# Novel Phosphor Specific Antibodies Developed by RIMMS Method Binds to Human Oestrogen Receptor Beta Phosphorylation Site at Position 105

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**Abstract:** Phosphorylation of oestrogen receptor alpha (ERα) has been proven to be involved in the progression of breast cancer and similarly increasing evidence shows that oestrogen receptor beta (ERβ) too phosphorylated at multiple sites upon ligand binding although the exact function of this site-specific phosphorylation is unknown. Nevertheless it is assumed that the site-specific phosphorylation of ERβ may be involved in the progression of breast cancer and targeting these sites could help in treatment strategies in the control of cancer progression. To test this hypothesis novel monoclonal antibodies have been developed against synthetic peptide specific for putative serine phosphorylation sites in human ERß (S105) adopting repetitive immunisation of multiple immunization strategy. The novel antibodies reacted strongly against phosphor peptide on ELISA and on the cancerous breast tissue samples providing clear evidence of phosphorylation of oestrogen receptor beta during the advanced stages of breast cancer. We believe that these antibodies could help in the advancement of breast cancer research.
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**Key words:**oestrogen receptor beta, Breast cancer, monoclonal antibodies, phosphor specific anti-peptide antibodies

## Introduction

Oestrogen is an important sex steroid hormone in the regulation of human physiology including growth of different tissues, immune system, nervous system and reproductive system. Among these tissues, oestrogen is an indispensable protein in the growth of mammary gland and uterine endometrium formation during pregnancy and the menstrual cycle (Keator et. al, 2011). In addition to its effects on cell proliferation on normal cells, oestrogen is implicated in the development or progression of cancer including breast cancer, ovarian, colorectal, and prostate cancer. Studies show that prolonged exposure to oestrogen i.e. early menarche, late menopause and oestrogen replacement therapy can increase the risk of breast and uterine cancers (Green et. al, 2012). Oestrogen conducts its mechanism of action through one of the oestrogen receptors oestrogen receptor alpha (ERα) or oestrogen receptor beta (ERβ). This interaction depends not only on the availability of oestrogen but also on the types of ERs and their levels in these cells. Oestrogen receptors are predominantly found in the nucleus and rightly classified as nuclear receptors, nevertheless fraction of these receptors are also found in the cell membranes (Jiang et. al, 1997). ER levels are low in normal breast tissue and high levels have been directly correlated with an increased risk of breast cancer (Su et. al, 2012; Khan et. al, 1994). Receptor levels increase with age in some ethnic groups and are usually higher in white women than in black or Japanese women (Chu et. al, 2002). Studies show that phosphorylation of ERα can be important in regulating gene expression and it has been proposed that this could contribute to cancer progression (Gustafsson et. al; 2010 and Shah et. al; 2005). To date several phosphorylation sites in the ERα have been identified (Murphy et al 2011; Lannigan, 2003 and Tremblay et. al, 1999), (Figure 1) and the hypothesis is that this may have roles in physiological and pathophysiological activities (Ali, et. al, 2002). More specifically, it is closely associated with the progression of human breast cancer and the development of resistance to endocrine therapies (Murphy et al, 2011 and 2006; Lam et al 2012). In the same way as ERα , several positions have been identified as phosphorylation sites in ERβ and have been proven to play an important role in the development and progression of human breast cancer (Palmieri et. al, 2002 and Lannigan, 2003) (Figure 2). A previous study by the author (Meerasahib et. al., 2011) has shown that phosphorylation of serine molecules in the N-terminus (Serine87) is involved in the progression of breast cancer whilst Hamilton-Burke et. al. 2010 used phosphor-S105 antibodies to demonstrate that phosphorylation of S105 is associated with good prognosis in breast cancer. Experts in the field of breast cancer believe that targeting ERβ phosphorylation pathways would be s

novel approach in the advanced endocrine therapy adopting ERβ as a better predictor of prognosis and treatment response in breast cancer. In this project, for the first time we have successfully developed highly specific novel phosphor monoclonal antibodies adopting Repetitive Immunization Multiple Sites (RIMMS) technique. In this procedure, mice were immunised with immunogen containing phosphorylated synthetic peptide in multiple sites close to draining lymph nodes for twenty five days before removing lymph nodes for fusion with myeloma cells. We believe that these phosphor antibodies to putative phosphorylation site (epitope containing Serine87) of oestrogen receptor beta would be of highly useful in further understanding of the role of oestrogen receptor beta in the progression of breast cancer. Whilst Hamilton-Burke et al (2010) used phosphor-S105 antibodies to demonstrate that phosphorylation of S105 is associated with good prognosis in breast cancer.

