4. MATERIALS AND METHODS
4.1 MATERIALS

4.1 Materials

4.1.1 Cell Lines

The BC cell line T47D (ATCC®-HTB-133™), MCF-7 (ATCC®-HTB-22™), ZR75-30 (ATCC®-CRL-1505™), supplied by American Type Culture Collection (ATCC) (Manassas, USA).

4.1.2 Commercial Assay Kits

- CA 15-3 specific ELISA kit was procured from Cal Biotech (Spring Valley, USA)
- CA 72-4 was sourced from DRG Instruments (Marburg, Germany)
- CA19-9 specific ELISA kit was sourced from Cal Biotech (Spring Valley, USA)
- CA125 specific ELISA kit was sourced from Cal Biotech (Spring Valley, USA)

4.1.3 Reference antigens

The reference antigens like CA125, CA15-3, CA19-9, and CA72-4 were procured from Meridian Life Science (Memphis, USA).

4.1.4 Standard Antibodies

The standard antibodies against CA125, CA15-3, CA19-9, and CA72-4 antigens were purchased from Fitzgerald Industries International Inc. (North Acton, USA).

4.1.5 Other major and minor materials

The major of the chemicals used in the study are listed in the Table-3 with description, purpose of use and specifications.
Table 3: Listing of Major Requirements for the Study.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Description</th>
<th>Purpose</th>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Animals (Balb/c mice)</td>
<td>Immunization</td>
<td>Age: 6-8 weeks old</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sex: Female</td>
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<td>2</td>
<td>Fetal Bovine Serum (FBS)</td>
<td>(Media component-for Growing of cells)</td>
<td>Gibco/Cat 10270106/1x500</td>
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<td>3</td>
<td>Dulbecco’s Phosphate Buffered Saline(10.X)</td>
<td>Immunization, cell washing, Cell injection</td>
<td>Sigma/Cat No.D1408-100mL</td>
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<td>4</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
<td>Medium for cells growth</td>
<td>Sigma/Cat No D1152-1.0L/Lot No 081M8301</td>
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<td>5</td>
<td>Sodium Bicarbonate</td>
<td>For media preparation</td>
<td>Sigma/Cat No S4019-500Gms</td>
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<td>6</td>
<td>Glycine</td>
<td>IgG purification and buffer preparations</td>
<td>SRL/Cat No. 74937</td>
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<td>7</td>
<td>Trizma Base</td>
<td>IgG purification and buffer preparations</td>
<td>Sigma/Cat No. T6066</td>
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<td></td>
<td>Surface disinfectant</td>
<td></td>
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<td>8</td>
<td>Sodium phosphate dibasic anhydrous</td>
<td>IgG purification and buffer preparations</td>
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<td>9</td>
<td>Sodium phosphate monobasic dihydrate</td>
<td>Surface disinfectant</td>
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<td>10</td>
<td>NaCl</td>
<td>For Purification Antibody from ascites fluid</td>
<td>From Kim chemicals</td>
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<td>11</td>
<td>KCl</td>
<td>Buffer preperation</td>
<td>Sigma</td>
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<tr>
<td>12</td>
<td>Isopropyl Alcohol</td>
<td>Cleaning and disinfection</td>
<td>Commercial grade</td>
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<td>13</td>
<td>Protein G Purification Column</td>
<td>Purification</td>
<td>GE Healthcare/cat no 17-0618-02</td>
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<tr>
<td>14</td>
<td>Concentrated Hydrochloric Acid</td>
<td>For pH Adjustment</td>
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<td>1M NaOH</td>
<td>For pH Adjustment</td>
<td>Commercial grade</td>
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<td>Goat anti-mouse IgG Fc specific HRP</td>
<td>For pH Adjustment</td>
<td>Sigma/A0168/ secondary antibody</td>
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<td>17</td>
<td>TMB/H2O2</td>
<td>ELISA</td>
<td>Sigma/T0440/ substrate</td>
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<td>Sr. No.</td>
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<td>Purpose</td>
<td>Specifications</td>
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<td>18</td>
<td>Hypoxanthine aminopterin thymidine media (HAT)</td>
<td>Selection of Clones</td>
<td>Sigma Cat No. H0262</td>
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<td>19</td>
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<td>For initial stage growth of selected clones</td>
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<td>20</td>
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<td>Fusion</td>
<td>Sigma Cat No. P7181 5*5ml</td>
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<td>SDS-PAGE &amp; Western Blot</td>
<td>NEB Cat No. P77095</td>
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<td>SDS-PAGE</td>
<td>Genei 3.5KDa to 205KDa (Cat No. 623110475001730)</td>
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<td>Nitrocellulose membrane</td>
<td>Western blot analysis</td>
<td>Pall Corporation Cat No. P/N 66485</td>
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<td>TMB/H2O2 For Localization</td>
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<td>Skim milk powder</td>
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<td>Freezing of vials</td>
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<td>27</td>
<td>Aqua guard 1 and 2</td>
<td>Sterilizing agent in water bath and CO2 Incubators</td>
<td>Promokine Cat # PK-CC01-867-1B, PK-CC01-916-1E</td>
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<td>Anti- Anti</td>
<td>Antibiotic in media</td>
<td>Gibco Cat # 15240-062</td>
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<td>Horseradish peroxidase</td>
<td>Conjugation of antibodies</td>
<td>Amresco Cat # 9003-99-00</td>
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<td>Conjugation of antibodies</td>
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<td>Desalting process</td>
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<td>Sodium borohydride</td>
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<td>33</td>
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<td>Biorad</td>
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4.2 BUFFERS

