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Synthetic Antigen Controls for Immunohistochemistry

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TITLE:

Synthetic Antigen Controls for Immunohistochemistry

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SUMMARY:

This work documents a simple method to create synthetic antigen controls for immunohistochemistry. The technique is adaptable to a variety of antigens in a wide range of concentrations. The samples provide a reference with which to assess intra- and inter-assay performance and reproducibility.

ABSTRACT:

Immunohistochemistry (IHC) assays provide valuable insights into protein expression patterns, the reliable interpretation of which requires well-characterized positive and negative control samples. Because appropriate tissue or cell line controls are not always available, a simple method to create synthetic IHC controls may be beneficial. Such a method is described here. It is adaptable to various antigen types, including proteins, peptides, or oligonucleotides, in a wide range of concentrations. This protocol explains the steps necessary to create synthetic antigen controls, using as an example a peptide from the human erythroblastic oncogene B2 (ERBB2/HER2) intracellular domain (ICD) recognized by a variety of diagnostically relevant antibodies. Serial dilutions of the HER2 ICD peptide in bovine serum albumin (BSA) solution are mixed with formaldehyde and heated for 10 min at 85 °C to solidify and cross-link the peptide/BSA mixture. The resulting gel can be processed, sectioned, and stained like a tissue, yielding a series of samples of known antigen concentrations spanning a wide range of staining intensities.

This simple protocol is consistent with routine histology lab procedures. The method requires only that the user have a sufficient quantity of the desired antigen. Recombinant proteins, protein domains, or linear peptides that encode relevant epitopes may be synthesized locally or commercially. Laboratories generating in-house antibodies can reserve aliquots of the immunizing antigen as the synthetic control target. The opportunity to create well-defined positive controls across a wide range of concentrations allows users to assess intra- and inter-laboratory assay performance, gain insight into the dynamic range and linearity of their assays, and optimize assay conditions for their particular experimental goals.

INTRODUCTION:

Immunohistochemistry (IHC) allows the sensitive and specific, spatially precise detection of target antigens in formalin-fixed, paraffin-embedded (FFPE) tissue sections. However, IHC staining results may be affected by multiple variables, including warm and cold ischemia time, tissue fixation, tissue pretreatment, antibody reactivity and concentration, assay detection chemistry, and reaction times[1]. Accordingly, reproducible performance and interpretation of IHC reactions require rigorous control of these variables and the use of well-characterized positive and negative control samples. Frequently used controls include paraffin-embedded tissues or cultured cell lines known from independent analyses to express the antigen of interest, but such samples are not always available[1]. Furthermore, the expression levels of the target antigens in tissues and cell line controls are generally understood only qualitatively and may be variable. Controls containing reproducible, precisely known concentrations of target antigen can assist in the optimization of IHC reaction conditions. A general method, adaptable to a variety of antigen types in a physiologically relevant range of concentrations to create synthetic IHC control samples, has been described by the authors[2]. A detailed protocol is provided here for the creation and use of this type of standard.

Appropriate controls are essential for the valid interpretation of IHC assays[1,3,4]. Tissues, cultured cells, and peptide-coated substrates have been employed as IHC controls according to the investigators' specific needs. The advantages and limitations inherent in using tissues as IHC controls have been extensively discussed[1,4]. For many antibodies, appropriate controls can be chosen from selected normal tissues containing cell populations expressing the target antigen over a wide dynamic range. Tissue controls are less suitable when the target antigen is not well-characterized concerning expression site or abundance or when potentially cross-reacting antigens are co-expressed in the same cells or tissue sites. In these contexts, blocks of cultured cell lines expressing the antigen of interest can be helpful. For providing further evidence of target specificity, cell lines can be engineered to over-or under-express target antigens. For example, such an approach was recently used to evaluate a variety of anti-PD-L1 assays using a tissue microarray of isogenic cell lines expressing a range of PD-L1 antigen[5]. Practical limitations to the routine use of cell line blocks include the cost and time needed to produce sufficient cell numbers and the fact that the expression of some antigens may not be reliably consistent, even within clonal cell lines[6]. Synthetic peptides are a third option for IHC controls for antibodies that recognize short linear epitopes. Steven Bogen and colleagues have published extensively on the use of peptides coupled to the surface of glass slides[7,8] and glass beads[9]. One study by this group demonstrated that quantitative analysis of peptide-based IHC controls could detect staining

process variation missed by qualitative evaluation of tissue controls analyzed in parallel[10]. While standards using bead-based antigens could be widely applicable, many details are proprietary to the authors, limiting widespread adoption.

Another approach to IHC standards incorporates target antigens into artificially created protein gels. This concept was first demonstrated by Per Brandtzaeg in 1972 in a study in which normal rabbit serum was polymerized using glutaraldehyde[11]. Small blocks of the resulting gel were then soaked for 1–4 weeks in solutions containing the immunoglobulin antigens of interest at various concentrations. After alcohol fixation and paraffin embedding, sections of the resulting controls were shown to stain with intensities corresponding to the logarithm of the antigen solutions in which they had been soaked. Later, investigators prepared glutaraldehyde conjugates of specific amino acids in dilute BSA or brain homogenate solutions as positive controls in immune-electron microscopy studies[12,13]. More recent work investigated the use of gels made from formaldehyde-fixed protein solutions as surrogates for FFPE tissue in mass spectrometry analysis[14]. Another recent work investigated the structure and properties of gels formed by heating human or bovine serum albumin solutions at various concentrations and pH[15]. These authors found that serum albumin forms three types of gels differing in mechanical elasticity, secondary structure preservation, and fatty acid-binding capability depending on the experimental conditions. Together, these papers demonstrate the general feasibility of the approach employed here. Protein solutions of defined composition create tissue-like gels that can be further processed, sectioned, and stained using routine histological methods.

This protocol describes the formation of a synthetic IHC control made from bovine serum albumin (BSA) polymerized with heat and formaldehyde. The gels can incorporate a wide variety of antigens, including full-length proteins, protein domains, and linear peptides, as well as non-protein antigens, including oligonucleotides[2]. This demonstration uses an example antigen, a linear peptide encoding the C-terminal 16 amino acids of the human ERBB2 (HER2/neu) protein TPTAENPEYLGLDVPV-COOH (see **Table of Materials**). This sequence includes the epitopes recognized by three commercially available, diagnostically relevant antibodies, including the Herceptest polyclonal reagent (ENPEYLGLDVP) and the monoclonal antibodies CB11 (AENPEYL) and 4B5 (TAENPEYLGL) (see **Table of Materials**)[16].

The method demonstrated here employs readily available reagents using processes and techniques familiar to any practicing histology laboratory. The most significant limitation is the need to identify and purchase the target antigens, which can be accomplished in many cases at a relatively modest cost. Because these synthetic controls are of the wholly defined composition and made with simple methods, they can be implemented in many laboratories with reproducible results. Their use may facilitate the objective, quantifiable evaluation of IHC staining results and allow greater intra- and inter-laboratory reproducibility.

PROTOCOL:

1. Preparation of stock solution and tools

1.1. Prepare 20 mL of a 25% w/v BSA solution by mixing 5 g BSA powder in 14 mL of PBS, pH 7.2 in a 50 mL conical tube until evenly dispersed. Vortex as necessary to disperse the BSA powder.

1.1.1. Keep the solution overnight at 4 °C to allow complete dissolution. Adjust the final volume to 20 mL with PBS to make a 25% w/v stock solution.

1.2. Prepare 20 mL of a 31.3% w/v BSA solution by mixing 6.26 g BSA powder in 13 mL of PBS, pH 7.2 in a 50 mL conical tube until evenly dispersed. Keep the solution overnight at 4 °C to allow complete dissolution. Adjust the final volume to 20 mL with PBS to make a 31.3% w/v stock solution.

