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# Effects of slide storage on detection of molecular markers by IHC and FISH in endometrial cancer tissues from a clinical trial: an NRG Oncology/GOG pilot study

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Authors' contributions

TAG carried out the FISH study, generated and analyzed data and illustrations and wrote study protocol, design and manuscript. VLF performed statistical analyses and interpretation of the data, contributed to study protocol, design, figures and tables and was major contributor in writing the manuscript. AGM and MA performed study conception and design, acquisition of IHC data and critical revision. MJG and LM provided key technical support, database, sample acquisition and handling. NCR provided sample acquisition and tumor morphology verification, study database and critical revision. CS, JPK, SMS, MWM and NMS provided database, sample acquisition and handling. OIO contributed to study conception, design, presentations and intellectual and funding support, and was major contributor in writing the manuscript. GFF contributed to protocol conception and design, interpretation of data, presentations, and was major contributor in writing the manuscript. MJB contributed to study conception, design, presentations and intellectual support. All authors read and approved the final manuscript.

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## Abstract

We performed a pilot study in anticipation of using long-aged precut formalin fixed paraffin embedded (FFPE) tissue sections stored in real-world conditions for translational biomarker studies of TOP2A, Ki67, and HER2 in endometrial cancer. FFPE tissue blocks or unstained slides or both from GOG-0177 were collected centrally (1999-2000) and stored at room temperature. During 2004-2011 specimens were stored at 4°C. Matched pairs of stored slides and freshly cut slides from stored blocks were analyzed for TOP2A (KiS1), Ki67 (MIB1) and HER2 (Herceptest™) proteins. To assess DNA stability (*HER2* PathVision), FISH was repeated on stored slides from 21 cases previously shown to be *HER2*-amplified. IHC staining intensity and extent, mean FISH copies/cell, and copy number ratios were compared using the kappa statistic for concordance or signed rank test for differences in old cut *versus* new cut slides. IHC results reflected some protein degradation in stored slides. The proportion of cells with TOP2A staining was lower on average by 12% in older sections (p=.03). The proportion of Ki67 positive cells was lower in stored slides by an average of 10% (p<.01). Too few cases in the IHC cohort were FISH positive for any conclusions. *HER2* amplification by FISH was unaffected by slide storage. We conclude that use of aged stored slides for proliferation markers TOP2A and Ki67 is feasible but may modestly underestimate true values in endometrial cancer. Pilot studies for particular storage conditions/durations/antigens to be used in translational studies are warranted.

### Keywords

Slide storage; Immunohistochemistry; Protein degradation; Epitope preservation; FFPE specimens

## Introduction

Multi-institutional clinical trials commonly include biomarker analysis of formalin fixed paraffin embedded (FFPE) samples performed en masse at the end of the trial. As blocks may not always be released by institutions for clinical trial use, or may be recalled, central repositories often store pre-cut FFPE sections mounted on glass slides at room temperature. There are more than 60 pre-analytical variables that are capable of impacting immunohistochemistry (IHC) during tissue fixation and processing [1,2]. If significant loss of immunoreactivity is a consequence of storage of sections on glass slides [3,4], the use of aged cut sections may contribute to spurious conclusions. Prior to performing a larger correlative study assessing the predictive value of Topoisomerase 2A (TOP2A, which has been reported to have predictive benefit for use of anthracyclines in breast cancer)[5], Ki67, and Human Epidermal Growth Factor Receptor 2 (HER2) on the efficacy of anthracycline-based therapy in the therapy of metastatic endometrial cancer for women enrolled on GOG 177 (which compared doxorubicin/cisplatin to paclitaxel/doxorubicin/cisplatin), we performed a pilot study to compare IHC staining on years-old stored unstained slides with IHC staining on new cut slides from stored blocks of endometrial tumor specimens. Nearly half of available samples from the GOG-0177 cohort were in the form of pre-cut unstained slides. We also evaluated the effect of slide storage on quantitation of *HER2* gene copies by Fluorescent *in situ* hybridization (FISH).

## Materials and methods

### Tumor samples and storage conditions

This study was approved by the GOG/NRG Oncology Group (GOG protocol 8013) and the University of Chicago Institutional Review Board and conducted on FFPE tumor samples from research subjects enrolled on GOG protocol 0177 [6] (Fig 1). Informed consent was obtained from all patients on GOG-0177 before sample submission. Multiple institutions participated in this study; information related to pre-analytical conditions of sample processing is unknown.

