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RESEARCH

The Non-Crosslinking Fixative RCL2®-CS100 is Compatible with Both Pathology Diagnosis and Molecular Analyses

Florence Boissière-Michot · Amélie Denouël · Nathalie Boulle · Carole Guillaume · Béatrice Orsetti · Evelyne Lopez-Crapez · Marie-Christine Chateau · Frédéric Bibeau

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Abstract Formalin is the key agent for tissue fixation and pathological diagnosis. However, it poorly preserves nucleic acids and this can impair molecular studies. An alternative to formalin would be a fixative which can allow both morphologic and molecular analyses. To assess the suitability of such a fixative, breast (n=11) and colon (n=12) tumor samples were fixed in the non cross-linking RCL2®-CS100 fixative and compared to paired formalin-fixed and to frozen samples, the current standards for histology and molecular analyses, respectively. Sections from RCL2®-CS100-fixed samples showed good preservation of cellular and architectural morphology, suitable for routine diagnosis. Although some antibodies required change in the immunohistochemical procedures, quality of the immunohistochemical staining was comparable to that obtained after formalin fixation. HER2 chromogenic in situ hybridization was also successfully performed. High quality DNA could be isolated from RCL2®-CS100-fixed cancer tissues as evidenced by successful amplification of large DNA fragment, CGH array, *KRAS* and microsatellites genotyping. The quality of RNA from RCL2®-CS100-fixed samples was slightly decreased in comparison to that of RNA isolated from frozen samples, as evidenced by a decreased RNA integrity number but remained exploitable for molecular assays. Our results support the use of the RCL2®-CS100 fixative for histological diagnosis and recovery of high-quality nucleic acids for molecular applications. However, specific procedures for tissue handing and processing, essential to provide high-quality specimens, could limit its use to small target lesions which cannot be frozen without impairing their pathological evaluation.

Keywords Fixative · Formalin substitute · Nucleic acids · Morphology

F. Boissière-Michot (✉) · A. Denouël · M.-C. Chateau · F. Bibeau
Department of Pathology, Val d'Aurelle Cancer Institute,
Montpellier 34 298, France
e-mail: Florence.Boissiere@montpellier.unicancer.fr

N. Boulle
Department of Cellular Biology and Hormonology,
Arnaud de Villeneuve Hospital,
Montpellier 34 090, France

N. Boulle
INSERM U896, Institut de Recherche en
Cancérologie de Montpellier,
Montpellier 34 298, France

C. Guillaume · B. Orsetti
INSERM U896, Val d'Aurelle Cancer Institute,
Montpellier 34 298, France

E. Lopez-Crapez
Department of Biology, Val d'Aurelle Cancer Institute,
Montpellier 34 298, France

Introduction

Formalin, a buffered solution of formaldehyde, is the most widely used fixative in histology thanks to its ease of use, low cost, rather short fixation time and good tissue preservation leading to accurate routine diagnosis. As a consequence, most histological techniques are based on the use of formalin-fixed paraffin-embedded (FFPE) tissues. However, formaldehyde-based reagents display two major drawbacks. First, formaldehyde is toxic and has been classified as a human carcinogen by the International Agency for Research on Cancer and, therefore, changes in work practice are required to reduce exposure [1]. Second, formalin forms cross-links between proteins [2] and induces chemical modifications, significant fragmentation and extensive intermolecular cross-links with nucleic acids [3–5]. Consequently, it is difficult to extract adequate quantities of good quality DNA and RNA from FFPE tissues [6]. This is a major weakness nowadays, as many molecular analyses have

been developed for diagnostic, prognostic and predictive purposes. Specially optimized protocols have been implemented to recover genomic DNA from FFPE tissue sections [7], but DNA isolated for such samples is often highly fragmented [8] and this can affect the outcome of molecular assays. Some studies have reported that RNA from FFPE tissues might be used to study a limited number of target genes and that the deleterious effects of formalin could be counterbalanced by using qRT-PCR and short amplicons [9–13]. For instance, the clinical Onco*type* DX® assay was developed to allow quantitative analysis of the expression of 21 genes using FFPE tumor tissues [14–16]. To date, the only FDA-approved microarray-based gene expression assay is cleared for use with unfixed tissues [17–20]. Indeed, frozen tissues are widely acknowledged to provide the best source of intact macromolecules such as nucleic acids or proteins. However, freezing is essentially restricted to few laboratories that possess appropriate facilities for quick tissue acquisition, processing and banking. In addition, freezing is limited to bulky tumors for which both cryopreservation and routine processing for histological diagnosis and staging are possible, a condition which is less and less frequent in breast pathology due to early detection by screening mammography. Hence, in most pathology departments, formalin fixation is the standard for routine histological practice as it allows the preservation of tissue integrity for both accurate diagnosis and long term storage. Therefore, more reliable fixative methods that are compatible with both molecular analyses and histological diagnosis need to be identified and validated for applications that require genome-wide expression analysis.

We thus investigated whether fixation with RCL2®-CS100 (Excilone, Plaisir, France), a commercially available non-crosslinking formalin-free fixative, could allow both types of analysis by selecting residual samples from large surgical specimens of breast and colorectal tumor samples. Fixation in RCL2®-CS100 was carried out according to an optimized fixation protocol that can be used in routine and automated settings [21]. For optimal comparison, FFPE and snap-frozen tissues from the same samples were used as reference for histological diagnosis and nucleic acid evaluation, respectively. The suitability of the RCL2®-CS100 fixation was assessed following histopathological examination (morphology and immunohistochemistry using a large range of antibodies) and using various molecular assays used in breast and digestive pathology.

Materials and Methods

Tissue Sample Collection

The effects of RCL2®-CS100 fixation were assessed using residual samples from large surgical specimens of 11 breast tumors (8 ductal and 1 mucinous carcinomas, 1 malignant myoepithelioma and 1 phyllode tumor) and 12 colorectal adenocarcinomas (2 well differentiated, 8 moderately differentiated and 2 mucinous carcinomas) sent to the Pathology Department (CRLC Val d'Aurelle, Montpellier, France) for diagnosis. Patients were aware that their surgical specimens could be used for research purposes and data were anonymized. The analysis was approved by our local research board.