1.3. Preheat a heat block to 85 °C.

NOTE: The protocol below creates peptide/BSA gels with volumes of 1.26–1.4 mL formed in 1.5 mL microcentrifuge tubes. To use smaller volumes, for example, when antigen stocks are limiting, prepare the gels in PCR tubes and use a thermocycler set to 85 °C as a heat block.

1.4. Test that the BSA/formaldehyde mixture forms a gel as expected by mixing 700 µL of 25% BSA solution with 700 µL of 37% formaldehyde. Mix well by pipetting up and down 5 times within 5–10 s. Avoid creating air bubbles.

CAUTION: Concentrated formaldehyde is toxic; use with appropriate safety precautions.

1.5. Immediately after mixing the BSA and formaldehyde solutions, place the closed microcentrifuge tube in a heat block at 85 °C for 10 min. Remove the tube from the heat block and allow it to cool. Confirm that the gel has formed as expected.

2. Preparation and dilution of peptides

2.1. Obtain 5–20 mg of the lyophilized peptide of the desired sequence.

NOTE: The C-terminal 16 amino acids of the human ERBB2 intracellular domain recognized by 4B5 is TPTAENPEYLGLDVPV-COOH.

2.1.1. Add 4 amino acids to the N-terminus, acetyl-YGSG, and C-terminus, GSGC-amide to facilitate cross-linking of the peptide to BSA and provide spacing between the BSA molecule and the peptide epitope.

NOTE: The complete sequence is: acetyl-YGSGTPTAENPEYLGLDVPVGSGC-amide.

2.1.2. If desired, use other N- and C-terminal amino acid sequences to extend the core peptide epitope.

NOTE: The impact of different sequences varies with different antibody/epitope combinations. The addition of the C-terminal peptide reduces the binding of some antibodies to C-terminal epitopes. In such cases, omit this sequence.

2.1.3. Confirm that peptides from commercial sources are supplied at >95% purity, the composition of which is confirmed by HPLC and mass spectrometry analysis.

2.2. Calculate the necessary volumes for the 5x (1.25 E-2 M) peptide stock solutions. Referring to **Table 1**, columns C–E, enter values for the antigen molecular weight (g/mole), percent antigen purity (0–100), and antigen mass (mg).

NOTE: The volume of solvent (in µL) to resuspend the sample to achieve a stock solution of 1.25 E-2 M is 800 x antigen molecular weight x percent antigen purity/antigen mass.

2.2.1 Prepare and clearly label eight 1.5 mL microcentrifuge tubes.

NOTE: The tubes will contain 5x peptide stock in a solvent, 1x peptide stock in a solvent, five 10x serial dilutions of 2.5 E-4 M to 2.5 E-8 M peptide/BSA/formaldehyde gel, and a negative control gel containing BSA/formaldehyde lacking added antigen. All gel samples look identical. When preparing multiple sets of peptide dilutions at one time, take care to label and identify all tubes and processing cassettes correctly. Use color-coded microcentrifuge tubes and processing cassettes where possible to minimize misidentification.

2.3. Prepare a 5x peptide stock solution at 1.25 E-2 M by resuspending the entire mass of lyophilized peptide (20 mg for the ERBB2 peptides) in 60 µL of the appropriate solvent.

NOTE: In this example, dimethylformamide (DMF) was added directly to the vendor's container.

2.3.1. Inspect the solution to ensure that the peptide is completely dissolved. If necessary, add additional solvent and/or sonicate the sample until the peptide is completely dissolved, taking care not to exceed the volume calculated in **Table 1** for the 5x peptide stock.

CAUTION: DMF is toxic; use with appropriate precaution.

NOTE: Depending on the amino acid sequence, and the corresponding hydrophobicity and charge, peptides may be soluble in DMF, dimethyl sulfoxide (DMSO), pure water, or dilute solutions of acetic acid, formic acid, or ammonium bicarbonate. Peptide characteristics may be calculated using a variety of online tools[17]. Some peptide vendors may suggest solvents appropriate for specific sequences.

2.3.1. Add solvent as necessary to bring the volume of the 5x peptide stock to the final volume calculated in **Table 1**. Vortex for 5 s and centrifuge at room temperature at 5000 x *g* for 5 s. Peptide stock solutions can be stored at -80 °C.

2.4. Referring to **Table 2**, Column C, prepare 150 µL of 1x peptide stock solution (2.5 E-3 M) by diluting 30 µL of the 5x peptide stock into 120 µL of solvent (DMF this example). Vortex for 5 s and centrifuge at room temperature at 5000 x *g* for 5 s.

2.5. Referring to **Table 2**, Column D, prepare 700 µL of 5 E-4 M peptide/BSA solution (Dilution 1) by diluting 140 µL of 1x peptide stock into 560 µL of 31.3% BSA/PBS, pH 7.2. Vortex for 5 s and centrifuge at room temperature at 5000 x *g* for 5 s.

NOTE: The final BSA concentration of this solution is 25% (w/v).

2.6. Referring to **Table 2**, Columns E-H, prepare four successive 10x serial dilutions of the 5 E-4 M peptide/BSA stock by adding 70 µL of peptide/BSA solution to 630 µL of 25% BSA/PBS, pH 7.2. Vortex for 5 s and centrifuge at room temperature at 5000 x *g* for 5 s.

NOTE: After this step, there will be five 10-fold serial dilutions of peptide (5 E-4 M to 5 E-8 M) in 25% BSA/PBS, pH 7.2. The first four samples will contain 630 µL. The last sample will contain 700 µL.

2.7. Referring to **Table 2**, Column I, prepare a negative control BSA sample containing 700 µL of 25% BSA/PBS, pH 7.2 (**Figure 1A**).

3. Preparing BSA-peptide gels

3.1. Confirm that the heat block or thermocycler is stable at 85 °C.

3.2. Refer to **Table 3**, Columns B–E. Working one sample at a time, add to the first 25% BSA/peptide sample (Dilution 1) 630 µL of 37% formaldehyde. Mix well by pipetting up and down 5 times within 5–10 s. Avoid creating air bubbles.

CAUTION: Concentrated formaldehyde is toxic; use with appropriate safety precautions.

3.2.1. After mixing the peptide/BSA and formaldehyde solutions, place the closed microcentrifuge tube in a heat block at 85 °C for 10 min.

NOTE: Mix the BSA-peptide solution and formaldehyde thoroughly, but do not spend more than 10 s pipetting the mixture before placing the sample on heat. Since formaldehyde cross-linking begins immediately, the gel may form differently if the procedure is varied for different samples. The final BSA concentration in these gels is 12.5% (w/v). Final BSA concentrations less than 10% may yield gels that do not solidify; final BSA concentrations greater than 16% may produce gels more brittle and difficult to section after processing.

3.2.2. Repeat steps 3.2 and 3.2.1 for each of the dilutions 2–4.

3.2.3. Repeat steps 3.2 and 3.2.1 for dilution 5, but add 700 µL of 37% formaldehyde, a volume

equal to the 700 µL of BSA-antigen solution.

3.2.4. Refer to **Table 3** column Column G; repeat steps 3.2 and 3.2.1 for the negative control sample, adding 700 µL of 37% formaldehyde, a volume equal to the 700 µL of negative control BSA solution.

3.3. Remove the tubes from the heat block after 10–12 min. The heating time should be as consistent as possible for each sample. Allow the gels to cool on the benchtop for 5–10 min (**Figure 1B**).

3.4. Using a clean, flexible disposable laboratory spatula, remove the gel sample in one piece from the microcentrifuge tube, and place it in a sealed container containing at least 15 mL of neutral buffered formalin (NBF), using a separate container of NBF for each sample.

3.4.1. Alternatively, cut off the bottom of the microcentrifuge tube with a new single edge razor blade, and push the gel out from the bottom with air or a suitable probe (**Figure 1C–G**).