For IHC evaluation, 15 cases with specimens from primary endometrial tumors for which both tumor blocks and ≥ 3 precut slides were available were analyzed. Selection of number of cases was based on the desire to evaluate assays in stored precut tissue yet preserve as much tissue as possible for future correlative studies. Matched pairs of stored (>10 years) and new cut (2-3-weeks old) tumor tissues were prepared.

For detection of DNA stability, we used slides from a separate group of GOG-0177 cases that had previously (from slides cut in 2001) been determined to be HER2 amplified [7]. Left-over tissue sections from this set of 21 *HER2* amplified cases had been stored at room temperature and the analysis of *HER2* amplification status by FISH was repeated on these old cut slides in 2004 (22 months old). Histology on these cases was as follows: clear cell (n=3), serous (n=6), mixed, grade 3 (n=4), endometrioid (G1:1, G2: 1, G3: 5), other (n=1).

### Immunohistochemistry (IHC) and Fluorescence *in situ* hybridization (FISH) assays

IHC was performed on 5 μm-thick FFPE tissue sections mounted on positively charged slides. All tissues were stained simultaneously to ensure consistency of the procedure with the given antibody. The antibodies (Ab) used were anti-TOP2A (KiS1, DAKO Cytomation, Denmark, dilution 1:400), anti-Ki67 (MIB1, DAKO, Carpinteria, CA, dilution 1:100) and anti-HER2 (CerbB2, HercepTest Kit™, K5204, DAKO, Carpinteria, CA, ready to use). Stroma and inflammatory cells were negative internal controls for all biomarkers. Isotype staining with corresponding immunoglobulin instead of Ab was used as the negative control for antibody specificity. All IHC procedures were performed centrally at the University of Chicago Human Tissue Resource Center (HTRC) IHC Core Facility using standardized antigen retrieval protocols and appropriate controls. Tissue sections from normal testis and tonsil were used as positive IHC controls for TOP2A and Ki67, respectively, and were provided by the HTRC. HER2 IHC control slides were included in the HercepTest® Kit.

For this portion of the study 15 tumors were used, 3 endometrioid gr1, 2 endometrioid gr 2, 6 endometrioid gr 3, 1 clear cell, 2 mixed and 1 undifferentiated carcinoma. Histology was confirmed by two gynecologic pathologists.

Sections were initially deparaffinized in xylene and rehydrated via graded ethanol and then immunostained for the marker using the EN Vision + (DAKO) assay with an automated staining system (I 6000, BioGenex, USA) according to the manufacturer's instructions and as described previously [8]. To minimize loss of antigenicity, microwave heating (MWH) antigen retrieval was used [3,9]. For TOP2A and Ki67 immunostaining, pretreatment included

20 min in a steamer and the use of antigen retrieval buffer (S1699, DAKO); for HER2 immunostaining, pretreatment included 40 min water bath or MWH and the use of epitope retrieval solution (HercepTest Kit™, DAKO). Thereafter, sections were incubated with the primary Ab for 30 minutes at room temperature and then incubated with polymer for 30 minutes. The antigen-antibody reaction was visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB) as a chromogen substrate and counterstained with hematoxylin and eosin (H&E). New-cut and old-cut H&E-stained slides were paired with corresponding new-cut and old-cut IHC -stained slides. Image acquisition was performed using a Leica DMLB microscope with DFC450 camera and LASX software (Leica Microsystems).

Each entire slide was scored manually in a blinded fashion without knowledge of slide pairing or slide age by two pathologists (TM and MA) jointly in a semi quantitative manner using conventional bright-field microscopy (x10, x20, and x40 objectives). Scoring disagreements were resolved at the time of reading. For TOP2A, the nuclear pattern of intensity and percentage of positive cells were recorded; positivity was interpreted using ImmunoReactive Scoring system (IRS) as described by Faggad and colleagues [10]:

0 (negative), 1+ (weak), 2+ (moderate) and 3+ (strong). The percentage of immunostained cells was captured at each intensity level and scored as following: 0 (0% staining), 1 (staining in 1-10% of tumor cells), 2 (11–50%), 3 (51-80%) and 4 (> 80%). The intensity of staining multiplied by percentage of positive cells resulted in a combined score with a value between 0 and 12. Scores of 0-3 were designated as negative or low expression, whereas scores of 4-12 were designated as positive (high) expression.

