Application Note

Characterization of Estrogen Receptor α Phosphorylation Sites in Breast Cancer Tissue Using the SNAP i.d® 2.0 System

Introduction

A major focus of breast cancer research is to understand the mechanisms responsible for disease progression and drug resistance. Toward that end, it has been found that approximately two thirds of all human breast carcinomas overexpress the Estrogen Receptor α (ERα) protein and it remains the primary pharmacological target for endocrine therapy[1,2]. The normal cellular function of ERα is as a transcription factor that mediates a wide variety of physiological processes, many of which are dependent upon phosphorylation of the receptor at specific amino acid residues[3,4]. Indeed, ERα is known to be phosphorylated at a multitude of different sites, yet how these all correlate to disease remains unclear[5]. Here, we interrogated multiple sites of ERα for phosphorylation status by screening an extensive panel of different breast cancer patient samples and other non-breast cancer tissue microarray (TMA) slide samples to determine their relevance to disease.

To facilitate this analysis, we used the new SNAP i.d.® 2.0 Protein Detection System for multi-slide screening with immunohistochemistry (IHC). This system is a versatile, vacuum-driven system that can process up to 24 slides simultaneously of either formalin-fixed paraffin embedded tissue (FFPE) or fresh frozen samples. The system can accommodate any typical manual protocol but eliminates the need for Pap pen marking of microscope slides during testing, thereby saving valuable time. In addition, any precious antibody reagent solutions used during testing can be recovered, thus saving valuable probes for future studies. Finally, the vacuum-driven system allows

for faster, easier buffer removal, thereby facilitating washing and allowing additional washes as needed.

In this study, we aimed to compare ERα staining patterns in healthy and breast cancer tissues using this medium-throughput IHC test system. We completed a side-by-side comparison of traditional IHC to SNAP i.d.® IHC and found both to yield highly similar results in terms of staining intensity and background signal. Next, we were able to successfully screen hundreds of FFPE breast cancer tissue samples using a variety of polyclonal and monoclonal antibodies targeting different phosphorylation sites of ERα. To aid in the analysis, the patient samples were classified according to their clinical grade (ER- positive or negative, Her2, progesterone-positive or negative, and Ki67 index).

Finally, tissue microarray (TMA) samples were also screened to ascertain if non-diseased tissue showed similar phosphorylation profiles. Our results demonstrate unique staining patterns depending upon the tumor classification, which further research may show to be valuable in biomarker analysis. The high variability in staining patterns in breast carcinomas underscores the need for broader immunohistochemical analyses using higher throughput methodologies to improve accuracy and precision of staining.

Materials and Methods

Materials

Equipment:

SNAP i.d.® 2.0 base (Merck Millipore Cat. No. SNAP2BASE)
IHC frame (Merck Millipore Cat. No. SNAP2FRIHC)
Slide holder (Merck Millipore Cat. No. SNAP2SH)

Reagents:

Xylene (Merck Millipore Cat. No. XX0060-4)
Ethanol (Merck Millipore Cat. No. EX0276-1)
Milli-Q® water
(Merck Millipore Milli-Q® Integral 15 system)
Modified Mayer Hematoxylin (Sigma Cat. No. MHS16)
DAB chromogen
Blocking buffer
Anti-Rabbit Secondary
Anti-Mouse Secondary
DAPI

Antibodies:

Anti-Estrogen Receptor α
(Merck Millipore Cat. No. 06-935)
Anti-phospho-Estrogen Receptor α (Ser118),
clone NL44 (Merck Millipore Cat. No. 05-793)
Anti-phospho-Estrogen Receptor α (Ser167)
(Merck Millipore Cat. No. 07-481)
Anti-phospho-Estrogen Receptor α (Ser305),
clone 124.9.4 (Merck Millipore Cat. No. 05-922R)
Anti-phospho-Estrogen Receptor α (Ser305)
(Merck Millipore Cat. No. 07-962)
Anti-phospho-Estrogen Receptor α (pSer102)
(Sigma Cat. No. SAB4301359)
Anti-phospho-Estrogen Receptor α (pSer106)
(Sigma Cat. No. SAB4504398)
Anti-phospho-Estrogen Receptor α (pTyr537)
(Sigma Cat. No. SAB4301234)

