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# Synthetic Peptides Identified from Phage-displayed Combinatorial Libraries as Immunodiagnostic Assay Surrogate Quality-Control Targets

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***Background:*** **Quantitative immunohistochemical (IHC) assays currently lack optimal reference quality-control material for cellular protein targets. To address this problem, we identified peptides that mimic the site on the native analyte to which the primary (monoclonal) antibody binds and used them as surrogate peptide controls.**

***Methods:*** **We identified peptide candidates from a combinatorial peptide phage-display library that mimic the epitope for the 1D5 estrogen receptor (ER) monoclonal antibody (mAb). The peptide inserts of the phage clones were sequenced. Several phage-encoded peptides were then synthesized and analyzed for affinity and specificity.**

***Results:*** **We identified phage clones that specifically bound to the ER 1D5 mAb. The binding was specific, in that the phage clones did not bind to two other isotype-matched mAbs. Their ability to bind the ER 1D5 mAb was related to the presence of a consensus sequence. Binding analysis revealed a $K_d$ of $8.3 \times 10^{-8}$ mol/L. The peptide was not recognized by any of 15 other mAbs commonly used for clinical IHC testing. Moreover, the peptide was able to inhibit the binding of ER 1D5 mAb to native ER, indicating that the peptide bound to ER 1D5 mAb at or close to the antigen-binding site.**

***Conclusions:*** **Surrogate peptide controls behave like the native analyte in terms of affinity and specificity. This technology may be especially useful when the native analyte is in short supply.**

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The linkage of diagnostics and therapeutics is an important new paradigm in cancer research and patient management. From the diagnostics perspective of this paradigm, most oncology testing is performed using an in situ format, i.e., the cellular analyte is quantified by visual microscopic examination. For example, HER-2 measurement (for assessment before chemotherapy or Herceptin™ therapy) is performed by either immunohistochemistry or in situ hybridization *(1–4)*. Estrogen receptor (ER)[4] analysis (linked to tamoxifen therapy) is another example where in situ methods, primarily immunohistochemistry, have largely replaced the older (in vitro) dextran-coated charcoal test *(5–7)*. Many other directed biologic therapies, currently in pharmaceutical pipelines, will produce increasing demand for accurate, quantitative measurement of tumor cell-associated analytes.

The accuracy of conventional in vitro diagnostics is maintained, in part, by the availability of agreed-on international analytic reference standards *(8)*. These reference standards ultimately translate into manufacturer-supplied calibrators and controls that clinical laboratories use to check the accuracy of their reagents and instrumentation. Calibrators for biologic markers in a serum matrix are widely understood, commercially available, and effective in fostering interlaboratory standardization *(8)*. By contrast, there is a striking absence of practical, useful calibrators for the new wave of (in situ) cellular diagnostics. Consequently, interlaboratory reproducibility for immunohistochemical (IHC) methods is far lower than that of most other clinical analytic measurements *(9, 10)*. As a result, there is now widespread recognition of the need

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[4] Nonstandard abbreviations: ER, estrogen receptor; IHC, immunohistochemical; mAb, monoclonal antibody; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; and PR, progesterone receptor.

for effective quality-control systems for (in situ) cellular diagnostic assays *(9, 11–13)*.

Previous efforts toward standardizing IHC assays have generally sought to promulgate standardized assay methodologies *(11, 12)* and provide external quality-assurance standards *(13)*. External standards have generally been a tissue sample from a central laboratory. Although tissue sections are useful for periodic proficiency testing among participating laboratories, they are not suitable as routine calibrators for daily use. Any single tissue block would be rapidly exhausted if used to supply many institutions on a daily basis. This is in contrast to conventional serum-based calibrators for in vitro diagnostics. Many serum calibrators can be prepared in sufficiently large volumes through commercial suppliers to enable routine daily assay calibration.

In addition, tumor tissues suffer from other important limitations that limit their use as analytic calibrators for routine daily use. Most important among these are that tissue sections are susceptible to both analytic and preanalytic variability. Preanalytic variability encompasses fixation (typically with formalin) and antigen retrieval practices. Antigen retrieval is a procedure for reversing the effect of formalin fixation to allow antibodies to recognize antigenic epitopes that were denatured by formaldehyde cross-linking. Available evidence suggests that the predominant preanalytic source of error is in the antigen retrieval step, not tissue fixation *(10, 14)*. Therefore, variability in analyte measurement using centrally supplied tissue section calibrators can potentially be attributable to both the laboratory's antigen retrieval technique (a preanalytic variable) and to analytic sources of error, such as reagents, test methodologies, and instrumentation. The absence of reference standards, calibrators, and control material capable of sorting out these variables is a major obstacle to fostering reproducibility of cellular diagnostic testing.

Additional limitations to the use of tissue sections as reference or control materials are that their manufacture is labor-intensive and that they are fragile. A tissue section, typically cut at a 4-$\mu$m thickness and mounted on a glass microscope slide, is destroyed by even a light touch. Preparation of such sections involves cutting the sections, floating them in a water bath, and then scooping them up on a glass slide. There is no automation for mounting tissue sections on slides.

In this report, we describe a new technology for generating control material that addresses the above-mentioned problems. Our technology provides an inexhaustible, reproducible, quantitative, antigen-specific, stable, and inexpensive source of analytic control material. The technology works by creating a surrogate analytic target that is not susceptible to preanalytic sources of error. The surrogate target is a synthetic peptide that resembles the three-dimensional conformation of the epitope to which the antibody binds. Phage-displayed random peptide libraries *(15–19)* have recently been shown to be invaluable because of their potential for identifying both linear as well as discontinuous epitopes *(19, 20)*. High-affinity antigen mimics are identified by phage-display technology. These synthetic peptides are covalently attached to the glass slide by isocyanate coupling chemistry. As the tissue sample is stained, so is the control material. The precise amount of color that develops on the synthetic peptides directly reflects the efficacy of the IHC stain.

This technology will allow, for the first time, highly accurate inter- and intrainstitutional quantitative comparisons of staining efficacy. Moreover, these calibrators can be widely distributed without fear of exhausting a biologic resource. We believe that this technology will promote accuracy and reproducibility of (in situ) cellular diagnostic testing.