Ki67 expression was scored by recording the percentage of cells with nuclear staining. High (above the cutoff point) versus low (below the cutoff point) expression was determined based on a 10% cut off [11-13]. Other cut off points of 13% and 1% [12,14,15] were also explored.

HER2 expression was detected using the same antibody and scoring system as in GOG-0177 and according to manufacturer and FDA recommendations [7]. HER2 IHC results were correlated with previously published HER2 FISH data from these cases [7].

FISH assay and analysis were performed as described previously [7,16], using the Vysis *HER2/CEP17* DNA Probe Mixture according to manufacturer recommendations (Vysis/Abbott Molecular, Des Plaines, IL). Control FFPE breast cancer cell lines were provided by the manufacturer. The pretreatment step was adjusted for use in archival material [16]. Analysis was performed using a Zeiss AxioImagerZ2 fluorescence microscope with Axiocam MRm camera and Axiovision software (Carl Zeiss MicroImaging). The mean copy number per cell and the ratio of *HER2* to chromosome 17 centromere enumeration probe (*CEP17*) were compared. Cells with a gene to chromosome signal ratio ≥ 2 were considered amplified. FISH results in old slides (stained in 2004) were analyzed blindly without knowledge of previous *HER2* FISH scores or scores in new cut slides from the same cases (stained in 2001-2002) [7].

### Statistical evaluation

The kappa statistic for marker expression concordance (0.75-1.0 considered good to perfect; 0.40-0.75 considered fair to good; and below 0.40 considered poor agreement)[17] and signed rank test for differences in percentage cells staining positive in old-cut versus new-cut slides were applied. The differences in percentages of cells stained positive between new and old sections (vertical axis) were mapped against average values of percentages of positive cells from pairs of new and old sections (horizontal axis) in Bland-Altman plots.

## RESULTS

### TOP2A assessment

TOP2A nuclear staining ranged from weak (1+) to strong (3+) with the exception of one case showing no staining (0), and this was an old cut slide (Fig. 2D). The levels of intensity between old- and new-cut slides were concordant in 53% of cases (Fig. 2A-B; Table I).

When intensity levels were grouped as no/weak *versus* moderate/strong, the concordance reached 87%. Figure 3A shows the percentages of positive cells in old sections plotted against those in new sections. The proportion of cells with TOP2A immunostaining was lower by 12% on average in the older sections and differences ranged between 50% lower and 10% higher (Bland-Altman Plot Figure 3B); this reduction was statistically significant (p=.03).

Using the IRS scoring system [TOP2A was classified as either no to low expression (no/low, negative) or high (positive) expression (Table 2)], expression was concordant in 12 of 15 pairs. No or low expression of TOP2A in the older slide with high expression in the newer slide was observed in two of three discordant cases (2 of 15, 13%), (Fig. 2C-D). In the third case the opposite was observed: high expression in the older slide and low expression in the newer slide. The kappa statistic for TOP2A expression concordance was 0.57 (fair to good).

### Ki67 assessment

The Scatter Plot reveals a clear shift toward higher percentages of Ki67 positive cells in new cut slides (Fig. 3C). The percentage of positive cells varied between 1% and 60% in new slides and between 1% and 50% in 8 of 15 old slides; the other seven (47%) old slides demonstrated loss of staining (<1%). In the seven corresponding new cut slides staining was detected in 1-5% of cells; the seven other new cut slides had 30-40% cells staining positive and one additional case had 60% of cells staining positive. The Bland-Altman Plot (Fig. 3D) showed consistently lower expression of Ki67 in stored slides by an average of 10% (p<.01), ranging between 0% and 35% lower in stored slides.

Categorization of Ki67 using a cut-off of 10% to define no/low proliferation (negative) *versus* high proliferation (positive) was associated with moderate agreement between old and new cut slides (kappa =0.61; Table 2). There were 5 pairs of specimens that had over 10% positive cells. Three of those pairs had over 25% positive cells in both old and new cut slides. The other two pairs had moderate staining (10%-20%) in the old slide and a high level of staining (≥ 30%) in the new slide. Slide interpretation was concordant in 80% of

cases (Table 2). Importantly, complete or partial loss of immunoreactivity was observed for both Ki67 and TOP2A in the old slides of the same cases (Fig. 2C-F).

