Evaluation of Cell Line-Derived Xenografted Tumors as Controls for Immunohistochemical Testing for Estrogen Receptors and Progesterone Receptors

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A thesis submitted to my supervisory committee in fulfillment of the requirements for the Degree of Master of Science in Medicine

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Abstract

Current Quality Control (QC) practices for diagnostic immunohistochemistry (IHC) use archived breast cancer specimens and breast cancer cell lines as control materials for Estrogen Receptor (ER) and Progesterone Receptor (PR) testing in breast cancer. However, archived breast tumors show inherent heterogeneity within and between specimens and cell lines are not histologically representative of patient-derived-tumor controls. In an effort to generate standardized controls for ER and PR IHC testing, I hypothesized breast cancer cell line-derived xenografted tumors are representative of patient-derived-tumor controls that can be used for QC purposes. Well-characterized breast cancer cell lines with varying steady-state levels of ER and PR expression – MCF7, T47D and MDAMB468 – analyzed by immunoblot and IHC were implanted in immune-deficient mice to generate cell line-derived Xenografted (CDX) tumors. ER and PR expression of the Xenografted tumors was consistent with the derivative cell lines. Three pathologists expert in breast biomarker reporting assessed CDX tumors for reproducibility from different Mammary Fat Pad (MFP) sites in the mice. The CDX tumors were indistinguishable regardless of the MFP sites. The xenografted tumors from the three cell lines represented the range of biomarker expression levels exhibited by the patient-derived-tumor controls. These results suggest the possible potential applications of CDX tumor as controls to assist in reporting of ER and PR test results.

Acknowledgement

It has been an excellent opportunity and a delightful experience to work for Dr. Kao. He has supervised me with patience and care all throughout the project. He provided insightful advices and suggestions whenever I needed them. He has been an excellent critique of my work from the very beginning of the project to bring the best out of me.

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The results of this thesis has been recently published in the **Journal of Clinical Pathology 2015;0:1–6. doi:10.1136/jclinpath-2015-203066**. The title of the article is kept the same as the title of this thesis - Evaluation of Cell Line-Derived Xenografted Tumors as Controls for Immunohistochemical Testing for Estrogen Receptors and

Progesterone Receptors.

Contributions to the article:

I have designed and conducted the principal technical aspects of study, as well as testing methodology and provided manuscript figures and draft documents. Dr. Kao - principal investigator of the study is responsible for ethics clearances, study design and writing the manuscript. Dr. Gai, Dr. Denic and Dr. Power provided conceptual input on study design and performed analysis for data collection. Finally, Kim Vosey conducted assays and prepared specimens for data collection.

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Abbreviations

ASCO-CAP	American Society of Clinical Oncology and College of American Pathologists
CIT	Cold Ischemic Time
CDX	Cell line-Derived Xenograft
DAB	Diaminobenzidine
FFPE	Formalin Fixed Paraffin Embedded
ER	Estrogen Receptor
HR	Hormone Receptors
Her2	Human Epidermal Growth Factor Receptor 2
IHC	Immunohistrochemistry
NIH	National Institute of Health
NBF	Neutral Buffered Formalin
PDT	Patient Derived Tumor
QC	Quality Control
QM	Quality Management
RIPA	Radioimmunoprecipitation
SERM	Selective Estrogen Receptor Modulator
SCID	Severely Combined Immuno-Deficient
TMA	Tissue Microarray

1 Chapter 1 Introduction

1.1 Breast cancer biomarker testing

In clinical pathology laboratories, Immunohistochemistry (IHC) is used to evaluate breast cancer biomarker expression levels for prognostic and predictive testing. Estrogen Receptor (ER) and Progesterone Receptor (PR) expression levels can predict how a patient will respond to treatment for Hormone Receptor (HR) positive breast cancers[1]. The HR positive cases are treated with a wide range of drugs collectively called Selective Estrogen Receptor Modulators (SERMs) - Tamoxifen is one of the most widely used effective SERMs[2]. Despite its benefits to patient outcome, there remain ongoing issues with the reproducibility of ER and PR IHC test results between local and central laboratories[3]. Approximately 10 to 20 percent of tumors from a cohort of 1009 patients were falsely classified as ER-negative in a recent study[4]. Such misclassification can potentially change the treatment management schemes for patients, prompting the issuing by a joint committee of American Society of Clinical Oncology and College of American Pathologists (ASCO-CAP) of guidelines on ER and PR testing by IHC. The task force recommended exercising stringent quality control in every aspect of the assay to minimize unreliable test results[2].

1.2 Breast cancer biomarkers

The National Institute of Health (NIH) defines a biomarker as, "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention[5]". In oncology, biomarkers are categorized into prognostic and/or predictive markers. Prognostic biomarkers can objectively determine the outcome of a disease whereas predictive biomarkers can independently predict the therapeutic outcome of a disease[6]. In breast cancer, hormone receptors that include ER and PR are routinely used as biomarkers. Although the prognosis of the HRs is debatable, they have shown significant predictive benefit on patient outcome[7]. Another routinely used diagnostic biomarker is a growth factor receptor called human epidermal growth factor receptor 2, also called Her2/neu receptor. Her2 receptors are overexpressed in approximately 30 percent of invasive breast cancer patients. Her2 receptor positive breast tumors have poorer prognosis compared to breast tumors without Her2 overexpression. However, Her2 receptor testing followed by therapeutic intervention against Her2 positive cases with Trastuzumab has shown significant increase in disease free survival among this sub group of breast cancer patients[8]. With the advancement of technology used for detecting biomolecules, more promising biomarkers are becoming available for clinical use for better management of the disease. But so far, the challenge has been in reproducible determination of these biomarkers.

1.3 IHC processing

IHC is the gold standard technique used to test for ER and PR expression in breast cancer. IHC is a complex technique, with multiple stages including pre-analytic, analytic and post-analytic tumor processing. Briefly, the pre-analytic phase involves; fixing tumor specimens in a suitable preservative, such as 10 percent Neutral Buffered Formalin (NBF) followed by paraffin embedding. The Formalin Fixed Paraffin Embedded (FFPE) tumor is then sectioned and mounted on positively charged glass slides. The analytic phase involves the use of primary antibody designed specifically to bind to the antigen of interest followed by a series of detection molecules that catalyzes a chromogen called diaminobenzidine (DAB) and stain the sub-cellular regions brown, where the proteins of interest are localized. The post-analytic phase involves interpretation and reporting of the stained tumor sections by pathologists.

The ASCO-CAP guidelines for HR testing provide insights on the variables associated with these three stages of IHC processing that may adversely affect IHC reporting[2]. Hormone receptors are susceptible to variables associated with pre-analytic phase[9]. It is recommended to fix the specimens within an hour of surgical removal of the tumors. Delay in fixation may cause degradation of the HRs. A fixation time ranging from 8 hours to 72 hours is considered acceptable by ASCO-CAP guidelines but they also caution about under-fixation, that is, fixation less than 8 hours. Under-fixation may contribute to insufficient preservation of protein molecules[10]. The analytical variables include the primary antibody clones, antigen retrieval techniques and reference standard materials used for HR testing, that have

critical roles in contributing to testing accuracy[11]. The post-analytic phase involves interpretation and reporting of the test results by breast pathologists. The pathologists use recommended semi-quantitative scoring techniques such as - Allred Score, H-Score, Quick Score - to evaluate the HR test results. Although the scoring algorithms for all the methods takes account of the proportion of the cells stained and the intensity of the staining[2], they are calculated differently from each other[11]. Lack of quantitation makes it difficult to standardize the post-analytic phase of HR testing.

1.4 Scope of quality assurance in IHC processing

In order to ascertain if a diagnostic test or assay is running accurately, it is essential that the test be performed under a robust Quality Management (QM) program[2]. Every prognostic and especially predictive biomarker testing by IHC should have an individualized program. An integral part of QM is QC, which includes the use of control materials alongside the test samples to ensure the quality of the staining method. Clinical testing, much like scientific experimentation, requires parallel processing of material that demonstrates expected performance parameters to monitor the reliability of the test, or experiment. Hypothetically, an infinite number of controls can be run alongside a test, but practice dictates selecting controls that cover the testing range of the assay. Thus, a standardized assay would require uniformly generated controls to ensure accurate test-to-test performance. But there are, as of now, no commercially available standardized reference materials for ER and PR testing.

Standard QC protocols for IHC ER and PR testing use positive and negative controls along with the test specimens to minimize discordant findings and to monitor antibody and assay performance. The positive controls are tissue specimens that are positive for ER and PR antigens detectable by ER and PR antibodies. Negative controls are tissues that lack ER and PR antigens and hence are not detected by ER and PR antibodies. Positive controls are divided into two categories, external and internal positive controls. The external positive controls include archived breast tumor specimens and breast cancer cell lines containing ER and PR antigens. The internal positive controls are regions of normal tissue that express normally expected levels of ER and PR present within the test specimen. Positive reference controls monitor instrumentation error in automated IHC platforms, poor ER and PR antibody sensitivity and other assay analytic drift, overall controlling for false negative results. Negative controls are also divided into external and internal controls. The internal negative control is derived from the healthy part of the patient's breast and external negative control is derived from archived breast tumors. Another type of negative control called negative reagent control monitors for non-specific staining on test specimens by not applying the primary antibody to the test specimen. As the negative controls lack ER and PR antigens, the objective of using such controls is to monitor for false positive staining on the test specimens[12].

1.5 Effects of pre-analytic factors on IHC biomarker testing

The pre-analytical phase of biomarker testing starts with surgical removal of tumor from patient until the test specimen is prepared for analysis[13]. Protein

biomarkers in tumors are labile to conditions like sudden temperature difference, change in pH, and metabolic stress conditions during and after surgery[14]. A biomarker portends prognosis and in some cases predicts the course of a disease[15]. Standardizing pre-analytic factors that influence biomarker expression during sample preparation may preserve the quality of biomarkers and reflect true biomarker status after analysis[9,13]. The pre-analytic variables can be broadly divided into three categories: surgery, tissue processing and tissue storage.

Briefly, the factors associated with surgery involve ischemic temperature and delay in fixation. Some of the variables in tissue processing involve type of fixatives, duration of fixation, size of the tissue, and types of the tissue processors among others. Some of the factors affecting tissue storage are slide storage temperature and length of storage time[9,16]. Two of the pre-analytic factors that can be standardized are cold ischemic time and formalin fixation time.

1.5.1 Ischemic time

Restriction of blood flow to tissues is called tissue ischemia. Tumors undergo ischemia during surgical procedure when blood flow is restricted to the tissue by means of clamping blood vessels to control bleeding. The length of time it takes to remove the tissue from the patient and place it in the preservative is known as ischemic time. Ischemic time is divided into warm ischemic time and cold ischemic time[9]. Warm ischemic time is defined as the time between the surgical disruption of blood flow to the tumor until its removal from the patient. Cold Ischemic time is the duration between surgical removal of the tumor until it has been placed in the

fixative. During ischemic time, the tumor cells undergo stress conditions like hypoxia, autolysis and metabolic acidosis that may degrade the HRs and other biomarkers of breast cancer[14,17].