## Materials and Methods

### IHC STAINING

All IHC stains were performed on formalin-fixed, paraffin-embedded ER-positive human breast cancer tissues. Serial tissue sections were deparaffinized in xylene, dipped in decreasing concentrations of ethanol, and then rehydrated in water. Epitope retrieval was then performed by incubating the slides in a pressure cooker (Nordic Ware) for 30 min in a 0.01 mol/L citrate buffer (pH 6.0). Slides were immunostained either manually or with an automated slide stainer (Artisan™; CytoLogix Corporation, Cambridge, MA) using the manufacturer's IHC detection reagent set, a labeled streptavidin–biotin detection system. ER antigen-specific primary monoclonal antibody (mAb) 1D5 *(21)* was used at the standard concentration supplied by CytoLogix (~3 mg/L). The antibody was added to the tissue section and incubated at room temperature for 40 min. The slides were then rinsed, and the presence of ER-specific antibody was detected by the CytoLogix IHC detection method. Briefly, these steps included incubation with biotin-conjugated horse anti-mouse IgG (heavy and light chain specific) secondary antibody for 20 min at room temperature. Slides were incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 20 min at room temperature. The color was then produced with liquid-stable 3,3′-diaminobenzidine tetrahydrochloride–hydrogen peroxide for 10 min and enhanced with 50 g/L copper enhancer (cupric sulfate pentahydrate) for 10 min. For slides that were used for image quantification, no counterstain was applied to simplify image colorimetric quantification.

### PHAGE-DISPLAY LIBRARIES AND BIOPANNING

Phage libraries contained rationally designed combinatorial libraries of peptide sequences inserted into the $NH_2$ terminus of the pIII minor coat protein of the M13 bacteriophage. The libraries were prepared and supplied by Dyax Corp. (Cambridge, MA). The libraries (termed TN6 and TN10) contained two conserved cysteine resides separated by either four (for the TN6 library) or eight (for

the TN10 library) amino acids. The cysteines formed a disulfide bridge, creating a conformationally constrained ring *(22)*. The amino acids within the ring and the three amino acids on either side of the ring were diversified to allow all amino acid types (except cysteine) with equal frequency, using trinucleotide-mutagenesis technology *(23)*. Trinucleotide-mutagenesis technology involves controlled polymerization of preformed trinucleotides.

The libraries were screened by biopanning using standard methods *(17, 19, 24)* with a few modifications. Briefly, paramagnetic beads coated with anti-mouse IgG (Dynabeads; Dynal) were prepared by mixing with either the ER-specific mouse mAb clone 1D5 (for positive enrichment) or the polyclonal mouse IgG (for negative depletion) and incubating overnight at 4 °C on a rotator. Antibody-adsorbed Dynabeads were washed four to five times with phosphate-buffered saline (PBS) containing 0.5 mL/L Tween 20 and twice with PBS before use in biopanning of phage libraries. A TN6 or TN10 phage library containing $10^{11}$–$10^{12}$ plaque-forming units was negatively depleted by incubation with Dynabeads (100 μL) coated with polyclonal mouse IgG for 1 h at room temperature on a rotator. With this negative depletion step, phage that were bound to common, shared regions of mouse IgG were depleted from the library by attaching to the magnetic beads. The unbound phage (supernatant) were then positively selected on the (ER-specific 1D5) target mAb-adsorbed Dynabeads. The library was incubated with the 1D5-coated beads for 2–3 h on a rotator. The beads were washed 10 times with PBS containing 0.5 mL/L Tween 20 and three times with PBS to remove nonspecifically bound phage. Phage bound to the 1D5-coated beads were eluted with a buffer containing 0.1 mol/L glycine-HCl (pH 2.2) containing 1 g/L bovine serum albumin (BSA). After elution, the acidic buffer was neutralized with 1 mol/L Tris-HCl (pH 9.0). To ensure that high-affinity phage were completely eluted, the beads were serially eluted twice, and the supernatants were pooled. The eluted phage were then amplified and used in a second round of biopanning. After two rounds of positive selection, *Escherichia coli* were infected with the cultured phage and grown on agar plates. Individual phage colonies were picked and grown for further analysis.

### sandwich phage elisa

A sandwich-type ELISA was used to assay the reactivity of the enriched recombinant phage with the ER-specific mAb (clone 1D5). As a first step, the ER-specific mAb (or other isotype-matched control mAbs) were adhered to the plastic. The various antibodies against which phage were purified were available only as culture supernatants containing other irrelevant serum-derived proteins. Therefore, we first coated the ELISA plates with a mouse IgG-reactive antibody that could capture the ER 1D5 or control mAbs from culture supernatant. Briefly, three 96-well microtiter plates (Immulon 1; Dynex) were coated with goat anti-mouse IgG (Sigma; 2 mg/L in carbonate-bicarbonate buffer, pH 9.5; 100 μL/well) and incubated overnight at 4 °C. The wells were rinsed in PBS and blocked with 200 μL of 20 g/L BSA in PBS for 1 h at room temperature. The wells were then washed five times in PBS containing 0.5 mL/L Tween 20. These microtiter plates, coated with an anti-mouse IgG antibody, were then used to capture various mouse mAbs from culture supernatants.

The ER 1D5 mAb or irrelevant, isotype-matched mAbs were diluted to 1 mg/L in PBS containing 0.5 mL/L Tween 20, added to the anti-IgG-coated 96-well microtiter plates (100 μL/well), and incubated at room temperature for 3 h. As an additional negative control (in addition to an isotype-matched negative control), microtiter plate wells were coated with 20 g/L BSA in PBS (200 μL/well) and incubated at room temperature for 2 h. Antibody- and BSA-coated wells were washed eight times with PBS containing 0.5 mL/L Tween 20.