The use of a more stringent cut off point of 13% resulted in lowered concordance between old and new slides of 73% and a lower kappa value of 0.48. When three-group (no proliferation *vs* low proliferation *vs* high proliferation) comparison was performed, concordance was even lower; 40% and 33% for cut off points of 0 and 10% and 0 and 13%, respectively. The lowest cut off point of 1% had a concordance of 53% (Table S1 shows the effect on concordance of using 13% and 1% cut off points in new cut *versus* old cut slides).

### HER2 assessment

In the set of slides for which IHC staining was performed, one concordant pair of slides with moderately stained tumor cells and one discordant pair with a strongly stained new cut slide and a faintly stained old cut slide were found. All other slide pairs showed either no or faint staining in both new and old slides for a concordance of 93% (Table 1). Percentages of cells staining positive for HER2 between old and new slides were not significantly different (p=1.0). The interpretation of HER2 score confirmed concordance in all but one pair: the new slide was positive (score 3+) and old slide was negative (score 1+; Fig. 2G-H). Overall, the overexpression of HER2 protein in this set was detected in 2 (13%) of 15 cases and correlated well with HER2 gene amplification (Table S2).

The DNA stability was studied by *HER2* FISH on cases shown in Figure 1 (see also Table S3). In old slides, the number of *HER2* signals per cell ranged between 2.53 and 48.07 compared to between 2.69 and 62.66 in new slides. The baseline values of *HER2/CEP17* ratios were 2.32 and 2.1 and the maximal values reached 21.52 and 26.78 in old slides and new slides, respectively. The average amplification ratio in tumor cells of stored slides was comparable to the average amplification ratio in tumor cells of new cut slides (Fig 3 E-F).

## Discussion

Although the antigen loss with immunohistochemical analysis of aged slides is a known phenomenon, particularly for membrane and nuclear antigens, and a number of studies have reported on potential issues with stored paraffin sections used for biomarker research, many of these studies were conducted on breast cancer specimens and the majority were under controlled experimental conditions[18,19]. Little has been published about this phenomenon in tissues from other organs including the uterus, or in real-world settings with prolonged storage. Moreover, to our knowledge, the effect of slide storage duration on TOP2A antigenicity has not been reported previously.

We found that even after many years of storage there was only modestly lower protein expression in old cut *versus* new cut sections of endometrial cancer, with an average decrease in the percentages of stained cells for TOP2A and Ki67 in the range of 10% to12% for old cut slides.

The Ki67 antigen instability found in our study is supported by a number of reports on breast cancer samples stained with the same MIB1 clone and stored at ambient temperature

[19-28]. The mean decrease in the proportion of positively stained cells was 10% after 4.5 years in a study by Combs et al [21] and 10-25% after 10 years in a study by Ramos-Vara and colleagues [26] of slide storage followed by a plateau. Ki67 decay has been noted to occur either within first several weeks or months to one year [20,23,24]. Even at −80°C, prolonged storage (average 12.8 years), while it resulted in little loss of staining for HER2, showed only an agreement of 0.67 for Ki67 (using a cutoff of 20%)[4]. Data on susceptibility of HER2 to degradation are conflicting [21,27,29-32]. In our study, too few cases were HER2 positive to draw firm conclusions about changes in HER2 staining.

Unlike the situation in breast cancer where heterogeneity of HER2 expression is uncommon, significant heterogeneity of HER2 expression has been reported in endometrial cancer, with a recently published proposal that would define a IHC 2+ score (which should be followed by FISH testing) as “intense complete or basolateral/lateral membrane staining in ≤ 30%, or weak to moderate in ≥ 10% of tumor cells”.[33] Such a cutoff might be importantly affected by minor degrees of loss in staining.

Interestingly, in our study the obvious degradation of two different nuclear proteins (TOP2A and Ki67) was concordantly observed in old slides relative to matched new slides of the same cases. This suggests that archival effect on protein decay is nonrandom and may be enhanced by suboptimal tissue processing before sectioning. Overall, results of our study contribute to those in the literature showing that for most molecular markers, a moderate loss of immunoreactivity in stored tissue section is apparent [1,20,21,25-27], and provide an estimate of potential extent of such loss.

There is no consensus in the literature on the mechanism of loss of immunoreactivity over time. Exposure to oxygen during sectioning and exposure to light, high humidity and elevated temperature during storage may be involved [1,19,21,25,26,28,34]. While the efficacy of antigen preservation at −20°C, 4°C, or ambient temperature varies among reports, slide storage at 4°C (in sealed boxes) has been reported to be superior to storage at ambient temperature [1,3,9,35], and storage at −80°C was not been found to be superior to storage at −20°C.[36]

Our study confirmed earlier reports in breast cancer that DNA targets (*HER2* FISH assay) seem to be unaffected by slide storage. Differences between old and new slides in this study were non-significant. FISH was conducted in slides with almost two-year interval between repeats. This is one of the longest archival times studied to date in the analysis of DNA stability by *HER2* FISH [24,32].

