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IHC validation in clinical settings; how lab developed assays drive clinical therapies

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**IHC VALIDATION IN CLINICAL SETTINGS;
HOW LAB DEVELOPED ASSAYS DRIVE CLINICAL THERAPIES**

by

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**IHC VALIDATION IN CLINICAL SETTINGS; HOW LAB DEVELOPED
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ABSTRACT

Diagnostic laboratory tests are critical to patient care as they help dictate the most appropriate treatments and procedures. To that end, hospitals and laboratories must ensure their diagnostic assays are optimized so as to afford patients their best chance for recovery. The pathology laboratory at Boston Medical Center, like all testing labs, must validate their IHC protocols yearly according to ASCO/CAP guidelines. Furthermore, BMC has a diverse patient population similar to the Atlanta population-based study published by Lund, *et al.* in 2010. Based on the results of the yearly ASCO/CAP testing and compared with the results of the Atlanta study, it was found that BMC's HER2 testing was most likely not capturing all positive cases consistently. This prompted an optimization procedure for HER2 to be implemented. In addition to HER2 testing, BMC is looking at ways to optimize and implement PD-L1 IHC protocols as a way to identify those patients who might benefit from more targeted therapy. **Methods:** HER2 IHC was performed with an altered protocol to attempt a higher concordance rate with PhenoPath, a reference lab in Seattle, and the Atlanta study data. ER and PR validation IHC protocols were also performed to ensure adequate concordance with required yearly testing. **Results:** ER and PR protocols were found to be >95% accurate as compared to reference lab results. The new HER2 protocol yielded more vibrant staining results when compared

to known positive reference data and previous BMC testing of the same sample.

Discussion: Optimizing lab assays is a critical step in ensuring proper clinical therapies are being utilized. Regular testing of a lab's IHC output must be continuously verified, and continued data collection of the improved HER2 protocol is needed to make sure appropriate standards are being met. Further testing of PD-L1 IHC protocols will be warranted to maintain maximum efficiency.

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LIST OF ABBREVIATIONS

AKT	Protein Kinase 3
ASCO.....	American Society of Clinical Oncology
BMC.....	Boston Medical Center
CAP.....	College of American Pathologists
CC1	Cell Conditioner, proprietary reagent of Ventana
CDC	Centers for Disease Control and Prevention
CEP17	Centromere 17
DAB	3,3' diaminobenzidine
ER	Estrogen Receptor
ERK.....	Extracellular Signal-regulated Kinases
FDA.....	Food and Drug Administration
FISH.....	Florescence in situ Hybridization
HER2.....	Human Epidermal Growth Factor Receptor 2
IHC.....	Immunohistochemistry
MAPK.....	Mitogen-activated Protein Kinase
mTOR	Mammalian Target of Rapamycin
PDL1	Programmed Death Ligand Receptor 1
PI3K	Phosphatidylinositol 3-kinase
PKC.....	Protein Kinase C
PLC	Phospholipase C

PR..... Progesterone Receptor
TMA..... Tissue Microarray
TNBC..... Triple-negative breast cancer

INTRODUCTION

Validated lab assays are critical to patient health as they help to ensure appropriate and effective treatment. Through years of research a number of biological therapeutic targets have been identified for more precise treatment than previously prescribed. In breast cancer, three receptors have been identified as therapeutic targets: ER, PR, and HER2. Treatment of breast cancer is determined by the patient's subtype, with triple-negative cancers (those lacking all three) having the worst prognosis overall since current targeted drugs would be ineffective. Medicines that target receptor positive breast cancer have increased patient survival while decreasing disease recurrence. Other targets are currently being researched, such as the immune suppressor PD-L1. This receptor has been identified in a number of cancers, and will hopefully provide for a variety of targeted treatment options for affected patients.

These precise medications are only prescribed if there is positive confirmation from the appropriate lab assay, namely IHC. It is therefore essential that lab assays be robust enough to capture the presence of these receptors so that efficient and effective treatments can be deployed. Laboratories need to ensure their protocols are optimized for accuracy and sensitivity. This is achieved by comparing results to a validated reference lab as well as maintaining ongoing protocol quality checks per ASCO/CAP guidelines. By having potent lab assays, doctors can be sure they have the information they need to give their patients the best clinical therapy available. Therefore, the focus of this study is validate the current ER and PR IHC protocols, optimize the HER2 IHC protocol, and

seek to begin development of a viable PD-L1 IHC protocol to service the patient population of Boston Medical Center.

Estrogen Receptor

There are two variants of the estrogen receptor, ER α and ER β . They are coded by different genes, ESR1 and ESR2 respectively on chromosome 6, but are very similar in structure and function. ER is a transcription factor that is activated by the binding of estrogen and can have an effect on multiple signaling pathways, to include ERK/MAPK, p38/MAPK, PI3K/AKT, and PLC/PKC [18]. Once activated, ER translocates to the nucleus and binds to genes that are activated by estrogen responsive elements (EREs). In ER positive cancer, ER is overexpressed which causes increased cell proliferation and tumor growth.

Progesterone Receptor

The progesterone receptor is encoded by the PGR gene on chromosome 11 and results in two variants, PR α and PR β . PR β has an extra N-terminal domain called B-upstream segment (BUS) which contains a third transcription activation function (TAF3) which results in both variants effecting on different genes [18]. PR is a transcription factor which is activated by the binding of progesterone. Like ER, an activated PR will bind to progesterone responsive elements (PREs) in the nucleus and induce transcription of targeted genes. Similarly, in PR positive cancer, PR is overexpressed and results in cell proliferation and tumor growth.

Human Epidermal Growth Factor Receptor 2

HER2 is coded by the erb-b2 receptor tyrosine kinase 2 gene (ERBB2) on chromosome 17. Through the RAS-MAPK signaling pathway it promotes cell proliferation and inhibits cell death through the PI3/AKT/mTOR pathway [13]. Overexpression of HER2 results in increased cell proliferation and prevention of apoptosis through AKT enzymes. Therefore, in HER2 positive breast cancer, cells continue to proliferate without succumbing to programmed cell death which leads to tumor growth. HER2 overexpression is also implicated in some gastric cancers, however that is not the focus of this paper.