4.2.1 Transfer buffer:

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<th>Components</th>
<th>Quantity/1000 ml</th>
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<tr>
<td>Tris-buffer</td>
<td>3.02 gm</td>
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<tr>
<td>Glycine</td>
<td>14.4 gm</td>
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<tr>
<td>Methanol</td>
<td>200 ml</td>
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<tr>
<td>Distilled water (Final volume)</td>
<td>1000 ml</td>
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4.1.1 Tris-buffer-saline Tween (TBST): pH-7.4

<table>
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<th>Components</th>
<th>Quantity/1000 ml</th>
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<tr>
<td>20 mM Tris buffer</td>
<td>2.422 gm</td>
</tr>
<tr>
<td>0.9% NaCl</td>
<td>9 gm</td>
</tr>
<tr>
<td>0.05% Tween 20</td>
<td>4 mL</td>
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</tbody>
</table>

4.2.3 Blocking solution:

5% nonfat milk in TBST or 3% BSA Solution.

4.2.4 Carbonate – Bicarbonate buffer pH 9.6±0.2

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity/1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Solution A) Anhydrous sodium carbonate</td>
<td>21.2 gm</td>
</tr>
<tr>
<td>(Solution B) Sodium bicarbonate</td>
<td>16.8 gm</td>
</tr>
</tbody>
</table>

For pH 9.6±0.2, take 80ml of Solution A, 170ml of Solution B and 750ml of Milli-Q water (Final volume should be 1000ml) – 50mM Carbonate buffer

4.2.4 10mM PBS (pH 7.4±0.2)

1.402gm Na2HPO4.2H2O (Sodium Dihydrogen Phosphate Dihydrate) and 0.2gm KH2PO4 (Potassium Dihydrogen Phosphate), 0.2gmof KCl, 8gm of NaCl in 800ml of Mili-Q water was mixed thoroughly and pH was in tune to 7.4, with afinal volume of 1000 ml.
4.2.5 Loading buffer (6X)

To prepare 1ml of 6X loading buffer, 0.5M Tris of pH 6.8(600µl), Glycerol 0.3ml (300µl), 0.1g SDS, 60 µl β-mercaptoethanol was taken in 1.5 ml centrifuge tube, mixed properly and was used as required.
4.3 METHODS

4.3.1 Cell culture and cell banking

Three cell lines namely T47D (ATCC®-HTB-133™), MCF-7 (ATCC® -HTB-22™), ZR75-30 (ATCC®-CRL-1505™) procured from ATCC were used. The cell lines were revived in tissue culture graded T-75 flask after 2 weeks and repeated passaging was performed for maintenance of growth characteristics and cellular morphology of the cell lines. All the three cell lines were maintained in the controlled environment of 37°C, with 5% CO2 and 70-80% relative humidity. After reaching confluency level of 90%, the cells were detached by trypsin to subculture and maintenance for further use.

The cell lines were cryopreserved as master cell bank and working cell bank. For this, cells were cultured in optimum condition up to 60-70% of confluency. After reaching 70% confluency, the monolayer was trypsinized and cell counting was performed. The cells were reseeded in a T-175 flask with a density of 3x10⁶ cells in each flask. These flasks were placed in a CO2 incubator adjusted at 37°C and 70-80% relative humidity for growth.

After 24 hours of seeding, the media in the flasks was changed to ensure a minimum number of floating dead cells within the flask. On attaining 70% confluency, the cells were trypsinized. The detached cells were mixed gently with a pipette to ensure disaggregation of clumps. The activity of trypsin was blocked by adding 3 ml of DMEM media enriched with 10% FBS. The sample for cell count was aliquoted out and cell count was performed in Neubauer chamber by diluting the cell suspension. The cell count was calculated accordingly of equation below equation-1.

\[
\text{Total number of cells} = \frac{R1 + R2 + R3 + R4}{4} \times DF \times TV \times 10,000 \, \text{Cells}
\]

Where: \( R1, R2, R3, R4 \) are the cell counts of 4 different chambers, \( DF \) is the dilution factor, \( TV \) is the total volume.  \( (I) \)
Figure 6: Generalized process diagram of cell banking

After cell counting the cell, pellet post-centrifugation was collected and suspended in freezing medium containing 10% DMSO (Sigma Cat-D2650) and 90% FBS(Gibco/Cat 10270106). 1 ml of cell suspension containing $3 \times 10^6$ cells/ml was aliquoted in 1.8 mL-capacity cryovials. The cryovials were maintained at -20°C for 2 hours in the frosty box (filled with 100% methanol) and further transferred to -70°C deep freezer for overnight. After overnight storage at -70°C deep freezer the, vials were stored in liquid nitrogen storage tank (-196°C) for long-term storage and future use.

4.3.2 Calculation of Population doubling time (PDT) of cells

Each of the three cell lines was seeded in 5 separate T-75 cm² flasks (marked as Day-1, 2, 3, 4, 5). Each flask was containing $2 \times 10^6$ cells/flask/15ml of media. The day of seeding was considered as day 0, while the seeding density was considered as the preliminary cell
count for the calculation of doubling time. Cell count was performed by trypsinizing flask at every 24hrs. The cell count was noted and doubling time was calculated by equation (2).

\[
\text{Population Doubling Time} = T \times \frac{\ln(2)}{\ln(X_e/X_b)}
\]

(2)

Where: \( T \) is the duration of incubation, \( X_e \) is cell count at the end of the experiment, \( X_b \) is the initial cell count.

