HHS Public Access

Author manuscript

Lab Invest. Author manuscript; available in PMC 2014 October 01.

Published in final edited form as:

Lab Invest. 2014 April ; 94(4): 467–474. doi:10.1038/labinvest.2014.7.

A Tissue Quality Index – an Intrinsic Control for Measurement of Effects of Pre-analytical Variables on FFPE Tissue

Veronique M. Neumeister, **Fabio Parisi**, **Allison M. England**, **Summar Siddiqui**, **Valsamo Anagnostou**, **Elizabeth Zarrella**, **Maria Vassilakopolou**, **Yalai Bai**, **Sasha Saylor**, **Anna Sapino**, **Yuval Kluger**, **David G. Hicks**, **Gianni Bussolati**, **Stephanie Kwei**, and **David L. Rimm**

Department of Pathology and Surgery, Yale University School of Medicine. Department of Pathology, University of Rochester School of Medicine. Department of Biomedical Sciences and Human Oncology, University of Turin, Turin, Italy

Abstract

While efforts are made to improve tissue quality and control pre-analytical variables, pathologists are often confronted with the challenge of molecular analysis of patient samples of unknown quality. Here we describe a first attempt to construct a Tissue Quality Index (TQI) or an intrinsic control that would allow a global assessment of protein status based on quantitative measurement of a small number of selected, informative epitopes.

Quantitative Immunofluorescence (QIF) of a number of proteins was performed on a series of 93 breast cancer cases where levels of expression were assessed as a function of delayed time to formalin fixation. A TQI was constructed based on the combination of proteins that most accurately reflect increased and decreased levels of expression in proportion to delay time.

The TQI, defined by combinations of measurements of cytokeratin, pERK1/2 and pHSP-27 and their relationship to cold ischemic time were validated on a second build of the training series and on 2 independent breast tissue cohorts with recorded time to formalin fixation. We show an association of negative TQI values (an indicator for loss of tissue quality) with increasing cold ischemic time on both validation cohorts, as well as an association with loss of ER expression levels on all 3 breast cohorts.

Using expression levels of 3 epitopes, we can begin to assess the likelihood of delayed time to fixation or decreased tissue quality. This TQI represents a proof of concept for the use of epitope expression to provide a mechanism for monitoring tissue quality.

Keywords

epitope degradation; immunofluorescence; immunohistochemistry; pre-analytic variables

Corresponding author: David L. Rimm, MD, PhD, Department of Pathology, BML 116, Yale University School of Medicine, 310 Cedar St, PO Box 208023, New Haven, CT 06520-8023, david.rimm@yale.edu, 203-737-4204.

The authors declare no conflict of interest.

Over the past 5–10 years there have been a number of events that have caused concern related to quality assurance in laboratories performing diagnostic testing in oncology and the accuracy of these tests, which ultimately determine patient therapy (1). Our field has recognized the danger in the lack of standardization of processes between different laboratories, the variability inherent in the analytical phase and the lack of control over the pre-analytical phase of tissue processing. Biospecimen science, the study of variables affecting biospecimen analysis, has identified a range of factors affecting the quality of harvested tissue (2–5). These factors, grouped as pre-analytical variables, represent a critical problem for biomarker measurement on tissue specimens that can lead to errors that affect patient care.

This issue is most prominent in breast cancer where it has been shown that pre-analytical variables, especially delayed time to formalin fixation (cold ischemic time), have affected companion diagnostic testing (6–9). Overall, it has been estimated that delay to formalin fixation and subsequent protein degradation within the tissue are responsible for a 10–20% false negative rate for ER in breast cancer (1, 9). A similar effect has been shown for HER2 with loss of protein and RNA as a function of increasing time to fixation of the tissue. These issues have prompted the American Society of Clinical Oncologists (ASCO) and the College of American Pathologists (CAP) to publish guidelines for the evaluation of ER, PgR and Her2 in breast cancer, limiting cold ischemic time to 60 minutes (10, 11).

The issue of pre-analytic variables is not limited to the companion diagnostics of breast cancer. Data have been published about the loss of antigenicity of phosphorylated proteins (12–15) and increased levels of hypoxia induced factors as well as markers of posttranslational modification as a consequence of delayed time to formalin fixation of harvested tissue (16). Also RNA degradation and time dependent modulation of gene expression have been observed as a cellular reaction to stress and sub-optimal pre-analytical processing conditions (15). Finally, the effects of pre-analytical variables on harvested tissue do not only play an important role for companion diagnostic testing and development of new drugs, but also in retrospective studies using archived tissue collections, where documentation of tissue handling and processing is often minimal.

Therefore it was our goal to develop and construct an intrinsic control for formalin fixed, paraffin embedded (FFPE) tissue that could be used in order to assess suitability of a given specimen of unknown quality for immunological assessments. Here we describe proof of concept for the development of a tissue quality index (TQI).

Material and Methods

Study Cohorts

This prospectively designed study included 3 different cohorts of breast tissue, all of which have exact information on pre-analytical specimen handling and processing with focus on cold ischemic time. All tissue was used after approval from the Rochester Institutional Review Board, from the Yale Human Investigation Committee protocol #8219 or #25173, or from the ethic institutional review board for “Biobanking and use of human tissue for experimental studies” of the Pathology Services of the Azienda Ospedaliera Città della

Salute e della Scienza di Torino. Written informed consent was obtained from all patients for their tissue to be used in research.