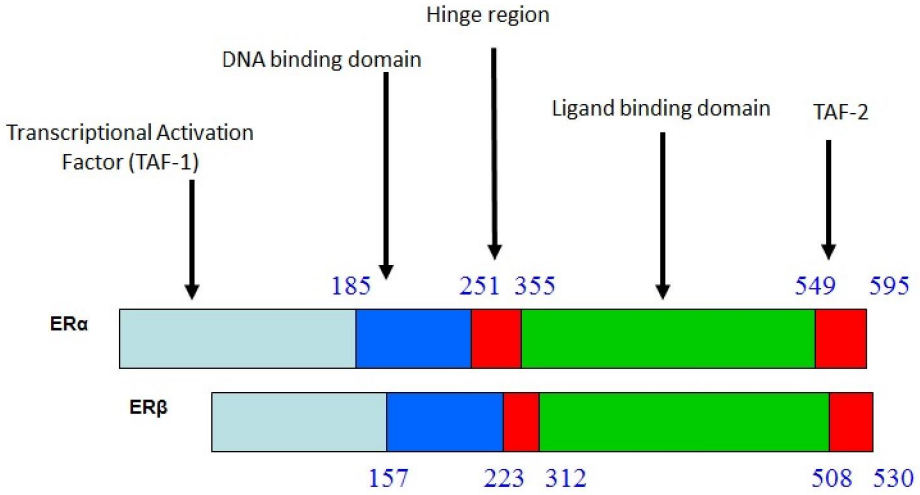


**Figure 1:** structure of oestrogen receptor alpha (595 aa) and beta (530 aa) genes. Homology of domains is presented in percentage (adopted from Lannigan, 2003).

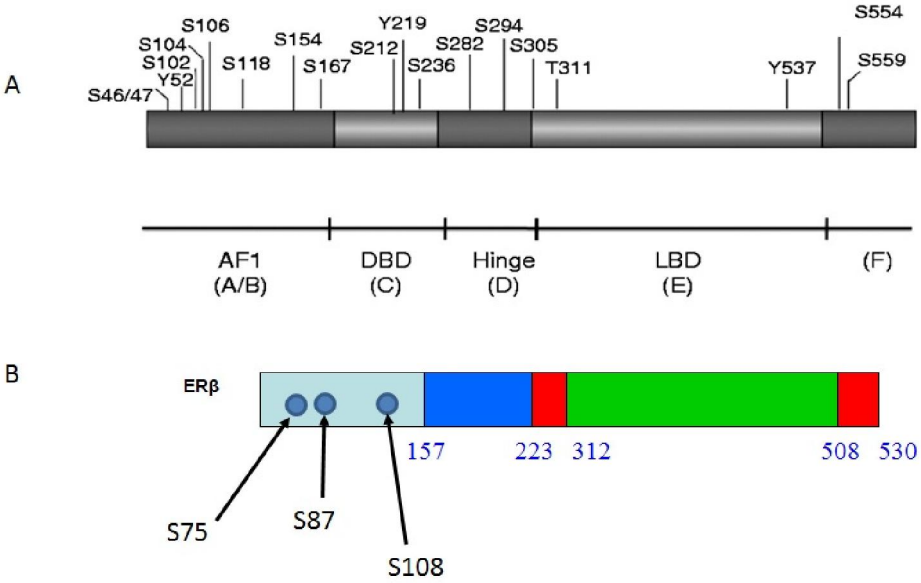


**Figure 2:** (A), Schematic diagram of human ERα multiple phosphorylated sites (A) (Murphy et al. 2011) Figure depicts different structural (A, B, C, D, E) and functional domains: activation function 1 (AF1), DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD). * S- serine; T- threonine; Y-tyrosine. (B), Identified Phosphorylation sites of oestrogen receptor beta (ERβ) receptor. Lam et al 2012 and Hamilton-Burk et al. 2010).

## 2. Materials and Methods

### Selection of antigens

An immunogenic peptide was selected from the ER beta protein sequence (putative phophorylation sites) to be used as immunogens. Phosphor peptide (includes phosphorylated Serine molecule) was commercially synthesised (BIOMOL International UK).