Immediately after surgical excision, a pathologist selected two samples of approximately 5×5×3 mm, leaving the rest of the specimen for routine examination. One sample was immediately put in a CryoTube™ vial (Nunc™) and snap-frozen in liquid nitrogen prior to storage at −80 °C. This sample was considered as gold standard for the nucleic acid-based assays. The other sample was fixed in cold (+4 °C) RCL2®-CS100 overnight, dehydrated, paraffin-embedded following the optimized protocol previously described [21] and stored at −20 °C until use. The FFPE portion of each tumor sample was used for routine diagnosis and was considered as reference for the morphology and immunohistochemistry studies.

Histopathological Analyses

Three-μm thick sections of paraffin-embedded tissues fixed either in formalin or in RCL2®-CS100 were mounted on SuperFrost Plus glass slides (Menzel, GmbH, Germany), deparaffinized before Hematein-Eosin-Saffron (HES) staining, or immunohistochemical studies. The pathological diagnosis and histological grading were established by two experienced pathologists (MCC, FB), blinded to the fixative used.

Immunohistochemistry was performed with the DAKO Autostainer system (DAKO, Denmark) and using antibodies that are classically employed in breast or colorectal pathology, namely anti-Estrogen Receptor-α (ER-α), -Progesterone Receptor (PR), -HER2, -Cytokeratin (CK) 5/6 and -E-cadherin antibodies (for breast tumors) and anti-CK7, -CK20, -hMLH1, -hMSH2, -hMSH6, -PMS2, -CDX2, -Villin antibodies (for colorectal tumors). Antibody clones, suppliers, antigen retrieval procedures, dilutions and staining protocols are listed in Table 1. For all tested antibodies, except HER2, immunoreactivity was assessed taking into account the percentage of marked cells (from 10 to 100 %) and the staining intensity (0: none, 1: faint, 2: moderate, 3: strong). HER2 status was determined according to the ASCO CAP guidelines [22].

Breast carcinomas that were scored as 3+ following HER2 immunohistochemistry were also assessed for *HER2* amplification by Chromogenic In Situ Hybridization (CISH). Briefly, sections were deparaffinized in xylene and rehydrated through a graded ethanol series. Heat pre-

Table 1 Antibodies and methods used for the 13 selected markers

	Marker	Antibody clone	Source	Antigen retrieval method	Antibody dilution	Staining protocol
Antibodies used for breast carcinomas	ER	6F11	Novocastra	EDTA pH 8	1/30	LSAB2
	PR	PgR 636	Dako	Citrate buffer	1/50	LSAB2
	Cerb-B2	Rabbit Polyclonal	Dako	Citrate buffer	1/600	LSAB2
	E-Cadherin	36	BD Bioscience	Citrate buffer	1/500	LSAB2
	CK 5/6	D5/16B4	Dako	EDTA pH 8	1/100	LSAB2
Antibodies used for colon adenocarcinomas	CK7	OV-TL 12/30	Dako	Citrate buffer	1/50	LSAB2
	CK20	Ks 20.8	Dako	Citrate buffer / EDTA pH9[a]	1/50	LSAB2
	MLH-1	G168-15	BD Bioscience	EDTA pH 9	1/30	ENVISION
	MSH-2	FE11	Zymed	EDTA pH 9	1/200	ENVISION
	MSH-6	44	BD Bioscience	EDTA pH 9	1/50	ENVISION
	PMS-2	A16-4	BD Bioscience	EDTA pH 9	1/200	ENVISION
	CDX2	CDX2-88	Biogenex	Citrate buffer	1/20	LSAB2
	Villine	ID2C3	Immunotech	Citrate buffer	1/80	LSAB2

[a] Citrate buffer for FFPE samples and EDTA pH9 for RCL2®-CS100-fixed samples

treatment was carried out in pre-treatment buffer at 100 °C for 15 min. Tissue sections were then digested with pepsin for 10 min at room temperature before dehydration through a graded ethanol series. Following denaturation at 95 °C for 5 min, hybridization with the ZytoDot SPEC HER2 probe (ZytoVision GmbH, Bremerhaven, Germany) was performed at 37 °C overnight. After stringent washing, the probe was immunodetected by incubating slides successively with a mouse anti-digoxigenin antibody, a Horse Radish Peroxidase (HRP)-anti-mouse antibody and diaminobenzidine as chromogen. Finally, slides were lightly counterstained with hematoxylin, dehydrated and mounted. Slide evaluation was performed by the first author, who was blinded to the fixative used, under a standard light microscope.

Nucleic Acid Extraction and Quality Assessment

Ten 10-µm thick sections from each paraffin-embedded sample were collected in RNA-free vials (Eppendorf, Le Pecq, France). The AllPrep DNA/RNA and the Allprep DNA/RNA FFPE Mini kits (QIAGEN, Courtaboeuf, France) were used for simultaneous purification of genomic DNA and total RNA from RCL2®-CS100 and FFPE samples, respectively, following the technical data sheet. As gold standard, ten 10-µm thick frozen sections of each sample were lysed and extracted in parallel. DNA was quantified using a spectrophotometer and loaded on 0.8 % agarose gels with a DNA ladder (1Kb DNA Ladder, Invitrogen, Carlsbad, CA, USA) to assess its quality.

Qualitative and quantitative assessments of the purified RNA samples were performed using an Agilent Bioanalyzer (Agilent technologies Inc., Santa Clara CA,USA). This technology evaluates RNA quality using a specific algorithm based on the ribosomal band ratio and on the presence or absence of degradation products visualized after capillary electrophoretic separation. The RNA Integrity Number (RIN) defines ten categories, ranging from 1 (totally degraded RNA) to 10 (intact RNA).

Additionally, to assess the stability of nucleic acids after long-term storing, nucleic acids from 3 breast and 3 colon tumor samples (FFPE, RCL2®-CS100 fixed and matched frozen samples) were also extracted 4 years after collection and subsequently analyzed.

PCR Amplification, KRAS Sequencing and Assessment of Microsatellite Instability (MSI)

The quality of DNA extracted from RCL2®-CS100, FFPE and snap-frozen samples was tested by performing amplification of an 84 base pairs (bp) fragment from exon 2 of KRAS, as described in Boissiere-Michot et al. [23] and of a fragment of 523-bp encompassing EGFR exon 15. The PCR products were visualized on a 3 % agarose gel stained with SYBR® Safe (Invitrogen).