Breast Cancer Incidence and Treatment

Breast cancer remains the most common cancer in women in the United States with 124.8 per 100,000 new cases being diagnosed in 2015 as reported by the CDC. For that same year the rate of death from breast cancer was 20.3 per 100,000 [2]. Generally, incidence of new disease increases with age with the average age at diagnosis being 61 years. The most common type of breast cancer overall is ER+, representing about two-thirds of all cases. White women tend to have the highest rates of breast cancer, however black women have a higher risk of death. This is partly due to the type of cancer more commonly found in black women: triple-negative. This type of breast cancer represents about 15% of all cases, except in premenopausal black women, where the number jumps to roughly 39% [8]. TNBC lacks overexpression of the three receptors, making hormone targeted therapy ineffective. It tends to be more aggressive than receptor positive cancer and is also more prevalent in women diagnosed under 40 years of age. HER2+ cancers

also tend to be aggressive and represent about 20% of diagnoses [15]. Given these statistics it is vital that lab assays can accurately diagnose patients so they may be assigned effective, lifesaving treatment.

Lab tests are preformed to determine if an individual's cancer is ER, PR, and/or HER2 positive, and those results determine which treatment option would be most beneficial to the patient. For those with triple-negative breast cancer, treatment generally includes lumpectomy, mastectomy, radiation, and/or chemotherapy. For those with receptor positive cancer, targeted medicines have been developed which can help streamline therapy and improve patient survival. These treatments attack the cancer cells directly by targeting the receptor itself, leaving normal cells alone. Although the rate of new cancers is steady, the 5-year survival rate for breast cancer is 89.7%, and the death rate has continued to decrease [14].

This is partly due to more targeted therapies that focus on specific receptors. There are many FDA approved medications available for patients with ER+ and PR+ breast cancers, a common example being tamoxifen which blocks the estrogen receptor, and/or aromatase inhibitors, which halts production of estrogen. For those with HER2+ cancer, trastuzumab has been the standard treatment, usually in conjunction with additional drugs and chemotherapy. Trastuzumab works by binding to HER2 receptors and blocking the intracellular signaling that results in tumor proliferation. These therapeutics generally increase survival time and decrease disease recurrence. For those with aggressive HER2+ cancer, these drugs are critical to reduce mortality. Prior to trastuzumab approval, HER2+ breast cancer had poor prognosis due to its fast-growing,

aggressive nature. If someone has HER2+ cancer but it remains undetected, that patient is missing out on potentially lifesaving treatment. It is therefore critical to ensure the lab assays used to detect breast cancer subtypes are adequately sensitive so that effective treatment can be administered.

Immunohistochemistry

The foundations of immunohistochemistry began with serum therapy, which used ‘anti-toxins’ from animals to confer passive immunity, and the discovery of antigen-antibody binding in the 1890’s [3]. Through the years, scientists worked to determine how to identify the correct antibodies that would adhere to the target antigen. In the early 20th century, Dr. Paul Erlich’s Nobel prize-winning research demonstrated that antibody-antigen binding was accelerated by heat and slowed by cold, and noted the high specificity of that binding. As the structure and chemistry of antibodies and antigens was discovered, labels were developed that would identify the targeted biochemical entities. Dr. Michael Heidelberger has been credited as the first to add dye in a solution to effectively label an antibody-antigen complex [3]. At first, immunoglobulin G (IgG) was the most commonly used antibody as its structure contained an antigen binding site and a free site with which a label could be attached. The label is a peroxidase enzyme conjugated with DAB which is what allows the pathologist to view the now colored antigen under a microscope and diagnose the sample. These previous decades of research has led us to the steps BMC uses today to conduct IHC assays for patient diagnosis.

Patients’ surgical samples are sent to the pathologist for microscopic observation of histological changes. For all invasive breast carcinomas found, an IHC test is

performed to determine the presence (or absence) of ER and PR, and/or overexpression of HER2 in the patient. There are two types of IHC: direct and indirect. In the direct method, a labeled antibody is used that will bind to the target antigen directly. Excess antibodies are washed away and DAB is added. DAB acts as the reporter conjugate and adheres to the target cell via the labeled antibody to allow for visual confirmation of the affected cells. This method is appropriate if there is a sufficient number of primary antibodies available. During the development of IHC, it was discovered that antibodies could also be thought of as antigens. This led to the development of the indirect method. In the indirect method, a primary, unlabeled antibody is added to the sample so that it binds to the target antigen. A secondary, labeled antibody is then added to the sample which will bind to the primary antibody. Just like the direct method, DAB is then added which will allow for visual confirmation of the target antigen should it be present.

If the target antigen, for example HER2, is overexpressed in the tumor cells being tested, the affected cells will look stained. This allows the pathologist to visually observe the presence of a specific target antigen and make a final diagnosis. There are two main components to IHC protocols: preparing the sample and subsequent staining. Excised tissues from biopsies or other surgical procedures are embedded in formalin-fixed paraffin blocks to preserve the specimen until they can be stained. Once the sample is ready to be tested the paraffin block is sliced for mounting on a glass slide. The paraffin is then removed and the stain with the appropriate antibody clone is administered.

IHC Breast Cancer Protocols

BMC employs specific IHC protocols to determine which type of breast cancer is present in a given patient. These protocols must be validated on an ongoing basis to ensure the lab assay is functioning at an optimal level. The ASCO/CAP have determined a set of guidelines for validating ER, PR, and HER2 assays to be implemented in laboratories. In 2007, the ASCO/CAP developed a set of guidelines for validating HER2 outcomes followed in 2008 by guidelines for ER and PR [5]. Both organizations convened an expert panel of researchers to review literature and evidence to determine recommendations for laboratory best practices. Input from industry experts and individuals from the FDA were taken into consideration to develop a comprehensive approach to breast cancer subtype validation. These guidelines are not updated annually, so testing labs must take into account their specific patient population and any new evidence that may come to light due to ongoing research.