The reactivity of the enriched phage clones with the ER 1D5 mAb or control mAbs was assessed by measuring binding to the respective mAbs immobilized in the microtiter wells as described above. Individual phage clones (~$10^8$–$10^9$ plaque-forming units/well), diluted in PBS–0.5 mL/L Tween 20–1 g/L BSA, were added to the microtiter wells (100 μL/well) and incubated at room temperature for 2 h. Wells were washed 10 times with PBS–0.5 mL/L Tween 20 to remove nonspecifically adherent phage. The presence and relative amount of plate-adherent phage were determined by adding HRP-conjugated sheep anti-M13 antibody (100 μL/well; Pharmacia Biotech) and incubating for 1 h at room temperature. The microtiter wells were then washed eight times with PBS–0.5 mL/L Tween 20 to remove nonspecifically adherent sheep anti-M13–HRP antibody conjugate. Finally, an HRP colorimetric enzyme substrate, 2,2-azino-di-[3-ethyl-benzthiaoline sulfonate] (0.5 g/L), was added to the microtiter wells. Color development in the microtiter wells was measured with a Bio-Tek EL311 (Bio-Tek Instruments, Inc.) microplate reader at a 450-nm wavelength. Data are expressed as absorbance. Phage clones that gave ELISA signals at least three-fold higher than the background value were considered positive.

### dna insert sequencing

Phage clones that gave high absorbance readings (considered to be specific) and several poorly binding clones were submitted for further analysis by sequencing the nucleotide inserts coding for the combinatorial peptides. The sequencing template was prepared by PCR amplification from an overnight phage culture. The primers used for sequencing were 5′-CGGCGCAACTATCGGTATCAAGCTG-3′ and 5′-CATGTACCGTAACACTGAGTTTCGTC-3′. Thirty rounds of PCR were performed on a MJ Research Tetrad thermocycler (MJ Research, Inc.). The PCR product was diluted 1:20 with distilled $H_2O$. Sequencing was performed in both the forward and reverse

directions with the following primers: 5′-GATAAAC-CGATACAATTAAAGGCTCC-3′ and 5′-GTTTTGTCG-TCTTTCCAGACGTTAG-3′. ABI Big Dye™ (Ver. 1.0) was used to perform a 5-μL sequencing reaction [2 μL of Big Dye, 1 μL of distilled $H_2O$, 0.5 μL of primer (at 3 pmol/μL), and 1.5 μL of diluted PCR product]. The samples were then cycled for 45 rounds on an MJ Research Tetrad thermocycler. After cycling, 2.5 volumes of absolute ethanol were added, and the mixture was centrifuged at 1850*g* for 30 min. The plates were inverted over paper towels, and then centrifuged at 100*g* for 30 min. The samples were resuspended in 5 μL of distilled $H_2O$ and detected on an ABI 3700 DNA Analyzer. The results were analyzed using Phred and Pharp to generate a consensus from the forward and reverse reads *(25, 26)*.

### synthetic peptides and peptide elisa

Three peptide sequences that represented consensus sequences and were associated with high absorbance readings from the phage ELISA were chosen for further analysis. The peptides were synthesized (SynPep Corp.) with slight modifications: the $NH_2$ terminus was acetylated, two lysine residues separated by a glycine residue were added to the COOH terminus, the inner lysine residue was labeled with fluorescein, and the COOH-terminal lysine was amidated. Sequences were chosen such that there were no internal lysine residues in the original sequence except those added.

The synthetic peptides were tested for binding to the ER 1D5 mAb (or irrelevant isotype-matched mAbs) in a "peptide ELISA". We covalently coupled the peptides through the ϵ amino group of the COOH-terminal lysine residue (100 μL/well; 100 μmol/L peptide in PBS) to maleic anhydride-activated polystyrene plates (Pierce Chemical Co.). The excess reactive groups on the plate were quenched with BSA (30 g/L in PBS). The wells were then incubated with one of the mAbs for 2 h at room temperature. The wells were washed (PBS–05 mL/L Tween 20) and incubated with goat anti-mouse IgG–alkaline phosphatase conjugate (Sigma) diluted 1:1000 in PBS–Tween 20. Color was developed with *p*-nitrophenylphosphate (Sigma 104; Sigma) and read on a microplate reader (Model 2550; Bio-Rad) at 405 nm.

### application of peptides to glass slides

Peptides were covalently coupled to the isocyanate-derivatized glass surface of microscope slides. Briefly, 1 μL each of various peptide concentrations (2–0.125 μmol/L) was spotted on activated, isocyanate-derivatized slides. The peptides were allowed to covalently couple to the glass surface (via the ϵ amino group of a COOH-terminal lysine) for 15 min. The slides were rinsed and further blocked with bovine γ-globulins (0.5 g/L; Sigma).

### affinity measurements of peptide

The affinity of ER peptide 3 was measured by fluorescence polarization (Tecan Polarion Fluorescence Polarization plate reader; Tecan), at room temperature. The binding constant was deduced from Scatchard analysis.

### peptide specificity and cross-reactivity

ER peptide was tested for binding to different mouse mAbs or a polyclonal antibody. Eighteen spots of ER peptide were applied to an isocyanate-activated microscope slide, each spot containing 2 pmol in a 1-μL volume. Various antibodies were then reacted with the spots: ER 1D5, progesterone receptor (PR) clone 636, p53 clone DO7, Ki-67 clone MIB-1, vimentin clone V9, leukocyte common antigen (CD45) clones PD7/26 and 2B11, cytokeratin clones AE1 and AE3 (cocktail), melanocyte-specific antibody clone HMB 45, S100 polyclonal antibodies (all at the manufacturer-supplied concentrations; CytoLogix), PR clone 1A6 (Dako), myeloma protein MOPC 141, and mouse polyclonal IgG (both at 1 mg/L; Sigma). Each spot was separated from the others on the slide by hydrophobic barriers drawn with a Pap pen (Research Products International Corp.). This prevented cross-contamination of the reagents from one spot to the others. After incubation with the primary antibodies, all 18 spots were then developed with an IHC detection reagent set (CytoLogix), as described previously.

### inhibition of 1D5 mAb binding by peptide

We tested the ability of the peptides to inhibit the binding of ER-specific mAb 1D5 to native ER antigen (in breast cancer tissue sections). If a peptide inhibits binding of mAb to native antigen, the result will be an absence of IHC staining on the tissue section. The ER 1D5 mAb or other irrelevant mAbs were incubated at room temperature (100-μL total volume; ~350 ng of mAb) with various concentrations of peptides. The IHC staining assay was as described above for the IHC staining method and was performed in triplicate on serial tissue sections. Tissue staining was quantified by a microscope-based image analysis program (Image Pro Plus; Media Cybernetics).

