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THE USE OF

IMMOBILIZED METAL-ION AFFINITY CHROMATOGRAPHY

IN IgG-RPE CONJUGATION

A Thesis

Presented to

The Faculty of the Department of Chemical Engineering

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science

by

Claudia Lissette Melara

August 1999

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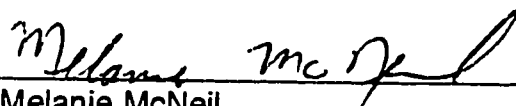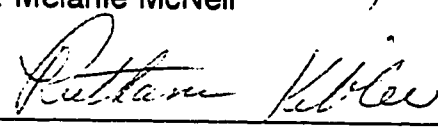
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ABSTRACT

THE USE OF
IMMOBILIZED METAL-ION AFFINITY CHROMATOGRAPHY
IN IgG-RPE CONJUGATION

by Claudia Lissette Melara

The applicability of Immobilized Metal-ion Affinity Chromatography (IMAC) in the conjugation reaction between CD20 antibody and R-Phycoerythrin (RPE) fluorochrome was tested for favoring the isolation of conjugates with an F/P ratio of 1. Nickel ions immobilized the antibody while the RPE was kept in the mobile phase of IMAC at pH 6. A 100 mM imidazole step gradient was used for the desorption of proteins.

A 2^2 Design of Experiments tested RPE loading concentration and reaction time as the factors. The responses were A_{280} and fluorescence of the reaction species. Statistical analysis verified that RPE concentration between the range of 5 to 15 mg/ml had a significant effect on 1:1 conjugate formation. All runs produced CD20-RPE conjugate and when purified resulted in cleaner product than current methods of production. Flow cytometry demonstrated that the experimental conjugate was equivalent to the reference conjugate in identifying the target B cell population.

This thesis is dedicated to:

JEREMY WYLD, my husband,
computer problem solver,
and number one supporter.

MY PARENTS, I succeed
because of your example.

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APC	Allophycocyanin
BD	Becton Dickinson Immunocytometry Systems
CV	column volumes
DOE	design of experiments
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
EX	excitation
EM	emission
F/P	fluorochrome to protein ratio
FITC	Fluorescein Isothiocyante
IDA	iminodiacetic acid
Ig	immunoglobulin, generic term for antibody
IgG	immunoglobulin, class G
IMAC	Immobilized Metal-ion Affinity Chromatography
kD	kilo daltons
MEA-HCl	mercaptoethylamine hydrochloride
2-ME	2-mercaptoethanol
PerCP	Peridinin Chlorophyll Protein
PMT	photo-multiplier tube
R	resolution
RPE	R-Phycoerythrin
scFv	single chain Fv antibody fragment
SH	sulfhyryls

SH/IgG	moles of sulfhydryl sites per moles of IgG
SMCC	succinimidyl-(N-maleimidomethyl)cyclohexane-1-carboxylate
TED	tris(carboxymethyl)-ethylenediamine
TNB	2-nito-5-thiobenzoate
TREN	tris(2-aminoethyl)amine
t_R	retention time
W	width of peak on a chromatogram

INTRODUCTION

This thesis investigates the relationship between antibody immobilization, using Immobilized Metal-ion Affinity Chromatography (IMAC), and efficiency of maleimide mediated fluorochrome conjugation to antibody. Antibodies are powerful tools in diagnostic techniques and cell biology due to their exquisite specificity for discreet molecular features, called antigenic determinants. In many cases, an antibody reaction is identified by a fluorescent molecule, or fluorochrome, attached to the antibody. The success of immunofluorescence often depends on the purity and quality of the antibody-fluorochrome conjugate. This technique of immunofluorescence has brought applicability to techniques such as fluorescent microscopy, fluorescence immunoassay, and flow cytometry; and is widely used in research, drug discovery, diagnostics, and therapeutics.

Two examples of clinical applications of immunofluorescence are lymphoma phenotyping and AIDS monitoring. In both cases, the clinical treatment depends on the stage of the disease. For lymphomas, clear definition of antigen expression is needed to classify the cancer. For HIV infection, the quantification of CD4+ cells is necessary to monitor the progression of the disease. Antibody-fluorochrome conjugates are required tools in both cases. To provide clear and definite results, each type of antibody-fluorochrome conjugate must be specific toward a single antigen and must provide the required intensity of fluorescence.

The logical starting point to ensure quality of the antibody-fluorochrome conjugate is the coupling reaction. The coupling of fluorochrome to antibody has the same pitfalls as other chemical reactions. Most chemical reactions do

not produce only one product. Instead, reaction mixtures contain reagents, products, and by-products. The conjugation reaction mixture contains free fluorochrome as well as conjugated product with different levels of bound fluorochrome. When R-Phycoerythrin (RPE) is used as a fluorochrome, the reaction produces not only the desired conjugate but also conjugates where two RPE molecules are attached to one antibody or where two antibodies are attached to one RPE molecule. The number of RPE molecules bound to the antibody is quantified as the fluorochrome to protein (F/P) ratio. The ideal antibody-RPE conjugate has an F/P ratio of one. The degree of conjugation (high or low F/P) depends, among other things, on the molar ratios of reactants, the availability of target groups, the reaction conditions, and size of the fluorochrome.

The F/P ratio of the conjugate is important not only in the performance of the antibody-fluorochrome conjugate but also in the efficiency of the reaction. As the F/P ratio increases, conjugate brightness generally increases, although fluorescence efficiency can degrade at higher conjugation levels due to self-quenching. Higher F/P ratios can cause non-specific binding of the conjugate as well as conjugate instability. Non-specific staining is an undesired quality when trying to identify specific cell populations.

The current method of conjugating RPE to antibody occurs in solution. The reaction produces significant by-products as well as the desired conjugate with an F/P ratio of 1. If one of the reactants is immobilized on a surface and presented with the other reactant in a sufficiently controlled manner, reaction efficiency could be greatly improved.

This investigation studied the applicability of using IMAC as an antibody

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immobilizing substrate for the conjugation reaction in an effort to improve the conjugation purification process. IMAC uses a metal ion to selectively bind to exposed electron donating pendant groups on the protein's surface. The antibody is a protein with several pendant groups that can interact with IMAC. IMAC was used as a matrix to hold the antibody in the solid phase while the fluorochrome remained in the liquid phase. In this manner, the antibody was bound to the column before the fluorochromes were introduced into the reaction mixture. By binding and immobilizing the antibody and, subsequently, the conjugate, IMAC allowed for the isolation of a purer conjugate. IMAC is a relatively new technique used to purify proteins. To narrow the scope of this investigation, the fluorochrome RPE and the IgG antibody were used as reagents in the conjugation reaction on IMAC.

BACKGROUND

This section provides general information on RPE, antibody, the conjugation reaction, and the conjugate product. The main principles of IMAC, along with general chromatography theory, are also discussed.

R-Phycoerythrin, a Phycobiliprotein

Numerous fluorochromes are obtained from algae. In many species of algae, light is harvested by protein structures called phycobilisomes to drive photosynthesis. Each phycobiliprotein of the phycobilisome is able to absorb light energy of a certain energy range, remove some energy, and transfer a less energetic photon to the next protein in the chain: the definition of fluorescence. When the phycobilisome aggregate is separated into its pieces, the individual phycobiliproteins can be isolated and used as fluorescent tags. Once purified and isolated, the phycobiliproteins become highly fluorescent, because the molecules no longer have any nearby acceptors to which to transfer the absorbed energy. The phycobiliproteins are made up of polypeptides called alpha and beta. The alpha polypeptides are usually smaller than the beta polypeptides. The three main types of phycobiliproteins are phycoerythrin, phycocyanin, and allophycocyanin with their main differences being size, complexity and pigment content. Each type has a distinctive spectroscopic property. For this particular set of experiments, RPE was chosen due to its popularity as a fluorochrome. RPE has a molecular weight of 240 kD with an excitation (EX) maximum of 566 nm and an emission (EM) maximum of 575 nm. The excitation and emission spectra are shown in **Figure 1.**

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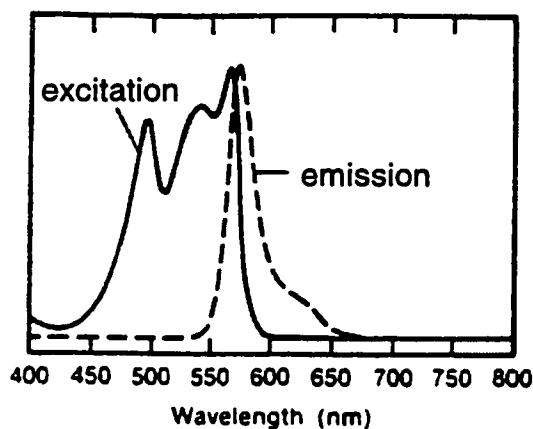
Figure 1: Excitation and emission spectra of RPE.

Phycobiliproteins are readily used as fluorescent labels due to their spectra being in the visible wavelength region and to the ease of coupling them to specific binding molecules like antibodies. Even though phycobiliproteins are large in size, they do not change the ability of the antibody to bind to the antigen. Furthermore, the fluorescent properties of phycobiliproteins are independent of pH over a broad range from pH 5 to 9 (Glazer, 1982).

Antibodies

Antibodies, or immunoglobins (Ig), are a natural part of the immune system. In humans, there are 5 classes of antibodies in the immune system: IgA, IgE, IgD, IgG, and IgM. The classes differ in structure and biological activity. The antibody IgG is the most abundant isotype found in serum (Kuby, 1994). An IgG antibody is a protein made up of four polypeptide chains. Two of the chains, designated heavy chains, are longer and weigh approximately 50 kD each. The other two chains, designated light chains, are shorter and weigh

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approximately 25 kD each. **Figure 2**, a schematic of the IgG antibody

structure, shows the two light chains and the two heavy chains joined by

disulfide bridges to create a structure similar to the letter "Y." Each heavy and

light chain contains a variable region of amino acids which form the epitope[1]

binding site.

Epitope
Binding
Site

Variable
Regions

Constant
Regions

▨▨▨ Heavy Chain

▥▥▥ Light Chain

▬▬ Disulfide Bonds
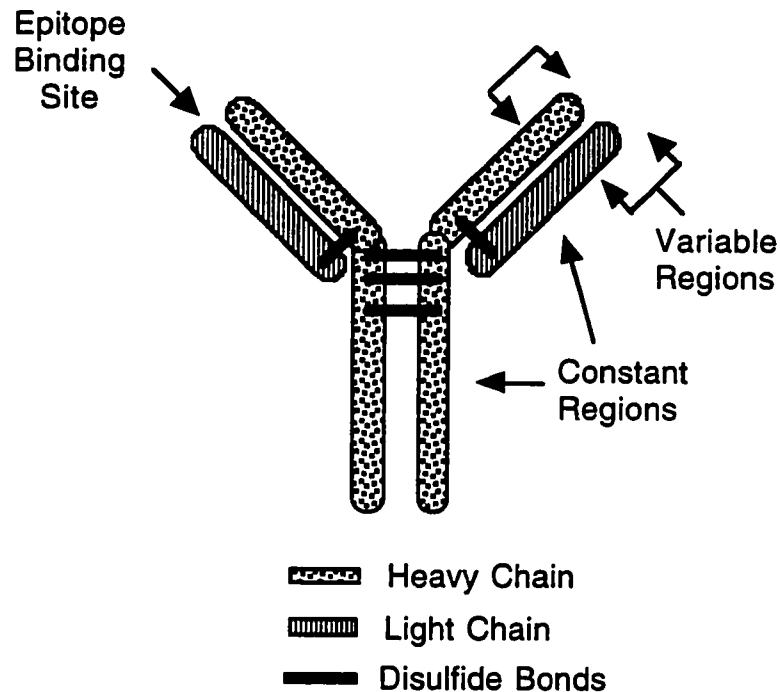
Figure 2: IgG antibody structure.

The most important characteristics of antibodies are their specificity and

diversity. The immune system contains B cells which are able to produce

antibodies if stimulated by antigen. An antigen is defined as foreign material to

the body, such as bacteria, viruses, or any substance that is recognized by the

immune system as non-self. The epitope is that small part of the antigen to

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[1] The epitope is 8-10 nucleic acids long and is the accessible binding site of the antigen.

which the antibody binds. The role of the antibody is to "tag" any foreign substance, which alerts other cells of the immune system to break down the non-self particles. A human is thought to be able to produce antibodies that recognize at least 10^8 different epitopes (Kuby, 1994). The binding of the antibody to the epitope is very specific; small structural changes in the epitope will prevent binding. Viruses fool the immune system by regular mutations to their outer envelope. Understanding how antibodies naturally work led scientists to believe that antibody production by the immune system can be achieved for any substance.

By exposing mice to an antigen of interest, the mice will naturally form antibodies toward that antigen. In this manner, scientists can create antibodies which are specific to the antigen. Monoclonal antibodies (antibody derived from a single B cell clone) can be produced by fusing immunized B cells with a myeloma partner and subsequently cloning an immortal cell line that makes a single kind of antibody. Therefore, monoclonal antibodies are a defined uniform population. A monoclonal antibody differs from the natural antibody only in how it is produced (Kuby, 1994).

Conjugate Production

In 1941, Coons introduced the idea of using fluorescent dyes for direct observation of antibodies (Malik and Lillehoj, 1994). A fluorescent dye like fluorescein offers visual color as well as fluorescence, making an antibody easier to detect. Fluorescein is known as the "green fluorochrome" and is still commonly used to make antibody-fluorochrome conjugates. However, for

research purposes it is useful to detect multiple epitopes on cells at the same time using different colors. It took 41 years before scientists coupled phycobiliproteins with molecules having biological specificity (Oi, *et al.*, 1982). They produced phycoerythrin-antibody conjugates with a fluorescent emission band extending into the red.

Phycobiliproteins are coupled to antibodies using common protein chemistry techniques. The use of heterobifunctional reagents allow different molecules to be linked covalently (Carlsson *et al.* ,1978). Heterobifunctional reagents contain reactive groups capable of reacting with two different targets and serve as the "linking arm" between the two molecules, in this case RPE and the antibody. Some of the most commonly used heterobifunctional reagents are:

- 2-iminothiolane
- S-acetylmercapto-succinic anhydride
- succinimidyl-3-(2-pyridyldithio)propionate
- succinimidyl-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)
- succinimidyl-4-(p-maleimidophenyl)butyrate

For this investigation, SMCC was used to covalently link RPE with an IgG mouse antibody. The heterobifunctional reagent SMCC is used by Becton Dickinson Immunocytometry Systems (BD), and this investigation revolves around improving this coupling reaction by using IMAC. The general reaction scheme can be seen in **Figure 3**.