The American Association of Clinical Oncology – College of American Pathologists (ASCO-CAP) recommends cold ischemic time less than one hour for HR testing[2]. The recommendation was based on publications that studied the effects of cold ischemic times on ER and PR expression. The studies were designed using breast cancer specimens. Comparing paired needle core biopsy specimens - considered as control – that usually have less than 1 hour delay in fixation, to excisional specimens showed that ER antigenicity decreased in excisional specimens with increase in delay in fixation[17–19]. The challenge has been ruling out variables like inherent tumor heterogeneity in clinical specimens that may contribute to the decrease in biomarker antigenicity.

1.5.2 Formalin fixation

As par ASCO-CAP guidelines for hormone receptor testing, breast cancer specimens should be immediately placed in 10 percent Neutral Buffered Formalin (10% NBF) post surgery and fixed for minimum of 8 hours and maximum of 72 hours[2]. Formalin fixation is a chemical process that preserves proteins in tumors from undergoing autolysis by cross-linking the lysine residues with the aldehyde groups of formalin. Penetration of NBF in tissues that takes place at a rate of 1 mm per hour is a relatively faster process than fixation of tissues. Fixation is a time consuming reaction due to the following - formalin is formed when formaldehyde is dissolved in water

until there is equilibrium between aqueous formaldehyde and methylene glycol. In this reversible reaction, conversion of methylene glycol to formaldehyde is the rate-limiting step[20,21]. In the fixation process, formaldehyde covalently links the amino acids of the adjacent protein molecules. Once the aldehydes are used up, more formaldehyde molecules are produced to maintain the equilibrium between formaldehyde and methylene glycol[22]. Given more time to the process, there would be sufficient aldehyde molecules to effectively fix the tissue. In order to adequately fix tissues, 24 hours of fixation is recommended[23,24] but for reliable ER IHC tests, Goldstein (2003), suggested a minimum of 6 to 8 hours of fixation. However, a recent study reported no difference in ER expression in mastectomy specimens when fixation time was varied from 30 minutes to 1 week[25]. Given the aforementioned pre-analytical considerations, a tumor model with consistent biomarker expression would be useful to design studies to determine exclusive effects of cold ischemic time and fixation time on biomarker antigenicity.

1.6 Study rationale

Derivation of External Control Material

External control materials for ER and PR IHC testing are derived from archived breast tumors with previously reported test results. Such archived specimens with a range of ER and PR expression levels are identified and used as external reference materials. Although these controls generally give satisfactory results, they have some limitations - archived breast cancer specimens used as controls have inherent heterogeneity within and between tissues. Moreover, once a control-tissue block is

exhausted, a new block has to be identified which will invariably have inconsistent staining and histological differences from the previous control specimens. Hence, due to inter and intra heterogeneity, these controls are not necessarily ideal standards. Furthermore, the pre-analytic processing conditions of such Patient-Derived-Tumor (PDT) controls are often not the same as test specimens. The use of cell line-derived controls may overcome the inconsistent staining reflected in PDT controls[21] but as they are grown *in vitro*, they are not histologically representative of PDT controls and also their pre-analytic preparation does not follow surgical specimen processing.

Cell line-derived Xenografted (CDX) tumors generated in immunodeficient mice have wide applications in studying *in vivo* tumor biology and cancer therapeutics[22]. Furthermore, CDX tumors are considered to be representative of the cell lines from which they are derived[23]. Unlike cell lines, CDX tumors show tumor morphology reflected in clinical tumor specimens and they are assumed to retain protein expression of the cell lines from which they are derived.

The purpose of this thesis is to demonstrate the possible applications of well-characterized breast cancer CDX tumors as standardized reference materials for ER and PR IHC testing in breast cancer. In an effort to generate standardized controls, I asked the question: ***"How can HRs testing be standardized?"*** **I** ***hypothesized that breast cancer cell line-derived Xenografted tumors are representative of patient-derived-tumors that can be used for QC purposes in HR IHC testing.***

The objectives of the study were:

1. To select breast cancer cell lines with varying steady-state levels of ER and PR

expression.

2. To determine if site of cell implantation - mammary fat pad - had any effect on ER and PR expression in the CDX tumors.

3. To assess if the CDX tumors retained ER and PR expression of the cell lines.

4. To evaluate if the CDX tumors can span the range of ER and PR expression levels of the PDT controls.

The results from this study suggested that ER and PR expression levels are consistent between the cell lines and their corresponding CDX tumors. The site of cell line implantation in the mice has no effect on ER and PR expression. And finally the range of ER and PR levels observed in the MCF7, T47D and MDAMB468 CDX tumors is comparable to the range of PDT controls routinely used for testing clinical specimens.

2 Chapter 2 Materials and Methods

2.1 Breast cancer cell lines

The breast cancer cell lines Hs-578Bst, Hs-578T, MDAMB231, SKBR3, MCF7, MDAMB436, MDAMB468, T47D, BT474 and MDAMB157 were purchased from ATCC (Manassas, VA). Cell lines were cultured in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY), 10% Fetal Bovine Serum (GIBCO) and incubated at 37°C with 5% CO_2.

2.2 Cell block preparation

Cell lines were resuspended in 3 mm sections of plastic drinking straws with 1% molten low melting point agarose at 37°C, allowed to set at room temperature and fixed in 10 percent Neutral Buffered Formalin (10% NBF) for 24 hours. Cell blocks were processed as routine clinical samples prior to paraffin embedding.

2.3 Protein preparation from cell lines and cell line-derived Xenografted tumors

Protein was extracted from approximately 4.0×10^6 cells for each cell line using the radioimmunoprecipitation (RIPA) cell lysis buffer. RIPA buffer is composed of sodium dodecyl sulfate 0.1%, triton X-100 1.1%, EDTA 1.2 mM, Tris-HCl pH (8.1) 16.7 mM, NaCL 16.7 mM and 1% protease inhibitor in distilled water. Protein was extracted from the CDX tumors by mincing the tumors in presence of the RIPA buffer followed by resuspending at 4°C until a homogenized mixture was obtained. The homogenate was centrifuged at 4°C for 10 minutes and the supernatant was collected and stored at -80°C.

2.4 Immunoblot analysis

Total protein concentration in the RIPA buffer extracted from the cell lines and the CDX tumors were determined using the BioRad assay reagent (Berkeley, CA). Approximately 30 µg of each lysate was resolved in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred to the Polyvinylidene Fluoride (PVDF) membrane (BioRad, Berkeley, CA). The proteins were immune-blotted for ER, PR and β-actin. ER was determined using ER (SP1 clone) monoclonal antibody from (Thermo Scientific™ Lab Vision™, Fremont, CA) and polyclonal ER antibody from (Sigma, St. Louis, MO). PR was determined using monoclonal PR antibody (Cell Signalling, Boston, MA), and β-actin with monoclonal β-actin (Sigma, St. Louis, MO). Protein expression was detected using enhanced chemiluminiscence (Amersham, Piscataway, NJ). β-actin was probed to ensure that equal quantity of the test proteins was loaded in each well.

2.5 Generation of cell line-derived Xenografted tumors

$1.2X10^6$ cells were mixed with Cultrex® - Basement Membrane Extract - (Trevigen® Gaithersburg, MD) in a ratio of 1:1 (V/V) and injected in the mammary fat pads of the severely combined immuno-deficient (SCID) Hairless Outbred (SHO™) mice from (Charles Rivers, Wilmington MA). For mice injected with ER-positive cells, a 0.72 mg of 17β-estradiol - 60-day extended-release tablet (Innovative Research of America, Sarasota, FL) was inserted using a 10-gauge trochar (Innovative Research of America). CDX tumors were harvested at 5 mm in longest dimension and immediately placed in 10% NBF for 24 hours at room temperature followed by routine processing

in paraplast blocks. For every CDX tumor, a 4µm section was stained with Hematoxylin and Eosin (H&E) to identify areas of viable tumor.

2.6 Tissue microarray construction

Tumors were first assessed using 4 µm H&E sections for suitability. Tissue Microarrays (TMAs) were constructed on an MT-1 Arrayer (Beecher™, Sun Prairie, WI) using 1 mm diameter x 3 mm cores from MCF7, T47D and MDAMB468 FFPE cell blocks and tumors. Two TMAs were constructed, one of an array of 6 MCF7 CDX tumors excised from 3 different mammary fat pad (MFP) locations from two mice, and the other of CDX tumors from each of the three cell lines. Pathologists scored one slide of each TMA, each containing three cores selected from each tumor. Cores from Human breast tumors previously assessed as either negative, low expression (<5%) and high expression (>95%) as part of the clinical breast service were used as controls and were placed amongst the CDX tumors so that their identity could not be revealed. Clearance for use of archived specimens was obtained from the regional Health Research Ethics authority (HREA 13.281).

2.7 Immunohistochemistry

The cell line microarray and CDX-TMAs were cut into 3µ sections and stained for ER with ER (SP1) rabbit monoclonal antibody (Ventana, Tucson, AZ) and for PR with PR (1E2) rabbit monoclonal antibody (Ventana, Tucson, AZ). The cell line microarray and CDX-TMA sections were stained in automated staining platforms that use the *iVIEW DAB* detection system and the *ultraView DAB* detection systems

(Ventana™ Medical Systems). In case of the *ultraView* system, the slides underwent antigen retrieval at 95°C for 8 minutes in the Cell Conditioner (CC) #1. For both ER and PR tests, the slides were incubated in pre-diluted primary antibody at 36°C for 4 minutes. The system uses a polymeric biotin free detection kit. Following the primary antibody incubation, the slides were treated with the UV HRP Universal Multimer for 8 minutes. Next the chromgen, UV 3,3-diamino-benzidine (DAB) was added to the slides. The HRP catalyzed the UV DAB for 8 minutes to stain the antigens of interest brown on the tissue surface. The slides were treated with UV Copper for 4 minutes to enhance the brown nuclear staining. Counterstaining was performed using the Hematoxylin II. In the *iVIEW* system, for ER tests, the slides underwent antigen retrieval at 95°C for 60 minutes in the CC #1. The slides were incubated with pre-diluted primary antibody at 37°C for 8 minutes. For PR test, the slides underwent antigen retrieval at 95°C for 30 minutes in CC #1. The slides were incubated in pre-diluted primary antibody at 37°C for 16 minutes. The *iVIEW* detection system consists of a secondary antibody conjugated to biotins, linking the primary antibody to the StreptAvidin conjugated HorseRadish Peroxidase (SA-HRP). Biotin has strong affinity for streptavidin. The HRP catalyzes the DAB to produce brown stain at the site, where the proteins are located. Counterstaining was performed using the Hematoxylin II.