4.3.3 Estimation of CA15-3

For estimation of secretory CA15-3, the flasks were incubated under the aforementioned condition without changing media after 0-24hrs. The cells were observed on a continuous basis to ensure the health of the culture and to check out any sign of contamination. After reaching 95% of confluence, the cell suspension was obtained. The culture supernatant from three different cell lines was collected and tested for secreted CA15-3 concentration.

Estimation of CA15-3 was performed with the help of commercially available CA15-3 sandwich immunoassay kit (Cal Biotech, USA) taking plane media as a negative control and CA15-3 antigen dilution as a positive control. 100µl of the culture supernatant and the standards were added to the individual well of ELISA plate. The plate was further kept for 1 hour at 37°C in the oven under moist and humid condition. Upon completion of the incubation, the solution from wells was aspirated out; wells were gently given three cycles of washing with the above mentioned washing buffer. The plate was tapped gently to remove air bubbles and water droplets. The kit detector was added to the wells and was incubated for 45 minutes at the same condition as previous. After completion of incubation the plate was washed thrice and 100µl of the kit developer was added to each well and incubated in dark at room temperature for 15 minutes, which produced blue colored complex on reaction. The reaction was stopped by adding 100µl of stop solution per well. The addition of stop solution
changed the blue color into yellow. The absorbance at 450nm was measured by Elisa reader and concentration of CA 15-3 was calculated by referring the calibration graph of standard antigens.

4.3.4. Immunolocalization of CA15-3

With the help of data received from the doubling time experiment, two cell lines T47-D and MCF-7 cell lines were narrowed down for further experimentations, out of which only one cell line was to be finalized for process optimization. With this reference, the localization of CA15-3 by immunofluorescence was performed to find out the expressive concentration of CA15-3 on the cellular surface.

For raising immunofluorescence image, 10000 cells were seeded with DMEM 10% (DMEM, Sigma/Cat No D1152) complete media in tissue culture graded 24well plate (Nunc). Next day of seeding, the media from the wells were aspirated out and fresh media was supplied to the cells. The cells attached to the base of plate were incubated in a CO₂ incubator for 56 hours at 37°C and 70-80% relative humidity to attain average confluence of 70%. Once the confluence level reached, the depleted media was aspirated out and the monolayer was washed thrice with 10mM PBS. The fixation of the monolayer was performed by 4% paraformaldehyde solution by incubating washed monolayer submerged in it presence for 30 minutes at ambient room temperature (ART). After the fixation of cells, paraformaldehyde was aspirated out and the fixed monolayer was given three gentle cycles of washing with TPBS (phosphate buffered saline with Tween) and the blocking was done with 2% BSA to restrict unspecific binding of antibodies. The fixed monolayer was allowed to react with anti CA15-3 commercial antibodies in the concentration of 50 µg per well per 1 ml of buffer. The plates were incubated in refrigerator overnight at 4°C. On next day, the plates were washed with TPBS solution and incubated with FC specific goat anti mouse FITC labeled antibodies.
(Sigma Aldrich Cat. No- F5387) labeled secondary antibody after the completion of incubation, the wells were gently washed with phosphate buffered saline and DPX (Distyrene-tricycle phosphate-xylene) mounting solution was added to ensure enhancement of fluorescence signal and preservation of stain (Figure.6).

**Figure7: Schematics of immunofluorescence procedure**

**4.3.5. Confirmation of cell line for CA15-3 in-vitro process development**

The objective was to identify the cell line which could secrete higher CA 15-3 content and express minimum relative doubling time. We waited for each cell line to reach 95% confluency and estimated CA 15-3 from the culture supernatant refer table: 2 for the results. With this aim the population doubling time, duration to reach 95% confluency was compared and cell for study was confirmed.
4.3.6 Low serum adaptation of T47-D cell

The T47-D cells line was preceded for adaptation process for low serum requirement in culture media. During low serum adaption cells were regularly trypsinized and reseeded in half concentration of FBS. After every FBS concentration change, cells were maintained at the same concentration of FBS containing media for at least three passages. The final concentration of FBS was maintained as 2.5% in complete media, up to this levels the cells grew well without any sign of stress and maintained proper morphology. The culture soup from adapted cells was further estimated for secretory CA15-3.

4.3.7 Population doubling time of T47-D cell at 2.5% FBS.

After low serum adaptation of T47-D, the PDT at 10% and 2.5% were compared (Table 8). Simultaneously, the secretory CA15-3 level was also estimated (Figure 15).

4.3.8 Augmentation of secretion

Under normal conditions, T47-D secretes 20 IU/ml of CA 15-3. With these secreted units the upscale was not feasible due to cost of production. For bulk production the secretion of CA 15-3 was needed to be augmented in such a way that production cost and time consumed should minimum. With these objective, different growth factors, hormones, chemicals and media components were tested in broadways to determine their effect on the secretion of cell derived CA 15-3.

