NUCLEIC ACIDS ISOLATION FROM ARCHIVE TISSUES

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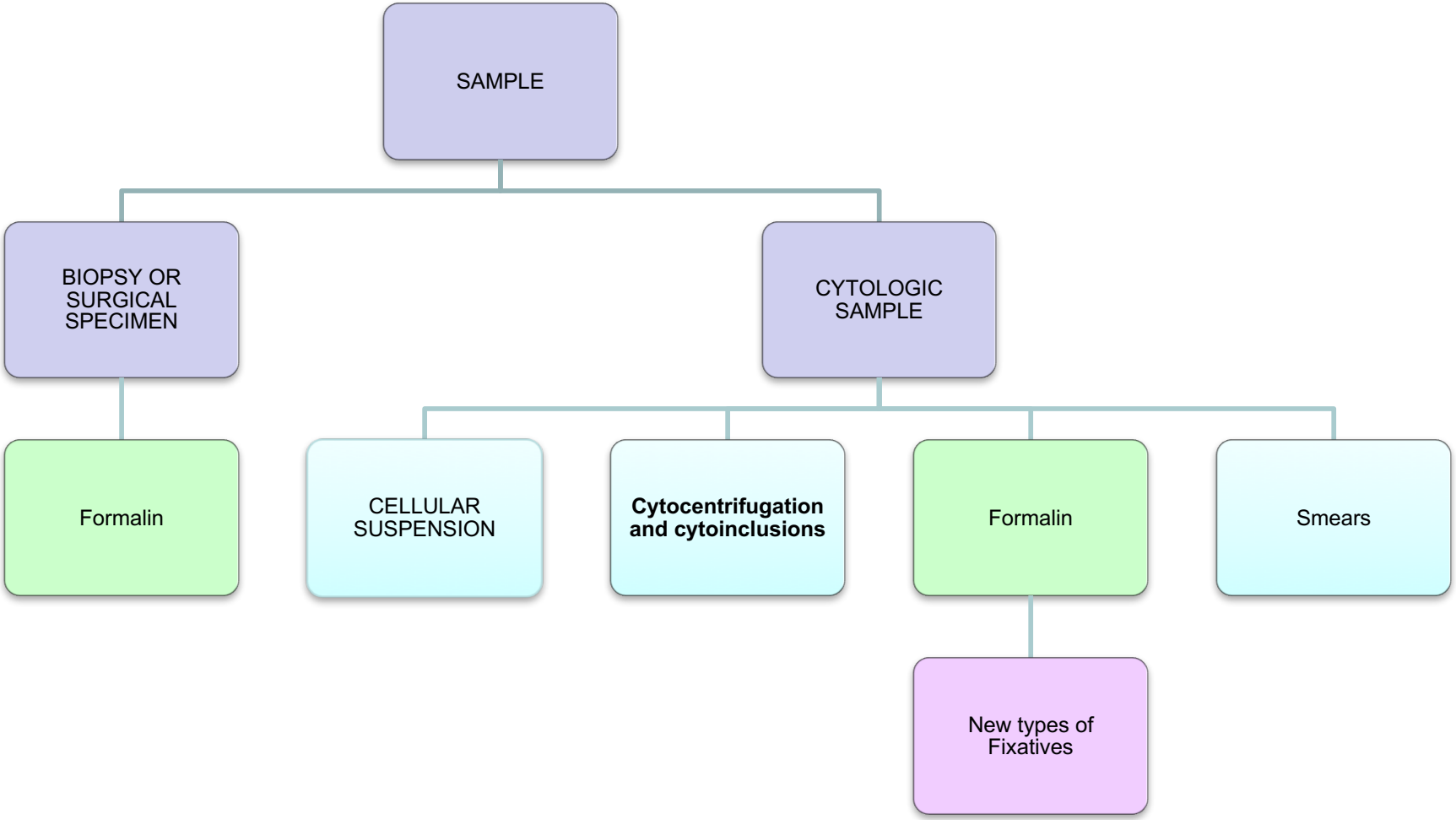
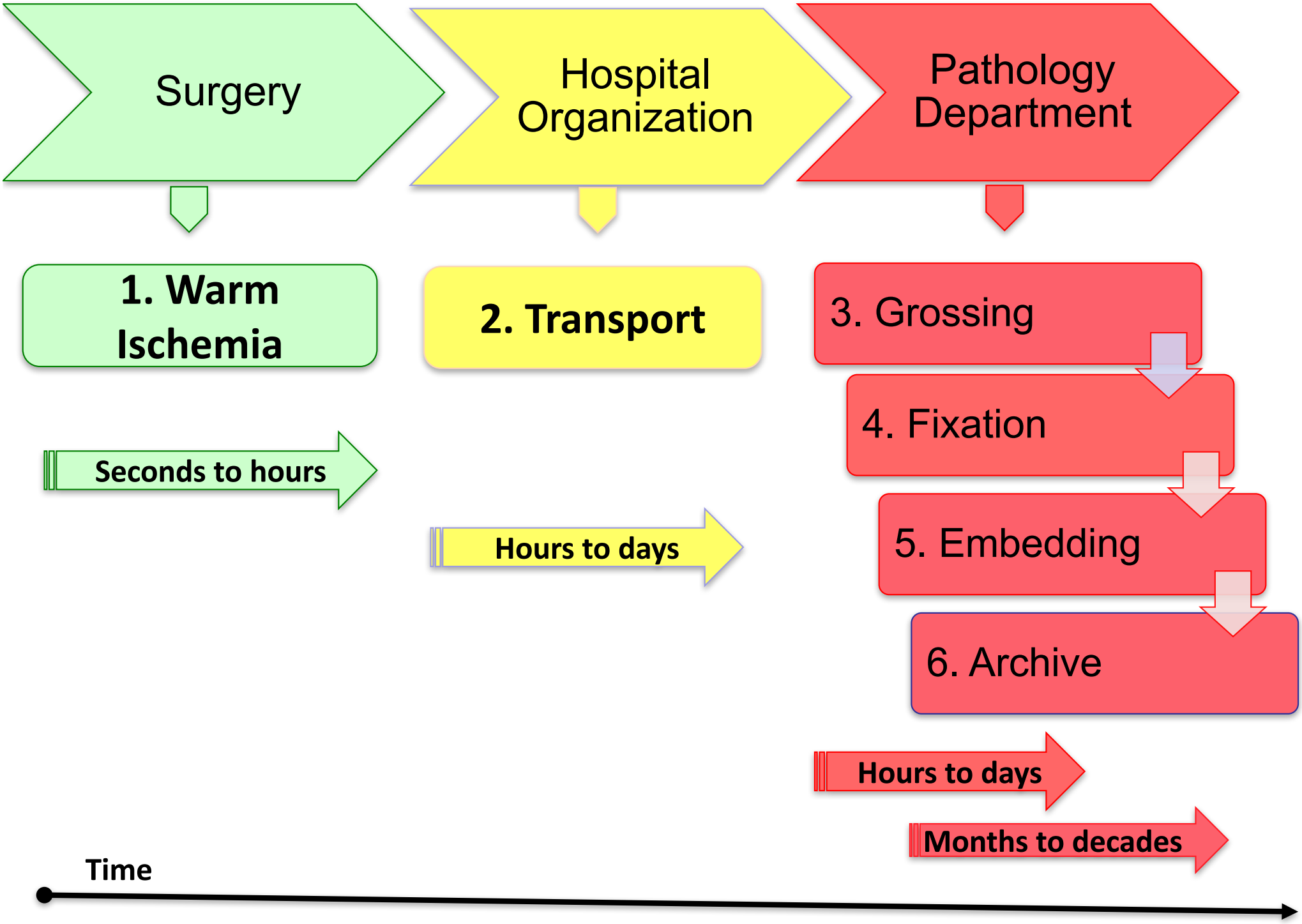
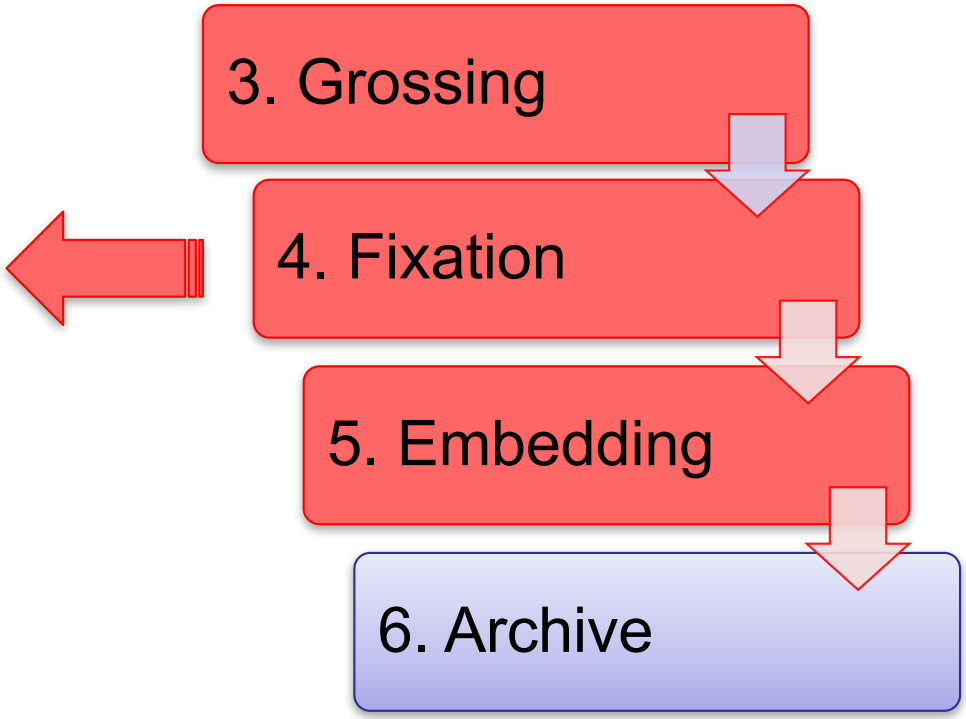

SAMPLE
BIOPSY OR SURGICAL SPECIMEN
CYTOLOGIC SAMPLE
Formalin
CELLULAR SUSPENSION
Cytocentrifugation and cytoinclusions
Formalin
Smears
New types of Fixatives


Surgery
Hospital Organization
Pathology Department
1. Warm Ischemia
2. Transport
3. Grossing
4. Fixation
5. Embedding
6. Archive
Seconds to hours
Hours to days
Hours to days
Months to decades
Time

The use of formalin leads to various reaction within the tissue, including the crosslinking of proteins through methylene bridge formation among proteins and nucleic acids.

Nucleic acids are degraded.

The longer is the permanence period in formalin the higher is the degradation.



Macromolecole in FFPE

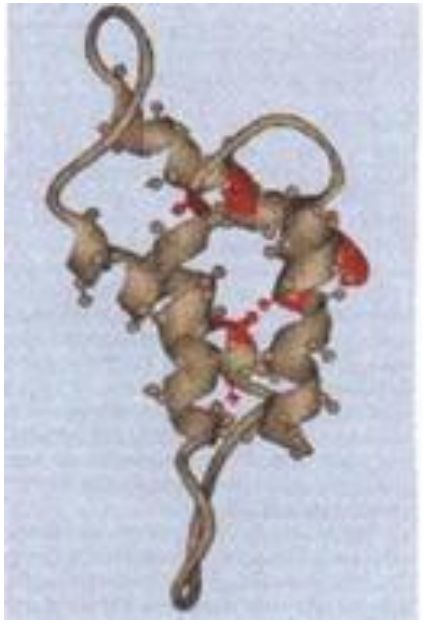
Formalin induces different reactions in tissues, including protein crosslinking due to the formation of methylene bridges between proteins and nucleic acids. The longer is the time time in formalin the greater the number of cross-links.

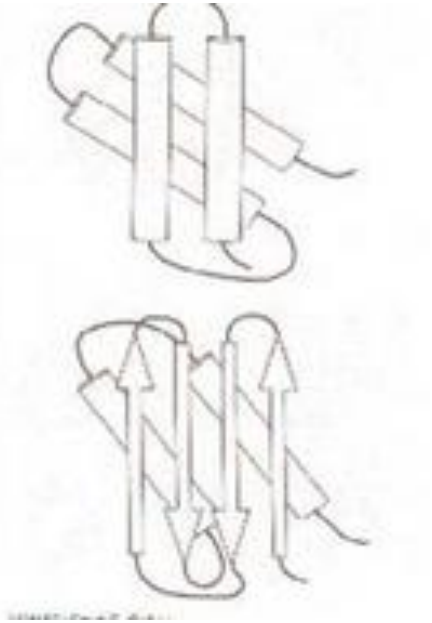
Nucleic acids are degraded

> is the fixation time> is the level of degradation

The presence of crosslinks determines the extraction processes in archival tissues. Proteolysis is essential for the extraction of Nucleic acids, but determines the loss of proteins.