General considerations for ER and PR validation include limiting the time from tissue excision to fixation so as to preserve the integrity of the specimen and keeping slides stored for not more than six weeks before they are stained and evaluated. For an initial assay validation the lab must compare its results against an already validated procedure from a different reference lab according to ASCO/CAP standards. The validating lab must test ≥ 40 positive and ≥ 40 negative cases and demonstrate $\geq 90\%$ concordance for positive results and $\geq 95\%$ for negative results. A positive test for ER and PR is defined as having $\geq 1\%$ of reactive cells. Pathologists who will be visually

diagnosing samples must also have their skills validated and demonstrate ≤ 2 incorrect assessments based on the 40 sample sets [4].

For ongoing validation, labs must calculate their results and compare them to approved ASCO/CAP standards at least twice annually. Generally ER+ results should represent about 70-85% of cases, while PR+ results should represent about 60-75% of cases. If those rates are not met, cases should be reviewed against age and histologic parameters to identify potential outlier influence, e.g. a sample population consisting of mostly younger individuals who are more likely to present with TNBC. Additionally, if gene expression analyses is performed then concordance must be $\geq 95\%$ against the ER and PR IHC results [4].

Testing for HER2 follows the same considerations as ER and PR testing regarding tissue fixation time and slide storage. From here the validation protocols as outlined by the ASCO/CAP vary from the previously described methods. HER2 results are graded as 0-1+, 2+, or 3+. Negative results (0-1+) are indicated by no staining or very weak, incomplete staining present in the sample. Borderline results (2+) are indicated by complete membrane staining that is weak or non-uniform. Circumferential differentiation is present in at least 10% of cells but less than 30%. Positive results (3+) are indicated by intense uniform membrane staining in at least 30% of cells.

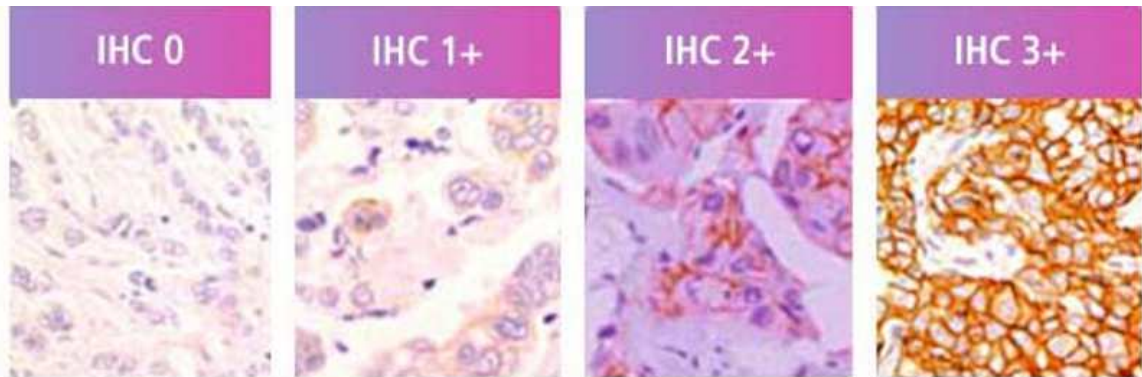


Figure 1: IHC HER2 results of increasing gradient.
(Image:her2support.org/pdf/her2testresults)

For borderline cases (2+), FISH is performed to determine if HER2 gene amplification (located on chromosome 17) is present so that a definitive diagnosis can be made. FISH technique utilizes colored probes which detect excessive amplification of a particular target gene. According to ASCO/CAP standards, the ratio of HER2 to CEP17 is evaluated. A positive FISH test for HER2 has a ratio > 2.2 , an equivocal test has a ratio of $1.8 - 2.2$, and a negative test has a ratio < 1.8 . Unlike ER and PR assays, HER2 outcomes are not binary but are instead on a gradient scale. Therefore, there is inherent variability in HER2 testing. These variations can be a result of different equipment used and how it is calibrated, differing fixation methods, materials used, and skill of staff. Labs conducting HER2 testing must evaluate their results two to four times per year in comparison to the results of a validated reference laboratory. ASCO/CAP requires a 95% concordance rate for lab assay validation protocols [20].

BMC uses Phenopath as a reference lab for IHC protocols. Phenopath is a pathologist run diagnostic lab located in Seattle, Washington which was recently acquired by Quest Diagnostics in September of 2018. They hold an accreditation from the College

of American Pathologists for a wide array of testing services which includes IHC and FISH. Although they must continuously monitor their lab functions to maintain accreditation, they are approved as a reference for comparison of testing results. This comparison is not blinded, however a detailed report of sample information is compiled so that retesting can be performed should statistically abnormal results present in the diagnostic lab.

BMC's original HER2 IHC assays were found to be lacking when compared with assays performed by PhenoPath as displayed in the images below:

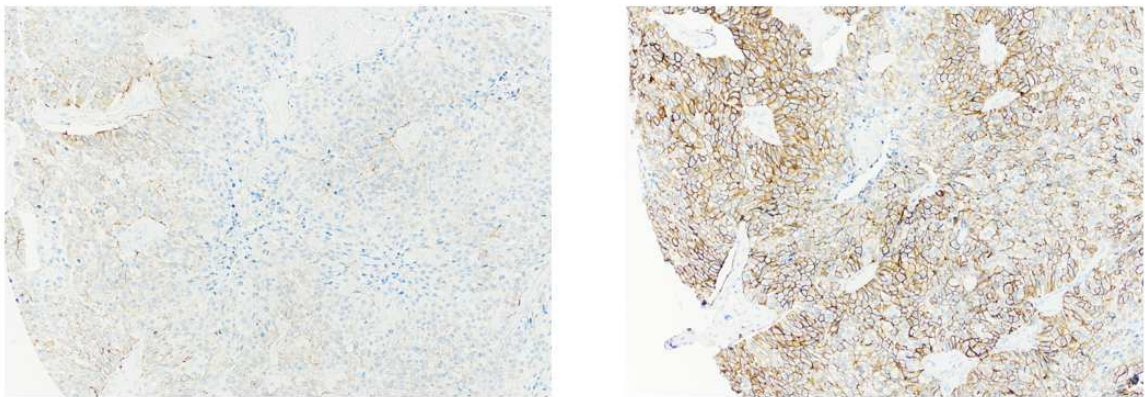


Figure 2: IHC assay for HER2 from BMC (left), score 1+ vs. PhenoPath (right), score 3+.

