# Estimation of Hormone Receptor Status in Fine-Needle Aspirates and Paraffin-Embedded Sections From Breast Cancer Using the Novel Rabbit Monoclonal Antibodies SP1 and SP2.

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**Abstract**

We describe a method of immunocytochemical assessment of estrogen receptor (ER) status on alcohol-fixed smears obtained by fine-needle aspiration (FNA) from breast cancer patients, using a commercially available rabbit monoclonal antibody anti-ER (SP1) without any antigen retrieval. A series of 40 aspirates were analysed and the results of ER status were compared with the respective formalin-fixed tissue using the same procedure, and with the assessment by the classical method using the mouse monoclonal antibody 6F11(anti-ER) with antigen retrieval on paraffin-sections. Twenty-four out of the 40 cases examined were positive at least by two methods and 16 were negative for all three determinations. The results obtained in the ER immunocytochemical assay on aspirates and paraffin-sections using the antibody SP1 and those obtained on paraffin-sections using the antibody 6F11 were quite similar. In one case the material was insufficient to interpret the reaction in the cytological specimen and only one case, with focal positivity reaction on paraffin-sections, was negative in the cytological specimen. The intensity of nuclei staining in cytological smears of breast cancer cells was stronger than the observed by the traditional methods. We also assessed progesterone receptor (PR) status on 40 paraffin-sections from breast cancer patients, using a commercially available rabbit monoclonal antibody anti-PR (SP2), with the same characteristics described for anti-ER (SP1). The results were compared with assessment by the classic method with mouse monoclonal antibody 1A6(PR) on paraffin-sections and a total agreement was observed. Of the 40 cases examined, 18 were positive and 22 were negative for the two determinations. We conclude that the application of ER method on alcohol-fixed smears / paraffin-sections with the rabbit monoclonal antibody SP1, and PR method on paraffin-sections with the rabbit monoclonal antibody SP2 provide several

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advantages, such as high sensitivity and specificity of the reaction, stronger immunostaining, shorter procedures times and avoidance of antigenic retrieval methods.

*Key Words:* FNA; Breast cancer; Estrogen receptor; Progesterone receptor, rabbit monoclonal antibodies

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**Introduction**

There is general agreement that the presence of estrogen (ER) and progesterone receptors (PR) in breast cancer are positively correlated with response to therapy [1]. Moreover, the absence of ER associated with other primary characteristics of the tumour (for example high histological grade) is associated with early recurrence and poor survival in breast cancer patients [2].

Although hormone receptor analysis has been traditionally performed on surgically removed specimens, FNA is a suitable alternative in a number of situations: a) inoperable cases and metastatic or recurrent tumors in which the size and the accessibility to surgical biopsy presents a problem; b) cases in which preoperative irradiation or presurgical therapy are the initial treatment options and c) advanced tumors in which serial hormone receptor studies may provide information regarding response to therapy [3]. Many studies have been performed on cytological material comparing the results of immunocytochemistry and biochemical assays [4], immunohistochemistry in frozen sections [3] and in paraffin-embedded sections [3,5]. Most of these studies showed a good correlation between all techniques. The application of these methods on alcohol-fixed smears eliminates the need for using a special fixation procedure and provides several advantages: improvement in morphological concomitant analysis, utilization whenever malignancy is found without necessity of re-aspirating the patient, and adequacy of archival material [3].

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Some years ago, our group showed the feasibility to performing ER analysis on routinely alcohol-fixed smears from breast cancer patients, using a commercially available monoclonal antibody to ER (1D5) [3]. All the procedure had a mean time length of eighteen hours and needed microwave heat-based antigen retrieval technique

[3]. Recently, a technology to produce rabbit monoclonal antibodies was developed [6]. These authors claimed that these antibodies were more specific and sensitive than the classical mouse monoclonal antibodies [6]. In attempt to verify the possibility of use these new antibodies on routinely processed material, we studied a series of 40 breast carcinomas using the rabbit monoclonal antibody against ER (SP1) in alcohol-fixed smears obtained from FNA of breast cancer. The results were compared with those obtained in paraffin-embedded sections of the respective tumours using the same antibody, and with the results obtained with a classical mouse monoclonal antibody anti-ER (6F1). We also test in paraffin-embedded sections a rabbit monoclonal antibody against PR (SP2).

## Material and Methods

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The study was carried out on primary breast carcinomas of 39 women and 1 man, with ages ranged from 41 to 82 year-old.

