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Evaluation of Protein Levels of the Receptor Tyrosine Kinase ErbB3 in Serum

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Abstract

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTK) consists of four members: EGFR1/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, and HER4/ErbB4. Signaling through these receptors regulates many key cellular activities, such as cell division, migration, adhesion, differentiation, and apoptosis. The ErbB family has been shown to be overexpressed in different types of cancers and is a target of several inhibitors already in clinical trials. ErbB3 lacks a functional tyrosine kinase domain and therefore has not been as extensively studied as the other members of this family, but its importance in activating downstream pathways, such as the PI3K/Akt pathway, makes this RTK a worthy investigation target, especially in urothelial carcinoma where the PI3K/Akt pathway is vital for progression. In recent times, ErbB3 overexpression has been linked to drug resistance and progression of various diseases, especially cancer. ErbB3 levels in the serum were shown in many cases to be reflective of its role in disease progression, and therefore detection of serum ErbB3 levels during treatment may be of importance.

Here we describe two methods for detecting ErbB3 protein in serum from patients who have undergone a clinical trial, utilizing two well-established methods in molecular biology—western blotting and ELISA, focusing on sample preparation and troubleshooting.

Keywords

EGFR; ERBB3; Serum; Urothelial carcinoma; Western blot; Elisa

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[4.]Notes

[13.]Serum samples will show a lot of background on western blots. There are kits to clean up IgG and albumin background that will appear around 50 and 65 kDa, respectively. Since we were looking for ErbB3, which is 180 kDa, we had no background influence in the desired bands. Also we suggest using a loading control that is away from the range of IgG and albumin sizes.

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1 Introduction

V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 3 (ErbB3), also known as Human Epidermal Growth Factor Receptor 3 (HER3), is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTK) [1]. While both EGFR and HER2/ErbB2, the first two members of this family that were discovered, have been well investigated in various diseases including cancer [2], in cardiac [3] or neural function [4, 5], as well as in other instances; ErbB3 and the fourth member, ErbB4/HER4, were not given due diligence, at least until recently. ErbB3, especially, has been under-investigated in cancer and other diseases because, unlike other members of this family, its tyrosine kinase domain is functionally defective [6]. Both ErbB3 and ErbB4 are activated by ligand binding with the neuregulin family of growth factors [7], and heterodimerize with other members of the family, especially ErbB2, for complete activation. However, despite the lack of kinase activity, it was discovered that ErbB3 signals effectively to downstream targets, especially the phosphoinositide-3-kinase (PI3K) pathway [8] through binding sites in the intracellular domain. Interest in this RTK really peaked when it was shown that overexpression of ErbB3 caused resistance of various cancers to inhibitors of EGFR and ErbB2 [9]. Since then, monoclonal antibodies to ErbB3 have been developed in an effort to target this RTK [10], and the role of this protein in cancer development and progression was granted a much closer look.

Muscle invasive bladder cancer (MIBC) constitute 33% of initial cases of urothelial carcinoma (UC) while the remainder are classified as non-muscle invasive bladder cancer (NMIBC) [11]. NMIBC is usually treated with transurethral resection of bladder tumor (TURBT) followed by either a single dose of intravesical chemotherapy or intravesical Bacillus Calmette-Guérin (BCG) [12]. In contrast, the majority of patients presenting with MIBC undergo radical cystectomy (RC), alone or following platinum-based neoadjuvant chemotherapy [13, 14]. Upon development of metastases, cytotoxic chemotherapy with the combination of cisplatin and gemcitabine (GC) as a first-line treatment is usually accepted [15].

The urothelium consists of three prominent layers—the superficial urothelium (umbrella cell layer), intermediate urothelial cells and basal urothelial cells. In the normal urothelium, ErbB3 is expressed primarily on the superficial cells but lower expression of ErbB3 may be seen in the other layers as well [16]. Multiple studies demonstrated a positive association between ErbB3 and tumor size, number, and histological grade [17–22]. Furthermore, ErbB3 was found to be a good predictor of first tumor recurrence [17]. ErbB3 expression may moreover be a good biomarker to detect the efficacy of ErbB inhibitors [23]. A phase II study of 59 patients with MIBC to determine the efficacy of the dual EGFR/ErbB2 inhibitor lapatinib as a second-line therapy following disease progression on prior platinum-based chemotherapy found that overall survival (OS) was significantly prolonged in patients with ErbB3 overexpressing tumors ($p = 0.001$) [24].

There are several ErbB3 transcripts that are transcribed in various tissues to form protein isoforms of different sizes. Full-length human ErbB3 is a 180 kDa glycoprotein [25]. As described in more detail in a previous publication [26], this RTK consists of an extracellular

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ligand-binding domain consisting of four subdomains (I, II, III, IV), a transmembrane domain (TM) and a cytoplasmic region consisting of a tyrosine kinase domain (TKD) and a C-terminal domain (CTD) [25, 26] (Fig. 1). *ErbB3* has been shown to encode two other alternate forms resulting from alternately spliced variants—a p85 protein formed by extracellular subdomains I, II, and III and part of IV, with addition of 24 unique C-terminal amino acids [27], and a p45 form that consists of extracellular subdomains I and II and part of subdomain III, plus 2 unique C-terminal amino acids [28, 29] (Fig. 1). Because these forms lack the transmembrane and cytoplasmic domains, they are easily secreted outside the cell and are labeled soluble ErbB3 (sErbB3). The p85 and p45 forms, similar to full-length ErbB3, bind neuregulins, but are unable to transduce signals to downstream targets inside the cell. Many investigators have therefore thought of these truncated forms of ErbB3 as negative regulators of neuregulin signaling; however, studies show that p45ErbB3 is a bone metastasis factor [30].