NOTE: The solidified formaldehyde/BSA gels can remain in the microcentrifuge tubes at room temperature for up to 24 h. Leaving the gels in the microcentrifuge tube for more than 24 h can cause them to become brittle and more difficult to process and section.

4. Trimming, processing, and embedding BSA gels

4.1. Trim the gel cone into cylindrical discs approximately 5 mm thick using a clean single edge razor (**Figure 1H,I**). Wrap the discs in a biopsy wrap, placing one larger gel disc into one cassette (to be used in the pilot study in step 5), and the remaining gel discs together into a second cassette (**Figure 2A,E**) for use in tissue microarray (TMA) construction in step 6. Place the wrapped gel discs in clearly labeled tissue processing cassettes.

4.1.1. Place the cassetted gels in at least 15 mL of 10% NBF per gel sample before processing, using a separate container of NBF for each sample. Gels can remain in 10% NBF for 6–48 h.

4.2. Process the gels in an automated histology tissue processor, following a large tissue schedule with pressure and vacuum. Each step takes 1 h: 10% NBF, 70% ethanol, 95% ethanol (repeat two times), 100% ethanol (repeat two times), xylenes (repeat three times), paraffin at 60 °C (repeat three times).

NOTE: For investigators choosing to process samples manually, follow the same sequence of reagents and times.

4.3. When the sample processing is completed, remove the cassettes from the tissue processor and move them to the paraffin embedding center.

4.4. Unwrap gels from the biopsy wrap and embed the gels in paraffin. For each sample, embed one disk of gel in a small 15 mm x 15 mm mold (**Figure 2B–D**), and the remaining gel discs together in a second larger mold (**Figure 2F–H**). The first block with one sample will be used to test the peptide gel in a pilot study. The second block can be used for TMA construction.

5. Pilot evaluation of the peptide dilution series

5.1. For each peptide dilution series, plan to create two glass slides containing a total of 6 separate sections: one section from each of the five dilution series samples, plus one section from the BSA-only negative control sample.

5.1.1. Onto the first glass slide, cut one 4 µm thick section from each of the smaller blocks containing one gel disc with the three highest peptide concentrations (2.5 E-4 M to 2.5 E-6 M).

5.1.2. Onto a second slide, cut one 4 µm thick section from each block with the two lowest peptide concentrations (2.5 E-7 M and 2.5 E-8 M) and one section from the BSA-only control block. Record the order of the samples on the slides.

NOTE: Expect the paraffin-embedded gels to cut smoothly, producing uniform sections without fragmentation, tearing, or chattering artifact. If particular paraffin-embedded gel samples are difficult to section, briefly soak the block face in ice-cold distilled water before sectioning. If necessary, experiment with different soaking times or with different solutions (e.g., ammonia water).

5.1.3. After sectioning, dry the slides at room temperature (about 23 °C) for 24 h followed by 60 °C for 30 min.

5.2. Stain the two slides prepared for each peptide with the desired antibody according to standard IHC protocols.

NOTE: The primary antibody on-slide concentration used for rabbit monoclonal 4B5 in this demonstration was 1.5 ug/mL.

5.2.1. Expect to see a relatively uniform signal within each gel section, with the different gel samples showing a range of signal intensity corresponding to the peptide dilutions.

5.3. If the results for the pilot study are satisfactory, construct a TMA from the gel donor blocks containing different concentrations of peptide antigen, as described in the next steps of the protocol.

6. BSA gel TMA construction

6.1. Construct a tissue microarray containing duplicate 1 mm diameter cores from donor blocks containing BSA gel alone and BSA gels containing all five dilutions of ERBB2 peptide.

NOTE: If desired, include BSA gels containing the same five dilutions of a non-target peptide as additional negative controls. If desired, include cores of representative ERBB2-expressing cell lines as positive controls.

6.2. Cut 4 µm thick sections of the TMA and stain with 1.5 ug/mL of anti-ERBB2/HER2/neu rabbit monoclonal 4B5 (see **Table of Materials**) according to laboratory-standard protocols.

6.3. Assess the resulting stain intensity qualitatively by inspection or quantitatively by digital image scanning and analysis (**Figure 3A,B**).

NOTE: As digital image analysis is not the focus of this protocol, these steps are left for the reader to perform according to their preference.

REPRESENTATIVE RESULTS:

Peptides should dissolve entirely in an appropriate solvent at room temperature to form an optically clear solution. If visible particulate material is still present after 30–60 min, it may be helpful to add additional volumes of the original solvent or an alternative solvent not exceeding the intended volume of the 5x peptide stock solution calculated in **Table 1**. Likewise, the combined peptide/BSA solution should remain translucent (**Figure 1A**).

Peptide/BSA gel samples should form an opaque rubbery mass after heating with 37% formaldehyde (**Figure 1B–I**). Minor fracturing of the gel samples may occur during removal from the microcentrifuge tubes (**Figure 3A**). These should not interfere with subsequent steps. Paraffin-embedded gel samples should section smoothly without tearing or chatter. Gels that show irregular tear-outs (**Figure 3B**) may benefit from less aggressive block facing and/or brief soaking in ice water or ammonia water. Areas of variably reduced signal in a watermark pattern may occur when the distribution of one or more staining reagents is uneven (**Figure 3C**). The focally increased macroscopic signal may be seen if there is reagent trapping under the gel sections at any stage of the staining process (**Figure 3C**). The use of positively charged glass slides and careful drying of slides after sectioning may reduce this artifact. Microscopic areas of the reduced signal may occur if there is an uneven distribution of antigen in the gel matrix or if variation in the local gel structure limits antibody-antigen interaction (**Figure 3D**). Incomplete peptide dissolution may result in scattered areas of focally intense signal in a low-intensity background (**Figure 3E**).

After reacting with an appropriate antibody, negative control BSA-only gel samples should show minimal signal (**Figure 4A**), optimally less than 2%–3% of the dynamic assay range. Very rarely, there may be significant antibody interaction with BSA gel material lacking any antigen that cannot be eliminated by changing reaction conditions. The signal intensity in an individual gel sample should be relatively uniform, with increasing signal in samples with increasing antigen concentrations (**Figure 4A,B**). The absolute signal intensity will vary depending on the antigen-antibody combination and conditions used for the IHC stain. Depending on the staining conditions, there may be a threshold antigen concentration below which the signal will be at the

background and an antigen concentration above which the signal will be saturated. In some assays, the signal above the background may be visible in samples with as little as 2.5 E-8 M peptide[2]. In replicate staining runs, the signal intensity for sections cut from each gel sample should be reproducible from run to run. In this application of anti-HER2 antibody, the widely used MDA-175 and SK-BR-3 cell line controls were stained in the same experiment to compare to the peptide controls. The cell lines show the expected distribution and intensity of signal: >10% of MDA-175 cells (HER2 1+) show faint (**Figure 4C,D**), incompletely circumferential membranous staining, and >10% of SK-BR-3 cells (HER2 3+) (**Figure 4E,F**) show intense completely circumferential membranous staining.

Possible reasons for the absence of signal include: (1) the peptide sequence is not a functional target for the antibody; (2) the staining protocol is not optimized to detect the antigen concentrations present in the gels; (3) the wrong peptide sample was stained; (4) reagent or instrument error. Possible reasons for signal in samples where it is not expected include non-specific reactivity of primary antibody or detection reagents with the BSA gel or peptide samples that were mislabeled during preparation.

FIGURE AND TABLE LEGENDS:

Figure 1: Peptide-BSA gel preparation. (**A**) 25% (w/v) BSA (without added peptide) in PBS, pH 7.2. (**B**) Gel formed by mixing equal volumes of 25% BSA and 37% formaldehyde solution and heating to 85 °C for 10 min. (**C–G**) After cooling at room temperature for 5–10 min, the BSA gel can be removed from the microcentrifuge tube using a flexible disposable spatula. (**H–I**) The intact gel is sliced into discs ~5 mm thick in preparation for processing and embedding. Scale bars are 1 cm.