Time to Fixation Breast Cancer Series (TFBC)—Formalin-fixed, paraffin-embedded (FFPE) tissues of 93 breast cancer patients, who underwent surgery at the University of Rochester, School of Medicine, (Rochester, NY), were collected. Time from surgical resection to immersion of the specimen in formalin was recorded and ranged from 25 to 415 minutes. A tissue microarray (TMA) was constructed, consisting of these 93 breast cancer specimens, cell lines and controls, all represented in two fold redundancy (two histospots per patient specimen).

Normal Breast Tissue Series (NBT)—Normal breast tissues from 11 breast reduction mammoplasties were collected. Tissue from each patient was divided into different parts and exposed to pre-defined and controlled pre-analytical variables, with time points until immersion of the tissue into formalin at 20 minutes, 2 hours, 24 hours and 48 hours after surgical removal of the tissue. A TMA was constructed representing these specimens with variable conditions in 4 fold redundancy.

Italian Breast Cancer Series (IBC)—Tissues from 100 breast cancer cases were collected and time until formalin fixation of these tissues was recorded, ranging from 1 to 72 hours. This cohort differs from the time to fixation breast cancer series in a way that tissues were vacuum sealed and stored at 4 degree Celsius (UVSC) right after surgical removal until gross dissection and immersion of the tissue into formalin (17). TMAs were constructed consisting of the 100 breast cancer specimens represented in two fold redundancy.

Cell Lines and Cell Culture

The cell lines T47D, BT474, SKBR3, MB231, MB468, CHO, A431, HT29, A59–195, and A82-68-B were purchased from ATCC (Manassas, VA), cultured in our lab, and used to create control cell line cell blocks for TMA standardization. Culture conditions and construction of cell pellets for TMAs have been described previously (18).

TMA Construction

The TMAs for the 3 breast tissue series were constructed as described previously (18). Representative areas from the FFPE breast tissue were placed in a recipient block using 0.6 mm cores for the TFBCS and the NBT sets and using 1 mm cores for the IBC series.

Antibodies, Immunofluorescent Staining and Quantitative Analysis using the AQUA method

These methods are all described in our previous work [9] and in the supplementary methods online.

Statistical Analysis

TQI design: The goal of this procedure was to identify pairs of markers such that the difference between their AQUA scores is indicative of the time to fixation of the corresponding tissue. To simplify the design we divided the times to fixation in two groups,

according to whether the time was longer or shorter than 60 minutes, which was roughly the median time to fixation in our dataset. We divided our dataset in 3 groups, two of which were used for training and one was used as the test set. We used the training set to construct and screen predictions rules for the time to fixation. We then selected the six prediction rules with the largest training performance and computed their test performance. Using the training set, for each pair of markers *j* and *k* computed a screening score based on the differences of AQUA scores for the two markers across all patients as follows:

$S_{jk}=\sum_{i=1}^{N}\tanh\left[(x_{ij}-x_{ik})\log_2(t_i/60)\right]$, where *N* is the number of samples (patients) in the training set, x_{ij} and x_{ik} are the AQUA scores of markers *j* and *k* marker for sample (patient) *i* and t_i is the time to fixation in minutes for sample (patient) *i*. The difference is weighted by the term $\log_2(t_i/60)$, which was used to favor pairs of markers whose difference had a different sign depending on whether the time to fixation (TTF) was longer or shorter than 60 minutes. The formulation as $\log_2(t_i/60)$ was chosen to reduce contributions to the score from samples with time to fixation around 60 minutes and thus reduced the effects of time measurement precision on defining whether a time to fixation was longer or shorter than 60 minutes. The logarithm was introduced to reflect the notion of exponential decays in markers half-lives, as well as the desired change of sign in the difference of AQUA scores in samples with time to fixation larger than the reference time of 60 minutes. The hyperbolic tangent represents a smooth threshold to limit spurious outliers. For each pair of markers, *j* and *k*, we derived a prediction rule R_{jk}. The rule R_{jk} predicts that the time to fixation is larger than 60 minutes when the AQUA score of marker *j* is larger than the AQUA score of marker *k*. For each prediction rule R_{jk} we computed its performance using the Area Under the receiver-operator characteristic Curve (AUC) on the training set. The AUC measured the ability of the difference between the AQUA scores of two chosen markers to correctly classify whether a TTF was longer or shorter than 60 minutes. Finally, we determined the test performance of the six rules with the largest training performance by computing their AUC on the test data. The chosen design addresses the inherent variability of biological samples by comparing two markers at a time. Our procedure favored pairs of markers such that the AQUA score of one increases with time to fixation, while the other decreases. For further validation of the TQI performance linear regressions were computed for $\log_2$ transformed time to fixation and the TQI components. 95% CIs were calculated using the bootstrapping approach (n=5000). Statistical analyses were performed using the R package (www.r-project.org).

Results

Quantification of protein expression according to delayed time to formalin fixation

To construct the TQI, the antibodies described in Table 1 were all tested on the TFBC series and their expression levels and possible changes as a function of pre-analytical variables were measured. These results have been described previously (16). Briefly – housekeeping genes do not lose antigenicity with increasing time to formalin fixation, while proteins of hypoxia and some posttranslational modifications significantly increase in expression. Other biomarkers, such as phospho-tyrosine or phospho-Erk1/2 are more labile and show significant degradation with increasing time to fixation.