2.8 Assessment of the cell line-derived Xenografted tumor microarray

Three pathologists experienced in reporting breast biomarker expression, independently scored the CDX-TMAs (Tables 3.3a, b, c, d, e, f and 3.4a, b, c, d, e, f). For each tumor core in the CDX-TMAs, the averaged proportion score was assessed

for 3 randomly selected fields regardless of the staining intensity. An average intensity score was assigned at high-power (40X). The Allred score (Tables 2.8a and 2.8b) was assigned by summing the proportion score and intensity score.

2.9 Statistical analysis

One-way ANOVA was performed to determine whether there was a significant difference in the ER and PR expression levels between the CDX tumors and controls derived from human breast tumors and represented with box-and-whisker plots using GraphPad Prism V6 (GraphPad Software, La Jolla, CA). A *P*-value of < 0.05 ($\alpha = 0.05$) was considered significant.

2.10 Microarray imaging

The microarrays were scanned using a digital slide scanner, ScanScope XT from Aperio® (Buffalo Grove, IL). Images of the arrays were taken using Aperio ImageScope v11.2.0.780. Representative image of each histospot in the microarrays was saved in TIFF format. The figures were prepared using Adobe® Photoshop®.

Table 2.10.a Allred Intensity Score

Intensity Strength	Intensity Score
Negative	0
Weak	1
Intermediate	2
Strong	3

Table 2.10.b Allred Proportion Score

Percentage Range	Proportion Score
0	0
>0-1	1
>1-10	2
>10-33	3
>33-66	4
>66-100	5

3 Chapter 3 Results

3.1 Expression of ER and PR in breast cancer cell lines

In order to select cell lines suitable for evaluation of CDX tumors, the relative expression levels of both ER and PR in ten breast cancer cell lines was first determined using Western (immuno) blots (Figure 3.1a). Of these, MCF7, T47D and MDAMB468 cells were selected for further utilization. Agarose cell blocks were made from MCF7, T47D and MDAMB468 and further tested for ER and PR expression using IHC. Human breast tumors previously assessed as either negative, low expression (<5%) and high expression (>95%) as part of the clinical breast service were used as controls to assign comparable levels of staining in the cell blocks. MCF7 cells displayed strong nuclear staining of both ER and PR while T47D cells showed relatively weak nuclear ER and strong nuclear PR staining. Staining for both ER and PR was negative in MDAMB468 cells (Figure 3.1.b).

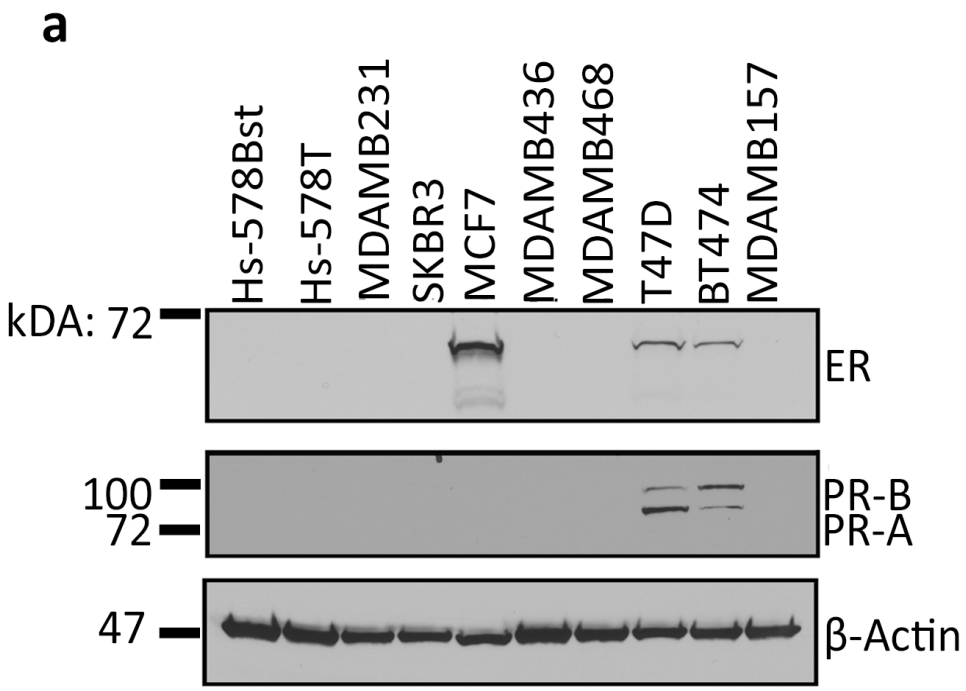


Figure 3.1.a Immunoblot analyses of ER and PR expression in breast cancer cell lines Steady-state levels of ER and PR expression were assayed by immuno-blot. β-actin was used as loading control.

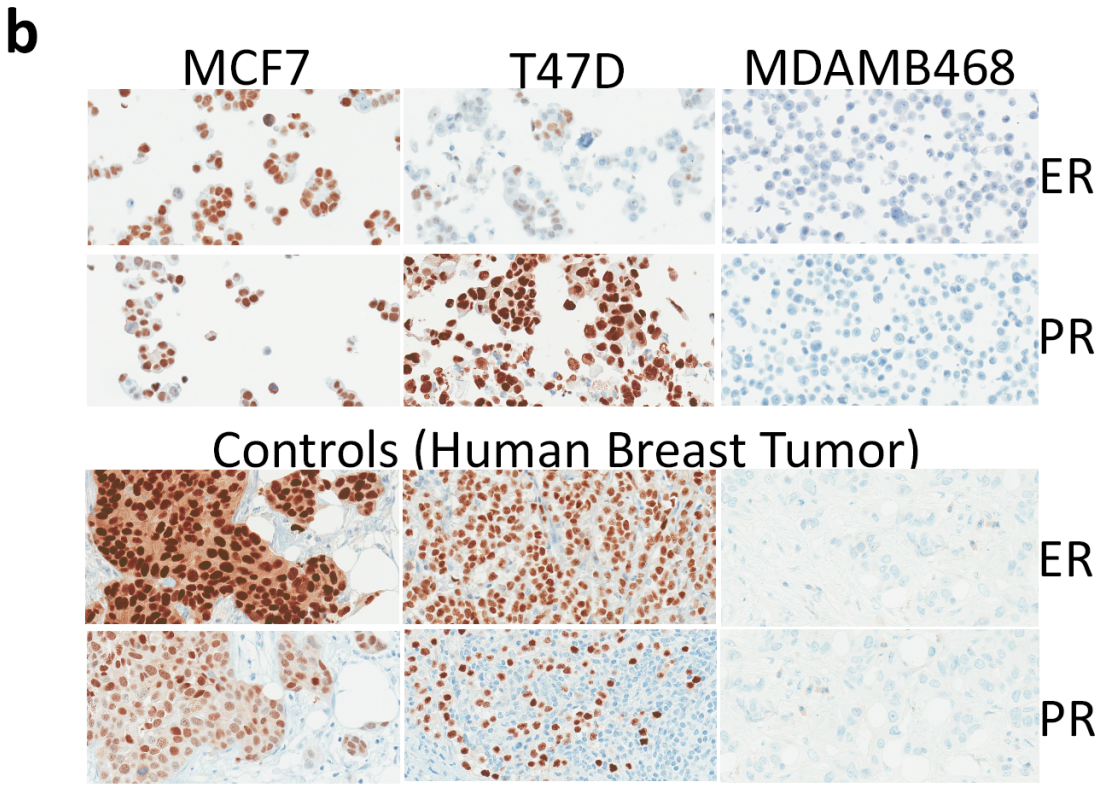


Figure 3.1.b IHC analyses of ER and PR expression in breast cancer cell lines
MCF7, T47D and MDAMB468 cell lines analysed by IHC using controls derived from human tumors with varying ER and PR staining intensity. Images were taken at (10X).

3.2 *ultraView* DAB detection system has superior signal to noise ratio over the i*VIEW* DAB detection system

In the IHC laboratory at Eastern Health, NL, estrogen receptor IHC tests are performed using *BenchMark XT*^{TM} and and *BenchMark ULTRA*^{TM} automated staining systems that use *iVIEW DAB* (avidin-biotin based) and *ultraView*^{TM} *Universal DAB* (polymer based) detection kits respectively. In order to determine the performance of the detection systems on the CDX tumors, we stained MCF7 CDX tumors alongside previously assessed human breast tumors (HBT) as controls for ER expression. The *ultraView* detection system showed relatively better signal to noise ratio than the *iVIEW* system in the MCF7 CDX tumors, notably observed in the negative ER control panels. Both *iVIEW* and *ultraView* produced relatively less non-specific background noise in the HBT controls. These observations suggested that the *ultraView* detection system produces superior signal to noise ratio in the CDX tumors in comparison to the *iVIEW* detection system.

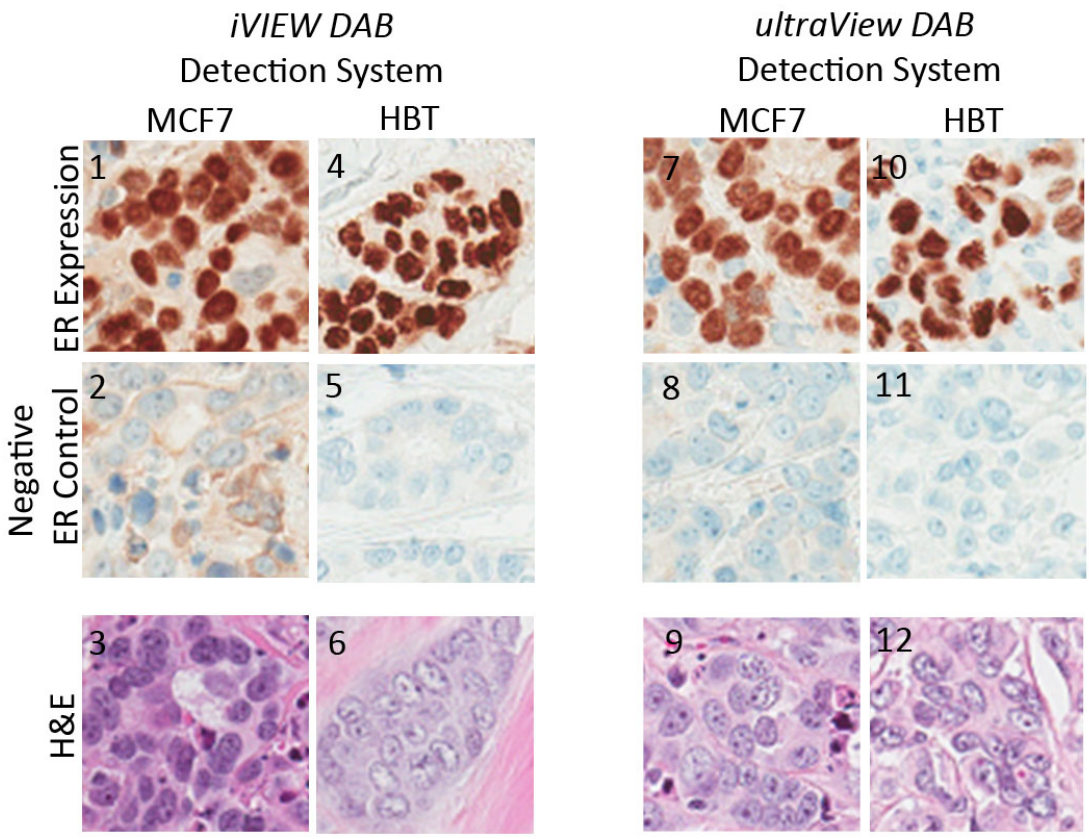


Figure 3.2 Comparison of detection systems on staining performance
Images 1 and 2 are of MCF7 CDX tumor and image 4 and 5 are of human breast tumors (HBT) stained with the *iVIEW* detection system with corresponding images 3 and 6 stained with Hematoxylin & Eosin (H&E). Images 7 and 8 are of MCF7 CDX tumors and image 10 and 11 are of HBTs stained with the *ultraView* detection system and H&E on 9 and 12. Images were taken at (40X).