DNA was further qualified by bidirectionnal DNA sequencing for detection of *KRAS* exon 2 mutations as previously described [24] and by using the MSI Analysis System (Promega, Charbonnières, France) according to the manufacturer's instructions. This kit is based on the co-amplification of two highly polymorphic pentanucleotide repeat markers (Penta C and Penta D) and five quasi-monomorphic mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27). The MSI status

is then determined by comparing the allelic profiles generated by amplification of DNA from paired normal and tumor samples. A tumor was considered MSI when at least two of the five mononucleotide markers analyzed were unstable.

Primers used for amplification and sequencing and PCR conditions are available upon request.

Array CGH Profiling

We used human *Integrachip V7* for genome profiling (IntegraGen SA, Evry, France, http://www.integragen.com). IntegraChip V7 is composed of 5984 bacterial artificial chromosome (BAC) clones that include 5514 sequenced clones with a median gap of 1 clone/0.478 Mb. DNA labeling and hybridization, were done as previously described [25] with slight modifications: 600 ng of DNA were labeled with the BioPrime Total Genomic Labelling System (Invitrogen SARL, Cergy Pontoise, France). Arrays were scanned using the Axon 4000B scanner (Molecular Devices, CA, USA) and images were analyzed using Genepix 6.0. Extracted values were then normalized, filtered and graphically represented using CAPweb 2.0 (Institut Curie, Paris, France) (http://bioinfo-out.curie.fr/CAPweb). Thresholds for losses and gains were the CAPweb default parameters (log2ratio ≤ -0.153; log2ratio ≥ 0.138 respectively). Clones were ordered according to the NCBI36/Hg18 assembly of the human genome.

RT-qPCR

Total RNA was subjected to reverse transcription using the Omniscript Reverse Transcriptase kit (QIAGEN). For each tissue sample and for the 3 experimental procedures (FFPE, RCL2®-CS100 and frozen samples), the same amount of total RNA was submitted to reverse transcription, based on the smallest RNA concentration obtained. Subsequent cDNA were then submitted to real-time PCR quantification using a LC480 instrument with LightCycler 480 SYBR Green PCR Master (Roche Diagnostics). Primers for β2microglobulin (β2M) and RS9 generated amplicons of 176 bp and 262 bp, respectively (primers available upon request).

This strategy allowed us to compare the β2M and RS9 cycle thresholds (Ct) obtained for the various studied conditions that are directly related to the amount of amplifiable DNA.

Statistical Analysis

Nucleic acid yields were analyzed using the non-parametric Wilcoxon matched pairs test. Statistical significance was set at <0.05.

Results

Effects of RCL2®-CS100 Fixation on Tissue Morphology

Microscopic examination was used to compare RCL2®-CS100-fixed and paired FFPE samples according to standard diagnostic procedures. Fixation with RCL2®-CS100 preserved tissue integrity and particularly the cellular and sub-cellular details. Tissue retractions were observed in few RCL2®-CS100- and formalin-fixed sections, but this artifact did not impair the appreciation of the global tissue architecture or of the cellular details. In all studied cases (*i.e.* 11 breast and 12 colorectal tumors), histological analysis was easy and an accurate diagnosis could be established. Representative examples are shown in Fig. 1. The same SBR grade was given to all paired tumor samples, excepted for two breast adenocarcinomas (samples 2 and 3) in which the SBR grade was lower in the RCL2®-CS100 tumor than in the FFPE counterpart due to higher tubular differentiation and lower nuclear pleomorphism (Table 2).

Effects of RCL2®-CS100 Fixation on Immunostaining and In Situ Hybridization

In breast tumors, expression of ERα, PR and HER2 by immunostaining was similar in paired formalin- and RCL2®-CS100-fixed tissue samples (Fig. 2) without adjustment of the immunohistochemistry protocols. Seven of the nine breast carcinomas were diagnosed as ERα-positive and two as negative (*i.e.* less than 10 % of stained nuclei) whatever the fixative used (Fig. 2a, b, i). Similarly, four tumors were diagnosed as PR-positive (*i.e.* more than 10 % of stained nuclei) and five as negative (Fig. 2c, d, j). Two of the nine tested breast carcinomas were scored as 3+ for HER2 expression (Fig. 2e, f) and *HER2* amplification was confirmed by CISH in both cases (Fig. 2g, h). Among the breast carcinomas with a 0 or 1+ score for HER2 expression, only one discrepancy (tumor 9) between fixatives was observed (0 for the formalin- and +1 for the paired RCL2®-CS100-fixed sample). Finally, expression of E-Cadherin and CK5/6, two other routinely tested breast tumor markers, was comparable whatever the fixative used (data not shown).

Similar results for both fixatives were obtained also when the expression of several colorectal tumor markers that are classically used for diagnostic purposes was assessed (Fig. 3). Only the immunostaining procedure used to evaluate CK20 expression required some modifications (*i.e.*, antigen retrieval with EDTA buffer pH9 for RCL2®-CS100-fixed tissues instead of citrate buffer pH6) to achieve comparable immunostaining in RCL2®-CS100 and FFPE samples (Fig. 3a, b). Of note, one of the 12 colorectal carcinomas displayed loss of MSH2 and MSH6 expression in both the formalin- and RCL2®-CS100-fixed tissue samples (data not shown).

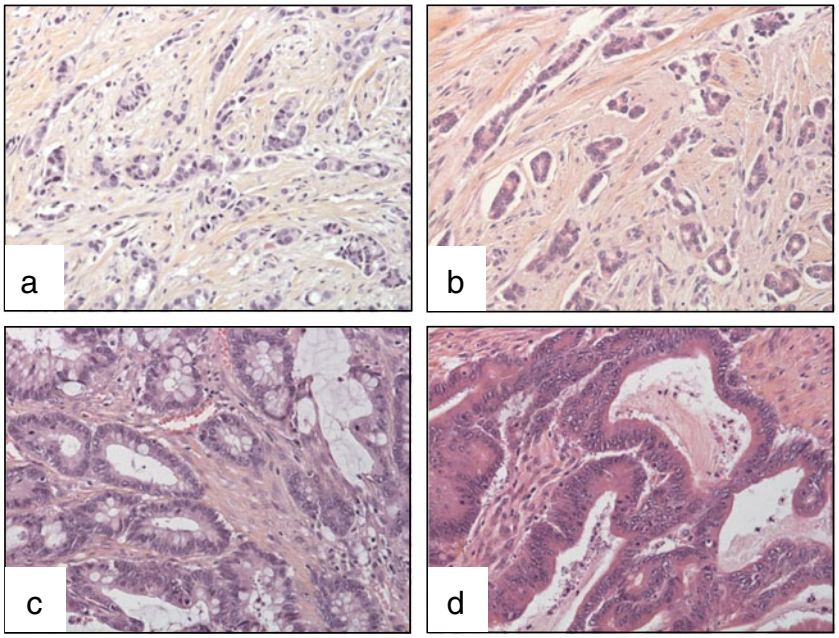

Fig. 1 Breast tumor (**a**, **b**) and colorectal adenocarcinoma (**c**, **d**) fixed in formalin (a, c) or RCL2®-CS100 (b, d). Preserved morphology on HES-stained sections (original magnification×200)