### colorimetric intensity measurement of peptide spots

The colorimetric intensity of the IHC-stained peptide spots was measured by scanning the slides with a flat-bed scanner (Perfection 1200U; Epson America, Inc.). The image was stored in Adobe Photoshop. The color intensities of the spots were then quantified by a Scion image program (Scion Corporation).

## Results

### identification of er-specific mAb 1D5 epitope mimics from a phage-display library

For many in situ (e.g., IHC) assays, the analyte is a cell-associated, oftentimes multisubunit, complex glycoprotein. Such proteins are usually in short supply or expensive to manufacture, challenging a manufacturer's ability to generate reproducible, low-cost quality-control material. It is certainly possible to produce such proteins

in recombinant form. The costs associated with such manufacture, however, can generate a product price that most customers would find excessive for a quality-control product. By contrast, short synthetic peptides can be manufactured relatively inexpensively and to high standards of reproducibility, both of which are important features of a quality-control product. We reasoned that if a synthetic peptide can be designed to mimic the antibody binding site of the native antigen, then it could serve as a quality-control target in lieu of the native antigen. This technology is therefore potentially an ideal source of quality-control material for antigens that are scarce or hard to manufacture. We used combinatorial peptide phage-display libraries to identify an ER epitope mimic, using the ER 1D5 mAb.

We sought to isolate phage (from a phage library) with combinatorial peptide inserts that were capable of specifically binding to the antigen-combining region of the ER 1D5 mAb. To do this, we first depleted from the library phage capable of binding to common, shared regions of IgG (see *Materials and Methods*). This was accomplished by panning the library using magnetic beads coated with polyclonal murine IgG. Subsequently, phage that bound to the ER 1D5 mAb were positively selected and enriched (see *Materials and Methods*). After two rounds of selection by biopanning, the input-to-output phage ratio reached a plateau (data not shown), indicating that most of the phage recognized the ER 1D5 mAb. Individual phage clones were then tested for their ability to bind to mAb 1D5 in a phage ELISA (see *Materials and Methods*). Representative data from the phage ELISA for clones from the TN6 and TN10 phage libraries are shown in Fig. 1, panels A and B, respectively. The selected phage clones, even after one round of panning, bound to the target mAb 1D5 (Fig. 1, column ER in group R1), but not to isotype-matched (IgG1) control mAbs (Fig. 1, columns PR and CM3 in group R1). The specificity of the pooled phage library was even better after the second round of enrichment (Fig. 1, column ER in group R2). Virtually all of the phage clones displayed specificity for the ER 1D5 mAb

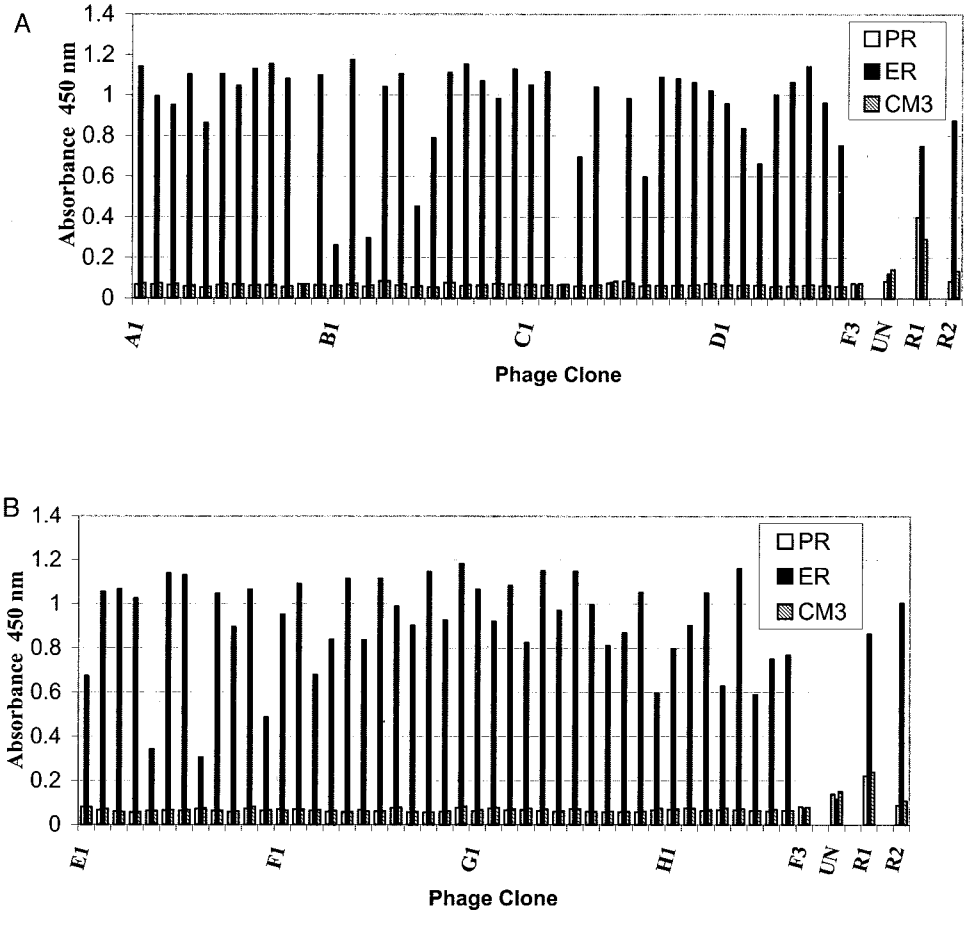


Fig. 1. Reactivity of representative phage clones from TN6 (*A*) and TN10 (*B*) libraries.

Shown are the reactivities of individual phage clones to three murine IgG1 mAbs from a second round of selection against ER 1D5 mAb. Each phage clone was tested for its ability to bind to immobilized PR mAb (□) clone 636 (IgG1), ER mAb (■) clone 1D5 (IgG1), or another IgG1 irrelevant mAb (*CM3*; ▨). Virtually all phage clones demonstrate high binding to ER and little to no specific binding to PR or CM3. Specific clones are listed on the *x axis*, starting at *A1*. There are 12 clones for each group (*A1–A12*, *B1–B12*, *C1–C12*, *D1–D12*). The reactivities of four additional phage samples are shown at the *right* in each *panel*. *F3*, irrelevant phage clone, not specific to ER, PR, or CM3. *UN*, starting phage library (unpanned), before affinity selection. *R1*, phage library pool after the negative depletion against polyclonal mouse IgG and one round of positive selection. *R2*, phage library pool after a subsequent second round of positive selection.