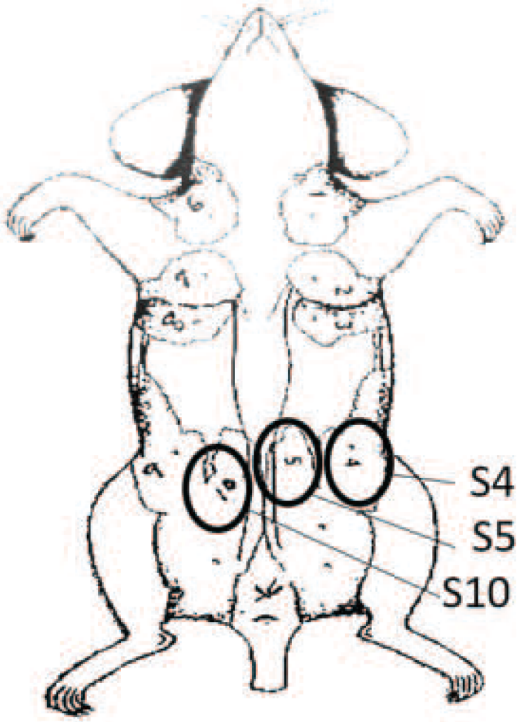
3.3 CDX tumors consistently maintain ER and PR expression levels irrespective of implantation sites

To construct CDX tumors, cell lines cultured *in vitro* were implanted in the mammary fat pads of recipient immunodeficient mice as described. In order to determine if the placement of the seeded cell lines had an effect on biomarker expression, CDX tumors were grown in different fat pad locations and in different mice and compared for ER and PR expression.

Six MCF7 CDX tumors were assessed from two mice from mammary fat pad (MFP) site 4, 5, and 10 as shown in (Figure 3.3.a). The expression of ER in MCF7 CDX tumors and human breast tumors (HBT) that are used as controls under standard operating protocols for biomarker testing, was assessed by three pathologists. The ER Allred score ranged between 7-8 and was consistent in the MCF7 CDX tumors regardless of the site of implantation and into which mice the tumors were produced. The three HBT controls were reported as strong, intermediate and negative for ER expression. ER expression in the MCF7 CDX tumors was concordant with the strong ER control shown in (Figure 3.3.b&c). Similarly, PR expression among the 6 MCF7 CDX tumors ranged between 6-8 (Figure 3.3d&e). These observations suggest that CDX tumors can be grown consistently and are comparable to patient derived controls.

a

S4: Site 4
S5: Site 5
S10: Site 10
M1: Mouse 1
M2: Mouse 2



M1/S4 M2/S4
M1/S5 M2/S5
M1/S10 M2/S10

Figure 3.3.a Mammary fat pad site numbers and the mouse numbers from where MCF7 CDX tumors were harvested

b

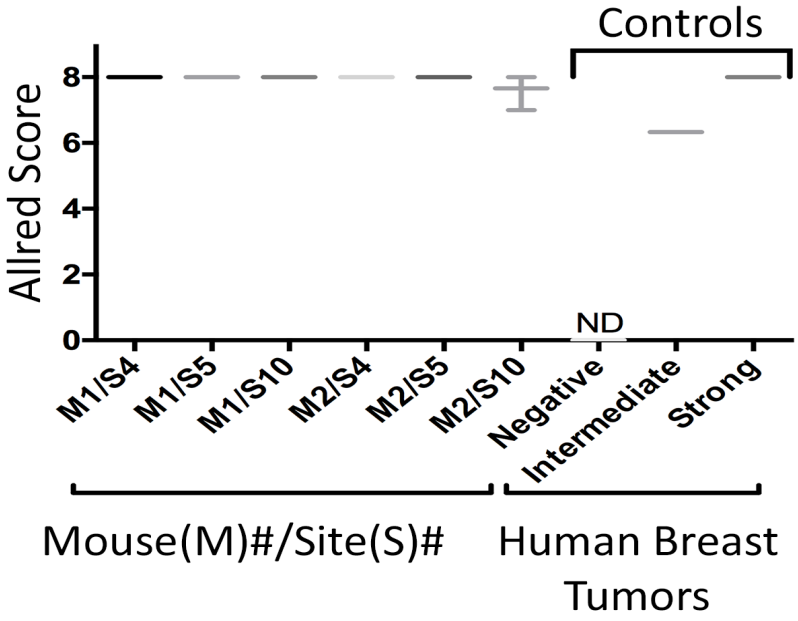


Figure 3.3.b Average Allred ER scores of the MCF7 CDX tumors
Box-and-whisker plot is used to represent the average Allred ER scores of the MCF7 CDX tumors (represented with the host and MFP site numbers) and three control human breast tumors. For each box, the middle line represents the median score, the lower whisker represents lowest score and the upper whisker represents the highest score. Non Detectable (ND).

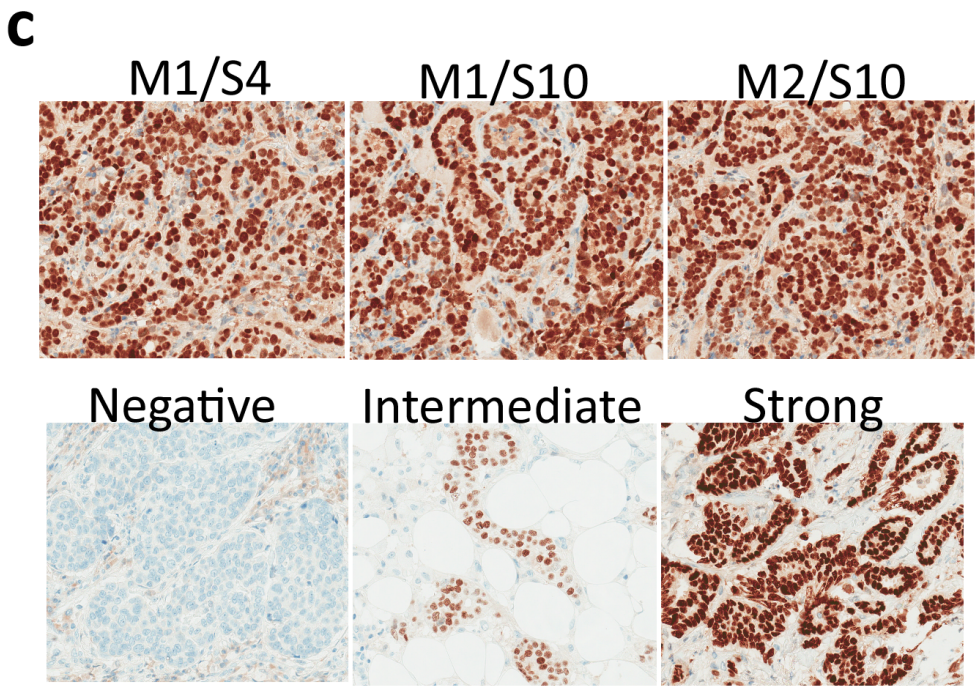


Figure 3.3.c ER Staining of MCF7 CDX Tumors
Representative IHC staining corresponding to part b. Images were taken at (10X).

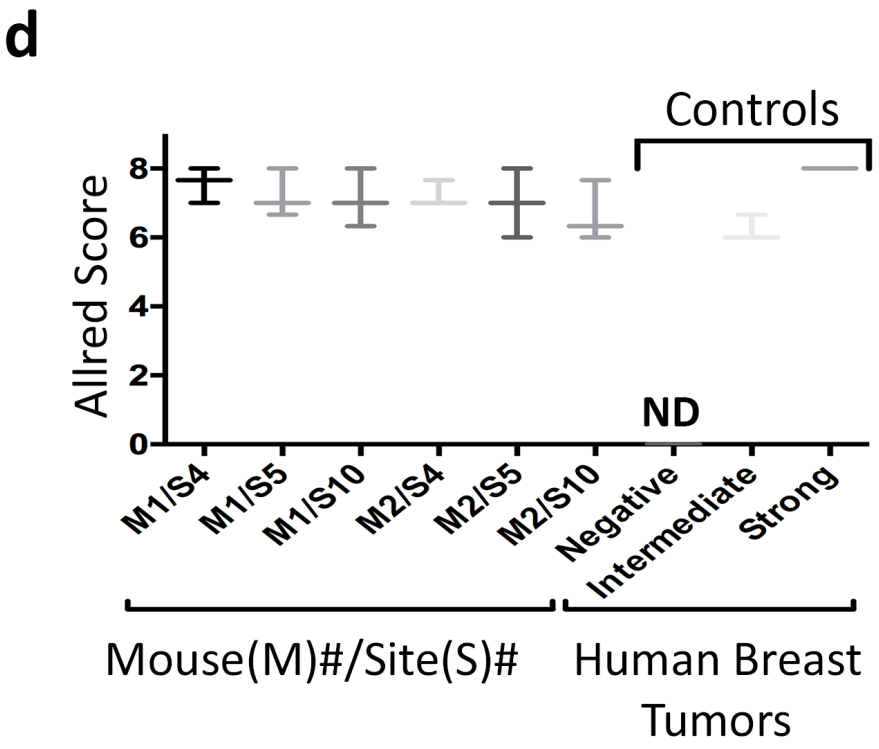


Figure 3.3.d Average Allred PR Score of MCF7 CDX Tumors
Box and whisker plot is used to represent the average Allred PR scores of the MCF7 CDX tumors (represented with the host and MFP site numbers) and three control human breast tumors. For each box, the middle line represents the median score, the lower whisker represents lowest score and the upper whisker represents the highest score. Non Detectable (ND).