In summary, FISH assay results for *HER2* were unaffected by slide storage for approximately two years. Protein expression by IHC for TOP2A and Ki67 was modestly decreased after many years slide storage, and results may still be informative. In cases where presence of protein (or gene) in borderline or small quantities is scientifically important, or when cut-points are used for endpoint determination, the degradation of signal is particularly relevant, and use of older pre-cut tissue sections may produce a false-negative result [25,26,37].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Grushko: current employee of Abbott

Method: current employee of Lilly and own Lilly stock

Fleming: Ad board for GSK, institutional PI for industry trials of Roche, Syros, GSK, Iovance, Sanofi, Sermonix, Incyte, Compugen, Abbvie, Eisai, Celldex, Astra Zeneca, Corcept, Merck, Plexxicon

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## Abbreviations

**ASCO**
American Society for Clinical Oncology

***CEP17***
chromosome 17 centromere enumeration probe

**CAP**
College of American Pathologists

**DAB**
3,3-diaminobenzidine tetrahydrochloride

**FISH**
fluorescence *in situ* hybridization

**FFPE**
formalin fixed paraffin embedded

**GOG**
Gynecologic Oncology Group

**H&E**
hematoxylin and eosin

**HER2**
human epidermal growth factor receptor 2

**HTRC**
Human Tissue Resource Center

**IHC**
immunohistochemistry

**IRS system**
ImmunoReactive Scoring system

**Ki67**
Proliferation marker protein Ki-67 encoded by MKI67 gene

**MWH**
microwave heating

**NRG Oncology**
National Surgical Adjuvant Breast and Bowel Project (NSABP), the Radiation Therapy Oncology Group (RTOG), and the Gynecologic Oncology Group (GOG)

**SDMC**
Statistics and Data Management Center

**TOP2A**
topoisomerase II alpha

**UCMC**
The University of Chicago Medical Center

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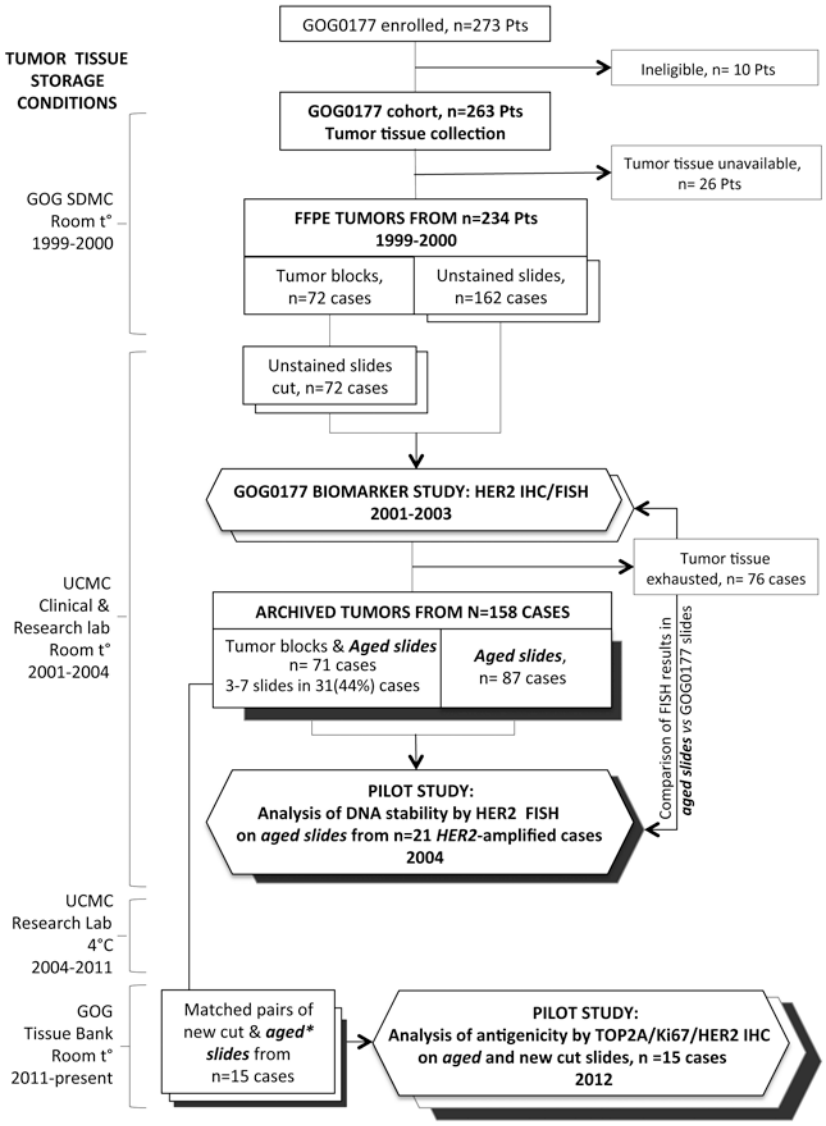


