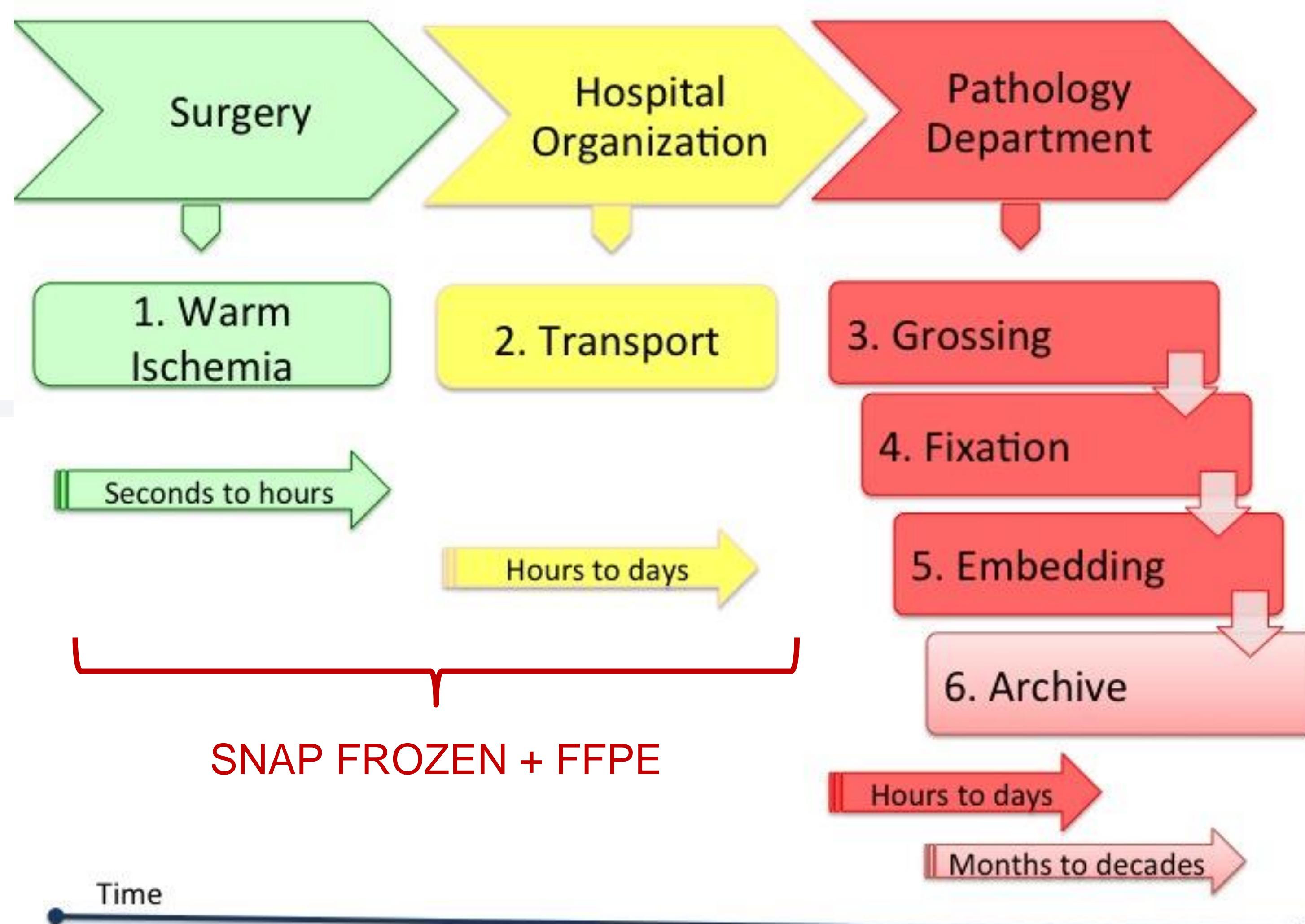
- ✓ an easier management of liquid samples for RNA analyses
- ✓ Storage at RT for longer time (days) without RNA degradation and RNA profile modifications

PRE-ANALYTICS FOR LIQUID BIOPSY



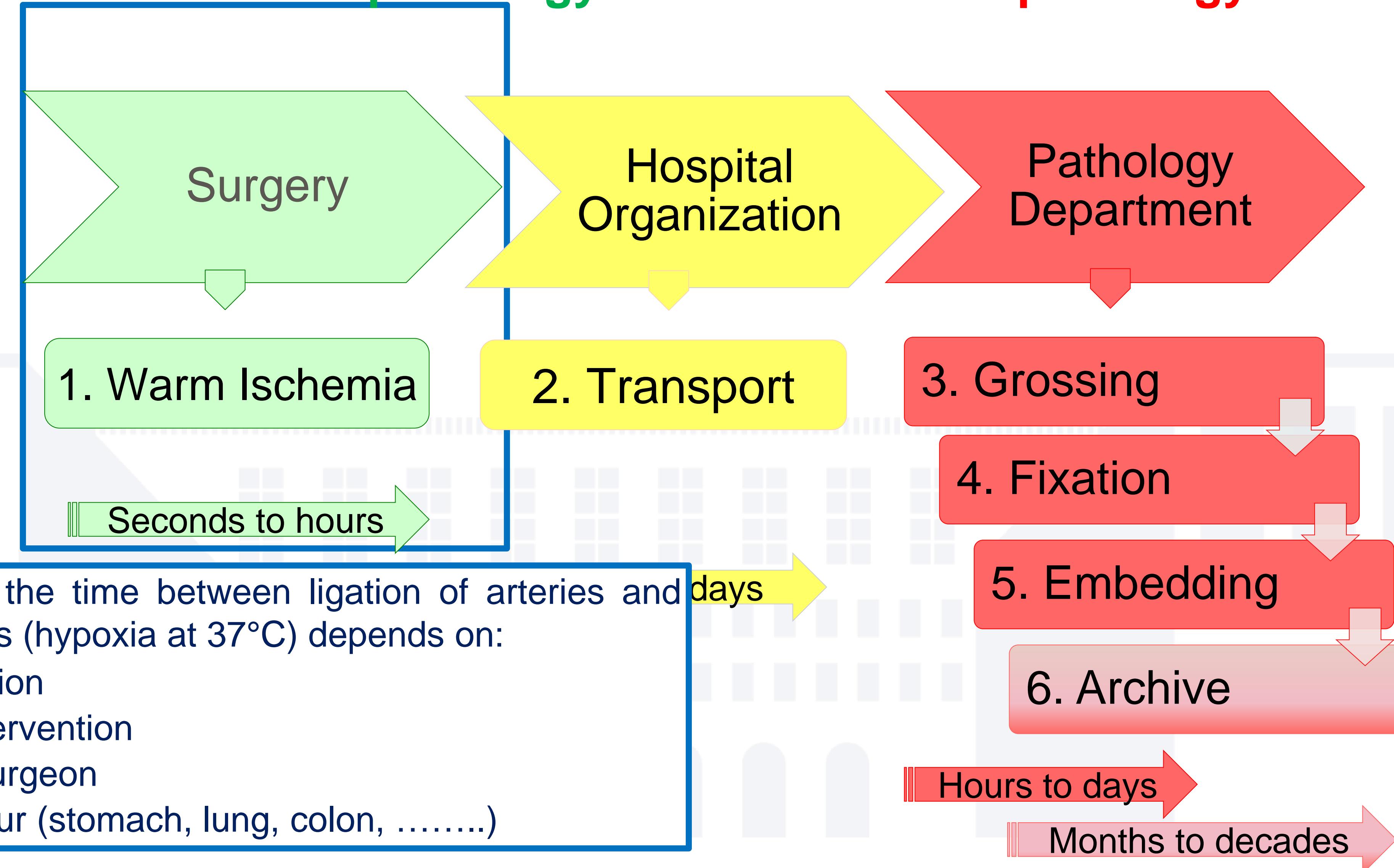
Tubes with stabilizers and EDTA tubes were used to collect blood samples for isolation of exosomes from plasma

TISSUE PREANALYTICS



Outside the pathology lab

Inside the pathology lab



Outside the pathology lab

Inside the pathology lab

Surgery

Hospital Organization

Pathology Department

1. Warm Ischemia

2. Transport

3. Grossing

4. Fixation

5. Embedding

6. Archive

Seconds to hours

Interval from the surgical table to the pathology lab

Alternatives to shorten cold ischemia:

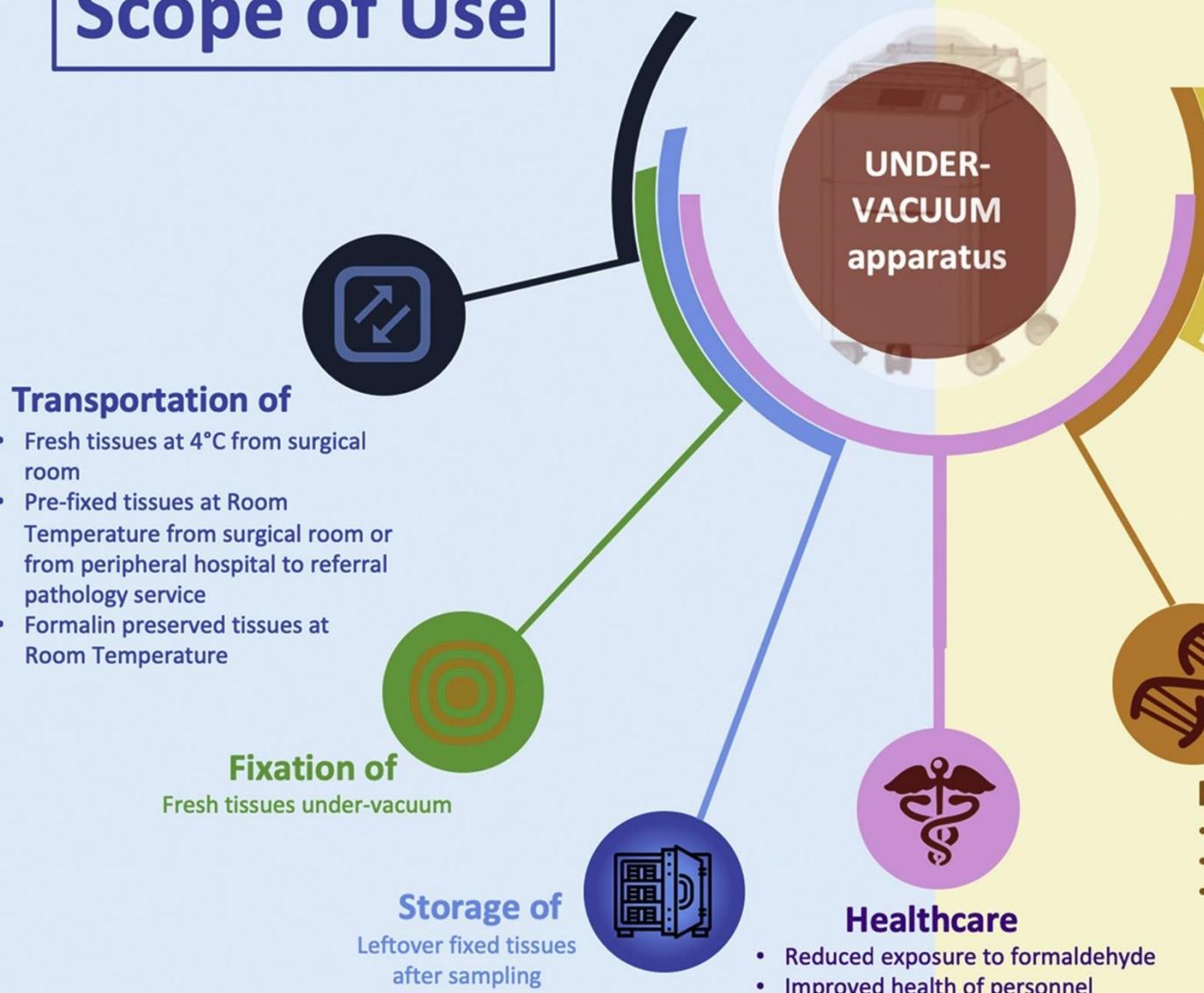
- a) Tissues left fresh transported at r.t. or in wet ice*
- b) Tissues immersed in formalin-small biopsies-annotation time
- c) Transport at 4°C under vacuum