Figure 2: Preparation of BSA gel samples for processing and embedding. (**A–D**) Preparation of a paraffin block containing a single disc of BSA gel. (**A**) The BSA-antigen gel is wrapped in biopsy tissue before processing. (**B**) After paraffin infiltration, the now-translucent gel disc is placed on the embedding center heated stage and unwrapped. (**C**) The single gel disc is placed in a 15 mm x 15 mm embedding mold containing liquid paraffin. (**D**) The completed paraffin block is ready for sectioning. (**E**-**H**) Preparation of a paraffin block containing multiple discs of BSA gel. As for Figures A–D above, the remaining gel samples are processed and embedded in one block to provide material for TMA construction. Scale bars are 1 cm.

Figure 3: Gel preparation and staining artifacts. (**A**) Cores may show minor fracturing introduced during processing and/or embedding steps; focal darkening may reflect reagent trapping under the section. (**B**) Tearing out of gel material (seen in stained sections as irregular voids) may result from over- or under-soaking the block before sectioning. (**C**) Irregular watermark staining patterns may be caused by uneven reagent distribution at one or more staining steps. (**D**) Microscopic patterning of the BSA-peptide structure may be seen, particularly at higher peptide concentrations. (**E**) Incomplete peptide dissolution may produce a starry sky pattern with a focal signal in a low-intensity background. Scale bars are 200 µm (**A–C**) or 20 µm (**D,E**).

Figure 4: Stained ERBB2/HER2 peptide TMA with ERBB2/HER2 1+ and 3+ cell lines. (**A**) Replicate TMA cores (top and bottom rows) containing no peptide (left column) or ERBB2 peptide at concentrations ranging from 2.5 E-8 M to 2.5 E-4 M were stained with anti-HER2/neu clone 4B5 according to the manufacturer's recommendations. Cores with increasing peptide concentrations show a graded increase in signal. (**B**) The TMA cores shown in panel A were digitally scanned, and the average pixel intensity [0.01 x (100 - % transmittance) x 255] is plotted vs. peptide concentration. (**C–F**) Cell line positive controls containing ERBB2/HER2-expressing cell lines MDA-175 (HER2 1+, **C,D**) and SK-BR-3 (HER2 3+, **E,F**) were included in the TMA containing the BSA-peptide gels so that the intensity of signal in the cell lines and BSA-peptide gels could be compared on the same slide. Scale bars are 500 µm (**A**), 250 µm (**C,E**), and 20 µm (**D,F**).

Table 1: Calculations for the preparation of peptide stock.

Table 2: Calculations for the preparation of BSA-peptide dilutions.

Table 3: Calculations for the preparation of BSA-formaldehyde gels.

DISCUSSION:

This method allows the user to create uniform samples of known composition and antigen concentration as standards in IHC reactions, using materials and techniques familiar to most histology laboratories. The most crucial step is to identify the epitope to which the antibody of interest binds. This protocol describes using a linear peptide antigen from the ERBB2/HER2 ICD. The same protocol can be used to form BSA gels containing oligonucleotides, fluorescent labels, protein domains, or full-length proteins. This latter approach can be helpful for antibodies binding to conformational epitopes that are not recreated by a single linear peptide sequence. For example, BSA gel standards containing 0.1 mg/mL naive IgG from mice, rats, and rabbits may be used as process controls to confirm that secondary detection and staining steps have worked as expected.

The antigen-BSA gel method described here complements other techniques to control and standardize IHC reactions. Cell lines and tissue samples with well-characterized expression levels of target antigens have been essential to well-controlled IHC protocols[1,3,4]. Peptides coupled to the surface of glass slides and glass beads have been proposed as quantitative controls[7–9]. Each of the potential methods has overlapping advantages and limitations. The BSA-antigen gels have several advantages; they are simple to make and adaptable to various antigen compositions and concentrations. They reproduce some of the three-dimensional architecture of tissue samples while controlling for the inherent heterogeneity found in biological samples. Because the synthetic antigen gels can be made with user-selectable antigen concentrations extending from below the limit of detection to the highest concentrations found in biological samples (~10^{-4} M)[2], they offer opportunities to calibrate and standardize assays performed with modern techniques, e.g., AQUA[18] and imaging mass spectrometry[19], whose dynamic range far exceeds traditional chromogenic IHC. In addition, the method allows controls incorporating more than one antigen, which can be used in multiplex assay development and standardization.

While the method requires the user to know the epitopes for the antibodies of interest, many antibodies bind to well-documented linear epitopes. Epitope mapping techniques[20–22] can often identify undefined linear epitopes, and synthetic peptides, including known linear epitopes, which can be purchased at a modest cost. Other antibodies bind to conformational epitopes that are not reproduced by linear peptides but are preserved in whole proteins or protein domains. For some of these antibodies, recombinant forms of the target antigens are commercially available.

For creating control samples with optimal uniformity and reproducibility, the peptide or other target antigen must be completely dissolved and carefully diluted into BSA solutions. It is also essential that efficient, rapid mixing of the BSA/peptide solution with 37% formaldehyde occurs before the sample starts to gel, and that the mixed sample is then placed promptly at 85 °C for 10 min. For avoiding over-fixation and processing artifacts, gels should be taken through processing and paraffin embedding according to the recommended schedule.

Variations on the method described here may be helpful in specific contexts. For instance, alternative N-and C-terminal peptide sequences may be used to optimize the detection of peptides bound to BSA[2]. It should be anticipated that antibodies recognizing epitopes from the extreme C-termini of proteins may be variably sensitive to C-terminal modifications of the native sequence. The peptide-BSA gel samples may also be formed by heating at 85 °C for 10 min without fixative, and then prepared as frozen blocks or post-fixed with a variety of chemistries[2]. In addition, peptide conjugation can be accomplished with maleimide chemistry or other methods than described here.

Biological samples such as cell lines and tissues have the advantage as controls of representing the complexity of the many variables found only in life. On the other hand, because tissue and cell standards are both internally heterogeneous and variable from sample to sample, the interpretation of variable staining from run to run is confounded. In addition, the antigen concentrations in tissues and cells are often known only qualitatively, though, with the increasing use of quantitative mass spectrometry, absolute protein concentrations are reported more frequently[23]. The BSA-antigen controls described here intentionally eliminate the spatial and biological context of proteins in cells and tissues. For this reason, the correlation between IHC signal intensity and antigen concentration found in these controls may imperfectly reproduce that seen in tissues. The synthetic controls described here may be helpful to optimize and standardize some aspects of IHC assay performance. Still, they do not prevent the need for the appropriate use of other control samples. For instance, potential non-specific staining in a target tissue can be evaluated only by the parallel use of a negative tissue control sample. In addition, BSA-antigen gel controls do not reproduce tissue-specific pre-analytic variables, including warm and cold ischemia time, proteolysis, or fixation and processing conditions that may affect IHC performance. Accordingly, and as has been discussed elsewhere in more detail[1,3,4], investigators should make thoughtful use of cell and tissue standards to precisely characterize the performance of the IHC system.

BSA-antigen controls may be used to develop or optimize staining protocols and serve as intra-

and inter-laboratory reference samples when assessing the reproducibility of established protocols. Access to well-defined standard samples should allow users to more rigorously characterize IHC reaction behavior, identify variation in staining performance, and optimize reaction conditions to achieve specific objectives.

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DISCLOSURES:

Charles A. Havnar, Kathy J. Hötzel, Charles A. Jones, Carmina M. Espiritu, Linda K. Rangell, and Franklin V. Peale are employees and stockholders of Genentech and Roche. Their affiliates produce reagents and instruments used in this study.