Effects of RCL2®-CS100 Fixation on DNA

Total DNA extracted from RCL2®-CS100-fixed tumors was quantified by spectrophotometry, submitted to electrophoresis and compared to the reference DNA extracted from the frozen portion of the same tumors. The mean (± SD) yield (ng of extracted DNA /mm^3 of tissue) of DNA isolated from RCL2®-CS100-fixed tumor tissues was lower than the one from paired frozen samples (breast: 880±864 *vs* 1180± 592 ng/mm^3, p=0.0409, n=11; colon: 871±471 *vs* 1562± 447 ng/mm^3, p=0.0047, n=12, respectively). The quality of the DNA extracted from RCL2®-CS100-fixed tumors was superior to that recovered from FFPE paired samples: it migrated as a smear showing fragments in size of several hundred bp whereas DNA from FFPE samples was much more fragmented on agarose gel. As expected, DNA extracted from frozen samples appeared to be of highest quality as it migrated as a band of high molecular weight (Fig. 4a).

DNA extracted from frozen and RCL2®-CS100-fixed tumors allowed amplification of both the 84-bp and the 523-bp amplicons. In contrast, we failed to amplify the 523-bp amplicon whereas successful amplification of the shorter amplicon was achieved with total DNA extracted from FFPE tumors (Fig. 4b).

KRAS sequencing and MSI assessment were successfully performed using DNA extracted from colorectal tumor samples fixed in RCL2®-CS100 (Fig. 4c and d). Moreover, the copy number alterations identified by microarray-based

Table 2 SBR grading in FFPE and RCL2®-CS100 fixed breast tumor carcinomas

#	Diagnosis	FFPE samples					RCL2®-CS100-fixed samples				
		Tubule Differentiation	Nuclear Pleomorphism	Mitotic Index	Mitotic Count[a]	SBR Grade	Tubule Differentiation	Nuclear Pleomorphism	Mitotic Index	Mitotic Count[a]	SBR Grade
1	Ductal Carcinoma	3	3	2	18	III	3	3	2	16	III
2	Ductal Carcinoma	2	3	1	6	II	1	2	1	4	I
3	Ductal Carcinoma	3	3	2	16	III	2	2	2	19	II
4	Ductal Carcinoma	3	2	1	5	II	3	2	1	2	II
5	Ductal Carcinoma	3	3	2	20	III	3	3	3	39	III
6	Mucinous carcinoma	3	2	1	2	II	3	2	1	2	II
7	Ductal Carcinoma	3	3	3	34	III	3	3	2	18	III
8	Ductal Carcinoma	2	2	2	14	II	3	2	2	22	II
9	Ductal Carcinoma	3	3	3	26	III	3	3	2	19	III