hours to days

Months to decades

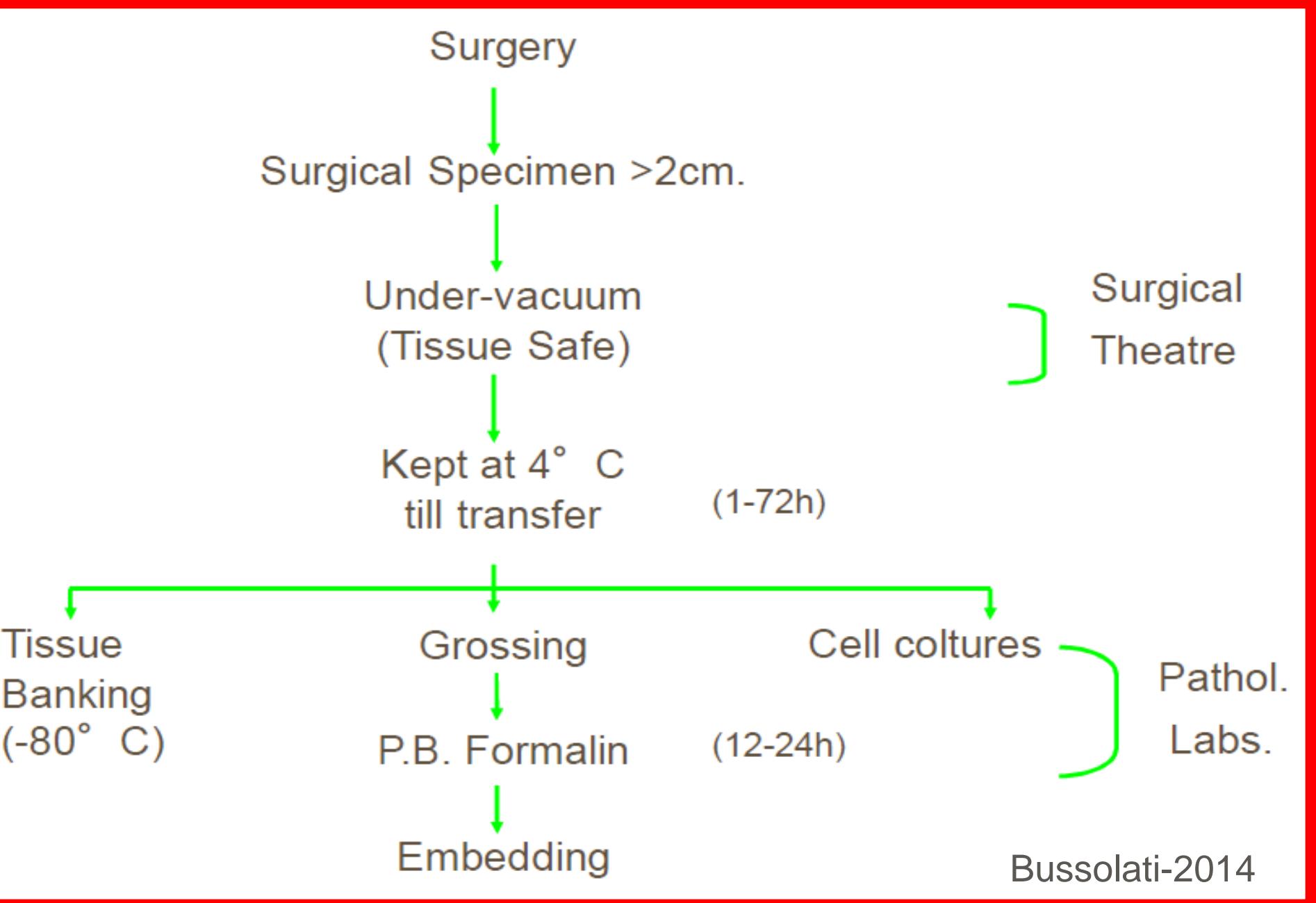
Tissue transport under vacuum

Scope of Use



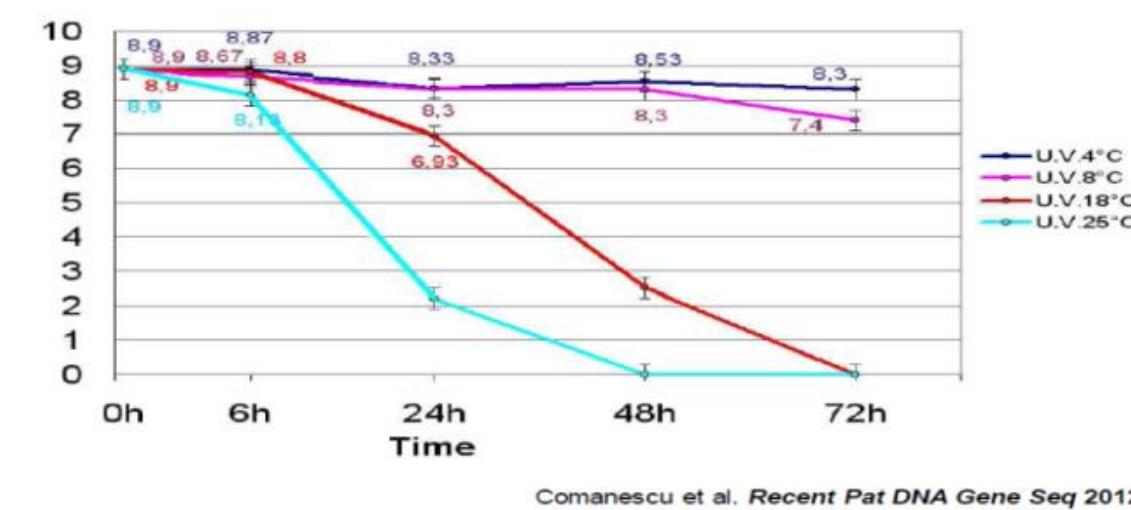
Benefits

- Monitoring of**
 - Cold ischemia time
 - Fixation time
- Assessment of**
 - Tissue morphology
 - Antigens/Proteins
 - Nucleic Acids (DNA, RNA)