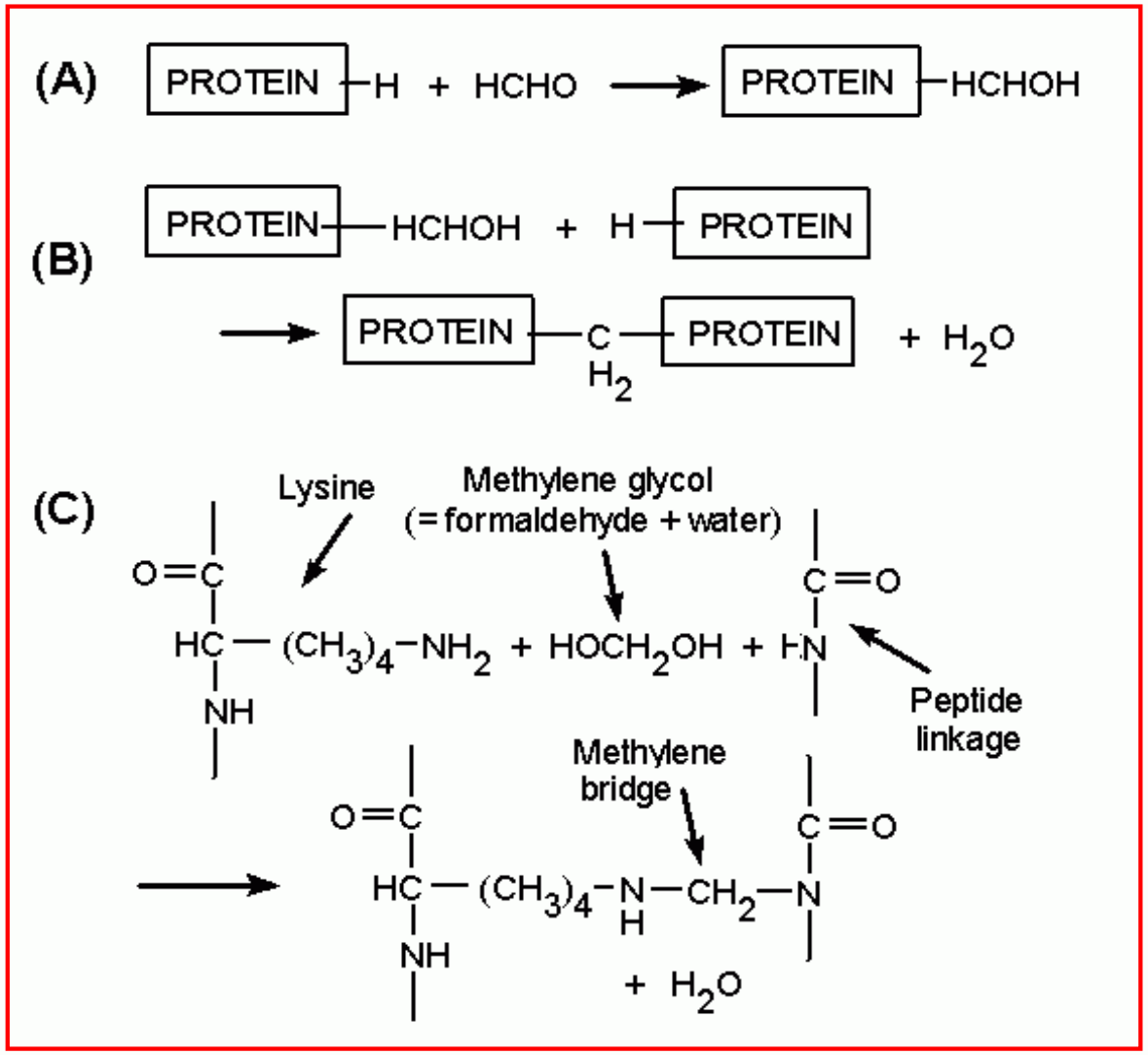
Formalin is a toxic and irritant reagent and it was defined as possible carcinogenic to humans (IARC 2004).





Degraded Nucleic Acids!

Effects of formalin fixation

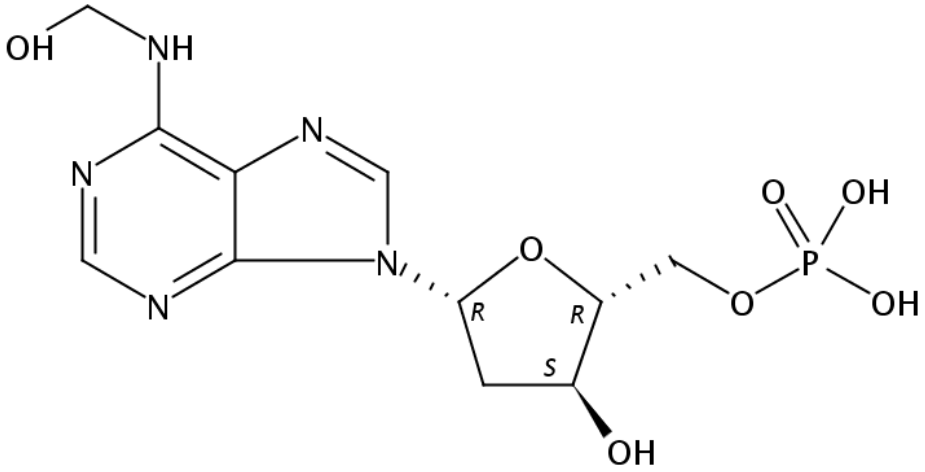


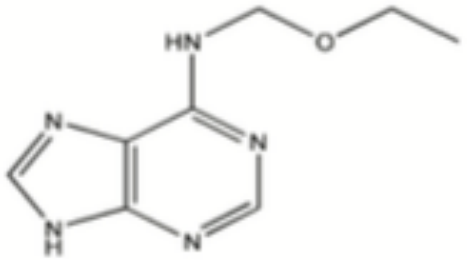
(A) Addition of a formaldehyd molecule to a protein.

(B) Reaction of bound formaldehyde with another protein molecule to form a methylene cross-link.

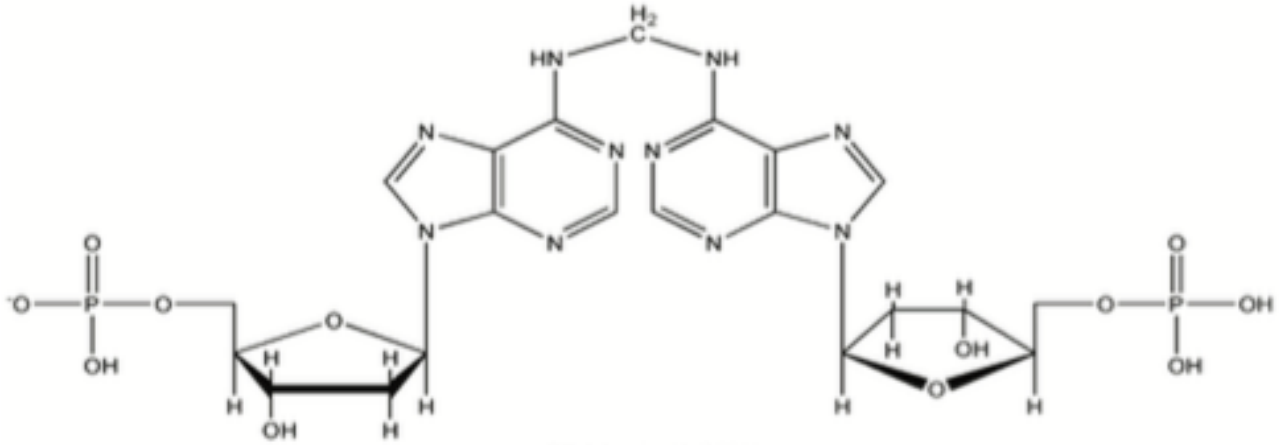
(C) A more detailed depiction of the cross-linking of a lysine side-chain to a peptide nitrogen atom.

Kiernan,JA. Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: What they are and what they do. Microscopy Today 00-1 pp. 8-12 (2000).

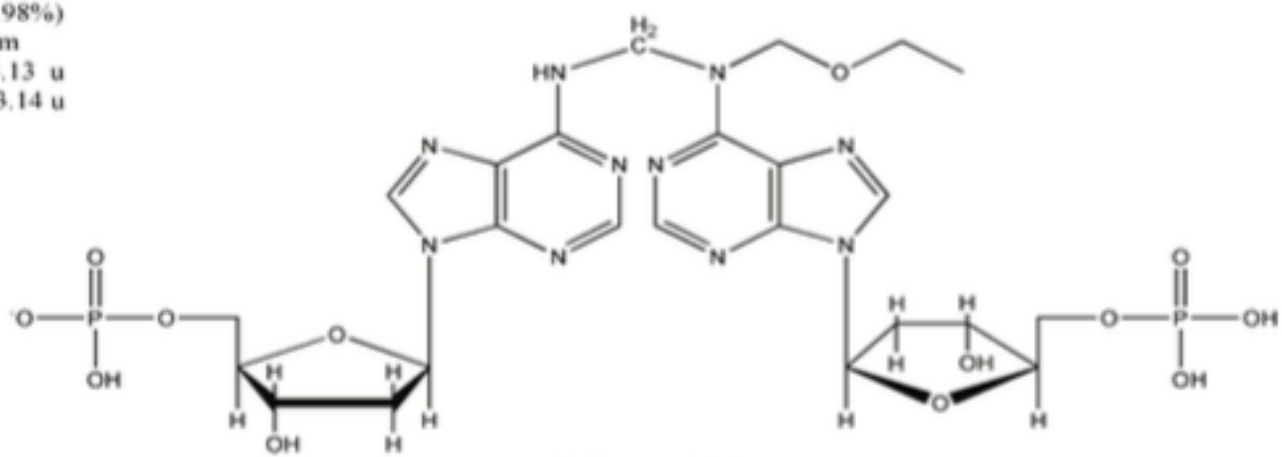




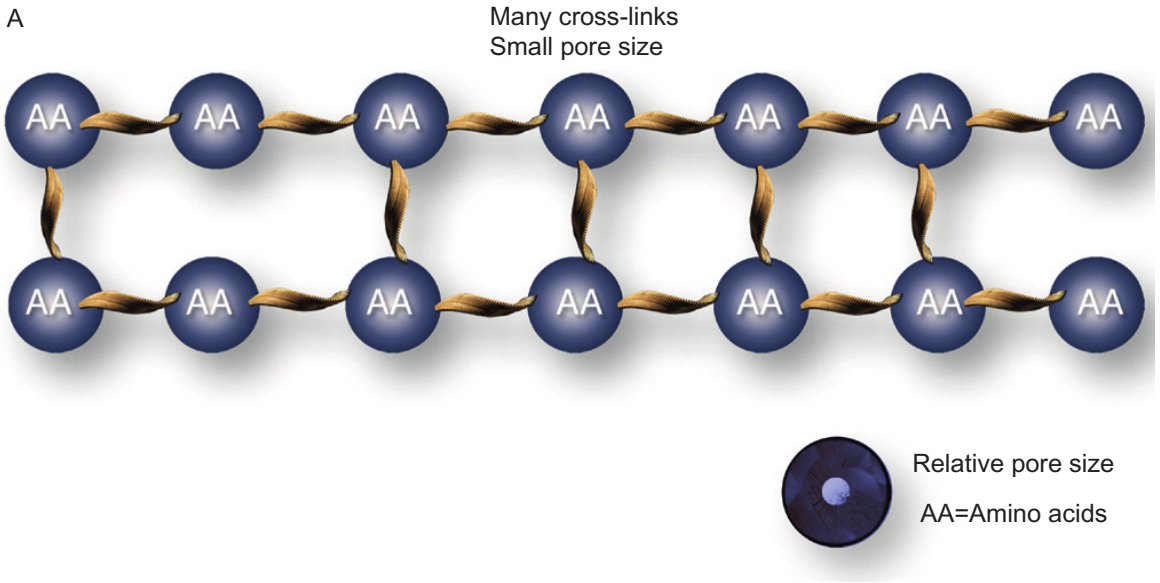
t_R 16.74 min (1.46%)
λ_{max} 263.8 nm
Exact Mass 193.10 amu
Found Mass 193.10 amu

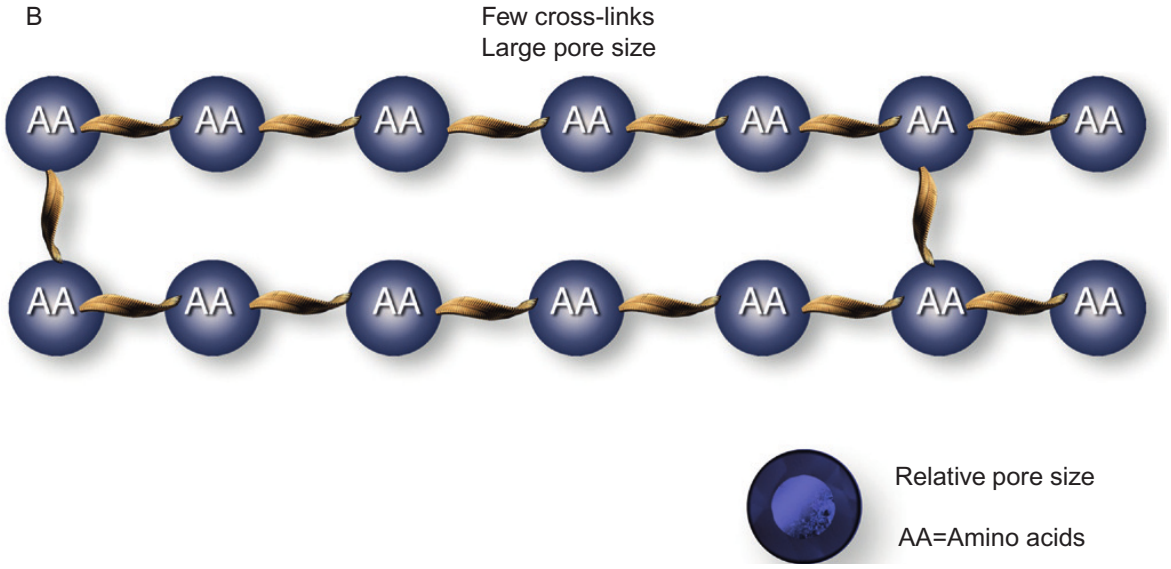


t_R 27.14 min (1.98%)
λ_{ma} 271.4 nm
Exact Mass 673.13 u
Found Mass 673.14 u



t_R 37.71 min (0.40%)
λ_{max} 272.4 nm
Exact Mass 731.17 u





The longer is fixation the higher is number of crosslinks

Is it possible to ameliorate formalin fixation for molecular diagnostics?