SNAP i.d.® Method:

Slide deparaffinization and antigen retrieval (Figure 1, steps 1 and 2, page 3)

Sections of formalin-fixed, paraffin-embedded ductal carcinoma tissue or TMAs were captured on microscope slides. The tissue sections were then de-paraffinized using 3 xylene washes, followed by rehydration in several changes of increasingly dilute reagent-grade ethanol (starting at 100% down to 30%), and finished with 2 washes of Milli-Q® water (step 1). The heat-induced epitope retrieval (HIER) was performed for 15 min at 110 °C in a pressure chamber (step 2).

SNAP i.d.® 2.0 IHC system operation (Figure 1, steps 3 to 5, page 3)

The microscope slides with tissue were then assembled in the SNAP i.d.® 2.0 IHC slide holder (Cat. No. SNAP2SH, step 3). The slide holder cassette was then inserted into the IHC frame (Cat. No. SNAP2FRIHC, step 4). To avoid tissue drying during the assembly of multiple slides, the slide holders were immediately filled with wash buffer (Tris-buffered saline containing 0.05% Tween® 20 surfactant; TBS-T). Wash buffer was flushed out by briefly initiating the vacuum for the entire unit (note: each subsequent removal of test solution was performed by briefly initiating the vacuum). The slides were then incubated with blocking solution for 10 minutes followed by 3 TBS-T washes. A variety of primary antibodies (from Merck Millipore and Sigma) were then added to the samples during testing. The antibodies were incubated for 1 hour then the slides were washed 3 times with TBS-T. After washing, the slides were incubated for 10 minutes with either anti-rabbit or anti-mouse HRP polymer. The slides were then washed 3 times with TBS-T. Detection included two steps, in which slides were incubated for 3 min with DAB and then counterstained with Modified Mayer's Hematoxylin (Sigma). Both detection steps were followed with 3 washes with Milli-Q® water. At this point the cassettes were removed from the SNAP i.d.® 2.0 system and the slides were removed from the holders.

Dehydration and clearing of slides (Figure 1, step 6, page 3) Slides were dehydrated in 100% reagent-grade ethanol, (4 times, 5 min each) and cleared with xylene (3 times, 3 min each)

Mounting and observation (Figure 1, Step 7, page 3)

Slides were permanently mounted and images at 20-40X magnification were obtained.

Traditional IHC Method:

Control tests were performed on tissue sections without the use of the SNAP i.d.® 2.0 system. The samples were treated with the same test reagents and equivalent incubation conditions but were processed manually by pipetting reagents onto slides. For these tests, a Pap pen was used to outline tissue sections prior to antibody incubations.