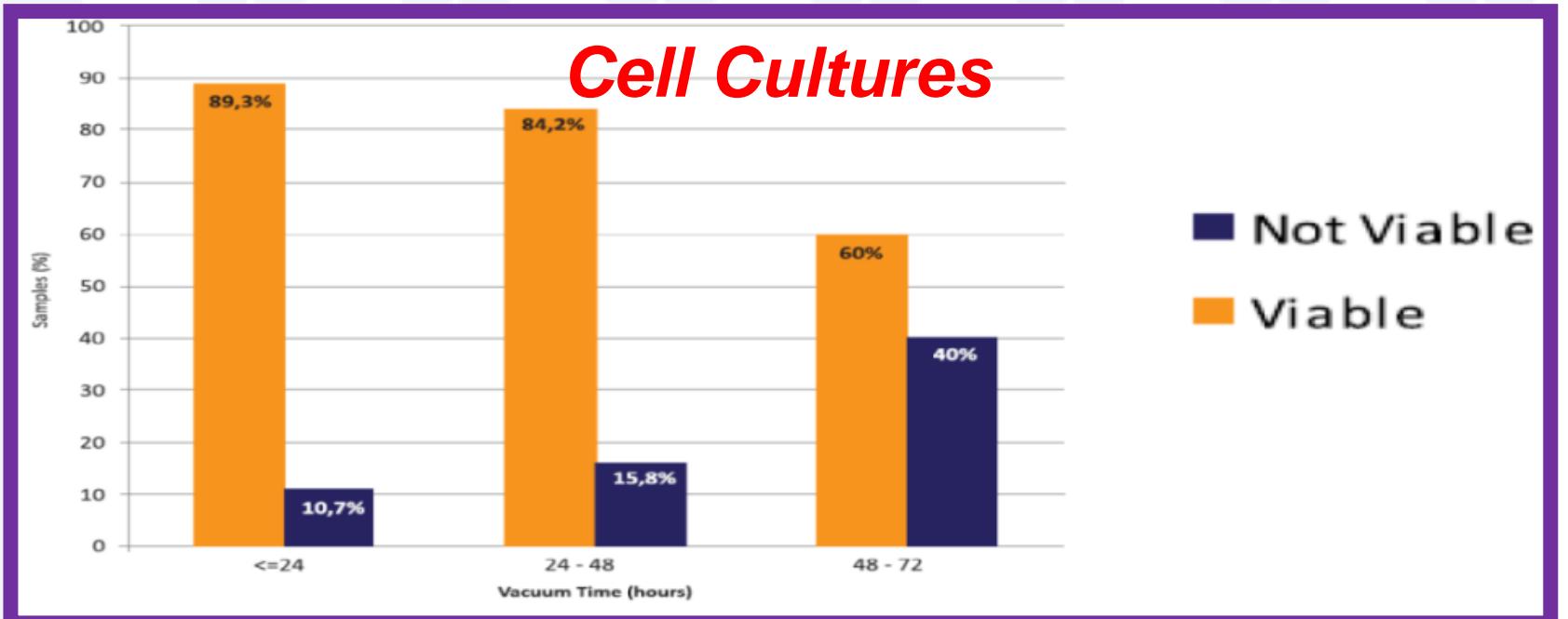


New Biotechnology 2019, 52:104-109

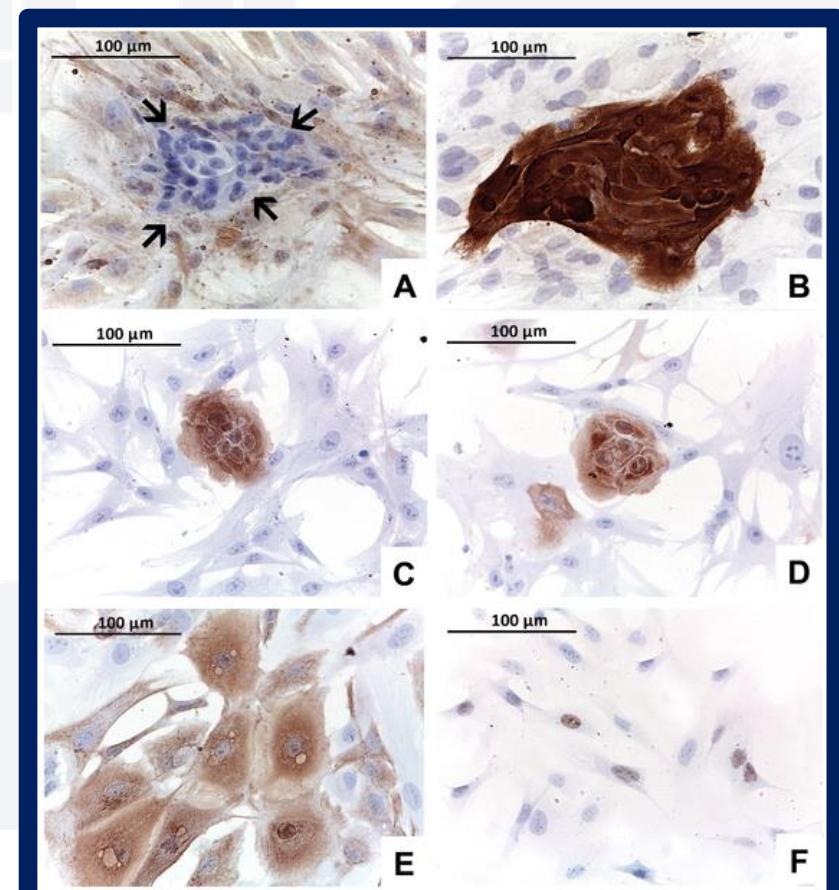
Validation test RIN Rat Liver U.V.



Cell Cultures

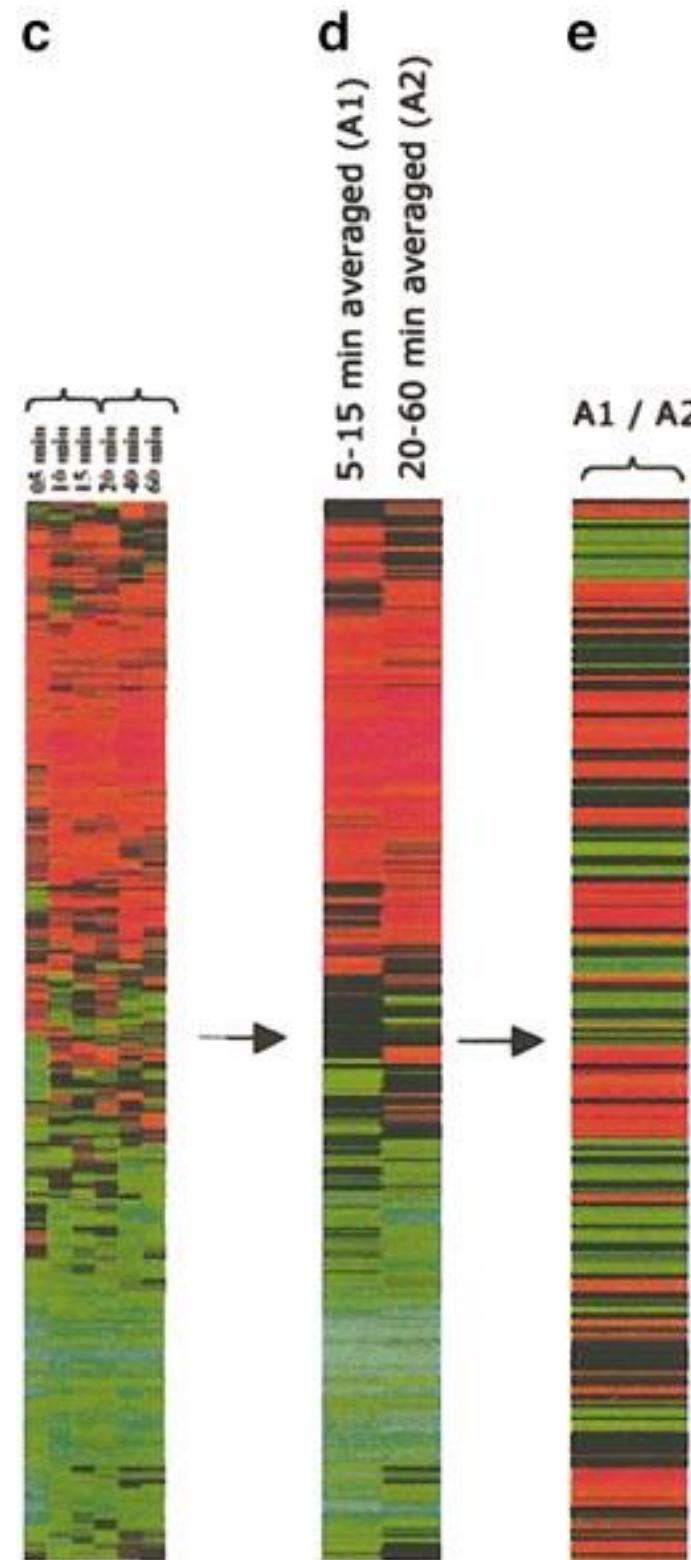


(2013) A Collection of Primary Tissue Cultures of Tumors from Vacuum Packed and Cooled Surgical Specimens: A Feasibility Study. PLOS ONE 8(9): e75193.



G. Bussolati Graz 2014

WARM AND COLD ISCHEMIA



Inducible genes: warm ischemia and cold ischemia can differently influence genes with increased or decreased expression, with changes in mRNA expression but also at the protein level.

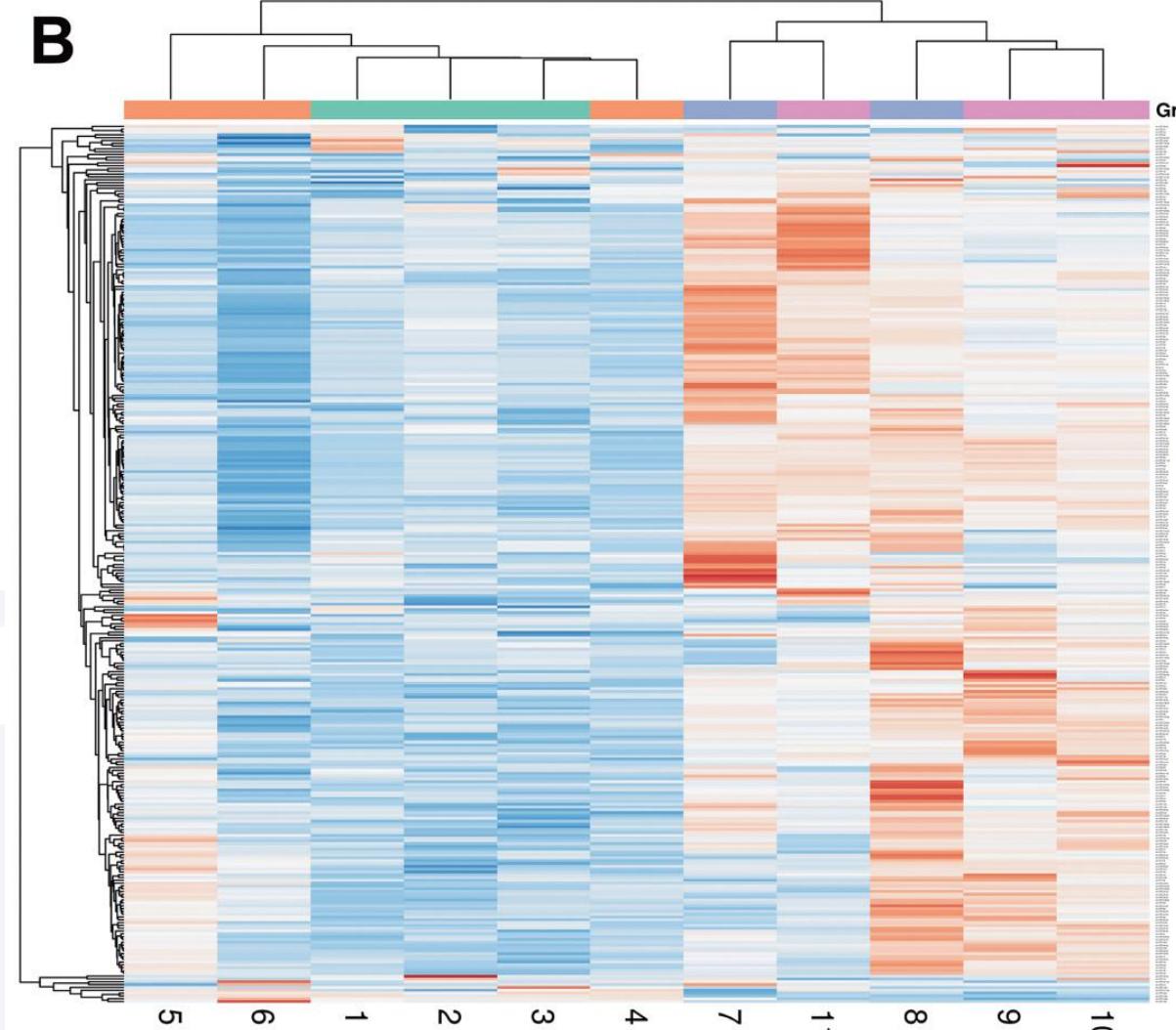
On the other hand, many genes can be totally indifferent to ischemia.