[a] Expressed as the number of mitotic figures per 10 high-power fields

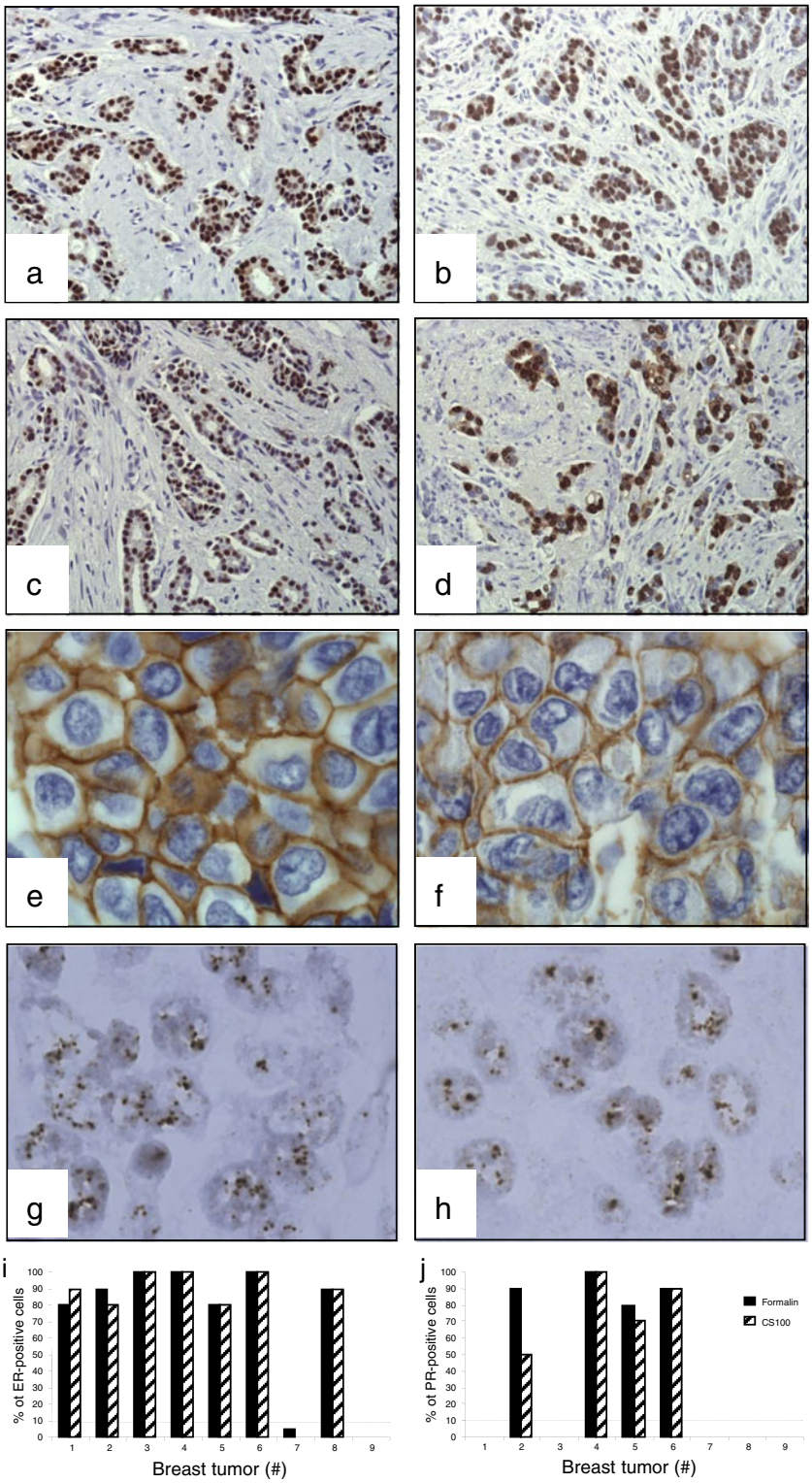

Fig. 2 Immunohistochemistry to assess ER (**a**, **b**), PR (**c**, **d**), HER2 (**e**, **f**) expression and *HER2* chromogenic in situ hybridization (**g**, **h**) in formalin- (a, c, e, g) and RCL2®-CS100 (b, d, f, h) -fixed paired ductal breast carcinomas. For each paired sample, the percentage of nuclei stained for ER (i) and PR (j) is reported (immunoperoxidase, × 200 (a, b, c, d) or× 1000 (e, f, g, h))

CGH assay, which requires high quality DNA, were similar in frozen and RCL2®-CS100-fixed colon and breast tumor samples, although a slight increase in the variance across ratios was observed in RCL2®-CS100-fixed samples as indicated by the noisier smoothing line (Fig. 4e and f).

Effects of RCL2®-CS100 Fixation on RNA

The effect of RCL2®-CS100 fixation on RNA yield and quality was assessed using the Agilent technology. Total RNA extracted from RCL2®-CS100-fixed samples was compared to the reference RNA extracted from the snap-

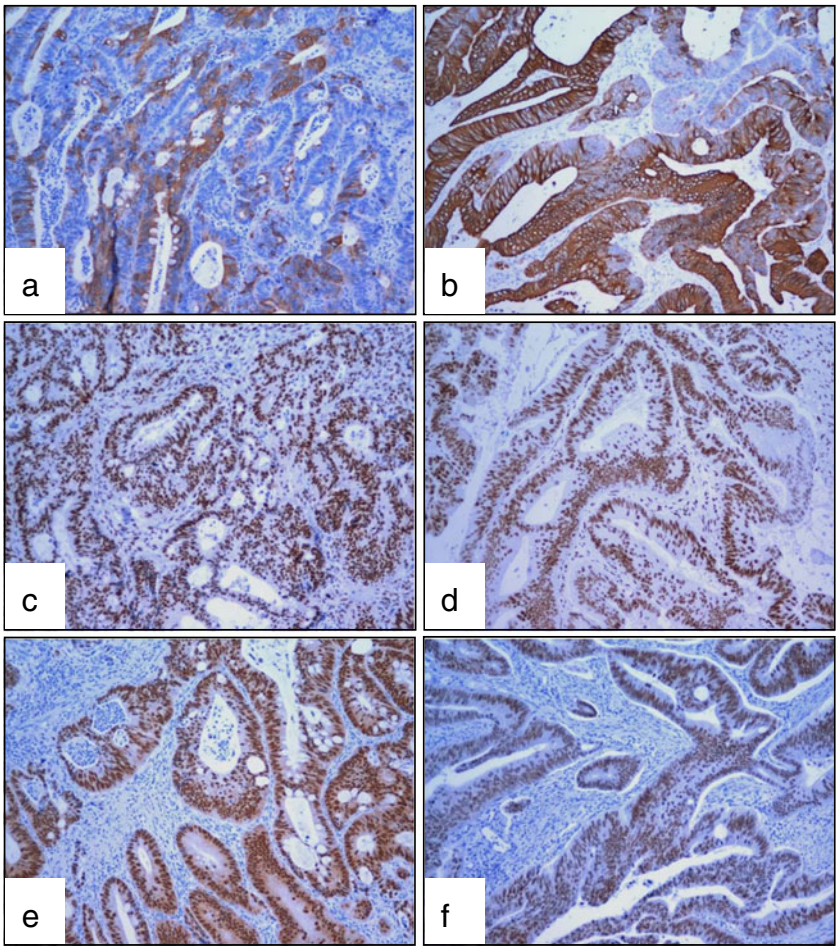

Fig. 3 Immunohistochemistry to assess CK20 (**a**, **b**), hMLH1 (**c**, **d**) and CDX2 (**e**, **f**) expression in formalin- (a, c, e) and RCL2®-CS100-fixed (b, d, f) paired colon adenocarcinomas (immunoperoxidase, × 100)

frozen portion of the same tumors. RNA yields from RCL2®-CS100-fixed tumors were highly variable in comparison to those from the paired frozen samples (breast tumors: from −73 to +80 %; colorectal tumors: from −92 to +101 %). Despite this high variation, the RNA yield (mean ± SD) was significantly lower for RCL2®-CS100-fixed colorectal carcinomas (1660±1586 *vs* 2748±1653 ng/mm^3 for frozen samples, p=0.0186; n=12) but not for RCL2®-CS100-fixed breast tumors (1753±1686 *vs* 1768±956 ng/mm^3, p=0.6566; n=11).

A slight but significant degradation of the total RNA extracted from RCL2®-CS100-fixed tumors was observed, resulting, for each sample, in a loss of about two RIN points (means ± SD): from 8.9±0.2 and 8.6±0.6 for RNA from frozen breast and colorectal cancer tissues to 6.9±0.4 and 6.3±0.4 for the RNA isolated from RCL2®-CS100-fixed breast and colorectal tumors, respectively (Fig. 5a for breast and 5b for colorectal tumors). Examples of electropherogram profiles are shown for RNA from breast and colon tumors (Fig. c, e and d, f, respectively).