To ascertain the effect of growth factors on the secretion of CA 15-3, the stock media was prepared containing growth factors such as D-glucose, interleukin 6, estrogen, and progesterone (mg/L). Diluent media (Media Code A) was prepared with DMEM low glucose medium containing 2.5% FBS. The overall detail of stock media and diluent are listed in Table-4.
4.3.8.1. The individual effect of different growth factors (D-glucose, estrogen, progesterone and IL-6)

To study the effect of individual components, media B, C, D, E and F were further diluted in different 24 welled plates with media A to attain altered concentrations of the individual growth factors for study. In each well of plate, $10^4$ cells were seeded, and incubated for 24hrs at 37°C with 5% CO2 and 70-80% relative humidity. Further media change was given to maintain same concentration of growth factors as initial stage, followed by the incubation of 96 hrs at aforesaid conditions. At the end of incubation of 96 hrs culture, the culture supernatant was evaluated for secretory CA15-3.

The composition of media and the respective component details are listed in Table 4.

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<thead>
<tr>
<th>Media Code</th>
<th>Media Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>DMEM low glucose complete media/ For dilution</td>
<td>DMEM+ 2.5% FBS</td>
</tr>
<tr>
<td>B</td>
<td>DMEM media Stock for D-glucose</td>
<td>DMEM low glucose + 20mg/L D-glucose + 2.5% FBS</td>
</tr>
<tr>
<td>C</td>
<td>DMEM media Stock for estrogen</td>
<td>DMEM low glucose + 2.5% FBS + 1mg/L of estrogen</td>
</tr>
<tr>
<td>D</td>
<td>DMEM media Stock for progesterone</td>
<td>DMEM low glucose + 2.5% FBS + 1mg/L of progesterone</td>
</tr>
<tr>
<td>E</td>
<td>DMEM media Stock for insulin</td>
<td>DMEM low glucose + 2.5% FBS + 1mg/ml of insulin</td>
</tr>
<tr>
<td>F</td>
<td>DMEM media Stock for IL-6</td>
<td>DMEM low glucose + 1mg/ml IL 6</td>
</tr>
</tbody>
</table>

4.3.8.2. Combined effect of two different growth factors (D-glucose and insulin, estrogen and progesterone)

The varying concentrations of D-glucose and insulin, estrogen and progesterone were prepared in four different plates by diluting it in the same way as in the previous reaction. Media from each plate was mixed together to reach the desired concentration of combination
two different growth factors of media in each well. In each well, $10^4$ cells were seeded and incubated for 24hrs. Further media change was given to maintain D-glucose and insulin, estrogen and progesterone concentration as initial stage followed by the incubation of 96 hrs. At completion incubation of 96 hrs, the supernatant was evaluated for secretory CA15-3. Throughout the experiment separate blank was also considered in which flask was containing only complete media with 2.5% FBS and $10^4$ cells.

4.3.9.1. Scale up of T47-D culture in roller bottle

For scaling up, the roller bottle culture was considered as a tool for large-scale production. Establishment of seeding density and revolution to be maintained during culture different RPMs and seeding density was tested. RPM shows a crucial part in the formation of monolayer during roller bottle culture. Increasing the RPM resulted in the formation of cellular clumps and detachment of monolayer if formed. Low seeding density will lead to formation of colony patches with vacant space.

1 liter of culture supernatant with high CA15-3 content (ie 200IU/ml of CA15-3) was collected by utilizing roller bottle culture. On the other side culture supernatant collected during small batch experimentations within the culture flask, were of low CA 15-3 content containing (20-30 IU/ml). These low CA15-3 containing supernatants were consumed for experimental procedures for purification process development and standardization.

4.3.9.2. Storage and preservation of culture supernatant (Raw material for the process)

5 liters of supernatant with low yield (20-30IU/ml CA15-3) and 1 liter of high yield.
(200IU/ml of CA15-3) was produced by roller bottle culture of T47-D cell lines. The culture supernatant was centrifuged at 4000 rpm to remove dead cell and debris. The processed
4.3.10. Acidic precipitation of contaminant proteins

The test volume of 100 ml from low-grade raw material was aliquoted in 3 separate test tubes. The use of precipitation agent was selected on the basis literature available. With reference to literature the 5% Trichloroacetic acid (TCA), 1.5M perchloric acid volume by volume in a ratio of 1:1 and 100% of ethanol volume by volume in a ratio of 1:1 was tested.

The treatment cycle was consisting of simple mixing for 5 min, holding for 5 min and centrifugation to settle down precipitated protein from raw experimental material and recover Cell-Derived CA15-3 in the supernatant after centrifugation. The collected supernatant was then stabilized at 7.3pH. And the supernatant was estimated for recovery by ELISA and contaminants left by SDS-Page.

4.3.10.1. TCA

TCA precipitation was carried out by adding 5 ml of 100% TCA to the 95 ml of culture supernatant. The TCA was added slowly for 10 minutes with intermittent mixing followed by centrifugation at 8000 rpm for 10 minutes. The supernatant was filtered and concentrated using 2 µm filters. pH of the filtrate was in tuned at 7.3 using NaOH. The desired protein (CA 15-3), left suspended in the filtrate was further estimated for recovery by ELISA and contaminants left was resolved by SDS-Page of the supernatant.

4.3.10.2. Perchloric acid (PCA)

PCA precipitation process was followed by 1.2M PCA. In brief the 100ml of 1.2M PCA was mixed with 100ml of culture supernatant. The precipitation process was followed
same as in TCA precipitation. The supernatants so collected were then estimated for suspended CA15-3.