The image on the left is a breast cancer sample that was given a HER2 score of 1+, while the image on the right is the same sample with a score of 3+ from PhenoPath. A discrepancy like this could drastically change a patient's treatment regime and subsequent prognosis if left unchecked. This illustrates the importance of assay validation and led to a reevaluation of BMC's HER2 IHC protocol with the goal of better alignment to PhenoPath's results.

The pre-optimized HER2 assay results also showed a difference from the Atlanta population-based study performed by Lund, *et al.* published in 2010 as displayed in Table 1. BMC patient IHC results from the previous year were tallied and compared to the results of the Atlanta study. Fortunately, the ER+ and PR+ rates are aligned with those from the Atlanta study which indicates those BMC assays are on target. Ideally, the rate of HER2 positive patients would be about 15% and match the Atlanta results closely as the tested population is similarly broken down by race, namely African-American and Caucasian.

Table 1: ER, PR, and HER2 IHC data from BMC compared to Atlanta study.

	BMC	Atlanta Study (Lund, <i>et al</i>)
Caucasian	34.5%	46%
African-American	42.8%	48%
Unknown	19.3%	N/A
Other	3.4%	6%
ER positive	78%	73.9%
PR positive	67%	63.5%
HER2 3+	9%	10.7%
HER2 2+	5%	13.5%
HER2 1+	16.8%	15.4%
HER2 0	68.5%	58.3%
HER2 Positive	12%	14.4%

The BMC data as shown in Table 1 exhibits a consistently lower HER2 positive number as compared to the Atlanta study data, an area with similar demographics. This, in conjunction with the results as shown in Figure 2, prompted a review of the HER2 protocol with a goal of aligning subsequent results to the reference lab and study data outcomes.

PD-L1

An emerging therapeutic target currently being studied is PD-L1. This protein receptor, located on antigen presenting cells, binds to PD-1, a member of the CD28 family, which is expressed on activated T, B, and natural killer cells [21]. PD-1/PD-L1 has a negative co-stimulatory effect on both naïve T cells and cytotoxic T cells during an immune response [10]. In healthy individuals this response maintains immune homeostasis by shutting down activated T cells, thereby preventing cellular damage and potential autoimmune disease. This downregulation is also a critical factor in maintaining maternal tolerance of a fetus during pregnancy. The presence of this immune suppressor has been found in a variety of cancers, namely non-small cell lung cancer and melanoma. When present, PD-L1 is typically overexpressed in these cancers which shuts down the immune response prematurely and allows the tumor cells to evade destruction. This is achieved by inducing T-cell exhaustion, and increased PD-L1 expression is generally associated with a poor prognosis.

The Blueprint project has worked to identify the most effective clones for use in PD-L1 IHC. In the first Blueprint phase, non-small cell lung cancer specimens were used to test staining with four different clones: 22C3, 28-8, SP142, and SP263. Researchers found that three of the four clones showed consistent staining of tumor cells, whereas the remaining clone, SP142, consistently displayed fewer cells expressing PD-L1 by comparison. In the second phase of the study researchers stained 81 lung cancer samples with five different clones: 22C3, 28-8, SP142, SP263, and 73-10. The results mirrored the previous phase, with SP142 showing the least sensitivity and 73-10 showing the greatest

sensitivity. Clones 22C3, SP263 and 28-8 were deemed relatively interchangeable. Using this data, laboratories must determine which clone would be most appropriate for their situation, taking into account the type of cancer being tested, the brand of instruments used, and the cost of the clones themselves [19].

Using this background information, BMC aims to adopt an efficient and cost effective PD-L1 assay as well as optimize their breast cancer IHC protocols. For now, BMC will have to utilize resources from other medical institutions throughout Boston in order to implement a working PD-L1 protocol. PD-L1 inhibitors are a fairly new class of drugs that are being developed by various pharmaceutical companies. With a suitable PD-L1 assay in place, BMC will be better able to offer physicians the correct information for them to prescribe the most cutting edge treatment to those in need. In the meantime, the breast cancer IHC assays that are currently being used for patient diagnoses need to be continuously examined to ensure compliance and optimized results. Given the effectiveness of targeted treatment against ER+, PR+, and HER2+ breast cancers it is the responsibility of diagnostic laboratories to ensure their assays are able to identify those patients who would benefit most. This is especially true given the aggressive nature of HER2+ breast cancer. Patient quality of life and survival is the ultimate goal of assay optimization, and the impacts of which will eventually affect all of us in one way or another.

METHODS

ER

For ER validation, TMAs were created from 100 patient samples from 2014 – 2018. To create the TMAs a puncher was used to remove a circular section from each fixed tissue sample that was to be tested. That circular section was then transferred into one well of the TMA block. Each TMA block contains 12 total wells, with one well containing smooth muscle as a control. This left 11 samples per TMA, which resulted in 10 TMA blocks. Slides were cut from each TMA paraffin block to be prepped for IHC. BMC used automated protocol #976 on the Ventana Benchmark Ultra immunostainer with clone SP1. Epitope retrieval was performed using CC1 reagent, with a maximum incubation of 36 minutes at 95°C. Antigen targeting was performed using clone SP1. Slides were incubated for 8 minutes at 20°C. Linker is added and slide is incubated for 8 minutes, followed by DAB application and additional 8 minutes incubation. Finally, chromogen is added, and slide is incubated for 8 minutes. Linker, DAB, and chromogen incubation is performed at 20°C. Slides are then visually inspected. Tonsil and cervix tissue was used for positive controls, while other tonsil tissue was used for negative controls.