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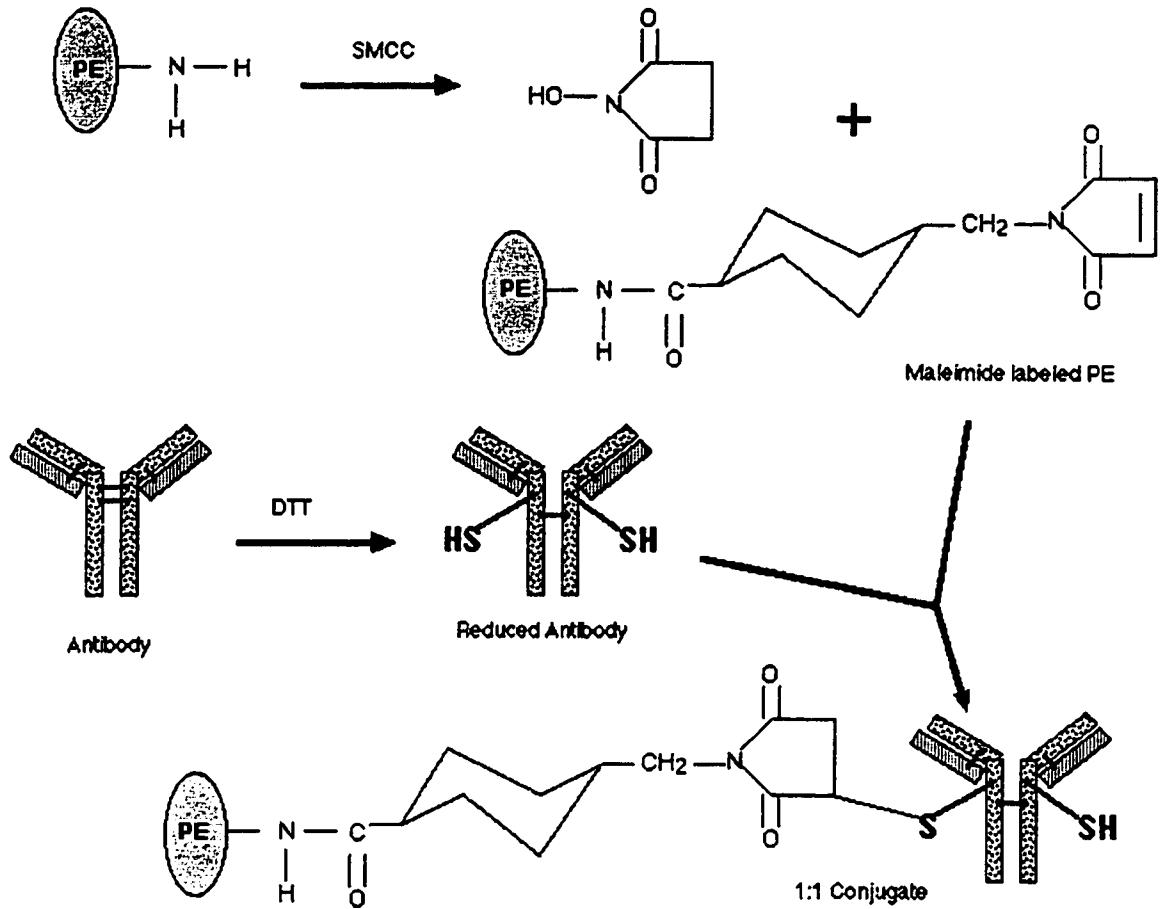
Figure 3: General conjugation reaction.

As **Figure 3** shows, the RPE protein is modified with SMCC to acquire a reactive maleimide group. The heterobifunctional reagent, SMCC reacts with primary amines of the fluorescent protein RPE. After the modification of RPE, free SMCC is removed from the solution via size exclusion chromatography. In a separate reaction, the antibody's disulfide hinge area is reduced by using dithiothreitol (DTT) to provide the sulfhydryl reactive site. To stop the reduction reaction, free DTT is removed by size exclusion chromatography. The conjugation reaction occurs when the modified RPE is mixed with the reduced

antibody. The maleimide groups preferentially react with sulfhydryl groups at neutral or slightly basic pH. This reaction is stopped by adding 2-mercaptoethanol (2-ME) which reacts with any free maleimides on the modified RPE. Then, the antibody-fluorochrome conjugate is purified using size exclusion chromatography. Fractionation by sizing can be difficult since the combined molecular weight of IgG-RPE is 400 kD compared to 240 kD of free RPE. Therefore, the purified 1:1 conjugate fractions will have some amount of free RPE. Free RPE is never desirable since it can cause non-specific staining.

The problem with the coupling reaction of fluorochromes and antibodies is the low efficiency of the reaction. **Figure 3** shows only one product, the ideal conjugate with one fluorochrome to one antibody. There are actually several by-products, including conjugates with one or more RPE molecules attached to an antibody or conjugates with one or more antibodies attached to an RPE. Other by-products include species with two joined RPE molecules or two linked antibodies. As efficiency of the reaction is increased, a statistical mix of species of three or more molecules will be produced as well the desired conjugate (Kronick, 1986). BD's manufacturing practices produce a one-to-one conjugate with a yield of about 25%. Their methods have a low efficiency, but the products are well characterized, the latter which is necessary for mass production.

CD20-RPE Conjugate

CD20-RPE is the antibody-fluorochrome conjugate used in this study. The CD20 designation refers to the epitope, which the mouse antibody identifies. The CD20 (Leu-16) epitope is a phosphoprotein with a molecular weight of 35 kD. The CD20 epitope is expressed on B cells in both the resting

and activated form, but not in plasma cells. A sub-population of T cells also has a low level expression of CD20 epitope. Thus, the CD20-RPE conjugate becomes useful in studies of B cell activation and in determining the population of B cells in peripheral blood.

This conjugate was chosen to be investigated in this study due to its ready availability at BD. For this study, the coupling of CD20 antibody to RPE was attempted using IMAC. The resulting product was purified and then compared to actual CD20-RPE conjugate manufactured by BD. The functions of both experimental conjugate and reference conjugate were compared in enumerating B cells in peripheral blood using flow cytometry.

General Chromatography Theory

Chromatography is a technique that allows for the separation of various compounds based on their differing solubilities between a mobile and stationary phase. A fluid (mobile phase) is the sample carrier and is forced through an immobile, porous, solid medium (stationary phase). The sample is comprised of several compounds that can be separated through the use of chromatography. Compounds that do not react significantly with the stationary phase pass through the column in the mobile phase. The mobile phase can be selected to enhance the absorption of sample onto the stationary phase. Components can be desorbed sequentially by reversing their affinity towards the stationary phase. The migration property of each compound is time dependent and can be measured and identified from chromatographic analysis.

Separation of several compounds within a sample requires varying adsorption properties of the compounds towards the stationary phase. The time

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between sample injection and when the component leaves the chromatography column is defined as retention time (t_R) and is illustrated in **Figure 4**. The degree of separation between two compounds in a mixture is measured by resolution. Resolution (R) between two peaks in a chromatogram (compounds A and B) is given by **Equation 1**. Where t_R is the retention time and W is the width of each peak. Resolution increases as the distance between the two peaks increases and decreases as the average band width increases.

$$R = \frac{2\,[\,(t_R)_B - (t_R)_A\,]}{W_A + W_B}$$

Equation 1
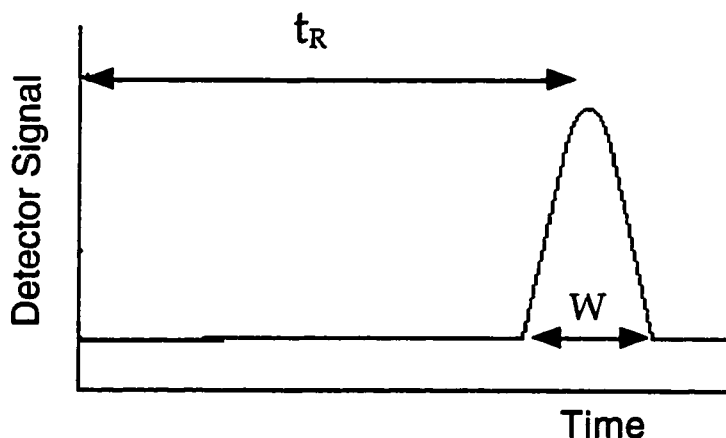
Figure 4: Illustration of retention time on a chromatogram.

To obtain optimal separation between compounds A and B, the chromatographic peaks must be sharp and symmetrical and the resolution should be R $\geq$1. Therefore, band broadening must be limited (W should be kept

as small as possible). Band broadening is undesirable because it makes separation more difficult.

The following factors can affect retention time, resolution, and band width: flow rate; mobile phase composition and temperature; column length; particle size and composition of stationary phase. Increasing the length of the column increases the retention time of each component. However, this can lead to an increase of band broadening, which is undesirable. Increasing the flow rate decreases the retention time of each component. A change in pore size of the stationary phase greatly changes the migration path of each component. Chromatography of a sample should be optimized for increasing resolution in order to easily separate components from a mixture.

Immobilized Metal-ion Affinity Chromatography

IMAC works on the principle that certain structures on proteins have an affinity for heavy metal ions. The antibody is a protein and will bind to the IMAC column. In the mid-1970's, Porath developed the concept of binding metal ions to a column for the separation of proteins. Their purpose was to exploit the different affinities of proteins for heavy metals. At the time, they hypothesized that the different affinity patterns of proteins for immobilized metal ions were due to different histidine and cysteine contents.   Current consensus is that three out of the twenty amino acids (histidine, cysteine and tryptophan) exhibit a strong affinity for IMAC (Hemdan and Porath, 1985; Belew and Porath, 1990; Porath, 1988).   The imidazole group of histidine, the thiol group of cysteine, and the indoyl group of tryptophan all are electron donating amino acids that interact with the immobilized metal. However, the binding strength not only depends

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on the number of exposed electron-donating amino acid residues, but is also affected by pH, salt type, salt concentration, immobilized metal, and protein size (Wong *et al.*, 1991).
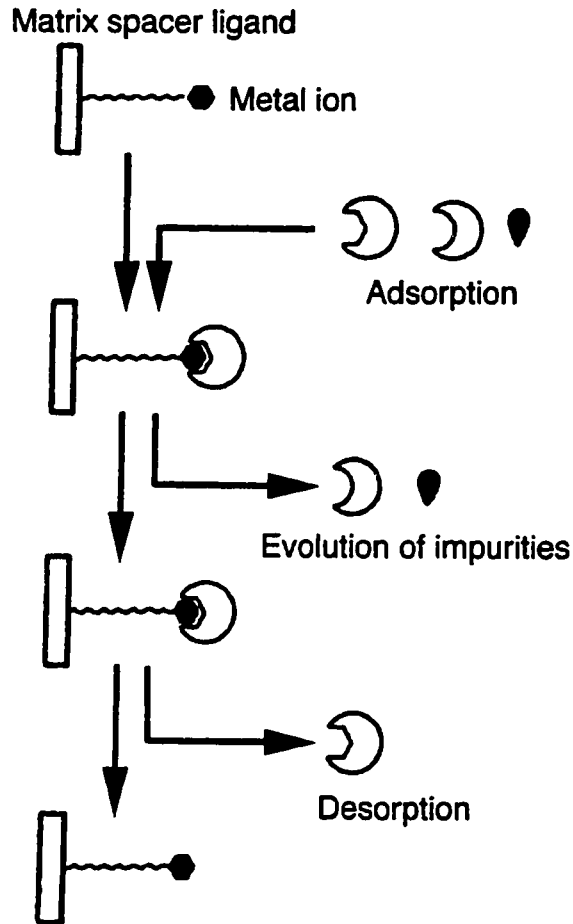
Figure 5: Principle of affinity chromatography (Asenjo, 1990, p. 403).

IMAC separates proteins based on their different affinities for the metal ion as seen in **Figure 5**. The immobilized metal is held in place by a chelating agent attached to a solid matrix support. The main function of the solid matrix is

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structural stability. The solid matrix should have large, uniform pores to allow the interaction of proteins with the metal. To avoid non-specific adsorption of protein to matrix, the solid matrix requires hydrophilic and uncharged properties (Davankov, 1988; Asenjo, 1990). The most common solid matrices are agarose and silica-based gels. Belew and Porath (1990) have reported differences in results due to the solid matrix used. However, each case will be different and largely depends on the types of proteins being separated. Most researchers do not test different solid matrices.

Chelating
agent   Metal ion

Solid phase
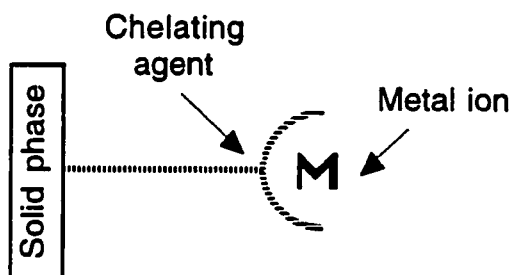
Figure 6: Schematic of immobilized metal ion.

The solid matrix offers a backbone to the column. The chelating agent is what actually immobilizes the metal ion as seen in **Figure 6**. There are also several choices of chelating agents. The two most common are iminodiacetic acid (IDA) and tris(carboxymethyl)-ethylenediamine (TED). Both the chelating agents must strongly hold the metal ion under the IMAC operating conditions and allow the easy removal of all metal ions for regeneration of column (Gooding and Regnier, 1990). The leaching of metal ions into the running buffer occurs when the protein has a stronger affinity for the metal ion than the chelating agent or when the interaction between chelating agent and metal ion

is weak. Porath (1988) suggests the column should not be loaded to capacity with metal ions. Instead, free chelating agent sites should exist to catch any metal ions that may be released during the chromatography run. The complete removal of metal ions from the column is usually done with ethylenediamine-tetraacetic acid (EDTA), which allows for reloading of other types of metal ions or regeneration of the column with the same metal ions. This ability to change the type of metal ions used makes IMAC very versatile since different metal ions will have different affinities for any given protein.

The ideal metals for use in IMAC applications fall into the "intermediate type of ions" as classified by Pearson (Porath, 1988). Copper, nickel, zinc, and cobalt ions interact with mainly nitrogen, but also with oxygen, and sulfur. Copper ion tends to bind histidines much more strongly than any other metal tested. The presence of one exposed histidine amino acid will bind a protein on an IDA-Cu^{2+} matrix at neutral pH (Sulkowski, 1985). Under comparable conditions, a nickel column requires at least two exposed histidines. The type of metal used will have a great effect on the overall separation of proteins. IMAC readily lends itself to the testing of several metal ions since the column can easily be regenerated.

Separation of proteins from a mixture occurs by changing the pH, changing the salt concentration, or by using a competitive eluting agent. A change in pH affects the ability of histidine, cysteine, or tryptophan to serve as an electron donating group. However, pH affects the behavior of the buffer components as well as the stability of the metal (Wong et al., 1991). At neutral pH, the immobilized metals tend to have a net negative charge. Thus, adsorption of proteins onto the column can be manipulated by varying the salt

concentration (Belew and Porath, 1990). Several authors have recommended the use of at least 0.5 M NaCl in the running buffer to eliminate any general ionic interactions between metals and all amino acids. The last technique takes advantage of the high affinity of most metals for the imidazole group in histidine. An increasing concentration gradient of imidazole decreases the retention time of proteins on the column.

LITERATURE REVIEW

This section reviews IMAC related articles, focusing on experimental conditions used to isolate proteins. The literature review captures the evolution of IMAC as an analytical tool. It covers the initial studies done on IMAC (interaction with amino acids) as well as the most recent IMAC applications (purification of recombinant proteins).

Separation of Amino Acids

Hemdan and Porath (1985) studied the behavior of individual amino acids on an immobilized Ni^{2+} column. The column (15 X 2 cm) was packed with Sephadex G-25 (Pharmacia) and the chelating agent used was IDA. The retention of amino acids was studied using an isocratic elution (same buffer throughout run) with 0.2 M N-ethylmorpholine buffer, pH 7, and a flow rate of 16 ml/hr. The effect of adding of 0.5 M K_2SO_4 or 4 M NaCl on individual amino acid retention with isocratic elution was studied. Results are shown in **Table 1**, where retention is measured as V_e/V_t. This experiment demonstrated that histidine, cysteine and tryptophan are retained more strongly than any other amino acids. Notice that the change in salt concentration and type affected amino acids in different ways. A change in salt type and concentration had no effect on cysteine and histidine; both bound tightly during the tests. However, the adsorption of tryptophan was greatly increased when a high salt concentration of 4 M NaCl was used in the buffer. Phenylalanine and tyrosine also had an increased affinity for the column with high NaCl concentration in the

buffer. The rest of the amino acids tested demonstrated little or no changes in adsorption.

Table 1: Retention of amino acids on IDA-Ni^{2+} column (Hemdan and Porath, 1985, p. 258).

Amino Acid	Ve/Vt with Buffer only	Ve/Vt with 0.5 M K2SO4	Ve/Vt with 0.4 M NaCl
L-Alanine	1	2.7	2
L-Arginine	7.6	3.9	11
L-Asparagine	4.9	8.8	9
L-Aspartic acid	1	1.6	2
L-Cysteine	>20	>20	>20
L-Cystine	>20	>20	>20
L-Glutamine	4.2	6	7.5
L-Glutamic acid	0.8	1.6	2.2
Glycine	1.1	3.5	2
L-Histidine	>20	>20	>20
L-Isoleucine	1.9	3.2	3.3
L-Leucine	2.8	3.2	3.3
L-Lysine	5.2	3	3
L-Methionine	4.7	4.7	5
L-Phenylalanine	4	6.8	>20
L-Serine	4.5	4.6	5
L-Threonine	4.9	5.9	6
L-Tryptophan	9.3	>10	>40
L-Tyrosine	6	6.3	>20
L-Valine	2.1	2.7	2.8

Belew and Porath (1990) studied the separation of amino acids on an immobilized Cu^{2+} column. The column (21 X 10 mm) was packed with Chelating Superose (Pharmacia) and the chelating agent used was IDA. Belew

and Porath (1990) eluted the amino acids with a decreasing pH gradient. The buffer used was 20 mM sodium phosphate buffer, 1.0 M NaCl, pH range 7.0 to 3.8, with a flow rate of 0.94 ml/min (56.4 ml/hr). Results shown in **Table 2** are in agreement with those of Hemdan and Porath (1985) in that histidine, tryptophan, and cysteine have stronger affinities for immobilized metal than the rest of the amino acids. In addition, Belew and Porath (1990) demonstrated that copper has a much stronger affinity than nickel for all the amino acids tested.