- Outside the laboratory:
Annotation of warm ischemia time: date and time of tissue removal and method of removal
Cold ischemia- transport in wet ice or Under Vacuum-fresh frozen
 - Preferable formalin-free for FFPE

Journal of Surgical Research 99, 222–227 (2001)

Importance of freezing tissue within 20 min.

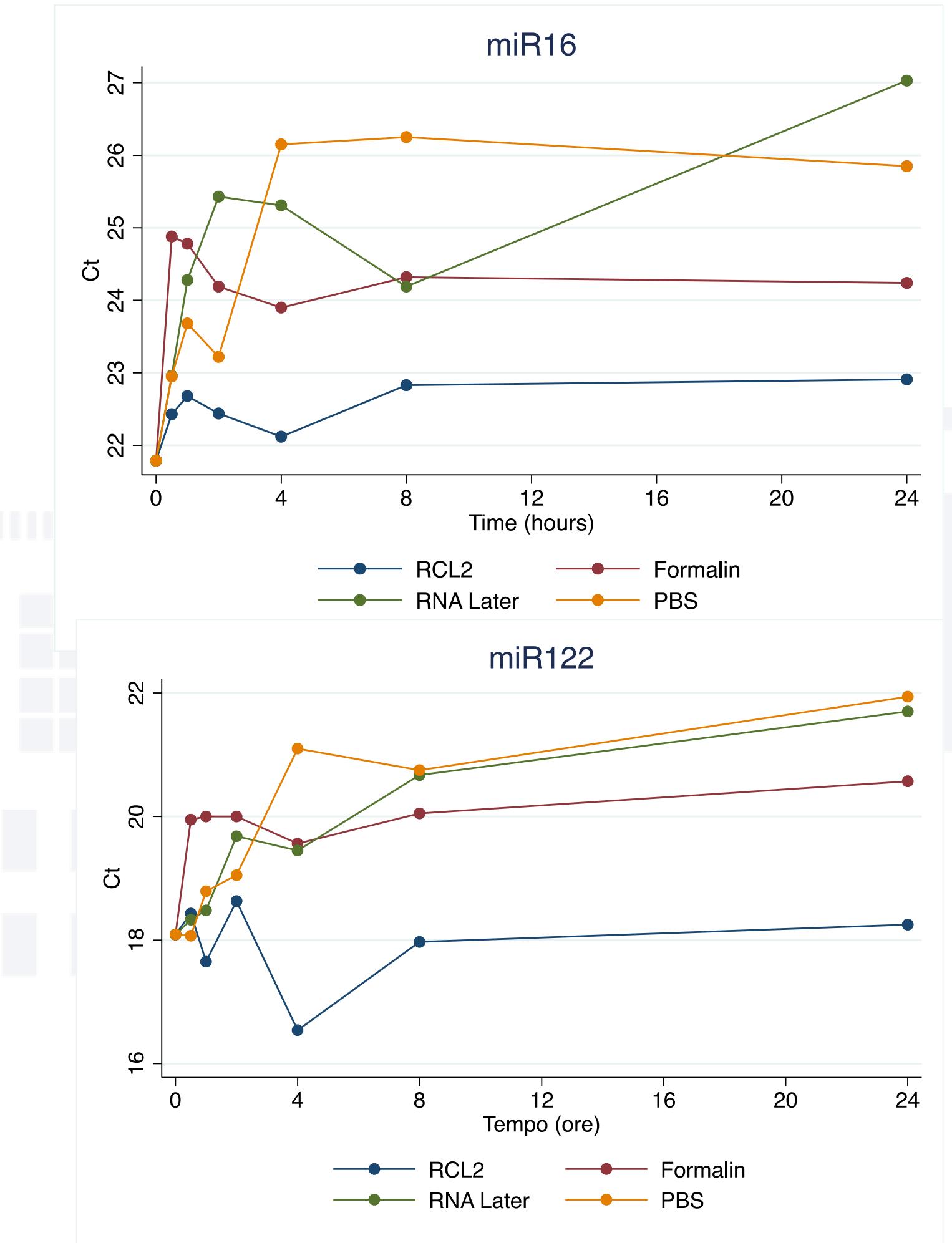
WARM AND COLD ISCHEMIA



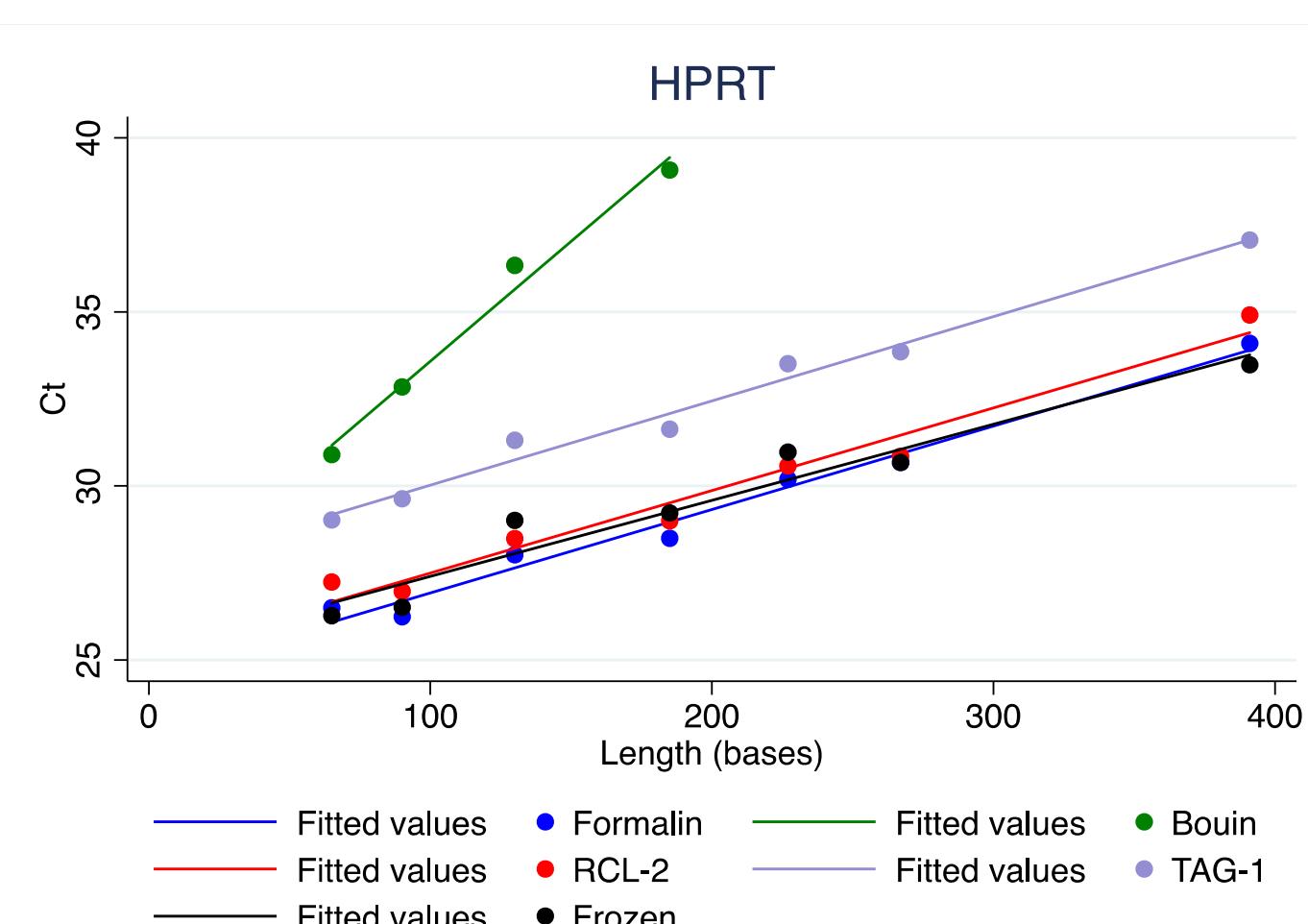
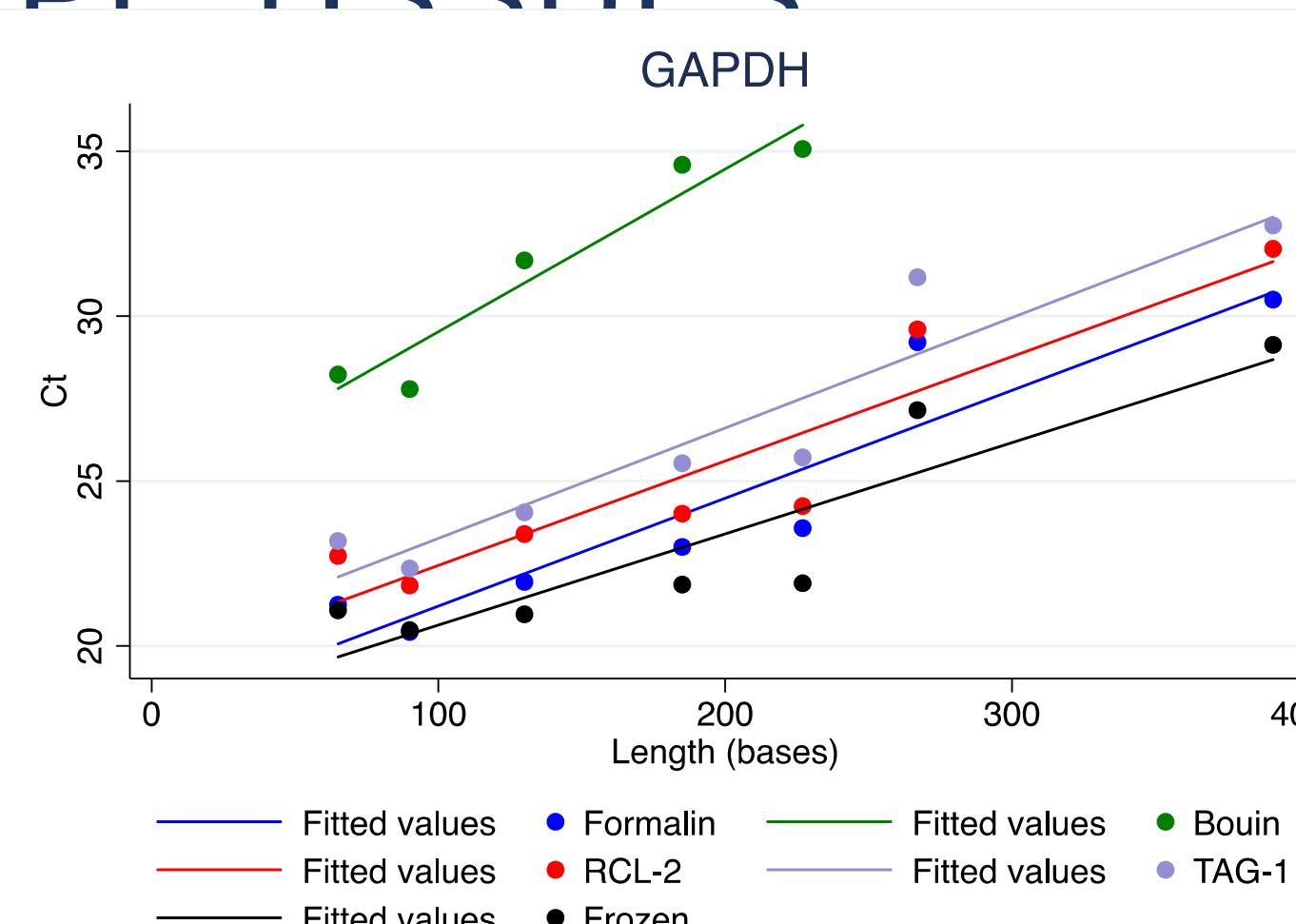
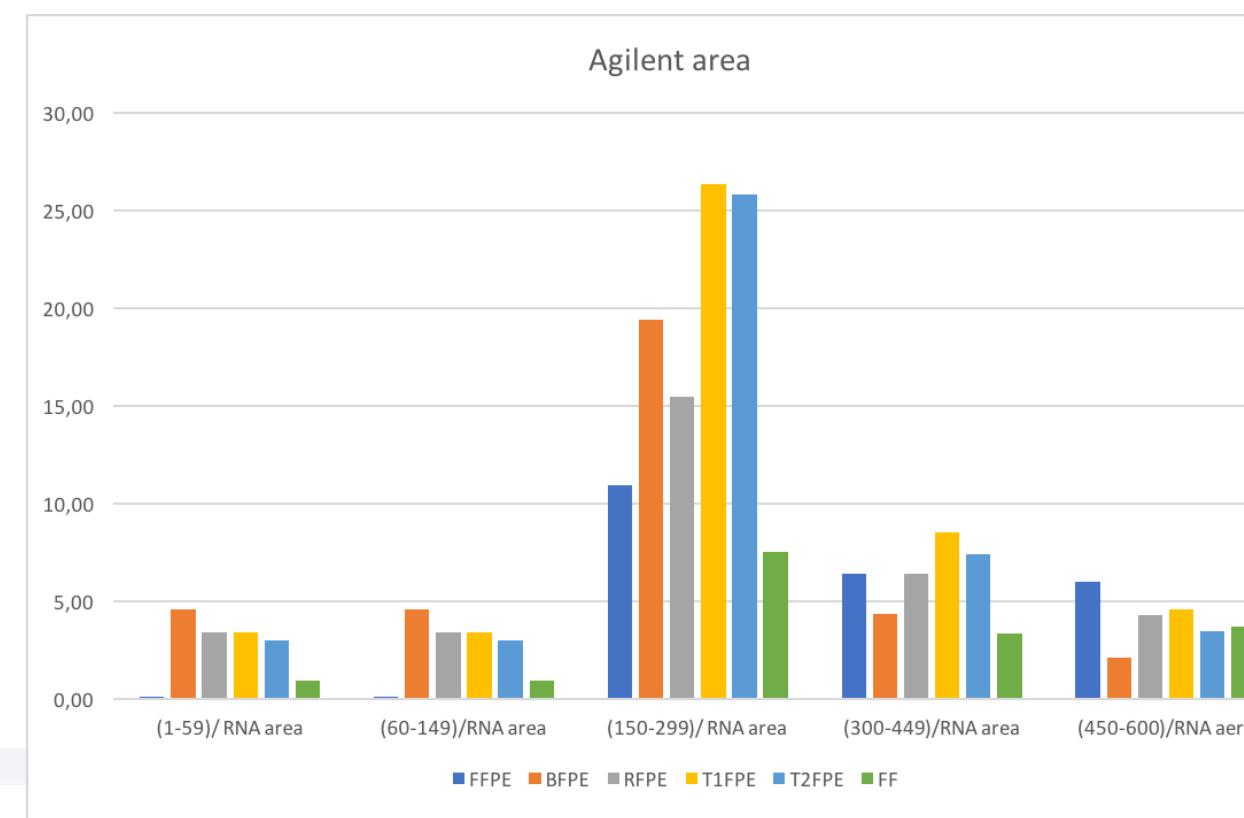
Diagnostics 2020, 10, 240;
doi:10.3390/diagnostics10040240