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Figure 1

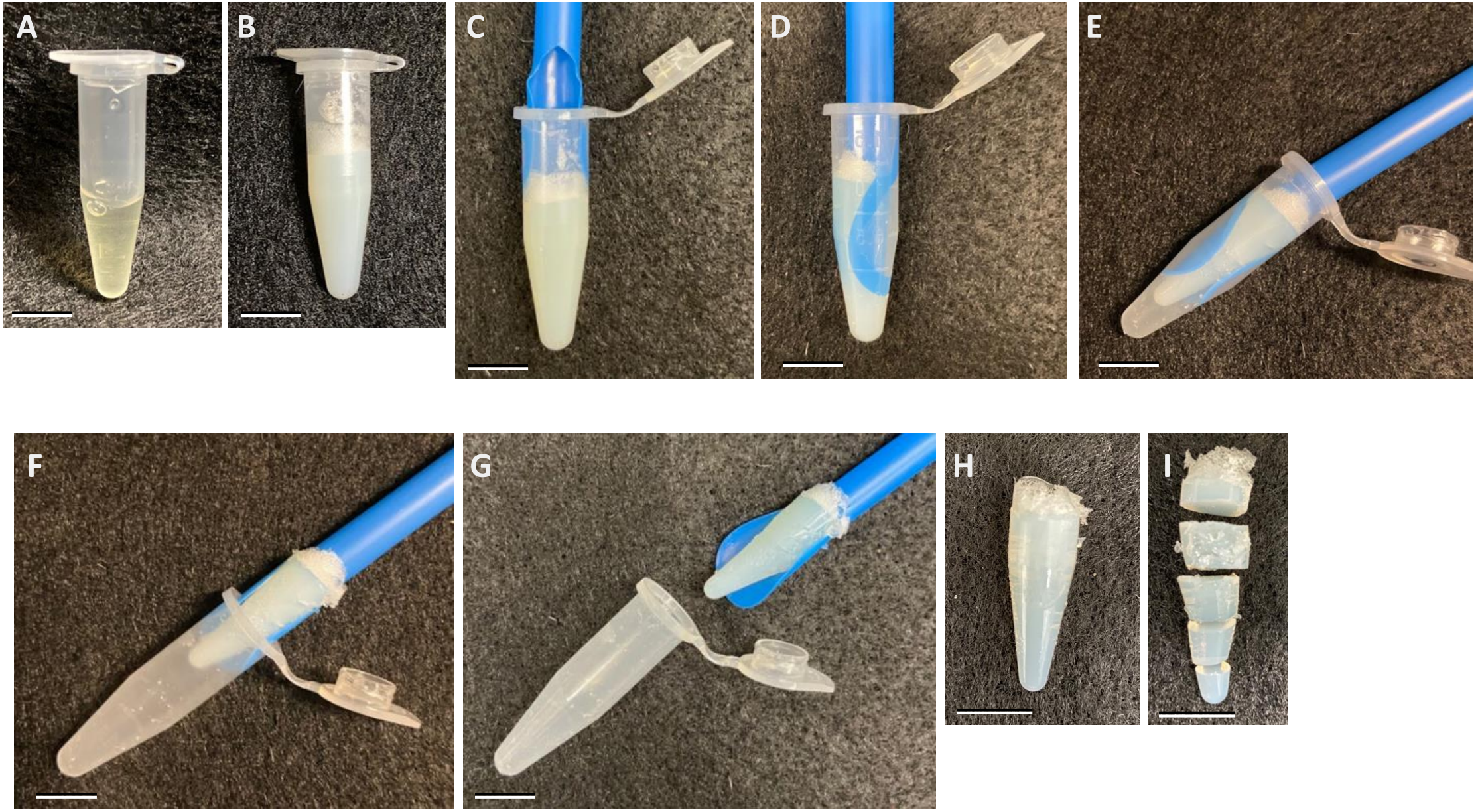


Figure 2

Click here to access/download;Figure;Fig2.pdf

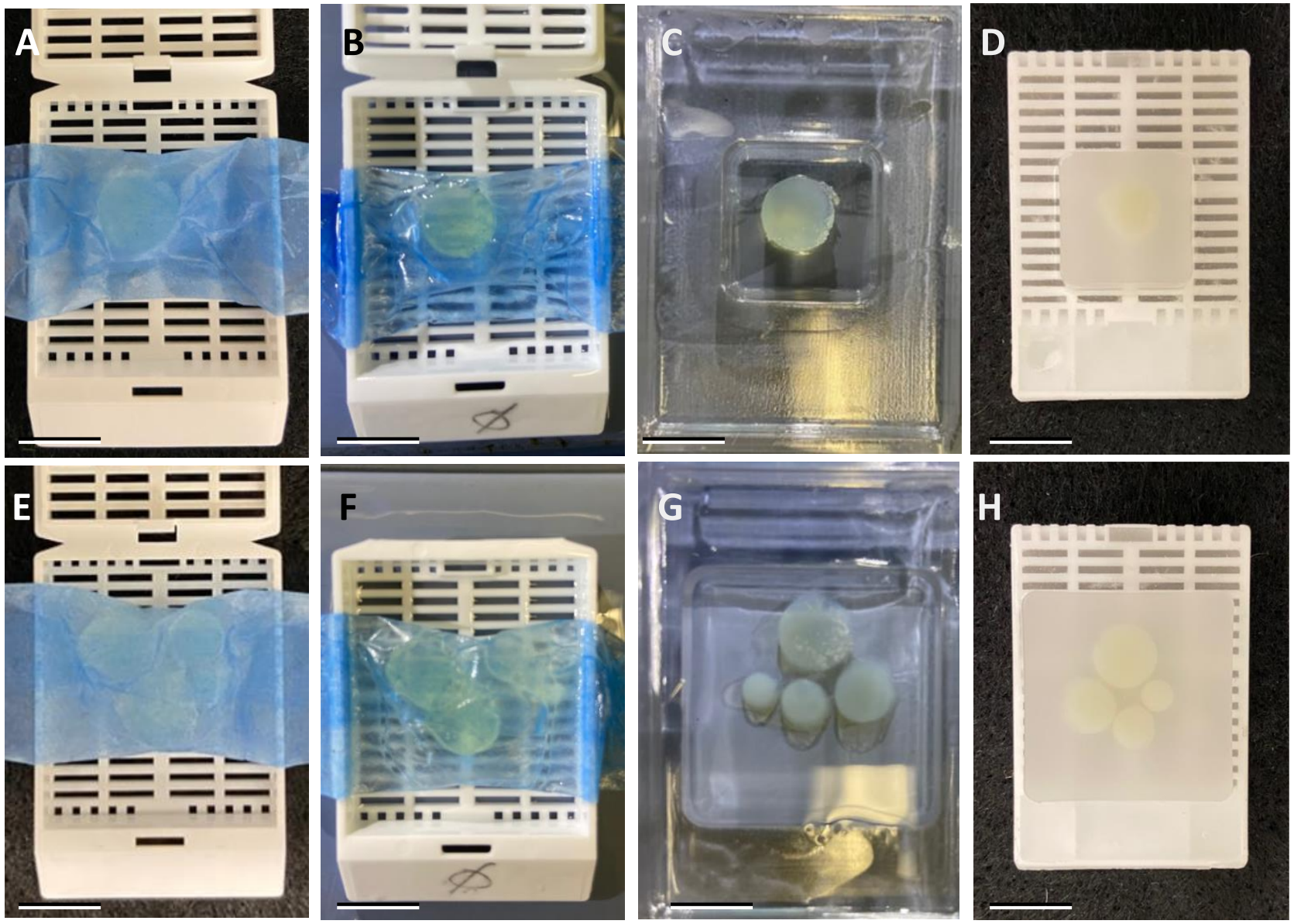


Figure 3

