

Validation of a Postfixation Tissue Storage and Transport Medium to Preserve Histopathology and Molecular Pathology Analyses (Total and Phosphoactivated Proteins, and FISH)

Michael M. Stumm, MD,¹ Maja R. Walker,¹ Caroline Stork,¹ Noelle Hanoteau,¹ Urs Wagner, MD,² and Terence M. O'Reilly, PhD¹

Key Words: 70% vol/vol ethanol; Histopathology; Molecular pathology; Immunohistochemistry; Fluorescence in situ hybridization; Preservation; Tumor; Overfixation

DOI: 10.1309/AJCPDZ4FAQ9BUEXC

Upon completion of this activity you will be able to:

- define the critical factors in tissue procurement and processing that have an impact on histopathology and molecular pathology analyses.
- discuss appropriate procedures to allow controlled tissue fixation and postfixation tissue storage and transportation.

The ASCP is accredited by the Accreditation Council for Continuing Medical Education to provide continuing medical education for physicians. The ASCP designates this journal-based CME activity for a maximum of 1 AMA PRA Category 1 Credit™ per article. Physicians should claim only the credit commensurate with the extent of their participation in the activity. This activity qualifies as an American Board of Pathology Maintenance of Certification Part II Self-Assessment Module.

The authors of this article and the planning committee members and staff have no relevant financial relationships with commercial interests to disclose. Questions appear on p 493. Exam is located at www.ascp.org/ajcpdme.

Abstract

Tumor biomarker studies are integral to oncology clinical trials but may yield artifactual results owing to variation in sample procurement and processing. Ethanol, 70% vol/vol, was validated as a sample transport medium using markers of the PI3K/Akt/mTOR pathway. BT474 tumor xenografts were excised and slices were immediately placed into formaldehyde and fixed for 24 hours. Fixed tissue slices were immediately processed into paraffin or transferred to 70% vol/vol ethanol and stored at room temperature for 1, 2, and 4 weeks before further processing. Freshly cut tissue sections were evaluated for pAKT^{S473}, HER2, pHER-2^{Y1248}, pS6^{S235/236}, and pS6^{S240/244}, Ki-67, and HER2 by fluorescence in situ hybridization and stained with H&E and Masson trichrome. No significant changes were observed when comparing samples stored in 70% ethanol for up to 4 weeks with immediately processed tissue. Ethanol, 70% vol/vol, provides a safe storage medium for formaldehyde-fixed tumor tissue, facilitating sample transport during multicenter clinical trials.

Tumor tissue collection has become integral to cancer clinical trials to correctly diagnose the tumor at the molecular level and to evaluate biomarkers. The correct assessment of biomarkers in tumor tissue to facilitate examination of proposed drug action and therapeutic outcome requires accurate and consistent results, which can be obtained only when tumor tissues are correctly procured and processed and the histoarchitecture and total and phosphoactivated proteins are preserved.¹⁻⁴ Managing all aspects of tissue analysis requires a reliable protocol for sample procurement, preparation, and transportation.⁵ Therefore, to optimize sample processing and analysis, shipment of samples promptly after collection to a central laboratory is preferable over local processing. In contrast with preclinical biomarker studies, in which tumor tissue procurement and processing can be highly standardized and well controlled,⁶ implementation of and achieving strict adherence to standardized tissue collection protocols in a clinical study setting is challenging, in particular with multicenter studies.^{7,8}

Although in their detection some cancer markers seem somewhat unaffected by variations in methods,⁹ other markers are not. In particular tissue size,¹⁰ the choice of fixative,¹¹⁻¹⁴ the volume of fixative, fixation temperature, delay in fixation,^{15,16} and time of fixation^{17,18} are assumed or have been shown to affect molecular pathology analyses in a negative way, in particular detection of phosphoproteins,¹⁵ which can degrade rapidly. In addition, fixative and storage procedures may also affect the detection of RNA^{10,19} and DNA^{20,21} in tissues.

Current clinical trials evaluating novel therapeutic interventions use multicenter designs to minimize recruitment time. Molecular pathology analyses (eg,

immunohistochemical analysis and fluorescence in situ hybridization [FISH]) are used with increasing frequency as methods to characterize tumors before inclusion of patients in a clinical trial and are used for monitoring the effects of treatment during or at the end of a clinical trial.²² Variation between centers in the quality of molecular pathology analyses prompts many researchers to use a centralized histopathology laboratory, where many variables affecting the quality of molecular pathology analyses can be controlled. However, although fixation time of tissue samples can be controlled in a multicenter trial, sample shipment times from contributing clinical centers to a centralized laboratory will vary and likely cannot be controlled. Accordingly, a relatively inert transport medium preserving sample integrity for a prolonged period, thus avoiding overfixation, would be useful.

We describe the results of the validation of 70% vol/vol ethanol as relatively inert sample storage and transport medium for tumor tissue. We assessed this by evaluation of biomarkers of the PI3K/Akt/mTOR pathway using tumor tissues from experimental animals.