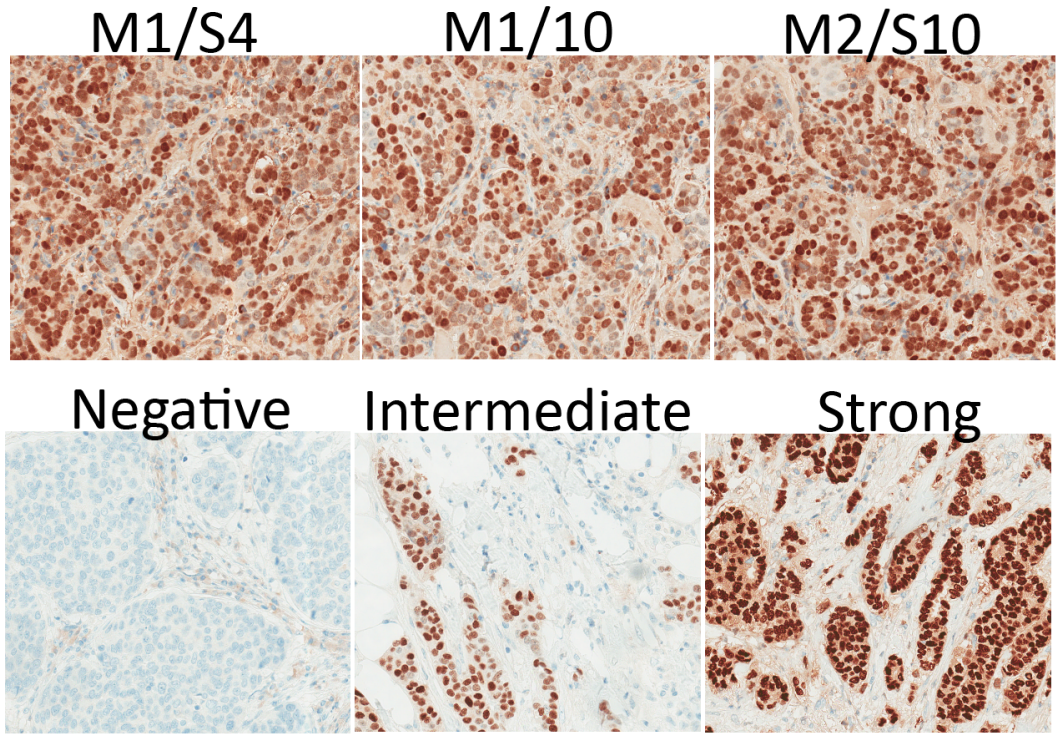


Figure 3.3.e PR Staining of MCF7 CDX Tumors
Representative IHC staining corresponding to part d. Images were taken at (10X).

Table 3.3.a ER Average Proportion Score (APS) – MCF7 CDX Tumors

Assessment of ER expression by three pathologists in 6 MCF7 CDX tumors denoted with Mouse/Site (M/S) numbers and three human breast tumor controls using the Proportion Score (PS) on three randomly selected fields (F). The PS of all three fields was averaged to obtain the Average Proportion Score (APS) for each tumor.

		MCF7 CDX Tumors						Human Breast Tumor Controls		
		M1/S4	M1/S5	M1/S10	M2/S4	M2/S5	M2/S10	Negative	Intermediate	Strong
Pathologist (P)	Field (F)	PS	PS	PS	PS	PS	PS	PS	PS	PS
P1	F1	5	5	5	5	5	5	0	5	5
	F2	5	5	5	5	5	5	0	4	5
	F3	5	5	5	5	5	4	0	4	5
	APS	**5**	**5**	**5**	**5**	**5**	**4.66**	**0**	**4.33**	**5**
P2	F1	5	5	5	5	5	5	0	4	5
	F2	5	5	5	5	5	5	0	4	5
	F3	5	5	5	5	5	5	0	5	5
	APS	**5**	**5**	**5**	**5**	**5**	**5**	**0**	**4.33**	**5**
P3	F1	5	5	5	5	5	5	0	5	5
	F2	5	5	5	5	5	5	0	4	5
	F3	5	5	5	5	5	5	0	4	5
	APS	**5**	**5**	**5**	**5**	**5**	**5**	**0**	**4.33**	**5**

Table 3.3.b ER Intensity Score - MCF7 CDX Tumors

Assessment of ER expression by three pathologists in 6 MCF7 CDX tumors denoted with Mouse/Site (M/S) numbers and three human breast tumor controls using the Intensity Score (IS).

Pathologist (P)	Table 5: MCF7 CDX Tumors						Human Breast Tumor Controls		
	M1/S4	M1/S5	M1/S10	M2/S4	M2/S5	M2/S10	Negative	Intermediate	Strong
	AS	AS	AS	AS	AS	AS	AS	AS	AS
P1	8	8	8	8	8	7.66	0	6.33	8
P2	8	8	8	8	8	8	0	6.33	8
P3	8	8	8	8	8	7	0	6.33	8

Table 3.3.c ER Allred Score - MCF7 CDX Tumors

Assessment of ER expression by three pathologists in 6 MCF7 CDX tumors denoted with Mouse/Site (M/S) numbers and three human breast tumor controls using the Allred Score (AS).

Pathologist (P)	MCF7 CDX Tumors						Human Breast Tumor Controls		
	M1/S4	M1/S5	M1/S10	M2/S4	M2/S5	M2/S10	Negative	Intermediate	Strong
	IS	IS	IS	IS	IS	IS	IS	IS	IS
P1	3	3	3	3	3	3	0	2	3
P2	3	3	3	3	3	3	0	2	3
P3	3	3	3	3	3	2	0	2	3

Table 3.3.d PR Average Proportion Score (APS) – MCF7 CDX Tumors

Assessment of PR expression by three pathologists in 6 MCF7 CDX tumors denoted with Mouse/Site (M/S) numbers and three human breast tumor controls using the Proportion Score (PS) on three randomly selected fields (F). The PS of all three fields was averaged to obtain the Average Proportion Score (APS) for each tumor.

Pathologist (P)	Field (F)	MCF7 CDX Tumors						Human Breast Tumor Controls		
		M1/S4	M1/S5	M1/S10	M2/S4	M2/S5	M2/S10	Negative	Intermediate	Strong
		PS	PS	PS	PS	PS	PS	PS	PS	PS
P1	F1	5	5	5	5	5	4	0	5	5
	F2	5	5	5	4	5	4	0	4	5
	F3	4	5	3	5	5	5	0	5	5
	APS	**4.66**	**5**	**4.33**	**4.66**	**5**	**4.33**	**0**	**4.66**	**5**
P2	F1	5	5	5	5	4	4	0	4	5
	F2	5	5	5	5	4	4	0	4	5
	F3	5	5	5	5	4	4	0	4	5
	APS	**5**	**5**	**5**	**5**	**4**	**4**	**0**	**4**	**5**
P3	F1	5	4	5	5	5	4	0	4	5
	F2	5	5	5	5	5	5	0	4	5
	F3	5	5	5	5	5	5	0	4	5
	APS	**5**	**4.66**	**5**	**5**	**5**	**4.66**	**0**	**4**	**5**

Table 3.3.e PR Intensity Score – MCF7 CDX Tumors

Assessment of PR expression by three pathologists in 6 MCF7 CDX tumors denoted with Mouse/Site (M/S) numbers and three human breast tumor controls using the Intensity Score (IS).

Pathologist (P)	MCF7 CDX Tumors						Human Breast Tumor Controls			
	M1/S4	M1/S5	M1/S10	M2/S4	M2/S5	M2/S10	Negative	Intermediate	Strong	
	IS	IS	IS	IS	IS	IS	IS	IS		IS
P1	3	2	2	3	3	2	0	2		3
P2	3	3	3	2	3	2	0	2		3
P3	2	2	2	2	2	3	0	2		3

Table 3.3.f PR Allred Score – MCF7 CDX Tumors

Assessment of PR expression by three pathologists in 6 MCF7 CDX tumors denoted with Mouse/Site (M/S) numbers and three human breast tumor controls using the Allred Score (AS).

Pathologist (P)	MCF7 CDX Tumors						Human Breast Tumor Controls		
	M1/S4	M1/S5	M1/S10	M2/S4	M2/S5	M2/S10	Negative	Intermediate	Strong
	AS	AS	AS	AS	AS	AS	AS	AS	AS
P1	7.66	7	6.33	7.66	8	6.33	0	6.66	8
P2	8	8	8	7	6	6	0	6	8
P3	7	6.66	7	7	7	7.66	0	6	8

3.4 Determination of ER and PR expression in Xenografted tumors derived from different cell lines

Each of the three cell lines consistently demonstrated their expected levels of biomarker expression, whether grown *in vitro* or as CDX tumors, as determined by immunoblot (Figure 3a). To assess whether the CDX tumors were comparable to the range of controls used for biomarker testing, pathologists blind-scored a tumor microarray of a series of cores that consisted of CDX tumors and one control each representing a negative, an intermediate and strong expresser of ER.

As can be seen in figure 3c, ER expression was undetectable in the CDX tumors derived from the MDAMB468 cell line, which were consistently scored for ER as most similar ($p<0.05$) to the negative tumor control. Similarly, CDX tumors derived from the most strongly expressing MCF7 cell line were most similar ($p<0.05$) to the strong tumor control. Likewise, T47D CDX tumors were most similar to the intermediate tumor control.