- Controlled fixation time
- Cold fixation (Longer fixation at low T)*
- Warm 2+2 fixation = 2h 4° C formalin + 2h 40° C formalin #

$$CH_2=O + H_2O \rightleftharpoons OH-(CH_2O)-H$$

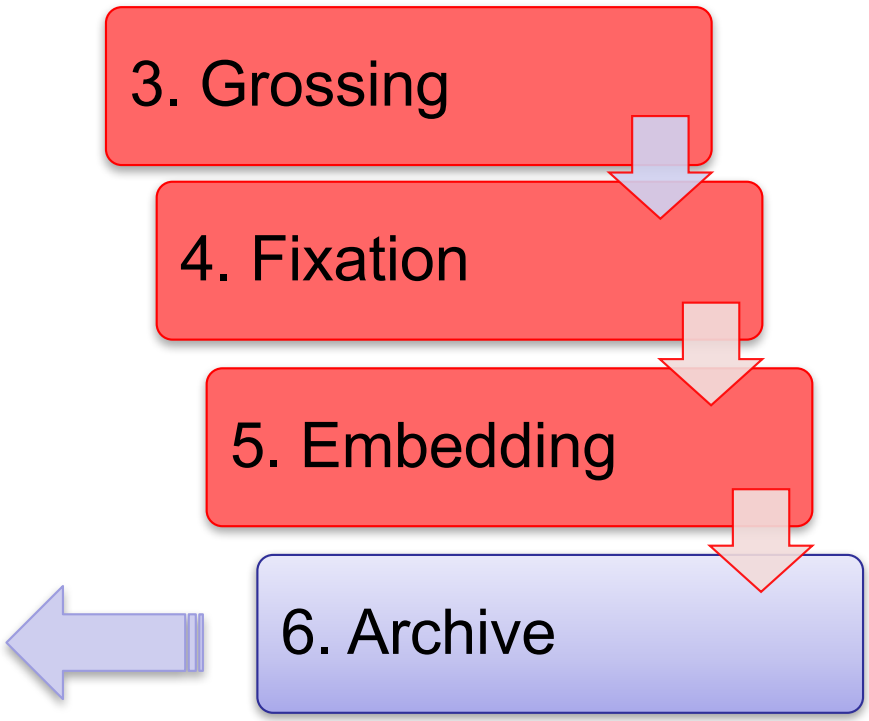
Heat likely speeds fixation : first, the formaldehyde-methylene glycol equilibrium shifts towards formaldehyde at higher temperatures and raises the effective concentration of the active molecule and, second, protein crosslinking should proceed more quickly at elevated temperatures.

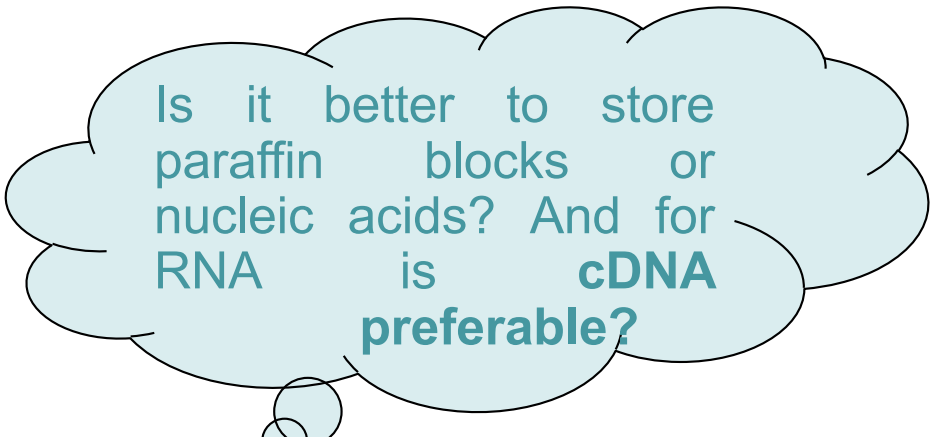
RNA degradation would be inhibited by maintaining low temperature through all the process of fixation.

RNA degradation in FFPE is the cumulative effect of RNAses activity during pre- and fixation, hydrolysis and mechanical rupture due to molecule stiffening from crosslinking. Every treatment which reduces those effects contributes to a better RNA preservation

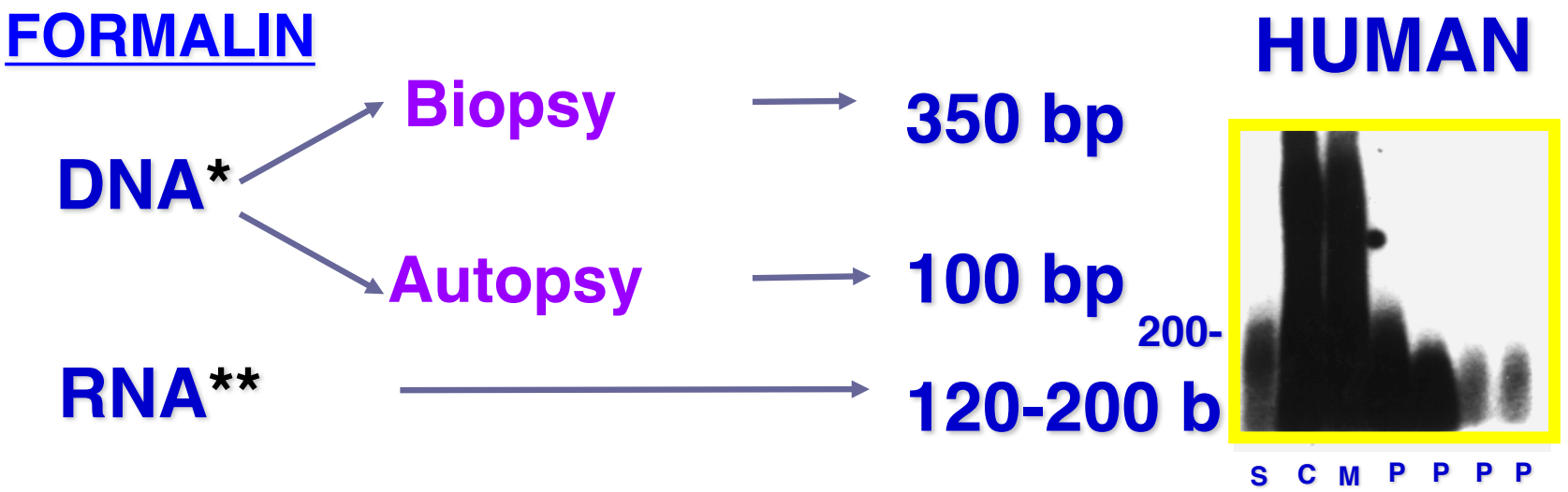
*Bussolati G et al. PLOS ONE 2011
#Chafin D et al PLOS OONE 2013

Even with extended processing times, at elevated storage temperatures and dry conditions, tissue sections demonstrate degradation.
Improper tissue processing leads to inadequate replacement of water by paraffin and retaining of endogenous water in FFPE tissue. Nucleic acids in tissue blocks degrade!





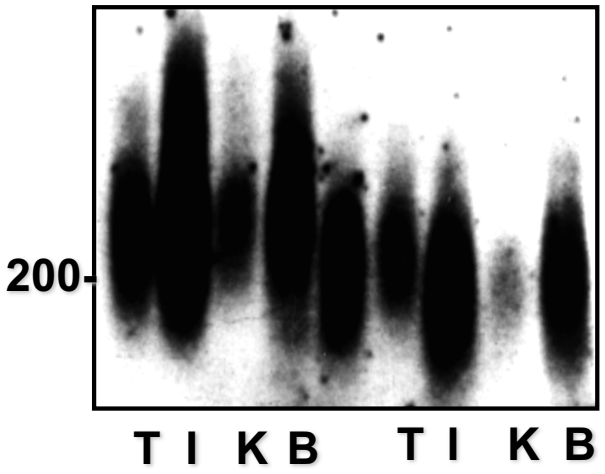
Nucleic acids have a wide range of degradation in FFPE tissues due to fixation processes and pre-analytical phase

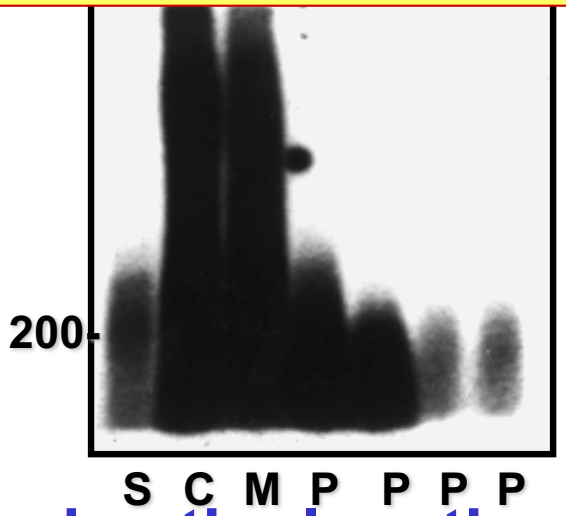


*-Bonin S., Petrera F., Stanta G. PCR and RT-PCR Analysis In Archival Postmortem Tissues. In Fuchs J, Podda M. Encyclopedia Of Diagnostic Genomics And Proteomics. M. Dekker, New York: 985-988; 2005

**-G. Stanta and C. Schneider, RNA extracted from paraffin-embedded human tissues is amenable to analysis by PCR amplification. Biotechniques, 11:304-308,1991.

RNA FROM FIXED AND PARAFFIN EMBEDDED TISSUES





The fixation and tissue type determine the length of the available RNA:

*-Formalin: Biopsy ~ 120-200 bases**

*Autopsy ~ 90 bases **

*-Bouin's fixative : ~ 75 bases **

*Alcholic Fixatives: ~ 600 bases **

•Bonin S., Petrera F., Stanta G. PCR and RT-PCR Analysis In Archival Postmortem Tissues. In Fuchs J, Podda M. Encyclopedia Of Diagnostic Genomics And Proteomics.
M. Dekker, New York: 985-988; 2005

G. Stanta and C. Schneider, RNA extracted from paraffin-embedded human tissues is amenable to analysis by PCR amplification. Biotechniques, 11:304-308,1991.

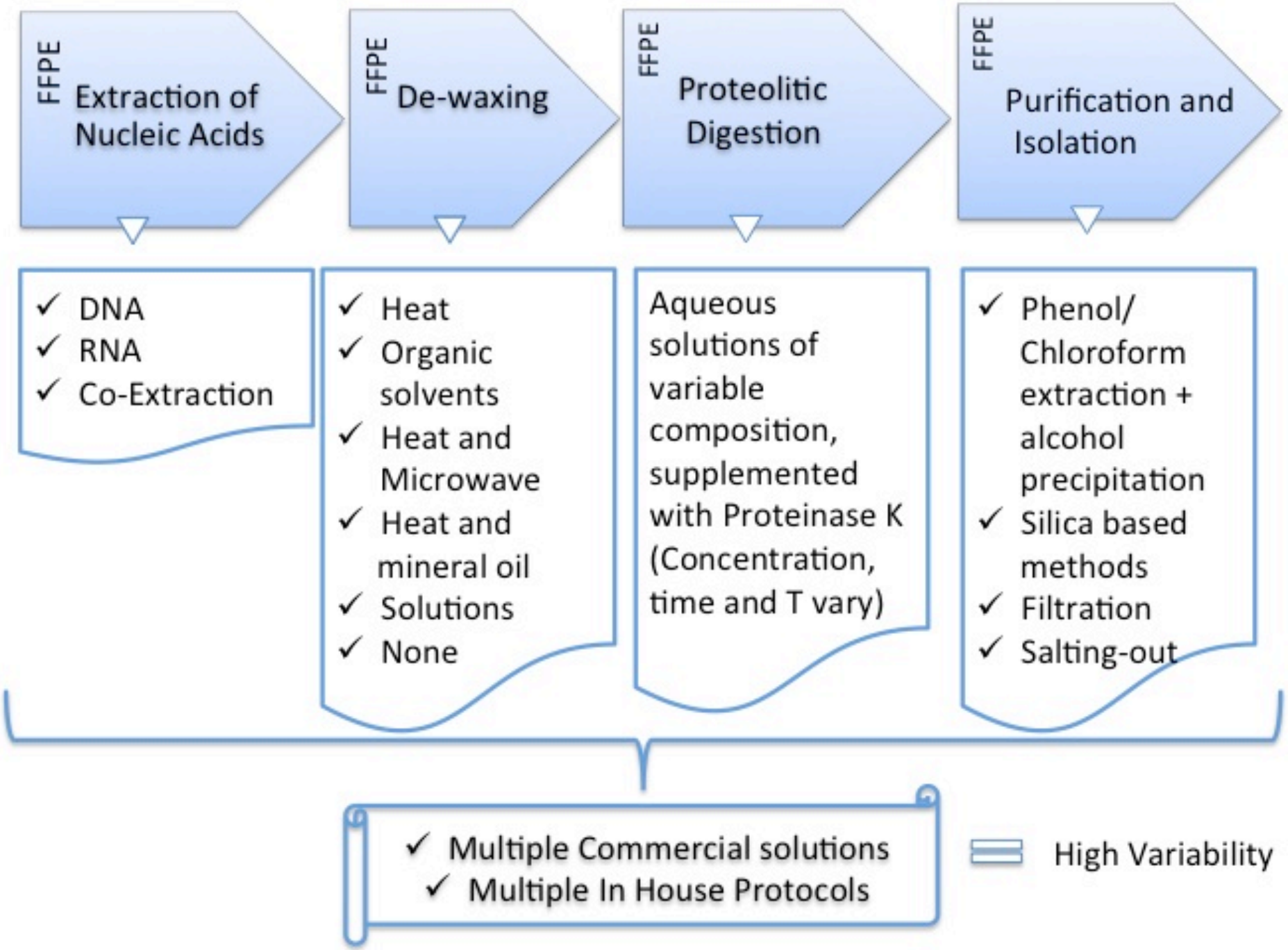
S Bonin, F Petrera, J Rosai, G Stanta, "DNA and RNA obtained from Bouin fixed tissues" J Clin Path , 58:313-316; 2005

Dotti I., Bonin S. et al. Effects of formalin, methacarn, and fineFIX fixatives on RNA preservation. Diagn Mol Patho, 19: 112-122; 2010

Isolation of Nucleic acids from FFPE tissues

- ***Tissue cut and microdissection***
- ***Dewaxing (xylene or heating)***
- ***EtOH washing and e re-hydration***
- ***Digestion with Proteinase K***
- ***Nucleic Acids Isolation***

* Stanta G et al, Biotechniques 1991, 11: 304-308
Specht K et al. Am J Pathol 2001, 158: 419-429
Nortvig Abrahamsen H et al. J Mol Diagn 2003, 5: 34-41.