Materials and Methods

Reagents

Cell culture materials were from Integra BioSciences (Wallisellen, Switzerland). Liquid media, fetal bovine serum, and media additives were from Life Technologies (Basel, Switzerland). Antibodies directed against Ki-67 (mouse monoclonal Mib-1, M7240), HER2 (HercepTest, rabbit polyclonal, K5204), and pHER-2^{Y1248} (mouse monoclonal PN2A, M7269) were from DAKO (Baar, Switzerland); antibodies directed against pAKT^{S473} (rabbit polyclonal, CST9277), pS6^{S235/236} (rabbit polyclonal, CST2211), and pS6^{S240/244} (rabbit polyclonal, CST2215) were from Cell Signaling Technology (BioConcept, Allschwil, Switzerland). Fluorescent DNA probes to detect *HER2* amplification (FISH, PathVysion) were from Vysis (Abbott, Baar, Switzerland) ■Table 1■.

■Table 1■
Overview of Staining Reagents Used

Name	Method	Antibody	Catalog No.	Supplier
Ki-67	IHC	Mouse (clone Mib-1)	M7240	DAKO, Baar, Switzerland
HER2 protein (HercepTest)	IHC	Rabbit polyclonal	K5204	DAKO
pHER-2 ^{Y1248}	IHC	Mouse (clone PN2A)	M7269	DAKO
pAKT ^{S473}	IHC	Rabbit polyclonal	CST9277	Cell Signaling Technology, BioConcept, Allschwil, Switzerland
pS6 ^{S235/236}	IHC	Rabbit polyclonal	2211	Cell Signaling Technology, BioConcept
pS6 ^{S240/244}	IHC	Rabbit polyclonal	2215	Cell Signaling Technology, BioConcept
HER2 amplification (PathVysion HER2/neu)	FISH	—	02J01-36	Vysis, Abbott, Baar, Switzerland

FISH, fluorescence in situ hybridization; IHC, immunohistochemical analysis.

Mouse Tumor Model

The human breast carcinoma cell line BT474, overexpressing ErbB-2, was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained and cultured according to established techniques as recommended by the supplier. Female athymic BALB/c nu/nu (nude) mice were kept in a pathogen-controlled environment (maximally 10-12 mice/type III cage) with free access to food and water. BT474 cells (4 × 10⁶ cells/50 μL) were injected into female athymic nude mice orthotopically in the mammary gland. At 46 days after tumor cell injection, the animals were killed and tumors harvested. Protocols involving animals were approved by the ethics committee and the Veterinaeramt, Kanton Basel-Stadt.

Tissue Collection and Processing

Tumors were excised immediately after animals were killed; two 3-mm-thick adjacent tissue slices were prepared and placed into fixative, keeping the time from excision to fixation at less than 3 minutes for each individual tissue slice. Fixation was carried out in 4% phosphate-buffered formaldehyde, pH 7.4 (J.T. Baker Formalin Solution, Medite, Nunningen, Switzerland), at 4°C for an exact duration of fixation of 24 hours (±5 minutes) for each individual tissue slice. After the end of fixation, 1 tissue slice was processed into paraffin without delay, whereas the other slice was transferred into 70% vol/vol ethanol without delay and stored at room temperature (20°C-27°C) for 1 (n = 3), 2 (n = 7), or 4 (n = 4) weeks before processing into paraffin. Tissue slices were processed using a formaldehyde-free standard tissue processing program on an automated tissue processor (TPC15Duo, Medite) consisting of an ascending alcohol series for dehydration, xylene series for clearance, and paraffin series for infiltration. Tissue embedding into paraffin followed tissue processing and was carried out manually on a tissue embedding station (TBS88, Medite).

H&E, Masson Trichrome, Immunohistochemical Analysis, and HER2 FISH Staining

For the study, 3-μm-thick serial tissue sections from each paraffin-embedded tissue slice were freshly cut and stained in pairs matching 70% vol/vol ethanol stored (1, 2,

or 4 weeks, respectively) with immediately processed tissue. Morphologic analysis was performed after routine H&E and Masson trichrome staining. Immunohistochemical analysis was performed using established protocols to detect Ki-67, HER2, pHER-2^{Y1248}, pAKT^{S473}, pS6^{S235/236}, and pS6^{S240/244}. In brief, tissue sections were dewaxed, hydrated, and subjected to heat-induced antigen retrieval. Following peroxidase block and protein absorption, tissue sections were incubated with primary antibody followed by detection with secondary antibody, chromogenic visualization, and counterstaining with hematoxylin. Stained tissue sections were permanently mounted for further evaluation.

HER2 FISH analysis was performed following strict adherence to the manufacturer's instructions (Vysis). In brief, tissue sections were dewaxed, hydrated, and pretreated with hydrochloric acid followed by protease digestion. Tissue sections were dehydrated and dried followed by incubation with DNA probes directed against HER2/*neu* (tetramethyl rhodamine isothiocyanate [TRITC]) and CEP17 (fluorescein isothiocyanate [FITC]) in an automated hybridizer (HYBrite, Abbott, Baar, Switzerland) overnight. Tissue sections were consecutively washed, counterstained with 4'-6-diamidino-2-phenylindole (DAPI), coverslipped, and stored at 4°C until evaluation.