Both the MCF7 and T47D cell line derived CDX tumors, which showed the highest levels of PR expression were most similar to the strongly PR-expressing control ($P<0.05$). The MDAMB468 CDX tumor, for which PR was undetectable, was also most similar to the negative PR expressing control. These observations suggested that CDX tumors are indistinguishable from archived specimens used for testing controls.

a

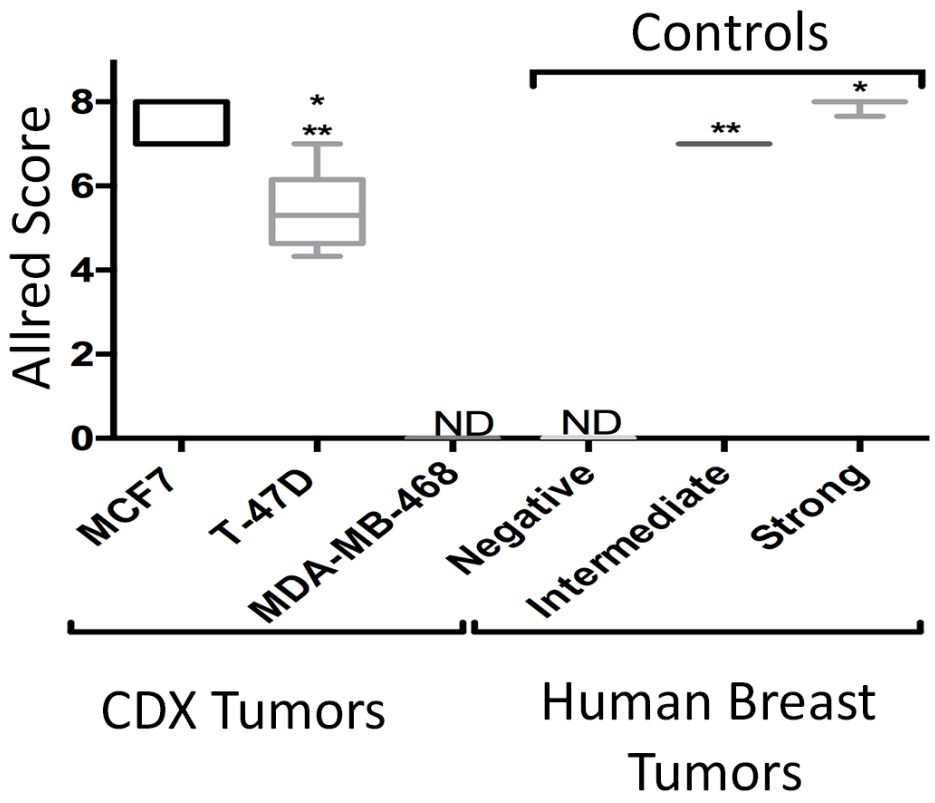


Figure 3.4.a Average Allred ER Score of CDX Tumors Generated from Different Cell Lines
Box-and-whisker plot is used to represent the average Allred ER scores of MCF7, T47D and MDAMB468 CDX tumors and three human breast tumors controls. For each box, the middle line represents the median score, the lower whisker represents lowest score and the upper whisker represents the highest score. P<0.05 compared to strong ER control. **P<0.05 compared to intermediate ER control. Non Detectable (ND).

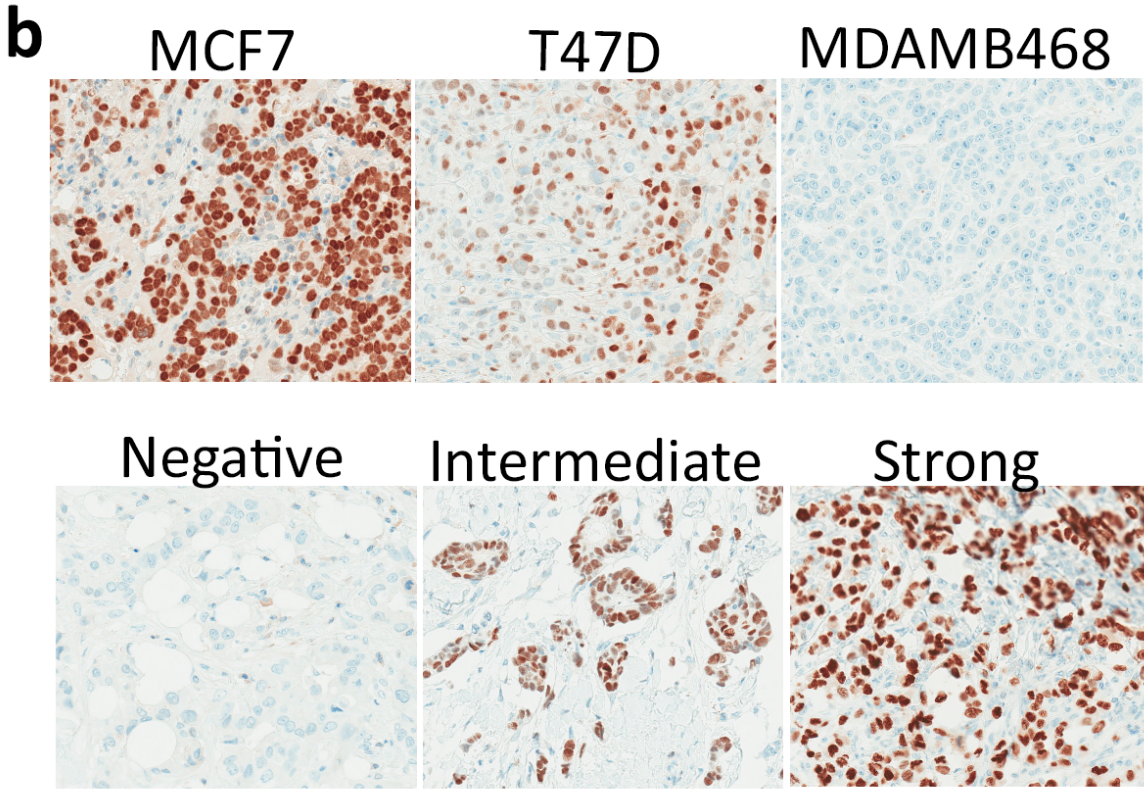


Figure 3.4.b ER Staining of CDX Tumors Generated from Different Cell Lines
Representative IHC images corresponding to part (a). The IHC images were taken at (10X).

c

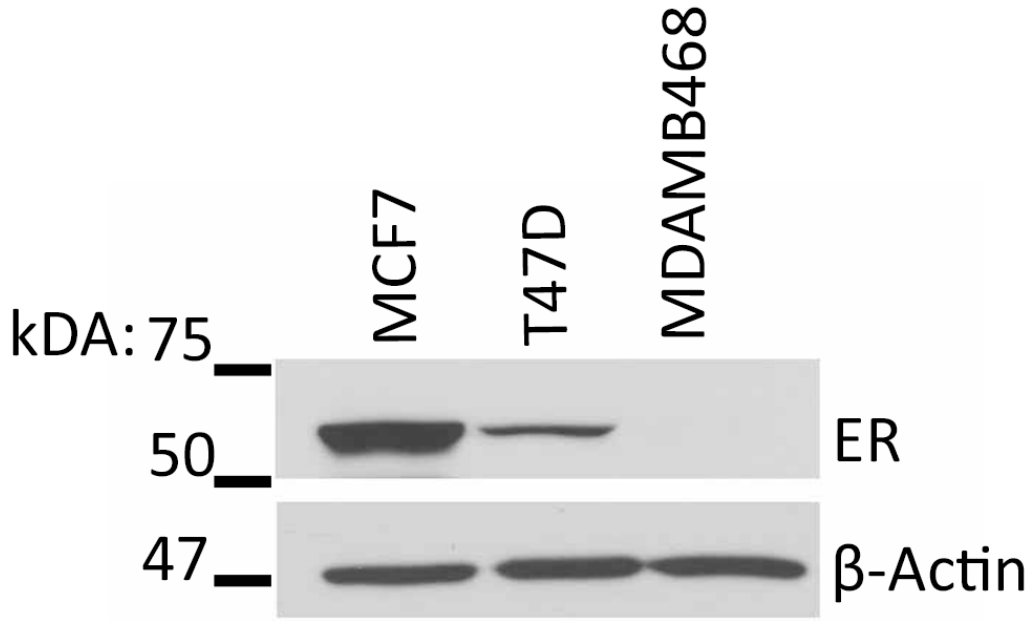


Figure 3.4.c Immunoblot analyses of ER expression in CDX tumors
ER expression levels in MCF7, T47D and MDAMB468 CDX tumors determined by immunoblot. B-actin used as loading control.

d

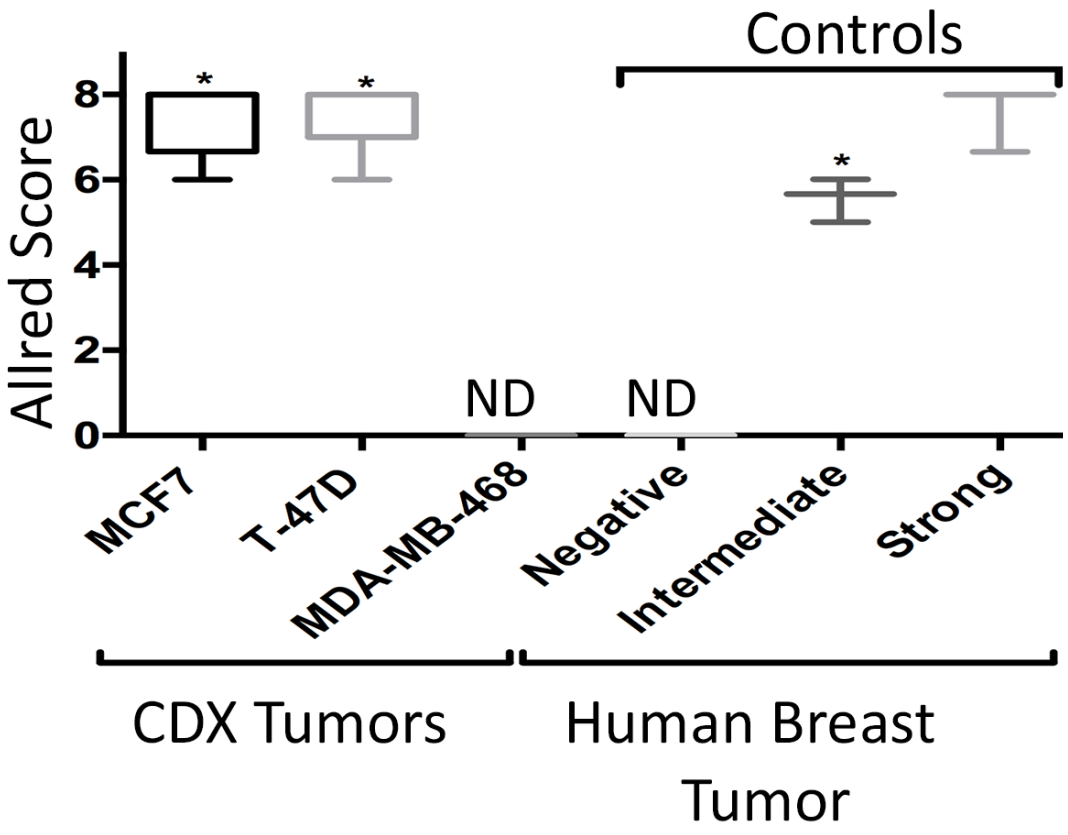


Figure 3.4.d Average Allred PR Score of CDX Tumors Generated from Different Cell Lines
Box-and-whisker plot is used to represent the average Allred PR scores of MCF7, T47D and MDAMB468 CDX tumors and three human breast tumors controls. For each box, the middle line represents the median score, the lower whisker represents lowest score and the upper whisker represents the highest score. *P<0.05 compared to intermediate ER control. Non Detectable (ND).