PR

For PR validation, TMA were created from 91 patient samples from 2014 – 2018 as previously described. Each TMA block contained 11 samples resulting in 9 TMA blocks. Slides were cut from the each TMA paraffin block to be prepped for IHC. BMC

used protocol #949 on the Ventana Benchmark Ultra immunostainer with clone 1E2. Epitope retrieval was performed using CC1 reagent, with a maximum incubation of 56 minutes at 95°C. Antigen targeting was performed using clone 1E2. Slides were incubated for 8 minutes at 37°C. Linker is added and slide is incubated for 8 minutes, followed by DAB application and additional 8 minutes incubation. Finally, chromogen is added, and slide is incubated for 8 minutes. Linker, DAB, and chromogen incubation is performed at 20°C. Slides are then visually inspected. Tonsil and cervix tissue was used for positive controls, while other tonsil tissue was used for negative controls.

HER2

For HER2 optimization, TMAs were created from 125 patient samples from 2014 – 2018 as previously described. Each TMA block contained 11 samples, resulting in 12 TMA blocks. Slides were cut from each TMA paraffin block to be prepped for IHC. BMC used protocol #1391 on the Ventana Benchmark Ultra immunostainer with clone 4B5. Previous protocols had epitope retrieval performed with CC1 for 8 minutes at 95°C. In an effort optimize results, multiple protocols were run with different incubation periods, both during the epitope retrieval and antibody binding steps.

Table 2: Experimental Incubation Times for HER2 IHC.

Epitope Retrieval Incubation at 95°C (minutes)	Antibody Incubation at 37°C (minutes)
8 (original protocol)	16 (original protocol)
20	12
36	8
36	12
52	12

Staining procedures remained the same for all variations tested. Hemotoxylin II was added and the slide was incubated for 12 minutes followed by Bluing reagent and an additional 12 minute incubation. Both staining steps were performed at 20°C. TMAs from resected breast carcinomas were used as controls.

Data Collection

After validation protocols were run patient data was obtained from CoPath, BMC’s internal records database. Race and subtype results were collected and organized into a spreadsheet for subsequent evaluation. This will continue to serve as a prospective database for all incoming test results to ensure breast cancer IHC assays remain valid.

PDL1

Preliminary IHC testing was performed on tonsil tissue with the Ventana Benchmark Ultra immunostainer using clone E1L3N. Multiple incubation times of epitope retrieval were performed to determine which would yield optimal results as displayed in Table 3. The incubation time for antibody binding was 12 minutes at 36°C for all samples tested. Staining procedures were the same for all, with two DAB application steps incubating for 8 minutes at 37°C each.

Table 3: Epitope Retrieval Times for PD-L1 IHC.

Epitope Retrieval Incubation at 100°C (minutes)
24
32
40
48
56

RESULTS

ER

BMC compares its results to PhenoPath. Out of 50 known positive cases tested, all 50 were found positive. Out of 10 known weak positive cases tested, all 10 were found to be weak positive. Out of 40 known negative cases tested, all 40 were found to be negative. Therefore, the BMC protocol returned 100% correlation with PhenoPath's accuracy on all cases tested. These results satisfy the re-validation requirement as set forth by ASCO/CAP.

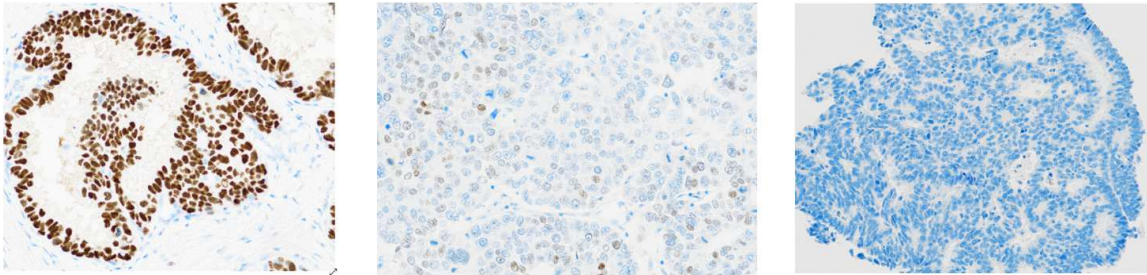


Figure 3: ER IHC Results – positive, weak positive, and negative (from left to right).

Figure 3 shows examples of the three different IHC results from the ER protocol. The image on the left shows distinct, dark staining which indicates a positive sample. The middle image shows inconsistent, light-colored staining indicating a weak positive sample. The image on the right shows no staining at all, which indicates a negative sample.

PR

Results from BMC's PR assay were compared to PhenoPath. Out of 41 known positive cases tested, all 41 were found positive. Out of 10 known weak positive cases

tested, all 10 were found to be weak positive. Out of 40 known negative cases tested, all 40 were found to be negative. Therefore, the BMC protocol returned 100% correlation with PhenoPath's accuracy on all cases tested. These results satisfy the re-validation requirement as set forth by ASCO/CAP.

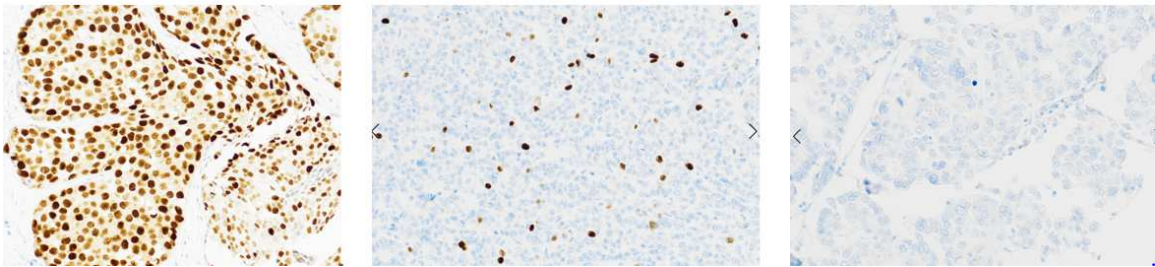


Figure 4: PR IHC Results – positive, weak positive, and negative (from left to right).