miRNA profiles vary during warm and cold blood STH2 cardioplegia during prolonged on-pump and off-pump

miRNA can alter their profile during cold ischemia time



STRINGENT STANDARD PROCEDURES GUARANTEE HIGHER RNA QUALITY IN FFPE TISSUES



Inside the laboratory

Grossing

Freezing

Fixation time

Validated extraction procedures

RNA BASED IN VITRO DIAGNOSTICS

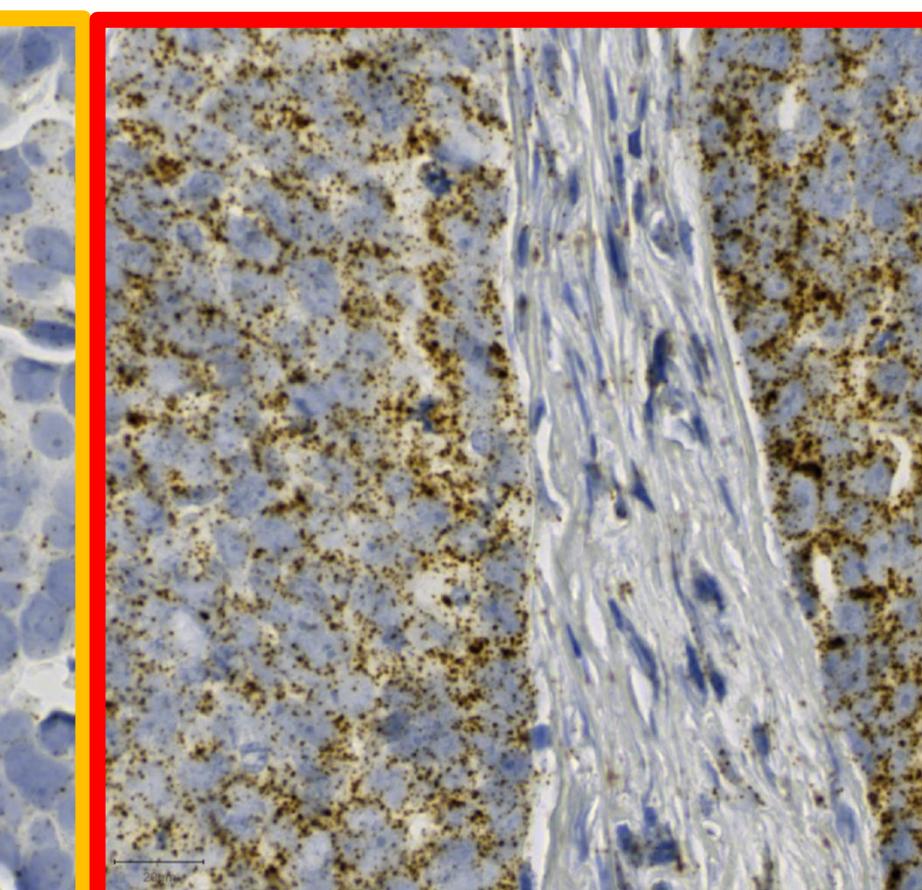
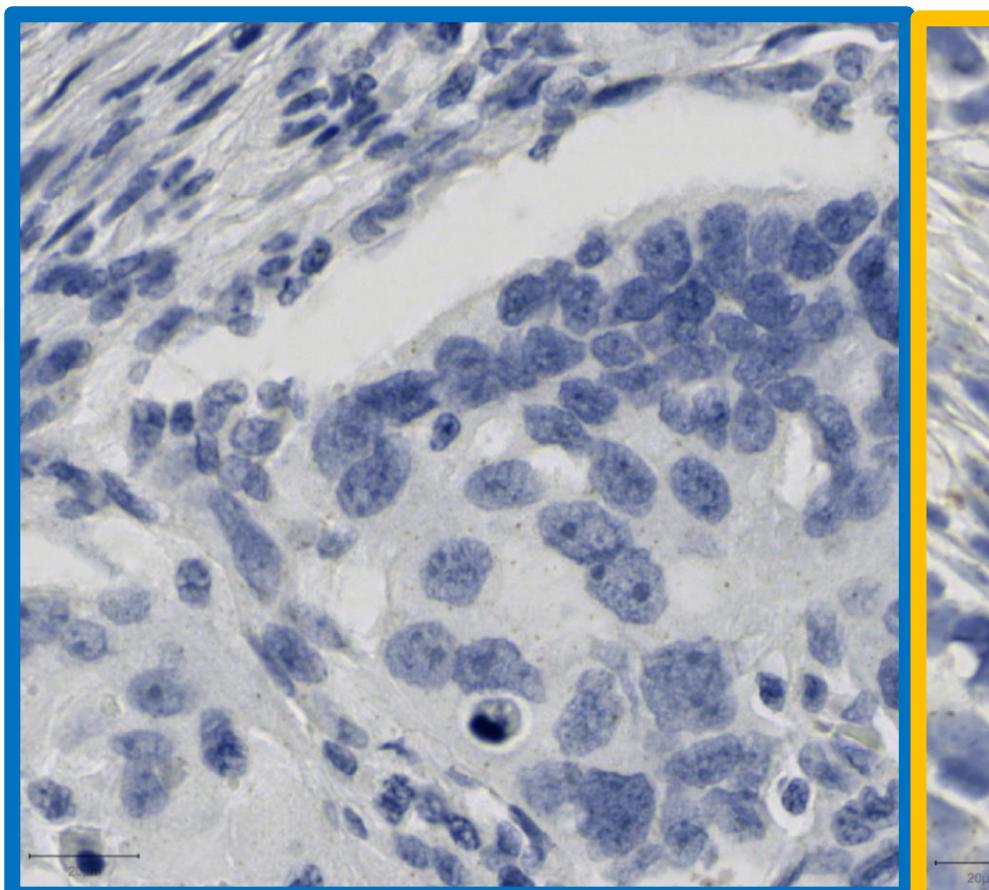
wet or in situ hybridization