Table 2: Retention of amino acids on IDA-Cu^{2+} column (Belew and Porath, 1990, p. 347).

Amino acid	Ve/Vt	Amino acid	Ve/Vt
Gly	12	Cys-Cys	26
Ala	11	Phe	17
Ser	14	Tyr	19
Thr	17	Trp	24
Val	11	His	31
Leu	13	Arg	15
Ile	12	Lys	13
Pro	14	Asp	14
Pro-OH	20	Glu	11
Met	16	Asn	17
Cys	26	Gln	14

Purification of Recombinant Proteins

IMAC has proven useful in the purification of recombinant proteins. Since these proteins are genetically engineered, an introduction of a histidine tail is easily accomplished. The gene that encodes the protein can be extended to include a multi-histidine domain at the beginning or end of its sequence. The histidine tail of the recombinant protein binds strongly to IMAC.

Recombinant techniques can be used to change the histidine content and available histidine sites of a protein. Several studies investigated the effectiveness of IMAC separation for histidine containing proteins. Canaan-Haden et al. (1995) achieved 93% purity of a single-chain Fv[2] (scFv) antibody expressed in E. coli. Casey et al. (1995) achieved 90% purity of another recombinant scFv antibody. Johnson et al. (1996) studied recombinant cytochrome c proteins which differed only in their histidine content.

Canaan-Haden et al. (1995) studied both immobilized copper and nickel metal ion columns. They used IDA as the chelating agent and agarose as the solid matrix. The purification of a cloned antibody fragment was done by a pH step gradient and by an imidazole step gradient. The recombinant scFv antibody was engineered to have 6 added histidines at the 5' end of the antibody. For both metal ions, the pH step gradient (pH 4, 5, and 6.3) used a running buffer of 0.1 M sodium phosphate, 0.5 M NaCl. For both metal ions, the imidazole gradient (100 mM and 250 mM steps) contained 0.5 M NaCl and a pH 7. Both metal ions achieved 93% purity of the scFv antibody, by using the different gradients. The IDA-Cu^{2+} column eluted product with 100 mM imidazole. The IDA-Ni^{2+} column eluted product with a pH of 4.0. The researchers also stated that imidazole eluted product for both metal ions but the pH gradient was incapable of eluting product when the copper ion was used. This again supports findings that copper has a stronger affinity for histidine than nickel.

Casey et al. (1995) also engineered an scFv antibody with 6 added histidines. They tested nickel, zinc, and copper ions. The column (10 X 2.5 cm)

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[2] Single chain Fv is an antibody comprised of only its variable region from its light or heavy chain.

was packed with Sepharose and used IDA as chelating agent. The running buffer employed was 0.5 M sodium phosphate with 1 M NaCl. Casey *et al.* (1995) used a 40, 60, 80, 100 and 120 mM imidazole step gradient. They found that protein binding strength of the metal ion ranked as Cu > Zn > Ni. They experienced leaching of nickel ions when the imidazole elution was started. The zinc ions did not leach, but the 80-100 mM imidazole gradient caused impurities when isolating the desired protein. This group of researchers acquired a 90% yield of pure product using copper ions and imidazole as the competitive agent.

Johnson *et al.* (1996) studied how histidine content and position of the histidine affect the affinity of cytochrome c proteins for metal ions. They used a series of yeast cytochrome c variants that differed in presence and location of histidine residues. The column (7.5 cm X 7.5 mm) was packed with TSK Chelate-G600XL (Tosohass) and used IDA as the chelating agent. The metal ion tested was copper with protein elution caused by a linear gradient of imidazole from 1 mM to 10 mM. The running buffer was 50 mM sodium phosphate, 0.5 M NaCl at pH 7.0. The imidazole gradient was able to separate the very similar cytochrome c proteins based on the number/placement of histidines. The separation can be seen in **Figure 7**. Johnson *et al.* (1996) also tested the effect of pH on binding of similar cytochrome c proteins. The buffer used was 50 mM sodium acetate, 50 mM sodium phosphate, 50 mM boric acid, 0.5 M NaCl with pH adjusted from 6.0-9.0. They hypothesized that under acidic conditions, IMAC binding of proteins was mostly due to histidine content. But if basic conditions were used, then IMAC behaved more like an ion-exchanger. In this case, the imidazole gradient proved to be more selective in differentiating

between very similar proteins.
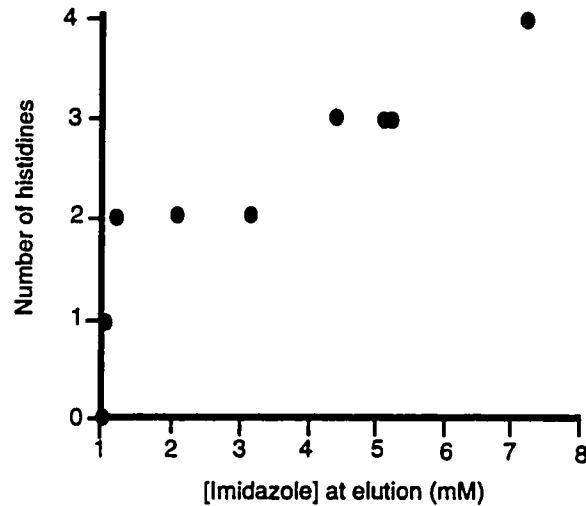
Figure 7: Imidazole gradient elution demonstrating separation of very similar cytochrome c proteins (Johnson et al., 1996, p. 228).

Binding of Antibodies

Since one of the main reagents of interest in this investigation is antibodies, the literature search was focused on antibody binding on IMAC. Boden et al. (1995) were able to separate goat antibodies from blood by using a decreasing linear pH gradient. The column (1 cm X 4.7 cm) was packed with Novarose SE1000/40 and TREN was used as a chelating agent. The metal ion used was copper. The running buffer contained 25 mM sodium acetate, 25 mM 3-(N-morpholino)propanesulfonic acid, 1.0 M NaCl with pH gradient from 7.0- 4.0 and a flow rate of 221 ml/hr. This group of researchers used was very high flow rate compared to the other groups. Boden et al. (1995) recovered goat

antibody with 95% purity near pH 5.5 elution.

Other reports did not focus on the isolation of antibodies, but did mention binding and eluting antibodies. Porath and Olin (1983), using conditions already described, found that Ni^{2+} column absorbed IgG at pH 8.1 while the Fe^{3+} column absorbed IgG more efficiently at pH 5.5. Furthermore, the choice of chelating agents also affected the results. The IDA column adsorbed the antibodies well while the TED column did not work as well.

HYPOTHESIS AND OBJECTIVES

The purpose of this study was to determine the applicability of IMAC as an immobilizing agent for antibody to facilitate the RPE and antibody conjugation reaction. This study has several discrete objectives:

- Immobilized nickel ions were tested for the ability to differentiate between free RPE and free antibody. Differentiation occurred when adsorption onto the column was significantly different for the antibody and free RPE.

- IMAC was tested for the ability to support the conjugation process. Free antibody, free RPE, and IgG-RPE conjugate were loaded onto IMAC individually to determine if adsorption occurs at the pH required for the conjugation reaction. The A_{280} and fluorescence of IMAC effluent were measured continuously for each experiment.

- Conjugation was optimized through the use of statistical Design of Experiments analysis.

- Final product was tested for equivalence to conjugate from liquid phase conjugation.

Hypothesis

Free antibody and free RPE will have different IMAC adsorption conditions at pH 6. Conjugation between reduced antibody and modified RPE will be possible on IMAC. Purification of IgG-RPE conjugate will result in cleaner product by using IMAC, since RPE will not be adsorbed onto the column.

MATERIALS AND METHODS

All the necessary equipment and materials for this investigation were provided by BD. All experiments were conducted at BD.

Immobilized Metal-ion Affinity Chromatography

The IMAC experiments were performed on a HR 5/50 glass column (Pharmacia, Upsalla, Sweden) with a bed volume 1 ml. The column was packed with MC Poros gel (PerSeptive Biosystems, Cambridge, MA) which has IDA as the chelating group and a pH stability of 3-13. The column used is designed specifically for high performance chromatography. Its glass construction was very useful in this experiment, because it allowed for easy observation of the fluorescent dye on the column.

The experiments were performed at room temperature (20 -25°C) using a BioCAD RPM workstation (PerSeptive Biosystems). The workstation consisted of:

1. Buffer/solvent pump system with six buffer channels
2. Sample loader with manual injection
3. Columns and plumbing
4. Spectrophotometer and fluorometer detectors
5. Conductivity, pH and pressure monitors
6. Computer control

Figure 8 shows a schematic of the BioCAD. The BioCAD can continuously monitor pH, A_{280}, fluorescence, and conductivity of IMAC effluent. The BioCAD can be programed to perform gradient elutions with a continuous

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or discontinuous buffer system.
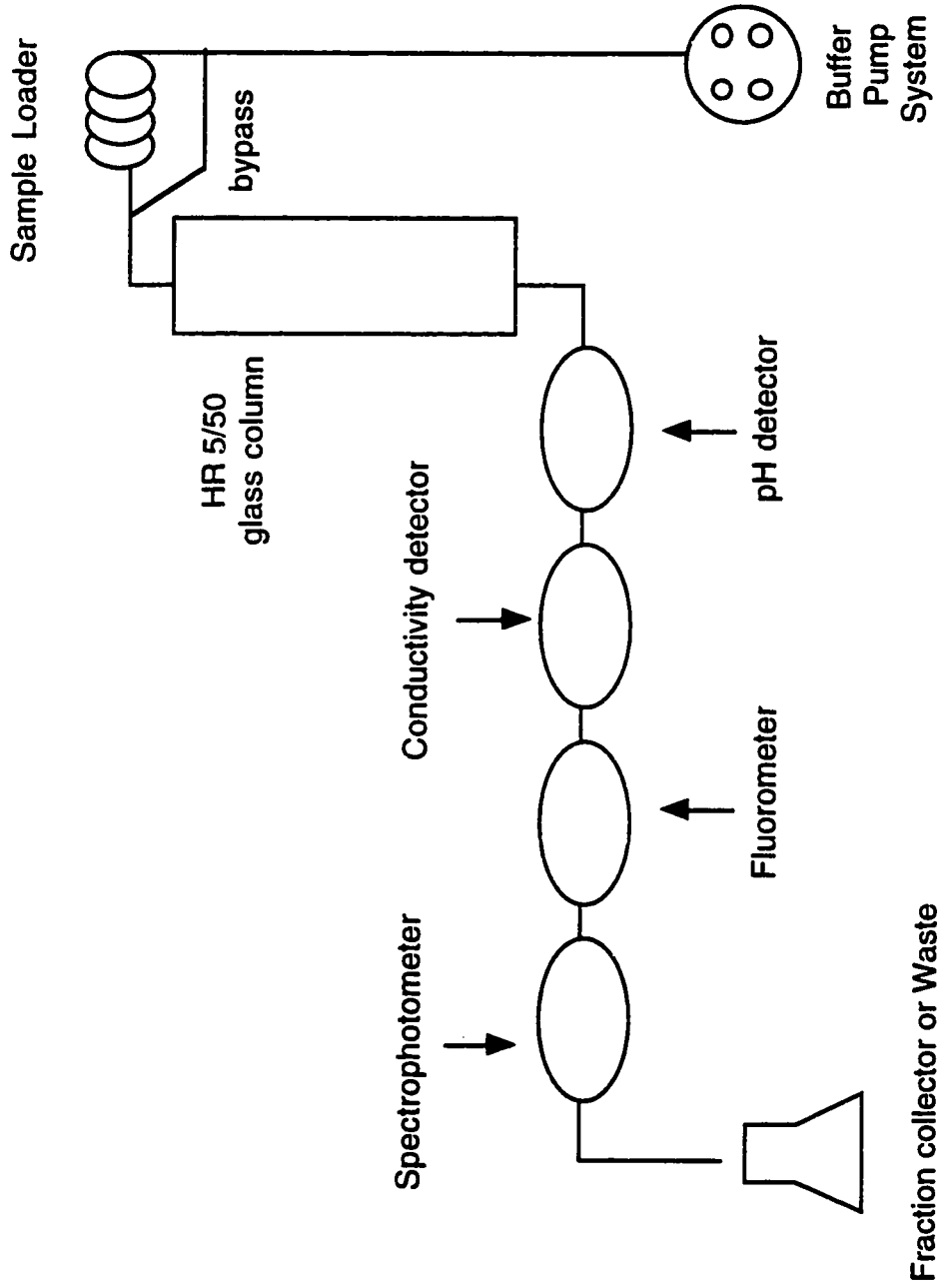
Figure 8: Schematic of BioCAD workstation.

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The following buffers/solvents were used and are referred to in abbreviated form:

- Solution A (column priming solution) - 50 mM sodium acetate buffer, 0.5 M NaCl, pH 3.8

- Solution B (metal ion solution) - 50 mM NiCl$_2$ in Solution A, pH 4.0

- Buffer C (equilibration buffer)- 50 mM MES buffer, 0.5 M NaCl, pH 6.0

- Buffer D (elution buffer) - 200 mM imidazole dissolved in Buffer C, pH 7.0

- Solution E (regeneration solution) - 100 mM EDTA, 50 mM sodium phosphate buffer, pH 6.0

Solution A was used to prime the column before the introduction of metal ions. Solution B provided the metal ions to the column. Buffer C was the running buffer and was used to load samples. Buffer D contained the competitive agent, imidazole, and was used in a gradient or step elution to remove bound proteins from the column. Solution E contained EDTA which stripped the column by removing the metal ions and any bound protein. Thus, solution E regenerated the column and guaranteed a clean column for each run.

This section describes the procedure required to perform an IMAC experiment. **Figure 9** shows the process flow chart for the IMAC experiments. The first step was to pack the HR 5/50 glass column with MC Poros gel to give a total bed volume of 1 ml. The packing of the gel was done with a 5 ml/min flow rate of a 30% ethanol solution in RO/DI water. The gel was then washed with RO/DI water for 10 column volumes (CV) to remove any ethanol. The activation of the gel with metal ions was done with a flow rate of 2 ml/min. First, Solution A was run through the gel for 12 CV. Solution B was then pumped through the gel for 15 CV to allow adequate loading of metal ions. The IMAC column was

then equilibrated with Buffer C for 15 CV. At this point the IMAC column was ready to accept the sample.