### *Fine-Needle Aspiration*

Percutaneous FNA biopsies were performed with a 0,6 mm (23 gauge) diameter needle using the procedure described by Zajicek [7]. The aspirated material was expelled on uncoated glass slides. The smears were either air-dried or fixed immediately in absolute ethanol. May-Grünwald Giemsa-stained slides were used for morphologic evaluation and alcohol-fixed smears were stained by the Papanicolaou method and used for morphologic and immunocytochemical studies.

*Surgical Specimens*

In all cases there was a previous cytological diagnosis of malignancy and every patient was submitted to surgery. The surgical specimens were received fresh in the pathology laboratory. The specimens were placed into phosphate-buffered formalin and were fixed for approximately 24 hr. Representative blocks of tumour were taken, embedded in paraffin, cut and stained with hematoxylin-eosin. Histopathological classification of the tumours was performed.

*Immunocytochemistry*

The coverglass of previously Papanicolaou-stained smears were removed with immersion in xylene and the slides were afterwards carried through xylene and grades of alcohol without destaining. Automated immunohistochemistry was carried out (Labvision Autostainer LV-1, Labvision Corporation, Hoersholm, Dennmark) according to the streptavidin-biotin-peroxidase technique as described elsewhere [8,9]. Briefly, after washing with tap water and blockage of peroxidase (5 minutes), the slides were incubated with the primary rabbit monoclonal antibody anti- ER (clone SP1, dilution 1:20, Labvision Corporation, Fremont, CA, USA) for 10 minutes, followed by incubation with the secondary antibody, biotinylated goat anti-rabbit (Labvision) for 5 minutes and the LV's Ultravision kit (Labvision) for 5 minutes. Color reactions were developed with diaminobenzidine (Labvision) for 10 minutes. No pretreatment was used. The reaction total time was 40 minutes.

The formalin-fixed, paraffin-embedded breast tissues were immunostained for anti-ER using the same rabbit monoclonal antibody SP1 and for anti-PR , using the

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clone SP2 (dilution 1:20, Labvision Corporation, Fremont, CA, USA) using the same protocol described above for the cytological material. No pretreatment was performed. We also immunostained the paraffin-sections of the respective cases using two classical mouse monoclonal antibodies against ER (clone 6F11, dilution 1:30, Novocastra, Newcastle, UK) and PR (clone 1A6, dilution 1:100, Novocastra, Newcastle, UK) using standard protocols in the automated LabVision Autostainer (Labvision, Dennmark), as described elsewhere [10], including antigen retrieval methods (Dako target retrieval solution, Dako Corporation, Carpinteria, California) with wet-heat at 98ºC for 30 minutes.

Positive and negative controls were always included with each bath of staining to ensure consistency between consecutive runs. A case of invasive breast carcinoma of known positive estrogen and progesterone receptors reactivity detected by standard immunocytochemistry methods and the manufacturers control cell slides were used as positive controls. Incubation of slides with omission of primary estrogen receptor antibody was performed as a negative control.

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*Staining Interpretation*

Positivity was indicated by the presence of dark brown nuclear staining and negativity by no nuclear staining. For evaluation, at least 100 nuclei of tumour cells were considered and areas with air-drying artifacts were not include in the scoring [5]. The cases were also assessed semi-quantitatively according to a previous system published by our group on cytological material [3]. All cases were scored independently by three cytopathologists (GC, FM,FS) in cytological and formalin-fixed materials. The

assessment of ER and PR in paraffin-sections with 6F11 and 1A6, respectively, was used as a “gold standard”.

*Statistical Analysis*

The data obtained were statistically analysed using accuracy, sensitivity, and sensibility. Accuracy referred to the closeness with which obtained values agree with true values, as defined by the assessment of ER and PR in paraffin-sections with 6F11 (ER) and 1A6 (PR). Sensitivity was considered as the percentage of positive cases with 6F11 (ER) or 1A6 (PR), that were positive using anti-ER (SP1) – in cytological and histological material – or anti-RP (SP2) – in histological material. Specificity was considered as the percentage of negative cases with 6F11 (ER) or 1A6 (PR) that were negative using anti-ER (SP1) – in cytological and histological material – or anti-PR (SP2) – in histological material. The correlation studies were performed using the Spearman’s rank coefficient test (95% confidence intervals).