RT-qPCR based

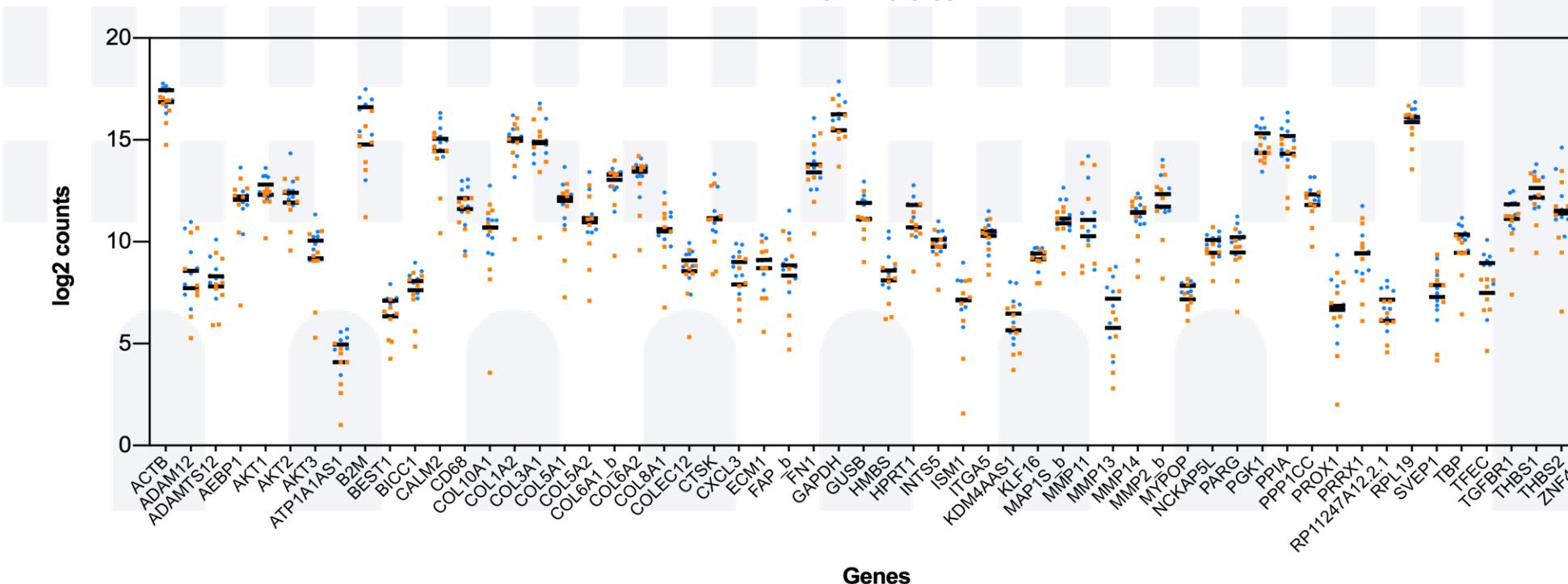
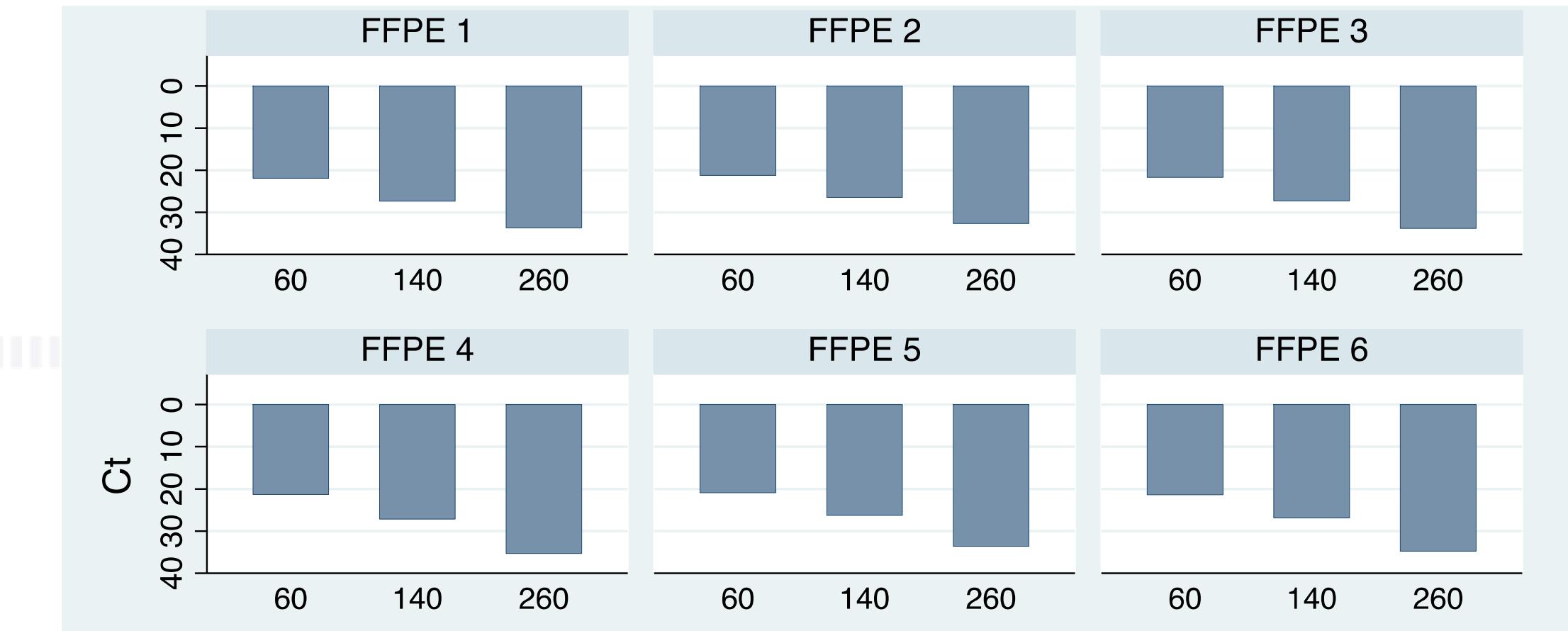
RNA seq



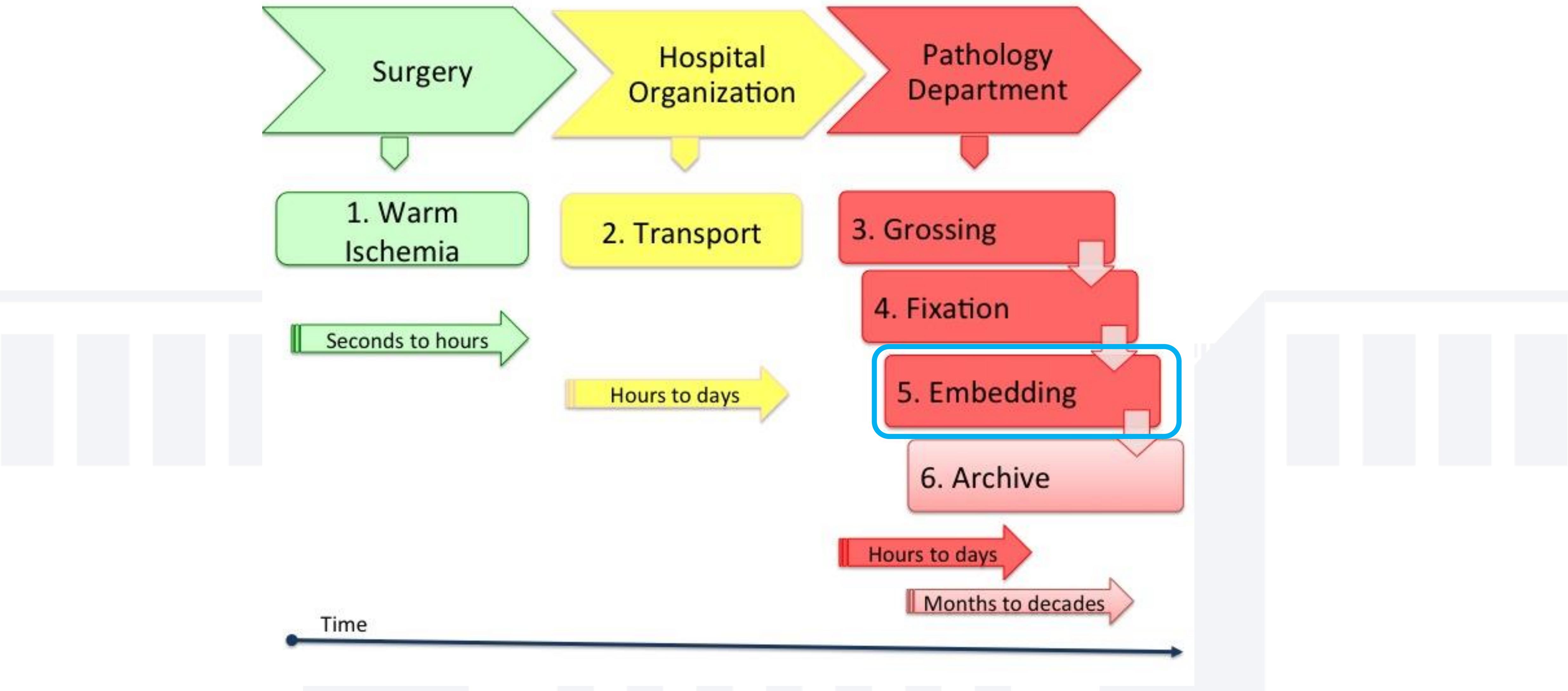
RNA QUALITY CONTROL!!!!