e

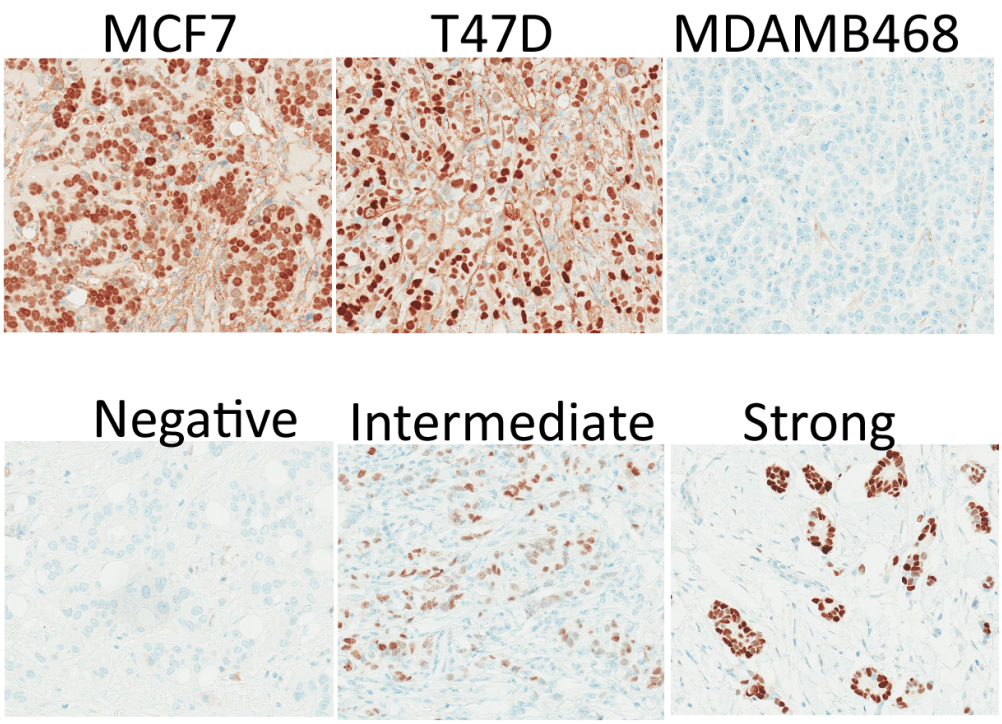


Figure 3.4.e PR Staining of CDX Tumors Generated from Different Cell Lines
Representative IHC images corresponding to part (d). The IHC images were taken at (10X).

Table 3.4.a ER Proportion Score - CDX Tumors

Assessment of ER expression by three pathologists in three biological replicates of each CDX tumor type and three human breast tumor controls using the Proportion Score (PS) on three randomly selected fields (F). The PS of all three fields was averaged to obtain the Average Proportion Score (APS) for each tumor.

			CDX Tumors			Human Breast Tumor Controls		
			MCF7	T47D	MDAMB468	Negative	Intermediate	Strong
Pathologist (P)	Replicate (R)	Field (F)	PS	PS	PS	PS	PS	PS
P1	R1	F1	5	4	0	0	5	5
		F2	5	4	0	0	5	5
		F3	5	5	0	0	5	5
		APS	**5**	**4.33**	**0**	**0**	**5**	**5**
	R2	F1	5	4	0	n/a	n/a	n/a
		F2	5	3	0	n/a	n/a	n/a
		F3	5	3	0	n/a	n/a	n/a
		APS	**5**	**3.33**	**0**	n/a	n/a	n/a
	R3	F1	5	4	0	n/a	n/a	n/a
		F2	5	4	0	n/a	n/a	n/a
		F3	5	4	0	n/a	n/a	n/a
		APS	**5**	**4**	**0**	n/a	n/a	n/a
P2	R1	F1	5	5	0	0	5	5
		F2	5	5	0	0	5	5
		F3	5	5	0	0	5	5
		APS	**5**	**5**	**0**	**0**	**5**	**5**
	R2	F1	5	4	0	n/a	n/a	n/a
		F2	5	4	0	n/a	n/a	n/a
		F3	5	4	0	n/a	n/a	n/a
		APS	**5**	**4**	**0**	n/a	n/a	n/a
	R3	F1	5	5	0	n/a	n/a	n/a
		F2	5	5	0	n/a	n/a	n/a
		F3	5	5	0	n/a	n/a	n/a
		APS	**5**	**5**	**0**	n/a	n/a	n/a
P3	R1	F1	4	4	0	0	5	4
		F2	4	3	0	0	5	5
		F3	4	4	0	0	5	5
		APS	**4**	**3.66**	**0**	**0**	**5**	**4.66**
	R2	F1	5	3	0	n/a	n/a	n/a
		F2	5	3	0	n/a	n/a	n/a
		F3	5	3	0	n/a	n/a	n/a
		APS	**5**	**3**	**0**	n/a	n/a	n/a
	R3	F1	5	4	0	n/a	n/a	n/a
		F2	5	4	0	n/a	n/a	n/a
		F3	5	3	0	n/a	n/a	n/a
		APS	**5**	**3.66**	**0**	n/a	n/a	n/a

Table 3.4.b ER Intensity Score - CDX Tumors

Assessment of ER expression by three pathologists in three biological replicates of each CDX tumor type and three human breast tumor controls using the Intensity Score (IS).

Pathologist (P)	Replicate (R)	CDX Tumors			Human Breast Tumor Controls		
		MCF7	T47D	MDAMB468	Negative	Intermediate	Strong
		IS	IS	IS	IS	IS	IS
P1	R1	3	2	0	0	2	3
	R2	2	2	0	0	n/a	n/a
	R3	3	2	0	0	n/a	n/a
P2	R1	3	2	0	0	2	3
	R2	2	2	0	0	n/a	n/a
	R3	2	2	0	0	n/a	n/a
P3	R1	3	1	0	0	2	3
	R2	2	2	0	0	n/a	n/a
	R3	3	2	0	0	n/a	n/a

Table 3.4.c ER Allred Score - CDX Tumor

Assessment of ER expression by three pathologists in three biological replicates of each CDX tumor type and three human breast tumor controls using the Allred Score (AS).

Pathologist (P)	Replicate (R)	CDX Tumors			Human Breast Tumor Controls		
		MCF7	T47D	MDAMB468	Negative	Intermediate	Strong
		AS	AS	AS	AS	AS	AS
P1	R1	8	6.33	0	0	7	8
	R2	7	5.33	0	n/a	n/a	n/a
	R3	8	6	0	n/a	n/a	n/a
P2	R1	8	7	0	0	7	8
	R2	7	6	0	n/a	n/a	n/a
	R3	7	7	0	n/a	n/a	n/a
P3	R1	7	4.66	0	0	7	7.66
	R2	7	5	0	n/a	n/a	n/a
	R3	8	5.66	0	n/a	n/a	n/a

Table 3.4.d PR Proportion Score - CDX Tumors

Assessment of PR expression by three pathologists in three biological replicates of each CDX tumor type and three human breast tumor controls using the Proportion Score (PS) on three randomly selected fields (F). The PS of all three fields was averaged to obtain the Average Proportion Score (APS) for each tumor.

Pathologist (P)	Replicate (R)	Field (F)	CDX Tumors			Human Breast Tumor Controls		
			MCF7	T47D	MDAMB468	Negative	Intermediate	Strong
			PS	PS	PS	PS	PS	PS
P1	R1	F1	5	5	0	0	5	5
		F2	5	5	0	0	3	5
		F3	5	5	0	0	3	5
		APS	**5**	**5**	**0**	**0**	**3.66**	**5**
	R2	F1	5	4	0	n/a	n/a	n/a
		F2	5	4	0	n/a	n/a	n/a
		F3	5	4	0	n/a	n/a	n/a
		APS	**5**	**4**	**0**	n/a	n/a	n/a
	R3	F1	5	4	0	n/a	n/a	n/a
		F2	5	4	0	n/a	n/a	n/a
		F3	5	4	0	n/a	n/a	n/a
		APS	**5**	**4**	**0**	n/a	n/a	n/a
P2	R1	F1	5	5	0	0	4	5
		F2	5	5	0	0	4	5
		F3	5	5	0	0	4	5
		APS	**5**	**5**	**0**	**0**	**4**	**5**
	R2	F1	5	5	0	n/a	n/a	n/a
		F2	5	5	0	n/a	n/a	n/a
		F3	5	5	0	n/a	n/a	n/a
		APS	**5**	**5**	**0**	n/a	n/a	n/a
	R3	F1	5	5	0	n/a	n/a	n/a
		F2	5	5	0	n/a	n/a	n/a
		F3	5	5	0	n/a	n/a	n/a
		APS	**5**	**5**	**0**	n/a	n/a	n/a
P3	R1	F1	4	5	0	0	4	5
		F2	4	5	0	0	4	4
		F3	4	5	0	0	4	5
		APS	**4**	**5**	**0**	**0**	**4**	**4.66**
	R2	F1	5	4	0	n/a	n/a	n/a
		F2	5	4	0	n/a	n/a	n/a
		F3	5	4	0	n/a	n/a	n/a
		APS	**5**	**4**	**0**	n/a	n/a	n/a
	R3	F1	4	4	0	n/a	n/a	n/a
		F2	4	5	0	n/a	n/a	n/a
		F3	5	4	0	n/a	n/a	n/a
		APS	**4.33**	**4.33**	**0**	n/a	n/a	n/a

Table 3.4.e PR Intensity Score - CDX Tumors

Assessment of PR expression by three pathologists in three biological replicates of each CDX tumor type and three human breast tumor controls using the Intensity Score (IS).

Pathologist (P)	Replicate (R)	CDX Tumors			Human Breast Tumor Controls		
		MCF7	T47D	MDAMB468	Negative	Intermediate	Strong
		IS	IS	IS	IS	IS	IS
P1	R1	3	3	0	0	2	3
	R2	3	3	0	n/a	n/a	n/a
	R3	3	3	0	n/a	n/a	n/a
P2	R1	2	3	0	0	2	3
	R2	3	2	0	n/a	n/a	n/a
	R3	3	3	0	n/a	n/a	n/a
P3	R1	2	2	0	0	1	2
	R2	2	2	0	n/a	n/a	n/a
	R3	2	3	0	n/a	n/a	n/a

Table 3.4.f PR Allred Score - CDX Tumor

Assessment of PR expression by three pathologists in three biological replicates of each CDX tumor type and three human breast tumor controls using the Allred Score (AS).

