

ORIGINAL ARTICLE

Protocol for HER2 FISH determination on PAXgene-fixed and paraffin-embedded tissue in breast cancer

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SUMMARY

Molecular diagnostics in personalized medicine increasingly relies on the combination of a variety of analytical technologies to characterize individual diseases and to select patients for targeted therapies. The gold standard for tissue-based diagnostics is fixation in formalin and embedding in paraffin, which results in excellent preservation of morphology but negatively impacts on a variety of molecular assays. The formalin-free, non-cross-linking PAXgene tissue system preserves morphology in a similar way to formalin, but also preserves biomolecules essentially in a similar way to cryopreservation, which markedly widens the spectrum, sensitivity and accuracy of molecular analytics. In this study, we have developed and tested a protocol for PAXgene-fixed and paraffin-embedded tissues for fluorescent *in situ* hybridization (FISH). The implementation of a 24-h formalin postfixation step of slides from PAXgene-fixed and paraffin-embedded tissues allowed us to use the assays approved for formalin-fixed and paraffin-embedded tissues. The equivalence of the methodologies was demonstrated by FISH analysis of HER2 amplification in breast cancer cases. The 24-h postfixation step of the slides used for FISH can be well integrated in the routine diagnostic workflow and allows the remaining PAXgene-fixed and paraffin-embedded tissue to be used for further molecular testing.

Keywords

breast cancer, FISH, HER2, molecular diagnostics, Postfixation of PAXgene tissue

Developments in personalized medicine and companion diagnostics have generated increasing demand for tissue stabilization methods that enable classical histopathological diagnostics, immunohistochemistry and a broad range of molecular diagnostics from one tissue sample. Formalin fixation is the gold standard for tissue fixation for histopathological diagnostics and medical research (Fox *et al.* 1985; Oosterhuis *et al.* 2003). However, formalin modifies proteins and nucleic acids by forming methylol adducts and cross-links which negatively interfere with several molecular analyses (Masuda *et al.* 1999; Metz *et al.* 2003; Evers *et al.* 2011).

The recently developed PAXgene tissue system (PAXgene) is a formalin-free, non-cross-linking fixative for tissues resulting in excellent tissue quality for molecular diagnostics as well as preserving tissue features for classical morphological diagnosis. A broad spectrum of analytical technologies

have already been investigated for their applicability on PAXgene-fixed and paraffin-embedded (PFPE) tissues, such as histopathology, immunohistochemistry (Kap *et al.* 2011; Viertler *et al.* 2012; Groelz *et al.* 2013; Gundisch *et al.* 2014), DNA- or RNA-based analyses, such as PCR and gene sequencing as well as proteomic analyses (Ergin *et al.* 2010; Viertler *et al.* 2012; Groelz *et al.* 2013; Gundisch *et al.* 2014). However, attempts to use protocols developed for fluorescence *in situ* hybridization (FISH) on formalin-fixed and paraffin-embedded (FFPE) tissues have so far failed on PFPE tissues.

FISH is used as a detection technique for a broad range of chromosomal alterations, in particular translocations, sub-microscopic deletions and amplifications, and has become a cornerstone of molecular cancer diagnostics. Several drugs in personalized medicine (e.g. trastuzumab for the treatment of breast cancer) require a FISH test listed as companion

diagnostics by the FDA (<http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm>). Therefore, usability of fixatives for classical histological diagnosis as well as FISH is an essential requirement for their relevance in molecular diagnostics in personalized medicine. For this reason, we have investigated whether FISH developed for FFPE can be used for PFPE tissue using FISH analysis of the human epidermal growth factor receptor 2 (HER2), which is one of the most commonly applied FISH analysis, as example. Amplification and overexpression of HER2 gene have been documented in approximately 20% of invasive human breast cancers (Pauletti *et al.* 1996; Owens *et al.* 2004; Wolff *et al.* 2007) and are associated with prognostic and predictive implications (Press *et al.* 1997; Yamauchi *et al.* 2001). As a consequence, the detection of HER2 gene amplification by FISH is a frequently required analysis in the context of anti-HER2-targeted personalized cancer therapy (Wolff *et al.* 2007).

In this study, we present a protocol for FISH on PFPE tissues by adapting the FISH assay for HER2 (ZytoLight HER2/CEN17 Dual Color Probe), which has been developed and validated for FFPE tissues.