Stained tissue sections were documented by taking digital images using a digital camera (JVC KY-F70, Gloor

Instruments, Uster, Switzerland) mounted on a combined bright-field and fluorescent research microscope (Nikon E600, Nikon, Kusnacht, Switzerland) equipped with filters for FITC, TRITC, and DAPI. Images were processed using image documentation and analysis software (analySIS FIVE, Gloor Instruments). Representative images obtained from stained tissue are presented.

Evaluation and Validation

For each ethanol storage condition, all tumor slices stored in 70% vol/vol ethanol for 1, 2, or 4 weeks or immediately processed were reviewed and compared for staining differences. Evaluation of each stained tissue section was done without the evaluator being aware of the preparation details. Staining intensity for immunohistochemical analysis was rated on a scale ranging from 0 to 4, whereas DNA amplification was assessed qualitatively.

Results

Histoarchitecture

No significant changes in histoarchitecture could be observed when comparing immediately processed tissues with those stored in 70% vol/vol ethanol for 1, 2, or 4 weeks before processing **Image 1**.

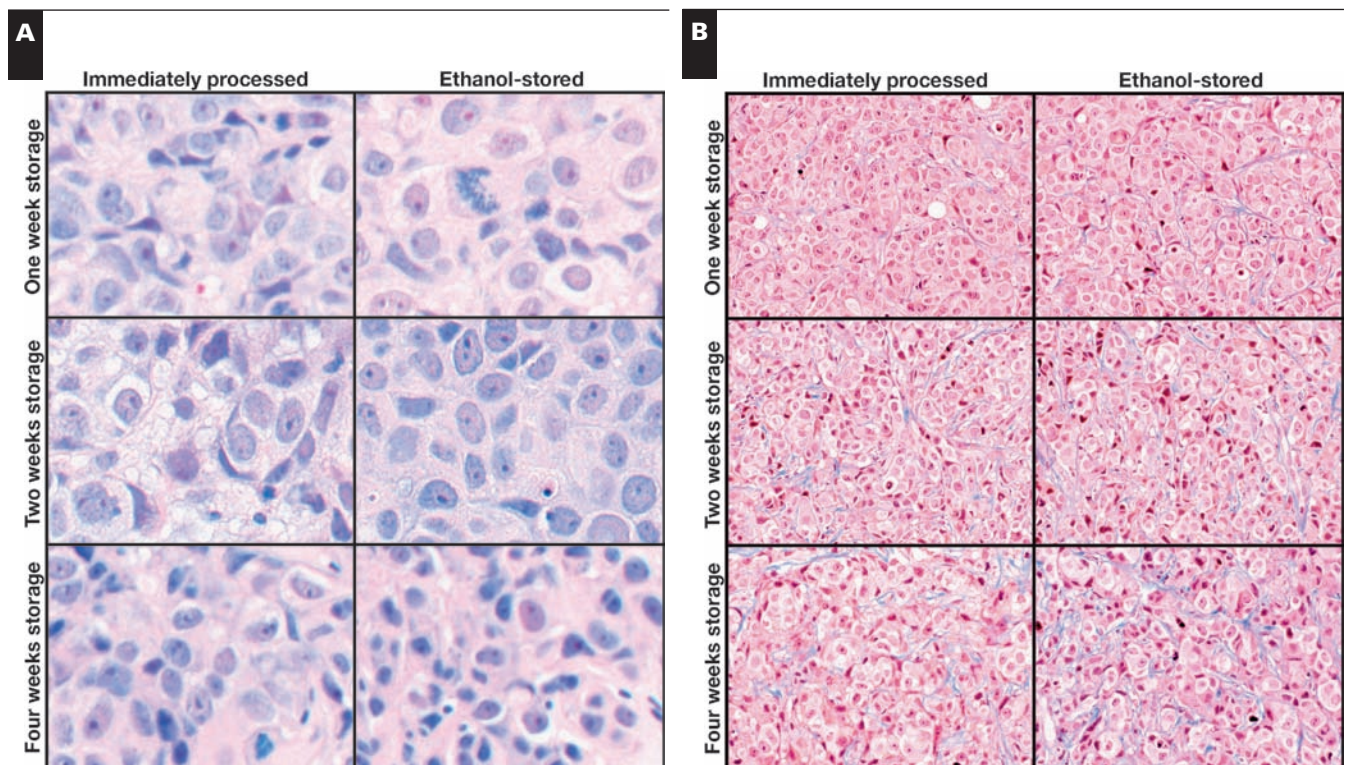


Image 1 Comparison of storage on the histologic features of experimental BT474 tumor tissues. Tissue sections from BT474 tumor slices were fixed in formaldehyde and immediately processed or stored in 70% vol/vol ethanol for 1, 2, and 4 weeks before processing. **A**, H&E staining. **B**, Masson trichrome staining.