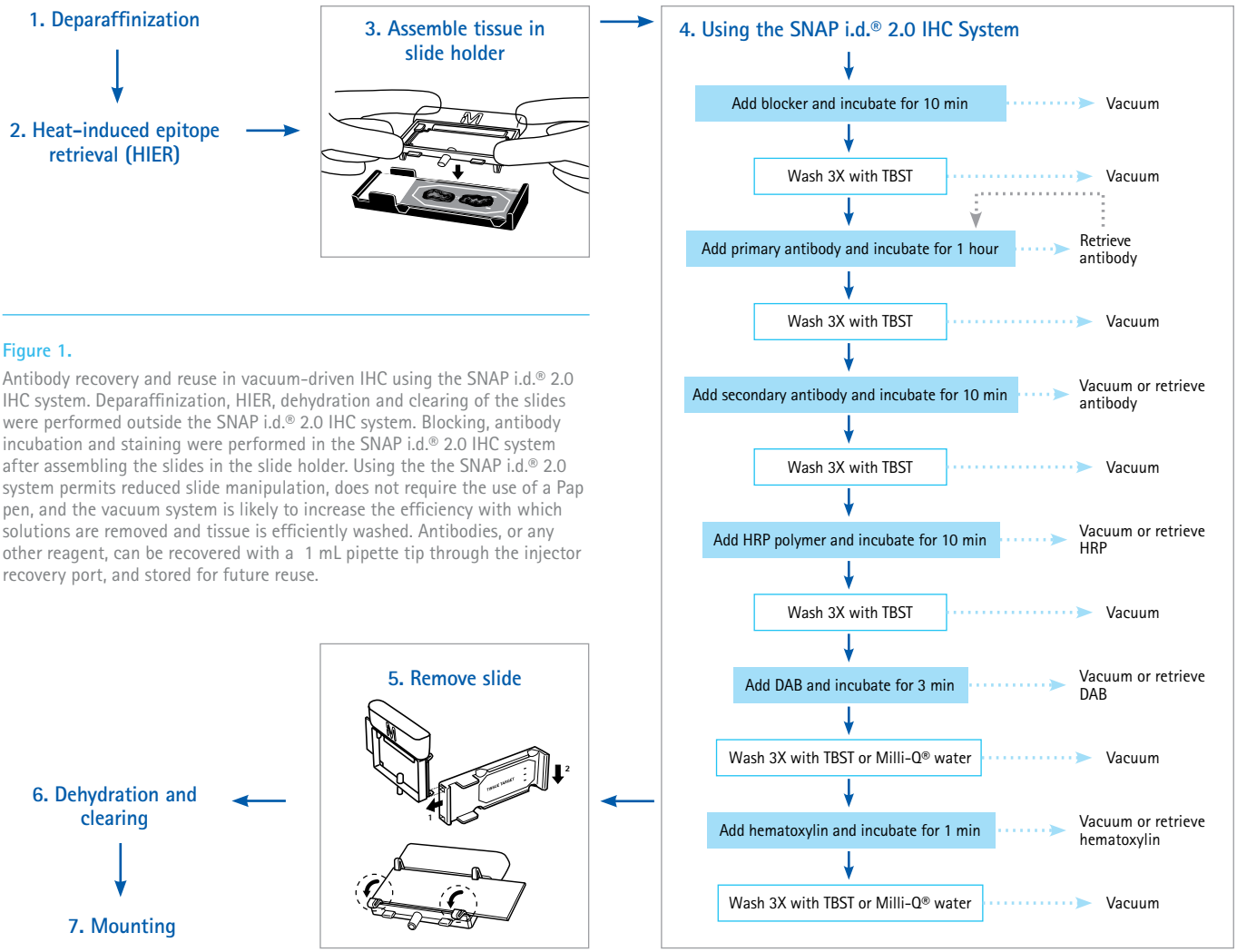

Figure 1.

Antibody recovery and reuse in vacuum-driven IHC using the SNAP i.d.® 2.0 IHC system. Deparaffinization, HIER, dehydration and clearing of the slides were performed outside the SNAP i.d.® 2.0 IHC system. Blocking, antibody incubation and staining were performed in the SNAP i.d.® 2.0 IHC system after assembling the slides in the slide holder. Using the the SNAP i.d.® 2.0 system permits reduced slide manipulation, does not require the use of a Pap pen, and the vacuum system is likely to increase the efficiency with which solutions are removed and tissue is efficiently washed. Antibodies, or any other reagent, can be recovered with a 1 mL pipette tip through the injector recovery port, and stored for future reuse.