The effect of fixation on RNA quality was further studied using an RT-qPCR approach, by comparing the Ct obtained for 3 breast and 3 colon carcinoma samples, frozen or fixed in both conditions (RCL2®-CS100 and formol fixed). Two housekeeping genes, RS9 and β2M, were evaluated at the time of tissue fixation (T0) and 4 years later (T4). Results are presented in Table 3. Reproducible Ct values were obtained after real time RT-qPCR for β2M and RS9 genes from frozen samples, with mean Ct values at T0 around 19 and 21, respectively. When comparing RNA from RCL2®-CS100-fixed and frozen tissue on T0, the average shift in real-time RT-qPCR was in the range of 0.4 - 4 Ct, suggesting that some mRNA degradation occurred after RCL2®-CS100 fixation, in line with the results obtained from RIN analysis. However, this degradation was far less important than that observed for the formol fixed tissues as results obtained from FFPE tissues indicated very low levels of β2M and RS9 mRNA levels (Ct > 33 when amplifiable, no amplification products for 1 and 5 out of 6 samples, for β2M and RS9 genes, respectively). Moreover, analysis of data indicated that mRNA from frozen tissues stored for 4 years is slightly more degraded than those extracted shorter after freezing. We observed an average shift in real-time RT-qPCR in the range of 0.7 and 1.8 for β2M and RS9, respectively. Both housekeeping genes mRNA appeared more stable in the RCL2®-CS100-fixed samples between

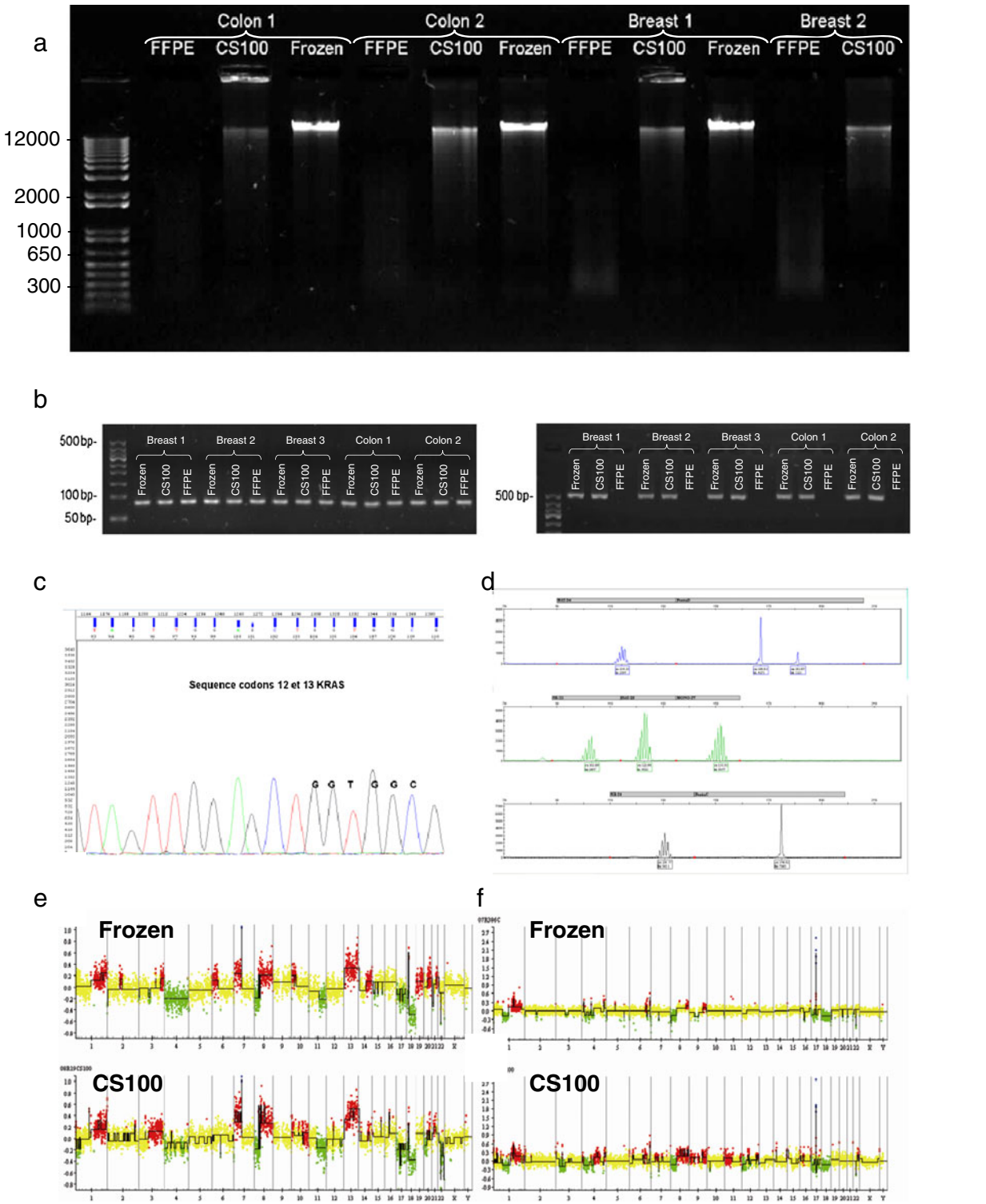

Fig. 4 Effects of RCL2®-CS100 fixation on DNA. Electrophoresis of DNA extracted from colon and breast carcinomas fixed in RCL2®-CS100 (CS100) and compared to paired frozen and FFPE tumor samples (200 ng of DNA were loaded in each well) (**a**). A 84 bp (left panel) or a 523 bp (right panel) fragment was amplified using DNA extracted from frozen samples or samples fixed in RCL2®-CS100 (CS100) or formalin (FFPE) (**b**). Representative *KRAS* sequencing (**c**) and microsatellites analysis (**d**) of DNA extracted from a RCL2®-CS100-fixed colon adenocarcinoma. Whole genome array CGH profiles obtained from paired colon (**e**) and breast (**f**) tumor samples to compare frozen (top) and RCL2®-CS100-fixed (bottom) tissues. Chromosomes are separated by grey vertical lines. Copy number alterations are represented as follows: green dot, loss; red dot, gain; yellow dot, no copy number alteration; blue dot, amplification. The black line corresponds to the smoothing line showing the segmented regions of gain and loss along the chromosomes