Significantly, it was found that many of these isoforms of ErbB3 could be detected in the serum or plasma [27, 30, 31]. Since ErbB3 overexpression has been associated with resistance to a large number of therapies in some cancers [32–34], whereas other cancers are thought to be sensitized to certain therapies by ErbB3 expression [35, 36], a blood marker of ErbB3 expression would be useful, as it is noninvasive and can be detected relatively easily. Therefore, we determined to identify methods for detecting ErbB3 levels in the serum, especially in patients undergoing therapy for cancer.

Here we describe techniques to detect ErbB3 levels in samples obtained from patients on a clinical trial at the UC Davis Comprehensive Cancer Center. The blood from these patients was collected at the time of treatment and separated into two parts—one was fractionated to serum and the other to plasma and peripheral blood mononuclear cells (PBMC). Our laboratory received samples of separated serum for analysis. The serum samples were frozen immediately following collection and stored at 80 $^{\circ}$C in aliquots of 0.5 mL or less, to avoid freeze–thaw cycles, until the time of the analysis. To detect the levels of ErbB3 in the serum samples we utilized two methods of protein detection commonly used in molecular biology: western blotting and enzyme-linked immunosorbent assays (ELISA). These are described in detail in the protocols in Subheadings 2 and 3.

ELISAs were developed for the detection of a target substance within a liquid sample, in this specific case ErbB3 protein in the serum following outlines described by others [37]. ELISAs rely upon relatively specific antibody–antigen interactions, and reporter-linked antibodies for detection and quantification of the analyte. It is therefore a rapid test to quantify or detect a specific antibody (Ab) or antigen (Ag).

There are four types of ELISAs: direct, indirect, competitive, and sandwich:

1. In a direct ELISA, the antigen-coated plate is detected by an antibody that is already conjugated with an enzyme ready for detection.
2. In an indirect ELISA, an unlabeled antibody is used first to bind to the antigen-coated plate, and then a secondary antibody, now conjugated with an enzyme, binds to the first antibody.

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3. In competitive ELISA, the solution is pre-mixed with a known amount of enzyme-conjugated antigen that will then compete in the plate for the coated capture antibody.
4. In a sandwich ELISA, the plate is coated with the Ab against the desired Ag, the sample is added and then the detection Ab is allowed to bind to any captured Ag; next, a secondary enzyme-linked Ab is added to the mix and allows the substrate to be chromatographically detected.

Of the four, the sandwich ELISA was deemed by us to be the most sensitive for our current needs (Fig. 2). It utilizes two primary antibodies—the detection antibody and the antibody against the desired antigen, the "capture" antibody. Because one capture antibody can bind to multiple detection antibodies, this assay amplifies the signal, making it extremely sensitive. Such a sensitive assay would be needed for the detection of small amounts of protein in serum samples.

While ELISA is easy to use, it has certain disadvantages. The single biggest problem is that if the antibody recognizes more than one isoform of the protein, as is the case for a multi-isoform protein such as ErbB3, it is impossible to determine which isoform is being expressed. The capture antibody in the ELISA used above is directed against the N-terminal region of ErbB3, so theoretically it would recognize all three isoforms identified in Fig. 1. To distinguish between the three isoforms of ErbB3 in the serum, we used Western blotting. Western blotting or simply immunoblotting is an easy method to analyze the presence of specific proteins in a tissue lysate or sample extract. This method utilizes electrophoresis to separate proteins in a polyacrylamide gel based on their isoelectric point, molecular weight or electric charge in a one-dimension gel or combination of these properties in a two-dimension gel. These proteins are then transferred onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane for detection. In our laboratory, we utilize molecular weight to identify specific proteins in a membrane and the steps for this protocol will be discussed below.

Comparison of the results for ErbB3 levels over time from a single patient shown in Fig. 3 illustrates that ELISA and Western blotting yield similar results. The ELISA capture and detection antibodies were against the N-terminal ErbB3 where all three isoforms had identical sequences. Therefore, the ELISA would not distinguish between the three isoforms. In contrast, the Western blot analysis revealed the three isoforms, however, the similarity between the 180 kDa band of ErbB3 in the Western blot with the ELISA, but not the other two isoforms, indicates that the ELISA is detecting p180 ErbB3 and not the other isoforms.

2 Materials

2.1 Determination of Protein Concentrations

1. Pierce bicinchoninic acid assay (BCA assay).
2. Spectrophotometer with capacity to read 450 nm.
3. 96-well plate.

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4. 4× Laemmli Sample Buffer Stock; 10 mL separating buffer, 40 mL glycerol, 10 g sodium dodecyl sulfate (SDS), water to complete 100 mL. Aliquoted in 1.5 mL tubes and kept at −20 °C.