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        ┌────────────────────────┐
        │   Pack column with gel  │
        └────────────────────────┘
                    │
                    ▼
        ┌────────────────────────┐
  ┌────▶│      Add metal ions     │
  │     └────────────────────────┘
  │                 │
  │                 ▼
  │     ┌────────────────────────┐
  │     │ Equilibrate with running buffer │
  │     └────────────────────────┘
  │                 │
  │                 ▼
  │           ┌──────────────┐
  │           │  Add sample   │
  │           └──────────────┘
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  │                 ▼
  │     ┌────────────────────────┐
  │     │   Remove loosely bound  │
  │     │  sample with running buffer │
  │     └────────────────────────┘
  │                 │
  │                 ▼
  │     ┌────────────────────────────┐
  │     │ Elute with increasing concentration │
  │     │ of competitive agent (imidazole)    │
  │     └────────────────────────────┘
  │                 │
  │                 ▼
  │     ┌────────────────────────┐
  │     │ Remove metal ions with EDTA │
  │     └────────────────────────┘
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  └─────────────────┘
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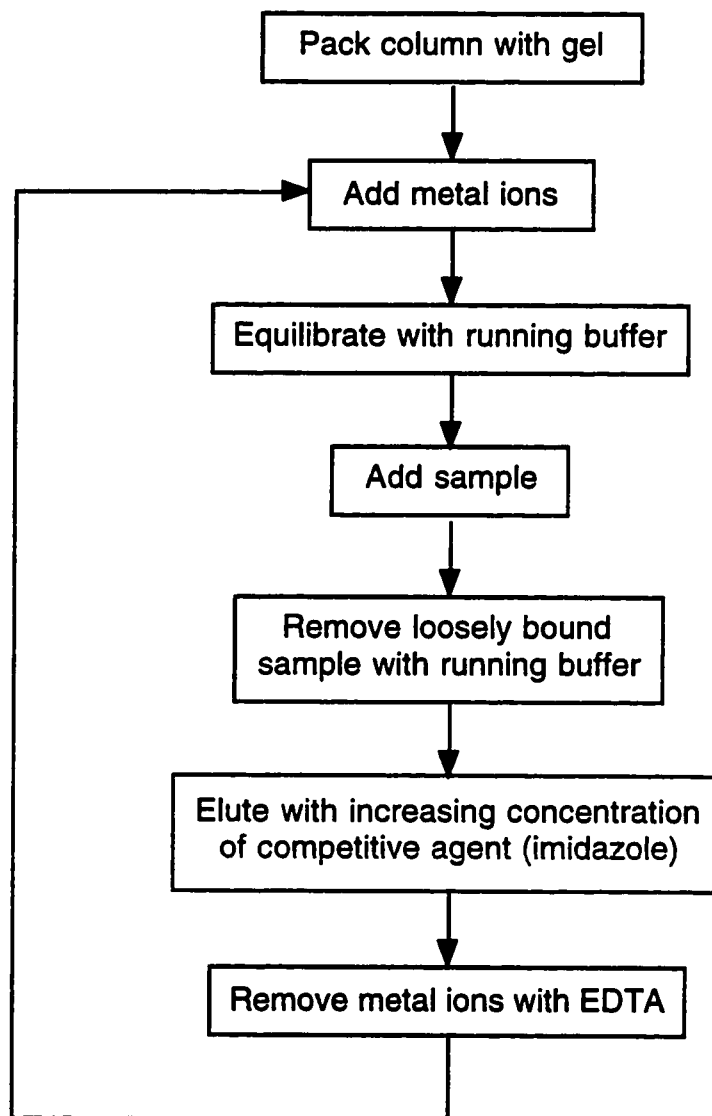
Figure 9: Process flow chart of initial IMAC experiments.

The adsorption of sample was done at a flow rate of 1 ml/min. After the injection of sample, Buffer C was pumped for 10 CV to remove any loosely bound sample. A step or linear gradient with Buffer D was then used to force the desorption of sample. Finally, the regeneration of the column occurred by pumping Solution E through the gel at 1 ml/min for 15 CV.

Since the column used was made of glass, changes in the IMAC gel media were easy to detect. Sometimes, depending on the amount of fluorescent species loaded, a trace of color was seen throughout the column even after Solution E was used. A 20 to 100 µl injection of 0.5 M NaOH was used to remove all traces of the color. RO/DI water was used as the running buffer. After the NaOH injection, the solution sequence used was 5 CV of water, followed by 5 CV of 30% ethanol solution in RO/DI water, and back to 5 CV of water. At this point, the IMAC column was regenerated as specified previously.

Design of Experiments

The use of Design of Experiments (DOE) allowed for evaluation of multiple factors with few experiments. Statistical analysis for DOE was performed with Design-Ease software (STAT-EASE Inc. Minneapolis, MN). Analysis of the results included a statistical evaluation of the significance of single factors and of factor combinations for a given response. The statistical results included the ANOVA table, the model coefficients with associated t-tests, and a case table that included actual values, predicted values, residuals, and calculated statistics for use in validating the model. The Design-Ease software performs the required statistical computations. Sometimes the statistical properties of the data require a transformation to improve the fit of the data to

the model. The Design-Ease program allows for numerous transformations as seen in **Table 3**. Usually, the data do not require transformation.

Table 3: Data transformation options of Design-Ease.

Transformations	Formulas
Square Root	$y' = \sqrt{y}$
Natural Log	$y' = \ln (y + k)$
Base 10 Log	$y' = \log_{10} (y + k)$
Reciprocal Square Root	$y' = \dfrac{1}{\sqrt{y}}$
Inverse	$y' = \dfrac{1}{y}$
Power	$y' = (y + k)^x$
Logit	$y' = \ln \left(\dfrac{y - \text{lower limit}}{\text{upper limit} - y} \right)$
ArcSin Square Root	$y' = \arcsin (\sqrt{y})$

However, if the residuals versus predicted response plot shows a pattern, then the data should be transformed to correct the problem. A pattern in the plot of residuals versus predicted values suggests that the variance (standard deviation squared) is dependent on the predicted response levels. This is a violation of the assumption made when computing the ANOVA results. For

each of the responses tested, the ANOVA assumptions are checked to verify that the analysis of the results is valid.

Another important step in the analysis is to determine the significance of the factors. For the results to be significant, the F value must be larger than the F critical value. The F value compares the variance of two sample groups. The Design-Ease program automatically determines the probability of the F value being greater than the F critical value due to chance alone. The probability is desired to be as small as possible since it predicts the percentage of time that the value of F would occur due to chance. A 10% significant level requires that the Probability>F number to be under a value of 0.1. When significance is found, the null hypothesis stating that the treatments do not differ must be rejected. Analysis of how the factors affect the responses is valid only when significance has been established.
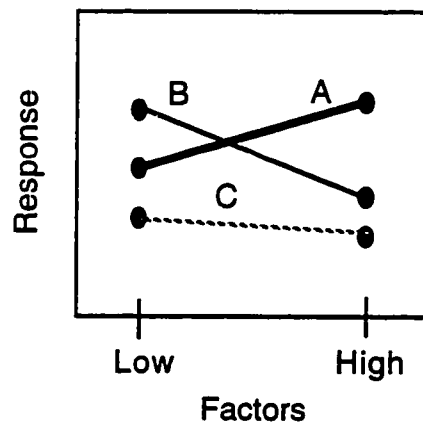

Figure 10: Example of Design-Ease single interaction analysis output.

The analysis of how the response changed with factor levels was made upon the validation of the model and the determination of significance levels. Design-Ease created graphs as shown in **Figure 10** for a single interaction analysis. In the example shown in **Figure 10**, factors A and B have opposite effects, while factor C has the least effect (based on a comparison of slopes). In that case, factor C can be chosen and kept constant in future studies. Correct analysis of the initial DOE results makes the second DOE more focused in understanding IMAC applicability in conjugate production.

Experimental Procedure

The adsorption conditions on IMAC were examined for the two reagents of the conjugation reaction, free RPE and antibody. Each was individually loaded onto the IMAC column with column stripping and activation prior to each load. Once the sample was loaded, the effluent was monitored for fluorescence and absorbance. Five bed volumes of Buffer C were monitored before introducing the competitive elution gradient. A linear gradient from 0 to 100% of Buffer D in 10 CV was employed to elute sample from the column. The linear gradient determined if a 0.2 M imidazole concentration of Buffer D was high enough to elute the species of interest. The imidazole concentration proved adequate since desorption of species occurred before 100% Buffer D was reached. Subsequently, a step gradient to Buffer D was used in the DOE studies so as to not dilute the eluent collected. The last step was to use Solution E to remove metal ions (stripping) and to regenerate the column.

Ellman's reagent was used to test if the antibody's sulfhydryl groups were physically available while the antibody was bound to IMAC. Initially, 100 µl of

14.9 mg/ml of reduced antibody was loaded at 1 ml/min onto IMAC without the removal of DTT[3]. This was done to test if IMAC could also act as the buffer exchange column. Approximately 8 CV of Buffer C were used to wash excess antibody off the column. 0.01 M Ellman's reagent was injected at a volume of 100 µl and at a flow rate of 0.2 ml/min. The flow rate was increased to 1 ml/min once the spectrophotometer, set at A_{412}, detected Ellman's reagent in the effluent. Buffer C was pumped through the column until the absorbance stabilized back to zero. A step gradient to Buffer D was used to elute the antibody from the column. Fractions were collected during Ellman's injection and Buffer D elution.

The conjugation reaction was attempted on IMAC by loading the antibody first and then introducing RPE to the column. First, the required prep work of the antibody and RPE was accomplished before loading them onto IMAC. The reduction of antibody and the modification of RPE were performed as stated in **Appendix A**. The RPE molecules were modified to react with the sulfhydryl sites of the antibody. The modification of RPE was done in one large batch that could be stored for up to two weeks. Each experimental run required a new batch of reduced antibody because the sulfhydryls tend to reform a disulfide bridge, thereby voiding the site for RPE conjugation. For the reduction of the antibody, a 1 ml sample containing 12 mg of IgG was incubated with 20 µl of 1 M DTT for 30 minutes. The reduced antibody was then purified using a buffer exchange column packed with Sephadex G-25 fine media (Pharmacia, Upsalla, Sweden). During the buffer exchange procedure, the antibody became diluted to less than 2 mg/ml. A 2 ml injection was used to ensure that

[3] DTT is the reducing agent used to break the disulfide bridges of the antibody to create reactive sulfhydryl sites.

the column was filled to capacity with antibody. The effect of the antibody loading conditions on overall conjugate production was not studied in this investigation. A 2 ml sample of reduced antibody, average concentration 1.82 mg/ml, was injected into the IMAC column at 1 ml/min for each run. The concentration of the reduced antibody varied slightly per run with a high of 1.98 mg/ml and a low of 1.65 mg/ml. The available sulfhydryls per antibody (SH/IgG) also varied for each batch of reduced antibody. The assay for determining how many available sulfhydryls per reduced antibody was followed as stated in **Appendix A.**

The actual conjugation was attempted on IMAC once the column was activated. After the introduction of the reduced antibody, the IMAC column was washed with 10 CV of Buffer C to remove any loosely bound antibody. Meanwhile, the injection port and needle were washed with Buffer C to remove any reduced antibody. A 1 ml sample of modified RPE (5 mg/ml, 10 mg/ml, or 15 mg/ml) was then injected onto the column at 0.2 ml/min. The IMAC column was saturated with approximately 1 CV of RPE sample solution and then the flow was stopped. The glass column was covered with aluminum foil to protect the fluorescence of the RPE. After the desired reaction time (30 min., 60 min., or 90 min.), the column was washed with 10 CV of Buffer C to remove the non-adsorbed RPE. A step gradient from Buffer C to Buffer D was used to elute the bound species. The eluent for each of the runs was collected upon the introduction of Buffer D to the column. A volume of approximately 3 ml was collected per run. To stop the conjugation reaction, 5 µl of 10 mM 2-ME was immediately added to the elution samples. 2-ME reacts with the maleimide on the RPE and stops the formation of conjugate while the elution samples are

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stored. The elution samples were stored at 4°C until all the runs were completed.

Samples were evaluated individually by analytical gel filtration. For flow cytometry, the eluted samples were pooled to obtain sufficient conjugate and purified by size exclusion chromatography (**Appendix A**). The final product is referred to as the experimental conjugate of CD20-RPE. Finally, the experimental conjugate and the actual bottled product manufactured by BD, the reference conjugate, were tested for purity and B cell enumeration.

Analytical Methods

To measure if adsorption and desorption of protein occurred, the effluent of IMAC was continuously monitored for A_{280} and fluorescence. The fluorescence was monitored since free RPE and conjugate show emission at 573 nm when excited at a wavelength of 488 nm. Furthermore, RPE was visually detected on the column by its bright, pink color. The spectrophotometer detector monitored the absorbance of antibody, RPE, conjugate, metal ion, and imidazole at a wavelength of 280 nm. The spectrophotometer detector was set at 412 nm when monitoring the Ellman's assay on IMAC. These were the basic tools that identified protein adsorption and desorption on IMAC. **Table 4** shows how species were monitored throughout the experiment. Once sample was collected from the effluent, it was further analyzed using high performance liquid chromatography. The chromatographic fractions were analyzed to study if IMAC improved the isolation of 1:1 IgG-RPE conjugates. The HP1100 Liquid Chromatography System (Hewlett Packard, Palo Alto, CA) was used to determine free RPE, free antibody, and conjugate composition of the fractions

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collected from IMAC based on size exclusion. The HP1100 was also used to analyze the fractions collected during the Ellman's assay on IMAC. Analysis of the fractions determined the amount of free antibody and Ellman's reagent in the effluent sample.

Table 4: Monitoring of species throughout conjugation experiments and Ellman's assay conducted on IMAC.

	Buffer C	Absorbance	Fluorescence EX 488, EM 573	Buffer D	Sample collection of effluent
Conjugation					
Inject sample:					
IgG	yes	A_{280}	no	-	-
PE	yes	A_{280}	yes	-	-
Non-adsorption or flow-through of excess sample:					
IgG	yes	A_{280}	no	-	-
PE	yes	A_{280}	yes	-	-
Forced Elution:					
IgG	-	A_{280}	no	yes	yes
Conjugate	-	A_{280}	yes	yes	yes
PE (trace levels)	-	A_{280}	yes	yes	yes
Ellman's Assay					
Inject sample:					
IgG	yes	A_{280}	no	-	no
Ellman's	yes	A_{412}	no	-	yes
Non-adsorption or flow-through of excess sample:					
IgG	yes	A_{280}	no	-	no
Ellman's	yes	A_{412}	no	-	yes
Forced Elution:					
IgG	-	A_{280}	no	yes	yes
Ellman's	-	A_{412}	no	yes	yes

Analysis using the HP1100 Liquid Chromatography System was systematic. The running buffer was 50 mM sodium phosphate + 0.15 M NaCl + 2 M Urea + 0.1% NaN3 with a flow rate of 1 ml/min. The columns used were Zorbax 250 and Zorbax 450 (MAC-MOD, Chadds Ford, PA) in series. The fluorescence detector was set at excitation of 488 nm and emission of 573 nm to detect any free RPE and any conjugate. The absorbance detector was set to 280 nm to determine any non-fluorescent species. A 50 μl, 100 μl, or 200 μl injection of sample was used in each analysis. The PMT value for the fluorescence detector was adjusted so the fluorescence signal was on scale.

The experimental conjugate was tested against the reference conjugate in its ability to enumerate B cells in peripheral blood. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used to check the performance of the IgG-RPE conjugated on the IMAC column. Staining of the whole blood was done by the lysed whole blood method. One hundred microliters of blood was reacted for 15 minutes at room temperature with the following conjugates:

- CD8-FITC, 0.125 μg/test (BD)
- CD20-RPE, 0.5 μg/test for reference conjugate (BD) or 0.25 μg/test for experimental conjugate
- CD3-PerCP, 0.125 μg/test (BD)
- CD19-APC, 0.5 μg/test (BD)

Erythrocytes were fixed with 2 ml of FACSlyse (BD) and then centrifuged. The dual lasers on the FACSCalibur were set at 488 nm and 650 nm. The flow data software used was PAINT-A-GATE[PRO] (BD). Lymphocytes were gated using forward and side scatter fluorescence analysis.

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RESULTS AND DISCUSSION

The results of this study are discussed in this section. The initial set of experiments explored the possibility of coupling fluorochrome to the bound antibody on IMAC. It was found that CD20 antibody bound to the metal ions of IMAC at the coupling pH of 6. Furthermore, the fluorochrome RPE did not bind to the column at that pH. The initial set of experiments showed that conjugation could occur on the column and a DOE was created to test RPE concentration and reaction time as the important factors of the design. The ten runs performed on IMAC all produced CD20-RPE conjugate. However, free antibody was the major component derived from IMAC's Buffer D eluent, as verified through high performance liquid chromatography. Further processing was required to isolate the CD20-RPE. The conjugate was purified in the same manner as bottled product. The purity of experimental conjugate was compared to the reference conjugate. Lastly, the experimental conjugate was tested against reference conjugate for ability to react with B cells.

Antibody Adsorption and Elution

Initially, pure CD20 antibody was loaded onto IMAC and tested for adsorption at the required conjugation reaction pH of 6. The antibody without reduction was adsorbed onto the column (results not shown) using the IMAC methods described previously. Forced elution using Buffer D caused a distinct absorbance peak detected at 280 nm. These were the desired results and further testing on IMAC continued.

