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Original contribution

# Optimal protocol for PTEN immunostaining; role of analytical and preanalytical variables in PTEN staining in normal and neoplastic endometrial, breast, and prostatic tissues ☆☆

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**Summary** In some tumors, phosphatase and tensin homolog (*PTEN*) inactivation may have prognostic importance and predictive value for targeted therapies. Immunohistochemistry (IHC) may be an effective method to demonstrate *PTEN* loss. It was claimed that PTEN IHC showed poor reproducibility, lack of standardization, and variable effects of preanalytical factors. In this study, we developed an optimal protocol for PTEN IHC, with clone 6H2.1, by checking the relevance of analytical variables in normal tissue and tumors of endometrium, breast, and prostate. Pattern and intensity of cellular staining and background nonspecific staining were quantified and subjected to statistical analysis by linear mixed models. The proposed protocol showed a statistically best performance ($P < .05$) and included a high target retrieval solution, 1:100 primary antibody dilution (2.925 mg/L), FLEX diluent, and EnVisionFLEX+ detection method, with a sensitivity and specificity of 72.33% and 78.57%, respectively. Staining specificity was confirmed in cell lines and animal models. Endometrial carcinomas with *PTEN* genetic abnormalities showed statistically lower staining than tumors without alterations (mean histoscores, 34.66 and 119.28, respectively; $P = .01$). Controlled preanalytical factors (delayed fixation and overfixation) did not show any statistically significant effect on staining with optimal protocol ($P > .001$). However, there was a trend of significance for decreased staining and fixation under high temperature. Moreover, staining was better in endometrial aspirates than in matched

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hysterectomy specimens, subjected to less controlled preanalytical variables (mean histoscores, 80 and 40, respectively; $P = .002$). A scoring system combining intensity of staining and percentage of positive cells was statistically associated with *PTEN* alterations ($P = .01$).


## 1. Introduction

Phosphatase and tensin homolog (*PTEN*) is frequently somatically mutated or deleted in tumors [1]. Among other activities, PTEN antagonizes the phosphoinositide 3 kinase/alpha serine-threonine kinase (*PI3K/AKT*) pathway by dephosphorylating PIP3, resulting in a decreased translocation of AKT to cellular membranes [2,3]. *PTEN* can be inactivated by several different mechanisms, including gene mutation, deletion, epigenetic silencing, transcriptional repression, micro-RNA regulation, disruption of competitive endogenous RNA networks, posttranslational modifications, and aberrant PTEN localization [4]. *PTEN/PI3K* pathway activation may be a strong biological correlate of metastasis and poor prognosis in some tumors [5-7]. Moreover, a new functional role for PTEN in genomic instability has been proposed [4] suggesting that PARP (poly ADP ribose polymerase) inhibitors may be useful in patients with *PTEN* mutant tumors [8]. Furthermore, *PTEN* expression has also been related to response to several drugs in anticancer targeted therapies [9-11]. It has been suggested that immunohistochemistry (IHC) may be an effective method to demonstrate loss of *PTEN* function, but some variability and poor reproducibility have been observed with different antibodies and techniques [12-16].

In this study, we optimized a protocol for PTEN IHC and assessed the impact of analytical and preanalytical factors, to check the usefulness of IHC as a method for evaluation of *PTEN* alterations. We assessed these variables in 3 tissues (endometrium, breast, and prostate), in which *PTEN* is expressed in normal cells and frequently abnormal in tumor cells. The optimal protocol was tested in cell lines and animal models as well as in tumors with known molecular status of in *PTEN*.

## 2. Materials and methods

### 2.1. Case selection and tissue microarray construction

Five tissue microarrays (TMAs) were constructed from formalin-fixed, paraffin-embedded (FFPE) tissue, corresponding to normal prostate (PN; 30 cases), prostatic carcinoma (PC; 30 cases), normal breast tissue (BN; 30 cases), breast carcinoma (BC; 30 cases), normal endometrium (30 cases), and endometrial carcinoma (EC; 45 cases). Samples were obtained from the surgical pathology files of Hospital Universitari Arnau de Vilanova, Lleida, Spain (HUAV). The study was approved by the local ethics committee. Informed consent was obtained from each patient. A tissue arrayer device (Beecher Instrument, Sun Prairie, WI, USA) was used. All tissue samples were histologically reviewed by 3 members of the team, and representative tumor or nontumor areas were marked in the corresponding paraffin blocks. Two selected cylinders (0.6 mm in largest diameter) from 2 different tumor or nontumor areas were included for each case.

### 2.2. Tissues for assessment of preanalytical variables

Eight surgical specimens were used, obtained from the pathology files of HUAV. Three of them corresponded to hysterectomy specimens for EC, 4 were breast excisions for BC, and 1 was a partial prostatectomy for prostatic hyperplasia. They were obtained from the operating room, immediately after resection, and were selected because the size of the specimens allowed obtaining a high number of small fragments to check all possible combinations of the controlled, preanalytical variables. The tissue was obtained from the exceeding material, after having selected tissue samples for appropriate diagnosis. The influence of additional, uncontrolled, preanalytical variables was also checked in 4 TMAs constructed from FFPE tissues corresponding to matched endometrial aspirates and hysterectomy specimens. These tissues had been collected from a total of 100 patients with EC, from the surgical pathology files of HUAV. The study was approved by the local ethics committee. Informed consent was obtained from each patient. Although preanalytical conditions were not controlled in these cases, this material was considered interesting for assessing the influence of uncontrolled preanalytical factors because endometrial aspirates are usually immersed in formalin, immediately after being obtained while hysterectomy specimens are usually subjected to variable conditions of delayed fixation and time fixation.

### 2.3. Case selection for validation

We used 2 different series of EC with known *PTEN* molecular status. One of them corresponded to 33 cases, from Hospital Santa Creu i Sant Pau, included in a TMA, which had been previously assessed for *PTEN* alterations, by mutation, promoter hypermethylation, and loss of heterozygosity (LOH) analyses [6]. The second series corresponded