Figure 4 shows examples of the three different IHC results from the PR protocol, which are very similar to the ER results as shown in Figure 3. The left image shows dark, consistent staining indicating a positive sample. The middle image shows sparse staining which indicates a weak positive sample. The right most image shows no staining, which indicates the sample is negative.

HER2

Figure 5 shows the results of the various incubation times tested. The 8ep/16ab and 20ep/12ab protocols were too weak and did not match the PhenoPath result as shown in the bottom right image. Use of these protocols would result in positive samples not being detected. Conversely, the 36ep/12ab and 52ep/12ab yielded results that were too strong as there is more prominent staining compared to the PhenoPath control. If used in practice, these protocols would result in too many false positives. It was therefore noted that the 36ep/8ab incubation configuration (upper right image) yielded results most

closely resembling that of PhenoPath, and was subsequently deemed the optimal protocol.

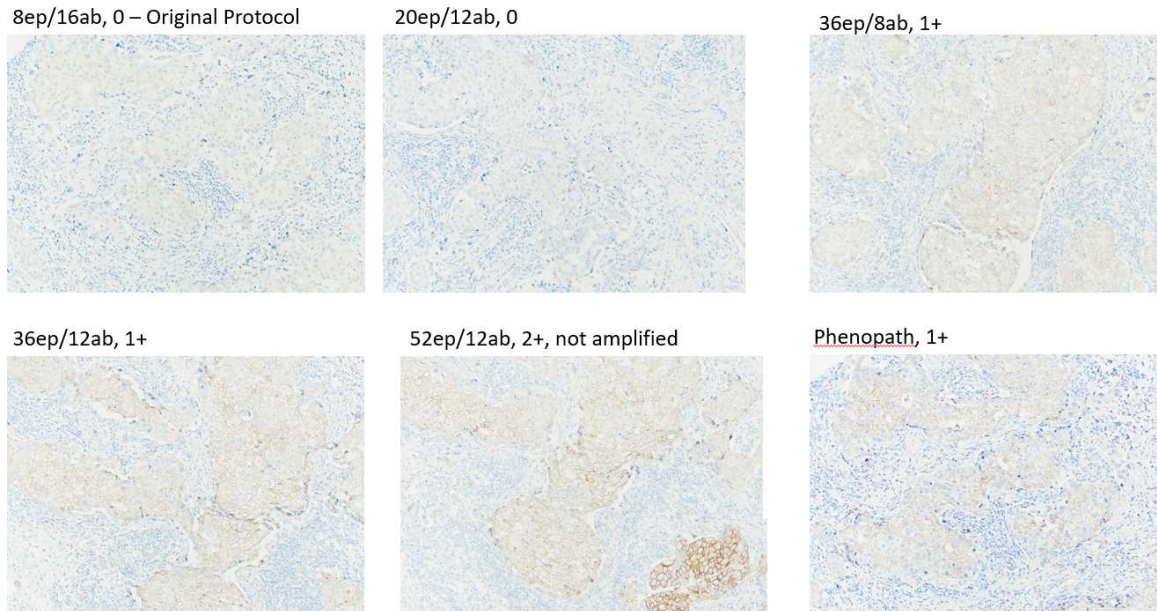


Figure 5: HER2 IHC results from various incubation times. (Ep = epitope retrieval time; ab = antibody binding time).

Using the optimized protocol, there were 33 positive (3+) cases from the IHC protocol which was verified by FISH. This demonstrates 100% concordance of positive screening tests. There were 30 known negative cases, 29 of which were verified by FISH as being non-amplified. This demonstrates 96.7% concordance of negative screening tests. Therefore, overall concordance for all HER2 was 98.4%. This satisfies the validation requirement as set forth by ASCO/CAP, and demonstrates an optimized protocol.

The images below show the increase in staining as a result of the optimized protocol. The left image, from BMC, shows similar staining patterns as the PhenoPath stain on the right. This sample is now rightly classified as HER2 3+.

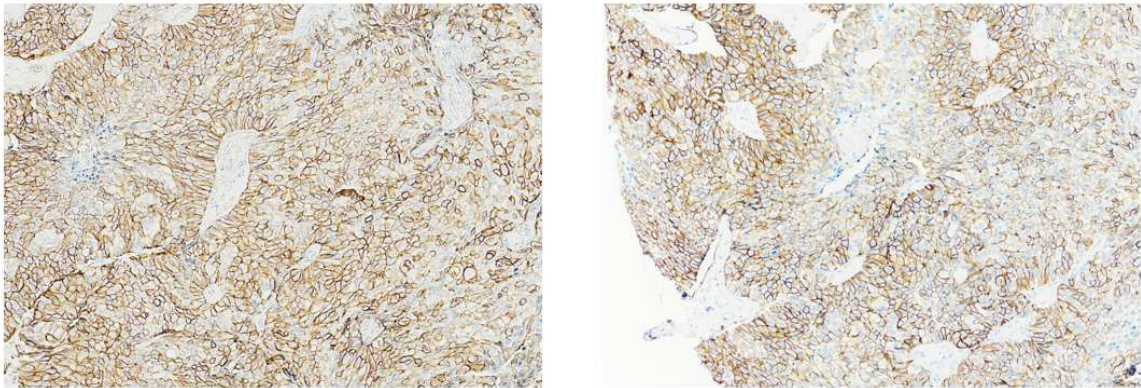


Figure 6: IHC assay for HER2 from BMC’s new protocol (left), score 3+ vs. PhenoPath (right), score 3+.