4.3.10.3. Ethanol (EtOH)

During ethanol precipitation the HPLC grade 100 ml EtOH was mixed with 100ml of culture supernatant. The solution was kept for 15 min at ART under gentle mixing. After the incubation it was centrifuged at 4000 RPM and the supernatant collected was dialyzed against PBS overnight before estimation of suspended CA15-3 recovery.

4.3.10.4. Optimum percentage of TCA to use

To find optimum TCA concentration, 10 different samples were precipitated with different concentration of TCA. In brief, 10 falcon tubes with 10ml of culture supernatant were added with 1%, 2% up to 10% TCA respectively. The further process was same as in previous precipitation procedures. After the completion of pH neutralization and centrifugation supernatant from each test tube was taken out and evaluated for suspended CA15-3 by Cal-biotech CA15-3 estimation immunoassay.

4.3.11. Preparation of Partially purified CA15-3(Pp. CA15-3)

The partially purified cell-derived CA15-3 was prepared by acid precipitation of contaminant proteins within culture supernatant. Briefly 100% TCA solution was prepared and 5% of TCA (V/V) was used to precipitate down major contaminants. The steps involved centrifugation, pH neutralization, and concentration and buffer exchange. At the end of the process, there was a significant reduction of volume from 1L to 30 ml.
4.3.12. Preparation of High pure CA15-3 (Hp CA15-3)

The partially purified material containing 10Ku/10ml of CA 15-3 and the loading buffer was subjected to size exclusion chromatography with GE Healthcare AKTA prime plus machine in S400 column. In brief the column was equilibrated with 50mM PBS and 10ml of partially pure CA15-3 was loaded at flow rate of 500 microliter /min. After sample loading the void volume and different fractions were collected and estimated for suspended CA15-3. The fractions were then pooled containing 10 fractions in each pool. The pools were subjected to SDS-PAGE and with reference of Gel-profile, the fractions were finally pooled for calculating total recovery of suspended CA15-3.

4.3.13. Optimized purification process

The final purification process consisted of acid precipitation (5%TCA), centrifugation (8000rpm), pH neutralization (7.4), concentration (1000ml to 35ml) followed by size exclusion chromatography, finally TFF filtration of positive pools containing high concentration of CA15-3 (300ml to 25ml).

4.3.14. Characterization of purified Cell-Derived CA15-3

Characterization is the most important part of any protein purification process which confirms the purity and gives supporting data for qualifying eligibility of purified molecule to use on a commercial scale and for scientific purpose. The purified protein was characterized on the basis of reactivity (ELISA/ Western blot), the presence of other protein (SDS-PAGE) and FTIR spectra of native and cell-derived protein.
4.3.14.1. Reactivity of purified cell derived antigen CA15-3 with the commercially available antibody.

To determine the activity of purified antigen checker board indirect ELISA was performed. Briefly the coating of antigens was done in decreasing dilutions of 1:2 through columns. Column1 (A, B, C, D, E, F, G) was coated with 100IU of antigen. In the second Column it was 50 IU in each corresponding wells.

Similarly dilution was made up to 0.75IU. The coated plate was Kept overnight at 4°C. Next morning the antigen solution was discarded and the wells were blocked with 5% nonfat milk. Further after blocking, wells were added with CA15-3 specific commercial antibodies in dilution row wise. All columns of row 1 were getting 100ng of antibodies. Similarly row 2 was getting 50ng in all corresponding column. Similarly antibody dilution was decreasing 50% to each corresponding rows starting with 100ng and ending at 0.75ng, Figure illustrates the plan for addition of antigen and antibodies through columns. For example, Column 1(A, B, C, D, E, F, G) was coated with 100IU of antigen. In the second column it was 50 IU in each corresponding wells, similarly, dilution was made up to 0.75U as in previous column. The coated plate was incubated overnight at 4°C. Next morning the antigen solution was discarded and the wells were blocked with 5% nonfat milk. Further after blocking, wells were added with CA15-3 specific commercial antibodies in dilution row wise. For example, all columns of row 1 were getting 100ng of antibodies. Similarly, row 2 was getting 50ng in all corresponding column. Similarly, antibody dilution was decreasing 50% to each corresponding rows starting with 100ng and ending at 0.75ng, Figure-9 illustrates the plan for the addition of antigen and antibodies.
Figure 8: Detailed process diagram for Cell-Derived CA15-3 purification from Culture supernatant of T47-D cell line
4.3.14.2 SDS-PAGE of purified CA15-3

The purified material was resolved through SDS-Page. 1KU of purified protein was subjected to SDS-PAGE. In brief a gradient gel was prepared by mixing of 4% and 7.5% gel within gradient mixer. Simultaneously the liquid, mixed gel was casted in 1.0 mm gel plate for polymerization. The samples from purified protein, raw material, and other reference materials such as DMEM complete media, FBS, Commercial CA15-3 was mixed with loading buffer and resolved under the influence of continuous supply of 150 volt DC current provided by power pack. After the completion of resolution the gel was transferred for fixation in fixative media containing 50ml methanol, 50ml DM water and 60 µl of formaldehyde and Kept for 30 minutes.

After incubation the gel was washed with DTT and Kept 30 minutes with AgNO₃ for permeabilization. The bands were developed by adding 3.5% solution of sodium carbonate. The reaction was stopped by adding 2.5M citric acid.