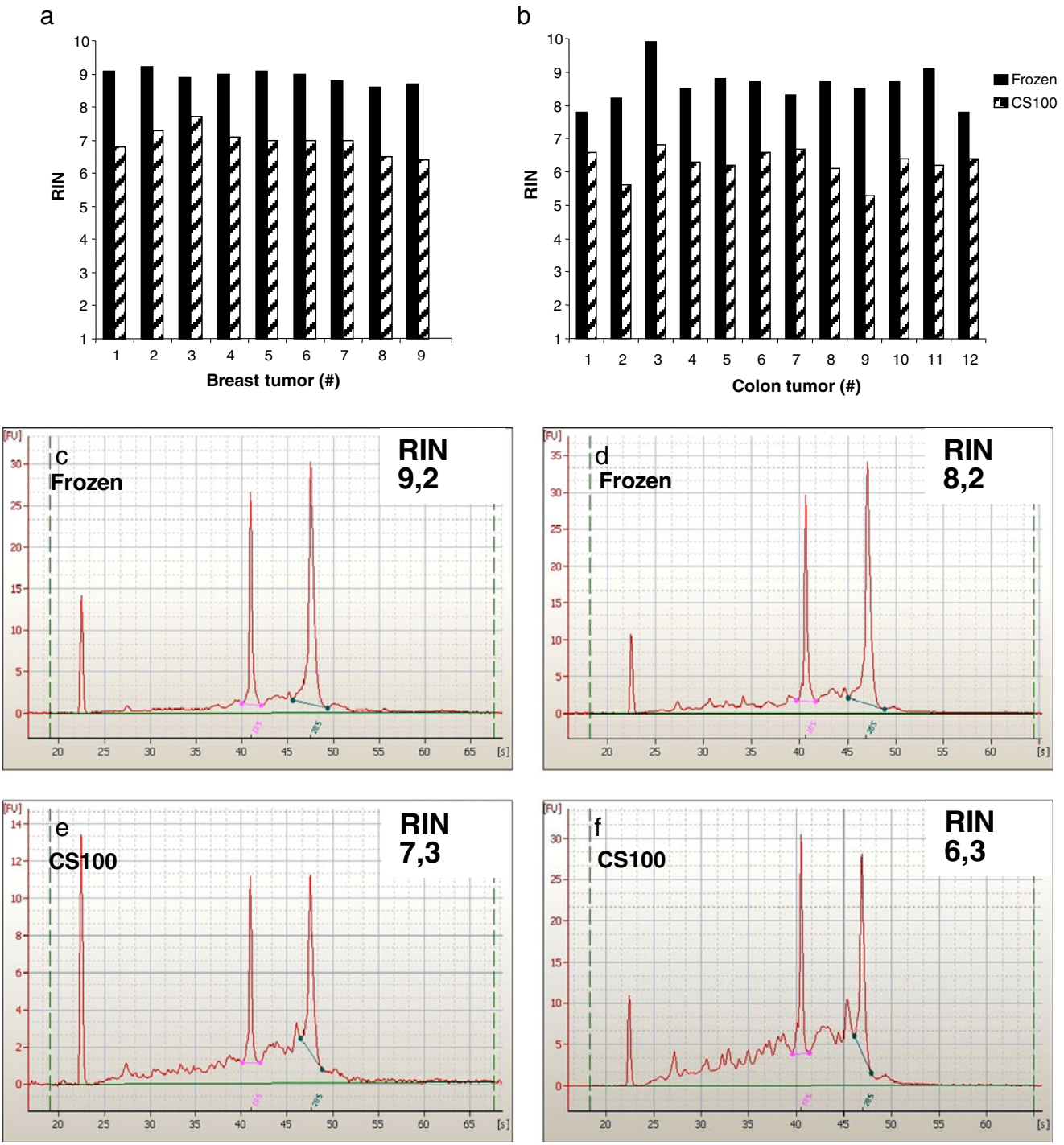

Fig. 5 Qualitative analysis of RNA extracted from paired frozen- and RCL2®-CS100-fixed breast tumors (a, c, e) and colon adenocarcinomas (b, d, f). The integrity of RNA extracted from breast (**a**) and colon (**b**) tumor samples was evaluated with the Agilent technology. Examples of electropherogram profiles of RNA from paired frozen (top) and RCL2®-CS100-fixed (bottom) breast (**c**, **e**) and colon adenocarcinomas (**d**, **f**)

T0 and T4 as suggested by Ct analysis, which was in contradiction with the slight decrease of RNA quality as evidenced by the reduced RIN in RCL2®-CS100-fixed samples stored for 4 years (Table 3).

Discussion

In this study, we tested the commercially available RCL2®-CS100 fixative in a routine, automated setting to assess

Table 3 Effect of fixative and long-term storage on RNA quality

		Sample	Frozen		CS100		Formol
		ID	T0	T4	T0	T4	T4
RIN		Breast #4	9.0	9.5	7.1	7.5	-
		Breast #7	8.8	9.9	7.0	6.7	2.2
		Breast #8	8.6	8.9	6.5	5.3	2.5
		Colon #4	8.5	8.7	6.3	4.9	2.3
		Colon #5	8.8	10.0	6.2	6.1	2.4
		Colon #6	8.7	9.7	6.6	4.9	2.5
Ct values	β2M	Breast #4	18.92	20.00	22.41	20.50	36.41
		Breast #7	18.94	19.62	21.54	22.18	33.25
		Breast #8	19.24	20.09	21.66	21.05	33.43
		Colon #4	19.76	20.01	23.50	22.40	35.61
		Colon #5	19.67	20.47	22.57	21.84	-
		Colon #6	19.83	20.37	22.41	22.06	40.00
	RS9	Breast #4	20.79	22.79	22.22	22.23	35.88
		Breast #7	22.56	23.84	22.99	23.89	-
		Breast #8	22.51	24.20	23.15	22.07	36.02
		Colon #4	22.63	23.82	23.72	23.97	-
		Colon #5	21.97	24.13	24.49	22.95	-
		Colon #6	22.39	24.64	24.35	23.77	-

- : Unsuccessful amplification

whether it could allow reaching a compromise between morphological integrity for correct histomorphological diagnosis and nucleic acid preservation. Our results indicate that the morphological diagnosis and the immunohistochemical investigations that are routinely carried out in case of breast and colorectal tumors are not compromised by fixation in RCL2®-CS100. DNA isolated from RCL2®-CS100-fixed tissues was successfully used for array CGH assays, assessment of the MSI status and *KRAS* mutations, and amplification of large DNA fragments which cannot be amplified in FFPE samples. Finally, most of the RNA extracted from RCL2®-CS100-fixed tissues displayed a relatively preserved integrity.