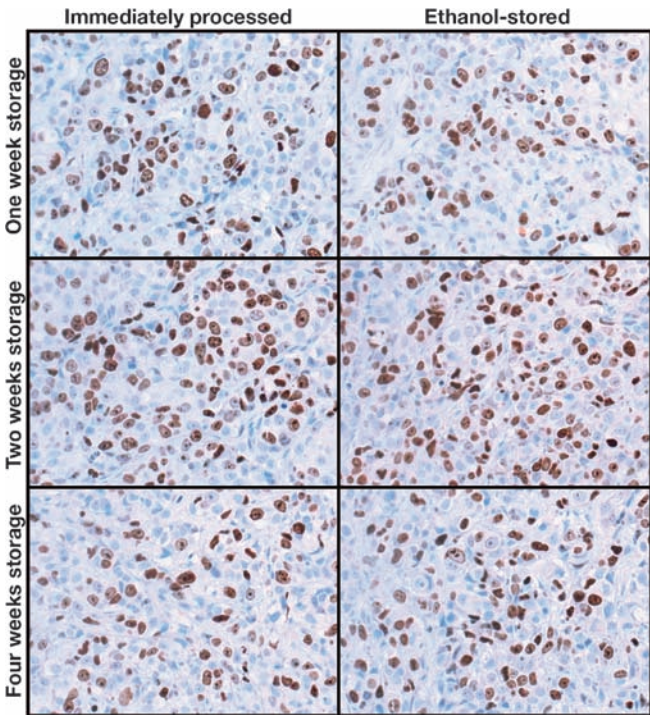


Image 2 Comparison of storage on the histologic features of experimental BT474 tumor tissues: Ki-67 staining. Tissue sections from BT474 tumor slices were fixed in formaldehyde and immediately processed or stored in 70% vol/vol ethanol for 1, 2, and 4 weeks before processing.

Immunohistochemical Analysis for Total and Phosphoactivated Proteins

No significant staining differences in immunohistochemical analysis for Ki-67, HER2, pHER-2^{Y1248}, pAKT^{S473}, pS6^{S235/236}, and pS6^{S240/244} could be observed when comparing immediately processed tissues with those stored in 70% vol/vol ethanol for 1, 2, or 4 weeks before processing **Image 2**, **Image 3**, **Image 4**, and **Image 5**.

FISH HER2 Amplification

No significant difference in the detection of FISH *HER2* amplification using probes detecting *HER2/neu* (TRITC) or CEP17 (FITC) could be observed when comparing immediately processed tissues with tissues stored in 70% vol/vol ethanol for 1, 2, or 4 weeks before processing **Image 6**.

Table 2 summarizes the semiquantitative analysis of the immunohistochemical markers chosen for the assessment of 70% vol/vol ethanol as an inert storage and shipment solution. There were no significant differences observed between immediately processed and stored tissue samples.

Discussion

The histopathologic and molecular pathologic analyses of tumor tissue are becoming increasingly relied on for the