2.2 Enzyme-Linked Immunosorbent Assay (ELISA) for ErbB3

We identified a sandwich ELISA kit from Abcam (ErbB3 Human ELISA kit ab100511) that had specifically been optimized for serum samples. In this kit is included:

1. ErbB3-coated plate: A 96-well plate coated with an anti-ErbB3 antibody that recognizes extracellular portion of the ErbB3 protein (the capture antibody).
2. 20× wash buffer (*see* TBST).
3. Assay diluents A and B (for serum/plasma (diluent A) or cells in suspension/ urine (diluent B), respectively. Since we did not use diluent B for our serum experiments, all reference to diluents are for diluent A).
4. Biotinylated anti-human ErbB3: This is the detection antibody.
5. Recombinant ErbB3 standards,
6. 200× horseradish peroxidase (HRP)-Streptavidin concentrate,
7. Tetramethylbenzidine (TMB) one-step stop solution.

2.3 Western Blotting

All reagents are kept in room temperature unless stated otherwise. Water used must be deionized water.

1. 30% Acrylamide.
2. Sodium dodecyl sulfate (SDS) (10% solution in water).
3. Ammonium persulfate (APS) (40% solution in water).
4. Glycerol (50% solution in water).
5. Tetramethylethylenediamine (TEMED).
6. Mini PROTEAN spacer plates and casting frame stand.
7. Isobutanol.
8. Bromophenol blue.
9. 2-Mercaptoethanol.
10. Stacking Buffer; 60.6 g Tris (0.5 M), 4 g SDS (0.4%), water to 1 L, pH 6.8.
11. Separating Buffer; 181.8 g Tris (1.5 M), 4 g SDS (0.4%), water to 1 L, pH 8.8.
12. 10× stock Running Buffer, 30.3 g Tris, 144 g glycine, 10 g SDS in 1 L water, pH 8.3 (if adjustment is needed). Dilute to 1× in water before use.
13. 10× stock Transfer Buffer; 24.2 g Tris, 45 g glycine in 1 L of water. Dilute to 1× and add 20% methanol before use. Store in 4 °C.

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14. 4× Laemmli Sample Buffer Stock; 10 mL separating buffer, 40 mL glycerol, 10 g SDS; water to 100 mL. Aliquoted in 1.5 mL tubes and kept at –20 °C.
15. 20× Tris-buffered saline stock with Tween (TBST); 242.2 g Tris, 210.4 g NaCl in 2 L of water, pH 7.4. Dilute to 1× in water before use. Add 10% Tween.
16. Skim milk powder.
17. Polyvinylidene difluoride (PVDF) membrane.
18. Chromatography paper.
19. Mini-PROTEAN 3 Electrophoresis Cell and Mini Trans-Blot Electrophoretic Transfer Cell ® with all accessories.
20. Pre-stained protein standards.
21. X-Ray film.
22. Supersignal West Femto maximum sensitivity Substrate (ThermoFisher scientific).

3 Methods

3.1 Determination of Protein Concentrations

The bicinchoninic acid assay (BCA assay) kit is used to estimate protein content in the serum samples. This assay determines protein concentrations from a standard curve with known protein contents.

1. Prepare the BCA standards by diluting the known protein sample (provided in the kit, typically bovine serum albumin). The solvent in which the standards are diluted is also provided as part of the kit and is used as the blank controls in the assay. The standards (0–2 μg/mL) are loaded in triplicate in a 96-well plate alongside the blanks which receive the solvent alone. It is advisable to have at least 5–6 standards with known protein concentrations in order to be able to accurately estimate the unknowns.
2. Serum samples are serially diluted in 1×Laemmli sample buffer to match the protein range compatible with the standards (*see* Note 1 below). Run triplicates of the undetermined samples on the same 96-well plate as the standards. As a general rule of thumb, samples in different plates cannot usually be correlated. A different set of standards should therefore be used for each plate. Read the 96-well plate using a spectrophotometer (Powerwave X plate reader, BioTek, Winooski, VT, USA) at 450 nm.
3. Determine the samples' protein concentration by calculating the mean of the triplicates of each standard and subtracting the average value of the blanks from this mean. Plot the results against the corresponding known concentration. The slope of the plot and the y-intercept can be calculated from the data

[1] For ELISA. Serum samples should be diluted in 1× sample buffer prior to use. We find that a 1:20–1:40 dilution provides best readings from the plate and it is within the protein range of the standards provided with the Abcam kit.

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(demonstrated in Fig. 4a). These parameters can then be used to determine the total protein concentrations of the unknown samples (Fig. 4b). Based on this concentration, the volume of sample required for each assay can be calculated and the samples can be used for ELISA or for Western blotting.