4.3.14.3. Western blotting of purified cell- derived CA15-3

The purified protein sample was resolved on SDS-PAGE and resolved gel was marked. Proteins were transferred to nitrocellulose membrane (GE Healthcare, UK) overnight at 20°C with constant supply of current (100 mA) using a Power Pack of GeNei (Bangalore Genei, India) in presence of gel transfer buffer (3.02 g/lt Tris-buffer, 14.4 g/lt glycine and 20% methanol).After washing the membrane with wash buffer containing 0.05% of Tween-20 (WBT), nonspecific sites were blocked using 5% (w/v) nonfat milk powder made in buffer (20 mM tris-buffer, 150 mM sodium chloride, 0.5 ml/lit Tween 20, pH 7.4±0.2) for 4 h at 20°C (Figure.10).
Figure 9: Antigen and antibody dilution plan during checkerboard ELISA

Figure 10: showing detailed process diagram for SDS-PAGE and Western Blotting
Nitrocellulose was then washed with WBT and Kept overnight at 4°C with primary anti CA15-3 monoclonal antibody (Fitzgerald Industries International Inc, USA). After washing with WBT, membrane was Kept for 1 h at 20°C with secondary antibody, HRP-conjugated Goat anti-Mouse IgG (Bangalore Genei, India). Detection was performed using TMB (3,3’,5,5’-Tetramethylbenzidine) as a substrate for peroxidase.

**4.3.14.4. Estimation of cross-contaminants in purified CA15-3.**

The cross contaminants such as CA19-9, CA72-4, CA125, CEA are always found with native CA15-3. In order to consider protein matrix to be high pure total cross contaminants level should be less than 5% of total CA15-3, then only the protein is said to be pure. The purified material was subject to Siemen’s CLIA platform to detect cross contaminants. The samples were diluted in the sample diluent provided by supplier and the estimation was carried out as guided by manufacturer.

**4.3.14.5. FTIR analysis of purified cell derived CA15-3 and commercially available native CA15-3**

The samples for FTIR analysis were processed as per buffer requirement for the analysis and were dialyzed in NaN$_3$ free buffer with a lower concentration of salts.

The FTIR spectra of purified CA15-3 in transmission mode was obtained with Bruker 3000 Hyperion Microscope Vertex 80 FTIR System under a focal plane array of 128 x 128 with a range of 4000-900 cm$^{-2}$ and single point detecting a range of 7500-450 cm$^{-2}$. With an analysis area of 128x128 in the 2D format on the sample plane 300x300 µm with a spectral resolution of 0.2 cm$^{-2}$. Each spectrum was recorded with a single scan, in step scan mode at 16 cm$^{-2}$ resolution.
4.3.15. Generation of anti-Cell Derived CA15-3 monoclonal antibody

4.3.15.1. Immunization of mice and estimation of antisera

The generation of monoclonal antibody (mAb) was achieved by immunizing four groups of six week old Balb-c female mice with each group containing 3 mice in. Test bleed (0.2mL max) before initiation of immunization was collected, labeled, and stored for reference by tail bleeding. The mice were immunized with cell derived CA15-3(Figure-11). With adjuvants Freund’s adjuvants system with the concentration as 2ku, 1ku of cell-derived CA15-3. Boosters were administered after 4 weeks of immunization. After 10 days of second booster tail bleeds from mice were collected and were tested for antisera secretion within mice by Indirect-ELISA. In brief, the antigen was Prepared by dilution into the PBS to give a final concentration of 100IU/100µl (need 100µl/well) and was mixed thoroughly to attain even distribution throughout the diluting buffer. 100µl of diluted antigen was added to each well, leaving few wells with no antigen (CONTROL) and Kept at the plate at 4ºC overnight. Next morning the plate was given three gentle wash with PBST for three times and remaining buffer by gentle tapping on a paper towel. Blocking with 5% skim milk was performed to prevent non-specific binding of primary antibodies and Kept the plate at 37ºC under the moist and humid condition for 1 hr. After completion of incubation blocking solution was decanted and wells were washed with PBST for three times and remaining buffer was removed by gentle tapping on a paper towel as previous. Now 100µl diluted mice sera diluted in PBS was added to the wells. Here commercial antibody was also added to separate wells in duplicates as positive controls and PBS as a negative control. Incubation of 1 hour was followed again at 37ºC under similar condition as previous. After incubation washing process as discussed above was followed. After assuring that there is no air bubble left in wells, 100µl secondary antibody goat anti-mouse IgG conjugated with HRP in dilution of 1:10000 in PBS was added to each well and incubate at 37ºC for 45 minutes. On completion of incubation time solution
in wells was decanted and the plate was washed two times with PBST one time with PBS, after removing the buffers droplet by gentle tapping, 100μl of TMB substrate was added to each well and the plate was kept in dark for 10 minutes. The reaction between TMB and HRP gave blue color and this reaction was stopped by adding 1NHCL as stop solution.

And OD was measured at the 450nm filter in ELISA plate reader. The booster dose was continued in same concentration at same interval till reaching of a quantifiable minimum titer of 1:62000. The immunization route throughout the procedure was subcutaneous (SC)-0.2mL max per mice (GPI0100-100ug/mice, Nabisco- 12ug/mice with the concentration as 2ku, 1ku of cell-derived CA15-3). The pre-fusion booster was administered by intravenous route (IV).