**Fig. 1.**

Flow chart depicting the tumor samples collection, storage and use in pilot study assays. Abbreviations: GOG SDMC, Gynecologic Oncology Group Statistics and Data Management Center; UCMC, University of Chicago Medical Center. Sent backward rectangles and hexagons: open indicate new cut slides; filled in indicate aged (old cut slides).

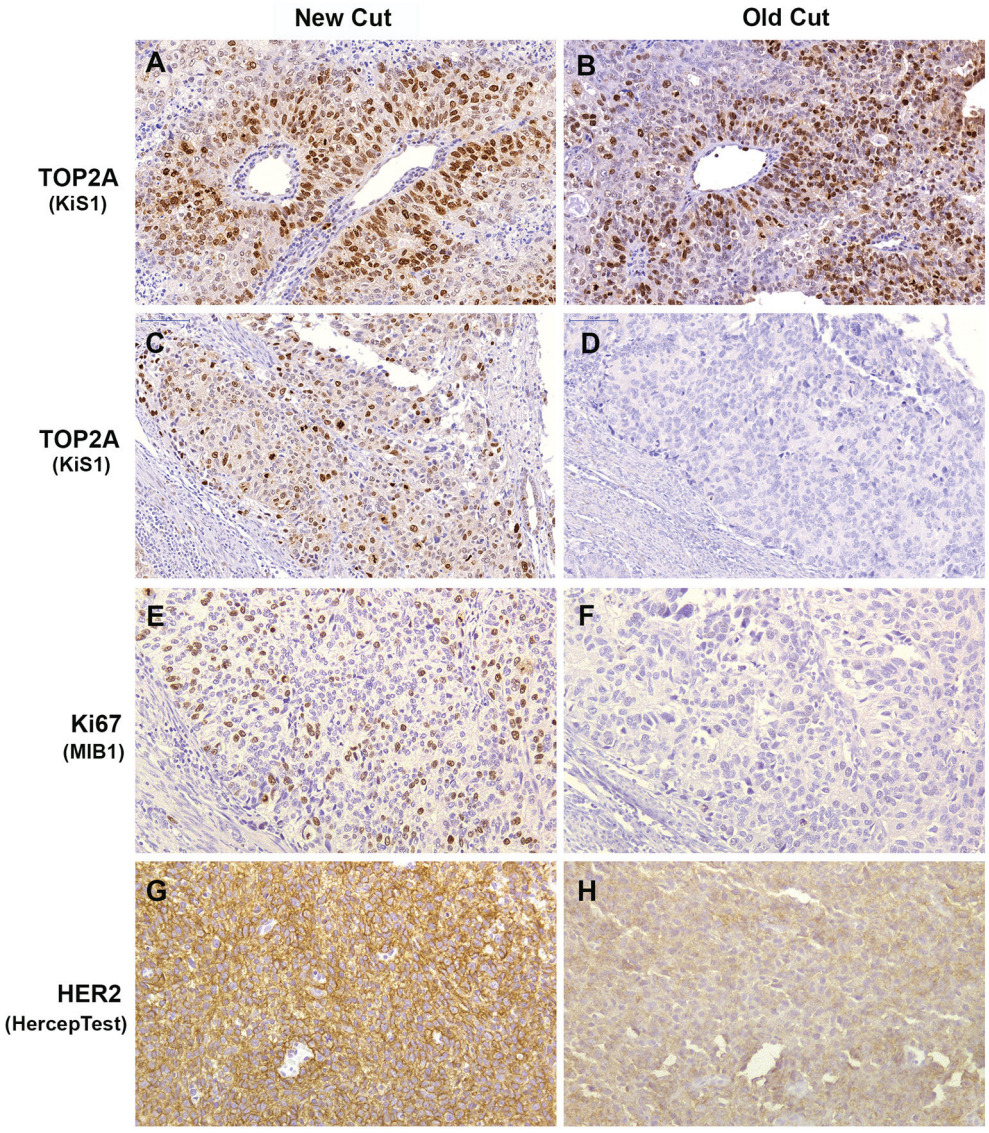


**Fig. 2.**

Photomicrographs of comparative TOP2A, Ki67 and HER2 IHC staining of new cut (3 weeks old) and stored (old cut, >10 years old) slides sectioned from the same FFPE tumor blocks of endometrial cancers. (A & B) Example of endometrial cancer demonstrating no effect of slide storage on TOP2A IHC interpretation. Strong (3+) positive nuclear staining was observed in both sections. (C – F) Staining patterns of TOP2A (C & D) and Ki67 (E & F) illustrating complete loss of immunoreactivity in old cut slides of the same case. In new cut sections of this tumor, the percentages of positively stained cells were 50% for TOP2A and 30% for Ki67. (G & H) Tumor showing change in HER2 intensity staining from strongly positive (3+; G) to weak (1+; H). Stroma and inflammatory cells were negative internal controls for all biomarkers. Original magnification x200.