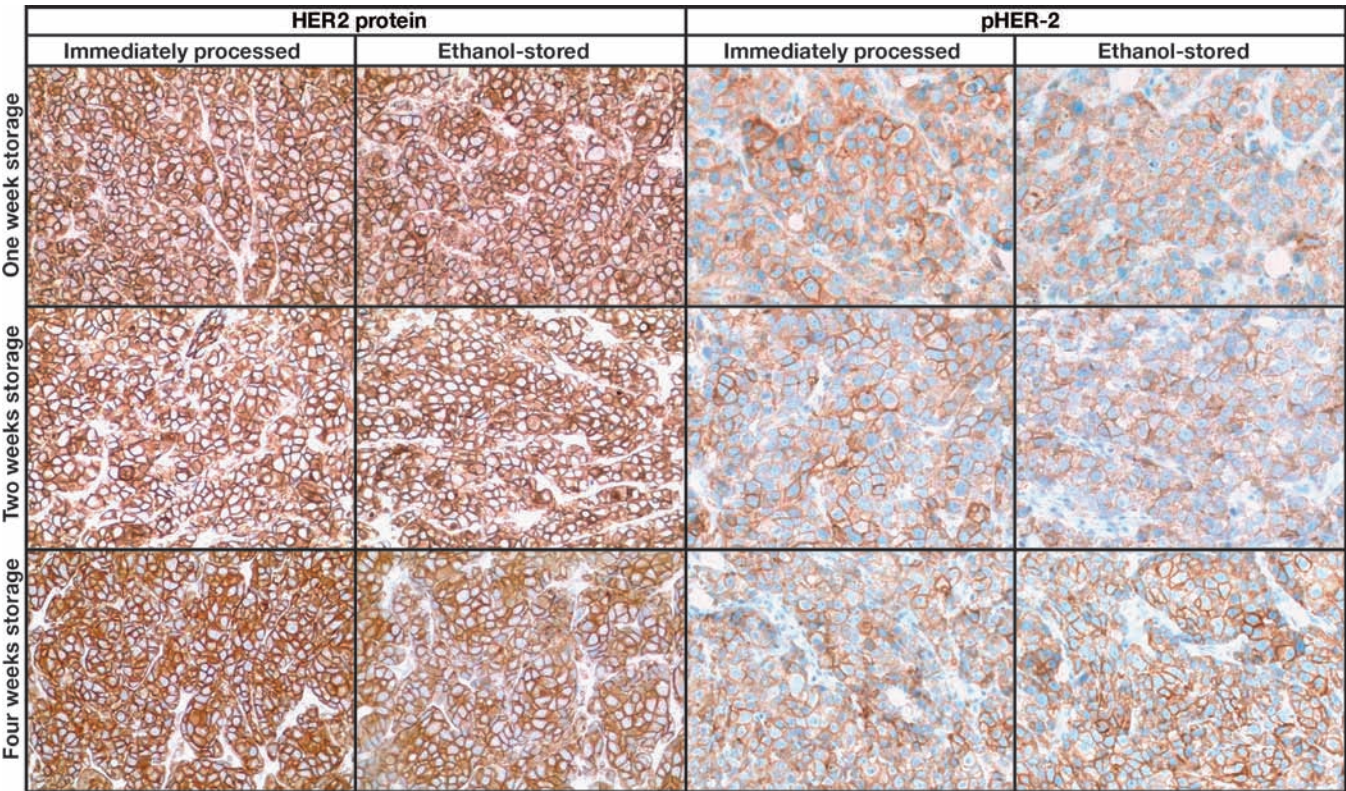


Image 3 Comparison of storage on the histologic features of experimental BT474 tumor tissues: HER2 and pHER-2^{Y1248} staining. Tissue sections from BT474 tumor slices were fixed in formaldehyde and immediately processed or stored in 70% vol/vol ethanol for 1, 2, and 4 weeks before processing.

precise characterization of tumor phenotypes and their suitability for targeted therapies. Furthermore, as part of contemporary oncology clinical trial design, obtaining tumor and surrogate tissue samples before therapy (for inclusion and stratification), during therapy (to determine potential drug efficacy based on target inhibition or molecular consequences resulting from target inhibition), and after therapy (to determine the effect of therapy on disease resolution biomarkers) is a desirable, if not mandatory, facet of new drug evaluation.²³

Management of tissue sample collection, processing, and analysis during multicenter clinical trials is a complex task in which many potential sources for introduction of aberrant results are possible. The easily identifiable factors that could affect tissue quality, such as time of tissue fixation and the possible continued fixation during a variable transport time to a centralized laboratory, prompted the present study. Our fear was that prolonged and variable shipping times may lead to artifacts due to tissue overfixation. Consequently, we studied the capacity of 70% vol/vol ethanol as a storage and transport medium to be used after standardized formaldehyde fixation duration in an experimental situation to determine whether this procedure would affect tissue sample quality.

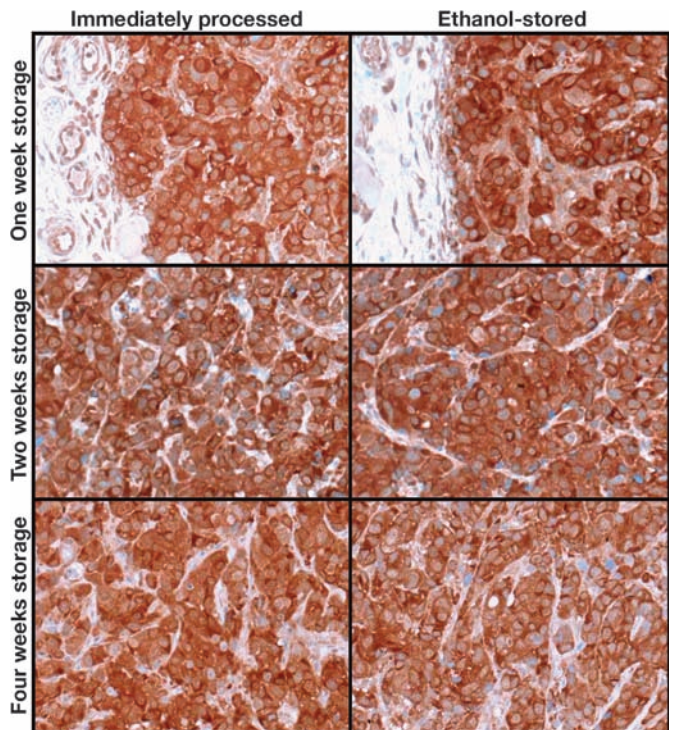


Image 4 Comparison of storage on the histologic features of experimental BT474 tumor tissues: pAKT^{S473} staining. Tissue sections from BT474 tumor slices were fixed in formaldehyde and immediately processed or stored in 70% vol/vol ethanol for 1, 2, and 4 weeks before processing.