The Tissue Quality Index (TQI) as an intrinsic control to assess delay to formalin fixation

The TQI was constructed using a subset of the TFBC series and its performance measured on a validation subset of the same array. Construction and performance of the TQI are described in Figure 1. The best candidate TQIs consisted of five pair wise combinations of proteins. The AQUA scores of all proteins were $\log_2$ transformed and subtracted from each other, with phospho-Tyrosine:phospho-HSP27 (pHSP27) performing best, the AUC value being 0.75, followed by Cytokeratin:pHSP27 and pERK1/2:pHSP27 with training set AUC values between 0.6 and 0.7 (figure 1A) and testing set somewhat lower. The predictive value of the TQI regarding delayed time to fixation was then assessed on the complete TFBC series (Figure 1B and C). A negative TQI value means that $\log_2$ normalized AQUA scores of pHSP27 are higher than the $\log_2$ normalized AQUA scores of phospho-Tyrosine or Cytokeratin or pERK1/2. This suggests that the tissue may have suffered loss of antigenicity caused through delay in formalin fixation or other less well defined pre-analytic variables. The number of specimens with negative TQI values appears to be associated with prolonged cold ischemic time on the TFBC series.

Validation of the TQI on two independent breast tissue validation cohorts

We then validated the TQI and its performance on two independent cohorts of breast tissue, the NBT series and the IBC series, both of which consist of breast tissue with recorded cold ischemic time. Due to the lack of lot to lot reproducibility of all tested phospho-Tyrosine antibody cocktails, and/or high levels of heterogeneity of the proteins targeted by this antibody, we were not able to reproduce our original results for phospho-Tyrosine and therefore omitted this epitope from the TQI. The other biomarkers, pHSP27, Cytokeratin and pERK1/2 showed robust reproducibility and were used in the validation of the TQI.

Before assessing the validation cohorts, we tested the TQI on a unique build (different TMA master block) of the TFBC series, which had initially served as the train/test cohort. We were able to show reproducibility of our original result, suggesting that this TQI is independent of biomarker heterogeneity seen for Cytokeratin, pERK1/2 and pHSP27. The performance of the TQI was also validated and measured on this new build of the TFBC series. Linear regressions between increasing time to fixation and the differences of AQUA scores of the TQI markers were computed. Larger differences of the TQI makers correspond with shorter time to fixation measures for a time window of 30 to 120 minutes (Figure 2A).

This proof of principle was followed by validation on the NBT series and the IBC series. Negative TQI values are correlated with increasing delay to formalin fixation on the NBT series, indicating potential for detection of pre-analytic epitopic degradation (Figure 2B). Chi Square analysis of each TQI and the combination of both shows a statistically significant association with time to fixation in this series (figure 2C). The performance of the TQI on build 2 of the TFBC series and the NBT series was also assessed by calculation of the area under the curve (AUC) of a ROC curve and showed values of 0.6 to 0.7 for these marker combinations recapitulating our original result (not shown). The IBC series consists of patient samples which were vacuum sealed and stored at 4° C (UVSC) to prevent degradation. While this is not the current standard of care, we used this cohort since this method represents an alternative approach to diminishing the effects of cold ischemic time.

The quantification of pHSP27, Cytokeratin and pERK1/2 and calculation of the TQI revealed mainly positive TQI scores (147 out of 169 readable samples have a positive TQI – the series consists of 100 patients, represented in 2 fold redundancy on the TMAs) suggesting better preservation of the tissue through vacuum sealing and storage at 4° C (Figure 2D).

TQI value and quantitative ER expression in breast tissue

Recent publications have shown that there is no loss of ER expression for tissue fixed within one hour of cold ischemic time (16). However, loss of ER antigenicity has been reported for longer cold ischemic times (6, 9). Here we determine if the TQI can indicate loss of ER reactivity. Specifically, we hypothesize that cases with a negative TQI should have lower ER scores. ER was measured by QIF with the clone SP1 on all 3 breast tissue series. We showed that negative TQI values are significantly correlated with lower ER AQUA scores on the NBT series and the IBC series (Figure 3 A and B), with p-values of 0.03. The correlation of TQI values and ER AQUA scores on build 2 of the TFBC series trends toward significance (p=0.067) but the majority of samples in this series were formalin fixed within 2 hours. The expression levels of the proteins included in this TQI (pHSP27, Cytokeratin and pERK1/2) do not show any correlation with ER expression as tested by Pearson's Correlation Coefficient of each protein separately (data not shown). Thus the association of the TQI and ER AQUA scores appears to be a function of tissue quality and not a coincidental correlation of the TQI proteins and ER expression.

Discussion

A clinical mishap in Canada and a series of papers focusing on pre-analytic variables have illustrated the need for a mechanism for tissue quality assessment (2, 5, 19). In response to these reports and new ER, PR and HER2 guideline recommendations from the ASCO/CAP panel, efforts have been made to control pre-analytical variables, especially cold ischemic time, to standardize companion diagnostic testing (10, 11). However, these standards cannot always be met and clinicians and researchers are often confronted with the challenge of accurate molecular characterization of tissue samples of unknown quality. Here we describe for the first time the construction of an internally calibrated tool consisting of 3 epitopes and their relative changes to assess the effects of cold ischemic time and the suitability of a given tissue for further immunological assessments.