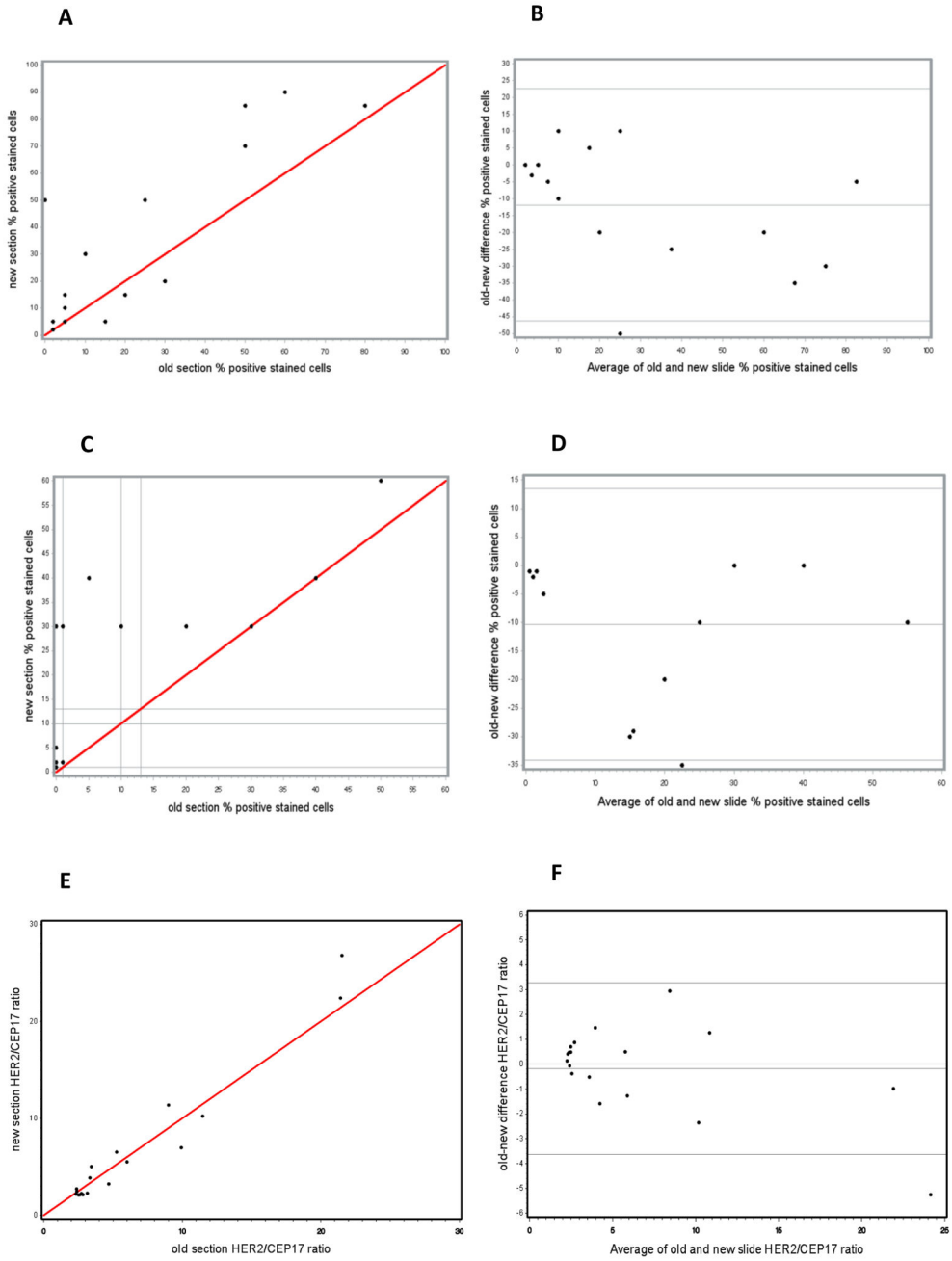


**Fig. 3.**

Graphical presentation of percentages of IHC positive cells for TOP2A (A & B) and Ki67 (C & D) and FISH *HER2/CEP17* ratios (E & F) in new and old cut sections for each case of endometrial cancer. (A, C & E) Scatter Plots of identity: red lines mark agreement. (B, D & F) Bland-Altman Plots of difference. Solid grey lines mark the average difference and the limits of agreement specified as an average difference ± 1.96 x Standard Deviation. (A & C) Percentages of stained cells in new slides were mapped against percentages of stained cells in old slides from the same cases for TOP2A and Ki67, respectively. (B & D) Differences in percentages of cells stained positive between new and old sections were mapped against

average values of percentages of positive cells from pairs of new and old sections for TOP2A (B) and Ki67 (D). In old slides, the difference from values in new slides ranged between 50% lower and 10% higher for TOP2A and between 0% and 35% lower for Ki67. Statistically significant loss of staining in stored slides by an estimated 12% for TOP2A and 10% for Ki67 was detected. (E) *HER2/CEP17* ratios in new slides were mapped against *HER2/CEP17* ratios in old slides from the same cases. (F) Differences in *HER2/CEP17* ratios between new and old sections were mapped against average values of *HER2/CEP17* ratios from pairs of new and old sections. In old slides, the variability from new slides ranged between 5.2 lower and 3.0 higher with an average estimated *HER2/CEP17* ratio lover by 0.1 showing no difference from new sections.