Pathologist (P)	Replicate (R)	CDX Tumors			Human Breast Tumor Controls		
		MCF7	T47D	MDAMB468	Negative	Intermediate	Strong
		TS	TS	TS	TS	TS	TS
P1	R1	8	8	0	0	5.66	8
	R2	8	7	0	n/a	n/a	n/a
	R3	8	7	0	n/a	n/a	n/a
P2	R1	7	8	0	0	6	8
	R2	8	7	0	n/a	n/a	n/a
	R3	8	8	0	n/a	n/a	n/a
P3	R1	6	7	0	0	5	6.66
	R2	7	6	0	n/a	n/a	n/a
	R3	6.33	7.33	0	n/a	n/a	n/a

4 Chapter 4 Discussion

4.1 Development of reproducible controls for ER and PR IHC testing

Accurate predictive breast cancer biomarker analysis by IHC is paramount in determining the correct treatment for the patients and also in deciding if the patients can be spared of the adverse consequences of ineffective treatment of the disease. Testing accuracy is contingent on the application of stringent QC on every aspect of the IHC assay[2,11]. Standardized reference materials are the core components of QC that can ensure the biomarker testing reliability.

In an effort to standardize breast cancer biomarker testing by IHC, we hypothesized breast CDX tumors are representative of patient-derived-tumor (PDT) controls and that they can be used for the QC purposes. In this study my data suggested that the CDX tumors are representative of the PDT and that they are consistent in expressing the breast cancer biomarkers. This feature of the CDX tumors potentiates their application as standardized QC materials for IHC ER and PR testing. We have specifically focused on the ER and PR expression in the CDX tumors in this study.

4.2 The benefits of CDX tumors as controls over PDT and cell line-derived controls

Archived breast tumors with known varying range of the ER and PR expression are used as external quality control materials alongside the test specimens. The ER and PR levels in the PDT controls should range from strong to negative expression. This range of expression in the PDT controls makes them graded calibrators of ER and PR IHC testing[12]. However, the PDTs used as external controls are not the ideal

reference standards because the ER and PR expression in such controls is not reproducible due to the invariable differences in tumors from different patients or the inherent heterogeneity that exist within the tumors[26]. For instance, when an intermediate or weak graded ER expressing control is exhausted, the technologists has to select another control from the archive and validate the new control before running it for clinical specimen testing. Although the newly selected control may provide satisfactory staining performance, the biomarker expression level may be slightly different from the previous control. This subtle difference in staining between the new and the previous controls may be difficult to determine using pathologist-based semi-quantitative assessment. Also such PDT controls may have pre-analytic processing different from the test specimens. Lastly, such practice of using the PDT controls destroys the archive tissue blocks.

The breast cancer cell line-derived controls have long been proposed as a reliable, relatively accurate, and abundant control materials that can gauge the ER and PR assay sensitivity due to their consistent biomarker expression[27,26]. MCF7 breast cancer cells have been used as the core component of “the Quicgel Method”. As a matter of fact, this technique was used as one of the initial attempts to quantitatively measure the ER expression in invasive breast cancer specimen tested by IHC[28]. However, the cell line-derived controls may not account for the changes in biomarker expression as a result of the pre-analytic variables as they are not histologically representative of the PDT controls. Pre-analytic tissue processing is a major area of concern in quality assurance of the HR IHC testing[11]. Ideally the control

materials should have the same pre-analytic processing as the test specimens, to monitor changes in antigenicity in the test specimens as a result of the pre-analytic variables.

In this study, controls were generated with consistent ER and PR expression as found in the cell lines but at the same time have the histology similar to that of the PDT controls. I generated breast cancer CDX tumors and determined their ER and PR expression levels in relation to human breast tumors with known ER and PR expression.

In an attempt to recapitulate the range of the ER and PR expression of the PDT controls in the CDX tumors, I selected three breast cancer cell lines with steady-state varying levels of ER and PR expression. The MCF7 cell lines strongly expressed ER and PR, the T47D showed intermediate ER expression but strong PR expression and the MDAMB468 expressed neither ER nor PR. In order to determine if CDX tumors can represent the PDTs, we orthotopically injected the MCF7 cells in various MFP sites in the immunodeficeint mice. The MCF7 CDX tumors showed consistently strong ER and PR expression regardless of the sites of cell implantation or the host. The characteristic strong ER and PR expression of MCF7 cell line was reflected in the corresponding CDX tumors. The strong ER expression of the MCF7 CDX tumors was comparable with the strong ER Human Breast Tumor (HBT) control. These observations suggest that ER and PR expression can be reproduced in the CDX tumors harvested from different MFP sites in the mice. In order to develop a control system that provides a range of ER and PR expression presented by the PDT controls, I

implanted the mice with MCF7, T47D and MDAMB468 cells. The MCF7 CDX tumors showed ER and PR expression comparable to the strong ER control. The T47D CDX tumors showed ER expression comparable to the intermediate ER control but PR expression comparable to the strong ER control and the MDAMB468 CDX tumors showed ER and PR expression comparable to the negative ER and PR control. These results suggested that staining patterns of the CDX tumors were consistent with cell lines from which they were derived and they also cover the range of ER and PR expressions of the PDT controls.

4.3 The use of tissue microarray to evaluate ER and PR antigen heterogeneity in the CDX tumors

The TMA has been employed as one of the QC tools for routine IHC tests to improve IHC staining performance[29]. The TMA approach was recently used to standardize ER tests. The concept is to consolidate multiple tissue cores with varying biomarker expression in an array to monitor the assay sensitivity and hence to calibrate the biomarker expression levels[4]. But such controls may not be considered standardized as tissues in the TMA block may have indefinite pre-analytical processing conditions. Tumor heterogeneity is another factor that confounds the functionality of such control system, as the tumor cores selected to construct the microarray may not represent the protein expression profile of the whole tumors.

Nevertheless, the TMA control methodology can be very useful if the tumors constituting the microarray have undergone pre-analytic processing recommended standard for the biomarker testing. This will ensure relatively better rescuing of the

antigen of interest. In this study, I constructed TMAs with the CDX tumors. The CDX tumors after excision were immediately placed in the fixative and fixed for 24 hours. These pre-analytic conditions followed are the standard protocol recommended by ASCO-CAP guidelines for ER and PR testing. The cores were withdrawn from random regions of CDX tumor blocks to construct the microarray. I found that ER and PR expression was consistently and reproducibly high in the MCF7 CDX tumor cores, consistently and reproducibly high PR but intermediate ER expression in the T47D CDX tumor cores and no ER and PR expression in the MDAMB468 CDX tumor cores in the TMA. This suggested that ER and PR antigens were consistently and uniformly distributed in the CDX tumors.

4.4 The applications of CDX tumors

The effect of pre-analytic variables such as cold ischemic time (time to fixation), duration of fixation, tissue processing is difficult to study prospectively with clinical specimens due to tumor heterogeneity and scarce tissue availability. The inherently inconsistent biomarker expression of the tumors makes them unsuitable subjects to study the effects of pre-analytic variables[30]. On the other hand, the consistent ER and PR expression of the CDX tumors and their histologic similarity to the clinical specimens make them useful subjects to perform prospective studies on ER and PR expression as a function of the pre-analytic variables.

Besides their possible application in studying pre-analytic variables, the CDX tumors can be used to study or validate sensitivity and specificity of new antibodies, detect assay analytic drifts as result of reagents or instrumentation errors. One of the

biggest challenges in the ER and PR IHC testing has been the quantitation of protein expression. The lack of quantitation makes it difficult to standardize reporting of the test results. Currently pathologists use scoring methods that give semi-quantitative values to the ER and PR expression to report the test results. Automated image analysis over two decades has shown considerable advancement in quantifying the ER and PR expression in the test specimens[31–33]. In our study, ER and PR expression in the CDX tumors was evaluated using one of the conventional pathologist-based scoring methods, the Allred score. The consistency in scoring among the three scorers makes the CDX tumors an ideal platform to validate the automated image analysis techniques with the conventional pathologist-based scoring techniques.

4.5 Weaknesses and merits of the CDX tumor approach

When stained using the *iVIEW* detection system, MCF7 CDX tumors showed significantly noisier background compared to the routine control specimens. But the non-specific background was remarkably improved when the MCF7 CDX tumors were stained using *ultraView* (UV) detection kit (Figure 3.2a). The *iVIEW* system is avidin-biotin based unlike UV, which is polymer based. A major problem with avidin-biotin system is that, if the specimen to be tested is rich in endogenous biotins then streptavidin would bind to them and produce the unwanted background noise. It may be possible that in the CDX tumors, the mouse stromal cells are rich in biotin that produces the high noise to signal ratio. On the contrary, the UV system uses a polymer backbone conjugated with secondary antibodies and multiple HRP molecules. The absence of streptavidin ensures no non-specific avidin-biotin

interaction. Such properties of the UV systems produces superior signal to noise ratio compared to the *iVIEW* system[34].

Generating CDX tumors is an expensive procedure and it requires technical expertise. If an IHC laboratory is interested to make in-house CDX tumors for QC purposes, it requires tissue culture and animal care facilities. Provided the availability of tissue culture facility, it is essential that the batch number of the cell lines used to generate the CDX tumors be documented because it has been reported that there are subtle changes in protein expression between different batches of the same cell lines[26]. It is possible that ER and PR expression level in the CDX tumors may show variation from expected staining intensity if undocumented cell lines are used. Hence regular validation of the cell lines is imperative.

Despite such limitations, there are substantial benefits of the implication of the CDX tumors for standardizing not only breast cancer biomarker testing but also other cancer biomarker testing. Multiple CDX tumors can be generated from one mouse - one mouse can generate 5 to 6 CDX tumors depending on the number of cell implantation MFP-sites. Furthermore, for the QC purposes, CDX-TMA can be constructed with only 0.5 to 1 mm core of CDX tumors. So one batch of the CDX tumors from one mouse can provide sufficient control materials that will last long enough to perform multiple tests.

4.6 Conclusion

In this study, I demonstrated a method of generating standardized control materials for ER and PR IHC testing. We showed CDX tumors are comparable to the

gold standard - PDT controls. We used semi-quantitative values of ER and PR expression to characterize each of the three CDX tumors; MCF7, T47D and MDAMB468. But in order to determine the near-exact quantity of proteins expressed in CDX tumors, the tumor sections should be assessed using automated image analysis techniques. As IHC has been increasingly used as a therapy decisive technique, it is essential that biomarker expression in test specimens be reported in exact quantity. This would require the biomarker expression in the control materials to be reported with quantitative values as well. If the CDX tumors are used as reference materials alongside the test specimens and the staining is assessed using automated image analysis, the exact values of controls and test specimens would not only assist the pathologists but the clinicians in deciding the appropriate therapy for the patients.

4.7 Future directions

The consistent expression of proteins in the CDX tumors makes them ideal tissues to study the effects of pre-analytic variables on biomarker expression. For example, CDX tumours can be exposed to defined cold ischemic time ranging from 0 hours to 24 hours and defined fixation times ranging from 1 hour to 120 hours. As stable fixation is achieved at around 24 hours[10,35], it will be possible to compare the biomarker expression status of these different time points with the stable pre-analytic conditions - CIT of 0 hours and fixation time of 24 hours - to suggest a time range for cold ischemia and fixation for the clinical specimens.

5 Chapter 5 References

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