While substantial efforts in our field have improved standardization and documentation of tissue harvesting and companion diagnostic testing, controlling and minimizing pre-analytical variables on tissue is still problematic and it is not hard to imagine a situation in which a TQI is needed. If for example, if a breast cancer specimen of unknown quality tests negative for ER, one could envision assessment of the TQI to discount false negative results caused through protein degradation. The TQI could also be envisioned to be applied to all specimens received for a clinical trial where tissue is collected from a wide range of sites around the world with less standardized laboratory settings. In this context the TQI could prove that the tissue had the capacity to inform for the companion diagnostic test or it could suggest elimination of that data point in the subsequent biomarker analysis due to poor

tissue quality. Finally, the TQI could be used on retrospective studies based on archived tissue collections where information on postsurgical tissue processing was not available. In each case, testing the quality of the tissue could provide better accuracy, reproducibility and applicability for research based on *in situ* biomarker evaluation.

Although the antibodies used for construction of the TQI were validated and the results of the TQI and its relationship to prolonged cold ischemic time and ER expression levels were reproducible on different breast tissue series, our work should be considered as pilot data and a proof of concept, rather than a definitive TQI test. This first study is subject to a number of limitations. Perhaps the most significant is the low sensitivity and specificity for prediction. The performance of the two marker combinations – Cytokeratin:pHSP27 and pERK1/2:pHSP27 – as measured by AUC value, ranges from 0.6 to 0.7 showing the assay is accurate only between 2/3 and ¾ of the times. Even though the TQI value is significantly correlated with increasing cold ischemic time, a performance of 0.6 to 0.7 AUC value suggests that several specimens in these breast tissue series are misclassified with respect to their potential loss of antigenicity.

A second major limitation of this TQI test is that it was constructed using only breast cancer tissues. Although it validated on two independent tissue sets, the IBC series were treated with different pre-analytical variables (UVSC conditions) (17) resulting in better preservation of biomarker expression as compared to the other 2 breast tissue series. In the future, we envision applicability of future TQIs in many other tissue types.

Finally, any TQI will always be limited by the variability in the epitope degradation rate between different epitopes. Here, we focused on ER where we observed a significant association of lower ER expression levels with negative TQI values in two out of the three breast cohorts used for this study. However, the TQI might be better or worse if other epitopes were assessed. It is possible that to be highly accurate in assessment of tissue quality, a unique index may be required for certain classes of proteins or even for individual proteins. For example, phospho-epitopes on tyrosine appear to be highly labile and might require a different TQI than more stable structural proteins like Tubulin or Actin.

The variable rate of degradation of epitopes raises the question of the time window for the TQI. It has been previously shown that the bulk of the degradation of ER does not occur within the first few hours of delay to formalin fixation but rather at a later time window (6, 8, 9). This observation may explain why we did not observe a significant loss of ER AQUA scores for patients with a negative TQI on the TFBC series where most of the cases where fixed within 2 hours. In comparison, in the longer NBT and IBC sets, where delay to fixation was stretched to 48 hours or more, the TQI performed better. In the future one can envision different TQIs constructed for different time windows, depending on the specific projected application for the TQI.

In summary, for the first time, we report the construction of a TQI which serves as an intrinsic control of tissue quality. While this work is preliminary and further optimization of this TQI is necessary to improve its performance and applicability to multiple tissue types, it represents a proof of concept for the potential for quantitative quality assessment of tissue

specimens. We are hopeful that this approach to quantitative measurement of 3 or 4 biomarkers and their relative changes/relationships to each other can provide a tool to monitor effects of pre-analytical variables on tissue specimens. In the future, we believe we will see more sensitive and specific tools for assessment of quantitative assessment tissue quality.

Acknowledgments

The authors would like to thanks Ms. Lori Charette and her team in at Yale Pathology Tissue Services for TMA construction and tissue sectioning.

Support for this research: This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. Also This work was supported by Ricerca Sanitaria Finalizzata RF-2010-2310674

Abbreviations

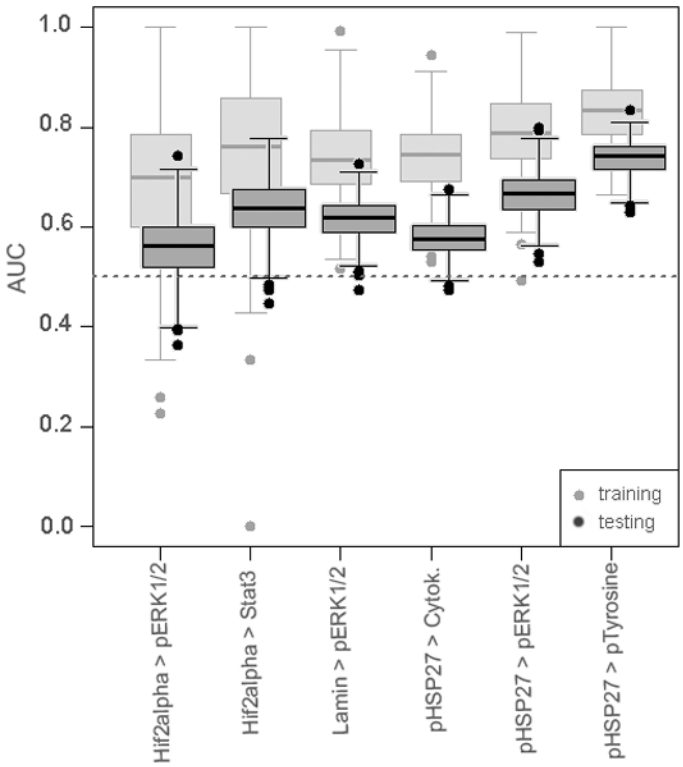
AUC	Area Under the receiver-operator characteristic Curve
AQUA	Automated Quantitative Analysis
ASCO	American Society of Clinical Oncologists
CAP	College of American Pathologists
FFPE	formalin fixed, paraffin embedded
IBC	Italian Breast Cancer Series
NBT	Normal Breast Tissue Series
TFBC	Time to Fixation Breast Cancer Series
TMA	tissue microarray
TQI	Tissue Quality Index
TTF	time to fixation
QIF	Quantitative Immunofluorescence
UVSC	under vacuum sealing and stored at 4º Celsius