The antibody was reduced and desalted as stated in **Appendix A**. The

reduction reaction was made up of 1.5 ml of 12 mg/ml CD20 antibody and 30 µl of 1 M DTT. After the required reaction time the antibody was purified to remove DTT. Finally, a 2 ml injection of 2.7 mg/ml of the reduced antibody was added to IMAC at a flow rate of 1 ml/min. The total concentration of antibody loaded onto the column was 5.4 mgs (2 ml * 2.7 mg/ml). 10 CV of Buffer C was pumped through the column to wash off loosely bound antibody. A total of 9 ml of the injection flow-through was collected. The concentration of this sample was measured as 0.54 mg/ml using **Equation A.3** in **Appendix A**. Therefore, the total milligrams of antibody that did not adsorb upon injection was determined as 4.9 mgs (0.54 mg/ml * 9.1 ml). Buffer D was used to elute the bound antibody from the column. This effluent was collected for further analysis. Measuring the concentration of the antibody in the Buffer D effluent required the entire sample to be dialyzed for the removal of imidazole. This was necessary since imidazole has an absorbance at 280 nm, like the antibody. The Buffer D effluent had a volume of 6.85 ml after dialysis. Then the antibody concentration could be determined by **Equation A.3** found in **Appendix A**. The concentration was read as 0.068 mg/ml for the Buffer D effluent. The total milligrams of antibody that adsorbed onto IMAC was determined as 0.47 mgs (0.068 mg/ml * 6.85 ml). To verify this value, a mass balance was computed as seen in the following equation:

$$\underset{\substack{\text{IgG}\\\text{injection}\\\text{load}}}{5.4 \text{ mgs}} = \underset{\substack{\text{IgG flow}\\\text{through}}}{4.9 \text{ mgs}} + \underset{\text{IgG adsorbed}}{0.47 \text{ mgs}} = \underset{\substack{\text{calculated}\\\text{total}}}{5.37 \text{ mgs}} \qquad \textbf{Equation 2}$$

The calculated value and the actual antibody injection load are very close. Therefore, 0.46 mg is an adequate estimate for the antibody adsorption capacity of the IMAC column and is used throughout subsequent studies.

Ellman's Assay on IMAC

The reaction conditions require IMAC to bind to the reduced antibody with the reactive sulfhydryl groups still accessible. Ellman's reagent, 5,5'-dithio-bis(2-nitrobenzoate), was used to prove the availability of the sulfhydryl sites. Ellman's reagent reacts with the reduced antibody's sulfhydryls to produce 2-nitro-5-thiobenzoate (TNB), which is quantified at an absorbance of 412 nm. As **Figure 11** shows, Ellman's reagent reacts to form one mole of TNB per mole of sulfhydryl groups on the antibody. The TNB is bright yellow and is easily detected with the naked eye. Therefore, a positive test for accessible sulfhydryl groups is indicated if the effluent turns bright yellow. The reaction can be monitored by spectrophotometry once the Ellman's reagent is introduced to the IMAC column containing the reduced antibody. This reaction is very fast and is not an exact duplicate of how the reduced antibody and the modified RPE react.

The antibody was reduced as specified in **Appendix A**. The concentration of the reagents were 0.25 ml of 14.9 mg/ml of IgG and 5 μl of 1 M DTT. After the required 30 minutes for reduction of antibody, 120 μl of the reaction mixture was injected onto IMAC. This was done to test if IMAC could act as the buffer exchange column and not adsorb DTT. Approximately 8 CV of Buffer C were used to remove the non-adsorbed species. At that point, 100 μl of 0.01 M Ellman's reagent was injected onto IMAC at 0.2 ml/min. A bright yellow band appeared near the bottom of the column and was detected in the effluent

at A_{412} with a peak max of 1.9. **Figure 12** shows the spectrophotometer A_{412} output for the IMAC effluent. The peak labeled Fraction 1 is the band observed and was collected for further analysis. The flow rate was increased to 1 ml/min once the spectrophotometer began detecting the peak. A duplicate Ellman's assay was made, 100 µl injected at a flow rate of 0.2 ml/min, and then increased to 1 ml/min once the peak was detected. This time, no yellow color was seen on the column. However, the spectrophotometer did detect a peak in the effluent.
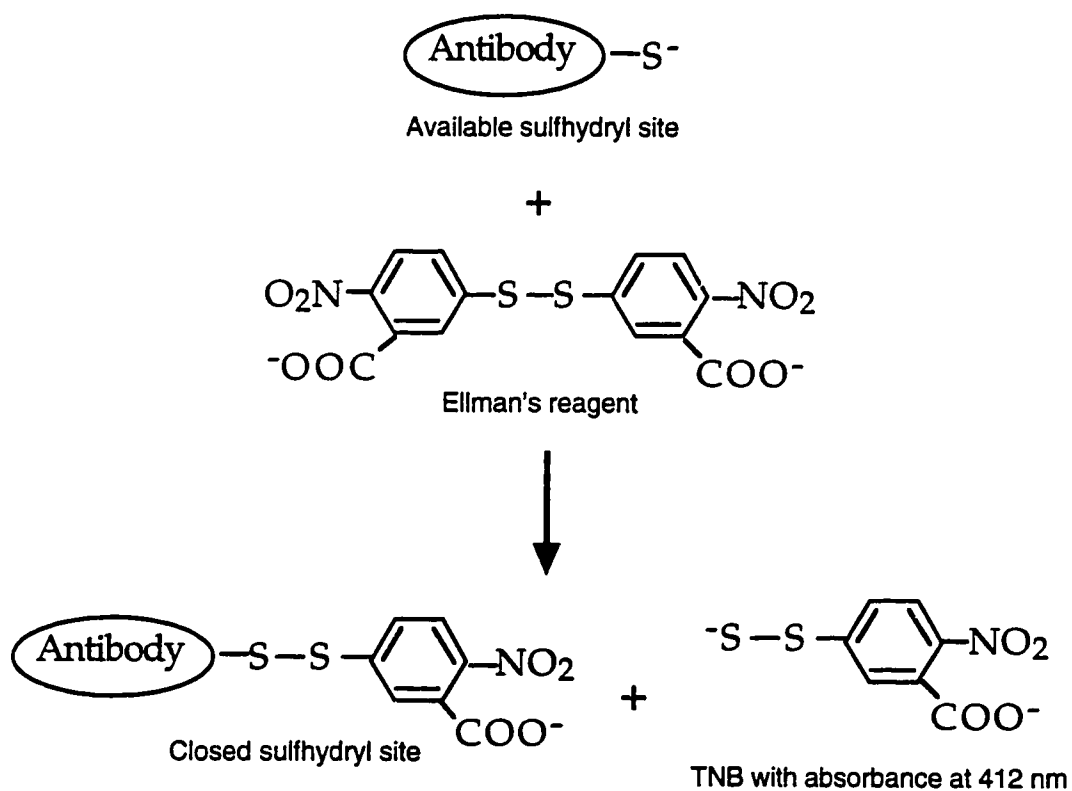
Figure 11: Reaction of Ellman's reagent with a sulfhydryl group.

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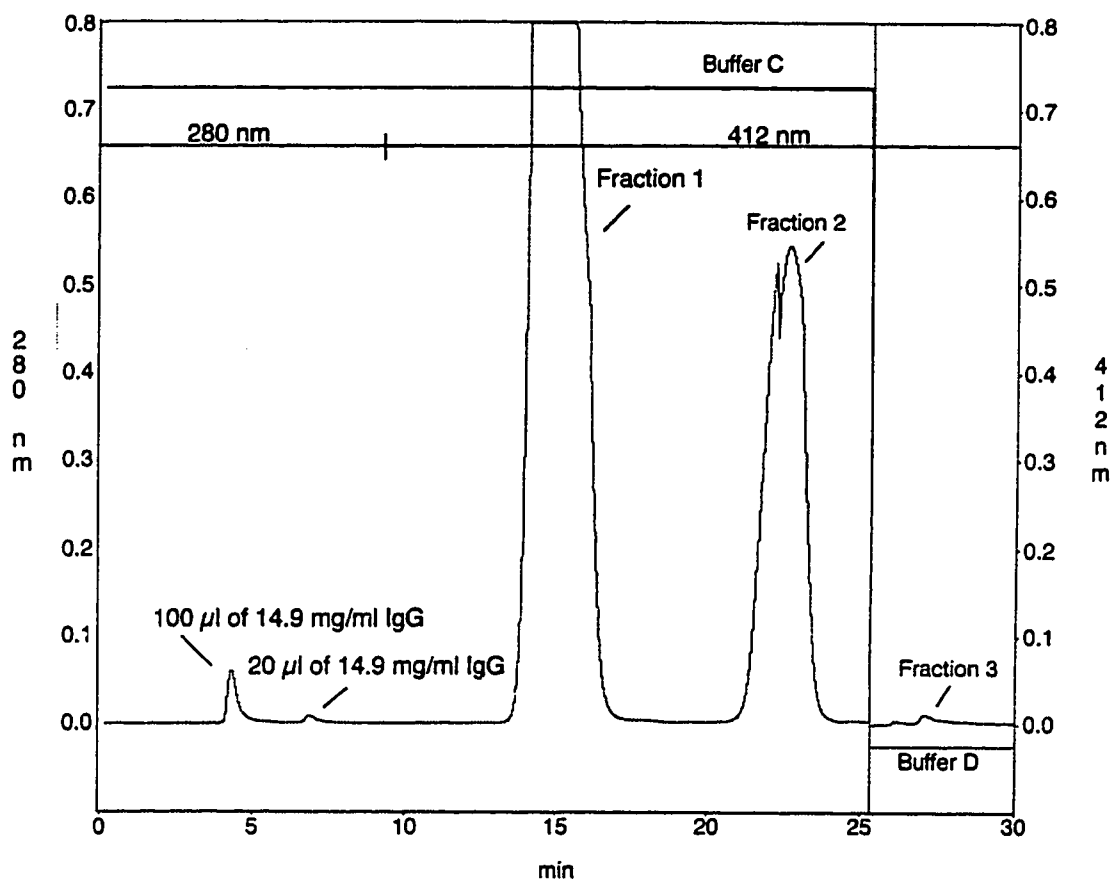
Figure 12: IMAC output during Ellman's assay. Absorbance was monitored at 412 nm.

This peak is labeled Fraction 2 in **Figure 12** and was also collected for further analysis. The slight double peak is explained by the change of flow rate. The absorbance value at 412 nm of the second Ellman's injection was much smaller than the first injection. This suggests that the first Ellman's injection encountered sulfhydryls and, as expected, these sites were closed off and TNB was formed. The first injection was able to encounter, most if not all, the available sulfhydryl sites since the second injection looked like the negative

control (results not shown). The negative control for the assay was the injection of the Ellman's reagent without antibody column. The following are possible explanations for why the first injection produced such a strong TNB band:

- The first injection of Ellman's reagent reacted like expected closing off sites on IgG. The second injection found fewer sulfhydryl reactive sites.

- DTT was still present in the column because not enough time was allowed between the loading of the reduction mixture and the introduction of Ellman's reagent. DTT molecules were on their way out of the column when the first Ellman's reagent injection was made. Thus, the bright yellow band near the bottom of the column.

Fraction 3, seen in **Figure 12**, was collected upon forced elution with Buffer D. The antibody was expected to elute at this time. The eluent was monitored at 412 nm, therefore no appreciable peak was detected since IgG absorbs at 280 nm.

The HP1100 Liquid Chromatography System was used to determine the composition of the fractions collected during the Ellman's assay on IMAC. **Figure 13** shows the HP1100 results of each fraction. **Table 5** shows the absorbance peak height for the species of interest in **Figure 13**. The antibody is expected to have a retention time of approximately 17.8 minutes on this column system. Notice that all three fractions analyzed have antibody present. The first two fractions, **Figure 13a and 13b**, have trace amounts of the antibody when compared to the forced elution, **Figure 13c**. The other major peak, seen at 26.5 minutes, can be deduced to be Ellman's reagent. Notice that **Figure 13a** has a smaller Ellman's reagent peak than **Figure 13b**.
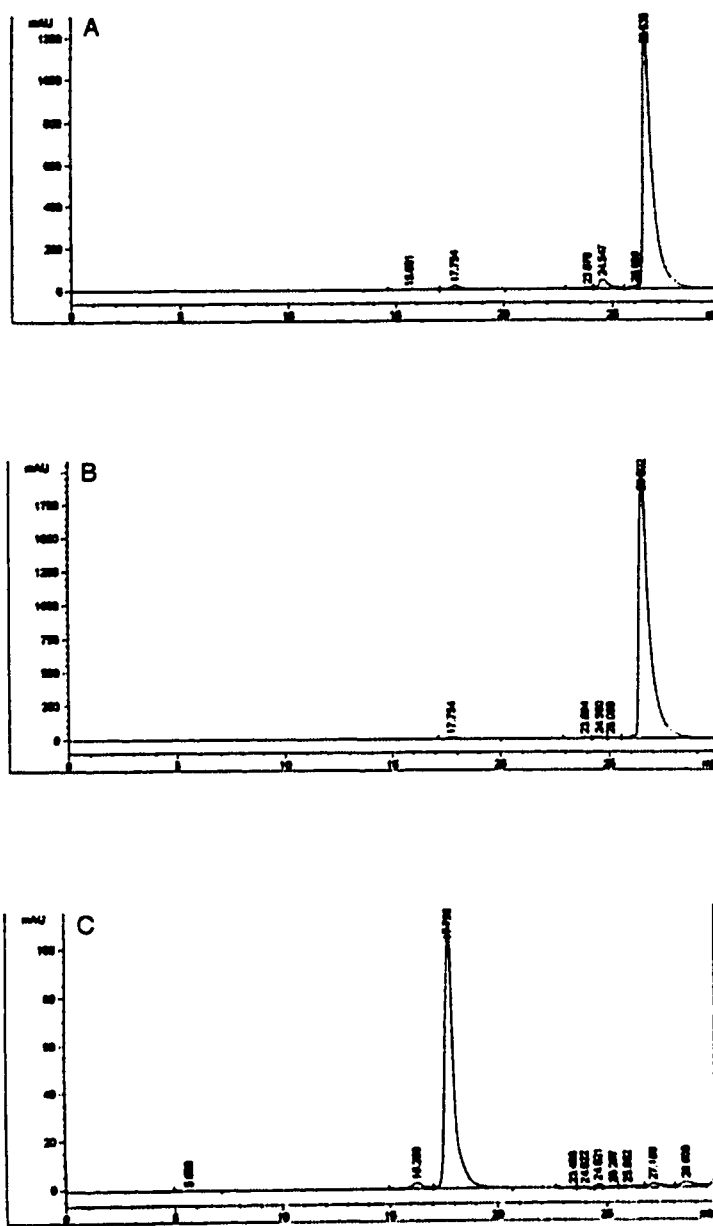
Figure 13: HP1100 Liquid chromatography analysis of fractions collected during Ellman's assay on IMAC (**Figure 12**). For all samples the volume analyzed was 100 µl and the absorbance was monitored at 280 nm on the HP1100 system. a) Fraction 1, eluent collected from first injection of Ellman's reagent. b) Fraction 2, eluent collected from second injection of Ellman's reagent. c) Fraction 3, effluent collected from desorption step with Buffer D.

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Less Ellman's is present in the first fraction collected, **Figure 13a**, because it reacted with available sulfhydryl sites on the IMAC column and became TNB. This was seen during the IMAC run as the bright yellow band near the bottom of the column. The second injection of Ellman's reagent on IMAC did not produce this band indicating that Ellman's reagent was still intact. **Figure 13b** shows more Ellman's reagent present in the second fraction analyzed. **Figure 13c** is the third fraction collected from the IMAC column, this time during the forced desorption with Buffer D. Buffer D caused the antibody to elute from the IMAC column as expected. There was no measured Ellman's reagent in this fraction indicating that the reagent did not stick to the IMAC column.

Table 5: HP1100 absorbance peak height comparison between the fractions collected during the Ellman's assay on IMAC.