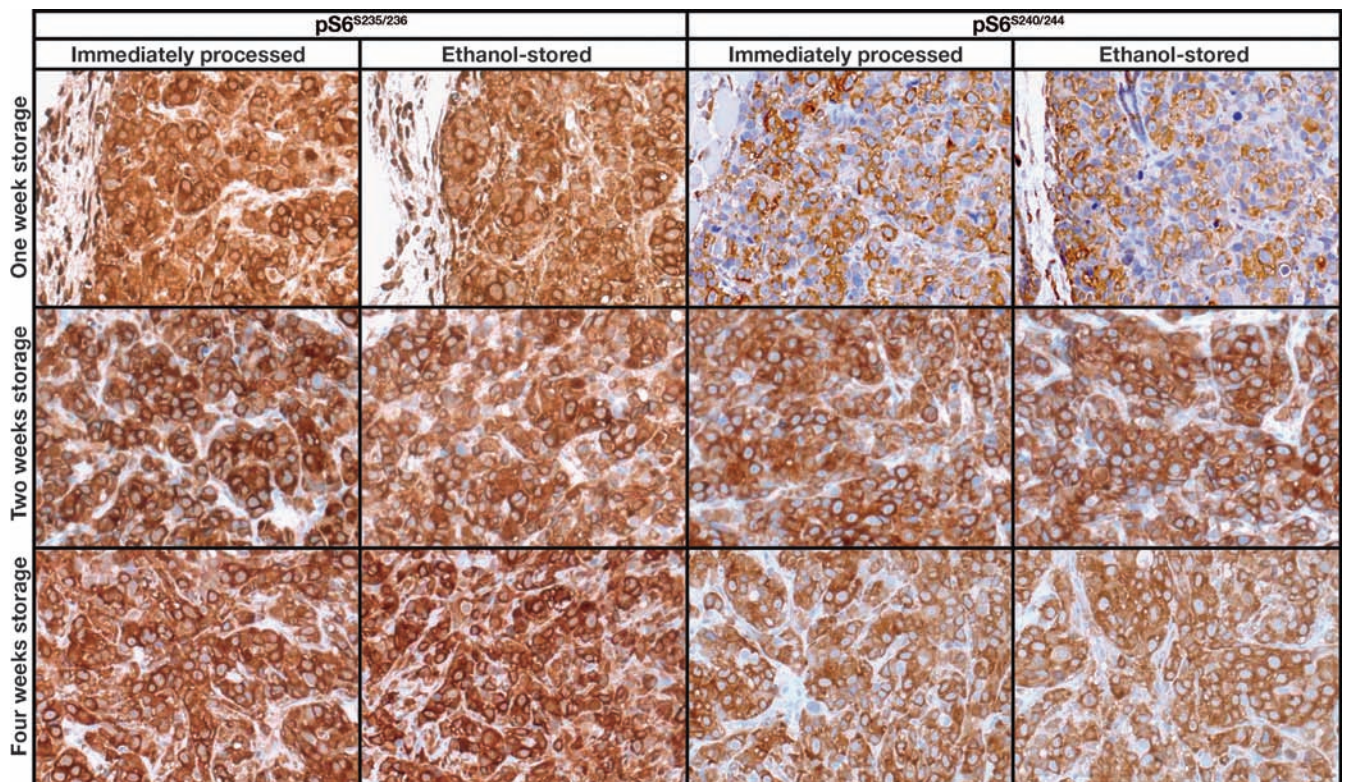


Image 5 Comparison of storage on the histologic features of experimental BT474 tumor tissues: pS6^{S235/236} and pS6^{S240/244} staining. Tissue sections from BT474 tumor slices were fixed in formaldehyde and immediately processed or stored in 70% vol/vol ethanol for 1, 2, and 4 weeks before processing.