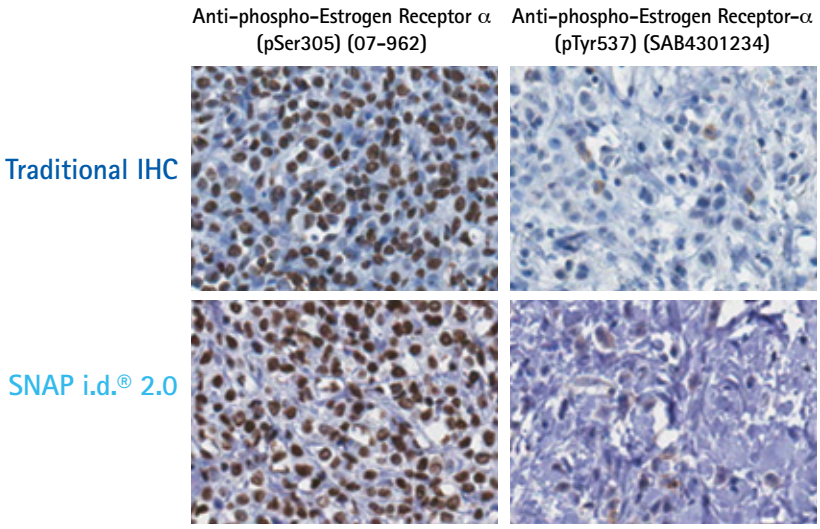

Figure 2.

Comparing IHC data obtained using traditional IHC with that using the SNAP i.d.® 2.0 immunodetection system.

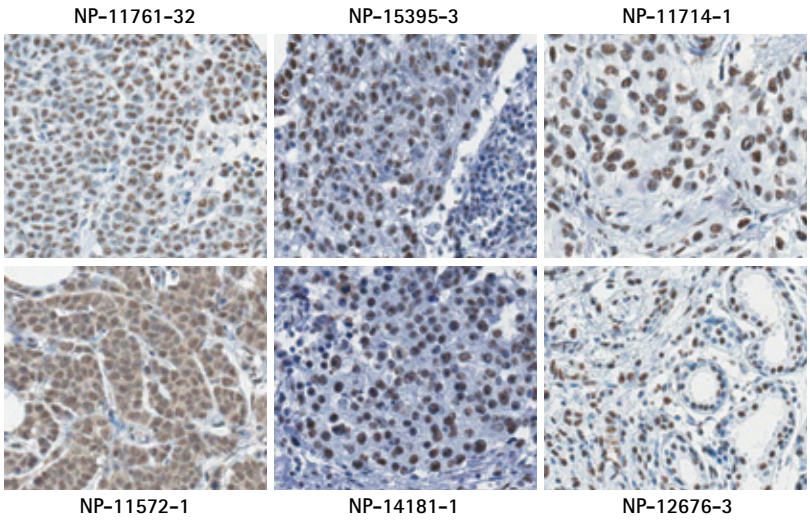

Figure 3.

Representative IHC data for all breast carcinoma tissues using pER-S305 antibody (05-922R).

Results

We initially started our testing by doing a western blot screening to identify antibodies that specifically recognize phosphorylated sites of ERα. To accomplish this, we analyzed a number of antibodies using lysates that were stimulated or unstimulated, samples that were lambda phosphatase treated or untreated, or tested recombinant constructs with wild type ERα or site-specific mutations (data not shown). From this analysis, we identified seven antibodies that were phospho-specific plus one for total ERα that were carried into the IHC phase of testing.

Previous testing of the SNAP i.d.® 2.0 Protein Detection System produced results highly analogous to traditional IHC methods using a variety of tissues and several different antibodies (Merck Millipore Literature No. AN6221EN00). To further validate those results, side-by-side comparisons were completed here using breast cancer tissue and testing it with two different phospho-specific ERα antibodies (targeting pSer305 and pTyr537). As seen in Figure 2, the samples tested both showed similar results, both in regard to target staining intensity and subcellular localization, and the amount of background staining. Given these results, plus the previous findings, we deemed it acceptable to proceed with all the subsequent testing on the SNAP i.d.® 2.0 Protein Detection System.