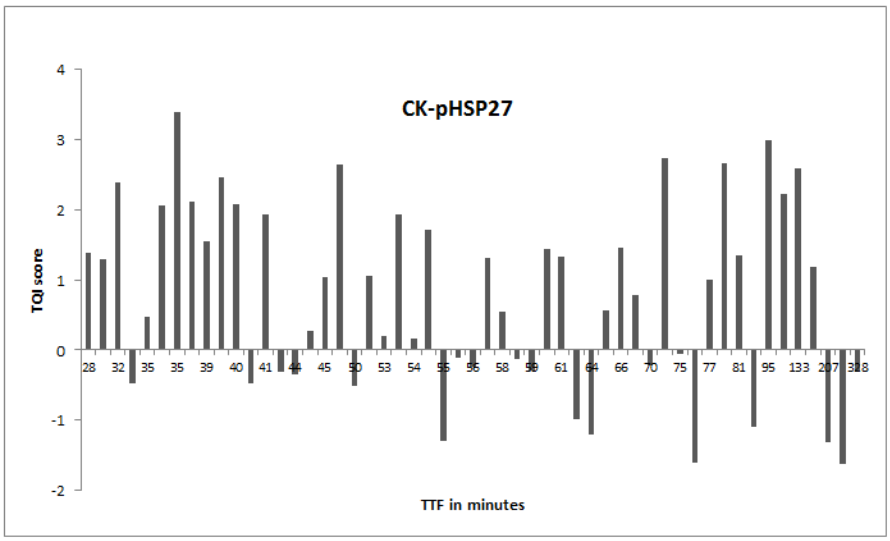
References

1. Hede K. Breast cancer testing scandal shines spotlight on black box of clinical laboratory testing. Journal of the National Cancer Institute. 2008; 100(12):836–7. 44.10.1093/jnci/djn200 [PubMed: 18544732]
2. Moore HM, Compton C, Alper J, Vaught JB. International Approaches to Advancing Biospecimen Science. Cancer Epidemiol Biomarkers Prev. 2011 1055-9965.EPI-11-0021 [pii]. 10.1158/1055-9965.EPI-11-0021
3. Vaught JB, Henderson MK, Compton CC. Biospecimens and biorepositories: from afterthought to science. Cancer Epidemiol Biomarkers Prev. 2012; 21(2):253–5. 21/2/253 [pii]. 10.1158/1055-9965.EPI-11-1179 [PubMed: 22313938]

4. Vaught J, Rogers J, Myers K, Lim MD, Lockhart N, Moore H, et al. An NCI perspective on creating sustainable biospecimen resources. J Natl Cancer Inst Monogr. 2011; 2011(42):1–7. lgr006 [pii]. 10.1093/jncimonographs/lgr006 [PubMed: 21672889]
5. Hicks DG, Boyce BF. The challenge and importance of standardizing pre-analytical variables in surgical pathology specimens for clinical care and translational research. Biotech Histochem. 2012; 87(1):14–7.10.3109/10520295.2011.591832 [PubMed: 21732745]
6. Khoury T, Sait S, Hwang H, Chandrasekhar R, Wilding G, Tan D, et al. Delay to formalin fixation effect on breast biomarkers. Mod Pathol. 2009; 22(11):1457–67. modpathol2009117 [pii]. 10.1038/modpathol.2009.117 [PubMed: 19734848]
7. Bai Y, Tolles J, Cheng H, Siddiqui S, Gopinath A, Pectasides E, et al. Quantitative assessment shows loss of antigenic epitopes as a function of pre-analytic variables. Lab Invest. 2011; 91(8): 1253–61. labinvest201175 [pii]. 10.1038/labinvest.2011.75 [PubMed: 21519325]
8. Yildiz-Aktas IZ, Dabbs DJ, Bhargava R. The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma. Mod Pathol. 2012 modpathol201259 [pii]. 10.1038/modpathol.2012.59
9. Nkoy FL, Hammond ME, Rees W, Belnap T, Rowley B, Catmull S, et al. Variable specimen handling affects hormone receptor test results in women with breast cancer: a large multihospital retrospective study. Arch Pathol Lab Med. 2010; 134(4):606–12. [pii]. 10.1043/1543-2165-134.4.606 [PubMed: 20367311]
10. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol. 2007; 25(1):118–45. JCO.2006.09.2775 [pii]. 10.1200/JCO.2006.09.2775 [PubMed: 17159189]
11. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med. 2010; 134(7):e48–72. [pii]. 10.1043/1543-2165-134.7.e48 [PubMed: 20586616]
12. Pinhel IF, Macneill FA, Hills MJ, Salter J, Detre S, A'Hern R, et al. Extreme loss of immunoreactive p-Akt and p-Erk1/2 during routine fixation of primary breast cancer. Breast cancer research : BCR. 2010; 12(5):R76.10.1186/bcr2719 [PubMed: 20920193]
13. Espina V, Mueller C, Liotta LA. Phosphoprotein stability in clinical tissue and its relevance for reverse phase protein microarray technology. Methods Mol Biol. 2011; 785:23–43.10.1007/978-1-61779-286-1_3 [PubMed: 21901591]
14. Espina V, Edmiston KH, Heiby M, Pierobon M, Sciro M, Merritt B, et al. A portrait of tissue phosphoprotein stability in the clinical tissue procurement process. Molecular & cellular proteomics : MCP. 2008; 7(10):1998–2018.10.1074/mcp.M700596-MCP200 [PubMed: 18667411]
15. De Cecco L, Musella V, Veneroni S, Cappelletti V, Bongarzone I, Callari M, et al. Impact of biospecimens handling on biomarker research in breast cancer. BMC cancer. 2009; 9:409.10.1186/1471-2407-9-409 [PubMed: 19930681]
16. Neumeister VM, Anagnostou V, Siddiqui S, England AM, Zarrella ER, Vassilakopoulou M, et al. Quantitative assessment of effect of preanalytic cold ischemic time on protein expression in breast cancer tissues. Journal of the National Cancer Institute. 2012; 104(23):1815–24.10.1093/jnci/djs438 [PubMed: 23090068]
17. Di Novi C, Minniti D, Barbaro S, Zampirolo MG, Cimino A, Bussolati G. Vacuum-based preservation of surgical specimens: an environmentally-safe step towards a formalin-free hospital. Sci Total Environ. 2010; 408(16):3092–5. S0048-9697(10)00400-6 [pii]. 10.1016/j.scitotenv.2010.04.022 [PubMed: 20444497]
18. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. Nat Med. 2002; 8(11):1323–7. nm791 [pii]. 10.1038/nm791 [PubMed: 12389040]