Methods

Study design and fixation processing of breast cancer tissue samples

The study included 13 cases with primary invasive breast cancer from Graz/Austria (eight) and from Hilden/Germany (five). All patients were women and the age range was 39–88 years (mean 64 years). For the study, a 4-mm-thick tissue specimen was cut from a breast tumour and divided into two approximately equal samples for different tissue fixation processes, formalin fixation and two-reagent PAXgene system (PAXgene Tissue System, PreAnalytiX, Hombrechtikon, Switzerland) (Fig. 2) consisting of non-crosslinking fixative and stabilizer. Each sample was placed into standard tissue cassettes (Simport Plastic Ltd., Beloil, QC, Canada) and completely submerged in a container filled with either 4% neutral buffered formaldehyde (NBF) (Sigma-Aldrich, Steinheim, Germany) for 24 h or PAXgene Tissue Fix (PreAnalytiX GmbH, Hombrechtikon, CH) with fixation solutions in a ratio of at least 20 parts fixative to one part of tissue (v/v) for 2–24 h at room temperature. PAXgene-fixed samples were then transferred into PAXgene Tissue Stabilizer for 2–72 h at room temperature. Tissue processing was performed in two different tissue processors for FFPE and PFPE samples respectively. After stepwise dehydration in 70%, 80%, 90% and 99% ethanol, followed by isopropanol and xylene, the PAXgene tissue-fixed and formaldehyde-fixed samples were embedded in paraffin. All blocks of FFPE and PFPE tissues were stored at 4°C in the dark until use. For deparaffinization, FFPE and PFPE sections (2 µm thick) were heated at 70°C for 30 min and rehydrated with successive applications of xylene

(2 × 15 min), 96% ethanol (2 × 15 min), 90%, 80%, 70% and 50% ethanol (2 min for each). Finally, samples were washed in distilled water (2 × 10 min) and PBS (1 × 10 min).

Postfixation of PAXgene-fixed and paraffin-embedded (PFPE) specimens

After deparaffinization, slides with PFPE sections were directly postfixed in 4% formalin for varying time periods of 1, 2, 4, 6, 8, 18 or 24 h at room temperature. Subsequently, excess formalin was removed by washing with PBS (3 × 10 min) and distilled water (2 × 10 min).

Fluorescence in situ hybridization (FISH)

For HER2 FISH testing, a ZytoLight[®] SPEC HER2/CEN17 Dual Color Probe Kit was used (ZytoVision GmbH, Bremerhaven, Germany) according to the manufacturer's instructions. Briefly, the FFPE, PFPE and the postfixed PFPE slides were incubated in a 98°C heat pretreatment solution citric for 15 min and washed in distilled water (3 min). Pepsin solution was applied on all slides for 10 min at 37°C, incubated for 5 min in wash buffer SSC and then rinsed in distilled water (1 min). Slides were dehydrated through graded alcohols (50%, 70%, 90%, 100%; 1 min each) and air-dried. For hybridization, 10 µl of HER2/CEN17 Dual Color Probe and specimen DNA were first codenatured on a hot plate at 75°C for 10 min and lastly hybridized at 37°C overnight. The slides were then incubated in 37°C prewarmed wash buffer A (5 min), dehydrated through graded alcohols (70%, 90%, 100%; 1 min each), air-dried and protected from light. Finally, 30 µl DAPI/antifade was applied and covered with a coverslip. Slides were stored at 2–8°C in the dark until evaluation by microscopy.

Confocal laser scanning microscopy (CLSM)

Breast cancer specimens were evaluated using a confocal laser scanning microscope LSM510 Meta (Zeiss). Images were acquired at 488 nm excitation wavelength using a BP 505–530 nm band-pass detection filter for the green channel and 543 nm excitation wavelength in conjunction with a LP 560 for the red channel.

Evaluation of HER2 amplification status

The HER2/CEN17 Dual Color Probe is a mixture of a red fluorochrome directly labelled CEN17 probe specific for the alpha satellite centromeric region of chromosome 17 (D17Z1) and a green fluorochrome directly labelled HER2 probe specific for the HER2 gene (probe location 17q12). Tumour specimens with a HER2:CEN17 ratio ≤2.0 per nucleus are scored as normal, whereas those with a HER2:CEN17 ratio ≥2.0 are scored as amplified (Wolff *et al.* 2007).