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**Results**

In 39 out 40 cases we obtained sufficient material to estimate ER content in aspirates. In one case the material was scant. Twenty-four of the 39 cases examined were positive at least by two methods and 16 were negative by all three determinations. All positive cases for the antibody SP-1 showed strong nuclei immunoreactivity of breast cancer cells in cytological and histological specimens (Fig 1 A and B). There was neither background nor cytoplasmic positivity in the cytological material. Regarding to the determination of PR, which was done only in histological material, we observed

a total agreement between the two antibodies used (SP2 and 1A6): 18 positive cases and 22 negative cases (Fig. 1 C).

Table I summarises the results obtained in the estrogen receptor immunocytochemical assay in FNA and paraffin sections, using the rabbit monoclonal antibody SP1 and paraffin sections using the mouse monoclonal antibody 6F11 (Fig. 1D). Table II summarises the results obtained in the progesterone immunocytochemical assay in paraffin sections using the rabbit monoclonal antibody SP2 and the mouse monoclonal antibody 1A6. The results obtained by the two methods in paraffin sections to ER and PR were similar, as showed in Tables III and IV. The ER detection in paraffin-sections using the antibody SP1 had a sensitivity of 100 %, a specificity of 100 % and a accuracy of 100 %, and the PR detection in paraffin-sections using the antibody SP2 had a sensitivity of 100 %, a specificity of 100 % and a accuracy of 100 %, (Tables III and IV). The results obtained by the three methods, in FNA and paraffin sections regarding to ER, were closely similar (Table III). ER detection in FNA using the antibody SP1 had a sensitivity of 91.7 % and a specificity of 100 %. Results for both methods agreed in 95 % of the cases. Indeed, in one case the material was non-sufficient in the cytological specimen and in other the positive reaction in the paraffin-section was only focal.

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The scoring results of the three cytopathologists using a previously published semiquantitative scoring system [3] were almost identical with a significant correlation between the assessment of ER using SP-1 in FNA and 6F11 in formalin-fixed material and between SP-1 in FNA and SP-1 in formalin-fixed material ($p<0.001$ for both). We observed more intense positivity with the SP-1 antibody in cytology and histology than using 6F11 in histology.

## Discussion

The assessment of hormone receptors in cytological material has always been performed by immunocytochemistry [3,4,5]. Since the first determinations of ER on cytological material using the ER-ICA method, it has been demonstrated a good correlation in the immunocytochemical assessment of ER content in FNA from breast cancer and the biochemical determinations (Dextran-coated charcol assay) of the respective surgical biopsies [11]. These results were confirmed more recently by Sauer et al [4] for ER and PR. However, the use of ER-ICA and PR-ICA immunoassay requires the use of a special sequence of fixation and storage of the slides at –20ºC in a solution for later processing [4,11,12] creating therefore, the necessity for at least one more aspiration for cytomorphological control and hampering the possibility of performing retrospective studies using archival material. In a previous study, we demonstrated that ER analysis can be performed on routinely alcohol-fixed smears using a commercially available mouse monoclonal antibody to ER (1D5), in conjunction with a microwave heat-based antigen retrieval technique [3]. In addition, this study also demonstrate that ER immunocytochemical determination on FNA material correlates well with the ER histological assessment both on formalin-fixed and frozen sections. Subsequently, other studies using other mouse monoclonal antibodies anti ER (6F11) and anti-PR (1A6) with heat-induced epitope retrieval, also showed similar results [5].

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The generation of monoclonal antibodies from species other than rats and mice has developed slowly over the last twenty years. The advent of antibody engineering and the concept that nonmurine antibodies can be superior in terms of affinities and specificities, has increased their relevance [6]. In general, rabbits produce

more sensitive and higher affinity antibodies than mice. In the other hand, mouse produces more specific antibodies. So, the production of a monoclonal rabbit antibody could result in a more specific and sensitive antibody. Recently, monoclonal rabbit antibodies against ER and PR were developed. Once these antibodies are more specific and sensitive and have more affinity than the mouse monoclonal antibody, the immunocytochemistry technique can be modified, for example, avoiding pretreatments to obtain the same good staining results.

Our study shows that there is a good correlation between the results obtained in the detection of ER on alcohol-fixed smears using the rabbit monoclonal antibody SP1 and those obtained with 6F11 using the classical procedure, with shorter processing times and no antigen retrieval,  We do not have "discrepant positive" cases, but only one "discrepant negative" case. In this case, the positive reaction in the paraffin-section was focal and it can explain the negative result on cytology. It is necessary to highlight that in cytological specimens, the intensity of positive immunoreactivity in the nuclei of breast cancer cells was stronger than the one observed by the traditional methods. We have also shown that is possible to perform ER assessment in paraffin-embedded sections using the same antibody, with total agreement of the results, and similar intensity of immunoreactivity. Similar results were obtained for PR using the rabbit monoclonal antibody SP2, also without the need for heat induced epitope retrieval.