Raw data

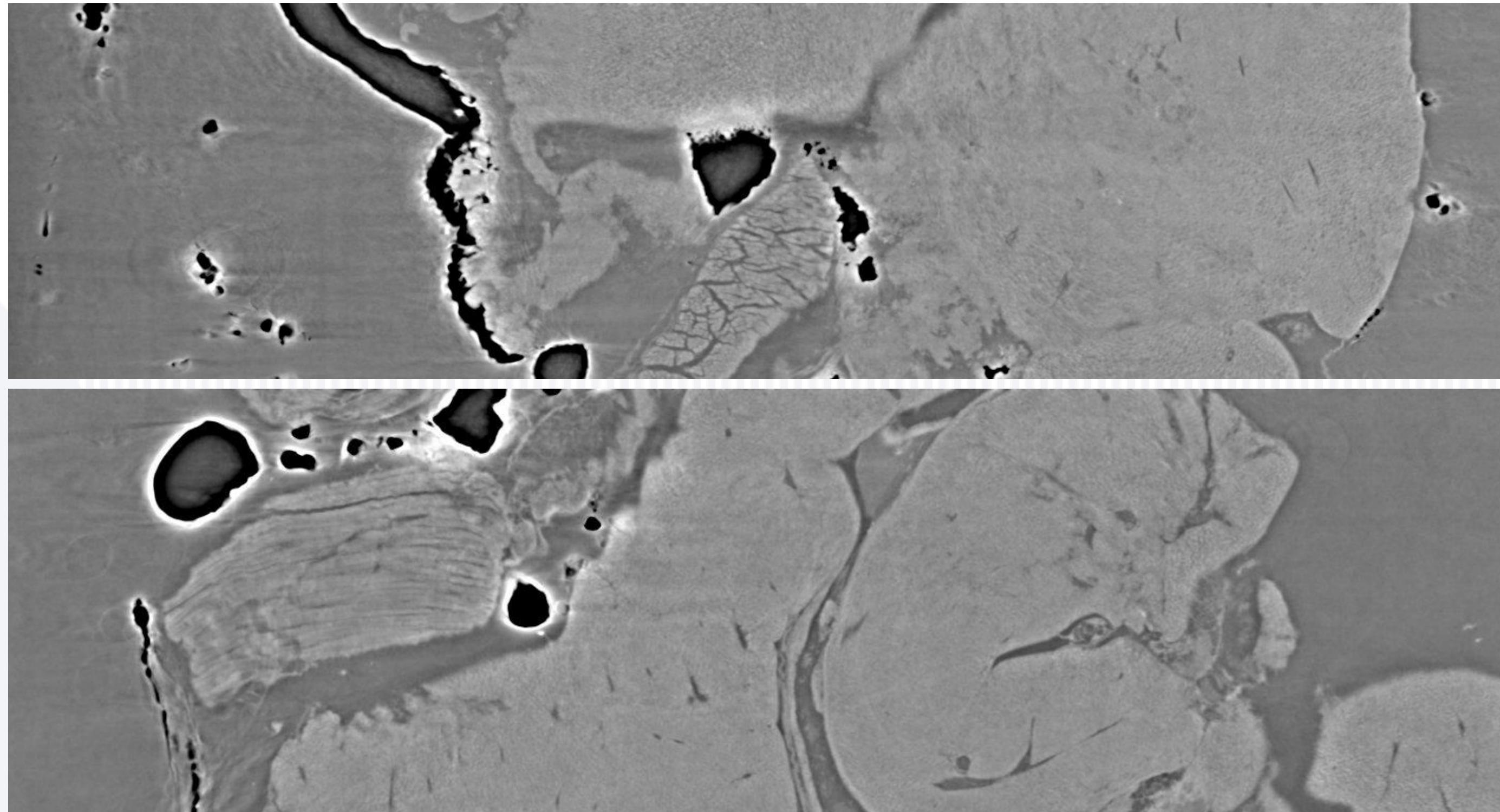


TISSUE PREANALYTICS

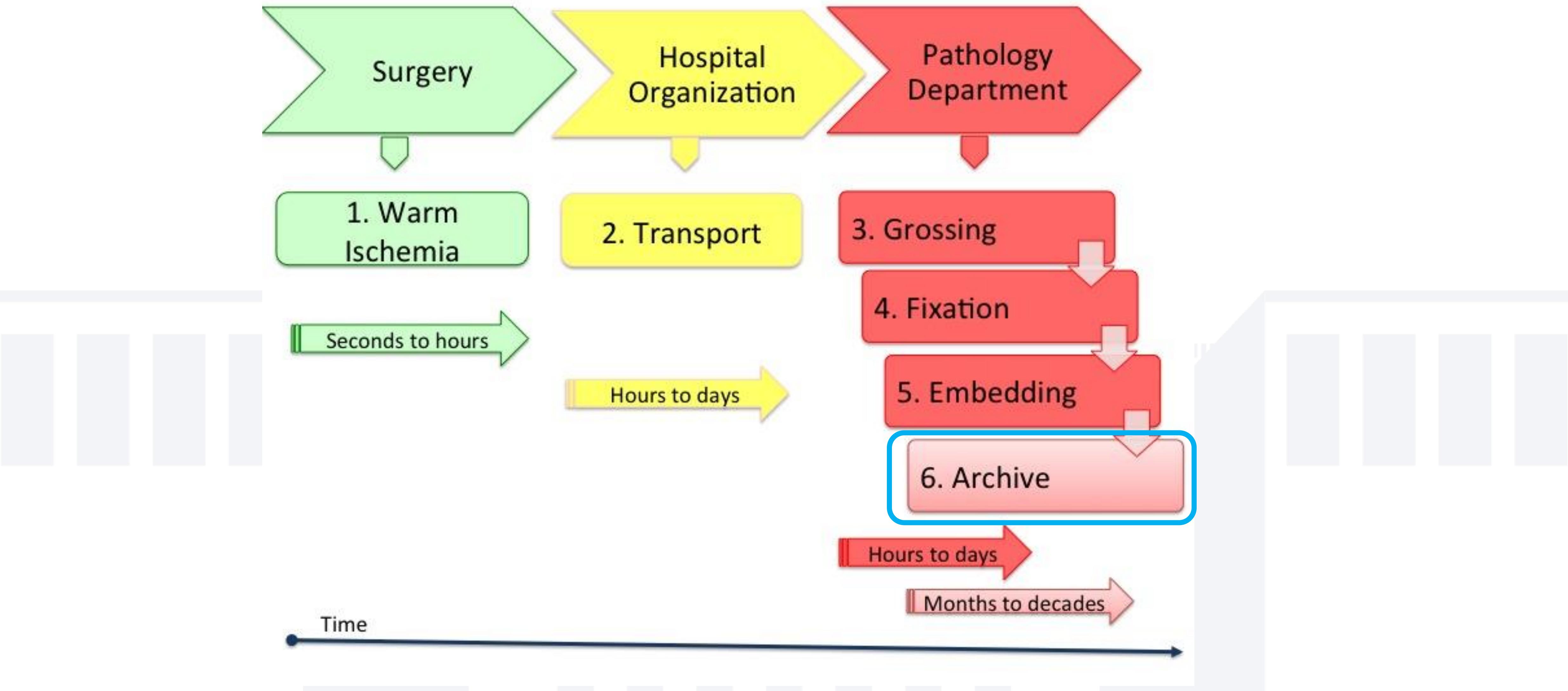


PARAFFIN EMBEDDING

In addition to the effect of temperature embedding...NON HOMOGENEITY IN THE PARAFFIN INCLUSIONS



TISSUE PREANALYTICS

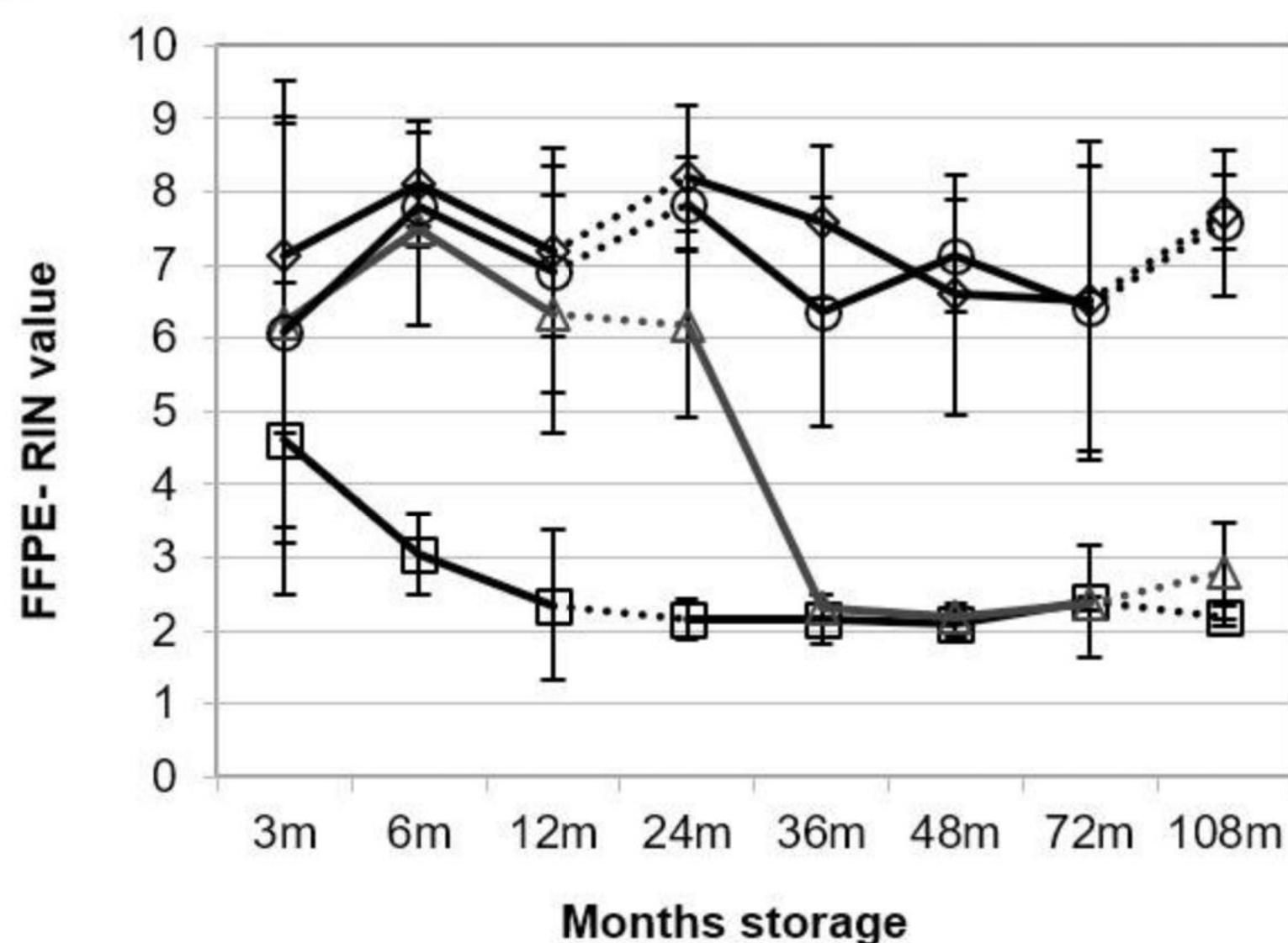


ARCHIVE STORAGE

While histology is not affected by storage, RNA degradation may increase with increasing storage time, especially for long time (Wolff, C.. (2011) PloS One Journal, 6, e16353)