19. Fergenbaum JH, Garcia-Closas M, Hewitt SM, Lissowska J, Sakoda LC, Sherman ME. Loss of antigenicity in stored sections of breast cancer tissue microarrays. Cancer Epidemiol Biomarkers Prev. 2004; 13(4):667–72. [PubMed: 15066936]





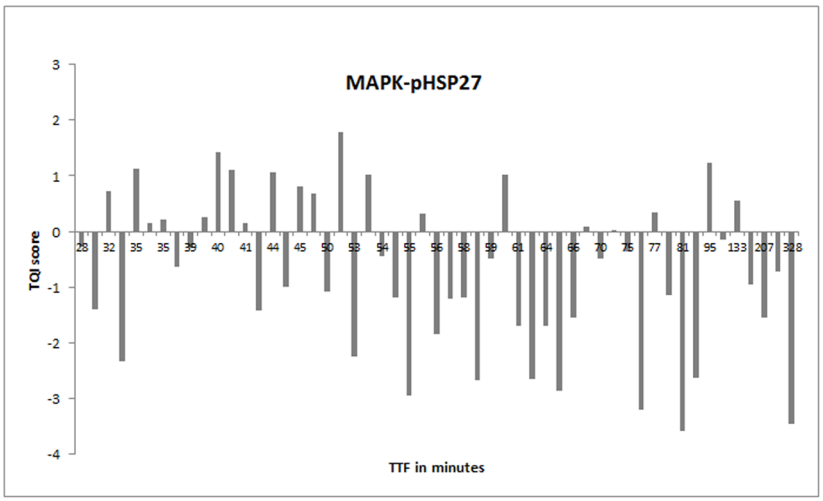
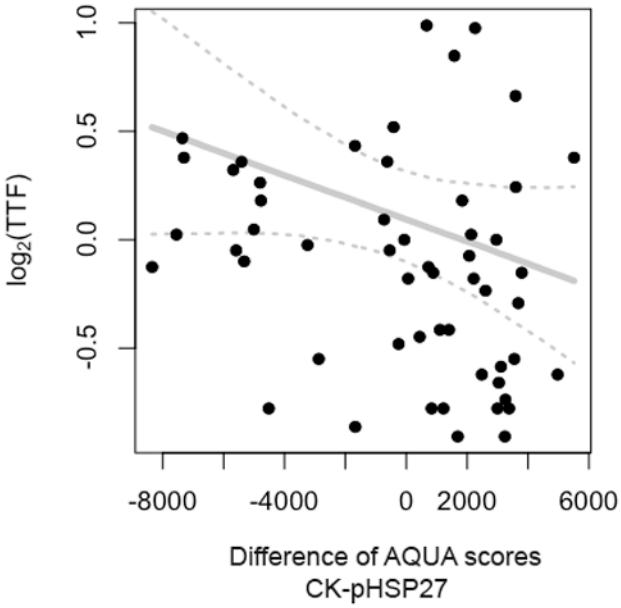
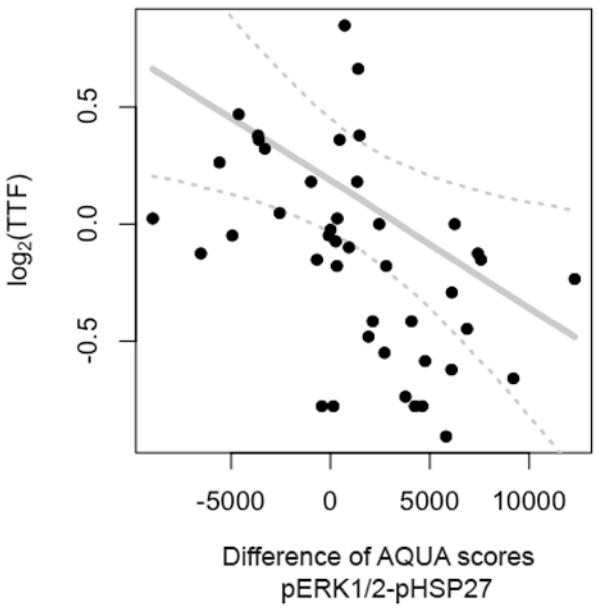
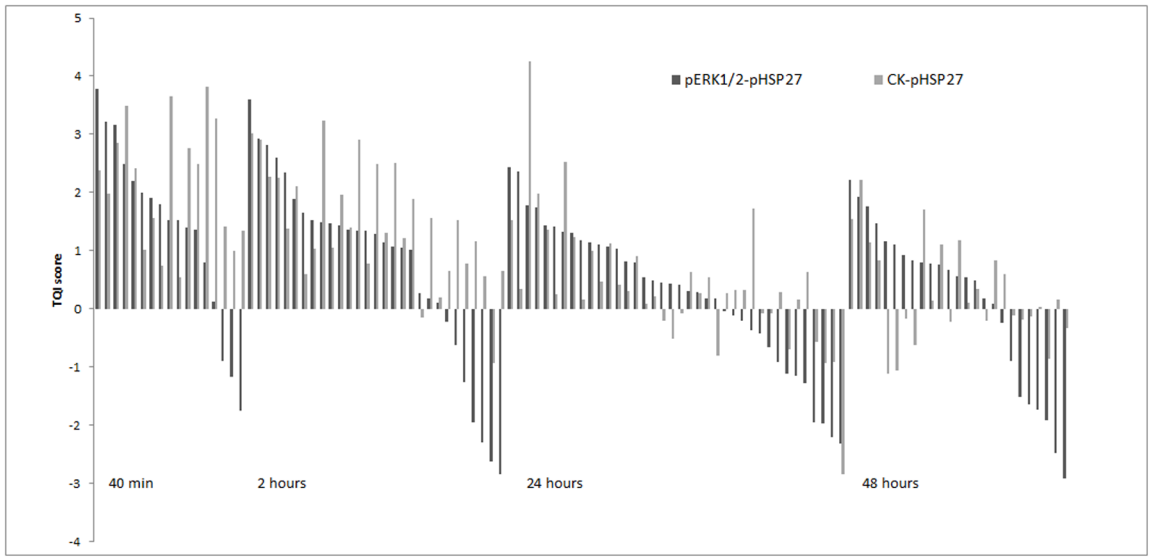


