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Characterization, Development and Immunological Application of Host Cell Protein Antibodies for Quality Assurance of a Recombinant Pharmaceutical PNU-214565

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2 ABBREVIATION

2-DE	2-Dimensional Electrophoresis
BSA	Bovin Serum Albumine
DTT	Dithiothreitol
ECP	*E.coli* proteins
ELISA	Enzyme Linked Immuno Sorbent Assay
Fab	Fragment of Immunoglobulin
HDCP	Host Cell Derived proteins
HRP	Horse Raddish Peroxidase
IEF	Isoelectric Focusing
PBS-Tw-20	Phosphate buffer with Saline and Tween 20
PNU	Pharmacia & (and) Upjohn
SADCC	Superantigen Antibody Dependen cell Cytotoxity
SEA	Staphylococcal Enterotoxin A
SDS	Sodium Dodecyl Sulfate

3 SUMMARY

The *E.coli* reference antigen preparation (Blank fermentation of PNU-214565) (1,2) was characterized by 2-dimensional electrophoresis (2-DE). The silver stained 2-DE gel showed approximately 200 proteins with varying pIs and molecular masses. Western blotting of the 2-DE gel showed that the antibodies raised against the *E.coli* protein (ECP) reacted with approximately 120 proteins. The proteins which were immunoreactive were evenly distributed over the entire 2-DE frame. That is, both acidic, neutral as well as alkaline proteins react as do proteins of high, medium and low molecular masses. The degree of coverage of the PNU 21-4565 ECP antibodies was approximately 60%.

Thus, the antibodies raised against the reference antigen preparation are suitable to detect contaminating proteins derived from the host organism both in the final product as well as in the in-process samples with for example, an enzyme linked immunosorbent assay (ELISA).

This report therefore also describes the development and application of an ELISA method using these antibodies and antigen for the quantification of contaminating ECP in samples from fermentation and purification steps from the production process of a recombinant biopharmaceutical, PNU-214565.

4 INTRODUCTION

Passive antibody therapy of cancer is one of the oldest and most prominent issues of tumor immunology. As early as 1895, Hericourt and Richet reported on their attempts to treat cancer patients with antisera prepared in dogs and donkeys. Paul Erlich with his antisera against plant toxins abrain and ricin, had shown the specificity of the newly induced serum substances and named them “Antikörper” (antibody). He became particularly intrigued by their potential use as specific weapons against cancer cells and coined for them the term “Zauberkugel” (magic bullets). Nevertheless, despite these early beginnings, antibody therapy of cancer has become a story of unending failures(3).

Colon cancer is the second most common cause of cancer death in U.S , only lung cancer kills more people. Cancers are staged Duke A to D with A and B (40%) without spread beyond the area of colon, Duke C (35%) with spread to local lymph nodes but not beyond and Dukes D (25%) with spread to organs outside the colon. See figure 1. The survival of patient is related to how well treatment can eliminate cancer. Duke A patients have a nearly 100% 5 year survival, Duke B 60-80%, Duke C 40-60%, while Duke D patients have almost no long term survivors.(4)

Figure 1.Dukes classification.

Pharmacia &Upjohn has developed a candidate drug, PNU -214565, for coloncancer therapy. PNU-214565 is a fusion protein, consisting of a Fab-fragment from a monoclonal antibody and a staphylococcal enterotoxin A (SEA) part. The total molecular weight for PNU 21-4565 is 75 kDa consisting of 51 kDa and 24 kDa for the heavy and light chain respectively. The pI for PNU-214565 is approximately 8.5.

The Fab-fragment is directed against epitopes expressed by certain tumor cells as colon cancer. The SEA binds to T-cell receptors and cells expressing the MHC class II antigen (5). The tumor cells killed by SADCC (Superantigen Antibody Depended Cell Cytotoxicity) See figure 2.

Figure 2. Outline of T cell mediated killing of tumor cells by antibody targeted superantigens. Tcells , with TcR expressing the appropriate Vβ region bind to the antibody-superantigen conjugate on the antigen positive target cell

In the protein, the constant and variable regions of the Fab-fragment are derived from the monoclonal antibody C 242. PNU-214565 consists of two polypeptide chains, one heavy and one light chain which are held together by hydrophobic interactions and one disulphide bond. The protein is expressed from recombinant E.coli containing plasmids coding for the fusion protein. The protein is released into the media. PNU-214565 is, after fermentation and separation of the cells, purified with a three steps procedure starting with an anion exchanger followed by an affinity chromatography step and as a final step a cation exchanger. See figure 3.