Samples from six different carcinoma patients were screened by IHC with eight different antibodies specifically targeting ERα. Figure 3 shows an example of the test results obtained using one antibody (pSer305) and all six breast cancer tissue samples. Although this target gave predominantly nuclear signal, there was some diversity among the samples in terms of the staining intensity and even some cytoplasmic staining. Similarly, Figure 4 (see page 5) shows an example of testing of serial tissue sections from one patient that was screened with all of the various antibodies used in this analysis. Again, the results show variable staining intensity and cellular localization depending upon the target modification. Finally, all of the IHC results were scored for staining intensity and subcellular localization, and the information was compiled in a table for easy reference (see Table 1 on page 5).

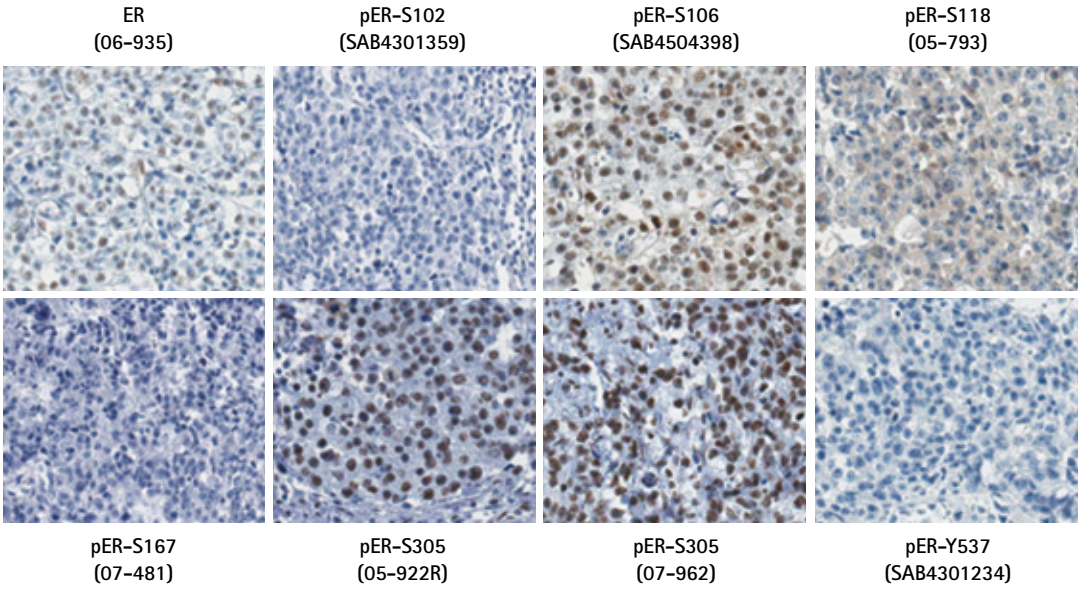

Figure 4.

Representative IHC data of all antibodies tested on ductal carcinoma tissue block NP14181-1.

	Ductal Carcinoma Tissue Sample											
	NP-11761-32		NP14181-1		NP15395-3		NP11714-1		NP11572-1		NP12676-3	
	PR-/Ki67 27%		PR-/Ki67 10%		PR-/Ki67 50%		PR+/Ki67 27%		PR+/Ki67 53%		PR+/Ki67 100%	
Target Tested	N	C	N	C	N	C	N	C	N	C	N	C
ERα (06-935)	+	+	++	+	++	+	+++	+	++	+	N/A	N/A
pER-S102 (SAB4301359)	-	-	-	-	-	-	-	-	-	-	-	-
pER-S106 (SAB4504398)	++	+	++	-	+++	+	+++	+	++	-	+++	-
pER-S118 (05-793)	+	++	-	+	++	++	++	+	+	++	N/A	N/A
pER-S167 (07-481)	-	-	-	-	-	-	-	-	-	-	-	-
pER-S305 (05-922R)	+	-	+++	-	++	+	++	-	+	-	++	-
pER-S305 (07-962)	+++	+	+++	-	++++	-	+++	+	++++	-	+++	-
pER-Y537 (SAB4301234)	-	-	-	+	-	-	-	+	-	+	-	-

Table 1.