Bonin S and Stanta G. Nucleic acid extraction methods from fixed & paraffin-embedded tissues. Expert Rev. Mol. Diagn. 13(3), (2013)

Table 1. Methods for DNA extraction compared in the literature and analyzed in the present study.

Study (year)	Methods, kit and manufacturer	Notes	Ref.
Bonin *et al.* (2010)	QIAamp® DNA Mini kit (Qiagen, CA, USA), in-house method (PCI), NucleoSpin® Tissue kit (Macherey Nagel, Düren, Germany), DNeasy FFPE kit (Qiagen)		[25]
Dedhia *et al.* (2007)	QIAamp DNA Mini kit, in-house method (PCI)		[53]
Funabashi *et al.* (2012)	In-house method (PCI), QIAmp DNA Mini kit		[26]
Huijsmans *et al.* (2010)	Heat treatment, QIAmp® DNA Blood Mini kit (Qiagen), easyMAG® (NucliSens, Biomerieux, NC, USA), Gentra-capture-column-kit (Gentra Systems, Qiagen)		[27]
Kotorashvili *et al.* (2012)	Trizol® (Invitrogen, Life Technologies Ltd, Paisley, UK), All Prep DNA/RNA FFPE kit (Qiagen), RecoverAll™ (Ambion, TX, USA), QIAmp® DNA FFPE kit (Qiagen)	Coextraction of RNA and DNA	[30]
Ludyga *et al.* (2010)	QIAmp DNA FFPE kit, in-house method (PCI), FFPE RNA/DNA purification kit (Norgen, Biotek Corp, ON, Canada)	Long-term preserved tissues	[28]
Munoz-Cadavid *et al.* (2010)	QIAamp DNA FFPE Tissue kit (Qiagen), TaKaRaDexpat (Takara Bio Inc., Otsu, Japan), PureLink®Genomic DNA Mini kit (Invitrogen, Life Technologies Ltd, Paisley, UK), Wax Free™ DNA (TrimGen, MA, USA), QuickExtract FFPE DNA Extraction kit (Epicenter Biotechnologies, WI, USA)	For detection of fungal DNA	[29]
Okello *et al.* (2012)	Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, MO, USA), TRI Reagent® solution (Ambion, TX, USA), RecoverAll, in-house method (PCI)	Archival autopsy tissues	[22]
Torrente *et al.* (2011)	In-house method (PCI), QIAmp DNA FFPE kit		[93]
Turashvili *et al.* (2012)	QIAmp DNA FFPE kit, RecoverAll, Wax Free DNA, in-house method (PCI)		[12]

FFPE: Formalin-fixed paraffin-embedded; PCI: Phenol chloroform isolation.

Bonin S and Stanta G. Nucleic acid extraction methods from fixed & paraffin-embedded tissues. Expert Rev. Mol. Diagn. 13(3), (2013)

METHODS FOR DNA ISOLATION FROM FFPE TISSUES

Different methods can be applied for DNA extraction from FFPE, but in any case they belong to one of the following types:

1. **DNA extraction without further precipitation or purification**
2. **DNA extraction with precipitation of the DNA either by NaAc/EtOH or by isopropanol**
3. **DNA extraction using silica based adsorption columns for purifying DNA**

DNA

Every procedure involves a step of proteolytic digestion with proteinase K to disrupt crosslinks and allow DNA solubilization.

☞**Digestion using Proteinasi K at 55° C (at least ON):**

50 mM Tris HCl pH 8,

1 mM EDTA,

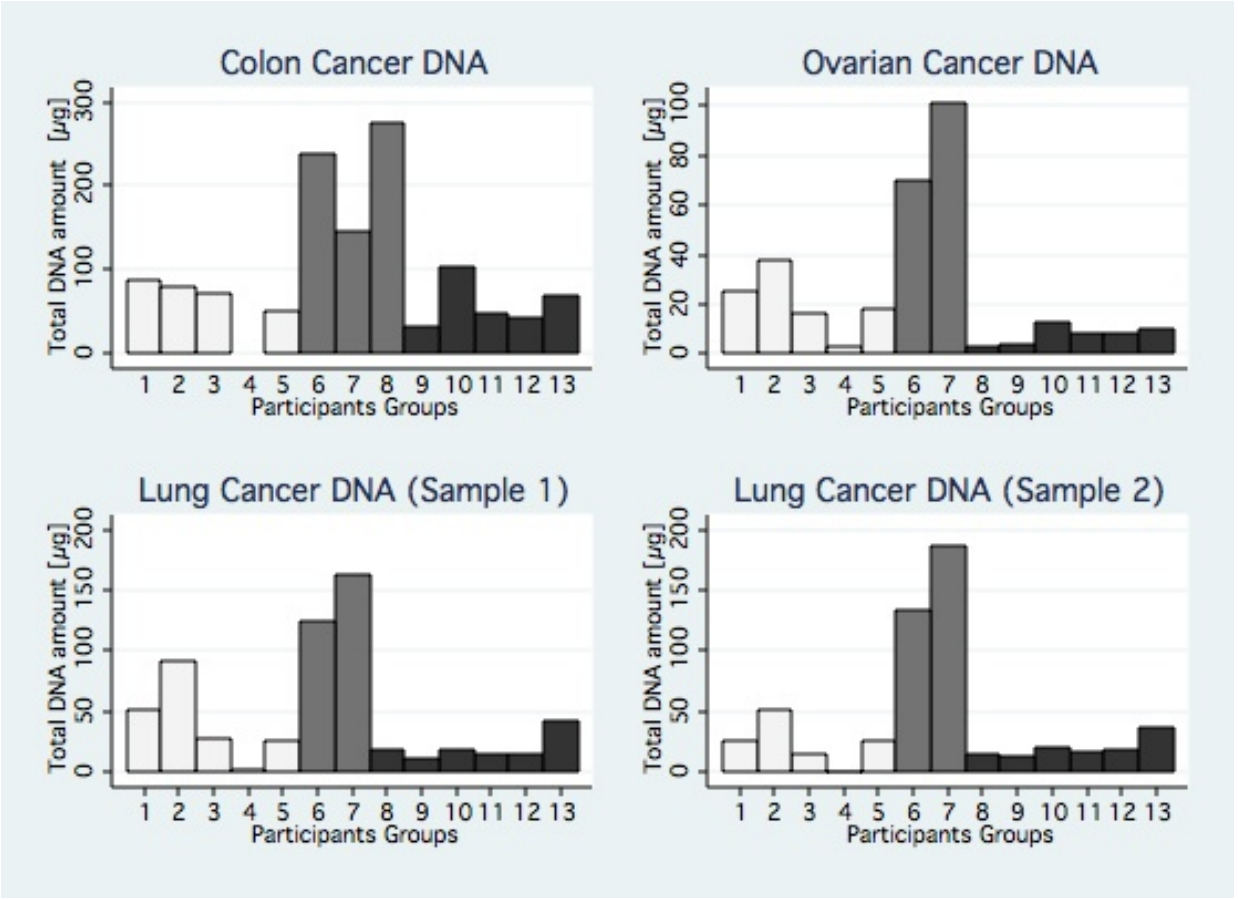
0.5% Tween 20, (*the use of non-ionic detergents is essential if no further purification is carried out)*

Proteinase K 1 mg/ml final.

The total amount of DNA depends on the **method** used and the **type of tissue**. In general, purification causes apparently loss of material, but greater purity.

From a multicentric study

Colon cancer: 100-200 bases;
Ovary cancer 100 bases;
Lung cancer 300-400 bases.

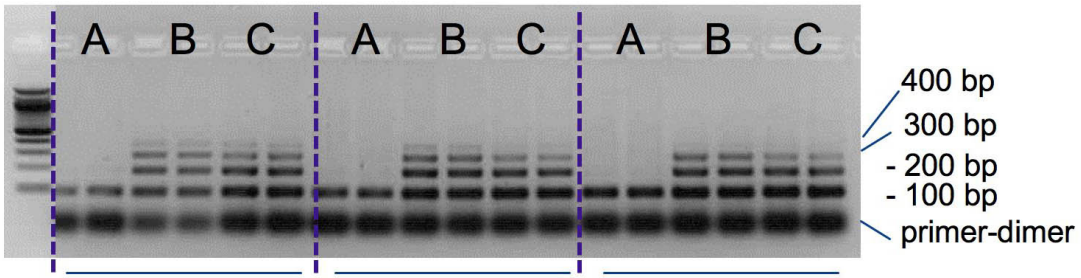


Virchows Arch (2010) 457:309–317
DOI 10.1007/s00428-010-0917-5

ORIGINAL ARTICLE

Multicentre validation study of nucleic acids from FFPE tissues

Serena Bonin · Falk Hlubek · Jean Benhattar · Carsten Denkert · Manfred Diet · Pedro L. Fernandez · Gerald Höfler · Hannelore Kothmaier · Bozo Kruslin · Chiara Maria Mazzanti · Aurel Perren · Helmuth Popper · Aldo Scarpa · Paula Soares · Giorgio Stanta · Patricia J. T. A. Groenen

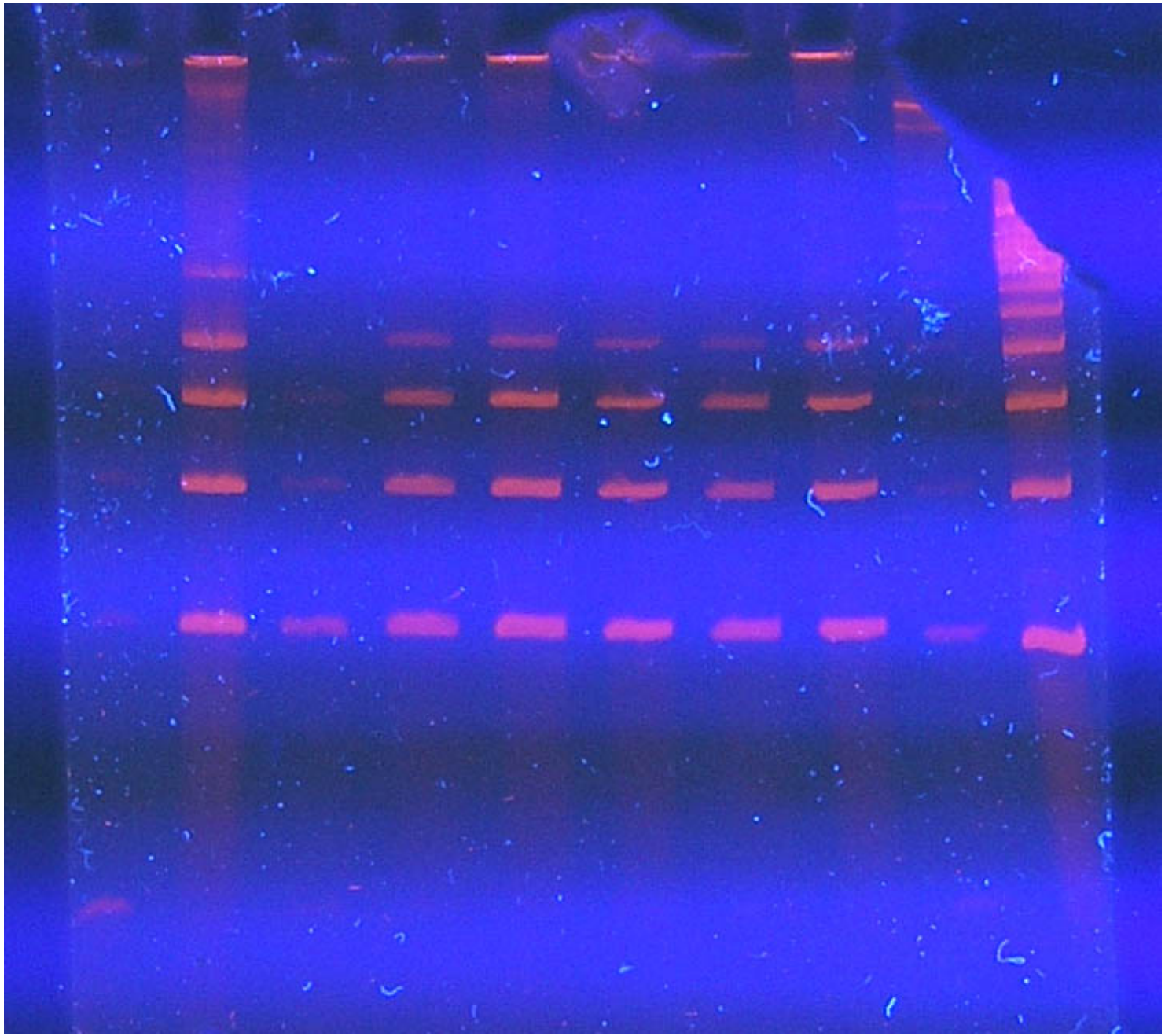


HOW TO STANDARDIZE?