A

| Clone# | | Amino acid sequence | | ELISA:OD |
|---|---|---|---|---|
| A07 | T G S | S W Q C P F W D C G D S | A P G | 0.985 |
| A05 | T G S | D K Q C P V I D C M E Y | A P G | 0.810 |
| A08 | T G S | . M Q C P V L N C S G D | A P G | 1.067 |
| A09 | T G S | A Q Q C P V K N C G I N | A P G | 1.091 |
| A10 | T G S | S H Q C P A L S C A V S | A P G | 1.019 |
| A12 | T G S | L I Q C P A F F C D N A | A P G | 1.040 |
| B02 | T G S | D F Q C P Y V E C V V N | A P G | 1.113 *3 |
| B05 | T G S | V S Q C P Y W E C D D Y | A P G | 1.046 |
| B08 | T G S | F W Q C P F F G C D N F | A P G | 1.053 |
| B07 | T G S | N Q H C P A Y A C Q K P | A P G | 0.744 |
| A06 | T G S | Y Q H C P Y Y D C D V D | A P G | 1.040 |
| A04 | T G P | F E L C K E N D C Q A P | A P G | 1.043 |
| B04 | T G S | V V Y C D A F L C M Y N | A P G | 0.986 |
| B06 | T G S | Y Q D C H K D D C G Q Q | A P G | 0.422 |
| B01 | T G S | F G D C N F M S C Q N P | A P G | 0.245 |
| B03 | T G S | W V S C E H Q M C Q A A | A P G | 0.274 |
| A11 | T G S | V D D C E D Y W C Y Y R | A P G | 0.069 |

B

| Clone | | Amino acid sequence | | ELISA:OD |
|---|---|---|---|---|
| F05 | S | D W A C D Q E P F F T L C S Y H | A | 1.061 *4 |
| F06 | S | D W A C D Q E P F F T L C S Y H | A | 0.792 |
| E02 | S | N H N C D Q S P Y Y L A C V N P | A | 1.001 |
| E05 | S | S L N C H Q S P Y L S Y C H Y P | A | 0.320 |
| E11 | S | Y F D C Q Q S Y Y L P N C F N N | A | 1.006 |
| F07 | S | H D S C A F N Q S P Y F C D H N | A | 1.060 |
| E12 | S | H L H C Q A P Y H N E G C H H F | A | 0.457 |
| F02 | S | H S H C Q A P Y L S M A C L P P | A | 1.039 *6 |
| E10 | S | H H S C Q A P F Y D R D C R N N | A | 0.844 |
| F01 | S | H D F C Q A P W F D E N C N S N | A | 0.905 |
| E09 | S | L S D C D K F Q A P Y V S C A F | N A | 0.993 |
| E03 | S | H S H C G S Q A P Y Y M C S D H | A | 1.013 |
| E08 | S | H L D C Y H Y S Q A P Y C Q S Y | A | 0.287 |
| E06 | S | H P F C D S N Q T P Y Y C F N N | A | 1.083 |
| E07 | S | H D L C T H N Q V P Y F C D N N | A | 1.076 |
| F04 | S | N H H C M N F Q Q P V Y C N N Y | A | 0.795 |
| F03 | S | N D D C Y V D N Q H P Y C H L L | A | 0.643 |
| E01 | S | N Y D C G M Q P W F M N C D Y Y | A | 0.634 |
| F08 | S | N Y V C E K S Y S P V E C D N P | A | 0.938 |

Fig. 2. Amino acid (single-letter codes) sequences of phage clone inserts of ER 1D5 antibody-binding phages from TN6 (*A*) and TN10 (*B*) peptide phage libraries.

The two cysteine residues that form the disulfide bond are *italicized*. Constant amino acids are shown as *regular font outside* the *dotted vertical lines*. Amino acids *within* the *two dotted vertical lines* are the peptide insert and are *bold*. Consensus amino acid sequences are *underlined*. Absorbance (450 nm) readings of individual clones from a phage ELISA are indicated to the *right* of each clone. ·, no amino acid located at this position. **3*, peptide 3; **4*, peptide 4; **6*, peptide 6. *OD*, absorbance.

after two rounds of enrichment. These data strongly demonstrate that the initial negative depletion effectively removed phage clones that recognize shared structural regions of IgG, common to many or all antibodies.

After the second round of positive selection, ~20 phage clones from each of the two libraries (ELISA absorbance readings ≥0.7) were picked for sequence analysis. A few clones with lower absorbance readings were also picked for sequence comparison. These sequences are shown in panels A (for the TN6 library) and B (for the TN10 library) of Fig. 2. Although the clones were rarely identical, the majority demonstrated consensus sequences that were shared among many phage clones. The TN6 consensus sequence was centered on the first conserved cysteine residue (Fig. 2A), whereas the TN10 consensus was within the cysteine ring (Fig. 2B). Both libraries had glutamine (Q), proline (P), tyrosine (Y)/phenylalanine (F), and alanine (A) as the predominant residues in the consensus sequence. As predicted, the low-affinity phage clones that produced only a weak signal in the phage ELISA (e.g., clones B01 and B03 in Fig. 2A) had little similarity to the consensus sequence.

Three of these cyclic peptides (19–21 amino acids long) were synthesized with a few additional modifications (Fig. 3): the $NH_2$ terminus of the peptide was acetylated, the COOH terminus was extended with a lysine-glycine-lysine sequence (KGK), and the COOH-terminal lysine was amidated. As a molecular tag, the penultimate lysine was labeled with fluorescein (Fl attached to sequences in Fig. 3). The ϵ amino group of the ultimate lysine residue was reserved for attachment to the isocyanate-activated glass slide.