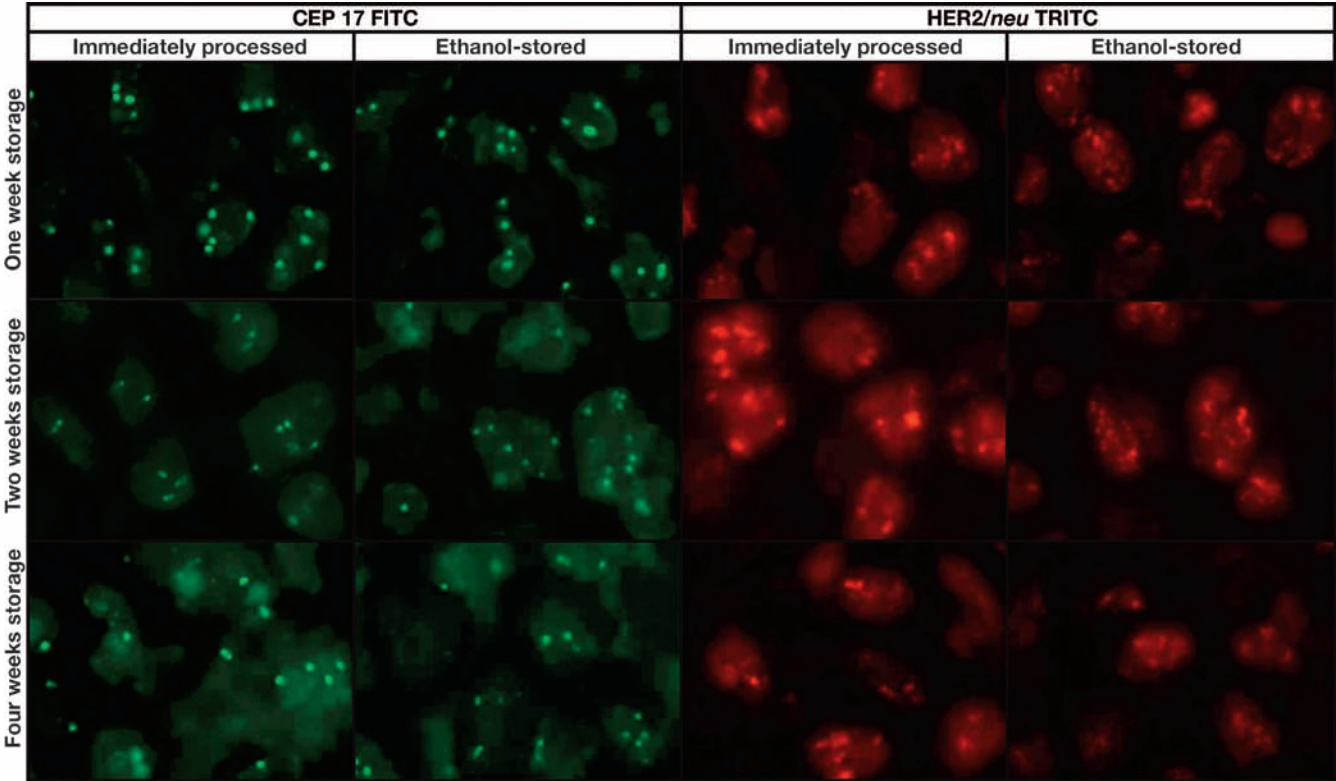


Image 6 Comparison of storage on the histologic features of experimental BT474 tumor tissues. Tissue sections from BT474 tumor slices were fixed in formaldehyde and immediately processed or stored in 70% vol/vol ethanol for 1, 2, and 4 weeks before processing and stained for CEP 17 (fluorescein isothiocyanate [FITC]) and HER2/*neu* (tetramethyl rhodamine isothiocyanate [TRITC]).

Although postfixation storage in 70% vol/vol ethanol has occasionally been indicated as a transfer storage step before tissue processing,⁶ to the best of our knowledge, this aspect of prolonged storage of fixed tissue samples in a suitable storage and transport medium has not been systematically addressed with respect to changes that may occur to the sample during prolonged and standardized postfixation storage that may consequently affect histoarchitectural

and molecular pathologic analyses (immunohistochemical analysis and FISH).

The results of the present study show that 70% vol/vol ethanol is a safe tissue storage and transport solution that preserves histopathologic and molecular pathologic features (as exemplified in immunohistochemical studies detecting total and phosphoactivated proteins and FISH detecting DNA) for up to 4 weeks. In addition, overfixation using

Table 2
Scoring of Immunohistochemical Analyses

	1 wk/Block						2 wk/Block							
	1		2		3		4		5		6		7	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Ki-67	4	4	4	4	4	4	4	4	4	4	4	4	4	4
HER2	4	4	4	4	4	4	4	4	4	4	4	4	4	4
pHER-2 ^{Y1248}	4	3-4	3-4	4	3	3	4	4	4	4	4	4	4	4
pAkt ^{S473}	4	4	4	4	4	4	4	4	4	4	4	4	4	4
pS6 ^{S235/236}	4	4	4	4	4	4	4	4	4	4	4	4	4	4
pS6 ^{S240/244}	4	4	4	4	4	4	4	4	4	4	4	4	4	4

Tissue sections from BT474 tumor slices fixed in formaldehyde and immediately processed (A) or stored in 70% vol/vol ethanol (B) for 1, 2, and 4 wk before processing were stained for the markers as indicated. Semiquantitative evaluation of the staining intensity was done by using a scoring system from 0 (no staining) to 4 (strong staining).

formaldehyde, known to negatively interfere with molecular pathologic analyses, can be avoided.

The use of 70% vol/vol ethanol as a postfixation sample storage and transport medium should help provide improved sample quality for molecular pathologic analyses (including biomarkers) during clinical trials. Indeed, this procedure has been successfully applied in a multicenter phase 2 oncology clinical trial evaluating everolimus (Afinitor) in breast cancer.²⁴

From ¹Oncology Research, Novartis Institutes of Biomedical Research, Novartis Pharma AG, Basel, Switzerland; and ²Head Pathology, Unilabs, Bern, Switzerland.

Address reprint requests to Dr Stumm: Guggeregge 6, 4303 Kaiseraugst, Switzerland.