Click here to access/download;Figure;Fig3.pdf

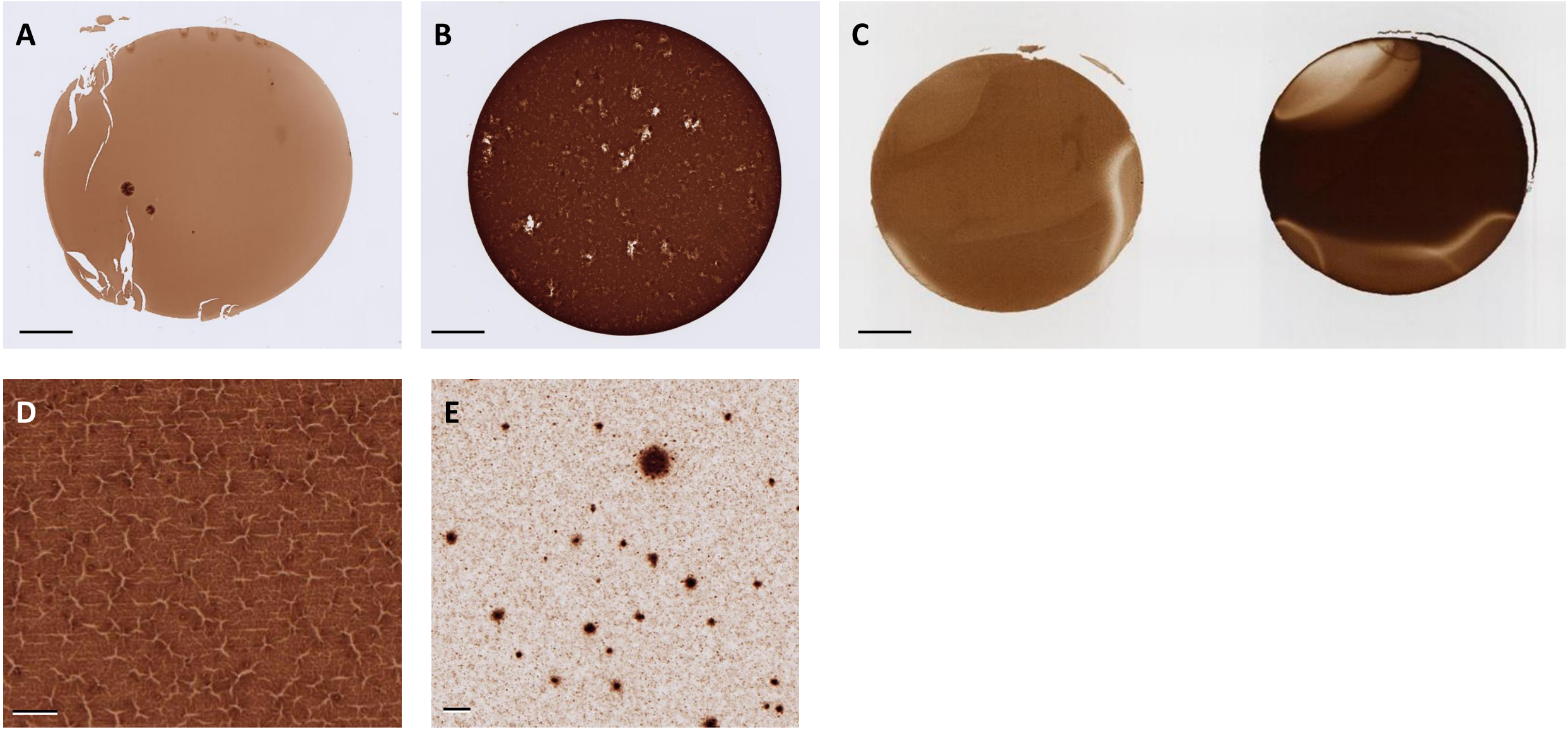
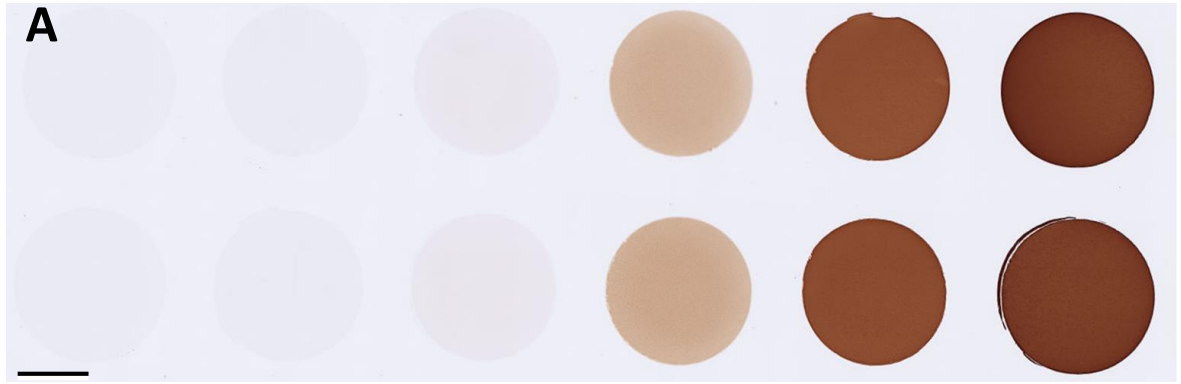
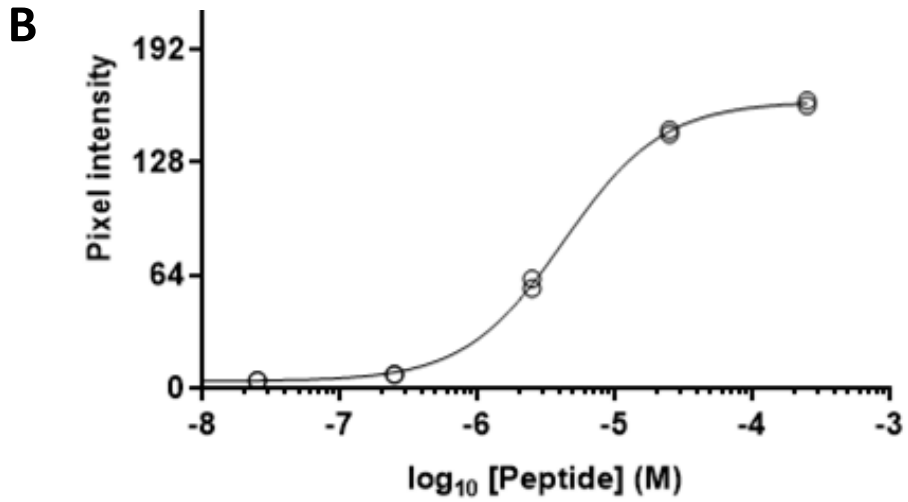
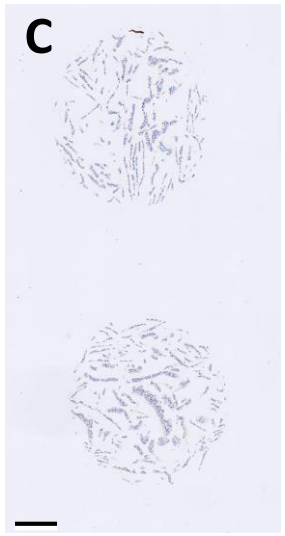