3.2 Enzyme-Linked Immunosorbent Assay (ELISA) for ErbB3

1. Dilute all reagents from concentrate to 1× before starting. The 50 ng/mL stock solution is prepared by adding 400 μL of 1× Diluent A into the **recombinant human ErbB3 standard.** Standards are prepared by serial dilution in diluent A as described in Fig. 5. Diluent A alone is used as control.
2. Serum samples should be diluted in diluent A to ensure the sample protein contents are in the range of the standards used. 100 μL of each standard and diluted samples should be added into appropriate wells (*see* Note 2 below), considering that at least three (or four) replicates/duplicates should be used for each standard and samples. Incubate the plate at 4 °C on a rocker overnight (*see* Note 3 below).
3. The next morning, discard the solution and wash each well by adding 200–300 μL of 1× wash buffer (diluted from 20× supplied), discarding the buffer and inverting the plate onto absorbent paper to remove the remaining buffer (*see* Note 4 below). The washing process should be repeated three times.
4. Add 100 μL of 1× **Biotinylated ErbB3 detection antibody** to each well and incubate for 1 h at room temperature on a shaker (*see* Note 5 below).
5. Discard the solution and repeat the washing steps.
6. Add 100 μL of **HRP-Streptavidin solution** to each well and incubate for 45 min at room temperature on a shaker, followed by three washing steps.
7. Add 100 μL of the One-step substrate reagent to each well and incubate for 30 min in the dark with light shaking. Do not discard this mixture.
8. Follow by adding 50 μL of TMB **stop solution** onto each well and immediately reading the preparation at 450 nm.
9. To analyze the data, the readings from the spectrophotometer are plotted against the corresponding concentrations for the standards (0–2500 pg/mL). Protein concentrations are calculated from the standard curve as explained above for BCA.

2. Using a reagent vessel helps facilitate the washes and reagent distribution if using a multichannel pipette.
3. Seal the plate with sealing film to avoid evaporation of reagents when incubating overnight.
4. In each wash, keep a stack of paper towels to blot the plate upside down after dispensing the wash buffer, making sure to remove all washing buffer from the wells before going into the next steps.
5. Try to avoid forming bubbles when adding the reagents by touching the side of the well when dispensing reagents into each well.

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3.3 Western Blotting

Polyacrylamide gels have two phases, a stacking phase where the proteins are packed in one band and a separating phase where the proteins are separated by molecular weight. These gels can have different polyacrylamide concentrations associated with large or small pores to separate proteins of different sizes. Full-length ErbB3 runs at 180 kDa. Therefore we recommend 6–8% SDS gels.

1. To cast a 6% resolution gel (*see* Note 6 below), add 2 mL of 30% acrylamide, 2.45 mL of Separating buffer, 5.4 mL of water, 0.1 mL of 10% SDS solution in water, 0.04 mL of 50% glycerol solution in water, 0.0135 mL of 40% APS solution in water, and 0.01 mL of TEMED to a 50 mL tube (*see* Note 7 below). Mix by inverting the tube and dispense the solution with a pipette between the plates in the casting stand leaving enough space for the casting gels and well combs. For more than one gel, adjust quantities accordingly.
2. To assure even polymerization, add 200–300 μL of water or isobutanol to the top of the gel and wait until gel sets.
3. In a separate tube, prepare the stacking gel by mixing 0.95 mL of 30% acrylamide, 1.25 mL of stacking buffer, 3 mL of water, 0.1 mL of 10% SDS solution, 0.005 mL of 50% glycerol solution, 0.01 mL 40% APS solution and 0.01 mL of TEMED and mix it by inversion. After dispensing the water or isobutanol from the top of the resolution gel, dispense the stacking gel on top of the separating gel and place the desired well comb to form the loading wells, allowing it to set.
4. Once gel has set, remove the plates from the casting stand and slowly remove the well combs, place the plates with the gel in the Mini-PROTEAN 3 Electrophoresis Cell and fill the chamber to indicated amount with 1×running buffer (*see* Note 8 below).
5. Prepare each sample by mixing the predetermined amount of serum sample to load 30–50 μg of protein (*see* Note 9 below) from the BCA assay with 1×sample buffer to bring volume to 19 μL and then adding 1 μL of bromophenol blue mixed in 2-mercaptoethanol (dip a clean pipette tip in bromophenol blue and mix in 400 μL of 2-mercaptoethanol.
6. Vortex the samples and heat at 95 °C for 5 min (*see* Note 10 below). Load the samples into the wells with appropriate protein standards and run electrophoresis at 150 V for 2 h or until desired protein standard separation.

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[6]. When putting the plates together for casting the gel, it is a good way to seal the bottom and sides of the plates using laboratory film before installing it into the clips and onto the stand. Simply cut a strip of the film and stretch it on the bottom of the plates making a seal, and then sliding it into the clips.

[7]. Polymerization of the gels is more even and faster if the 40% APS solution in water is freshly made. Prepare aliquots in small 0.5 mL tubes and replace them often.

[8]. For all buffers prepared, add half the amount of water to the graduated cylinder before starting to add any powder reagent. Allow the magnetic stir bar to stably stir and add reagents in small portions. Wear a mask when weighting powdered reagents.

[9]. Different well combs will produce different well sizes, and we found that a final sample amount of 20 μL fit most wells

[10]. Heat the samples at 95 °C for about 5 min before you load them into the gel, spinning them briefly afterwards to collect the entire sample in the bottom of the tube. Plan to load 2–3 μL less than final volume to account for pipetting errors.

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7. Following electrophoresis, separate the plates and remove the stacking gel portion with the preformed combs and move the gel into a container with 1× transfer buffer (*see* Note 11 below). Label a 2.5″3.5 ″ PVDF membrane and soak it in 100% pure methanol for 1 min, discard the methanol and keep it in transfer buffer.