Peptide -YAEPQK-Ser($PO_3H_2$)-PWCEAR

Below is the amino acid sequence of human oestrogen receptor beta protein. The Position of the peptide chosen as immunogen is shaded,

MDIKNSPSSL NSPSSYNCSQ SILPLEHGSI YIPSSYVDSH HEYPAMTFYS PAVMNYSIPS
61 NVTNLEGGPG RQTTSPNVLW PTPGHLSPLV VHRQLSHL**YA**
**EPQKSPWCEAR**SLEHTLPVN
121 RETLKRKVSG NRCASPVTGP GSKRDAHFCA VCSDYASGYH YGVWSCEGCK AFFKRSIQGH
181 NDYICPATNQ CTIDKNRRKS CQACRLRKCY EVGMVKCGSR RERCGYRLVR RQRSADEQLH
241 CAGKAKRSGG HAPRVRELLL DALSPEQLVL TLLEAEPPHV LISRPSAPFT EASMMMSLTK
301 LADKELVHMI SWAKKIPGFV ELSLFDQVRL LESCWMEVLM MGLMWRSIDH PGKLIFAPDL
361 VLDRDEGKCV EGILEIFDML LATTSRFREL KLQHKEYLCV KAMILLNSSM YPLVTATQDA
421 DSSRKLAHLL NAVTDALVWV IAKSGISSQQ QSMRLANLLM LLSHVRHASN KGMEHLLNMK
481 CKNVVPVYDL LLEMLNAHVL RGCKSSITGS ECSPAEDSKS KEGSQNPQSQ

### Peptide coupling:

Above purified peptide (10mg) was coupled to a carrier protein (tuberculin) as described in our previous studies (Meerasahib et al, 2011). A set of female mice (T/O or Balb/C strain mice) aged 8-12 weeks were immunized with antigen emulsified in Freund's incomplete adjuvant (FICA,GibciBRL, Grand Iisland, NY) and boosted with the same doses using RIBI adjuvant (Immun, Chem. Research, Inc., Hamilton, MT). Immunogen was administered subcutaneously into the upper area of forelegs and hind legs proximal to draining lymph nodes over the course of twenty five days (day 0, day 5, day 10, day 17 and day 21 and day 25). For each dose immunogen was diluted in equal amount of FCA and emulsified thoroughly before delivery (dose consisted of 10μg of immunogen in 0.1ml/ site). Mice were then boosted every five days for further 20 days with the same dose.

### Preparation of oestrogen receptor beta nuclear extracts:

Nuclear extracts were obtained from transiently transfected HEK293 cells expressing ERβ1 and ERβ2, this was then stimulated with EGF to phosphorylate the oestrogen receptors. The following steps were undertaken in the preparation of phosphorylated form of oestrogen receptor beta protein.

- Determination of ligand-dependent phosphorylation of ERβ
- Preparation of nuclear extracts
- Immunoprecipitation
- Immunoblotting to determine the presence of phosphorylated oestrogen receptor beta protein

### Determination of ligand-dependent phosphorylation of ERβ

Initially we investigated whether ERβ isoforms would be phosphorylated at serine residues in response to growth factors by transfecting HEK 293 cells with ERβ1 or ERβ2 GFP tagged plasmids and using an anti GFP antibody to immunoprecipitate GFP tagged proteins. This would allow only GFP tagged proteins to be run on a western blot which we could then use a nonspecific phosphor serine antibody to detect any phosphorylated serine molecule. By comparing EGF (epidermal growth factor) stimulated and un-stimulated cells we could determine if the expression of phosphor serine was increased in the stimulated cells.

### Preparation of nuclear extracts:

HEK 293 cells (ECACC no 85120602) obtained from the European Collection of Cell Cultures (Porton Down, UK) were maintained in Dulbecco's modified essential medium (DMEM, Sigma, Poole, UK) supplemented with 10% foetal bovine serum (FBS), 2mM glutamine, 100U penicillin, streptomycin and 0.25ug/ml fungizone (Invitrogen, Paisley, UK) at 37°C in 5%$CO_2$.