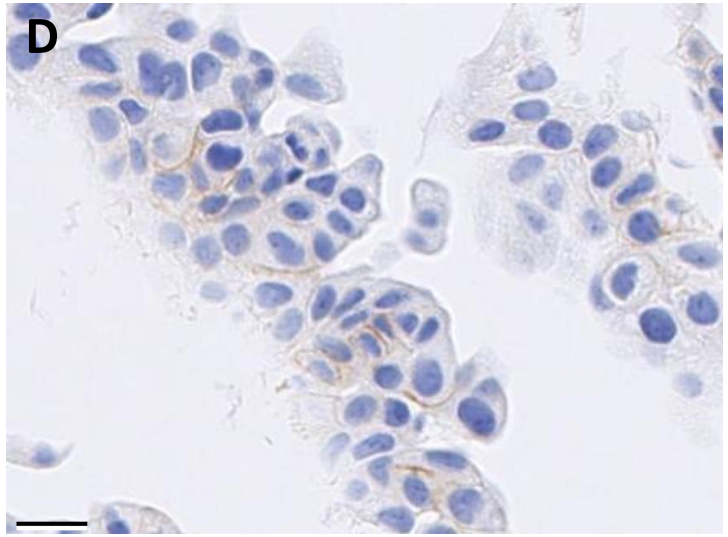
Figure 4R

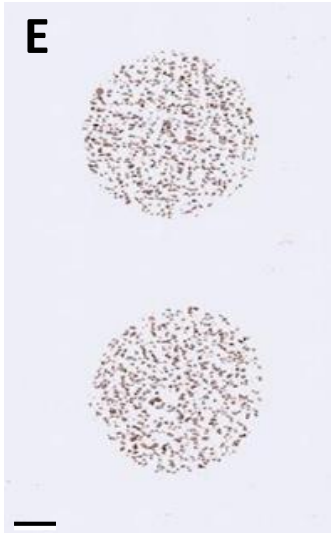
Click here to access/download;Figure;Fig4 R.pdf











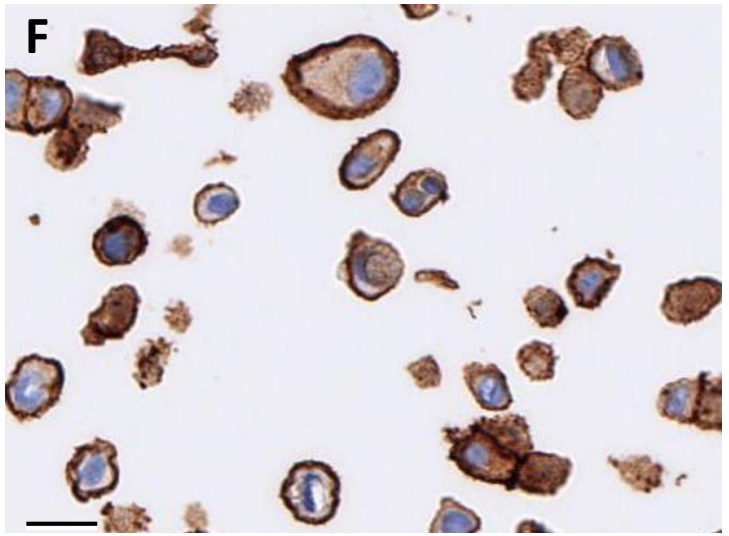


Table 1

A	B	C	D
Peptide name	Peptide sequence	MW (Da)	Peptide purity (%)
ERBB2 / HER2	Ac YGSGTPTAENPEYLGLDVPVGSGC amide	2424.6	95.0

E
Peptide mass provided (mg)
20.0

Table 2

Tube	5x Peptide stock	1x Peptide stock	Dilution 1	Dilution 2	Dilution 3	Dilution 4
[Peptide] stock (M)	**1.25×10^{-2}**	**2.5×10^{-3}**	5×10^{-4}	5×10^{-5}	5×10^{-6}	5×10^{-7}
Peptide solution (in solvent)	> 30 uL	30 µL from 5x (1.25 E-2 M) peptide stock	140 µL from 1x (2.5 E-3 M) peptide stock	70 µL from Dilution 1	70 µL from Dilution 2	70 µL from Dilution 3
Solvent volume		120 µL				
Final peptide stock volume		150 µL				
31.3% BSA/PBS volume			560 µL 31.3% BSA			
25% BSA/PBS volume				630 µL 25% BSA	630 µL 25% BSA	630 µL 25% BSA
Volume of diluted peptide in BSA			700 µL	700 µL	700 µL	700 µL

Dilution 5	**Negative control**
5×10^{-8}	None
70 µL from Dilution 4	None
630 µL 25% BSA	700 µL 25% BSA
700 µL	700 µL

Table 3

Tube	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Negative control
Volume remaining of peptide in diluted BSA	630 µL	630 µL	630 µL	630 µL	700 µL	700 µL
[Peptide] (M) in diluted BSA	5×10^{-4}	5×10^{-5}	5×10^{-6}	5×10^{-7}	5×10^{-8}	None
Volume of 37% Formaldehyde to add	630 µL	630 µL	630 µL	630 µL	700 µL	700 µL
Final [Peptide] (M) in gel	**2.5×10^{-4}**	**2.5×10^{-5}**	**2.5×10^{-6}**	**2.5×10^{-7}**	**2.5×10^{-8}**	**None**

Amit Krishnan, Ph.D.
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Aug. 3, 2021

Dr. Krishnan:

Thank you for your recent response to our manuscript submission. We considered the reviewers' comments carefully and have made adjustments to the manuscript to accommodate their suggestions and criticisms. Our detailed responses to each reviewer are below.

Reviewers' comments: (Authors' replies are in blue text.)

Reviewer #1:
Havnar and co-workers describe a method for preparation of synthetic antigens controls for immunohistochemistry. The method description appears to be in sufficient detail to permit reproduction by others.

Concerns
1. My main concern is that the authors do not properly discuss the roles of this method in relation to the specificity of antigen detection in biological samples. In particular, the limitations are hardly discussed at all: (A) Binding of the antibody to the antigen control is no guaranty that the observed labeling of the biological specimen is only due to the antigen of interest. In fact, it might not be due to that at all!

We thank Reviewer 1 for this comment, and we agree that binding of an antibody to a positive control sample does not guarantee that only antigen-specific binding will occur with the same antibody in all tissue sections. We note that this caveat applies equally to any type of positive control sample and is not a limitation specific to the synthetic antigen controls we describe in this manuscript. Appropriate use of negative and positive tissue control samples is required to interpret any positive tissue signal, has been discussed in detail by Stephen Hewitt, Emina Torlakovic and others, and the issue is clearly highlighted in our Introduction (references 2-4 in our manuscript). While we do not claim in the original manuscript that the synthetic controls eliminate the risk of non-specific tissue signal, we have added a sentence clarifying this limitation, as follows:

Lines 462 ff in the Discussion have been edited to:

"We emphasize that the synthetic controls described here may be useful to optimize and standardize some aspects of IHC assay performance, but they do not obviate the need for appropriate use of other control samples. For instance, potential non-specific staining in a target tissue can be evaluated only by the parallel use of a negative tissue control sample... Accordingly, and as has been discussed elsewhere in more detail[2-4], investigators should make thoughtful parallel use of cell and tissue standards to precisely characterize the performance of the IHC system."]

(B) The availability of the epitope in a biological sample might be very different from the availability in the BSA-gels described in the present manuscript. The availability will be affected by tissue structure, by crosslinks due to fixation and changes to the tissue (e.g. proteolysis) prior to fixation. These point should also be discussed.