8. Set up the transfer by placing the gel and PVDF membrane in between two 3" 4" filter paper and sponges inside a cassette accordingly to manufactures directions, and slide inside transfer cell stand. Fill the chamber with 1× transfer buffer, adding the cooling unit and running the electrophoretic transfer at stable 200 mA in 4 $^{\circ}$C room for 2 h.

9. Next remove the membrane from the apparatus and place it in a container for 5 min washes with TBST. Repeat the washes five times.

10. Block the membrane by submerging it in 10 mL of 5% skim milk dissolved in TBST for 1 h on a rocker followed by five TBST washes. The membrane is now ready to receive desired primary antibody (Santa Cruz (SC-285)) diluted as indicated by the manufacturer overnight on a rocker at 4 $^{\circ}$C (*see* Note 12–14 below).

11. Next day take the primary antibody off the membrane and wash it with TBST five more times.

12. Prepare the secondary antibody (Jackson Immunoresearch goat anti-rabbit IgG (111–035–045)) at 1:10,000 dilution in 2.5% skim milk solution in TBST and incubate on rocker for 1–2 h at room temperature. Wash blots once again with TBST five times.

13. To visualize the transferred proteins, mix in a 10 mL tube 1:1 parts of the two substrates found in the Supersignal West Femto kit and dilute it with water to 1:10 dilution to complete to 5 mL total volume (*see* Note 15, 16 for adjusting concentration). Allow the substrate to bind for 2 min on a rocker and place the membrane in between plastic sheets inside the cassette. In a dark room, place the x-ray film on top of the membrane for 1 min and pass it through the developer. Adjust time of exposure accordingly. (*see* Note 17 for quantitative analysis of western blots).

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[11]. Transfer buffer takes 20% pure methanol, which should be added right before preparing the transfer. Dilute the 20× transfer buffer in water to 1× leaving space enough for 20% methanol and allow it to cool down before using.

[12]. For primary antibodies dilution, start at 1:1000 in TBST and test the strength of the signal shown in the x-ray film. Adjust the concentration accordingly to save antibody. Secondary antibody dilution should be adjusted as well if too much background is found on the film.

[14]. When dealing with small sample quantity, a membrane which was already blotted for a specific primary can be blotted for another primary even if the size bands are similar. For that purpose, we recommend using a stripping solution such as Restore from ThermoFisher and incubating at 37 $^{\circ}$C for 15 min completely submerged and then washing with TBST five times before incubating with new antibody.

[15]. The 1:1 developing solution diluted to 1:10 in water should be adjusted depending on the signal strength of the primary antibody. If not sure of how strong the signal is, start the final dilution at 1:40 and increase as necessary.

[16]. When preparing the 1:1 developing solution, it is best to mix them inside the dark room since it is light sensitive. Keeping the solution in a dark place while working allows reuse of the solution for several membranes.

[17]. For Western blot quantitative analysis, we suggest the use of image quantification software such as ImageJ that can relatively quantify individual bands in a single gel to show fold increase/ decrease in band intensity. To quantify the bands, open the image in ImageJ then with the rectangular tool make a selection including all bands you wish to quantify. Go to *Analyze > gels > Select first*

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lane to highlight the selection. Then go to *Analyze > gels > Plot lanes.* A Plot with all the selected lanes will appear in a separate box. If the bands are well separated, there will be a clear depression in between the bands indicating the limits of each band. With the straight line tool, draw a line from the lower part of the peaks and the bottom of the graph. Then using the wand tool, click in each individual peak representing each band. A new window will open with the quantification values, which can be used for times fold calculations.

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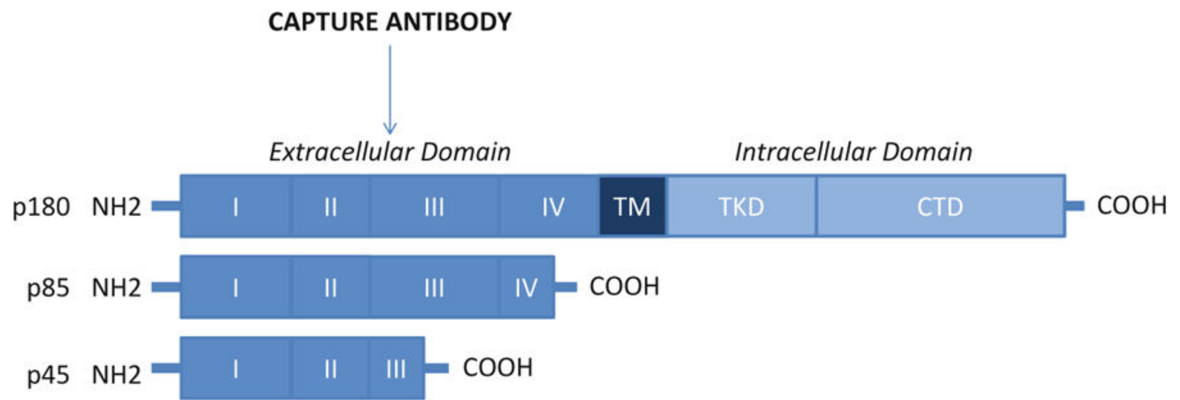