For nuclear extracts, cells were plated in 6 well tissue culture plates at $1x10^6$ cells/ml in phenol red free DMEM with 10% charcoal stripped FBS (CSFBS) for 24 hours prior to transfection. Cells were transiently transfected with 1ug of a plasmid expressing full length cDNA to ERbeta 1 tagged with GFP (pERb1GFP) or ERbeta 2 tagged with GFP (pERb2GFP using 2ul of JetPEI (Polyplus transfection Inc, New York, USA) in phenol red, serum and antibiotics free DMEM for 4 hours at 37°C in 5% $CO_2$. The media was removed and fresh media containing 10% CSFBS was added. The cells were incubated for 48 hours. Serum free media was added 24 hours prior to stimulation. Cells were stimulated with $10^{-8}$M epidermal growth factor (EGF, Sigma) for 30 minutes in order for the ERβ1 to be phosphorylated. Media was removed and the cells were washed in cold PBS before adding 0.8ml of ice cold NP-40 lysis buffer. Cells were scraped using a cell scraper into the buffer and transferred to an ice cold tube, vortexed for 10s followed by centrifugation at 13000rpm for 15 mins at 4°C.

**Immunoprecipitation:**

Samples in NP-40 lysis buffer were boiled for 5mins before the addition of 75ul Protein G Plus/Protein A-agarose (Calbiochem) and incubated overnight at 4°C. The lysates were centrifuged at 12000g for 20s at 4°C and supernatants transferred to a new cold tube. One ul of anti GFP antibody, (Molecular Probes cat no. A11122) was added to the lysate and incubated for 1hr followed by the addition of 20ul of protein G plus/protein A and a further incubation overnight at 4°C. The samples were centrifuged at 12000g for 20s followed by careful removal of the supernatant so as not to disturb the beads. The beads were re-suspended in 1ml of wash buffer 1 and incubated for 20 min on a rocker at 4°C.

The beads were again pelleted by centrifuging at 12000g for 20s at 4°C. The pellet was re-suspended in 1ml of wash buffer 2 and incubated for 20min on a rocker for 20mins at 4°C. A further spin at 12000g for 20s at 4°C was carried out with the pellet being re-suspended in 1ml of wash buffer 3 and incubated on rocker for 20 minutes at 4°C. A final spin at 12000g for 20s was carried out and the supernatant removed to a new tube. Loading buffer was added and the samples heated at 70°C for 10mins before loading onto a 4-12% polyacrylamide gel (see appendix for buffers).

**Immunoblotting to determine the presence of phosphorylated oestrogen receptor beta protein**

Gels were run at 120 volts in 1x SDS loading buffer for 50 mins. The separated proteins were transferred to Immobulin membrane and blocked in blocking buffer (5% milk in TBS/tween) for 1hr at room temperature on shaker. A 1:500 dilution of anti-mouse phosphor serine antibody (Sigma, UK) was added and the blots were incubated overnight at 4°C on a shaker. Membranes were washed 4x 5 mins in TBS tween at room temperature on shaker. Anti-mouse HRP antibody (Sapu) was added at 1:5000 in 5% milk/TBS/Tween for 1 hour at room temperature on shaker. Membranes were washed 3x in TBS/Tween for 5 mins each at room temperature before developing using ECL Plus (Amersham) and exposing to X-ray film. Membranes were stripped and re-probed with the anti-mouse GFP antibody (1:500) or 1:50 dilution of anti-mouse ER beta (Figure 3).

**Polyethylene glycol induced somatic cell fusion and the development of hybridoma**

After the final boost on day 25, lymph nodes of axillary, popliteal, superficial inguinal and brachial were harvested and pooled together in IMDM cell culture media. Lymph nodes were washed in IMDM cell culture medium for three times before it was further processed. These lymph nodes were then homogenized in order to extract a single cell suspension which then subsequently centrifuged at 2000RPM to collect lymphocyte cells. These cells were then mixed with myeloma cells (non-antibody producing mouse B cells) (SP2/0) at a ratio of 1:2 in a falcon tube to which 1.5ml of polyethylene glycol (PEGMW1500 Roche, USA) was added drop-wise while mixing over a period of 1minute in a water bath at 37°C. The cell suspension was continuously mixed for another minute to avoid cell aggregation and then 9ml of IMDM cell culture media was added drop-wise over a period of 3 minutes (1ml in 60seconds, 3ml in 60 seconds and 5ml in 60 seconds). Finally, the total volume was made up to 40ml and incubated at 37°C for 10 minutes (5% $CO_2$) after which period, cell suspension was centrifuged at 1000RPM for 10 minutes to separate the fused cell mixture. After discarding the IMDM, the palette was re-suspended in 3ml of HAT (HAT-hypoxanthine-aminopterin, thymidine)hybridoma culture medium (supplemented with 0.1% gentamycine and 15% of bovine fetal calf serum) and added to 90ml of methylcellulose medium (Clonacell-HY, StemCells Technologies UK) After mixing thoroughly this cell mixture was then transferred to a flask and incubated (incubator supplied with 5% $CO_2$ at 37°C) for an hour. Finally the cell mixture was plated out in Petri dishes and placed in the incubator (7-10 days) for hybridoma clones to develop. Subsequently individual colonies of clones were picked (1100 clones) and grown in HAT hybridoma culture medium in tissue culture plates in order for collecting supernatants. After four days into culturing hybridoma clones, 30µl of supernatants from each clone was screened on ELISA (described below) against phosphorylated oestrogen receptor beta peptide, nuclear extract that contained phosphorylated oestrogen receptor beta and nuclear extract with unphosphorylated oestrogen receptor beta protein.