Species	Retention Time (min.)	Absorbance Peak Height (mAU)
Fraction 1 IgG	17.754	18.71
Fraction 1 Ellman's	26.53	1245
Fraction 2 IgG	17.754	11.29
Fraction 2 Ellman's	26.5	1974
Fraction 3 IgG	17.798	110.71
Fraction 3 Ellman's	none	none

The trace levels of antibody found in Fraction 1 and Fraction 2 caused by the injection of Ellman's reagent were unexpected. Upon examination of the Ellman's reagent used, it was found to contain 1 mM EDTA as preservative. Recall that EDTA is a strong chelator capable of removing the metal ions from the column. Thus, the EDTA present in the Ellman's reagent caused trace levels of metal ions along with the attached antibody to be desorbed. The antibody released into the liquid phase is capable of reacting with Ellman's reagent, also found in the liquid phase, as it traveled through the column. However, it is unlikely that such trace levels of antibody resulted in the formation of the high levels of TNB detected at A_{412} for the first injection of Ellman's reagent. It does not explain why the bright color formation was seen only at the bottom of the column for the first injection. The antibody is to have adsorbed throughout the column, not just at the bottom. Any desorption would occur at the first point of contact, that being the top of the column. So if the desorbed antibody reacted with the Ellman's reagent then the TNB formation should have been seen starting at the beginning of the column. Furthermore, the second injection caused similar trace levels of antibody to be eluted from the column, yet a high level of TNB was not detected. These observations further suggest that the Ellman's reacted with the DTT.

The Ellman's assay was duplicated except this time 15 CV of Buffer C were used to see if DTT would exit the IMAC column. The concentration of the reduction reagents were 0.25 ml of 14.9 mg/ml IgG and 5 µl of 1 M DTT. After the 30 minute reaction time, 0.75 ml of Buffer C was added to dilute the IgG concentration to 3.7 mg/ml in 1 ml. **Figure 14** shows the actual BioCAD output for this run. The reaction mixture including DTT was introduced onto IMAC by a

total of six 100 μl injections. After close to twice as many column volumes used in previous attempt, a 100 μl injection of 0.01 M Ellman's reagent was added to IMAC at 0.2 ml/min. This time, a slight yellow front was seen starting at the top of the column which continued all the way out of IMAC. The peak absorbance value measured was 1.2 at 412 nm. A second injection of Ellman's resulted in no detectable front through the column and the Ellman's flow-through was similar to the negative control results. The spectrophotometer was changed to record at A_{280} to record the antibody coming off the column as a result of using Buffer D. This run demonstrated that there was a lot of antibody still on the column even though the Ellman's reagent contained 1 mM EDTA.

The second Ellman's assay attempted on IMAC did show a front at the top of the column caused by TNB. The second trial had an overall higher load of antibody (2.2 mg) than the first (1.7 mg), and that can explain why a front was seen traveling down the column during the second assay and not the first. The first assay demonstrated a strong yellow band only at the bottom of the column which was most likely caused by DTT and not the antibody.

The Ellman's assay proves that a small compound can react with the sulfhydryl groups of the reduced antibody. Recall that the RPE protein is very large when compared to the Ellman's reagent. The results with Ellman's reagent suggest the reduced antibody on the IMAC column still has available sulfhydryl groups and is binding to the metal ions through other sites besides the sulfhydryl reactive sites. Thus, the modified RPE should have an opportunity to conjugate with the reduced antibody. This assumption will have to be tested with actual conjugation on the column.
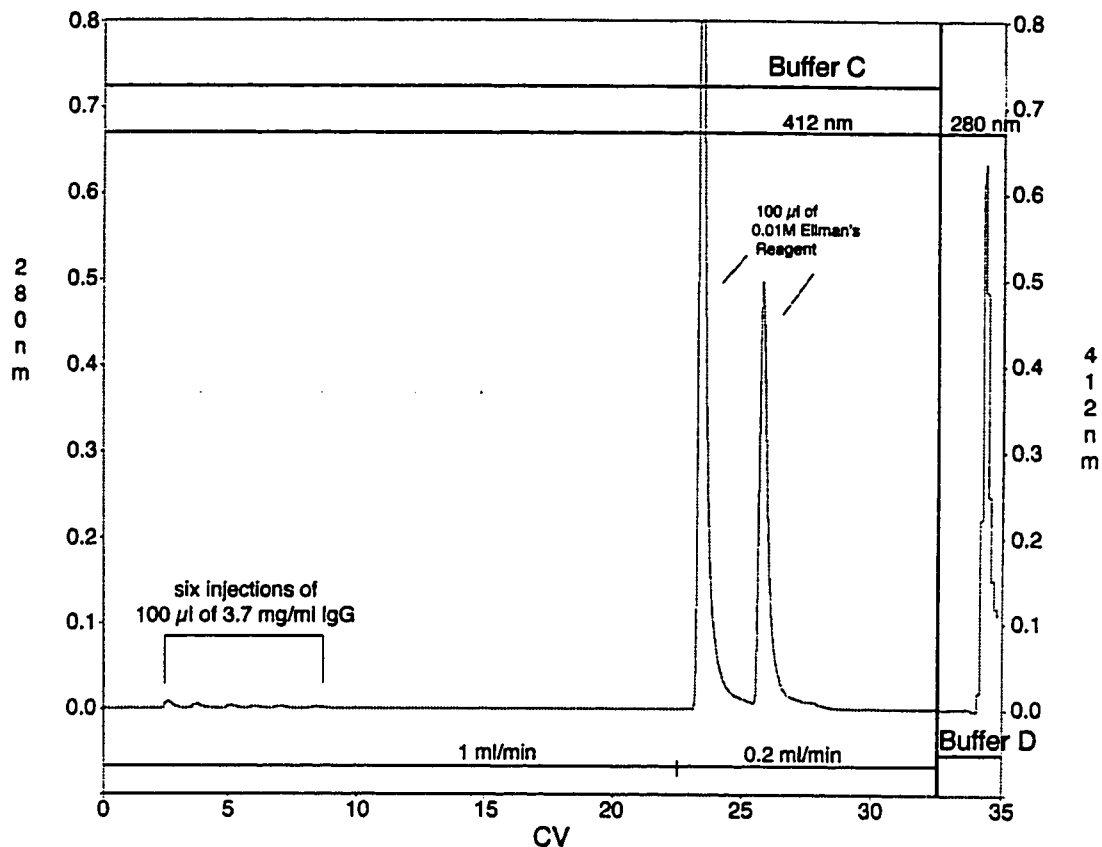
Figure 14: Second Ellman's assay attempted on IMAC. Absorbance was monitored at 412 nm and 280 nm.

Note that during column cleaning with NaOH, as described in the Methods section, a bright yellow band was detected. One mole of Ellman's reagent can break down into two moles of TNB under basic conditions. This finding suggests that Ellman's reagent is adsorbing onto the IMAC column. Solution E containing EDTA is strong enough to remove all metal ions from the column leaving the support matrix. This suggests that the Ellman's reagent is binding to the matrix since it remained on the column after Solution E was used. For this reason, Ellman's reagent was no longer added to the IMAC and a new

column was poured. It was also decided to purify the reduced antibody to remove DTT before introducing it into IMAC. In this manner, the SH/IgG value could be determined per run on IMAC.

RPE Non-adsorption

When modified, free RPE was injected onto the column at a high concentration using Buffer C. The bright pink solution eluted immediately (BioCAD results not shown). This suggests that RPE did not bind to the metal ions at this pH since the column media remained white after the injection had flowed through. To verify if any RPE remained on the column, the competitive agent was introduced. Buffer D caused some RPE to be detected in the effluent but it is in trace levels compared to the concentration injected. Therefore at pH 6, free RPE is not readily adsorbed onto IMAC and the experiments were continued as planned.

Liquid-Liquid Control Reaction

A liquid-liquid control reaction was performed to establish a better understanding of the 1:1 conjugate formation compared to the multiple formation. The conjugation procedure was followed as stated in **Appendix A.** The modified RPE used had a concentration of 10.3 mg/ml with 0.9 moles of maleimides per mole of RPE. The reduced antibody had a concentration of 1.15 mg/ml with an SH/IgG value of 8.1. The start time of the reaction was recorded when the reagents were combined. The total reaction mixture had a volume of 4.93 ml with the concentration of the reagents being 0.86 mg/ml IgG and 2.59 mg/ml RPE. Sampling of the mixture was done by taking 100 µl while

the conjugation reaction occurred and monitoring the time. The reaction of the
sample taken was stopped by adding 3 µl of 10 mM 2-ME. Seventeen
samples were taken in an hour. These samples were stored at 8 °C until the
last sample, taken two hours from the start of the reaction, was collected.

Table 6: Comparison of the amount of species present in the
conjugation reaction mixture as a function of time. The amount of
species in the mixture is determined by the HP1100 A_{280} peak
height measured.

Time (min)	Peak height (mAU) Antibody	Peak height (mAU) 1:1 Conjugate	Peak height (mAU) Multiples
3.25	170.4	17.75	0
5.75	166.46	23.28	0
7.9	161.22	25.63	0
10.08	161.42	32.37	0
13.08	159.76	38.39	3.78
15.6	158.17	42.38	4.53
17.6	155.22	45.12	4.9
19.43	153.31	47.89	5.45
22.85	150.12	53.09	6.67
26.42	147.15	57.04	7.8
30.4	143.18	60.72	8.98
34.65	139.56	65.1	10.52
38.43	137.31	67.94	11.65
42.17	136.36	70.54	12.79
46.17	133.05	73.77	14.14
50.15	130.16	76.14	15.3
54	129.03	79.71	16.84
127.92	110	99.39	35.39

The reaction samples were analyzed using the HP1100 Liquid

Chromatography System as stated in the Analytical Methods section. The

samples were analyzed using a 50 µl injection volume into the HP1100. The fluorometer detector was set at EX 488 nm and EM 573 nm with a PMT setting of 6 for all the samples analyzed. The spectrophotometer was set to measure at A_{280}. The HP1100 detector results are not shown. **Table 6** compares the relative amount of each species in the mixture based on the peak height acquired from the HP1100 analysis. **Figure 15** shows how the formation of 1:1 conjugate compares to the formation of multiples in the conjugation reaction. Notice that multiples don't begin to form until approximately 10 minutes into the reaction.
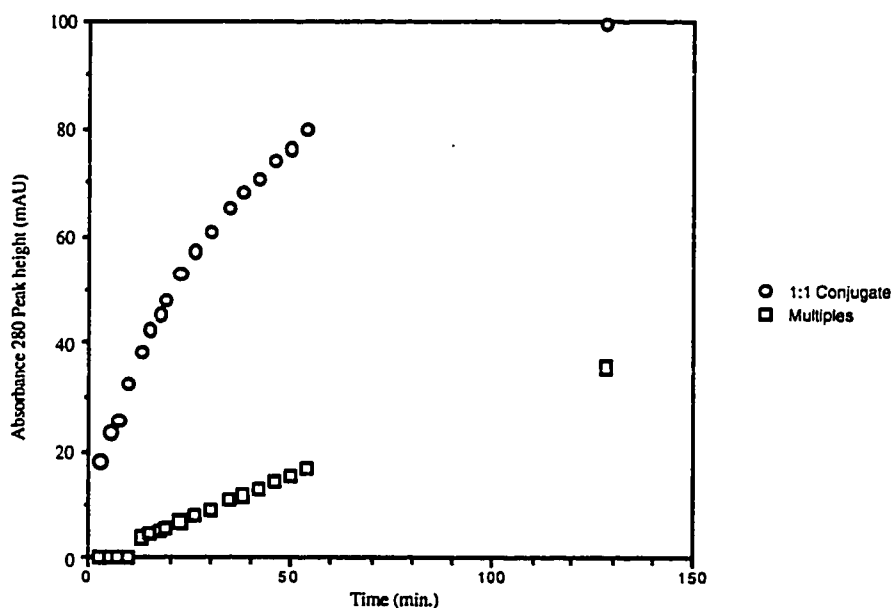
Figure 15: Formation of 1:1 conjugate and multiples as a function of time for the liquid-liquid reaction. The X axis is measured in minutes and the Y axis measures the A_{280} peak height determined using the HP1100 system.

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Design of Experiments

A strategic experimental design was used to maximize the efficiency of testing. The DOE study was necessary to identify important experimental factors and to identify optimal experimental conditions. Strategic experimentation uses two classes of experiments known as screening and optimization experiments. The initial set of experiments are classified as screening experiments because the results are used to determine the important from the the unimportant experimental factors. Screening is required because there are several experimental factors to consider and interactions among the factors is most likely to occur. The initial experiments are used to focus the experimental design in the direction of optimal experimental conditions. The optimal settings are those experimental conditions which produce the desired results. The experimental factors are the variables which can be controlled during the duration of the experiment and which may affect the values of a response. The response factors are those variables which are measured and used to determine the success of the experiment.

Each experimental factor is set to a high and low value and testing takes into account the interactions for each factor within the defined experimental high and low regions. Information is interpreted within this framework of the experimental design. Strategic experimentation tests combinations of factors at specific levels and generally requires the shortest amount of time to complete, when compared to other methods.

The main goal of this investigation was to determine any advantages of converting the liquid-liquid reaction of coupling antibody to RPE into a solid - liquid phase reaction. In theory, immobilized metal ions can bind the antibody

into a solid phase matrix while free RPE is introduced in the mobile liquid phase. The coupling of RPE to the bound antibody should result in bound conjugate. Since the antibody is hindered in movement, the formation of multiples might be hindered as well. Furthermore, since RPE is not adsorbed onto the column, IMAC can purify the product by eliminating one of the reagents from the reaction mixture. These are the reasons why IMAC was tested for favoring 1:1 conjugates.

The reaction conditions such as pH and buffer solutions used by BD to conjugate RPE and IgG were adapted to the solid-liquid phase on IMAC. The screening set of experiments were run to determine if antibody and free RPE bind to IMAC at the pH and buffer used for the coupling reaction. Simultaneously, desorption conditions were compared for the two proteins. The optimization set of experiments focused on time dependence of the coupling reaction and the concentration of the reactant free RPE.

Table 7: Factor levels used in the Design of Experiments.

Factors	High Level	Low Level	Middle Level
RPE (mg/ml)	15	5	10
Time (min)	90	30	60

The results from the screening set of experiments were used to set up the final set of experiments as a 2^2 design with a center point. The selected factors chosen were the coupling reaction time and the free RPE concentration. **Table 7** shows the high, low, and middle values tested in this design. The middle

level for the reaction time factor was based on the liquid-liquid reaction time used by BD. The high and low levels for the reaction time were chosen as ±30 minutes of the middle level. This time span provided an adequate range for testing. The RPE concentration factor was chosen as 5 mg/ml as the low level because at this concentration the RPE fluorochrome was easy to detect visually on the column. The middle and the high levels were then chosen as 5 mg/ml increments.

The responses measured in this design were the relative amounts of free RPE, free antibody, 1:1 conjugate, and multiples present in the final IMAC desorption step. The amount present of each of the species in the IMAC effluent was analyzed by liquid chromatography. The amount present of each of the species was not empirically determined but rather compared. The liquid chromatography output consisted of peak heights and peak areas based on absorbance or fluorescence which are proportional to actual concentration of each species present in the sample.

A full factorial DOE required a total of five runs. Each run was duplicated to achieve better statistical confidence when analyzing the results. **Table 8** shows the required experimental runs in the order they were performed.

The DOE was carried out as planned with the tested factors being RPE concentration and Reaction Time. **Table 9** shows the specifics of each run performed. As mentioned in the Materials and Methods section, the loading concentration of the reduced antibody was expected to vary due to the purification by size exclusion chromatography for each batch. The second row of **Table 9** shows the actual loading concentration of the reduced antibody per

run. The number of available sulfhydryl groups per antibody (SH/IgG) of each of the batches varied, as seen in the third row, even though the same procedure was followed each time. This variation can be attributed to inconsistent vortexing while adding DTT or to a deviation in DTT concentration since working with small quantities of reactants. The coupling procedure used by BD requires that the SH/IgG value be greater than 5 to continue with the conjugation reaction. All the measured SH/IgG values were greater than 5 so the conjugation procedure was continued. The last row is the recorded absorbance peak height (280 nm) of the effluent once Buffer D was used in a step gradient. **Figure 16** shows the spectrophotometer and fluorometer readings recorded during Run 7. Similar readings were recorded for the remainder runs (see the **Appendix B** for actual BioCAD printouts).

Table 8: Experimental run order performed on IMAC.