Storage conditions, such as humidity and temperature, can have an impact on protein and RNA amounts and quality (Thompson, S (2013). Proteomics - Clinical Applications, 7, 241–51)

A



- FFPE 22°C
- FFPE 4°C
- FFPE -20°C
- FFPE -80°C

Biomolecule Gene Fixative Ct L 2020 Ct L 2021 Ct M 2020 Ct M 2021 Ct S 2020 Ct S 2021

Mus-musculus mt-CO1
mRNA
Mus-musculus GAPDH
Mus-musculus HPRT

Biomolecule	Gene	Fixative	Ct L 2020	Ct L 2021	Ct M 2020	Ct M 2021	Ct S 2020	Ct S 2021
	<i>Mus-musculus mt-CO1</i>	FFPE	25.4±0.6	26.6±0.6	17.6±0.6	18.3±0.4	17.6±0.4	16.2±0.3
		RFPE	23.4±1.0	24.6±0.3	17.5±1.0	17.2±0.2	17.9±0.9	16.0±0.08
		FF	24.8±1.2		17.7±0.7		17.3±0.5	
		FFPE	29.4±0.4	32.6±0.6	22.8±0.3	24.2±0.3	20.3±0.3	24.1±0.2
mRNA	<i>Mus-musculus GAPDH</i>	RFPE	29.8±0.9	31.6±0.6	24.3±0.8	23.6±0.3	22.0±0.6	23.9±0.1
		FF	27.0±0.5		22.1±0.5		20.5±0.4	
		FFPE	33.7±0.7	41.1±0.8	30.1±0.6	34.6±0.4	26.3±0.3	27.3±0.3
	<i>Mus-musculus HPRT</i>	RFPE	35.0±1.3	41.3±0.7	31.3±1.3	34.6±0.3	27.6±0.8	27.4±0.2
		FF	33.4±1.8		33.6±1.3		26.5±0.4	

[Groelz D et al PLoS One. 2018; 13\(9\): e0203608](#)

DEDICATED REVERSE TRANSCRIPTASE REACTION

depends on the analyte (miRNA or mRNA)

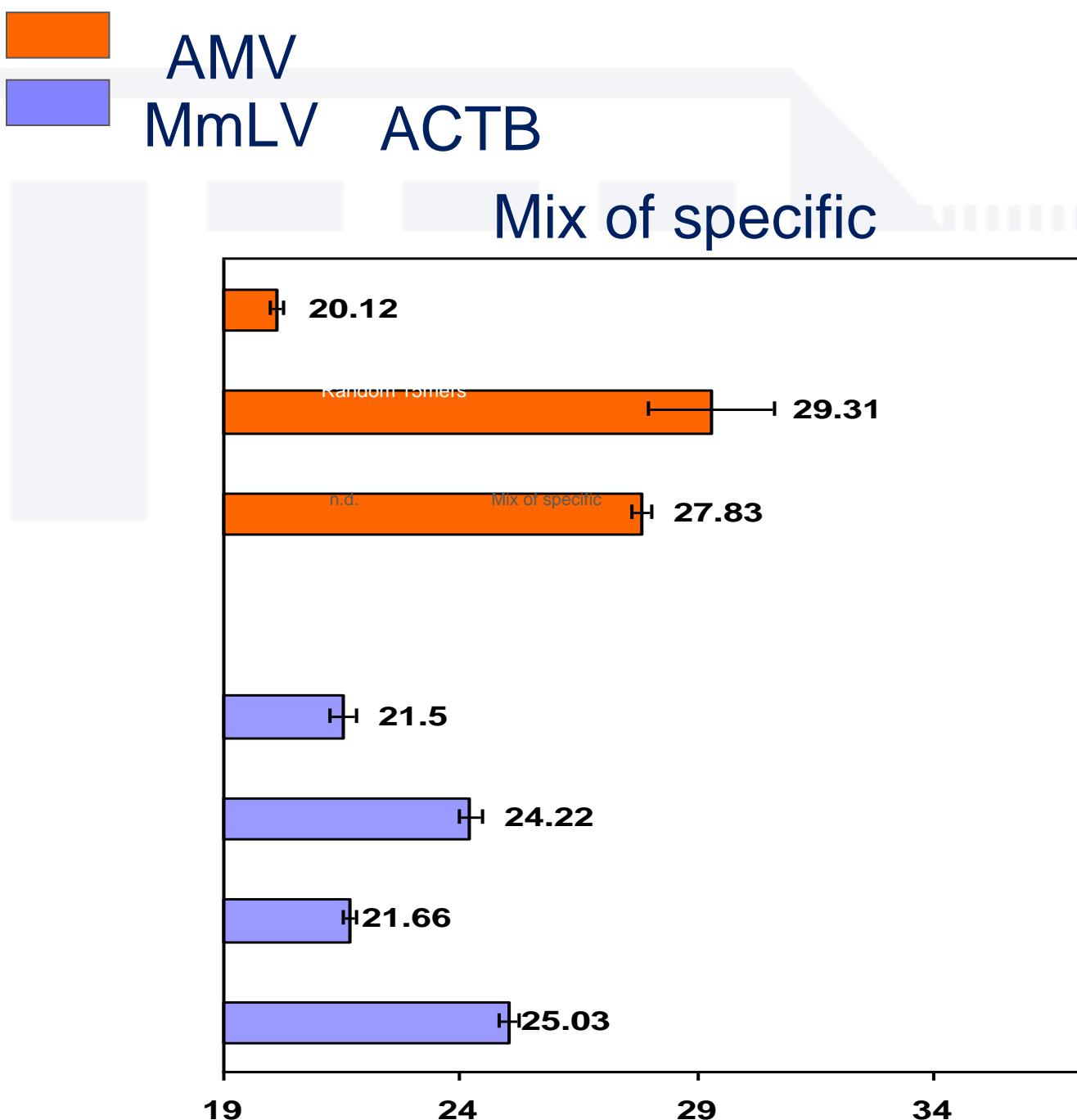
the abundance of the analyte (Montecarlo Effect)

the type of sample (FFPE, fresh frozen, liquid biopsy)



RT REACTION HAS NOT 100% YIELD!!

different priming strategies and RT enzyme



Method	cDNA dilution in PCR mix							E%
	1:10	1:40	1:160	1:640	1:2560	1:10240		
6-MERS M-MLV	TS	30.79 (0.01)	32.65 (0.04)	34.79 (0.33)	37.89 (0.42)	-	-	84
	TP	27.56 (0.04)	29.22 (0.04)	31.70 (0.05)	33.63 (0.15)	35.24 (0.35)	37.67 (0.40)	98
	DPD	28.97 (0.29)	30.82 (0.12)	32.71 (0.27)	34.99 (0.43)	-	-	100
	ACTB	20.43 (0.25)	21.30 (0.16)	23.20 (0.06)	25.43 (0.14)	27.76 (0.18)	30.09 (0.22)	100
15-MERS M-MLV	TS	29.37 (0.21)	30.61 (0.07)	33.34 (0.01)	35.98 (0.23)	-	-	85
	TP	28.30 (0.16)	29.54 (0.05)	31.64 (0.08)	33.38 (0.40)	35.96 (0.45)	38.02 (0.49)	100
	DPD	29.24 (0.14)	30.31 (0.14)	32.49 (0.31)	35.34 (0.48)	-	-	97
	ACTB	20.60 (0.13)	21.85 (0.17)	23.74 (0.21)	25.59 (0.11)	27.76 (0.19)	30.36 (0.20)	100
Mix of specific AMV	TS	-	29.88 (0.48)	31.66 (0.04)	34.17 (0.43)	35.80 (0.45)	38.60 (0.47)	91
	TP	-	26.76 (0.05)	28.86 (0.08)	31.01 (0.30)	33.45 (0.40)	36.26 (0.40)	80
	DPD	-	27.96 (0.08)	30.54 (0.18)	32.65 (0.28)	35.28 (0.35)	36.48 (0.48)	86
	ACTB	18.44 (0.04)	19.95 (0.14)	21.84 (0.05)	24.22 (0.23)	26.42 (0.02)	28.98 (0.11)	92

CONCLUSIONS

- # RNA preanalytical work flows depends both on the measurand and sample type
- # ***For liquid samples it is highly suggested the use of dedicated tubes with stabilizers, facilitating nurses and sample transport***
- # ***For solid tissue it is recommended a formalin free transport at low temperature (to minimize RNase activity)***
- # ***RNA isolation procedures should be matched and optimized with the analytical test***
- # ***Any deviation from the "conventional procedures" shall be reported, verified and validated***
- # ***RNA QUALITY CONTROL IS MANDATORY BEFORE ANALYTICAL TESTS***
- # ***Any RNA based test from FFPE tissues should rely on the detection of short fragments***
- # ***Reverse transcription reaction should be selected in agreement with specific RNA abundance***
- # ***FOLLOW STRICTLY ISO STANDARD AND CEN TECHNICAL DOCUMENTS FOR RNA IN VITRO DIAGNOSTICS***

Thank you for your attention!!

Ermanno Nardon
Eleonora De Martino
Eros Azzalini
Caterina Medeot
Domenico Tierno
Iris Zalaudek
Claudio Conforti.....



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