According to the patent application, RCL2®-CS100 is a non-aldehydic compound composed of trehalose, ethanol, acetic acid and water. This alcohol-based fixative is supposed to preserve macromolecules through denaturation and precipitation of proteins and should not alter nucleic acids. Previous studies have reported that RCL2 fixation of normal colonic mucosa [26], breast tumors [27], various thyroid pathologies [28] or brain tumor biopsies [29] does not seem to significantly compromise histological and immunohistochemical analyses and preserves macromolecules (DNA, RNA, proteins) in a better way than formalin fixation. However, some of these studies were based on the use of a non-commercial version of RCL2 and on manual fixation and/or embedding procedures that are unsuitable for a routine application. The strength of our study lies in the use of an optimized RCL2 fixation protocol [21] that can be implemented in routine and automated laboratory settings.

As previously reported for breast tumors [27], we found that RCL2®-CS100 fixative is suitable for morphological diagnosis of breast and colorectal tumors. All but two RCL2®-CS100-fixed breast adenocarcinomas were classified as having the same SBR grade than the FFPE counterpart. The SBR grade was lower in the two discordant cases, possibly due to tumor heterogeneity (the RCL2®-CS100-fixed samples were of smaller size than the ones used for the routine pathological diagnosis), or due to the part of subjectivity inherent to the SBR system [30].

Immunohistochemistry was successful for all the tested routine markers, without any modification of the protocols, except for the anti-CK20 antibody in colon tumors, since the procedure had to be optimized to get results comparable to those obtained in FFPE samples. In addition, *HER2* amplification in the two breast tumors that were scored 3+ was confirmed by CISH in both FFPE and RCL2®-fixed samples. Altogether, our data support the use of RCL2®-CS100 for breast and colon tumor histopathological diagnosis.

Although DNA isolated from FFPE-tissues has been often used for DNA amplification, mainly to assess single-nucleotide polymorphisms or DNA mutations, such DNA is often partially degraded and displays some sequence alterations [31]. Here, we demonstrated that DNA extracted from RCL2®-CS100-fixed tumors was always of better quality than the DNA extracted from FFPE tumors. Electrophoresis

performed with DNA extracted from RCL2®-CS100-fixed tissues demonstrated the presence of high molecular weight bands, suggesting the recovery of large DNA fragments. Similarly, Delfour and colleagues reported that a 850 bp long amplicon generated from DNA extracted from samples fixed in methacarn or an earlier version of RCL2 could be sequenced [27], and Preusser et al. could amplify high-molecular weight DNA from RCL2®-CS100 samples, even after prolonged fixation times [29]. In addition to the amplification of large DNA fragments, we also shown that DNA extracted from RCL2®-CS100 fixed samples was suitable for array CGH assays, an assay that requires high quality DNA, as well as for the assessment of the MSI status and of *KRAS* mutations, two relevant molecular assays that are routinely performed for colorectal cancer management. Importantly, 4 years storage of the samples did not impact the quality of the DNA. Altogether, these data strongly support RCL2®-CS100 fixation as a promising solution to preserve DNA.

RNA can be rapidly degraded by the almost ubiquitous RNase enzymes and due to its intrinsically low stability. Immediate freezing of tissue specimens after surgery is widely acknowledged to preserve RNA integrity. However, conservation of frozen tissues requires a precise logistic management and can only be performed for large tumors. Although the expression of a limited number of genes can be assessed using RNA extracted from FFPE tissues and specific technologies [15, 32–34], RNA in such tissues is often highly degraded and is not suitable for genome-wide expression assays [34, 35]. Moreover, RNA continues to degrade over time when archive storage is performed at room temperature [11, 36]. This is a major issue since RNA integrity has been shown to significantly impacts on the relative gene expression [37, 38] and a RIN $\geq$ 6 is recommended to provide reproducible microarray results [39]. Here we show that, despite a slight degradation observed in the RNA extracted from RCL2®-CS100-fixed tumors, all but two of the RNA samples isolated from RCL2®-CS100-fixed cancer tissues displayed a RIN > 6, a quality higher than what obtained for FFPE samples [34]. As for DNA, RNA extracted from RCL2®-CS100-fixed tumors was always of better quality than the RNA extracted from formalin-fixed tumors. Importantly, we observed a good preservation of mRNA integrity throughout several years, as limited variations in mRNA levels encoding two housekeeping genes were observed 4 years after samples collection. Altogether, our results are in line with those by Dotti et al. who recently reported that alcohol-based fixatives were a good solution for fixation of tissue samples by virtue of their effects on mRNA preservation [35]. We hypothesize that the quantitative and qualitative differences observed between nucleic acids extracted from frozen and RCL2®-CS100 samples are mainly linked to the obligatory additional steps required for obtaining tissue blocks. Indeed, although RCL2®-CS100 preserves nucleic acids, the mandatory steps of fixation, processing, paraffin-embedding and deparaffinization before lysis are all putative pitfalls that might affect their recovery.

Although tested in a routine setting, it must be emphasized that we used small 3–4 mm thick pieces of tumors for RCL2®-CS100 fixation. This procedure is mandatory as RCL2®-CS100 is an alcohol-based fixative and its mechanism relies mainly on tissue dehydration, leading to a slower penetration in tissues than with formalin [40]. We thus think that the major issue for obtaining both accurate morphology and nucleic acid integrity concerns the fixation of large surgical specimens. Indeed, large samples should be quickly cut into 3–4 mm thick sections and dipped in a large volume of cold RCL2®-CS100 to ensure a proper fixation. This step could be challenging for some specimens as it would require performing the macroscopic examination on fresh tissue. Another weakness of the RCL2 fixative is that an optimized fixation protocol must be employed to obtain high-quality nucleic acids. Overnight fixation at +4 °C before paraffin embedding and storage at −20 °C of the paraffin blocks until use are two steps which could limit the day-to-day application of RCL2®-CS100 in clinical practice.

On the whole, our results suggest that fixation with RCL2®-CS100 supports the feasibility of combining molecular analyses with histopathological diagnosis, particularly for the evaluation of small target lesions which cannot be frozen without impairing their pathological evaluation, or for specific clinical research protocols. Such a fixative could offer new perspectives for molecular pathology and translational clinical research.

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