References

- Mandell JW. Phosphorylation state-specific antibodies: applications in investigative and diagnostic pathology. *Am J Pathol.* 2003;163:1687-1698.
- Mandell JW. Immunohistochemical assessment of protein phosphorylation state: the dream and the reality. *Histochem Cell Biol.* 2008;130:465-471.
- Burford-Mason AP, MacKay AJ, Cummins M, et al. Detection of proliferating cell nuclear antigen in paraffin-embedded specimens is dependent on preembedding tissue handling and fixation. *Arch Pathol Lab Med.* 1994;118:1007-1013.
- Werner M, Chott A, Fabiano A, et al. Effect of formalin tissue fixation and processing on immunohistochemistry. *Am J Surg Pathol.* 2000;24:1016-1019.
- Leiva IM, Michael R, Emmert-Buck MR, et al. Handling of clinical tissue specimens for molecular profiling studies. *Curr Issues Mol Biol.* 2003;5:27-35.
- Golubeva Y, Rogers K. Collection and preparation of rodent tissue samples for histopathological and molecular studies in carcinogenesis. *Methods Mol Biol.* 2008;511:3-60.
- Olapade-Olaopa EO, Ogunbiyi JO, MacKay EH, et al. Further characterization of storage-related alterations in immunoreactivity of archival tissue sections and its implications for collaborative multicenter immunohistochemical studies. *Appl Immunohistochem Mol Morphol.* 2001;9:261-266.
- Troyer D. Biorepository standards and protocols for collecting, processing, and storing human tissues. *Methods Mol Biol.* 2008;441:193-220.
- Jensen V, Ladekar M. Immunohistochemical quantitation of oestrogen receptors and proliferative activity in oestrogen receptor positive breast cancer. *J Clin Pathol.* 1995;48:429-432.
- von Ahlfen S, Missel A, Bendrat K, et al. Determinants of RNA quality from FFPE samples. *PLoS One.* 2007;12:e1261. doi:10.1371/journal.pone.0001261.
- Vince DG, Thakhi A, Gaddipati A, et al. Quantitative comparison of immunohistochemical staining intensity in tissues fixed in formalin and Histochoice. *Anal Cell Pathol.* 1997;15:119-129.
- Atkins D, Reiffen KA, Tegtmeier CL, et al. Immunohistochemical detection of EGFR in paraffin-embedded tumor tissues: variation in staining intensity due to choice of fixative and storage time of tissue sections. *J Histochem Cytochem.* 2004;52:893-901.
- Burns JA, Li Y, Cheney CA, et al. Choice of fixative is crucial to successful immunohistochemical detection of phosphoproteins in paraffin-embedded tumor tissues. *J Histochem Cytochem.* 2009;57:257-264.
- Penault-Llorca F, Adelaïde J, Houvenaeghel G, et al. Optimization of immunohistochemical detection of ERBB2 in human breast cancer: impact of fixation. *J Pathol.* 1994;173:65-75.
- Baker AF, Dragovich T, Ihle NT, et al. Stability of phosphoprotein as a biological marker of tumor signaling. *Clin Cancer Res.* 2005;11:4338-4340.
- Spruessel A, Steimann G, Jung M, et al. Tissue ischemia time affects gene and protein expression patterns within minutes following surgical tumor excision. *Biotechniques.* 2004;36:1030-1037.
- Chiu KY, Loke SL, Ho FC. Immunohistochemical demonstration of c-erbB-2 oncoprotein in gastric adenocarcinoma: comparison of cryostat and paraffin wax sections and effect of fixation. *J Clin Pathol.* 1994;47:117-121.

2 wk/Block								4 wk/Block							
8		9		10		11		12		13		14		15	
A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
4	4	4	4	3	2	3-4	4	3	4	4	4	4	3-4	4	3-4
4	4	4	4	4	4	2-3	4	4	3-4	4	4	4	4	2	2
4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

18. Arber DA. Effect of prolonged formalin fixation on the immunohistochemical reactivity of breast markers. *Appl Immunohistochem Mol Morphol*. 2002;10:183-186.
19. von Smolinski D, Leverkus I, von Samson-Himmelstjerna G, et al. Impact of formalin-fixation and paraffin-embedding on the ratio between mRNA copy numbers of differently expressed genes. *Histochem Cell Biol*. 2005;124:177-188.
20. Ferrer I, Armstrong J, Capellari S, et al. Effects of formalin fixation, paraffin embedding, and time of storage on DNA preservation in brain tissue: a BrainNet Europe Study. *Brain Pathol*. 2007;17:297-303.
21. Selvarajan S, Bay BH, Choo A, et al. Effect of fixation period on HER2/*neu* gene amplification detected by fluorescence in situ hybridization in invasive breast carcinoma. *J Histochem Cytochem*. 2002;50:1693-1696.
22. Yap TA, Sandhu SK, Workman P, et al. Envisioning the future of early anticancer drug development. *Nature*. 2010;10:514-523.
23. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
24. Baselga J, Semiglazov V, van Dam P, et al. Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. *J Clin Oncol*. 2009;27:2630-2637.