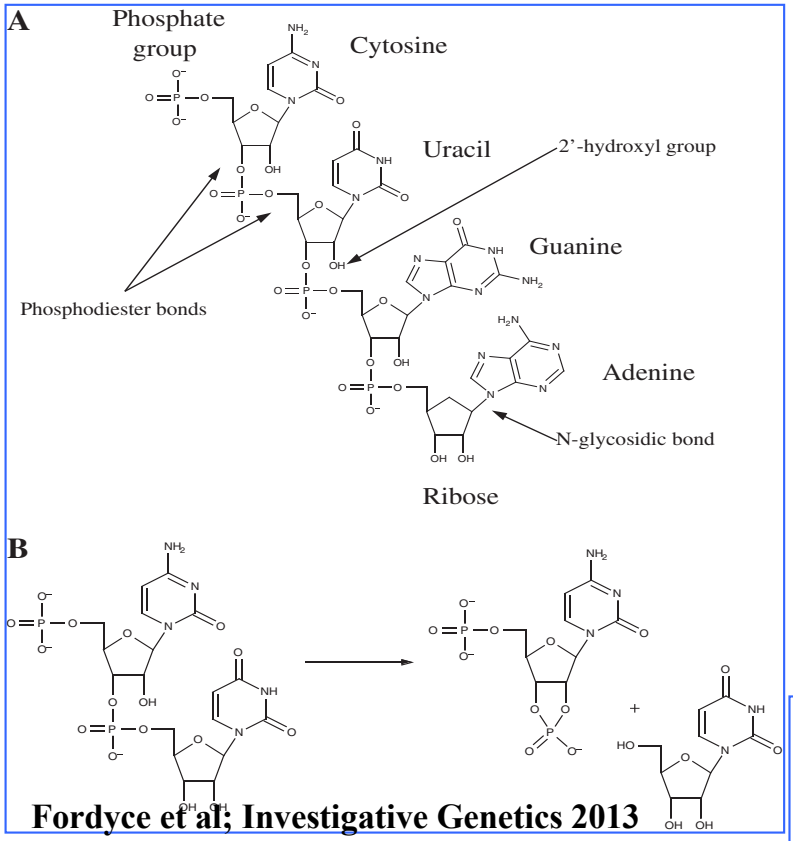
- The amount of extracted DNA varies greatly from protocol to protocol, but it is not related to quality in terms of maximum amplifiable length.
- The purity of spectrophotometric measurements is not related to quality as maximum amplifiable length
- Therefore, considering the quality as amplifiability there is not a single extractive protocol that prevails over the other. The choice depends on the analyzes to be carried out. When a good quantification of DNA is required, extractive protocols with purification steps must be chosen.
- Rather than evaluating a single extractive test to be considered as standard, it is more important to consider a standard quality assessment test:

Samples which do not pass the test should be re-extracted or excluded from the study

DNA FROM FFPE W/O PURIFICATION PROCEDURE

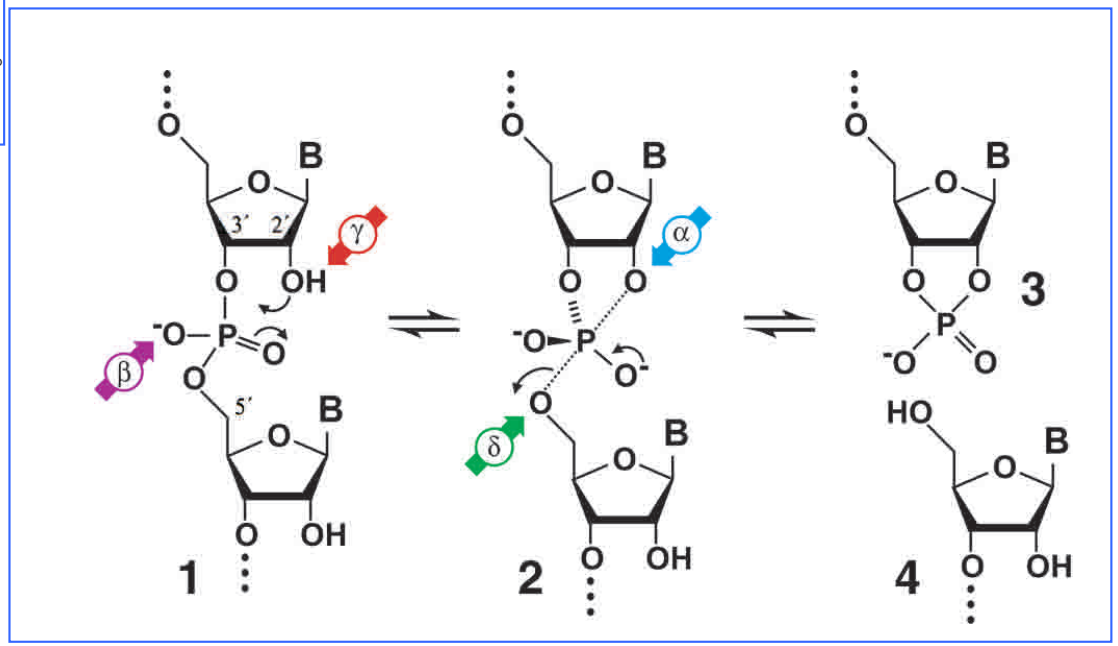


RNA degrades differently from DNA



- *The 2'-OH allows the RNA molecule to be more easily degraded via hydrolysis than DNA.*
- *The phosphodiester bond in RNA can be broken during hydrolysis.*
- *The N-glycosidic bond is stronger in RNA than DNA*
- *The chemical process of hydrolysis, where the 2'-hydroxyl group has attacked the adjacent phosphodiester bond, cleaving the backbone of the RNA.*

Fordyce et al; Investigative Genetics 2013



RNAse A increases the rate of RNA cleavage by internal phosphoester transfer

DNA DEGRADATION AND LOSS MECHANISMS DURING CONSERVATION

Depurination / ß elimination

In a water solution DNA can degrade as depurination /ß elimination. This reaction is cathalyzed in acid conditions with bond break and a ß-elimination. The pH of the conservation solution has an important role in the stability of DNA.

Free radicals oxydation

Nucleic acid bases and desossyriboses→are susceptible to degradation by free radicals oxydation.

Eso- and endonucleases

Temperature, pH and salt concentration influence enzyme activity of eso- and endonucleases.

MECHANISM OF DNA LOSS IN PLASTICWARE

- **DNA binds to the plasticware**

-For low or moderate ionic strength (common conditions of DNA conservation) polypropilene tubes bind less than 5% of DNA, but there can be some tubes binding over 25%.

-The conservation for short stretches of DNA is more problematic, they tend to denaturate and it is possible to form complex DNA structures and polypropilene interaction.

DNA CONSERVATION

- **DNA in solution:**

-In sterilized H_2O or TE buffer(10 mM Tris, pH 7.5-8.0, 1 mM EDTA), Temperature from 4° C to -20° C, for few months with low level of degradation.

Freezing and thawing do not cause further degradation.

- **DNA precipitation:**

Conserved in EtOH covered with chloroform at RT or 4° C

(at -20° C after long time problem with rehydration.)

- **In Buffers with EDTA:**

In EDTA solution at RT low level of degradation for over 2 years.

High level EDTA concentration and adding Ethanol, DNA stability is increased for very long time.

RNA EXTRACTION PROBLEMS

↓

Low level quality and quantity of RNA

↓

RNA can be degraded during the extraction!

Precautions:

- **RNase free reagents (DEPC treatment, RNAse inhibitors)**
- **Gloves**
- **Glassware RNase free……**
- **Use of disposables**

RNAses

For a successful extraction no RNAses contamination is necessary.

RNAses are small enzymes (15 kDa single chain with very rapid renaturation) that do not need cofactors, very resistant to high temperature, active in a wide pH range.

Sources: external (mostly from operators), internal (the same tissues, the richest are pancreas and spleen).

RNAses inhibitors

Specific: ***Rnasin (40 kDa -from human placenta or rat liver).***

Not specific: ***heparin, DEPC, polivynil sulfate.***

-Water treatment with DEPC: 0.1% final in H_2O at 37° C O.N. Then in autoclave DEPC degrades at high temperature to Ethanol+CO_2, that are volatile.

-DEPC reacts with Tris, solution must be performed after water treatment.

-DEPC reacts with adenine residues with inactivation of in vitro translation.

-Glassware treatment at 250 - 300° C for at least 3hrs.

RNA

Every procedure for RNA extraction is based on proteolysis step.

The extraction protocols can be roughly divided in 3 major groups as follows:

1) **RNA extraction with phenol extraction and isopropanol precipitation- home made protocols**
2) **RNA extraction by the use of monophasic commercial solutions**
3) **RNA extraction using commercial kits with adsorption based columns for purification**

☞ **Proteinase K digestion at 55° C (at least ON or as described by the manufacturer):**

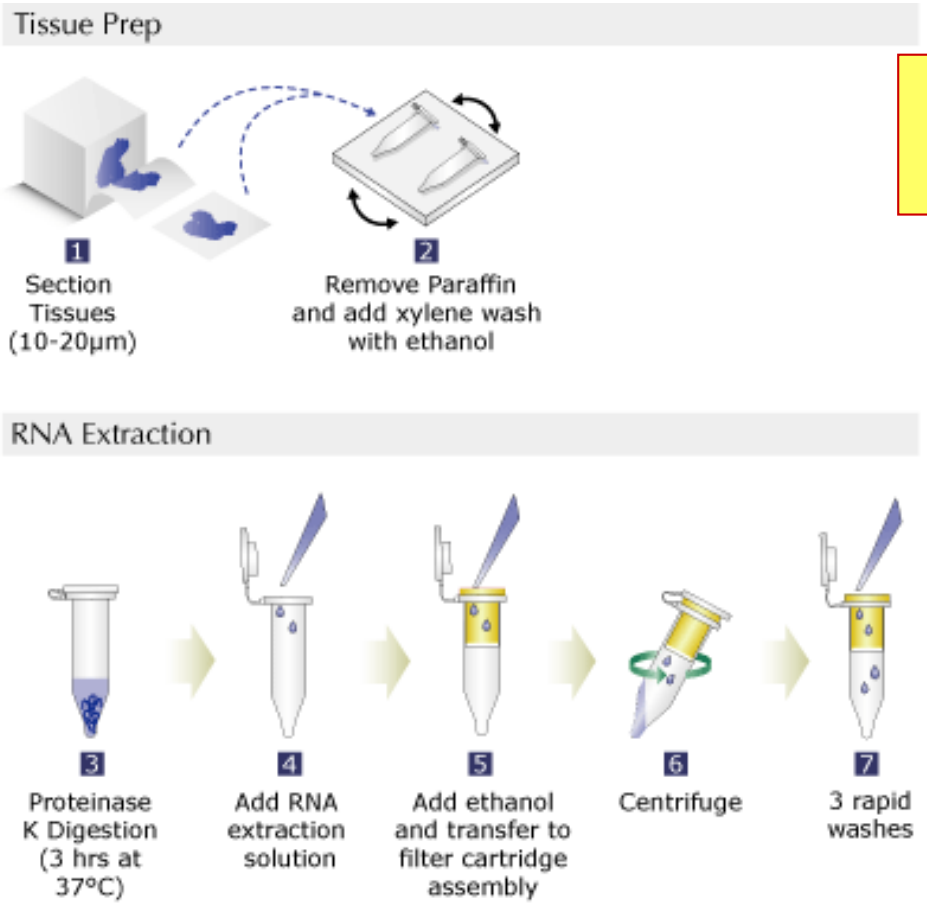
RNA yield, as expected, is tissue dependent. Regarding the protocol type, the higher RNA yield is obtained using commercial kits based on silica column purification, followed by the use of commercial solutions and then home made protocols.

Table 2. Methods for RNA extraction compared in the literature analysed in the present study.