**Table I.**

TOP2A and HER2 staining intensity in new cut *versus* old cut slides

| Old cut Slides | New Cut Slides | | | |
|---|---|---|---|---|
| | **0, 1+** | **2+** | **3+** | **Total** |
| | | **TOP2A** | | |
| 0, 1+ | 1 | 2 | 0 | 3 |
| 2+ | 0 | 4 | 4 | 8 |
| 3+ | 0 | 2 | 2 | 4 |
| Total | 1 | 8 | 6 | 15 |
| Concordance 87% (in 13 of 15 pairs) by groups (0,1+) *versus* (2+, 3+) | | | | |
| | | **HER2** | | |
| 0, 1+ | 13 | 0 | 1 | 14 |
| 2+ | 0 | 1 | 0 | 1 |
| 3+ | 0 | 0 | 0 | 0 |
| Total | 13 | 1 | 1 | 15 |
| Concordance 93% (in 14 of 15 pairs) by groups (0,1+) *versus* (2+) versus (3+) | | | | |

NOTE: Staining intensity: (0,1+), no staining/weak; (2+), moderate; (3+), strong

**Table 2.**

TOP2A and Ki67 IHC interpretation in new cut *versus* old cut slides

| Old cut Slides | New Cut Slides | | | |
|---|---|---|---|---|
| | No/Low | High | Total | Kappa 95% CI |
| **TOP2A**[a] | | | | |
| No/Low | 4 | 2 | 6 | |
| High | 1 | 8 | 9 | 0.57 (0.14, 1.00) |
| Total | 5 | 10 | 15 | |
| Concordance 80% (in 12 of 15 pairs) | | | | |
| **Ki67**[b] | | | | |
| No/Low | 7 | 3 | 10 | |
| High | 0 | 5 | 5 | 0.61 (0.24, 0.97) |
| Total | 7 | 8 | 15 | |
| Concordance 80% (in 12 of 15 pairs) | | | | |

[a] Expression categories defined by combined score of IRS system

[b] Expression categories defined by cutoff point of 10% positive cells