Fl $NH_2$

*ER Peptide 3*. Acetyl-**D F Q C P Y V E C V V N** A P G G K G K (19 a.a)

Fl $NH_2$

*ER Peptide 4*. Acetyl-**D W A C D Q E P F F T L C S Y H** A G K G K (21 a.a)

Fl $NH_2$

*ER Peptide 6*. Acetyl-**H S H C Q A P Y L S M A C L P P** A G K G K (21 a.a)

Fig. 3. Amino acid sequences and modifications of the synthetic peptides.

$NH_2$, ϵ amino group belonging to the ultimate lysine, which is the attachment site of the peptide to the isocyanate-activated glass slide. *Fl*, fluorescein group attached to the penultimate lysine. *a.a.*, amino acids.

### SYNTHETIC PEPTIDES SPECIFICALLY BIND THE 1D5 mAb

Initially, the specificity of the ER synthetic peptides was tested in a peptide ELISA, where the peptides were covalently coupled to maleic anhydride-activated polystyrene plates (see *Materials and Methods*). Because small peptides of this size usually do not passively adsorb to unmodified polystyrene wells, we used maleic anhydride-activated polystyrene wells to couple peptides via a free reactive amine (COOH-terminal lysine residue). All three ER peptides specifically bound to the ER 1D5 mAb and not to two other isotype-matched (IgG1) mAbs (Fig. 4). Peptide 3 had the highest binding to ER.

The binding affinities of peptides 3 and 6 were measured in a fluorescence polarization assay. ER peptide 3 had a $K_d$ of $8.3 \times 10^{-8}$ mol/L, whereas ER peptide 6 had a $K_d$ of $3.2 \times 10^{-7}$ mol/L. Antigen-antibody (or ligand-receptor) affinities in this range ($10^{-7}$–$10^{-8}$ mol/L) are generally considered to be moderate. Because ER peptide 3 had the higher binding affinity for the ER 1D5 mAb, it was used for further work.

For the final product, an IHC control or calibrator, peptides need to be covalently attached to glass slides. Therefore, the specificity of the ER peptide was further tested after immobilization to a glass slide, simulating its actual use in the final product. Peptides were applied to isocyanate-activated glass slides (1 $\mu$L per spot), 18 spots per slide. The peptides were allowed to couple to the isocyanate groups on the glass (see *Materials and Methods*). Using a wax pencil, we drew lines between the peptide spots to create a hydrophobic barrier between them. Each spot was then treated with 1 of 15 other mAbs, a polyclonal antibody, or the ER 1D5 mAb. The presence or absence of antibody binding to the slides was demonstrated by IHC detection (see *Materials and Methods*). As was apparent visually (Fig. 5), only the ER 1D5 mAb bound to the peptide (two spots at opposite corners of the slide). Most of the other mAbs that we tested are commonly used for IHC assays and react with various cell-associated antigens.

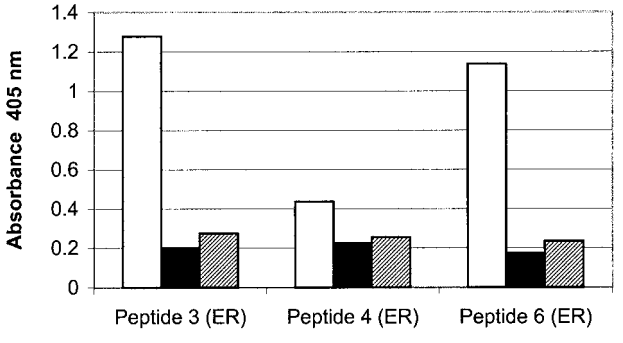


Fig. 4. Immunoreactivity of each of three mAbs with each of three synthetic peptides selected for ER antibody (ER 1D5; □) reactivity.

Each *column* represents the mean absorbance (*y axis*) of triplicate measurements. PR 636 mAb (▨; specific to human PR) and CM3 mAb (■; of irrelevant specificity, IgG1) are isotype-matched murine mAbs. SEs were <5% of the mean and therefore are not shown.

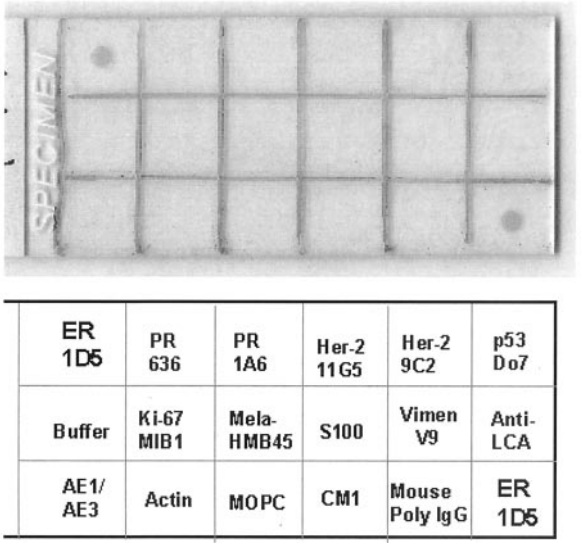


Fig. 5. Specificity of peptide binding to the ER 1D5 mAb on glass slides.

Peptide 3 was applied as a 1-$\mu$L spot, for covalent binding, to the center of each grid location. Various antibodies and controls were subsequently applied to the different grid locations, as described in *Materials and Methods*. The *bottom panel* describes each of the antibodies or controls that were applied to each grid location (see *Materials and Methods* for a description of the antibodies). The ability of the various antibodies to bind to peptide 3 was tested by immunohistochemistry (see *Materials and Methods*). The presence of antibody bound to a peptide spot is therefore revealed as a colored dot. *Her-2 11G5* and *9C2*, human epidermal growth factor receptor type 2 clones 11G5 and 9C2; *Mela-HMB45*, melanocyte-specific antibody clone HMB45; *Vimen V9*, vimentin clone V9; *Anti-LCA*, anti-leukocyte common antigen clones PD7/26 and 2B11, combined as a cocktail; *Mouse poly IgG*, mouse polyclonal IgG.