Study (year)	Methods, kit and manufacturer	Note
Abramoviz *et al.* (2008)	RecoverAll™(Ambion, TX, USA), High Pure Paraffin kit (Roche, NJ, USA), RNeasy® FFPE (Qiagen, CA, USA), ArrayGrade™ FFPE RNA isolation (SuperArray, Qiagen)	Application to DASL assay
Bonin *et al.* (2010)	RNeasy FFPE kit, High Pure FFPE RNA (Roche), in-house methods (PCI and Trizol® [Invitrogen Life Technologies Ltd, Paisley, UK] purification)	
Doleshal *et al.* (2008)	RNeasy FFPE kit, Absolutely RNA® FFPE kit (Stratagene, Agilent Technologies, CA, USA), High Pure FFPE RNA Micro (Roche), PureLink® RNA Isolation kit (Invitrogen), RecoverAll	For miRNA expression analyses
Jacobson *et al.* (2011)	RNeasy FFPE kit, High Pure FFPE RNA, RecoverAll	Application to microarray
Kotorashvili *et al.* (2012)	Trizol, All Prep DNA/RNA FFPE kit (Qiagen), RecoverAll	Coextraction of RNA and DNA
Linton *et al.* (2009)	Optimum FFPE RNA isolation (Ambion), RNeasy FFPE kit	Optimized
Ludyga *et al.* (2012)	RNeasy FFPE kit, in-house method (PCI); FFPE RNA/DNA purification kit (NorgenBiotek Corp, ON, Canada)	Long-term preserved tissues
Okello *et al.* (2012)	Absolutely RNA FFPE kit, TRI Reagent® solution (Ambion), High Pure FFPE RNA, High Pure FFPE RNA Micro kit (Roche), in-house method (PCI), Wax Free™ RNA kit (TrimGen, MA, USA), RecoverAll	Archival autopsy tissues
Ribeiro-Silva *et al.* (2007)	RecoverAll, High Pure FFPE RNA, Absolutely RNA FFPE kit, FormaPure kit (Agencourt Bioscience Corporation, MA, USA)	
Roberts *et al.* (2009)	RecoverAll, Paradise® Whole Transcript RT reagent system (Arcturus Bioscience, CA, USA), High Pure FFPE RNA, PureLink® FFPE RNA (Invitrogen), FormaPure RNA (Agencourt Bioscience Corporation)	
Ton *et al.* (2011)	RNeasy FFPE kit, High Pure FFPE RNA	Application to DASL assay
Turashvili *et al.* (2012)	RNeasy FFPE kit, Wax Free RNA kit, RecoverAll, in-house method (PCI)	

DASL: cDNA-mediated annealing, selection, extension and ligation; PCI: Phenol chloroform isolation.

4-RNA EXTRACTION FROM FFPE commercial kits



- ☞ ***Proteinase K digestion***
- ☞ ***Microfilter cartridge for extraction****
- ☞ ***RNA elution***
- ☞ ***Cell and Tissue Lysis with Proteinase K***
- ☞ ***Specific Precipitation of DNA and proteins*****
- ☞ ***RNA recruitment***

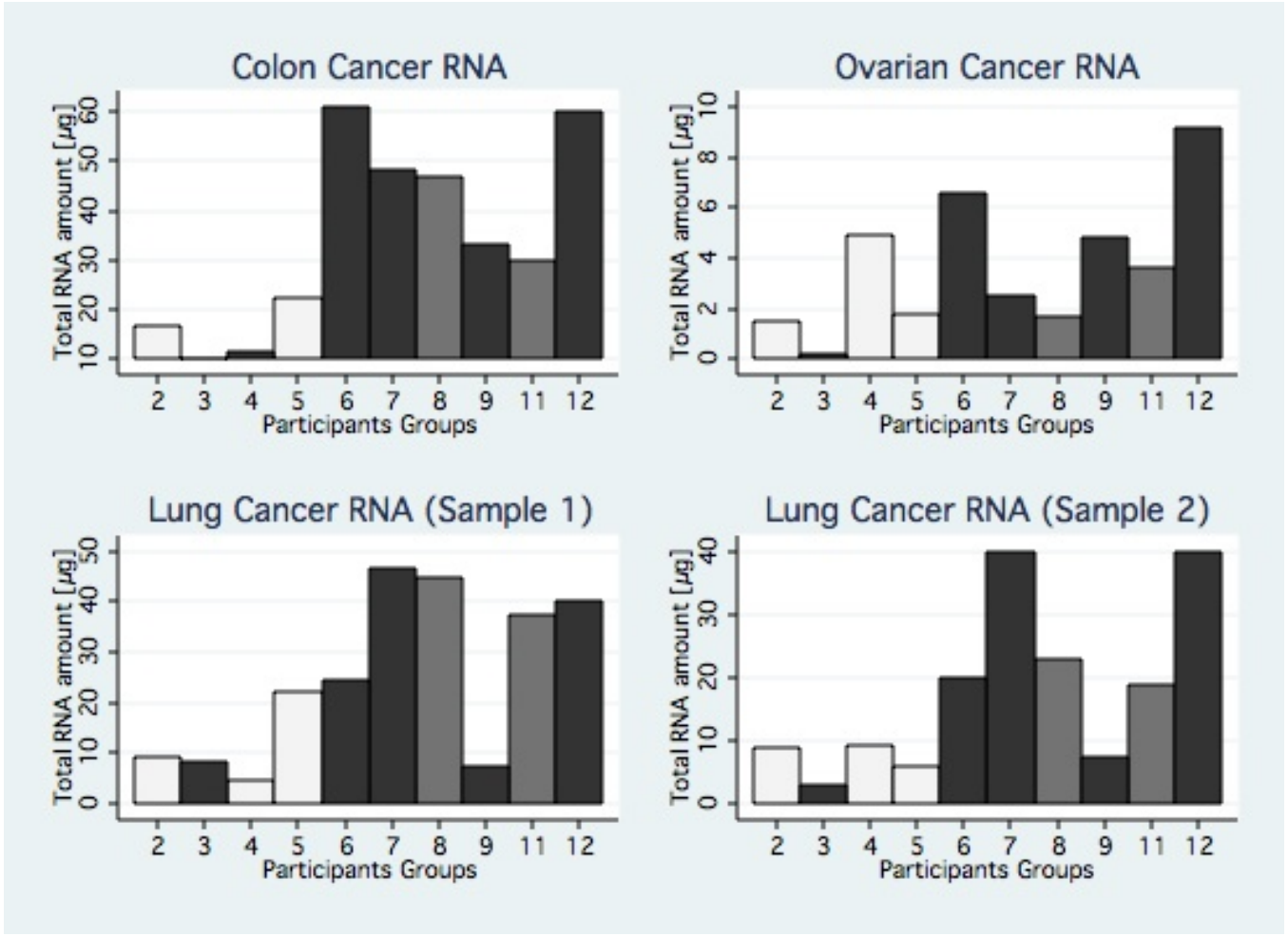
*RNA: Arcturus, Ambion; DNA: Qiagen, Roche, Intergen….

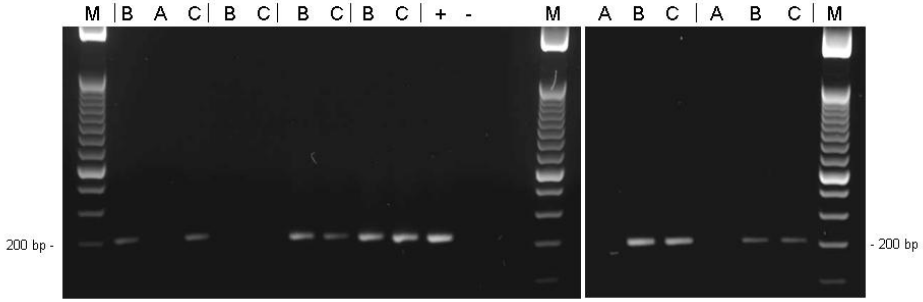
**Gentra Systems

FROM IMPACTS MULTICENTRIC VALIDATION

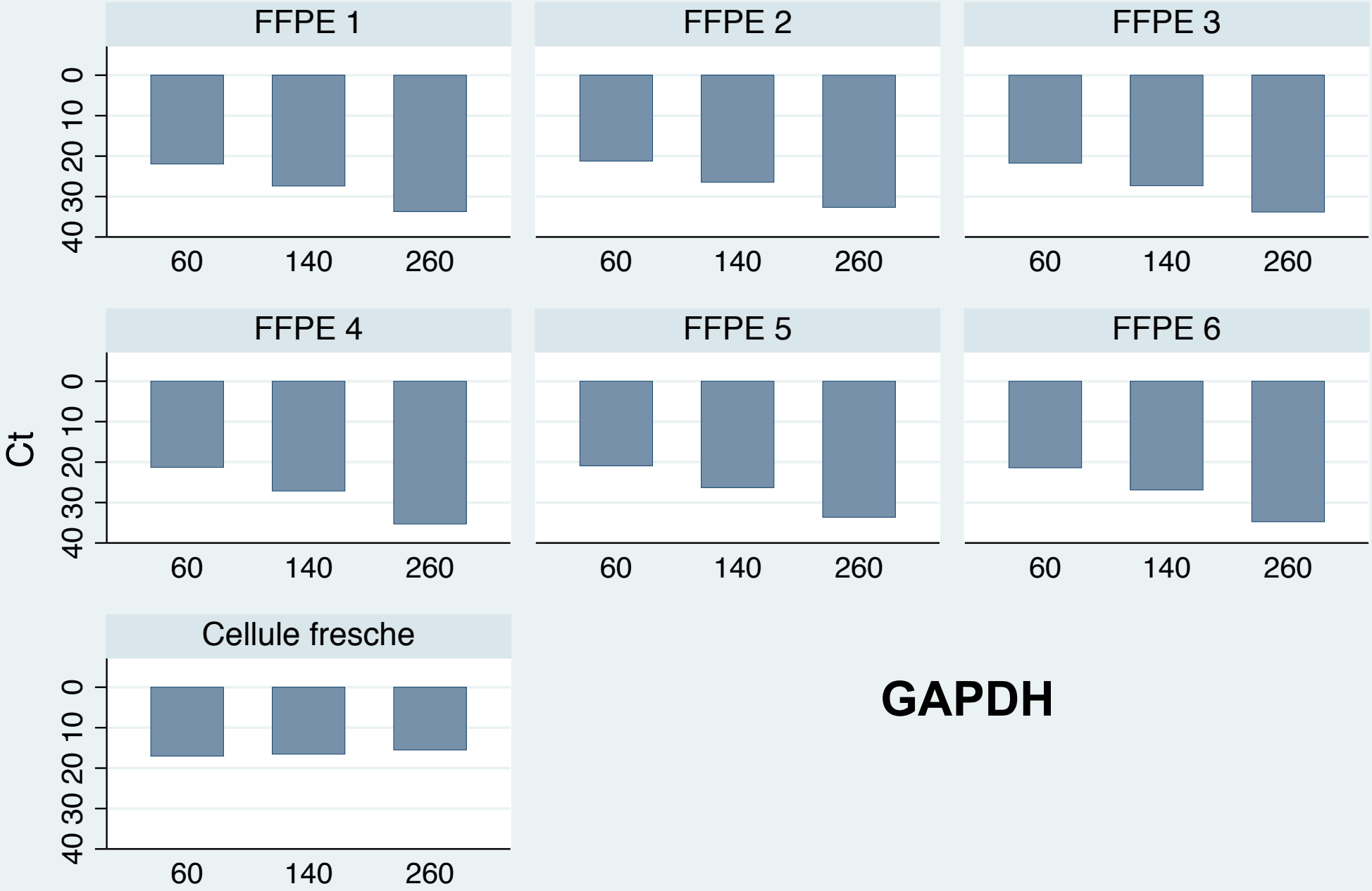
Column based commercial kits yielded the best RNA quality.

S. Bonin et al. (2010) Multicentre validation study of nucleic acids extraction from FFPE tissues. Virchows Arch. 457:309–317.





Real time PCR da cDNA



GAPDH

How to Standardadize?

- The amount of extracted RNA varies from protocol to protocol, obtaining on average higher yields with the use of commercial kits.
- The purity of spectrophotometric measurements is not related to quality understood as maximum amplifiable length.
- Considering the quality as maximum amplifiability, the use of commercial kits allows extracting more RNA and more intact, but it is not SUFFICIENT TO HAVE A STANDARDIZATION OF THE METHOD.
- Even using the same commercial product there can be variations in terms of quantity, but also quality due to small adjustments and / or the operator.
- It is essential to use a common method to test the quality of the extract to standardize the results. NO AGILENT BIOANALYZER !!!
- Samples that do not pass the quality test should be re-extracted or excluded from the study

RNA CONSERVATION

- **Conservation of the extract dissolved in H_2O DEPC at -70° C:** ***It is the most common method, but the RNA stored in H_2O tends to degrade because of not optimal pH and possible traces of RNAses.***

A better result can be obtained with sodium citrate 1mM or TE buffer pH<7. In this way hydrolysis of bases is partially prevented.

- **Stabilized in formamide**: ***The RNA pellet is solubilized in concentrated deionized formamide solution, this allows the preservation at -20° C for ~1 yr (formamide solution has to be substituted with H_2O before any enzymatic reaction).***
- **As ethanol precipitate**: **it can be stored as ethanol precipitate at RT, -4° C or -20° C**

- **As lyophilised powder : After quantification, a certain volume of the RNA solution could be evaporated at room temperature (no heating) in a speed-vac until complete dryness (very important) of the RNA. The tube could be kept at room temperature or at 4°C for weeks. Before using the RNA, it is just necessary to add water according to known quantity of RNA before the evaporation and wait few minutes for dilution.**

LOSS OF RNA EXTRACTS

⇒ **Attention has to be paid to avoid recontamination of specimens with RNAses during manipulation.**

⇒ **To avoid RNA degradation due to repeated freezing and thawing, it is advisable to store RNA in small aliquots rather than in a large single one.**

RNA from CROSSLINKING FIXATIVES

- It is Modified from the addiction of mono-methylol grouops to the bases. As a result the molecules are rigid and more prone to breakage.
- It is degraded reduced yield and quality if compared to fresh or frozen tissues
- Unsuitable to traditional methods such as the Northern Blot.
- It requires standardization for quantitative analytical methods, both in real-time and PCR end-point.
- Relative quantification.