This new optimized protocol was put into practice starting December 19, 2018. Since that day, breast cancer subtype data has been recorded and organized according to race. To date, 53 total patient cases have been examined which have yielded HER2 scoring results as displayed in Table 2.

Table 4: HER2 scoring broken down by race.

HER2 Score	African-American	Caucasian	Asian/Pacific Islander	Unknown
HER2 negative (score 0 or 1)	15	9	2	5
HER2 2+/FISH Negative	5	1	1	6
HER2 2+/FISH Positive	1	1	0	0
HER2 3+	4	1	1	1

There have also been four triple-negative cases found since the implementation of the new protocol. Two are African-American, one is Caucasian, and one is of unknown race.

The data collected so far shows a HER2 3+ rate of about 13%. This is an improvement over the previous rate of 9%, however data collection will need to be continued throughout the year in order to obtain a complete picture of how well the assay is performing.

PDL1

The incubation configuration of 48 minutes for epitope retrieval and 12 minutes of antibody binding yielded the desired results as shown in Figure 6. There is strong staining in the squamous cells on the left side of the image indicating PD-L1 overexpression and weak staining in the macrophages on the right side of the image. More tests will need to be conducted with different tissue types, namely lung and melanoma, however this is a promising first step in BMC's objective to run a PD-L1 IHC protocol on site.

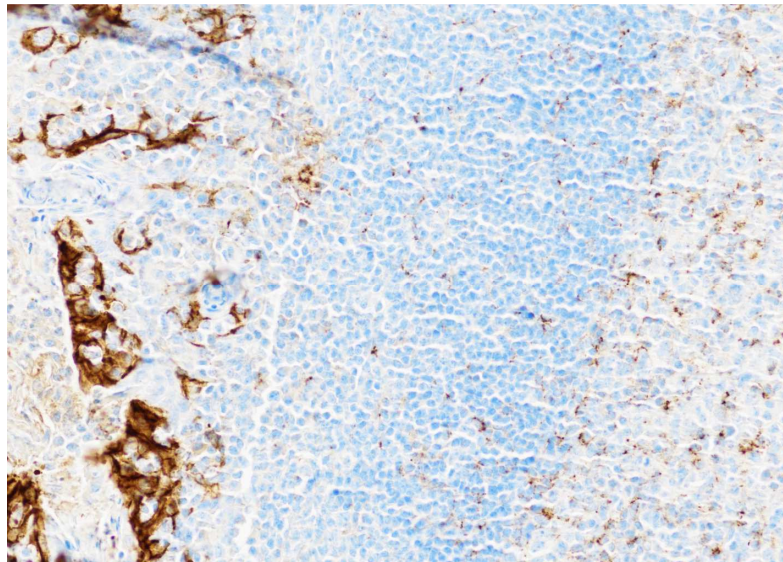


Figure 7: PD-L1 staining of tonsil tissue.

DISCUSSION

Lab assay validation is a pivotal component of patient care as it informs healthcare professionals how best to treat their patients. Immunohistochemistry remains an efficient and accurate way to identify molecular targets for precise treatment. It has the capacity to test for a number of molecular entities simply by changing the antibody clone and adjusting for optimal incubation time and temperature. This adaptability makes IHC a potent tool which can be adapted to assess an array of maladies. With validated IHC protocols, pathology labs can diagnose patients quickly so that proper treatment can be administered.

PD-L1 remains a promising therapeutic target with multiple pharmaceutical companies working on development of applicable medicines. BMC's preliminary IHC testing on PD-L1 shows promise, however more assays need to be run in order to implement a working protocol for patient samples. Next steps will include comparison of reference samples performed by PhenoPath on melanoma and non-small cell lung cancer tissues against tests run at BMC. Once BMC is able to demonstrate consistent proficiency with the optimal PD-L1 protocol and sufficient concordance according to ASCO/CAP guidelines, the lab will be able to implement the procedure as part of their diagnostic program. Patient data will need to be continuously collected and analyzed to ensure ongoing veracity of results.

The ER and PR protocols have maintained their validated status as demonstrated by the high level of concordance with the known reference lab samples, so there were no changes needed. The newly optimized HER2 IHC protocol has so far demonstrated better

results than previous protocols. This was achieved by testing various incubation configurations to find the result that most closely resembled that of PhenoPath. By increasing the epitope retrieval incubation time from 8 minutes to 36 minutes during the epitope retrieval step, more antigen was present for antibody binding, which in turn yielded a stronger stain than previously shown in other BMC results. Conversely, it was also important to make sure the stain was not too strong which would result in false positives. This was achieved by reducing the antibody binding incubation time from 16 minutes to 8 minutes. This toggling of incubation times highlights the nuanced nature of HER2 IHC. Unlike the binary outcomes of ER and PR (i.e. positive and negative), HER2 IHC can be open to interpretation, most notably if the sample shows a borderline result (score 2+). This, coupled with the possible variations of different pathologists' interpretations, underlines the gravity of having accurate, validated IHC protocols in order to eliminate erroneous testing outcomes.

Altering the incubations times is a relatively small change which has aligned BMC to ASCO/CAP guidelines and will provide physicians the correct information to care for their patients. It is no more work intensive than previous protocols, which will help maintain short turnaround time in the lab, about 24 hours from surgery. Since the new protocol was only implemented three months ago, continued data analysis will need to be conducted in order to maintain sufficient assay potency. This involves periodically comparing HER2 IHC results to PhenoPath as well as keeping a log of HER2 results as found in the BMC patient population. Since implementation there have only been 53 patients tested for HER2. Right now, the breakdown is generally in line with expected

outcomes, however more data points need to be collected in order to gain a complete picture of testing accuracy.