Fig. 1.
Schematic representation of different splice variants of ErbB3 (p180, p85 and p45). Full-length ErbB3 consists of an extracellular ligand-binding domain consisting of four subdomains (I, II, III, IV), a transmembrane domain (TM) and a cytoplasmic region consisting of a tyrosine kinase domain (TKD) and a C-terminal domain (CTD). The p85 isoform of ErbB3 is formed by subdomains I, II and III and part of IV, with addition of 24 unique C-terminal amino acids, whereas the p45 isoform consists of extracellular subdomains I and II and part of domain III, plus 2 unique C-terminal amino acids. Note that all three forms are capable of binding the common ligands of ErbB3—neuregulins 1 and 2, but only the full-length one is capable of transmitting intracellular signals. The capture antibody coated in the 96-well plate recognizes the extracellular domain common to all three isoforms

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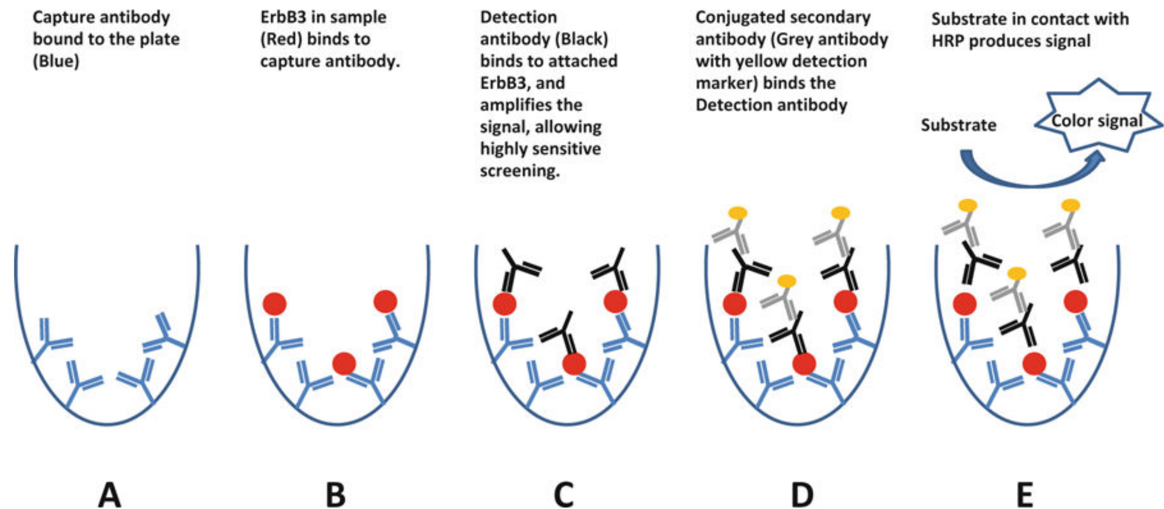

Fig. 2.

Schematic representation of a sandwich ELISA using a pre-coated plate with the capture antibody. (**a**) The walls of the well are pre-coated with the antibody. (**b**) Sample is added to the wells and the antigen binds to capture antibodies. (**c**) After washing nonattached antigens, a primary detection antibody (biotinylated ErbB3) is added to amplify the signal (**d**) followed by the conjugated secondary antibody (HRP-conjugated streptavidin). (**e**) The TMB substrate solution is added to each well developing a color signal with intensity proportional to the amount of bound ErbB3 from the sample

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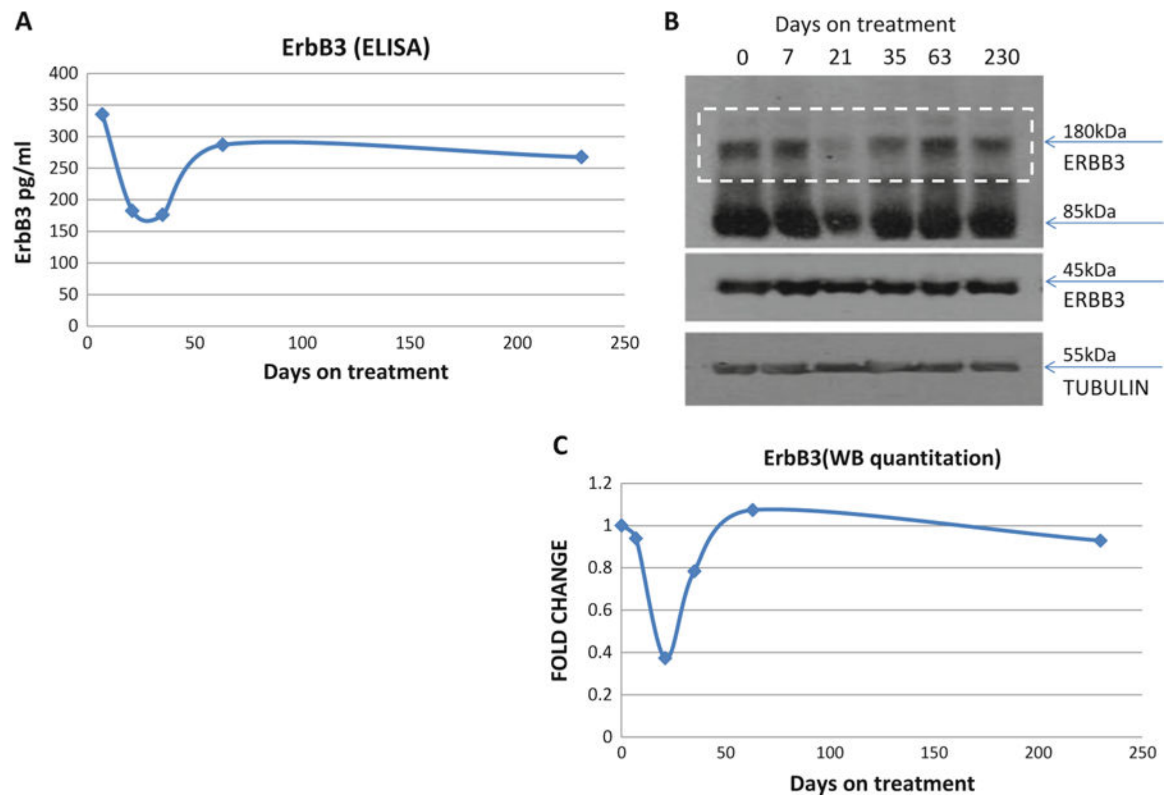