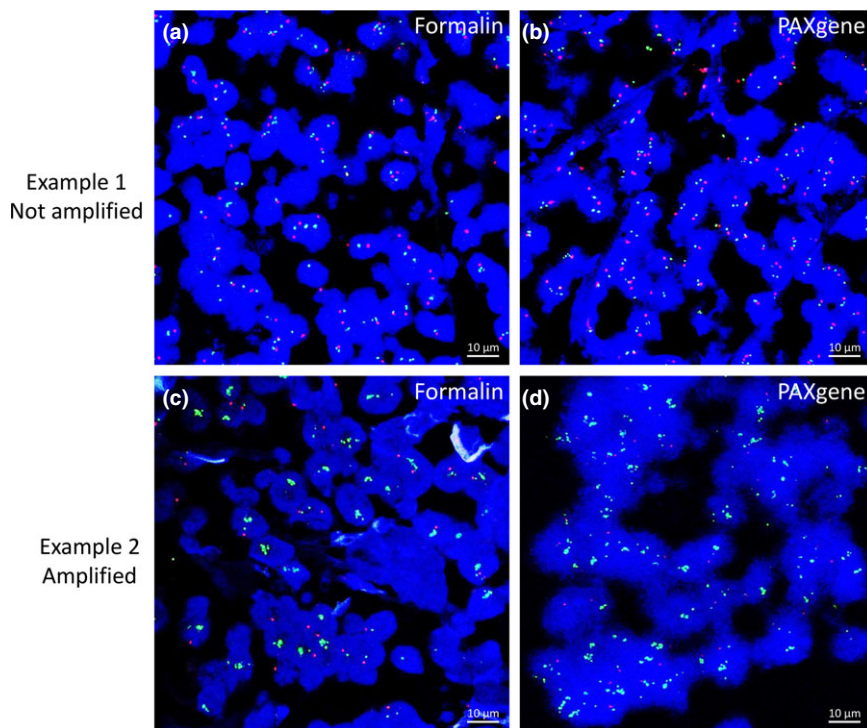


Figure 1 FISH evaluation of HER2 status in breast cancer. In normal interphase nuclei (blue), two red (CEN17) and two green (HER2) signals are expected. In cells with amplified HER2 gene locus, multiple copies of green signals or green signal clusters are detected. Within our analysis, signals of HER2 and CEN17 indicate normal interphase cells in example 1 (a,b) and HER2 amplification in example 2 (c,d). HER2 status was identical in corresponding FFPE (a,c) and PFPE (b, d) tissue sections. Scale bar 10 μm.

Ethical approval

Sample donors provided written informed consent, and sampling was approved by the Ethics Committee of the Medical University of Graz, Austria (reference number 20-066; 15.12.2015) and from Cureline (South San Francisco, CA).

Results

In this study, we tested different formalin postfixation treatments of PFPE tissue sections ranging from 60 min to 24 h

Table 1 Determination of HER2 status. Identical results were obtained by analysis of 13 breast cancer cases in corresponding FFPE and PFPE tissues

Breast cancer cases	HER2 Status
1	Not amplified
2	Not amplified
3*	Not amplified
4	Amplified
5	Not amplified
6	Not amplified
7	Amplified
8	Not amplified
9	Not amplified
10	Not amplified
11	Not amplified
12*	Amplified
13	Not amplified

*Images of cases 3 and 12 are represented in Figure 1. Analyses of 13 breast cancer cases indicated HER2 amplification in 3 cases.

postfixation. Tissue sections with postfixation times up to 8 h showed weak and uneven signal intensities for the two probes as well as increased background in comparison with the corresponding FFPE sections. However, postfixation times of 18 and 24 h resulted in FISH signals, which were comparable to FFPE tissues. In total, 3 of 13 cases showed HER2 amplification (Table 1), and in all cases tested, the HER2 amplification status was identical in both FFPE and the corresponding postfixed PFPE slides. FISH signals of

Workflow of HER2 determination

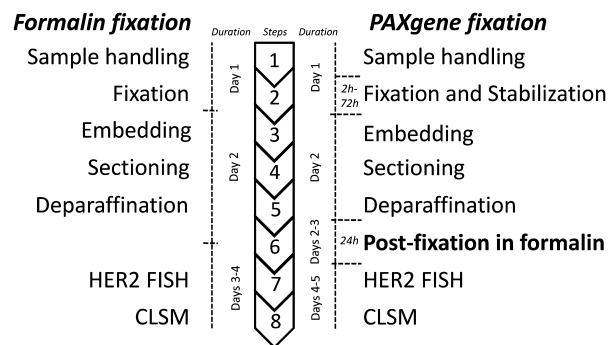


Figure 2 Similar workflows of HER2 determination of formalin-fixed and paraffin-embedded (FFPE) and PAXgene-fixed and paraffin-embedded (PFPE) tissues. This figure shows the preparation and analysis of breast cancer tissues fixed in both fixation procedures. A standard workflow of a FFPE HER2 FISH protocol is shown (steps 1–5, 7–8). An additional formalin postfixation step (6) of 24 h is applied to PFPE tissues, allowing the FISH test to be performed using the same time frames as established for FFPE but with a 24-h delay after step 5.