Cancer affects everyone, either directly or indirectly, and it is imperative that pathology departments are equipped to properly diagnose affected individuals. These lab assays are relied upon to give accurate results so that targeted therapy can be prescribed to those who would benefit. Precision medicine has gained a lot of attention recently as it promises to streamline therapy and improve survival. Traditional chemotherapy can be effective, but it is plagued with unpleasant side effects and a diminished quality of life for those undergoing treatment. Given the prevalence of cancer, it is in society's best interest for laboratories to maintain optimized standards for diagnostic assays and for scientists to remain committed to advancing targeted clinical therapies for continued patient survival.

REFERENCES

1. Abbas, A., Lichtman, P., and Pillai S. *Basic Immunology*. St. Louis: Elsevier, 2016 5th edition.
2. Centers for Disease Control and Prevention. United States Cancer Statistics: Data Visualizations. (2015) <https://gis.cdc.gov/Cancer/USCS/DataViz.html>
3. Childs G.V. (2014) History of Immunohistochemistry. In: Linda M. McManus, Richard N. Mitchell, editors. *Pathobiology of Human Disease*. San Diego: Elsevier; p. 3775-3796.
4. Fitzgibbons, Patrick, et al. (2010). Recommendations for Validating Estrogen and Progesterone Receptor Immunohistochemistry Assays. *Archives Pathology & Laboratory Medicine*. Vol.134, pp.930-935.
5. Hammond, M. Elizabeth, et al. (2010). American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer. *Archives of Pathology & Laboratory Medicine*. Vol.134, pp. 907-1101.
6. Hirsch, F., et al. (2017). PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project. *Journal of Thoracic Oncology*. Vol12(2), pp. 208-222.
7. Howlader, N., Altekruse, S., Li, C., Chen, V., Clarke, C., Ries, L., & Cronin, K. (2014). US Incidence of Breast Cancer Subtypes Defined by Joint Hormone Receptor and HER2 Status. *Journal of the National Cancer Institute*, Vol.106(5).
8. Ismail-Khan, R. and Bui, M. (2010). A Review of Triple-Negative Breast Cancer. *Cancer Control*, Vol.17(3), pp. 173-176.
9. Kumar, P., Bhattacharya, P., & Prabhakar, B. (2018). A comprehensive review on the role of co-signaling receptors and Treg homeostasis in autoimmunity and tumor immunity. *Journal of Autoimmunity*, Vol.95, pp.77-99.
10. Liechtenstein, T., et al. (2012). PD-L1/PD-1 Co-Stimulation, a Brake for T cell Activation and a T cell Differentiation Signal. *Journal of Clinical and Cellular Immunology*, Supp.12: doi:10.4172/2155-9899.S12-006.
11. Lund, M.J., Butler, E., Hair, B., Ward, K., Andrews, J., Oprea-Ilie, G., Bayakly, A., O'Regan, R., Vertino, P., & Eley, J. (2010). Age/Race Differences in HER2 Testing and in Incidence Rates for Breast Cancer Triple Subtypes. *Cancer*, Vol.116(11),pp. 2549-2559.

12. Luongo de Matos, L., Trufelli, C., Luongo de Mato, M., and Pinhal, M. (2010). Immunohistochemistry as an Important Tool in Biomarkers Detection and Clinical Practice. *Biomark Insights*, Vol.5, pp. 9-20.
13. Munzone, Elisabetta. (2017). Anti-HER2 Therapies in the Adjuvant and Advanced Disease Settings. *Breast Cancer Innovations in Research and Management*. Switzerland. Springer International Publishing, Chapter 47.
14. National Cancer Institute: Surveillance, Epidemiology, and End Results Program. Cancer Stat Facts: Female Breast Cancer.
<https://seer.cancer.gov/statfacts/html/breast.htmlv>
15. National Cancer Institute. Trastuzumab after Chemotherapy is Effective in HER2-Positive Breast Cancer. (2011)
<https://www.cancer.gov/types/breast/research/trastuzumab-after-chemo>
16. Reisenbichler, E., Lester, S., Richardson, A., Dillon, D., Ly, A., & Brock, J. (2013). Interobserver Concordance in Implementing the 2010 ASCO/CAP Recommendations for Reporting ER in Breast Carcinomas: A Demonstration of the Difficulties of Consistently Reporting Low Levels of ER Expression by Manual Quantification. *American Journal of Clinical Pathology*, Vol.140(4), pp.487-494.
17. Stocker, A., Hilbers, M., Gauthier, C., Grogg, J., Kullak-Ublick, G., Seifert, B., Varga, Z, and Trojan, A. (2016). HER2/CEP17 Ratios and Clinical Outcome in HER2-Positive Early Breast Cancer Undergoing Trastuzumab-Containing Therapy. *PLOS One*, Vol.11(7):e0159176. Doi:10.1371/journal.pone.0159176.
18. Suijkerbuijk, KPM., van der Wall, E., and van Diest, PJ. (2016). Hormone Receptors in Breast Cancer. *Molecular Pathology of Breast Cancer*. Switzerland. Springer International Publishing, Chapter 4.
19. Tsao, M. et al. (2018). PD-L1 Immunohistochemistry Comparability Study in Real-Life Clinical Samples: Results of Blueprint Phase 2 Project. *Journal of Thoracic Oncology*. Vol.13(9), pp. 1302-1311.
20. Wolf, Antonio, et al. (2007). American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. *Archives of Pathology & Laboratory Medicine*. Vol.131, pp. 18-43.
21. Zhang, M., Li, G., Wang, Y. Wang, Y., Zhao, S., Haihong, P., Zhao, H., and Wang, Y. (2017). PD-L1 expression in lung cancer and its correlation with driver mutations: a meta-analysis. *Nature: Scientific Reports*. Vol.7(10255) doi:10.1038/s41598-017-10925-7.

22. Zhang, Y., Tian, M., Tang, M., Liu, Z., and Liao, A. (2015). Recent Insight into the Role of the PD-1/PD-L1 Pathway in Feto-Maternal Tolerance and Pregnancy. *American Journal of Reproductive Immunology*. Vol.74(3), pp. 201-208.

CURRICULUM VITAE