DNA AND RNA ANALYSIS IN AUTOPSY TISSUES: PARAMETERS INVOLVED IN DEGRADATION

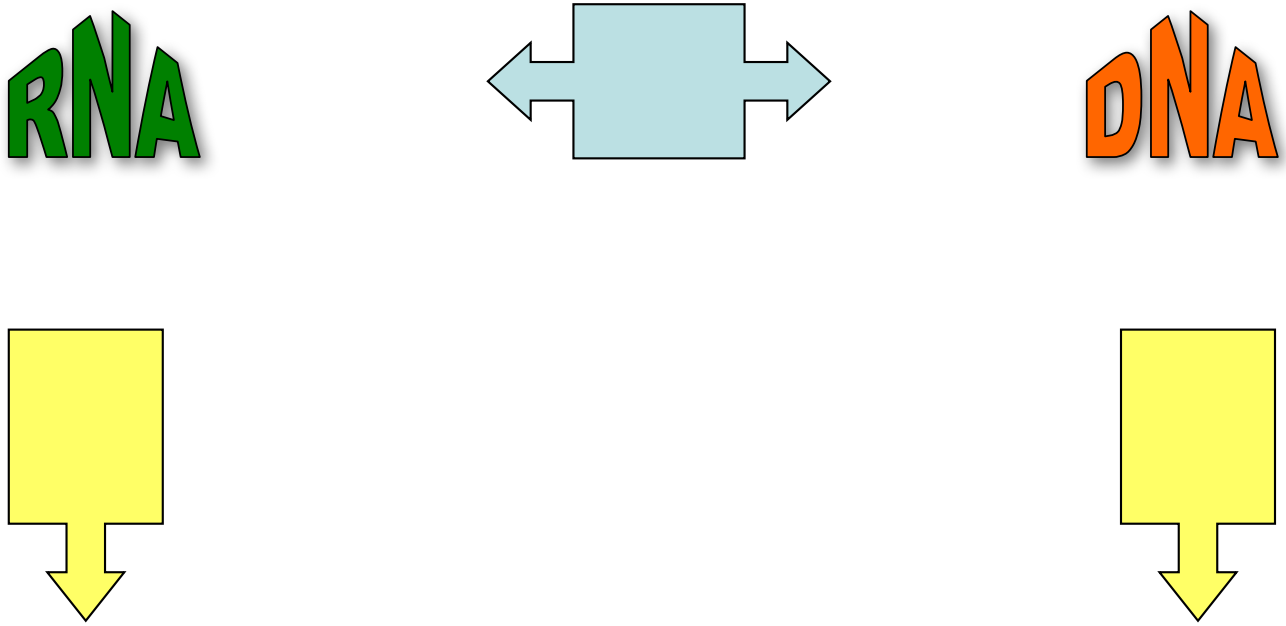
-Fixation in autoptic tissues is usually performed for a much longer period than in biopsies.

-Postmortem interval (PMI - between death and tissue collection) is often long and variable with major autolysis events.

-The level of degraded nucleic acids is related to the duration of PMI (especially for DNA) and to a longer time of fixation procedures.

-Bonin S., Petrera F., Stanta G. PCR and RT-PCR Analysis In Archival Postmortem Tissues. In Fuchs J, Podda M. "Encyclopedia Of Diagnostic Genomics And Proteomics". (Pp. 985-988). New York: Marcel Dekker Inc (United States) 2005.

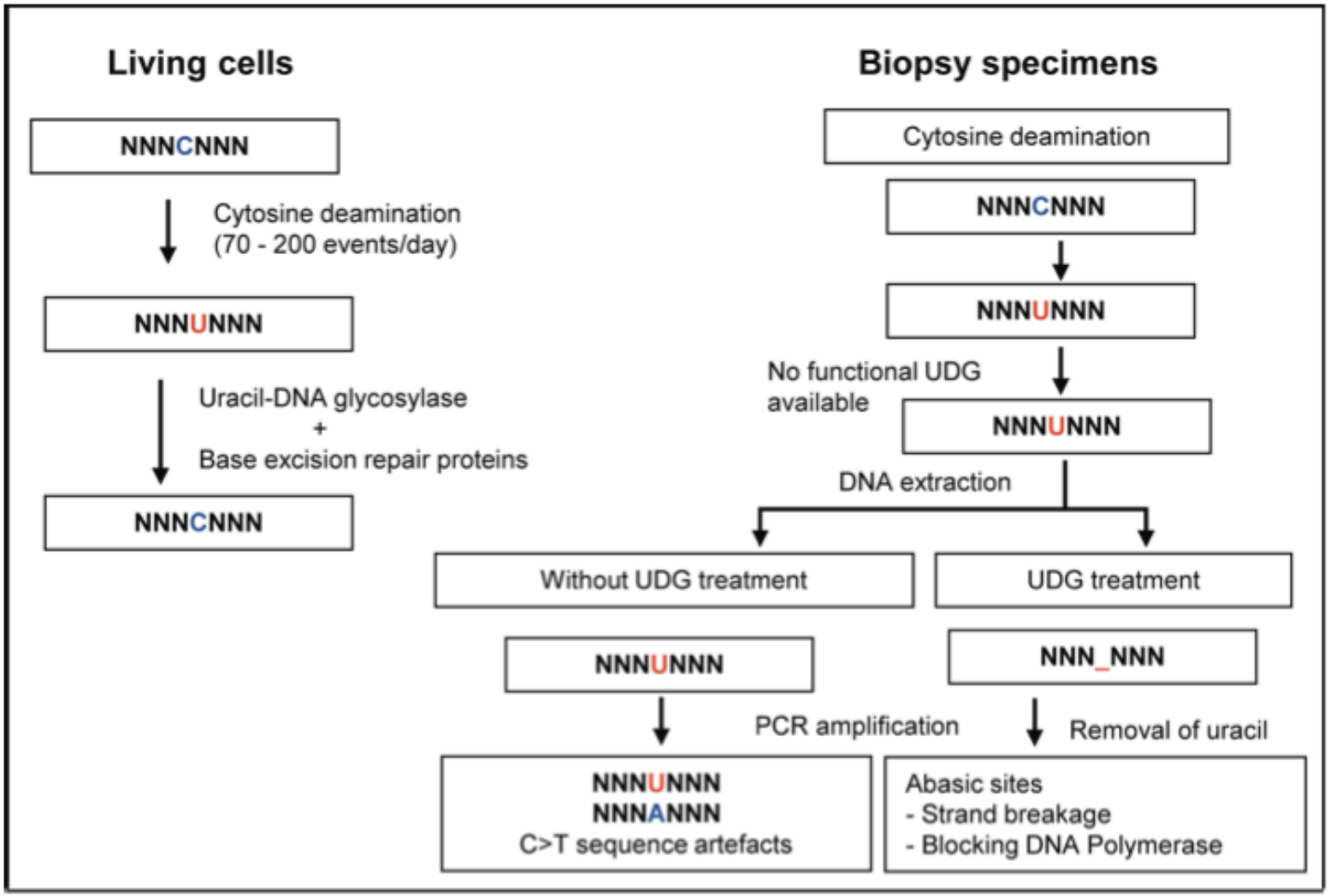
Is it possible to increase the quality of extracts?



DEMODIFICATION

PARTIAL RECONSTRUCTION

- **Cytosine deamination to uracil is a common form of DNA damage in ancient DNA**
- **Treatment of FFPE DNA with uracil-DNA glycosylase (UDG) would lead to the reduction of C>T (and G>A) sequence artefacts**



Do H and Dobrovic A Oncotarget 2012; 3: 546-558

DNA RESTORATION

- **DNA degradation is related to random breaks on a single strand.**
- **Longer DNA fragments can be obtained by rehybridizing DNA with itself, in order to use the other strand as a template.**
- **Pretreatment →DNA denaturation and incubation at 55° C for 1h in 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 2% Triton X100, and 200 μM dNTPs.**
- **Then add 1unit of Taq for "reconstruction" → at 72° C for 20 minutes.**
- **Specimens can be stored at -20° C.**
- **Before PCR long denaturation at 95° C for 5 min is needed!**

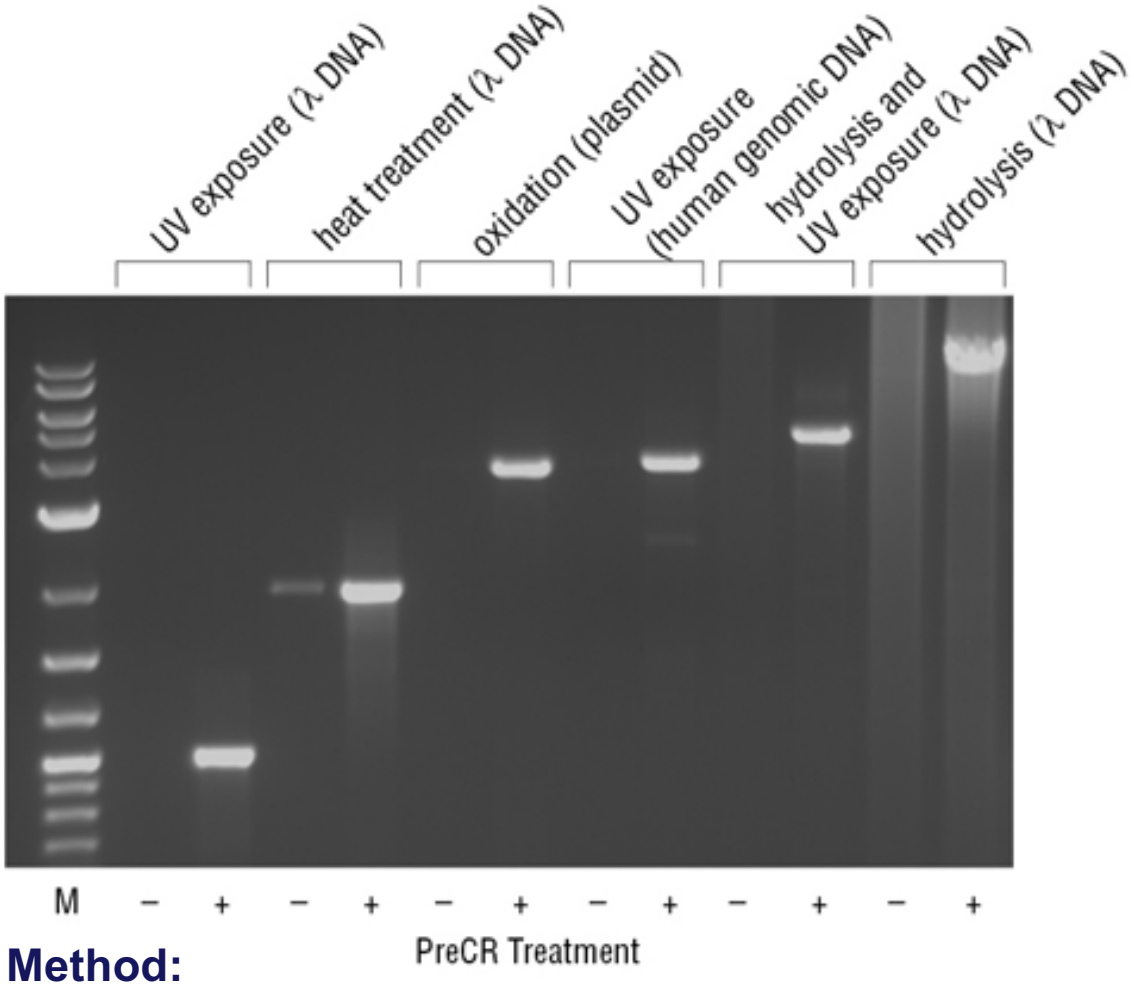
- -S Bonin,F Petrera,B Niccolini,G Stanta, "PCR analysis in archivial postmortem tissues." Mol Pathol.:56:184-186;2003

DNA RECONSTRUCTION-2

- *PreCR repair Mix is an enzyme cocktail to repair damaged template DNA prior to its use in PCR, microarrays or other techniques.*
- *It repairs damaged template DNA prior to its use in the PCR, microarrays or other DNA technologies. PreCR is active on a broad range of DNA damages, including those that block PCR (e.g. apurinic/apyrimidinic sites, thymine dimers, nicks and gaps) and those that are mutagenic (e.g. deaminated cytosine and 8-oxo-guanine). In addition, it will remove a variety of moieties from the 3´end of DNA leaving a hydroxyl group. The Mix will not repair all damages that inhibit/interfere with PCR.*