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In a few cases, we observed a faint cytoplasmic staining for ER, that did not cause interpretation problems. Although cytoplasmic staining for ER had been considered as an artefact during long time, recently, Kumar R et al. [13] showed that naturally occurring short form of the metastatic tumour antigen 1 (MTA1), sequesters ER in the cytoplasm, which can be detected by using sensitive monoclonal antibody.

Therefore, our cases with cytoplasmic reaction for the antibody SP1 can not be consider as unspecific staining.

In conclusion, our study indicates that ER analysis can be performed on routinely alcohol-fixed smears and paraffin-embedded sections from breast cancer patients, using a commercially available rabbit monoclonal antibody to ER (SP1). Our study also demonstrates that ER immunocytochemical determination on FNA material correlates well with the ER histological assessment in formalin-fixed sections both using the rabbit monoclonal antibody SP1 and mouse monoclonal antibody 6F11. We also demonstrate that the rabbit monoclonal antibody provides a technical procedure with an excellent quality of staining, a simplification in the immunocytochemistry protocol, high sensibility and specificity, saving time and costs.

We also test the rabbit monoclonal antibody anti-PR (SP2) in formalin-fixed specimens with a good correlation with the classical method using the mouse monoclonal antibody 1A6. This procedure may be a valuable tool to analyse PR content in FNA of primary breast cancer, with the same advantages demonstrated for ER evaluation. This study must be carried out in a near future.

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**Legends to figures**

**Fig 1. A:** FNA from breast carcinoma showing strong nuclear staining for estrogen receptor with the rabbit monoclonal antibody SP1. **B:** Histological section correspondent to the smear represented in A, stained with the rabbit monoclonal antibody SP1. Observe the strong nuclear positivity. **C:** Histological section of breast carcinoma stained for the rabbit monoclonal antibody SP 2 (anti-PR). **D:** Histological section of breast carcinoma stained for the mouse monoclonal antibody for estrogen receptor (6F11).

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**Tables**

**Table I.** Summary of the Results Obtained in the Detection of Estrogen Receptor (ER) in Cytological Specimens (FNA) and Formalin-Fixed Specimens (FF) Using the Rabbit Monoclonal Antibody SP1 and FF Using the Mouse Monoclonal Antibody 6F11.

| *Reaction* | *FNA-SP1* | *FF-SP1* | *FF-6F11* |
|---|---|---|---|
| Positive | 22 | 24 | 24 |
| Negative | 18* | 16 | 16 |
| *Total* | *40* | *40* | *40* |

* In one case the material was insufficient in the cytological specimen and in other the reaction on paraffin-section was focal.

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**Table II.** Summary of the Results Obtained in the Detection of Progesterone Receptor (PR) in Formalin-Fixed Specimens (FF) Using the Rabbit Monoclonal Antibody SP2 and the Mouse Monoclonal Antibody 1A6.

| *Reaction* | *FF-SP2* | *FF-1A6* |
|---|---|---|
| Positive | 18 | 18 |
| Negative | 22 | 22 |
| *Total* | *40* | *40* |

**Table III.** Accuracy, Sensitivity and Specificity of Estrogen Receptor (ER) Immunocytochemical Assay Using the Rabbit Monoclonal Antibody SP1 in Fine-Needle Aspirate Specimens (FNA) and Formalin-Fixed Specimens (FF) Compared With ER Detection Using the Mouse Monoclonal Antibody 6F11 in Formalin-Fixed Specimens (FF).

| | *FF-6F11 N (%)* | | |
|---|---|---|---|
| Reaction | Accuracy | Sensitivity | Specificity |
| FNA-SP1 | 38/40 (95) | 22/24 (91.7) | 16/16 (100) |
| FF-SP1 | 40/40 (100) | 24/24 (100) | 16/16 (100) |

**Table IV.** Accuracy, Sensitivity and Specificity of Progesterone Receptor (PR) immunocytochemical Assay Using the Rabbit Monoclonal Antibody SP2 in Formalin-Fixed Specimens (FF) Compared With PR Detection Using the Mouse Monoclonal Antibody 1A6 in Formalin-Fixed Specimens (FF).

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| | FF-1A6 | | |
|---|---|---|---|
| Reaction | Accuracy | Sensitivity | Specificity |
| FF-SP2 | 40/40 (100) | 18/18 (100) | 22/22 (100) |