Figure III. The purification steps of PNU-214565

Minor deleterious impurities such as host cell derived proteins (HCDP), DNA or endotoxins pose potential health hazards and therefore must be reduced to parts-per-millon level or less,(6,7) in the finished biopharmaceutical product.
In the biological manufacturing of recombinant proteins it is necessary to have an analytical method for determination of HCDP. The analytical method, in this case an ELISA method (8,9,10),is used to show the reduction of HDCP during the purification process.

In order to produce antibodies against HDCP a blank fermentation is utilized.

The principle for a blank fermentation is that microorganism or mammalian cell cultivations are performed in the same way as in an ordinary production run. The exception is that no structural gene encoding the recombinant protein of interest is present. In addition one or more purification steps may also be performed before a mix of HCDP (host cell derived proteins) is sampled. This mix of HCDP is then used to immunize animals for the generation of antibodies.(2)

In order to characterize the reference antigen preparation (Blank fermentation of PNU-214565) (1,2) and the antibodies raised against the antigen preparation, two-dimensional polyacrylamide gel electrophoresis was performed followed by western blotting using the antibody preparation employed in the ELISA procedure.

5 MATERIAL AND METHODS

5.1 Reference antigen-standard

Reference antigen -standard (1,2 mg/ml) from PNU-214565 blank fermentation was used for developing this ELISA method (1,2). The protein content of the reference antigen was determined by amino acid analysis. The sample was hydrolyzed with 6M HCl for 45 minutes at 155°C.The hydrolyzed sample was analyzed on a Beckman 6300 amino acid analyzer using an ion exchange column for separation of the amino acids and ninhydrin for detection.

5.2 Antisera

The preparation of HDCP was performed by a blank fermentation of E.coli containing a plasmid lacking the structural gene for PNU-214565. A small part of the harvest cell broth that was subsequently microfiltered was sonicated in order to distrupt cells. The reason was to minimic the cell lysis that occurs during normal production run. The fermentation was followed by cross-flow microfiltration and a cartridge filtration. The cartridge filtration was used as the HDCP mix.(2) The mixture was freeze-dried before delivery for immunization. The immunization was made by Peter Owen, Department of Microbiology, Moyne Institute, Univerisity of Dublin, Ireland.Twelve rabbits was immunized with 4 mg of the mixture per rabbit for each primary and booster injection. The antisera was purified by

ammonium sulphate precipitate, acetate dialysis, phospate dialysis and was concentrated by ultrafiltration using a PM 10 filter. The protein concentration was determinated to 105 mg/ml by Lowry method (1). The same antisera was also biotinylated described by manufacturer from PIERCE (14).

5.3 2-Dimensional Electrophoresis (2-DE) Method

A 2-DE gel is the product of two sequential separations performed in an acrylamide gel media: isoelectric focusing (IEF; carried out in an acrylamide gel support under denaturing conditions) as the first dimension, and SDS(sodium dodecyl sulfate) slab gel electrophoresis as the second dimension. Isoelectric focusing separates proteins according to amino acid composition (mainly the ratio of acidic to basic chemical groups), and can be accomplished using a pH gradient established by carrier ampholytes (CA-IEF) or an immobilzed pH gradient (IPG-IEF). SDS electrophoresis separates by molecular size. In typical current practice, a small sample of protein (10-50µg in ~10-50µl) is applied to the top of an IEF gel, and separated over a period of 20 hours at high voltage, using the fact that proteins migrate in the applied electric field until they reach the pH zone at which they have zero net charge, where they focus. After the run, the gel is applied along the edge of an acrylamide gel. The buffers involved contain sodium dodecyl sulfate, a detergent that unfolds each protein into a rod whose length is related to the mass of the protein; the proteins then migrate under an applied field into the slab, perpendicular to the first (IEF)

gel, where they are separated by the sieving action of the gel support (small proteins move fastest). A two-dimensional pattern of spots, each consisting of a specific

protein, is formed. The spots are subsequently detected by staining, or by radiographic methods if the proteins incorporate a radioactive label. (11)

The Pharmacia Biotech system was employed essentially as described by the manufacturer (instruction 18-1038-63 Pharmacia Biotech) (12).