Figure 1.

A) The performance of 6 marker combinations on the testing and validation subgroup of the Time to Fixation Breast Cancer Series as measured by ROC curves and AUC values. The TQI was then calculated on the complete Time to Fixation Breast Cancer Series. Panel B illustrates the TQI values of Cytokeratin:pHSP27 and Panel C pERK1/2:pHSP27 in relationship with increasing cold ischemic time.







I pERK1/2 TQI

TQI <0

	no	yes	Totals
0.66 hours	14	3	17
2 hours	22	7	29
24 hours	24	14	38
48 hours	17	8	25
Totals	77	32	109

Chi-square: p=0.045

II Cytokeratin TQI

TQI <0

	no	yes	Totals
0.66 hours	17	0	17
2 hours	27	2	29
24 hours	27	11	38
48 hours	13	12	25
Totals	84	25	109

Chi-square: p=0.0003

III Combined TQI

TQI <0

	no	yes	Totals
0.66 hours	14	3	17
2 hours	21	8	29
24 hours	20	18	38
48 hours	11	14	25
Totals	66	43	109

Chi-square: p=0.0299

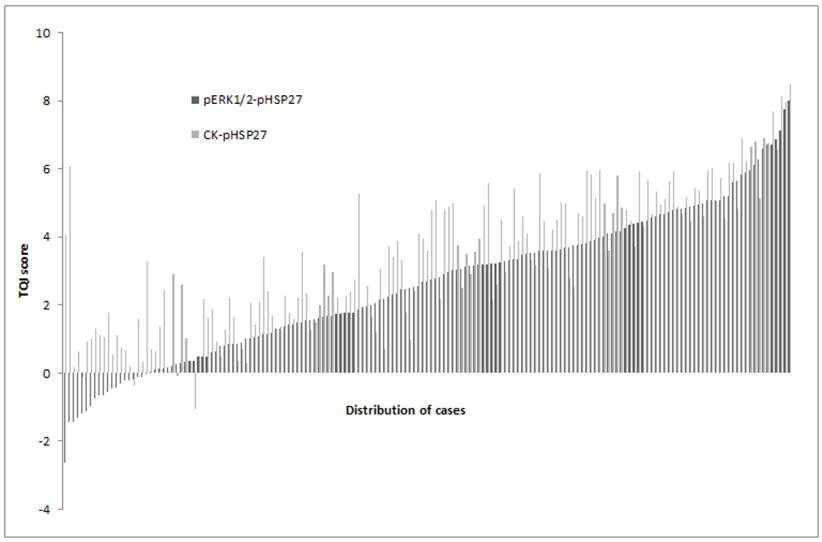


Figure 2.

Validation and performance of the TQI on the TFBC, the NBT and IBC series: Panel A: Linear regressions between increasing time to fixation (log2 transformed) and the differences of AQUA scores of the TQI markers as performance measurement on an independent built of the TFBC. Higher values correspond with shorter time to fixation. The dotted line shows the 95% CI of the regression line. Panel B: TQI pairs on each time-point of the NBT series. Panel C: Chi squared analysis of the different TQI components. While each marker combination by itself shows a significant correlation to increasing cold ischemic time, the combination of the separate TQI components facilitates the identification of a larger number of samples, which may have compromised tissue quality. Panel D: The TQI performance on the IBC series where special fixation conditions appears to result in significantly less epitope degradation.