**Step I: coating a microplate with the peptide (uncoupled peptide)**

Peptide was dissolved in 0.2M sodium carbonate-sodium bicarbonate buffer, pH9.4 which was then added to a microplate (96 well-NuncMaxisorb plates-SLS, UK) at a concentration of 1µg/ml (50µl/well) and incubated at room temperature on a shaking platform for two hours. The plate was dried by inverting vigorously onto a soft tissue and blocked with ELISA blocking buffer (1%BSA (w/v) in PBS) (100µl/well) and agitated for 30 minutes at room temperature.

**Step II: Addition of primary antibody**

Dilution of supernatants (primary antibody) were made in Tris conjugate buffer (25mM Tris buffer pH 7.4, 0.05% w/v Tween-20 and 0.5% w/v BSA) (1:1 supernatant: Tris buffer). Before the addition of samples, plates were washed with wash

solution (0.05M Tris-HCl buffer containing 0.15M NaCl and 0.05% (w/v) Tween-20 (Sigma) for 3-5 times in order to remove unbound and loosely bound proteins. Samples (50µl/well) were added to duplicate wells on the ELISA plate and incubated at room temperature on a shaking platform for an hour.

**Step III: Addition of Secondary antibody (anti-mouse antibody)**

After a thorough wash with wash solution (3-5 times), the secondary antibody was added at 50µl/well (Peroxidase conjugated goat anti mouse IgG –1 in 1000 dilution in Tris conjugate) and incubated for 30 minutes at room temperature.

**Step IV: Addition of substrate**

Plates were washed again thoroughly with wash solution five times and this was followed by washes with distilled water for another five times. The plates were dried and equal amounts of substrate solution A and B (hydrogen peroxidase substrate and tetramethylbenzidine -Insight biotechnology) was mixed and added to the wells (50µl/well) and left in the dark for 5-10 minutes for the reaction to develop. The reaction was stopped by the addition of ortho-phosphoric acid (6% v/v; 50µl/well). Finally, the absorbance reading at 450nm was measured by using a plate reader (Benchmark-Bio-Rad). Serum samples from two unimmunised mice were used as controls in these experiments.

**Selection of highly specific clones**

Highly positive clones identified in the initial screening of supernatants were grown in 24 well cell culture plates in HAT media supplemented with methyl cellulose. Each well contained several hundreds of cells derived from a single clone in 1.5ml of media. Conditioned media were harvested from each well when medium is turned orange/yellow (acidic) colour and these wells were replenished with fresh media for continued cell growth. Serial dilutions [($10^{-2}$, $10^{-3}$,$10^{-4}$, $10^{-}5$, $10^{-6}$ and$10^{-7}$) of each conditioned media was made with Tris conjugate buffer ((25mM Tris buffer pH 7.4, 0.05% w/v Tween-20 and 0.5% w/v BSA) and added to micro plate coated with nuclear extracts that contained phosphorylated oestrogen receptor beta protein (figure 5). Finally ELISA was developed as described above and absorbance readings were taken at 450nm using a microplate reader (Benchmark-Bio-Rad, UK).