Enzyme	Substrate	Function
Endonuclease IV	AP sites in ss or dsDNA	Cleaves phosphodiester bond at 5´ of AP site; removes unsaturated aldehyde and phosphate from 3´ termini; leaves 5´ deoxyribose-5-phosphate and 3´ hydroxyl groups
T4 Pyrimidine-dimer-glycosylase (T4 PDG)	Cis-syn-cyclobutane pyrimidine dimers	Cleaves N-glycosydic bond at 5´ end of dimer to create AP site; removes one thymine from dimer (second thymine remains as first base at 3´ end of AP site)
Endonuclease VIII	Damaged pyrimidines in dsDNA	Cleaves N-glycosydic bond at 5´ end of damaged base to create AP site; cleaves at 5´ and 3´ end of AP site to create 3´ phosphate (via 3´ a,b-unsaturated aldehyde) and 5´ phosphate termini, respectively
Formaminidopyrimidine-DNA-glycosylase (FPG)	Damaged purines in ss or dsDNA	Cleaves N-glycosydic bond at 5´ end of damaged base to create AP site; cleaves at 5´ and 3´ end of AP site to create 3´ phosphate (via 3´ a,b-unsaturated aldehyde) and 5´ phosphate termini, respectively
Taq Ligase	dsDNA	Catalyzes formation of phosphodiester bond between juxtaposed 5´ phosphate and 3´ hydroxyl termini in dsDNA; cannot repair ss nicks; activity less optimal at 37°C
Bst DNA Polymerase	dsDNA	DNA polymerase with 5´-3´ exonuclease nick translation; no 3´-5´ exonuclease proof-reading; no strand displacement
Uracil-DNA-glycosylase (UDG)	Deaminated cytosines in ss or dsDNA	Cleaves N-glycosydic bond of uracil to create AP site

DNA RECONSTRUCTION-2



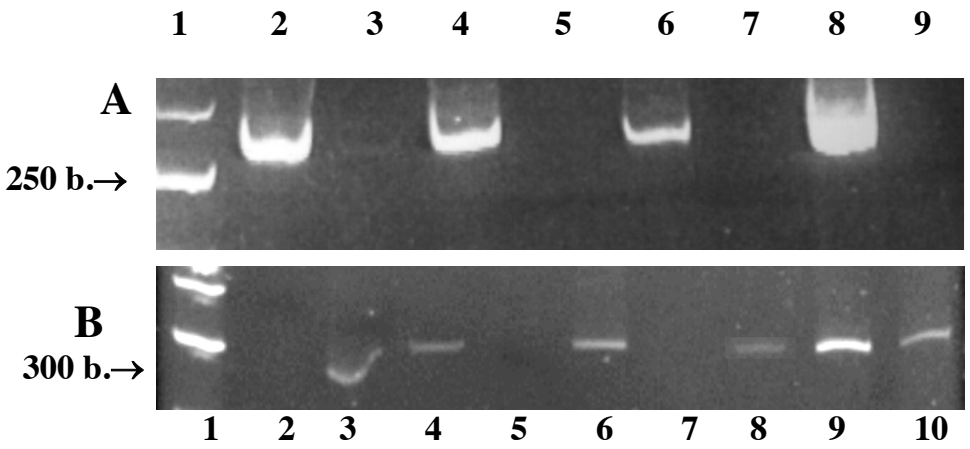
DNA Damage	Cause	Can it be repaired by the PreCR Repair Mix?
abasic sites	hydrolysis	yes
nicks	hydrolysis nucleases shearing	yes
thymidine dimers	UV radiation	yes
blocked 3´-ends	multiple	yes
oxidized guanine	oxidation	yes
oxidized pyrimidines	oxidation	yes
deaminated cytosine	hydrolysis	yes
fragmentation	hydrolysis nucleases shearing	no
Protein-DNA cross-links	formaldehyde	no

Method:

- **At rt, combine 1X Buffer, 100 µM dNTPs, 1X NAD+, damaged template DNA and H_2O to 46 µl.**
- **Add 1 µl of the PreCR Repair Mix, and mix gently.**
- **Incubate the repair reaction for 15-20 minutes at 37° C.**
- **Place the reactions on ice.**
- **Add the primers, a second aliquot of dNTPs (another 100 µM) and the PCR polymerase directly to the repair reaction mix.**
- **Proceed with the PCR amplification protocol.**

DNA analysis in autopsy tissues

PCR ANALYSIS IN BIOPSY AND AUTOPSY TISSUES



PCR products of DNA obtained from autopsy archive tissues. (A) Postmortem sample analysis for the Apolipoprotein E gene (287 bp). Size marker (lane 1), postmortem samples with the restoration (lanes 2, 4, 6 and 8), the same postmortem samples without the restoration step (lanes 3, 5, 7 and 9). (B) Postmortem samples analysis for the TTR gene (291 bases). Size marker (lane 1), postmortem samples with the restoration treatment (lanes 2-10).

Table 1: PCR amplification of DNA of autopsy and biopsy origin

Amplicon	Restored and denatured autopsy DNA	No restored biopsy DNA
ApoE[1] (287 bp)	4/4	4/4
TTR1[2] (291 bp)	6/9	9/9
TTR1 (339 bp)	1/9	9/9

1 Apolipoprotein E

2 Human prealbumin gene

-Bonin S., Petrera F., Stanta G. "Encyclopedia Of Diagnostic Genomics And Proteomics". (Pp. 985-988). New York: Marcel Dekker Inc (United States) 2005.

RNA analysis in autopsy and biopsy tissues

RT-PCR IN BIOPSY AND AUTOPSY TISSUES COMPARISON

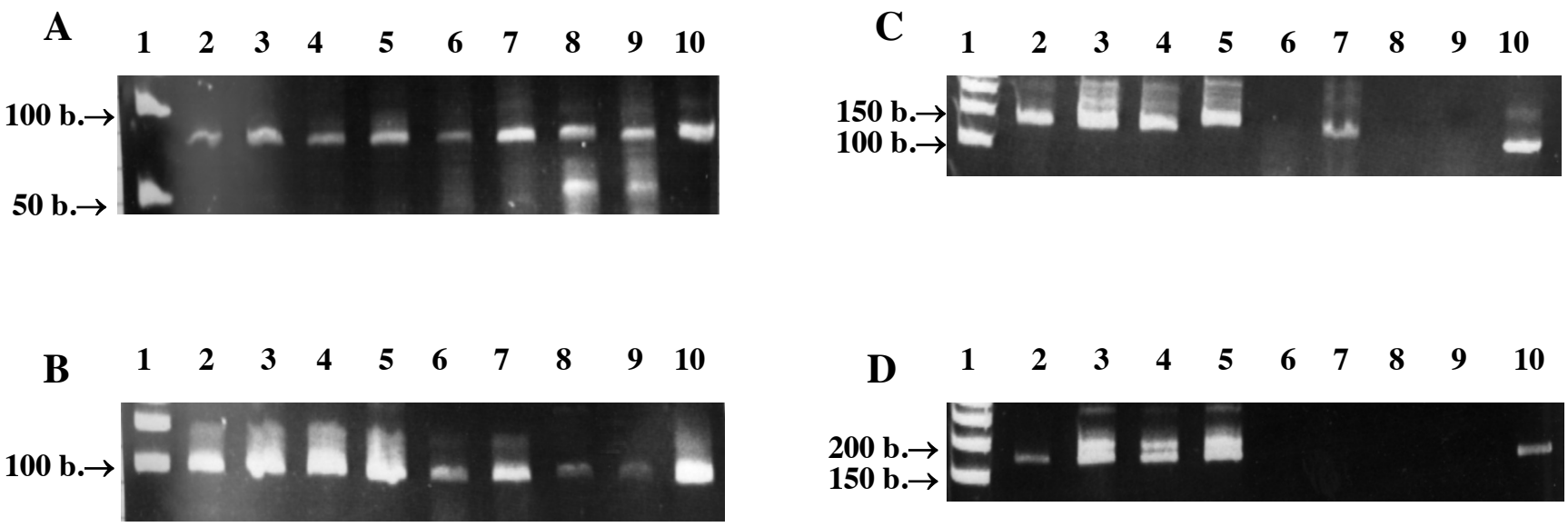


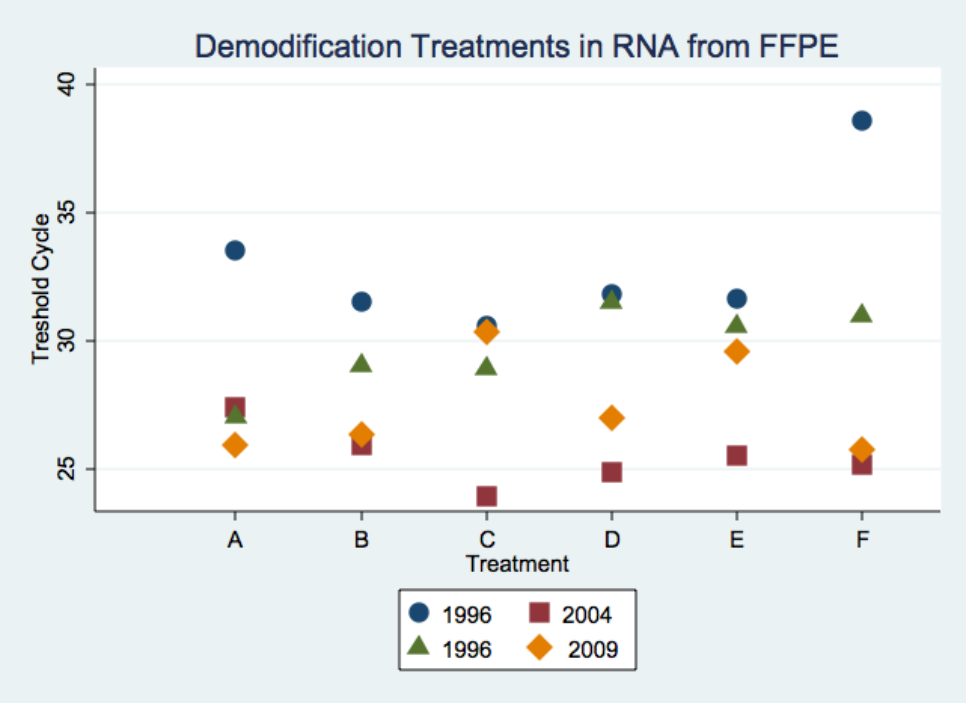
Table 2: RT-PCR amplification of b-Actin RNA of autopsy and biopsy origin

b Actin Amplicon size	Autopsy RNA	Biopsy RNA
77 bp	4/4	4/4
100 br	4/4	4/4
120 bp	1/4	4/4
170 bp	0/4	4/4

RNA DEMODIFICATION

- The addition of CH_2-OH confers to RNA resistance to RT.
- The level of modification of the 4 bases is different (40% A ÷ 4% U).
- Those grous can be removed by heating in buffers w/o formalin

- **Method:** dissolve the extract in TE buffer 1X pH 7.5 and incubate for 20 min. at 70° C. The method allows obtaining lower Ct in real time PCR, it is suggested for old samples.



miRNA Isolation

- Methods for RNA from FFPE have been optimized to extract longer RNAs.
- Extensive crosslinking of miRNAs with proteins in the fixation makes miRNAs more resistant to extraction. Enzymatic degradation before and during fixation and chemical degradation results in a reduction in the quantity and integrity of RNAs.
- The accessibility of miRNAs from FFPE does not seem to depend on fixation and appears to be similar to that of fresh tissues.

They are analyzed after RT in relatime with:

-TaqMan MicroRNA reverse transcriptase kit or TaqMan MicroRNA assay kit (Applied Byosistems, California, CA)

-Alternatively: il Locked nucleic acid (LNA) based miRNA array .

CONCENTRATION OF NUCLEIC ACIDS

SPECTROPHOTOMETRIC METHOD FOR DNA AND RNA

For ds DNA ***[DNA]= A_{260}* dilution factor*50*10^{-3} (µg/µl)***

For ss DNA ***[DNA]= A_{260}* dilution factor*33*10^{-3} (µg/µl)***

For RNA ***[RNA]= A_{260}* dilution factor*40*10^{-3} (µg/µl)***

The ratio A_{260} / A_{280} is used to evaluate the purity level of nucleic acids with respect to proteins, phenol, etc. Contamination by other nucleic acids is not considered. A ratio higher than 1.5 and ≤2 means good purity.

The ratio A_{260} / A_{230} is used to evaluate the contamination level by carbohydrates and salts. A ratio higher than 1.5 means that A_{260} reflects the real concentration of the nucleic acid.

FLUOROMETRIC METHOD FOR DNA OR RNA

Incubation with fluorochrome (Hoechst 33258 or PicoGreen for DNA and RiboGreen for RNA) and comparison with standard curve. Fluorimeter based.

ETHIDIUM BROMIDE METHODS

Agarose plate or minigel (comparison with known standards). Spotting directly the tested DNA on agarose gel prepared in a small Petri capsule together with 4 or 5 reference DNAs, of known concentration. Evaluation at a UV transilluminator by direct comparison.

QUALITY OF NUCLEIC ACIDS

Electrophoresis:

Agarose gel electrophoresis for DNA and denaturing agarose gel electrophoresis for RNA

PCR based methods

Amplification of fragments of increasing length (both for DNA and RNA).

3':5' assay using a highly expressed housekeeping gene (only for RNA) (RNA from FFPE could be deaminated!)

LAB on a chip methods

Agilent Bioanalyser 2100: electropherogram (manual method); for RNA, 28S/18S ratio (ratio method-it is the ratio of the 28S peak value to the 18S peak value. A proportion of the ribosomal bands (28S:18S) between 0.7 and 2.5 is considered to be typical of good quality RNA. The second one is the RIN number (RIN 10 is referred to intact RNA).

APPLICATIONS

Nucleic acids from FFPE are available for both qualitative and quatitative analysis using highly sensitive methods such as:

1. **End point PCR based analyses**
2. **Real-time PCR based analyses**
3. **In situ Hybrization**
4. **Microarrays technologies (only for research and followed by partial real-time PCR validation)**