![Image](image.jpg)

**Figure 11: Showing immunization schedule and titer estimation for mice**

4.3.15.2. Raising hybridoma cell

After reaching a reactive titer of 1:64000, the fusion was planned and the requirement of fusion was prepared two days prior to fusion. A generalized process of hybridoma generation is shown in Figure: 12.
4.3.15.3. Pre-fusion booster of mice

The pre-fusion booster was administered to mice with antigen only with PBS and left for rest. The booster was ensured to be administered 3 days prior to fusion to give proper resting time to animal and to get a maximum number of dividing splenocytes in the spleen at the day of fusion.

4.3.15.4. HAT sensitivity of myeloma cells (Sp2)

One flask of Sp2 was subcultured and divided into two flasks. Flasks were marked as A, B continued by date of passage. Out of these two flasks, A was treated with HAT-containing media to check HAT sensitivity of Sp2 cells. After 24 hours the cells in the first flask were observed to dead as a result of HAT treatment. The flask B was sub-cultured and used for fusion.

4.3.15.5. Preparation of feeder layer

Feeder layer was produced by naive splenocytes one day prior to fusion. The significance of feeder layer was to provide an ambient condition to enhance fusion efficiency through secretion of various factors and scavenging of dead cells which helps for the growth of hybridoma cell. For the preparation of feeder layer, fresh splenocytes were harvested from normal unimmunized healthy mice.

The collection of splenocytes was performed by sacrificing mice and removing a spleen in aseptic condition. The spleen after recovery from mice was transferred to a petri dish containing 10 mL DMEM media without FBS, washed gently and again transferred to another Petri dish containing plane media. The fat and connective tissues were trimmed out and the spleen was placed on a sterile cell strainer in a new Petri dish containing 10 mL media. Using the back of a 5mL syringe, the spleen meshed to get all splenocytes in
suspension leaving the spleen transparent. The strainer was washed with media, and the cells were transferred to suspension with the help of a cell strainer into a 50 mL falcon. Further, the cells were centrifuged at 900 rpm for 5 minutes. On completion of centrifugation, the pellet was dislodged by gentle tapping and 5 ml of ice cold RBC lysis buffer was added to dislodge cells prior to incubation of 5 min at 4°C. On completion of incubation, cells were centrifuged at 900 rpm for 5 minutes. The pellet was washed with 10 ml media. The cell count was taken and dilution was made in such a way that per well there must be 5000 cells/100µl of complete media. 10 plates with feeder layer were prepared and Kept in a CO₂ incubator for next day use.

4.3.15.6. Fusion

Before initiating the fusion process, Sp2 cell flask was observed for confluence, contamination. Well grown healthy cells were observed within flask without any sign of contamination. All 96-well plates with feeder layer were free of contamination. The autoclaved fusion box, sterile DM water, miniature water bath, DMEM plain media, and other essential materials were kept in the places of their need during the fusion process.

Blood from G2RC mice was collected. The mouse was sacrificed and spleen was isolated under aseptic condition. The recovery of splenocytes was performed in a similar was as, during feeder layer preparation, the only difference during the process was that to ensure minimum loss of splenocytes. The splenocytes were counted (21.7X10⁶ Sp2 count).

The monolayer of Sp2 cells was detached from the surface of the flask using a bent pipette and observed under a microscope to confirm detachment. Cells were pooled from all the 3 flasks into a 50 mL Falcon and centrifuged at 900 rpm for 5 minutes. The supernatant was decanted. Cells were washed with 25-30 ml of plain DMEM medium thrice. Cell count was noted down (96.7x 10⁶). The splenocytes and myeloma cell were mixed at a ratio of 1:5
(myeloma: splenocytes) and centrifuged 1000 rpm for 5 minutes. The pellet was washed twice with excess 30 ml of plain DMEM medium. After 3rd wash, the cell pellet was added with 1 ml of PEG dropwise in the duration of 1 min with keeping the tube at 37°C in warm water. The tube was gently shaken while adding PEG/DMSO. The cells were mixed with gentle shaking for 1 min at 37°C. After the 1-minute interval, 1 ml of plain DMEM was added dropwise within a period of 1 min at 37°C provided by miniature warm water. Then 5 ml of plain DMEM was added within same condition and same duration of time. The tube was gently shaken throughout the procedure. After this, the cells were centrifuged at 900 rpm for 5 minutes. The whole pellet was mixed evenly with 100 ml of DMEM with 20% FCS + 2X HAT. 100µl/well suspension was added to plates seeded earlier with feeder layer. The plates were kept in the CO₂ incubator. The plates were observed daily for microbial contamination and formation of hybrids (live duplicating cells). 100µl of spent media was aspirated from these plates. After 48 hours fresh 100µl DMEM with 20% FCS + 2X HAT was added to each well. After three such replacements, DMEM with 20% FCS + 1X HT was added to all wells. After HT treatment the hybrid clones started growing. The screening was performed at 60% confluence.

4.3.15.7. Screening of positive clones

The first screening was performed for Native, Cell-derived CA15-3 antigen by indirect ELISA as in titer estimation. In brief 22 plates containing 100IU/well/100µl were coated with native and cell derived antigen (11 with native, 11 with cell derived). Indirect ELISA was performed and clones with higher absorbance were selected for further screening.

Next cycle of screening was against other cancer antigens such as CA19-9, CA72-4, CEA, CA125 etc. After screening with other cancer antigens the specifically reacting clones were chosen for single cell cloning and monoclonal antibodies production.
4.3.15.8. Single cell cloning

Limiting dilution procedure for Single cell cloning was performed. The dilutions of selected hybridoma clones were prepared after counting cell from detached parent clone. The dilution was made in a way that total of 80 cell was available to 10 ml of media. Cells were added to the reservoir containing 10ml of the cloning medium. Cells were mixed well and 100μl from this was added to each well of 96 well plate containing feeder layers. Within the duration of 7 days, the single cell colonies begin to appear in the well. The colonies were observed using an inverted microscope and single cell clones were further screened for antigen sensitivity and were expanded gradually for antibody production.