We thank Reviewer 1 for this comment, and we agree with the statement. We agree that the BSA gels do not fully reproduce the complexity of tissue composition, and do not mimic the variables inherent in tissue collection and processing. This is intentional, and it creates both strengths and limitations. The extent of concordance between IHC staining of BSA-antigen gel standards and tissues is still incompletely understood (though we have not yet found significant discordance); it will become clearer with time as more data become available. We believe the text, modified as noted above (line 459 ff) and below now makes this point more clearly:

"The BSA-antigen controls described here intentionally eliminate the spatial and biological context of proteins in cells and tissues. For this reason, the correlation between IHC signal intensity and antigen concentration found in these controls may imperfectly reproduce that seen in tissues…. In addition, BSA-antigen gel controls do not reproduce tissue-specific pre-analytic variables, including warm and cold ischemia time, proteolysis, fixation and processing conditions, that may affect IHC performance. Accordingly, and as has been discussed elsewhere in more detail[2-4], investigators should make thoughtful parallel use of cell or tissue standards to precisely characterize the performance of the IHC system."

2. As the authors point out, the idea of embedding antigens at different concentrations to use as controls is not new. The authors, however, should say a bit more about this. Long time back (in the 1990s, I think), OP Ottersen and co-works and also Peter Streit and co-workers made similar antigen controls. But in contrast to the method in present manuscript, they added antigens to tissue homogenates in order to try to mimic the biological samples as much as they could. Thus, the authors should broaden their discussion supported by a few more citations.

We thank Reviewer 1 for this comment, as we were not familiar with these authors' work. We find two references that are consistent with this comment (Matute and Streit, 1986; Ottersen 1987). These describe the preparation and use of immune-electron microscopy positive controls in the form of amino acids conjugated to dilute BSA solutions and brain homogenates, respectively. We have added mention of these to the Introduction (Line 85 ff), and revised the References list accordingly:

"Later investigators prepared glutaraldehyde conjugates of specific amino acids to dilute solutions of BSA or brain homogenates for use as positive controls in immune-electron microscopy studies[12,13]."

[Reviewer 1 continues...]
In conclusion, the method description in the present paper is nice, but as the paper is written now, it is misleading and will not contribute to the badly needed improved immunohistochemistry culture.

We appreciate that Reviewer 1 believes our method is “nice”, but emphatically disagree with two aspects of the concluding statement.

Reviewer 1 states that our manuscript is misleading. From the more precise comments made previously, we infer that Reviewer 1 believes we are minimizing limitation(s) of the method, chiefly that the BSA-antigen gels do not serve as negative tissue controls, and do not fully reproduce the molecular and spatial complexity of tissue samples. As detailed above, the original manuscript does describe both limitations, and our revised text (details above) discusses these points more clearly. We intentionally do not revisit in detail those topics related to positive and negative tissue control samples that are discussed fully in previously published work cited in the text.

Reviewer 1 further states (paraphrasing) that the method we describe here does not contribute to improving the practice of immunohistochemistry. In contrast, we believe the method provides unique capabilities that have been long-sought but not previously implemented. One application for these materials is as test samples to develop and optimize IHC staining procedures for new antibodies. This is a particularly valuable attribute when the target antigen has an unknown tissue distribution and/or is expressed at low levels in target tissues; in that context, tissue positive controls are not available. Multiplex immunohistochemistry is increasingly used in research and in clinical trials, and our method provides the opportunity to create well-controlled test samples to optimize these complex protocols. In a second application, relevant even for well-characterized antibodies used in established IHC procedures, the BSA-antigen gels provide reproducible, uniform target samples that can be used to monitor the performance of an IHC assay over time, or at different laboratories. These applications are valuable even if (and there is not yet enough data to know) the synthetic antigen gels do not fully reproduce the staining behavior of all target tissues.

The synthetic control method adds significant capabilities to the existing suite of control tissue and cell line samples that can be used to bring IHC reactions closer to reproducible, well-controlled laboratory assays. Our previously published paper in the Journal of Histochemistry and Cytochemistry discussed these capabilities and limitations in more detail than we do in the current manuscript. On accepting that manuscript, Stephen Hewitt, the editor in chief wrote, “I will note the reviewers view the manuscript as a substantial step forward that many in the community have envisioned and attempted, but failed.” These synthetic controls have been of substantial value in our own immunohistochemistry laboratory. Our goal in this manuscript is to explain as clearly as possible to a naive user how to produce and use these materials, which we expect can also be of value to many other investigators.

Reviewer #2:

Manuscript Summary:

The manuscript entitled "Synthetic Antigen Controls for Immunohistochemistry" is development of a method for reproducible and quantitative immunohistochemistry by including Paraffin-embedded gels campsite of BSA-peptide antigen as a positive control.

Minor Concerns:

The authors should correct the title of manuscript "Synthetic Antigen Controls for Immunocytochemistry". Because they used the cell lines rather than tissue samples for the experiment.

We appreciate the suggestion from Reviewer 2 to change the title, but we believe the current title is appropriate. We intend the proposed title to mean “Synthetic Antigen Controls [for use] in Immunohistochemistry”, where “immunohistochemistry” is understood to mean the application and detection of antibody reagents to tissue samples intended for microscopic analysis. In this context, it is unimportant that some samples in the manuscript are paraffin-embedded blocks of cell lines. These were included because they are well-characterized with respect to Her2 antigen expression, and are widely used in breast cancer research. We anticipate that changing the title to say “Immunocytochemistry”, as Reviewer 2 suggests, might create a different misunderstanding, that our method is relevant to the analysis only of cell lines, but not tissues. Accordingly, we respectfully propose to keep the title as originally suggested.

Reviewer #3:
Manuscript Summary:
The authors provide a well-considered explanation of the need for good controls when performing IHC staining. Additionally, they consider the objective limitations of the current methods used and outline the need for an easy-to-adopt approach that employs widely available reagents. The stated goals of the authors are to provide a method that enables highly reproducible results, that is cost effective and enables objective and quantifiable evaluation of IHC results across experiments and different labs.

The protocol is easy to follow and very clear. Authors incorporate helpful notes/tips and calculation tables throughout to facilitate quick problem solving. Representative Results section includes additional points and expected observations throughout the protocol.

Figures are helpful and explanatory. Figure 3 provides particularly useful examples of artifacts.

Discussion is sufficient to place the newly developed protocol in context and to explain its overall utility.

Major Concerns:
None

Minor Concerns:
Line 26 - ICD should be defined first before writing as an abbreviation
Line 53 - typo ICH should be IHC
Line 233 - typo "processing13" should be corrected
Step 4.1.2 - the wording of 'no less than 10 volumes (15ml per gel sample)' is confusing. Consider re-wording.
Step 4.2 - can this be done manually, if so, it would be useful for authors to provide a note with direction.
Line 368-369 - bolding is extended too far into the legend.

We thank Reviewer 3 for the careful review of our manuscript. The request changes have been made in the text.

In addition, we carefully reviewed the editorial comments 1-18 in your note and have made many adjustments and corrections accordingly. Particular details are listed below:

2. All authors' email addresses are now provided on the title page.

3. All personal pronouns were removed from the text.

4. All protocol statements are in the imperative tense.

5., 6. All SI and time units are corrected to your standard form (e.g., mL, min).

8. All protocol steps other than "Notes" are appropriately numbered.

9., 10. The dilution of the 4B5 antibody used in this manuscript is specified.

12. As requested, the following are detailed in the Discussion: critical steps (lines 469-474), possible modifications (lines 476-483), protocol limitations (485-504), and future applications (506-510).

13. References are in your preferred format.

15. A scale bar is now included in panel A of Figure 4, and in all other Figure panels. We note that some figure panels (e.g., Fig. 3C) include more than one tissue microarray core. In these panels, the same scale bar applies to all samples illustrated in the panel.

16. Pixel intensity units are now described in the text of Figure 4, panel B.

17. SI units in Tables 2 and 3 are now consistent with your standards.

18. The Table of Materials has been sorted in alphabetical order.

We respectfully submit the revised manuscript with the hope that these corrections and revisions fully address the points made by the reviewers and meet the goals of your journal.

Please feel free to contact me if you require any further changes.

Regards,

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Research Pathology
Genentech, Inc.