An additional test of the specificity of the ER peptide involved using the free peptide to inhibit the binding of the ER 1D5 mAb to native ER. This tested whether the peptide was truly binding at or very close to the antigen-binding site on the ER 1D5 mAb. For native ER, we used tissue sections from a breast carcinoma previously demonstrated to express high amounts of ER. ER peptide 3 was premixed, in various concentrations, with the ER 1D5 mAb before application to the tissue section (see *Materials and Methods*). Other irrelevant peptides (specific for other mAbs) were used as negative controls. The ER 1D5 mAb (with various peptides) was then used as the primary antibody in an IHC staining assay (see *Materials and Methods*). We found that ER peptide 3 was capable of inhibiting the binding of ER 1D5 mAb to native ER in a dose-dependent fashion (Fig. 6). Other peptides, at comparable concentrations, failed to inhibit the binding of ER 1D5 mAb to native ER in tissue. Therefore, ER peptide 3 specifically binds at or very near the antigen-binding site on the ER 1D5 mAb.

### PEPTIDE SPOTS AS CALIBRATORS ACROSS A LINEAR ASSAY RANGE

By applying various molar amounts of ER peptide 3 to isocyanate-activated glass slides, we could generate a calibration curve against which tissue staining could be assessed. To do this, doubling dilutions of peptide were

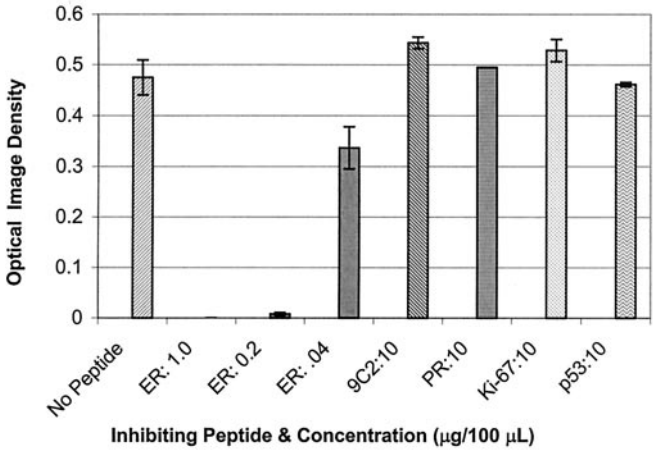


Fig. 6. Inhibition of binding of ER 1D5 mAb to native ER by peptide 3.

The 1D5 mAb was first incubated with or without a peptide (see *Materials and Methods*) and then used to stain an ER-positive breast carcinoma tissue section. IHC-stained tissue images were quantified and plotted as optical image density against the inhibiting peptide concentration. *Number* after the *colon* in each *column label* indicates concentration in μg/100 μL. *Bars*, SE.

applied to the activated glass surface, the highest concentration being 2 μmol/L. This range produced a colorimetric signal that included the linear range for the assay (Fig. 7). The intensities of the spots representing the calibration curve were measured on a flat-bed scanner (see *Materials and Methods*). As can be seen in Fig. 7, the curve began to plateau at the 2 μmol/L range.

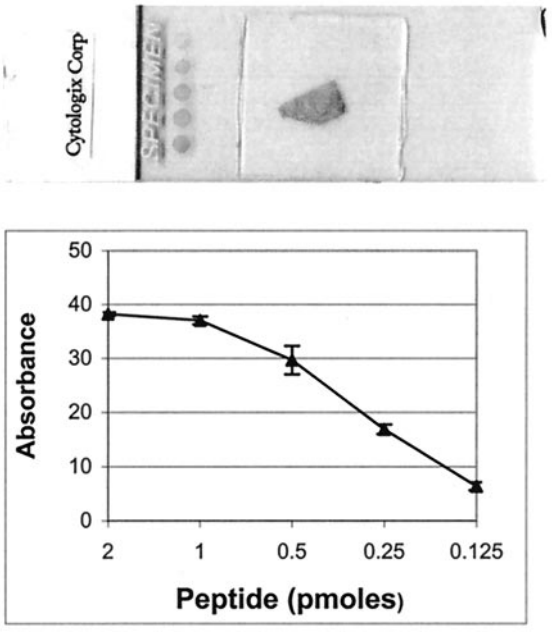


Fig. 7. Linearity of ER 1D5 IHC staining at various concentrations of peptide 3.

(*Top*), photograph of a representative slide showing stained calibrator spots. To show where the tissue section would be mounted relative to the spots, an ER-positive tissue section was also mounted on the same slide and stained. (*Bottom*), mean absorbance (*bars*, ± SE) of spots plotted against the concentration of the peptide. Various concentrations of ER peptide 3 (1 μL of each concentration) were coupled to glass slides and subjected to a standard IHC staining procedure, using the ER 1D5 antibody. Data in the graph are representative of three independently stained peptide slides.

## Discussion

In this report, we introduce a novel approach for creating quality-control material for immunoassays. The approach entails the use of peptides that mimic the mAb binding site of the native antigen. It is potentially useful for analytes that cannot be readily isolated or manufactured at a cost that is commercially practical. Rather than trying to manufacture the analyte by recombinant methods, our approach requires only that a relatively small peptide be chemically synthesized. The peptide is attached to the solid-phase matrix, a glass slide in our case, in lieu of the native antigen. In this fashion, the peptide simulates the native antigen throughout the entire assay procedure. Peptides are far simpler and less expensive to manufacture than most of the analytes of importance in clinical immunohistochemistry.

The major challenge in such an approach is in identifying a peptide sequence that will be recognized in the immunoassay with a high affinity similar to that of the native antigen. Most epitopes recognized by mAbs are conformational rather than linear. Therefore, analysis of overlapping peptides or fragments of the native antigen that represent the linear sequence of the native antigen will not be helpful in identifying the epitope. Phage display is an established, direct method of identifying suitable peptide binders that does not require any prior knowledge about the analyte.

Analysis of the sequences in Fig. 2 reveals a recurring amino acid motif of QXPY, where X can be one of several amino acids. In the TN6 library, X was usually a cysteine (C), a constant residue that is intentionally engineered into the peptide to form a cyclic peptide. In the high-affinity binders from the TN10 library, X was most commonly an alanine (A) or a serine (S). Comparison of this consensus motif to the reported amino acid sequence for human ER (α chain) revealed a close match at positions 127–130. At those positions, native ER contains the sequence QVPY. This region is within the B domain of the ER protein, in agreement with the manufacturer's information that the antibody reacts within the A or B domains of ER. It is unclear how the three-dimensional structure of our cyclic peptides would relate to the native conformation of the true epitope.