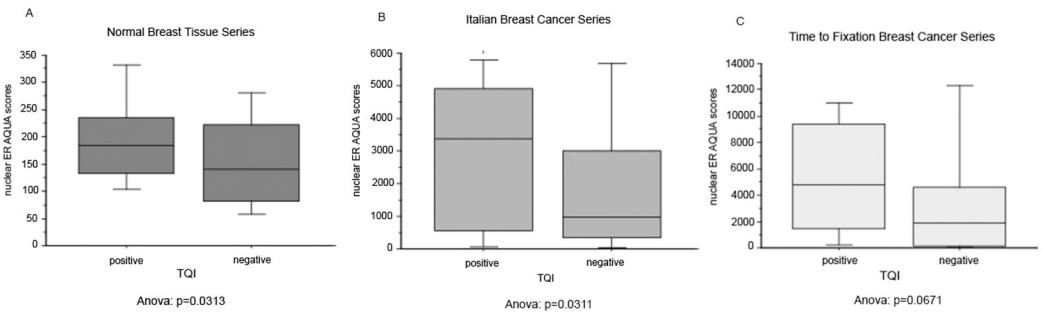


Figure 3.

Measurement of the TQI performance as a function of ER expression levels quantified by AQUA. Negative TQI values are significantly associated with lower ER AQUA scores on the NBT series and the IBC series (A and B), while the correlation between TQI values and ER expression does not reach significance on build 2 of the TFBC series.

Table 1

Antibodies tested for the TQI

Symbol	Description	Antibody	Supplier
		Clone/Isotype	
Markers of Cold Ischaemia			
ACTB	Beta-Actin	13E5/IgG	Cell Signaling Technology
TUBB	Beta-Tubulin	pF3/IgG	Cell Signaling Technology
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	14C10/IgG	Cell Signaling Technology
HIST4	Histone 4	L64C1	Cell Signaling Technology
HIST3	Histone 3	96C10/IgG1, kappa	Cell Signaling Technology
LMNA/C	Lamin A/C	polyclonal	Cell Signaling Technology
LDHA	Lactat Dehydrogenase	IgG, C4B5	Cell Signaling Technology
ERalpha	Estrogen Receptor alpha	SP1/IgG	Thermo Scientific
CK	Cytokeratin	AE1/AE3/IgG1	DAKO
CK	Cytokeratin	polyclonal	DAKO
p53	Anti-Human p53 protein	IgG2b. DO-7	DAKO
Markers of Hypoxia			
CCND1	Cyclin D1	IgG/SP4	Thermo Fisher Fremont
Caspase	Cleaved Caspase 3 (Asp175)	polyclonal	Cell Signaling Technology
HIF1	Hypoxia Inducible Factor 1	polyclonal	Novus Biological
AKAP13	A-kinase anchoring protein13	IgG2a/ZX-18	Santa Cruz Biotechnology
CDC42		IgG3/B-8	Santa Cruz Biotechnology
CCNB1	Cyclin B1	GNS-11/IgG2	BD Biosciences
HIF-2alpha	Hypoxia inducible factor - 2alpha	ep190b/IgG1	abcam
CA9	Carbonic Anhydrase IX	polyclonal(aa581–592)	Lifespan Biosciences
Markers of phosphorylated proteins			
pAKT 473	phospho-Akt (ser473)	D9E/IgG	Cell Signaling Technology
pERK1/2	Phospho-p44/43MAPK (Erk1/2) (Thr292/Tyr204)	IgG	Cell Signaling Technology
pER	Phospho-Estrogen Receptor alpha (Ser118)	16J4/IgG2b	Cell Signaling Technology
Anti-Phosphotyrosine	4G10 Anti-Phosphotyrosine	IgG2b	Millipore
Anti-Phosphotyrosine		p-Tyr-100	Cell Signaling Technology
pHSP27 (pS78)	phosphorylated Heat Shock Protein 27	Y175	Epitomics
pHer2 (Tyr1248)	Phospho-Her2/ErbB2 (Tyr1248)	PN2A	Thermo Scientific
Phospho-Stat3 (Tyr705)	Phospho-Stat3 (Tyr705)	D3A7/IgG	Cell Signaling Technology
p-S6 Ribosomal Protein (Ser235/236)	Phospho-S6 Ribosomal Protein (Ser235/236)	D52.2.2E/IgG	Cell Signaling Technology
Phospho-Jak2 (Tyr1007/1008)	Phospho-Jak2 (Tyr1007/1008)	polyclonal	Cell Signaling Technology
Phospho-Met (Tyr1234/1235)	Phospho-Met (Tyr1234/1235)	IgG	Cell Signaling Technology
Phospho-Sapk/Jnk	Phospho-Sapk/Jnk	IgG	Cell Signaling Technology
Phospho mTor (Ser2448)	Phospho mTor (Ser2448)	49F9/IgG	Cell Signaling Technology
Markers of posttranslational modification			

Symbol	Description	Antibody	Supplier
Sumo1	small ubiquitin related modifier 1	Y299/IgG	abcam
Acetylated-Lysine	proteins posttranslat. Modified by acetylation	polyclonal, purified	Cell Signaling Technology
NEDD8	neural precursor cell-expr. devel. Downreg. protein9	IgG, 19E3	Cell Signaling Technology