5.3.1 First dimension

1-D isoelectric focusing was performed using 180 mm Immobiline® DryStrip, precast polyacrylamide gels (T=4%, C=3%) with linear pH range 3-10. The Immobiline® DryStrip was rehydrated overnight with 8M Urea, 0.5% Triton X-100, Pharmalyte 3-10 and DTT. The Immobiline® DryStrip was run using a Multiphore II and Power supply EPS 3500 from Pharmacia Biotech, according to the instruction from Pharmacia Biotech except that the temperature on the cooling plate was set to 25°C. 50µl of the sample, (Blank fermentation of PNU-214565) (1,2) with the concentration of 0.3 mg/ml was loaded in the sample application cup. The Immobiline® DryStrip was run for 3500 V for a total of 56000Vh.

5.3.2 Second dimension

The Immobiline® DryStrip was equilibrated in two steps, the first step with DTT (100 mg/10 ml equlibration solution) and the second equilibration step with iodoacetamide (0.45 g/10 ml equilibration solution) (12)

An Excel Gel XL SDS (Pharmacia Biotech) with 12-14% acrylamide gradient supplied with buffer ions from precast ExcelGel SDS buffer strips was used. The gel was run at constant power (40W) at 15°C for 45 minutes, the current was 20 mA, and subsequently at 40 mA for approximately 2 hours. As molecular weight marker LMW 14.4-94.0 kDa from Pharmacia Biotech was used. The gel was then silver stained using Pharmacia Biotech Silver staining Kit-Plus One.

5.3.3 Western blotting

A complementary gel was subjected to western blotting. The gel and a nitrocellulose Hybond membrane, (Amersham RPN 2020 D) were first soaked in transfer buffer (48 mM Tris, 39 mM glycine, 0.02% SDS and 20% MeoH) and then the proteins were transferred (Semidry-Nova Blot) to the nitrocellulose membrane (250 mA 1.5H). After transfer the membrane was blocked with 5% non fat dry milk (BIORAD) in PBS-Tween-20 pH 7.4 for 1 hour at room temperature. The membrane was then rinsed or/and washed 3 x 20 minutes in PBS-Tween-20 before incubation for 16 hours at 4°C with 100 ml of primary antibody, rabbit anti-ECP antibody (1,2) derived from PNU-214565 blank fermentation, 105 mg/ml, diluted 1:40 000 in PBS-Tween-20. The membrane was then washed 3 x 25 minutes in PBS-Tween-20 and subsequently incubated for 1 hour at room temperature with 100 ml goat anti-rabbit IgG-HRP conjugate (BIORAD 170-6515) solution diluted 1:10 000 in PBS-Tween-20 followed by 3 x 25 minutes wash with PBS-Tween-20. Detection was performed as described for Amersham ECL ™ Kit (13). Development time was 20 minutes and exposure time 20 sec.

5.4 ELISA method

Microtiter plates (Dynatech M 129 B) were coated with antibodies against *E.coli* proteins (1,2) in Na-bicarbonat buffer pH 9,6 (SIGMA) and were incubated overnight in a refrigerator(+ 2-8°C). After washing with PBS-Tween-20 in an automatic plate washer (Wellwash 5000) the plates were blocked with 1% bovine serum albumin (BSA) (PIERCE) in phosphate buffer with saline and were incubated at room temperature (+ 20-24°C) for 2 hours. After washing with PBS-Tw-20, the samples, blanks and standard dilutions were added and the plates were incubated at +37°C for 2 hours on a platform shaker. After washing the biotinylated antibody (14) against *E.coli* (1.2) was added to the microtiter plates. The plates were incubated overnight in refrigerator. After incubation, horseradish peroxidase conjugated avidin (PIERCE) was added to the plates. A colour was developed when TMB (3,3´,5,5´-tetramethylbenzidine) (BIORAD) was added as substrate. Absorbance were read at 450 nm with a microtiter plate reader (Molecular Devices E-max) The intensity of the colour obtained is proportional to the amount of *E.coli* proteins in the sample of PNU-214565.

5.5 Evaluation of the coating concentration of rabbit-anti-ECP antibody.

In order to determine the optimal coating concentration, the microtiter plates were incubated in a refrigerator overnight with 100 µl of coating buffer solution containing 1,875-30 µg/ml rabbit anti-ECP antibody (1.2). Tests made with high (50 ng/ml standard material) and low control (5 ng/ml of standard material) and blanks (only PBS-Tw-20). In this study, the dilution of biotinylated rabbit anti-ECP antibody was 1/6000 and the dilution of HRP-Avidin was 1/10000.