Phage display, using combinatorial peptide libraries, is a relatively straightforward method for identifying suitable peptides for binding to the antigen-binding site of mAbs *(27)*. The combinatorial libraries that we used had $\sim 10^8$ different peptide combinations. We used the ER-specific 1D5 mAb to select a high-affinity peptide from the library. Our primary concern was whether the peptide would be specific for only the 1D5 mAb. With such a high degree of diversity ($10^8$), there were peptide sequences capable of binding to many different regions of the IgG molecule. To identify peptides specific for only the anti-

gen-combining region of 1D5, we first performed a subtractive panning step. We initially removed those phage having peptide inserts that recognized the common, shared structural features of IgG. The phage library was then positively selected with the desired mAb (the ER 1D5 mAb).

The ER was chosen as a first subject of this approach because it is an important, quantitative analyte measured by clinical immunohistochemistry laboratories. Currently, there are no commercially available quantitative ER controls. Although ER quantification has important therapeutic implications, studies have shown that interlaboratory reproducibility is suboptimal *(9–11, 13)*. One of the reasons for this problem is the inability of laboratories to quantitatively detect out-of-range assay conditions. This problem is partly a result of the need for each laboratory to obtain and validate its own control material. Moreover, there are no internationally agreed-on reference materials. Even if there were, there is no practical way to disseminate those standards to laboratories through the use of standardized control material. It is our hope that these surrogate peptide analytes might serve that purpose, thereby improving patient care. We have subsequently developed peptides to mAbs that recognize the PR, the Ki-67 antigen, p53, and HER-2. These analytes are used for determining tumor prognosis and/or therapy, especially in breast cancer.

Peptides have previously been proposed as assay surrogates, although to our knowledge, this has not been reduced to practice in a clinical context *(28)*. A limitation of using peptides as surrogate controls is that this approach is useful for only mAbs. As a small molecule, the peptide is capable of simulating only a single epitope, such as one recognized by a mAb. Our approach will therefore not be suitable for immunoassays that use polyclonal antibodies because such antibody preparations are directed against multiple epitopes of a protein. Fortunately, virtually all antibodies that are used in clinical immunohistochemistry laboratories are monoclonal in nature.

Another drawback of peptide surrogate controls is that they will not be useful for other ER mAbs. In the field of clinical IHC testing, this is not likely to be a major problem. Most clinical immunohistochemistry laboratories use one of two clones for ER testing, either the 1D5 or the 6F11 mAb *(10, 11)*. Thus, there is relatively little diversity in mAb clone selection among clinical immunohistochemistry laboratories. This is a result of the relatively stringent performance requirements for mAbs in clinical IHC testing. Not only do the mAbs need to recognize their target analyte, they must further react with the target after formalin fixation and paraffin embedding *(29)*. Relatively few mAbs have that capability, even with the use of antigen-retrieval techniques. Therefore, a single peptide, such as described here for the ER 1D5 mAb, will have broad applicability for a large percentage of clinical immunohistochemistry laboratories.

Peptides as surrogate controls offer major advantages over the use of tissue biopsies as controls. Most importantly, the scarcity of standardized tissue resources precludes their wide dissemination for daily clinical laboratory use *(30)*. Tissue sections have been used successfully for proficiency surveys, such as those sponsored by the College of American Pathologists (US) or National External Quality Assessment Scheme (UK). However, such surveys are sent out only a few times each year and therefore place a much more limited demand on tissue resources. By contrast, no single central laboratory or manufacturer has a tissue bank large enough to supply the daily needs of clinical laboratories. Even if such tissues could be procured, the challenge of validating each biopsy specimen would place an untenable burden on the central supply facility.

Recently, there has been interest in the concept of using immortalized cell lines in lieu of tissue sections as IHC controls *(31, 32)*. Such cell lines can be grown in vitro and then embedded in paraffin or agarose for sectioning and mounting on glass microscope slides. Cell lines offer the advantage that they are potentially available in unlimited quantities. This feature overcomes a critical limitation of tissues as controls. Unlike tissue biopsies, cell lines can be grown in vitro to any desired quantity. Despite these features, however, cell lines have several major drawbacks as quality-control material. Generally, the amount of an analyte, such as ER, within a tumor cell line will be somewhat heterogeneous, depending on cell cycle and growth conditions. Consequently, different batches of cells may not express consistent amounts of the analyte. In addition, the amount of any particular analyte expressed by the cells can drift over time. This phenomenon has been attributed to the outgrowth of subclones that express higher or lower amounts of the analyte. Therefore, a manufacturer will need to periodically subclone the cells to force them to express a predefined amount of analyte. This need further complicates their manufacture and increases the cost. Furthermore, sectioning and mounting sections of cell blocks is labor-intensive and therefore relatively expensive.

The peptide spots lend themselves well to quantitative analysis. We have found that decrements in antibody concentration, simulating antibody failure, are reflected in less intense color formation on the peptide spot (data not shown). This technology is therefore well suited to identifying out-of-range assay conditions attributable to errors in technique, instrument failure, or reagent degradation. If the amount of peptide on the slide is consistent and stable across production lots, then the resulting color intensity of the peptide spots after IHC staining can be quantified and compared among institutions. This technology can therefore be useful in fostering interlaboratory standardization. Moreover, the color intensity can be logged over time within a single institution, thereby creating a Levey–Jennings type of charting capability *(33)*.

Unlike most other clinical laboratory tests, this capability does not currently exist for IHC assays.

In conclusion, we present a novel approach toward developing quality-control material for analytes that are scarce or difficult to manufacture. The peptides are analyte specific because they are mAb specific. The peptide has a specificity and an ability to bind to the mAb comparable to those of the analyte itself. As a source of unlimited and highly reproducible target material, synthetic peptides may be valuable in developing quality-control products for the immunohistochemistry laboratory. We foresee the possible use of this approach to solve numerous quality assurance challenges in developing reproducible and standardized quantitative IHC methodologies. The same approach may also be useful for in vitro diagnostic assays when the analytes are scarce, biohazardous, or otherwise difficult to manufacture.

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