**Immunolocalisation of novel phosphor-specific monoclonal antibodies**

Cancerous breast tissue samples of diverse clinical stages (purchased from Bioserve, Beltsville USA) were serially sectioned at 5 µm thick and mounted on poly-l-lysine (VWR International Ltd, Lutterworth, UK) coated slides. These slides were then de waxed twice in Histoclear (National Diagnostic, East Riding, UK) for 5 minutes each and rehydrated through an alcohol series (BDH Analar; 100%, 100%, 70%, 2 minutes each), followed by antigen retrieval in sodium citrate buffer (pH 6.0)at high temperature for 20 minutes. Then, sections were quenched for 30 minutes in 3% hydrogen peroxide in methanol to block endogenous peroxidases activity. Sections were incubated with 20% (v/v) normal goat serum in PBS supplemented with 4% (w/v) BSA to reduce nonspecific antibody binding. After washing with PBS, sections were incubated with tissue culture supernatants of mouse anti phosphor antibodies (oestrogen receptor beta) overnight at 4°C. Next day, sections were washed with PBS and a secondary antibody, goat anti-mouse immunoglobulin (1:200 in PBS; DakoCytomation Ltd, Ely, UK) was added and incubated at room temperature for 30 minutes. After which stage, sections were washed with PBS and incubated for 60 minutes with peroxidase-conjugated avidin biotin complex (ABC; diluted 1:200 in PBS; Vector Labs, Peterborough, UK). Following washing with PBS, the peroxidase activity was developed with 0.07% 3, 3'-diaminobenzidine tetrahydrochloride (ABC; Zymed labs, Cambridge, UK). Finally, sections were counterstained for 2 minutes with Erlich'shaematoxylin. One section served as a negative control (omitting the addition of supernatants). Slides were examined using an E600 microscope (NikonUK Ltd, Kingston-Upon-Thames, UK) (figure 6).

**3. Results:**

Upon completion of the immunization stage of the procedure, fusion of pooled peripheral lymph nodes cells with myeloma cells (SP2/0) produced large number of hybridoma clones. The primary screening was carried out on the phosphorylated and un-phosphorylated peptides in order to select clones that are specific to the phosphorylated peptide. Out of 1100 supernatant screened, 876 (79.6%) displayed specificity to the phosphorylated peptide and 54 (4.9%) clones reacted against both phosphorylated and un-phosphorylated peptides. The secondary screening was designed to select the clones which are specific to the native protein. This was carried out using nuclear extracts which contained of phosphorylated oestrogen receptor beta. We determined the presence of phosphorylated form of oestrogen receptor beta in the nuclear extract preparation through immunoblotting, which showed a strong black band corresponding to the site where anti phosphor serine antibody detected a phophorylated serine (figure 3).This outcome is a clear demonstration that the serine molecule at position 105 of the oestrogen receptor beta protein contained in the nuclear extract preparation is

phosphorylated. The positive clones identified in the primary screening, [876 (79.6%) of clones] were tested on phosphorylated and unphosphorylatednuclear extracts side by side. There were 792 (90.4%) supernatants positively reacted exclusively to the nuclear extract which contained phosphorylated oestrogen receptor protein (figure 4).

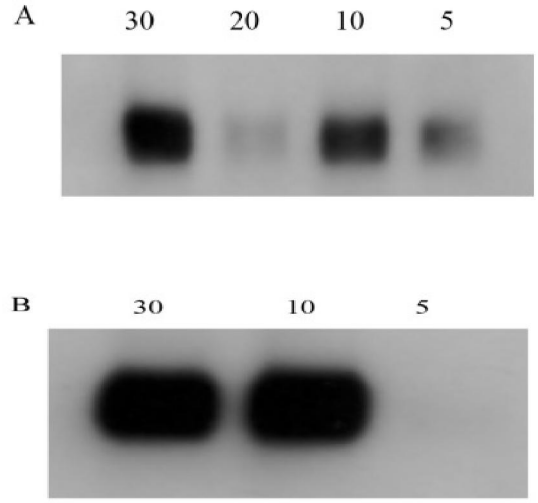


**Figure 3:** Each lane was loaded with 20ug of total protein extracted from HEK 293 cells that had been transiently transfected to express either (**A.** ERβ1) or (**B.** ERβ2) and had been stimulated with EGF for 5, 10, 20 or 30 mins. The black band corresponds to where the anti-phosphor serine antibody has detected a phosphorylated serine.

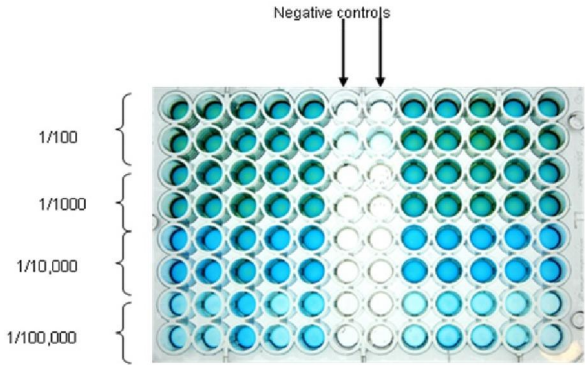


**Figure 4:** Hybridoma culture supernatants titrated against phosphorylated oestrogen receptor beta peptide on ELISA. Test showed that serial dilutions of all samples reacted strongly against oestrogen receptor phosphor-peptide. Serum samples from two unimmunised mice were used as negative control in this experiment did not show any reaction.