5.6 Evaluation of proper dilution of biotinylated rabbit anti-ECP antibody

In order to select a proper working dilution of the biotinylated rabbit anti-ECP antibodies, different dilutions (1/500-1/10 000) of the biotinylated antibodies were added to the wells of a microtiter plate that after coating with rabbit anti-ECP antibodies had been incubated for 2 hours with high control, low control and blanks. In this study the coat concentration was 20 µg/ml and the dilution of HRP-Avidin was 1/10000.

5.7 Evaluation of proper dilution of HRP-Avidin

In order to select a proper working dilution of the HRP-Avidin antibody, different dilutions in range of 1/2500-1/40 000, were added to the wells of a microtiter plate that after coating with rabbit anti-ECP antibodies had been incubated for 2 hours with high control, low control and blanks. In this study, the coat concentration was 20 µg/ml and the dilution of biotinylated rabbit anti-ECP antibody was 1/6000.

6 RESULTS

6.1 2-Dimensional Electrophoresis (2-DE) and Western Blotting

6.1.1 Silver stained 2-DE gel

Figure 4 shows the silver stained two-dimensional electrophoresis gel which was obtained with the reference antigen preparation. As can be seen in the figure, a number of proteins are present in the reference antigen preparation. From approximately 15 µg of total protein, which was loaded onto the gel, approximately 200 spots were observed on the silver stained gel. These spots were evenly spread over the entire 2D frame.

Figure 4. Silver stained two-dimensional electrophoresis gel obtained for the reference antigen (Blank fermentation of PNU-214565).

6.1.2 Western blotting

Figure 5 shows the western blot complementary to the silver stained gel in figure 4. As can be seen, approximately 120 protein spots reactive to the antibodies raised against the reference antigen preparation. The proteins which were immunoreactive were again evenly distributed over the entire 2-DE frame. That is, both acidic, neutral as well as alkaline proteins react as do proteins and peptides of high, medium and low molecular masses.As seen in figure 5 the majority of the polypeptides in the vicinity of where the PNU-214565 polypeptides would be expected are detected.

Figure 5. Western blotting using the antibodies raised against the blank fermentation of PNU-214565, complementary to the silver stained gel shown in figure 4

6.1.3 Covering degree

The covering degree was calculated by deviding the number of spots obtained after Western blotting analysis with the number of spots obtained after silver staining multiplied with 100 to give the value in per cent. Number of spots were determined by visual inspection. The degree of coverage of the PNU-214565 ECP antibodies was thus found to be approximately 60% (120/200).

6.2 ELISA method

6.2.1 Determination of optimal rabbit anti-ECP antibody concentration

As shown in figure 6, both with high and low control, a plateau value was obtained of absorbance (450 nm) values at coating concentrations of 7,5-30 µg/ml. 20 µg/ml anti-ECP antibody was chosen as optimal for the ELISA assay.

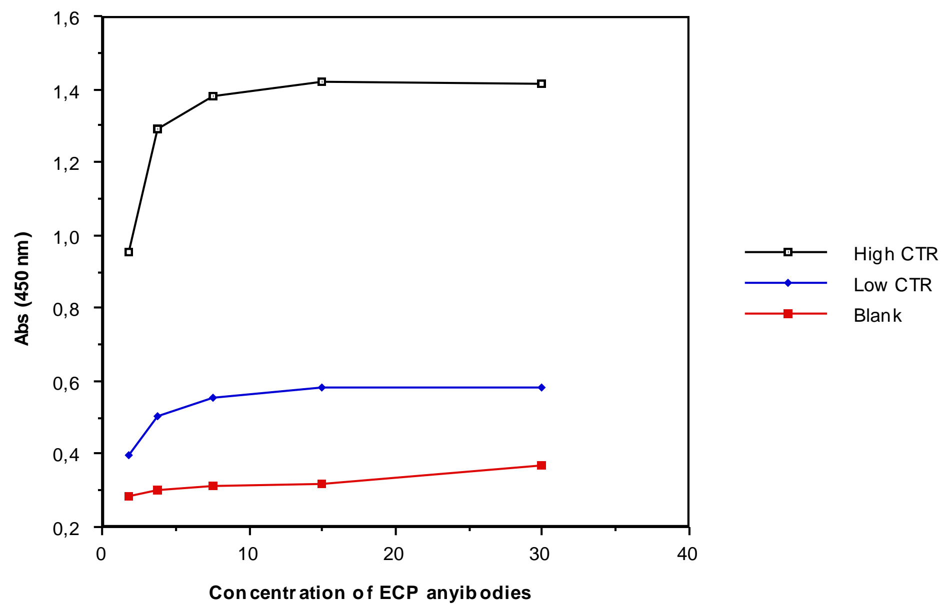

Figure 6. Determination of optimal coating concentration with high control, low control and blank as samples. The concentration of the rabbit anti-ECP antibodies ranged between 1.875-30 µg/ml.