Run	RPE (mg/ml)	Time (min)
1	5	90
2	15	90
3	5	30
4	15	30
5	10	60
6	5	90
7	15	90
8	10	60
9	5	30
10	15	30

Table 9: The recorded results for each run of the Design of Experiments performed on IMAC. The factors tested were concentration of RPE and the conjugation reaction time. The last row is the absorbance value of the peak recorded once protein desorption occurred using Buffer D.

Run Number	1	2	3	4	5	6	7	8	9	10
IgG (mg/ml)	1.73	1.78	1.79	1.98	1.87	1.91	1.8	1.84	1.65	1.78
SH/IgG	8.8	8.4	9.2	7.8	7.4	10	7.8	8.1	6.9	7.4
RPE (mg/ml)	5	15	5	15	10	5	15	10	5	15
Reaction Time (min)	90	90	30	30	60	90	90	60	30	30
Eluent Peak (280 nm)	0.25	0.3	0.39	1	0.7	0.6	0.6	0.3	0.55	0.5

The left Y axis is for the fluorometer which excites at 488 nm and reads fluorescence at 576 nm in Volts. The right Y axis is for the spectrophotometer which reads absorbance at 280 nm. The X axis measures flow through IMAC in column volumes. As stated in the Methods section, 2 ml of the reduced antibody was injected at 1 ml/min. Some of the reduced antibody was absorbed by IMAC but most of the antibody eluted as seen in the first peak in **Figure 16**. This large peak detected by A_{280} was expected since the injection volume was twice as large as the bed volume of the column. The injection volume was chosen to establish detectable amounts of antibody onto IMAC. The column was washed with 10 CV of Buffer C to remove any loosely bound antibody. Thus, the absorbance value of the effluent decreased as less antibody eluted from the column. For Run 7, 1 ml containing 15 mg of modified RPE was injected at 0.2 ml/min for 1 CV. The flow rate was stopped once the column was saturated with modified RPE and the timer was set for 90 minutes. The flow rate of 1 ml/min was started after the reaction time was completed for Run 7. Since free RPE does not bind to IMAC it eluted from the column as monitored by the high absorbance peak (pegged at a value of 3) and the dual fluorescence peaks

seen in **Figure 16**. The dual fluorescence peaks occurred because of the high concentration of RPE in the eluent. The fluorometer could not detect emission readings for the highest elution concentration of RPE so it recorded zero readings instead. The IMAC column was washed with 10 CV of Buffer C to make sure all the RPE had eluted. The final step for the IMAC run was to switch to Buffer D causing any bound protein to be eluted from the column. For Run 7, the Buffer D step gradient caused protein to elute having both fluorescence (peak value of 0.15 V) and absorbance (peak value of 0.6 280 nm). Those readings suggest that conjugate is present in the effluent since both the fluorescence and absorbance peaks occurred simultaneously. As the eluent was collected, 5 µl of 10 mM 2-ME was added to the sample to stop the conjugation reaction. This step was done to ensure the conjugation happened while the antibody was attached to the metal ions of the column and not while in liquid solution. There is a time span of less than a minute from the moment of IMAC desorption to the adding of 2-ME. There is a possibility that conjugate was formed during that time, but it is expected that most of the conjugate present in the elution samples was formed on IMAC.

The samples collected were stored at 8°C and protected from light. When the runs were completed, they were analyzed on the HP1100 Liquid Chromatography System. The same system settings were used for all the samples. A control sample of just free antibody was also analyzed. **Figure 17** shows the HP1100 fluorometer and spectrophotometer output for Run 6. Run 6 was chosen as a representative of the results (see **Appendix C** for the remaining runs). The analysis is based on size exclusion so the the larger species are expected to be detected first. The fluorometer detects those

species which contain RPE molecules, while the spectrophotometer detects all species in the reaction.
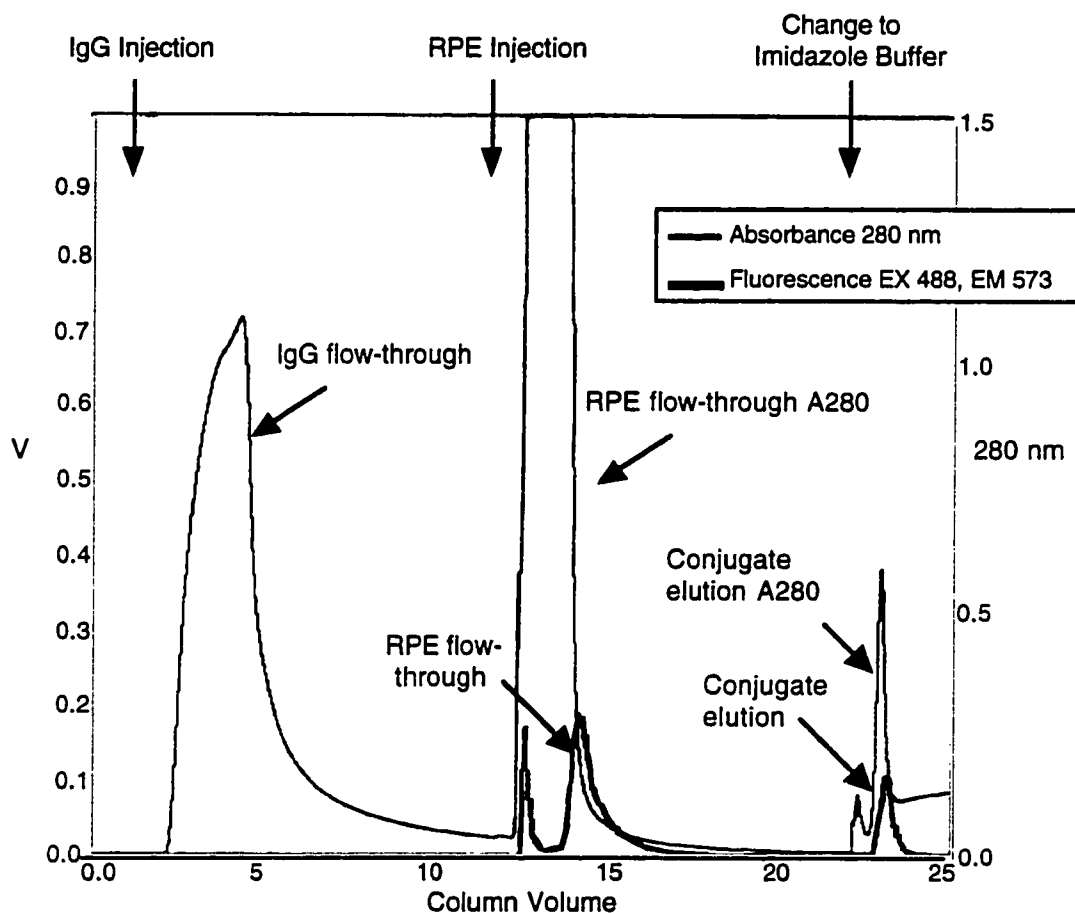
Figure 16: Fluorometer and spectrophotometer recordings of Run 7. The Factors were RPE concentration of 15 mg/ml and reaction time of 90 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.

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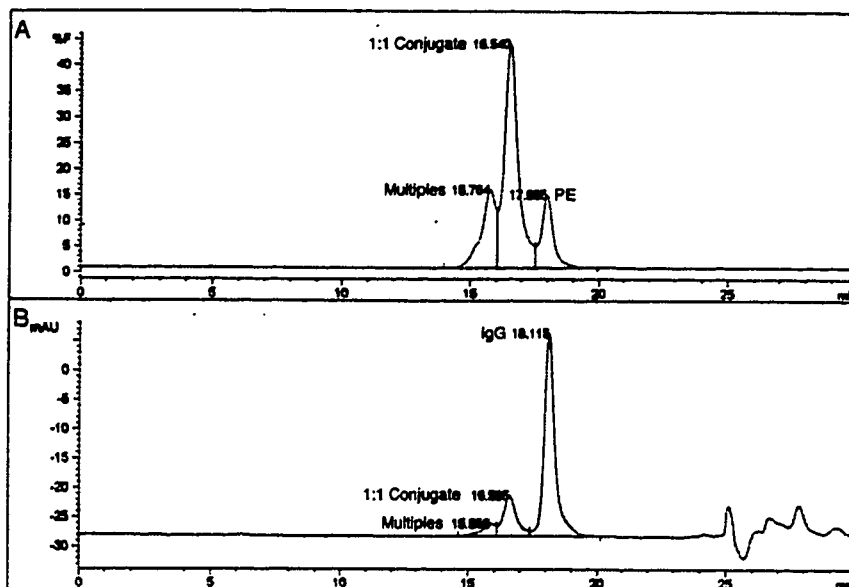
Figure 17: HP1100 liquid chromatography analysis of Run 6. The factors tested were 5 mg/ml RPE and 90 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A_{280}.

For the fluorometer output (**Figure 17a**), the first peak, with retention time of 15.7 minutes, represents those compounds that have multiple RPE molecules or conjugates with joined antibodies. The second peak, with retention time of 16.5 minutes, represents the 1:1 conjugate. The third peak, with retention time of 17.9 minutes, represents the free RPE that did not conjugate. The height of the peaks is related to the concentration present in the sample. **Table 10** shows the signal readings of the fluorometer. Thus, the concentration of the conjugate is about twice as much as the other fluorescent species.

Table 10: Fluorometer signal readings for Run 6, analyzed on the HP1100 Liquid Chromatography System.

Peak #	Species	Time (min.)	Height (%F)
1	Multiples	15.764	15.067
2	1:1 Conjugate	16.54	43.129
3	Free RPE	17.985	14.023

For the spectrophotometer output (**Figure 17b**), the first two peaks are the expected multiples and the conjugate. **Table 11** shows the signal readings for the spectrophotometer. The third peak cannot be the free RPE since its concentration was established as less than the conjugate. Therefore, the third peak must be the free antibody that did not conjugate. Note that the antibody is the most expensive reagent in the conjugation reaction. The HP1100 results show that performing the conjugation reaction under the conditions tested does not improve the yield. There is too much antibody wasted under these conditions. Further fine tuning might improve the conjugation yield.

Table 11: Spectrophotometer signal readings for Run 6, analyzed on the HP1100 Liquid Chromatography System.

Peak #	Species	Time (min.)	Height (mAU)
1	Multiples	15.856	2.168
2	1:1 Conjugate	16.595	6.866
3	Free IgG	18.115	34.577

Purity of Conjugate

The purity of the experimental conjugate was compared against the

reference conjugate. The IMAC elution samples collected from each run of the conjugation reaction were pooled together. The BD's method of producing conjugate includes purification by size exclusion chromatography and fractionization as stated in **Appendix A**. The experimental conjugate was purified in the same manner. The fractions which contained mostly 1:1 IgG-RPE conjugate, determined by electrophoresis, were pooled together. Lastly, the experimental conjugate was dialyzed into the correct buffer and concentrated.
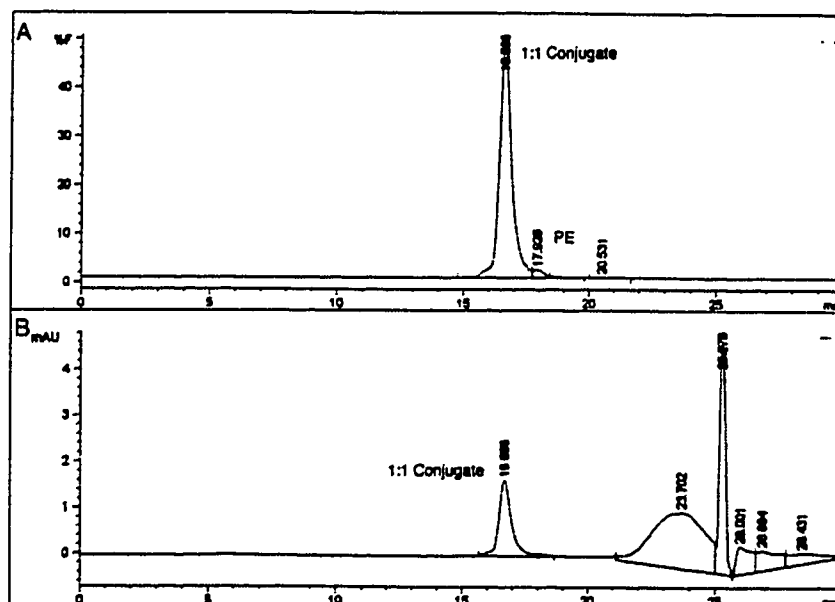
Figure 18: HP1100 analysis of experimental conjugate. a) Fluorometer detector set at EX 488 nm and EM 573 nm. b) Spectrophotometer detector set at A_{280}.

The purity of product was determined by using the HP1100 Liquid Chromatography System. Comparison of **Figure 18** and **Figure 19** show

that the experimental conjugate is purer.   Note the species seen after 20

minutes retention time are caused by the buffer used and can be ignored in this

analysis.   The fluorescence output for the experimental conjugate, **Figure 18a**,

shows a trace level of RPE present.   Such a small amount of free RPE will not

affect the staining performance of the conjugate.   The reference conjugate,

Figure 19, is composed of mostly multiples with retention times <15 minutes.

Surprisingly, this reference conjugate contains high levels of free RPE as

determined by the fluorescence output, **Figure 19a**.   The free RPE in the

reference conjugate might cause non-specific staining when used in flow

cytometry applications.
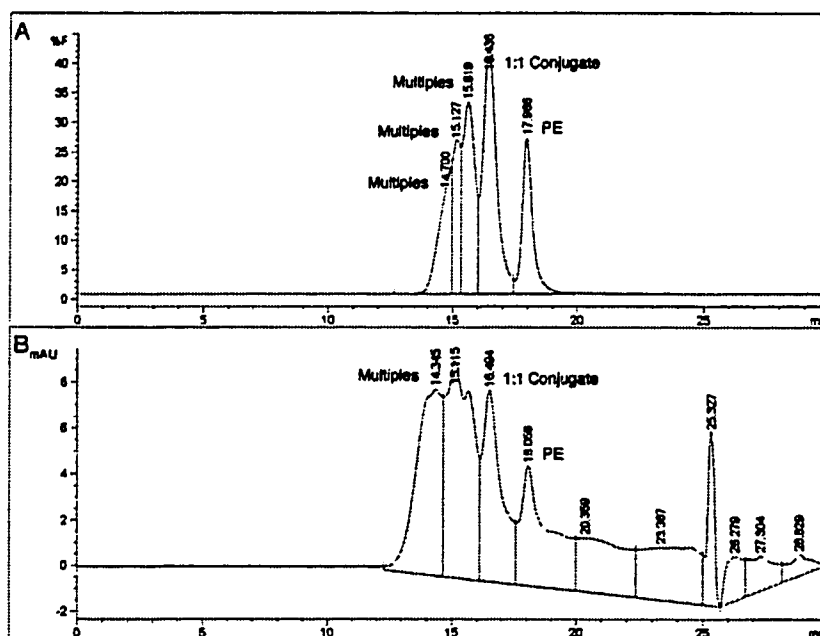
Figure 19: HP1100 analysis of reference conjugate.   a)
Fluorometer detector set at EX 488 nm and EM 573 nm.   b)
Spectrophotometer detector set at A280.

Statistical Analysis

So far, the actual responses have been discussed. The statistical analysis measures how the factors affected the responses. Recall that for this DOE study, the factors tested were reaction time and RPE concentration while the responses were free RPE, free antibody, 1:1 conjugate, and multiples based on fluorescence (%F) and absorbance (mAU) measured. Another important response is the yield of the reaction defined as conjugate/(conjugate + IgG) based on absorbance peak area. **Table 12** shows the measured responses per run with duplicate runs paired. These values were entered into the Design Ease Program which computes the ANOVA. As mentioned in the Design of Experiments section, the significance level must be determined to make valid conclusions. **Table 13** shows the Probability >F values for the responses studied. Originally, none of the data was transformed. As seen in **Table 13**, only the amount of RPE, multiples, and 1:1 conjugate in the product elution from IMAC were found to be statistically affected by the factors within an appropriate significance level. However upon validating the model, a pattern was found in the residual versus predicted values for all the responses tested except for 1:1 Conjugate (mAU). **Figure 20** shows the residual versus predicted values for the RPE response tested. The X axis measures the predicted RPE values and the Y axis measures the student residual values. An obvious pattern of a parabolic shape can be detected. This pattern is more obvious when the absolute value of the student residuals versus the predicted values was plotted as seen in **Figure 21**. Then, the 10 points reduce to 5 since the zero line of the y axis divided the symmetrical parts of the parabola. The RPE response analysis was chosen as a representative of the results (see **Appendix D** for

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the remainder statistical output). The other responses did not necessarily have a parabolic pattern in their residual versus predicted value plots, but symmetry at the zero y line was prevalent. Therefore, transformation of the data was done to correct this problem.