This test also identified 179 clones (20.4%) that cross reacted with both phosphorylated and un-phosphorylated nuclear extracts. Overall, 613 (55.72%) clones (out of 1100 clones) were identified as specific to the phosphorylated oestrogen beta receptor protein. After the preliminary assessment of the supernatants as described above, high titreantibody secreting hybridomas (39 clones) were selected for further characterisation. Through repeated titration on microplates coated with nuclear extract that contained phosphorylated version of oestrogen receptor beta protein, 12 highly sensitive, highly specific hybridomas with titres of up to $10^{-6}$ were selected (figure 5).

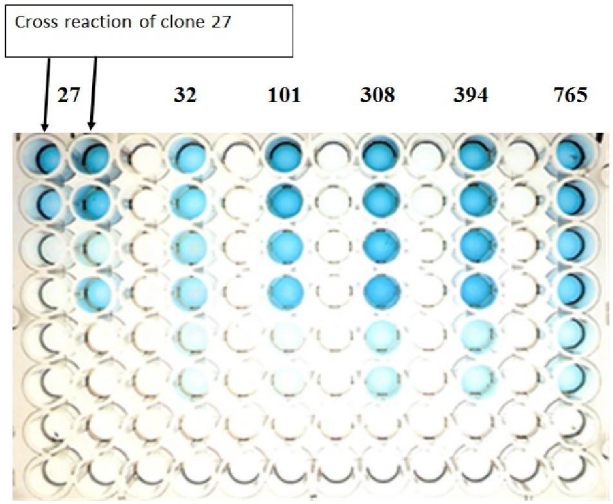


**Figure 5:** Titratration of selected clones (hybridoma supernatants derived from specific clones 27, 32, 101, 303, 394 and 765) against un-phosphorylated and phosphorylated (alternative columns respectively) nuclear extracts (contained oestrogen receptor beta protein) coated side by side. Plate shows cross reaction of clone 27 against both phosphorylated and un-phosphorylated proteins.

These 12 clones were again tested on a selection of negative controls. No cross reaction was observed with any of the supernatant on unrelated phosphoserine containing peptides (VTA-Ser($PO_3H_2$)-RTWAAVLC or ATW-Ser($PO_3H_2$)-VTAWLWC) or an unrelated phosphorylated protein (histone) (not shown). To further establish the specificity of the novel monoclonal antibodies, all high titre antibody producing clones (39 clones) were subjected to immunohistochemical tests. Undiluted hybridomasupernatants were tested on sections of breast tissues that are at various progressive stages of breast cancer. This immunolocalisation test showed strong binding of these antibodies against the putative phosphorylation sites of oestrogen receptor beta protein (figure 6). The test further demonstrated that the amount of oestrogen receptor beta phosphorylation in breast cancer varied according to the severity of the clinical stages of breast cancer.