Fig. 3.

Graphic representation of two different results to detect (**a**) ErbB3 levels in one patient collected on different days of treatment. (**b**) Western blots of serum from the same patient immunoblotted for ErbB3. (**c**) Graphic representation of fold change from western blot results using tubulin as a control and imageJ for quantification

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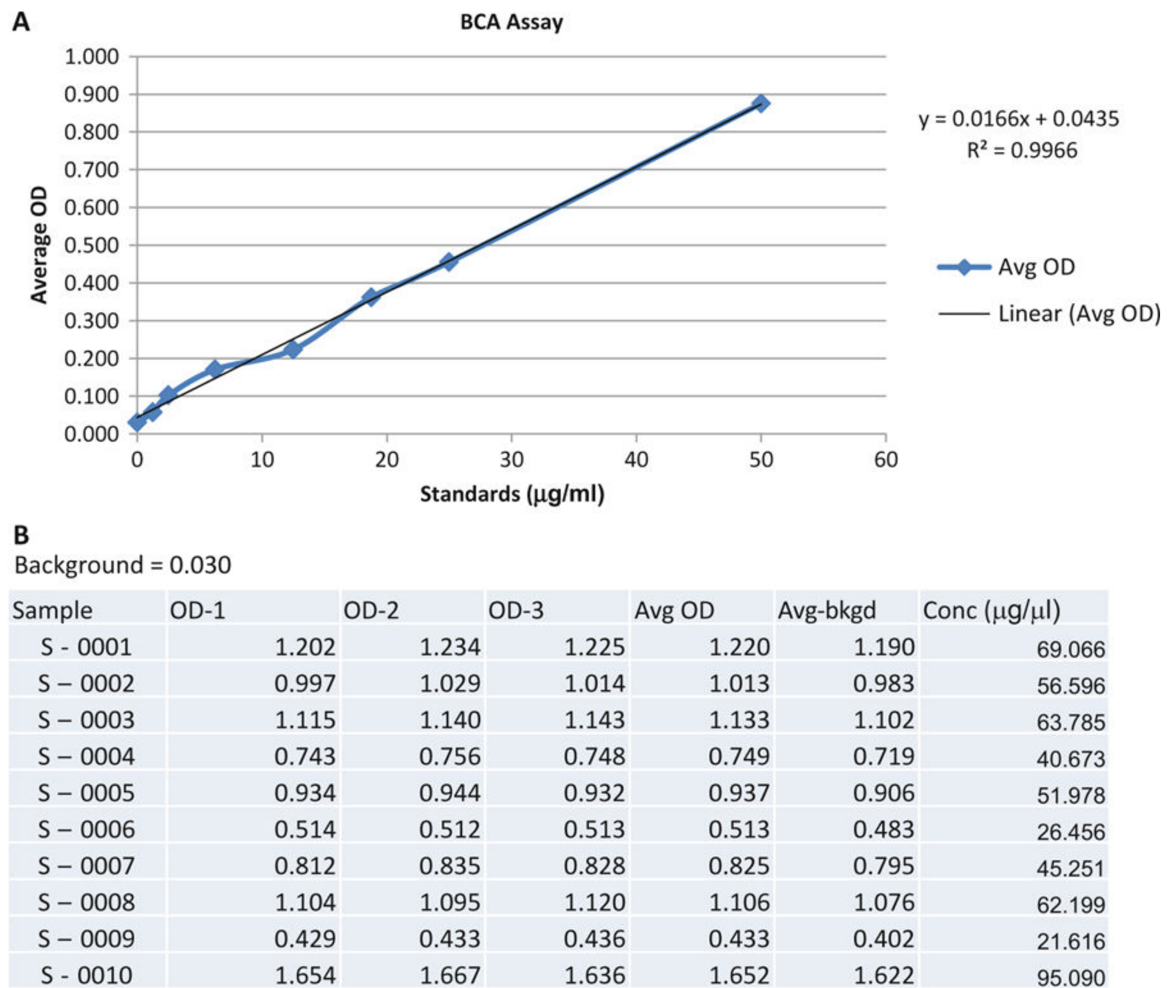