Compilation of IHC results for all antibodies tested with all the carcinoma samples.

Conclusions

In this study, a collection of antibodies specific for individual phosphorylation sites on ERα protein were analyzed by IHC on a panel of breast cancer patient tissue samples. The SNAP i.d.® 2.0 protein detection system was used to facilitate this analysis, as the system provided consistent and reproducible IHC staining results that were comparable to traditional/manual staining methods. The higher throughput and ease of use afforded by the SNAP i.d.® 2.0 system was used for staining analysis of all of the samples. Staining intensity and subcellular localization of ERα phosphorylation varied with respect to specific phosphorylation site, as summarized below:

- Two phosphorylation sites showed strong staining in the nucleus (S106 and S305)
- One phosphorylation site (Y537) exhibited no nuclear staining and was only found at low levels in the cytoplasm
- Some sites (S102 and S167) showed little if any phosphorylation
- Finally, phosphorylation at S118 showed a mixed phenotype with low to moderate staining in the nucleus as well as the cytoplasm

Furthermore, it is worth noting that no reactivity in tissues that are not known to be sensitive to estrogen was detected during analysis of a large variety of non-ductal carcinoma TMAs (data not shown).

In conclusion, our data indicate that phosphorylation of ERα is highly variable in breast carcinoma. Samples from different patients showed that certain phosphorylation sites showed high levels of phosphorylation whereas others showed essentially none. In addition, most of the staining observed was found to be in the nucleus, but not exclusively so, as some cytoplasmic staining was present. Further analysis of more samples would be required to make definitive conclusions about the significance of each signal and to determine if a particular staining pattern could be used as a definitive marker for breast cancer.

While the manual immunohistochemistry method can be performed with small volumes of antibody and reagents, the process can be long, tedious, and sometimes challenging. The vacuum-driven SNAP i.d.® 2.0 IHC system offers an alternative for reduced slide manipulation, easy format, no need for pap pen, reproducibility, efficient washing, the opportunity to recover and reuse the antibodies with minimum volumetric loss and, most importantly, no decrease in sensitivity.

Ordering Information

SNAP i.d.® 2.0 system
Base System

The SNAP i.d.® 2.0 Systems for IHC contain everything you need to get started, including the detection base, IHC frame and incubation cover, slide holders, an assembly fixture, vacuum tubing and a Quick Start User Guide.

Description	Catalogue No.
SNAP i.d.® 2.0 Protein Detection System – Single IHC	SNAP2IHC
SNAP i.d.® 2.0 Protein Detection System – Double IHC	SNAP2IHC2

SNAP i.d.® 2.0 consumables

Description	Qty	Catalogue No.
SNAP i.d.® 2.0 IHC Frame	1 EA	SNAP2FRIHC
SNAP i.d.® 2.0 IHC Slide Holders	24/pk	SNAP2SH

1. Jordan, V. C. (2015, Oct 22) The new biology of estrogen-induced apoptosis applied to treat and prevent breast cancer. Endocr. Relat. Cancer, 22(1), R1-31.
2. Roy, S. S. and Vadlamudi, R. K. (2012, Jan) Role of estrogen receptor signaling in breast cancer metastasis. Int. J. Breast Cancer, 2012(1), 654698.
3. Murphy, L. C., Seekallu, S. V., Watson, P. H. (2011, Feb 1) Clinical significance of estrogen receptor phosphorylation. Endocr. Relat. Cancer, 18(1), R1-14.
4. Lee, H. R., Kim, T. H., Choi, K. C. (2012, Jun) Functions and physiological roles of two types of estrogen receptors, ERa and ERb, identified by estrogen receptor knockout mouse. Lab. Anim. Res., 28(2), 71-76.
5. Bruce, M. C., McAllister, D., Murphy, L. C. (2014, July 23) The kinome associated with estrogen receptor-positive status in human breast cancer. Endocr. Relat. Cancer, 21(5), R357-70.

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