PFPE specimens were well detected after postfixation times of 18–24 h (Figures 1 and 2). Dual-probe FISH staining resulted in clear and discernible colours for HER2 (green) and CEN17 (red) in both FFPE and PFPE tissues. In example 1 of Figure 1, the majority of tumour cells have nuclei with two green dots representing two HER2 signals and two red dots representing two CEN17 reference probe signals indicating a normal diploid status. Example 2 (Figure 1) shows HER2 amplification, where most of the nuclei have multiple green dots indicating the presence of extra copies of HER2 in these tumour cells.

Discussion

FISH protocols used in medical diagnostics have been optimized and validated for use on FFPE tissues. Since in particular the prehybridization steps have to be tuned to the properties of the tissue, such as fixation type and times, new fixatives typically require substantial modification of the prehybridization and possibly also of the hybridization or washing procedures. Since the chemical modification and cross-linking properties of formalin are a major difference between formalin and PAXgene fixation, we tested whether a postfixation step of sections of PFPE tissues could generate tissue properties similar to FFPE tissues. The observed efficiency of the postfixation protocol for FISH, although simple, was not obvious since a similar postfixation step was not suitable to use immunohistochemistry protocols developed for FFPE on PFPE tissues (data not shown). Interestingly, short postfixation times (8 h and less) had no positive effects on the FISH results. Only postfixation times of 18 or 24 h resulted in properties equivalent to FFPE tissues. Formaldehyde is a small molecule (MW 30) and penetrates quite rapidly through tissues and cells, typically reaching a penetration depth of 5 mm in about 2 h (Baker 1958). Consequently, the penetration time of formalin is not limiting in the experimental conditions applied on 2- μ m-thick sections. The observed requirement of a postfixation time of 18–24 h can be better explained by the time required to achieve equilibrium of formalin-induced chemical modifications. The chemical mode of action of 14 C formaldehyde as a fixative has been investigated extensively. Formalin leads to the formation of intra- and intermolecular cross-links by chemical reaction with proteins, and the longer the fixation time, the greater the degree of cross-linking (Gustavson 1956). We confirmed these reported observations, where postfixation with formalin required approximately 18 h of reaction time to be effective which is well in line with the incubation time already demonstrated experimentally, where tissue sections fixed in formaldehyde reached equilibrium in 24 h at room temperature (Fox *et al.* 1985).

Since a postfixation time of 18 h would result in a workflow that is difficult to implement in routine diagnostics, we focussed in our study on a 24-h postfixation time, which would allow maintaining, with a 24-h delay, the same timing for the workflow as established for FFPE tissues (Figure 2). The procedures for FFPE and PFPE tissues are

summarized in Figure 2 starting from sample handling (Figure 2, step 1) via both fixation methods (step 2), through to deparaffinization (step 5). A 24-h formalin postfixation step of sections from PAXgene-fixed and paraffin-embedded tissues enabled the use of FISH protocols validated for formalin-fixed tissues (step 6). Finally, HER2 FISH and confocal laser scanning microscopy (CLSM) analysis (steps 7–8) can be performed, and a 24-h delay in diagnosing the HER2 amplification status should be acceptable in the planning of breast cancer therapy. At the same time, the preservation of the cancer tissue using PAXgene would be of major advantage compared to formalin for a variety of additional molecular analyses, such as gene expression studies, protein phosphorylation status, to be performed from the same PFPE tissue sample.

Thus, our study demonstrates that a simple postfixation step of sections from PFPE tissues results in identical HER2 FISH results as those from FFPE tissues. This allows the use of the same protocol as originally established, validated and approved for FFPE and brings the additional benefits of PFPE tissues for a broad range of molecular analysis, both for routine diagnostics and for research applications.

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Conflict of interest

D.G. and R.W. are employed by Qiagen and have stock options or bond holdings in the company's pension plan. The data presented in this manuscript refer to the PAXgene tissue system, which has been developed in a joint effort by Qiagen and PreAnalytiX. Qiagen is industrial partner of the public-funded Christian Doppler Laboratory for Biospecimen Research and Biobanking Technologies. All authors have no conflict of interest.

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Authors' contribution

C.V., K.Z. and M.L. contributed to the conception of the study. L.O.W. performed the research. K.Z., L.O.W. and

M.L. implemented analysis and presentation of data. K.Z., L.O.W., M.L. and D.G. drafted the article. K.Z. and L.O.W. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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