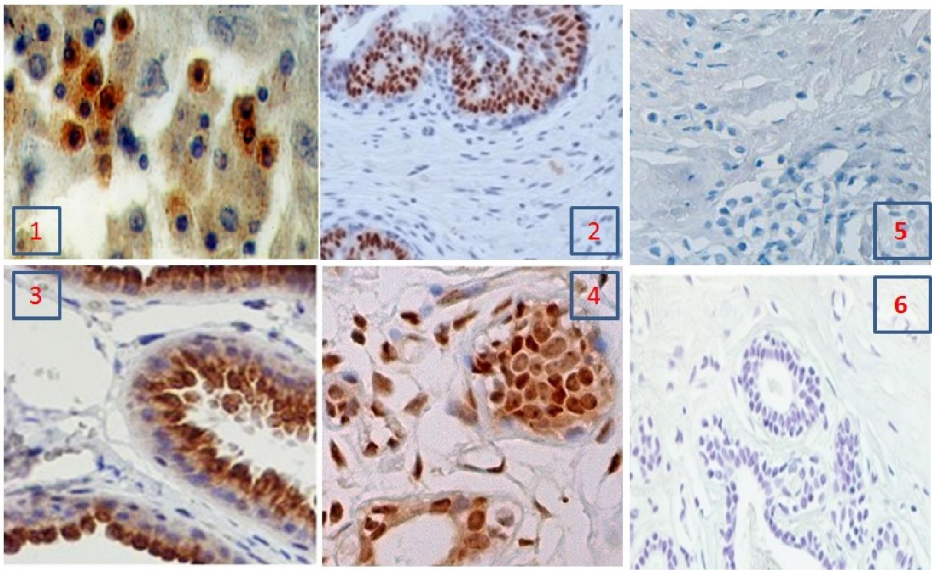


**Figure 6:** Immunolocalisation of phosphorylated oestrogen receptor beta in the advanced stage of breast cancer tissues. Slides shows the novel phosphor specific antibodies (clones 101, 308, 394 and 765; slides 1, 2, 3 and 4 respectively) bind to denatured form of phosphorylated oestrogen receptor beta protein on tissue samples. Slides 5 and 6 used as controls

**4. Discussion:**

There are two important factors that facilitate in the rapid development of IgG secreting hybridomas, first the fact that affinity maturation of immunoglobulin's takes place in peripheral lymph nodes three days earlier than spleen (Szakal*et. al.* 1989) and the other is the use of Fruend's adjuvants that helps in the modulation of humoral responses and in the recruitment of T helper cells (Kuus-Rcichel*et. al,* 1994). Adopting this multiple site immunization technique a set of mice were hyper-immunised in order to collect affinity matured immunoglobulin secreting B lymphocytes from draining lymph nodes such as popliteal, superficial inguinal, axillary and brachial. Our initial objective of this experiment was to collect the immunologically active lymph nodes upon completion of the immunization procedure.

Subsequent fusion of the peripheral lymph node cells with mouse origin myeloma cells (non-antibody producing cancerous B cells) produced large number of hybridoma clones. This outcome is a complete contrast to our previous several experiences in monoclonal antibody production adopting conventional method of fusing myeloma cells with hyper immunised spleen cells in which only a fifth of the number of hybridoma clones were produced. Several other studies also have proven similar outcomes with peripheral lymph node and spleen cell fusion (Kilpatric*et. al,* 1997).

The cell culture supernatants (collected form several hybridoma clones) tested on ELISA identified several positive clones that reacted strongly with phosphor peptide but, serum samples collected from un-immunised mice used in this same experiment as negative controls did not show any reaction (figure 4). This is clear evidence that the novel antibodies raised were specific to serine phosphorylated peptide. Further it demonstrates that the peptide used for immunisation is highly immunogenic and had triggered an active immune response. This can be attributed to the adjuvant used in this multiple site immunisation technique that has helped in the rapid expansion and maturation of antigen-specific peripheral lymph node B lymphocytes.

When the supernatants were screened side by side with phosphorylated and un-phosphorylated nuclear extracts, a majority of the clones showed a strong reaction against the phosphorylated form of nuclear extract and conversely small fraction of clones cross reacted with un-phosphorylated form of nuclear extract (figure.5). These cross reaction could be attributed to an epitope or part of an epitope that lies outside of the phosphorylated serine molecule of the peptide used as immunogen (YAEPQK or PWCEAR of the immunogen). Further, no cross reaction was observed when tested on any unrelated phosphor-serine containing peptides or an unrelated phosphorylated protein against these novel monoclonal antibodies. This test is further evidence of the specificity of the novel antibodies developed. When high positive monoclonals were subjected to immunohistochemical tests on human cancerous breast tissue sections obtained from various stages of

cancer progression showed strong reaction to advanced stage of the cancerous tissue section (figure 6). A study by Shah *et. al*. (2005) proposed that phosphorylation of oestrogen receptor could contribute to cancer progression; and our study is a clear evidence of the hypothesis proposed. The novel phosphor-serine antibodies reacted exclusively to cancerous tissues where high amount of phosphorylated form of oestrogen receptors were found, on the other hand, control tissue used in this experiment (non-cancerous breast tissue of various ages) did not show any reaction on immunohistochemical tests. This is a clear proof so far to confirm that there is a positive correlation between the phosphorylation of oestrogen receptor beta and breast cancer progression. We believe that these novel hybridoma clones that secrete highly specific monoclonal antibodies to putative phosphoryoation sites (phosphorylation at serine 105) of oestrogen receptor beta could be used in further understanding of the role of oestrogen receptor beta phosphorylation in breast cancer progression.

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