6.2.2 Determination of optimal biotinylated rabbit anti-ECP antibody concentration (conjugate)

As seen in figure 7, the absorbance values (450 nm) gradually decreased with increasing dilution of biotinylated rabbit anti-ECP antibody conjugate. At conjugate dilution´s of 1/4000 or less the background (=blank) slightly increased. Regarding the development time a 1/6000- fold dilution of the conjugate was found suitable. This experiment should be repeated after each labeling of the ECP antibodies.

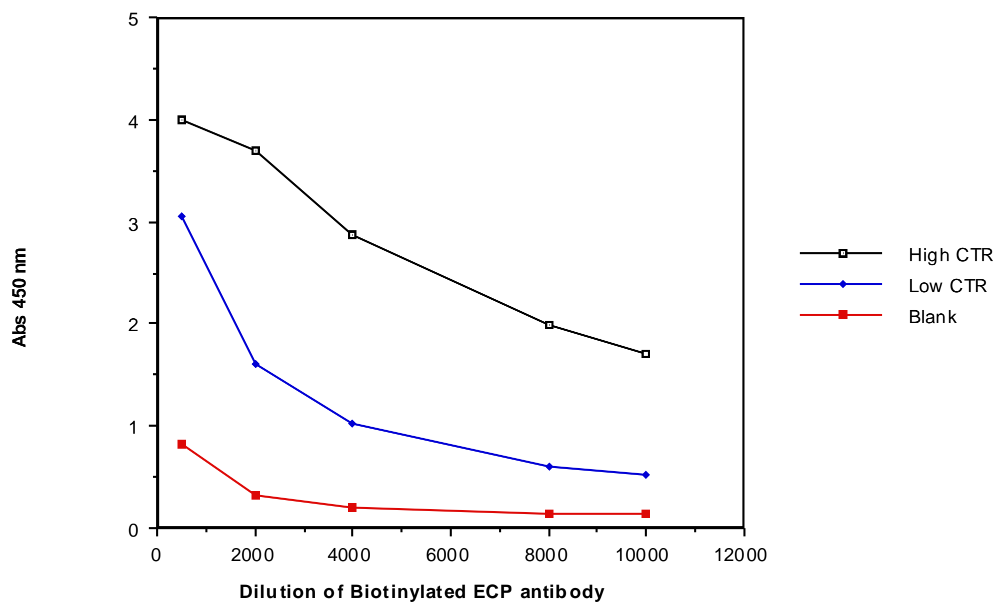

Figure 7. Determination of optimal biotinylated ECP antibody concentration with high control, low control and blank as samples. Biotinylated ECP antibodies were diluted 1/500-1/10000.

6.2.3 Determination of optimal HRP-Avidin concentration

As seen in figure 8, the absorbance values (450 nm) gradually decreased with increasing dilution of HRP-Avidin. At dilution´s of 1/10 000 or less the background (=blank) slightly increased. Regarding the development time a 1/10 000- fold dilution of the HRP-Avidin was found suitable.