4.3.15.9. Antibody production

To have sufficient amount of antibodies Clones 8e4 and 9A05 were upscaled in tissue culture system. In brief, the clones were expanded in 5 separate T-175 cm² tissue culture flasks.

4.3.15.10. Antibody purification

The culture supernatants were first harvested by centrifugation and 0.1% of NaN₃ was added to the supernatant. Prior to affinity purification, it was filtered through 0.45 μ filter. The antibodies from harvested supernatant were purified by Hi-Trap Protein-G affinity column (GE Healthcare) according to the manufacturer’s instructions. In brief, column was washed by passing 5 CV of 20% ethanol to get rid of bubbles at the flow rate 2ml/min. Equilibration of the column was performed by passing 10 CV columns volume of Washing Buffer at the flow rate at 2ml/min. after equilibration, the harvested culture supernatant The filtered culture supernatant was passed through the column at flow rate of 1 ml/min in 3 cycles. Flow through and the unbound protein was collected in a separate beaker. The
column was then washed with PBS to eliminate unbound trapped protein. The captured antibody was eluted with glycine-HCl buffer (0.1 M, pH 2.7). Eluted antibodies were dialyzed against PBS (pH 7.5) at 4°C overnight. Ultrafiltration was performed for concentrating the harvested antibodies.

4.3.15.11. Cross-reactivity analysis with cancer antigen

To test the cross-reactivity purified antibodies the antibodies were allowed to react with antigens (CA72-4, CA19-9, CEA, CA125, NHS) on ELISA plates. And the complete indirect ELISA was performed. Absorbance at 450 nm was noted down.

Figure 12: Generalized protocol for hybridoma development and antibody synthesis.

4.3.15.12. Characterization of purified antibodies

For characterization, the western blot analysis and reactivity with antigen were taken as a base.
4.3.15.13. Western blot from purified antibodies

To ascertain the activity of purified antibodies, western blot was performed on native and cell derived CA15-3, along with high concentration of other cross contaminants. In brief, proteins were resolved by SDS-Page and were transferred to nitrocellulose membrane. Further nitrocellulose membrane was treated with 5% blocking grade nonfat milk to prevent unspecific binding of antibodies. Nitrocellulose was then washed with WBT and kept overnight at 4°C with primary anti CA15-3 monoclonal antibody. After washing with WBT, membrane was kept for 1 h at 20°C with secondary antibody, HRP-conjugated Goat anti-Mouse IgG (Bangalore Genei, India). Detection was performed using TMB (3,3’,5,5’-Tetramethylbenzidine) as a substrate of peroxidase.

4.3.16. Sandwich assay development

The produced antibodies were compared with the capture antibodies within commercially available immunoassay kit. The sets of reaction were performed in the kit and same reactions were performed by synthesized antibodies.

To determine capture efficiency the ELISA plate was coated with 3F10 and 4B05 antibodies (100ng/100µl/well) overnight at 4°C. Further, they were blocked with 5% nonfat milk and washed with PBST. Then these coated wells were allowed to react with standards provided with the kit. Simultaneously the strips from the kit were also allowed to react with same standards in the same volume. The all three plates were allowed to react with kits detector and developed with provided solution of the kit. The absorbance was measured after the reaction. The absorbance produced from all three sets of the reaction was compared by plotting standard curve graph.

Similarly, 8E4 was conjugated with HRP and the detector efficiency was estimated by comparing with the results of kit. In brief, the set of reaction performed on kits plate one with
the kit detector and other with HRP conjugated 8E4. The detector efficiency with new conjugated antibody was compared by plotting a standard graph with kits standard and with antigens of known concentration. In final the efficiency of CA15-3 detection was compared in between kit and combination of 3F10, 4b05409 (capture) and HRP conjugated 8E04 as detector.

4.3.16.1. Capture antibody identification and optimization

To ascertain the efficiency of 3F10 and 4B054B09 as a capture both antibodies were coated on separate ELISA plates. 100µl of kits standard of different concentrations were added to wells. Simultaneously kits plate was also added to standards. Further, the kit detector was allowed to react with all three. And absorbance was measured at 450 nm. (Table-13).

In next experiment, the same reaction was followed as in the previous case but here the antigens were of known concentrations other than kits standard. The absorbance table (Table1-14) so produced was plotted down against a standard curve of kit and the respective antigen concentration was calculated.

4.3.16.2. Detector antibody conjugate optimization

For testing the detector efficiency of 8E04 first it was coupled with HRP enzyme. In two separate reactions, the absorbance between kit standards and HRP conjugated 8E04 was compared to determine the efficiency of 8E04 as detector (Table-15).

4.3.16.3. Comparison of CA15-3 detection with Commercial Kit

In house optimized CA15-3 estimation kit using antibodies during present study was compared with commercially available CA15-3 estimation kit (Cal Biotech, USA). In-house
assembly and the commercially available CA15-3 estimation kit were compared for their respective detection efficiency for different batches of biomedical fluid for the CA15-3 estimation. In a test run of 50 batches (Table-18), the detection efficacy was compared.