Background = 0.030

Sample	OD-1	OD-2	OD-3	Avg OD	Avg-bkgd	Conc (μg/μl)
S - 0001	1.202	1.234	1.225	1.220	1.190	69.066
S – 0002	0.997	1.029	1.014	1.013	0.983	56.596
S – 0003	1.115	1.140	1.143	1.133	1.102	63.785
S – 0004	0.743	0.756	0.748	0.749	0.719	40.673
S – 0005	0.934	0.944	0.932	0.937	0.906	51.978
S – 0006	0.514	0.512	0.513	0.513	0.483	26.456
S – 0007	0.812	0.835	0.828	0.825	0.795	45.251
S – 0008	1.104	1.095	1.120	1.106	1.076	62.199
S – 0009	0.429	0.433	0.436	0.433	0.402	21.616
S - 0010	1.654	1.667	1.636	1.652	1.622	95.090

Fig. 4.

Methodology to calculate protein concentrations. (**a**) Graphic demonstration of BCA assay data plotted in a scatter graph with average optic density (OD) in *y* and Standards concentration (μg/mL) in *x* axis. A trend line can be drawn from the points yielding an equation (**b**) Table showing individual readings from samples, average OD, average OD with background substracted and the concentration obtained from the trend line equation

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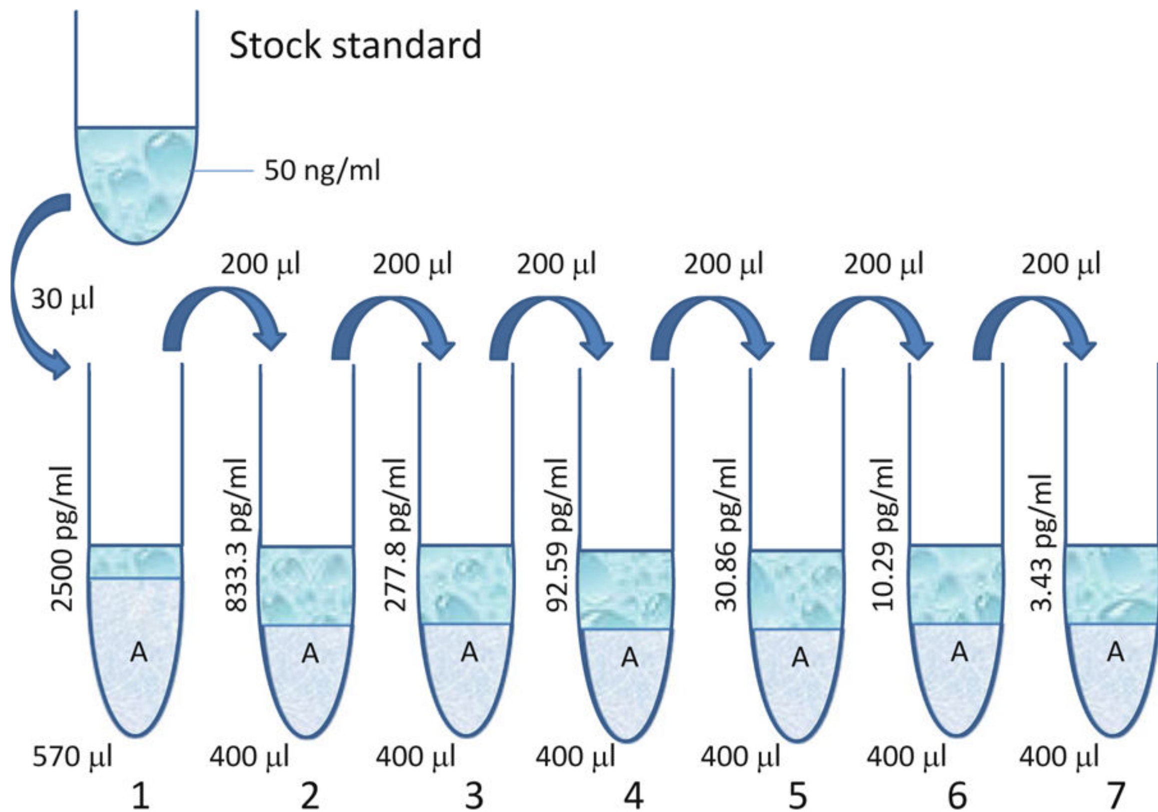

Fig. 5.

Dilution of recombinant human ErbB3 standard stocks for ELISA. The 50 ng/mL stock solution is prepared through serial dilution of the **recombinant human ErbB3 standard** with 1×diluent A. Label seven [7] tubes from 1 to 7, adding 570 μL of assay diluent A into tube #1 and 400 μL of diluent A into tubes #2–7. Prepare standard #1 by adding 30 μL of stock standard (to 600 μL) and mixing thoroughly (2500 pg/mL). Prepare standard #2 by adding 200 μL of standard #1 into tube #2 (which already has 400 μL, bringing the volume to 600 μL) and mixing it (833.3 pg/mL). Prepare tubes #3 to #7 by repeating the process of adding 200 μL from the previous one until tube #7