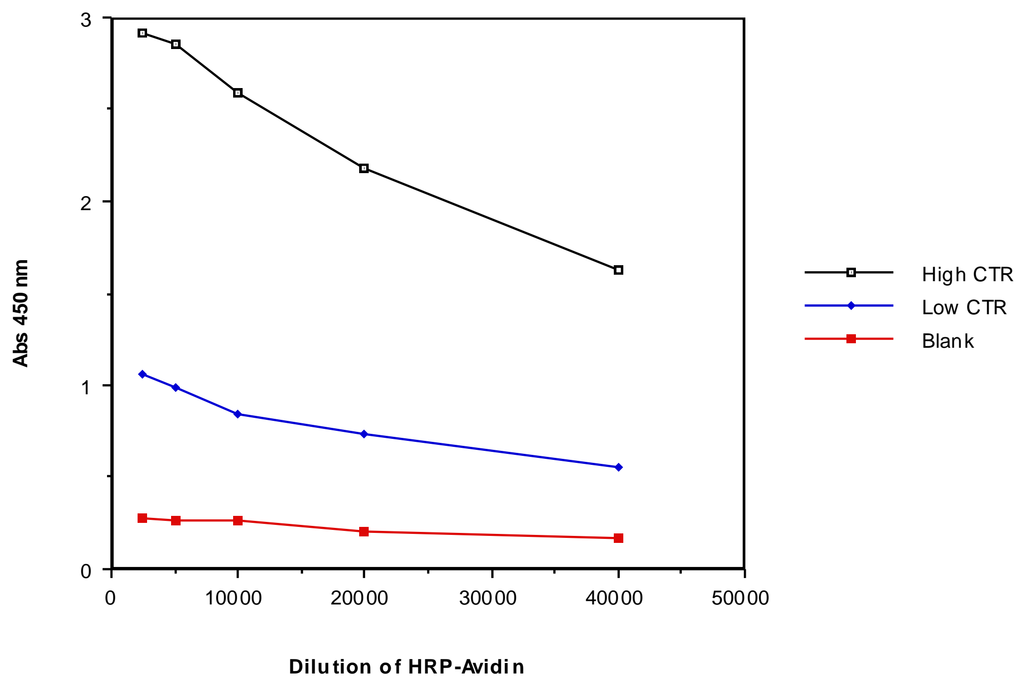

Figure 8. Determination of optimal HRP-Avidin antibody concentration with high control, low control and blank as samples. The HRP-Avidin antibody concentration was diluted 1/2500-1/40000.

6.2.4 Standard Curve

Using the optimal conditions determined 20ug/ml coating antibody, 1/6000 dilution of biotinylated antibody and 1/10000 dilution of HRP-avidin, a standardcurve was performed. A typical standardcurve is shown in figure 9.

Figure 9. A typical standard curve using the optimal parameters.

7 APPLICATIONS

The ELISA method was used for determination of the *E.coli* impurites in five different batches, each batch has three purification steps. Table I show the reduction of *E.coli* proteins during the purification process. The *E.coli* proteins reduced more than 100.000 times from the initial value of the last purification step.

Table I Reduction of E.coli proteins during the purification process

Purification steps	**Batch 245**	**Batch 246**	**Batch 247**	**Batch 248**	**Batch 249**
Initial value	2300000 ng/ml	3000000 ng/ml	3200000 ng/ml	3000000 ng/ml	3000000 ng/ml
1	347 ng/ml	751 ng/ml	612 ng/ml	672 ng/ml	630 ng/ml
2	29 ng/ml	42 ng/ml	50 ng/ml	41 ng/ml	70 ng/ml
3	20 ng/ml	22 ng/ml	22 ng/ml	25 ng/ml	39 ng/ml

8 DISCUSSION

The antibodies raised against the reference antigen preparation (Blank fermentation of PNU-214565) are suitable, as seen with 2-DE to detect contaminating proteins derived from the host organism which may occur both in the final product as well as in the in-process samples. The covering degree was calculated by visual inspection and it is not ensured that the spots from the silver stained gel correspond to the western blot gel.

The covering degree can be much lower, to ensured that it is necessary to scanned the gels and used a computer program to match the two gels together.

This report also describes the development and application of the enzyme linked immunosorbent assay (ELISA) based on these antibodies to be used for the quantification of the contamination of E.coli proteins (ECP) in samples from fermentation and purification steps of PNU-214565. The application shows that the reduction is more than 100.000 times from initial value to the last purification step. The last purification step has a value approximately about 25 ng/ml of *E.coli* related proteins (ECP) , it can seem to be high but the pharmaceutical PNU-214565 will be given only to patients with cancer and hopfully only once in the lifetime.

Further studies will be done to reduced the background of the ELISA method. One possible way is to change the substrate solution to another product, and change HRP-Avidin to HRP-Streptavidin.

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12. Pharmacia Biotech Instructions 18-1038-63
Immobiline® DryS trip Kit for 2-D elechtrophoresis with Immobiline® DryStrip and ExcelGel™ SDS.

13.Amersham: ECL Western Blotting protocol RPN 2106, 2108. 2109.

14. Instructions 21335 ImmunoPure ®Sulfo-NHS-LC-Biotin.