Table 12: The measured responses used in the statistical analysis of the Design of Experiments. Each value is based on area of the peak recorded during the HP1100 analysis, absorbance in mAU and fluorescence in %F. Results are organized with the duplicate runs paired.

Run	RPE Load (mg)	Time (min.)	IgG (mAU*)	RPE (%F)	1:1 Conj. (%F)	Mult. (%F)	Yield** (mAU*)
3	5	30	287	469.3	1232	671.9	0.3394
9	5	30	700.9	609.8	1354	661.6	0.2348
4	15	30	609.1	1374	3675	2821	0.4322
10	15	30	348.8	2122	2649	1952	0.4999
1	5	90	126.5	457.1	1147	652.4	0.5207
6	5	90	1006	471.2	1671	590.7	0.23
2	15	90	62.78	979.5	1559	1906	0.7461
7	15	90	653.2	1330	2933	1883	0.3791
5	10	60	839.6	1323	4263	3642	0.3959
8	10	60	231	906.6	2151	1535	0.5395

* IgG absorbance results have been corrected to remove excess absorbance caused by RPE molecules, see **Appendix D**.
** Yield is calculated as conjugate/(conjugate + IgG).

Table 13: Determining the significant results using the Probability>F values from the Design-Ease output with no transformations.

Response	PROB>F	Significant
Yield (mAU)	0.4414	NO
1:1 Conjugate (%F)	0.0522	5% Significance Level
1:1 Conjugate (mAU)	0.2237	NO
Multiples (%F)	0.0128	5% Significance Level
IgG (mAU)	0.9645	NO
RPE (%F)	0.0222	5% Significance Level
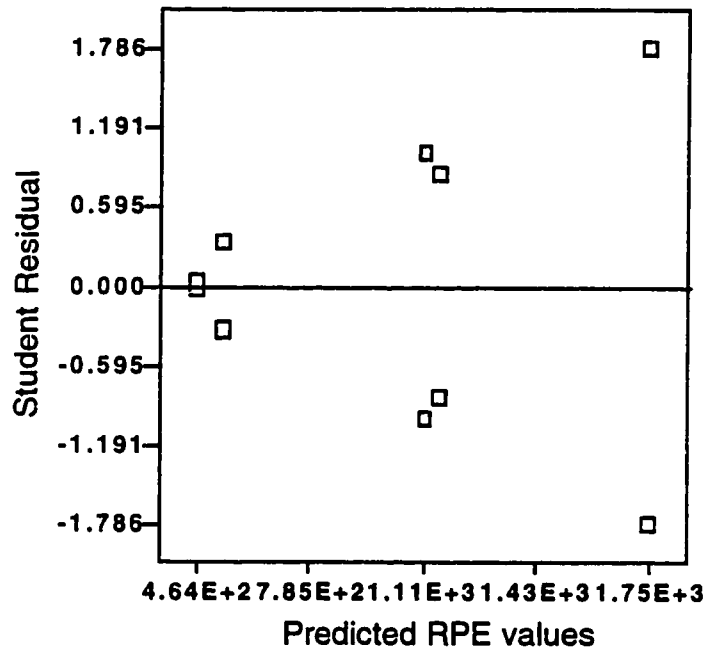
Figure 20: Design-Ease output of the student residual versus predicted values for the RPE response.
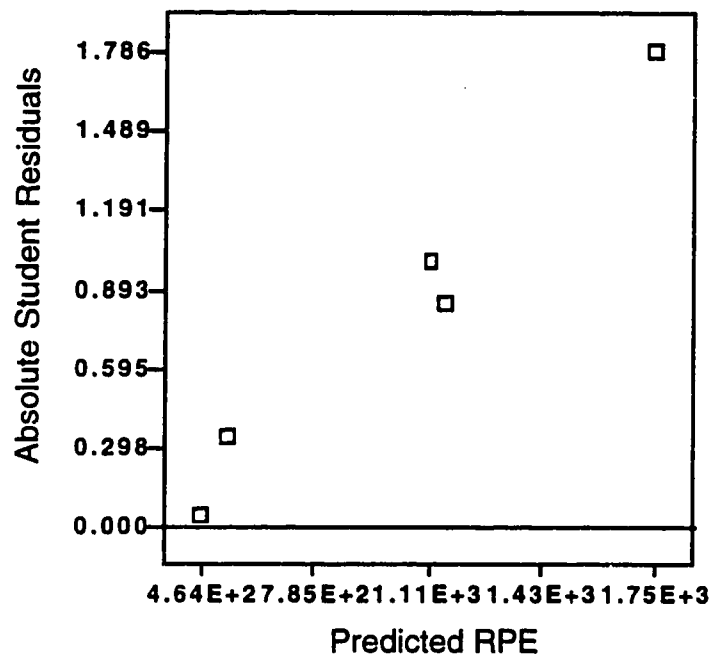
Figure 21: Design-Ease output of the absolute student residual versus predicted values for the RPE response.

The transformation of the response results is done to create a better fit of the data to the model. As shown in the Design of Experiments section, the Design-Ease program used for statistical analysis had several transformation options. The mildest transformation is to take the square root of the data and then perform the statistical analysis (Box *et al.*, 1978). This was the first transformation attempted on the responses tested. However, if this did not solve the pattern problem, the reciprocal square root was used. Finally, if a pattern still existed, the inverse transformation was attempted. **Table 14** shows the transformations used and the resulting Probability>F value. Notice that when

the Probability>F value was significant before the transformation, it became more significant after the transformation. This is as expected since the transformation is supposed to improve how the data fits the model. Also notice the responses that originally were not significant, remained that way after the transformation. From these results, it was established that further interpretation would be valid for 1:1 conjugate (%F) and RPE (%F), since a 5% significance level was found. The multiple formation demonstrates significance, but there is still a trend found in its corresponding residuals vs. predicted plot. As discussed before, this trend indicates a possible violation of the ANOVA assumption. Therefore, the statistical analysis of multiple formation will be discussed but should be considered inconclusive at this time. The Design-Ease report for the last transformation done on the data is found in **Appendix D**.

Table 14: Resulting Probability>F value after transformation calculated by Design-Ease software. Data was transformed only when the residual vs. predicted values graph showed a trend.

Responses	Not Transformed	Square Root	Reciprocal Square Root	Inverse
Yield (mAU)	0.4414	0.4335	0.4309	0.4373 *
IgG (mAU)	0.9645	0.9481	0.6657	0.6071 *
RPE (%F)	0.0222	0.0113	0.0002	Not Necessary
1:1 Conjugate (mAU)	0.2237	Not Necessary	Not Necessary	Not Necessary
1:1 Conjugate (%F)	0.0522	Not Necessary	Not Necessary	Not Necessary
Multiples (%F)	0.0128	0.0027	0.0001	0.0001*

* Inverse was the last transformation attempted and the residual vs. predicted values still showed a trend.

Once significance was established, the associated t-test is used to determine if the factors were significant in affecting the responses. The

Probability > I t I works under the same principle as the Probability > F value, only this time it specifically measures the significance of how each of the factors affected the responses. The value should be as small as possible since it predicts the probability that the results are due to chance alone. Increasing from low to high levels of RPE was found to increase the formation of 1:1 conjugate (%F) with a 5% significance level. The effect of reaction time on 1:1 conjugate (%F) was insignificant. The finding of free RPE in IMAC elution also was significant with increasing loading of RPE reagent onto the column. This is the obvious observation proven statistically significant with a 1% level. This result makes sense, because higher IMAC loading conditions of RPE generally cause higher trace levels of RPE to be found in the effluent upon using Buffer D. Reaction time was found insignificant for RPE detection upon elution from IMAC. Multiple formation is increased by increasing the loading conditions of RPE with a significance level of 1%. Once again, contact time did not affect the multiple formation. A trend observed, which was not statistically significant, was that yield formation increased slightly with increasing RPE concentration. Further fine tuning of the DOE might reveal the importance of this finding.

Statistical analysis demonstrates that concentration of RPE affected the responses under the IMAC reaction conditions, while the reaction time did not. A significant result was found for the formation of 1:1 conjugate (%F), multiple (%F), and RPE (%F) out of the responses measured. The finding that RPE affects itself is the obvious result and requires no further interpretation. It is known that the liquid-liquid·reaction does favor multiple formation rather than conjugate formation. This applies to the coupling reaction done on IMAC as well. However, the DOE should be optimized by future studies to verify if

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multiple formation is truly significant. The results acquired in this study pertaining to multiple formation suggest a violation of the ANOVA assumption. The results suggest these possible scenarios:

1. Non-reacted IgG is not affected by RPE concentration which suggests that the antibody is not available to react with the excess RPE molecules. This could be due to the sulfhydryl sites being sterically hindered by the binding of the antibody on IMAC or else the RPE molecules are too big to access the bound antibody.

2. The formation of 1:1 conjugate is affected by RPE loading which verifies that some antibody is available to react with the RPE. However, the amount of actual conjugate formed on the column is small compared to the amount of antibody loaded onto IMAC. This suggests that the excess RPE is not able to react with the bound antibody.

3. The trend that multiple formation increases with increasing RPE suggests that there is a group of antibodies easily accessible to the RPE molecules. As the loading of RPE increases, this same group of antibodies reacts with the RPE while the majority of antibody is not accessible to the RPE. The sites occupied by this non-reacted antibody are available to the smaller molecule imidazole which causes the antibody to elute from the column.

4. The fact that reaction time did not affect the responses suggests that the high and low values used were beyond the critical reaction time. However, if the sulfhydryl groups on the antibody are unavailable for the RPE, then no reaction time will change those results.

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Performance of Experimental Conjugate in Identifying CD20⁺ Cells

A FACSCalibur flow cytometer was used to check the performance of the experimental CD20-RPE. As described in the Introduction, the CD20-RPE conjugate is used to enumerate B cells in peripheral blood. The experimental conjugate and the reference conjugate were compared in enumerating the B cells of two human blood samples.

The amount of conjugate added to the blood was 0.25 µg/test of experimental CD20-RPE while the reference was added at the normal value of 0.5 µg/test. This was done because historically the RPE conjugate is bright enough to produce an adequate stain at the 0.25 µg/test. Since the immunofluorescence performance of the experimental conjugate was not known, the lower concentration of 0.25 µg/test was used to avoid getting fluorescent results above the scale detected by the flow cytometer.

The CD20+ cells consist of B cells and a small sub-population of T cells. To determine if the experimental CD20-RPE identifies the correct population of cells in peripheral blood, a four color immunofluorescence system was utilized. This entailed using monoclonal antibodies with specificity against other epitopes on the cells. Each of these antibodies was labeled with a fluorochrome emitting light at a different wavelength. Four color immunofluorescence allows for the determination of co-expression of antigens on the same cell. B cells also contain the CD19 epitope and thus can be identified by CD19-APC fluorescent antibody. All T cells contain the CD3 epitope, while only some of the T cells contain the CD8 epitope (T cytotoxic cell).
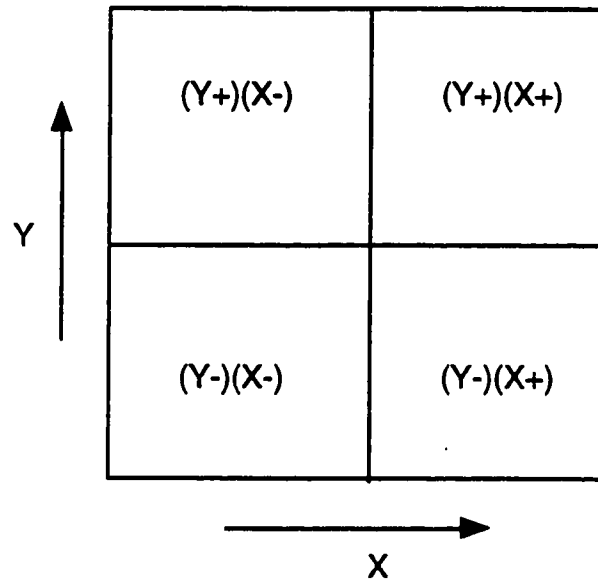
Figure 22: Two color immunofluorescence analysis.

To distinguish between these populations of T cells, CD3-PerCP and CD8-FITC were used. The three different fluorescent antibodies, along with the CD20-RPE, were used to determine the populations of B cells and T cells in the sample of blood. The results of experimental and reference CD20-RPE conjugates were then compared. Each graph shows a dual color analysis can be divided into four quadrants as seen in **Figure 22.** The Y axis is the RPE signal, related to the CD20 population count. An increase in the Y axis represents an increase in the fluorescence of cells which contain the CD20 epitope. The X axis represents the other antibody fluorochrome conjugate whether it is APC, FITC or PerCP. Again, an increase in the X axis represents an increase of fluorescence in the labeled cells. Each dot represents a cell labeled by the conjugates which is detected by the flow cytometer. The upper-

left corner of the quadrant identifies cells which contain the Y epitope. The upper-right quadrant identifies cells which contain the Y and X epitopes. The lower-right quadrant identifies cells which contain only the X epitope. Finally, the lower-left quadrant represents cells which do not contain either the X or Y epitope.

Figure 23 shows the four color immunofluorescence output of reference CD20-RPE results enumerating B cells and T cells of donor 1 using a FACSCalibur. Figure 23a shows the reference CD20-RPE results comparing B cells and T cells of donor 1. Since the CD20-RPE reference is in the Y axis and B cells contain the CD20 epitope but do not contain the CD3 epitope, then the upper-left quadrant represents B cells. The lower-right quadrant represents those cells which contain the CD3 epitope but do not contain the CD20 epitope, thus T cells are identified. Figure 23b is the reference CD20-RPE results comparing B cells and T cytotoxic cells by using CD20 and CD8 epitopes. Once again, the upper-left quadrant represents B cells since these cells are CD20+ and CD8-. The lower-right quadrant represents T cytotoxic cells since that population is CD8+ and CD20-. Also notice there is a population of cells that contain the CD8 epitope which are also labeled by the CD20-RPE fluorescent antibody. These cells are also seen in Figure 23a in the upper-right quadrant, thus are CD20+ and CD3+. These are cells that belong to the small population of T cells that contains the CD20 epitope for unknown reasons. Using these results, the total T cell population is identified as 54.28% of the cells gated for donor 1. Figure 23c verifies the B cell population by using the CD19 epitope which identifies only B cells. Thus, the B cells identified in the upper-right quadrant are CD20+ and CD19+. B cells account for 21.03% of the

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cells gated for donor 1. When comparing these results to those of the experimental CD20-RPE conjugated on IMAC (**Figure 24**), the experimental conjugate gave similar population distributions, but the CD20-RPE intensity was lower. FACSCalibur results for donor 2, seen in **Appendix E**, show similar trends. The tabulated results of the cell population identified by CD20-RPE reference and experimental are compared in **Table 15** (donor 1) and **Table 16** (donor 2).

Table 15: Comparison between reference and experimental CD20-RPE in identifying the population of cells of donor 1. Two color immunofluorescence analysis using CD3-PerCP as the second conjugate.

Cell Type	Reference CD20-RPE and CD3-PerCP	Experimental CD20-RPE and CD3-PerCP
CD3+ T Cells	54.28%	58.74%
CD20+ B cells	21.03%	20.08%
CD20+ T Cells	2.97%	0.00%

Table 16: Comparison between reference and experimental CD20-RPE in identifying the population of cells of donor 2. Two color immunofluorescence analysis using CD3-PerCP as the second conjugate.

Cell Type	Reference CD20-RPE and CD3-PerCP	Experimental CD20-RPE and CD3-PerCP
CD3+ T Cells	76.38%	78.14%
CD20+ B cells	5.13%	5.84%
CD20+ T Cells	3.46%	0.00%
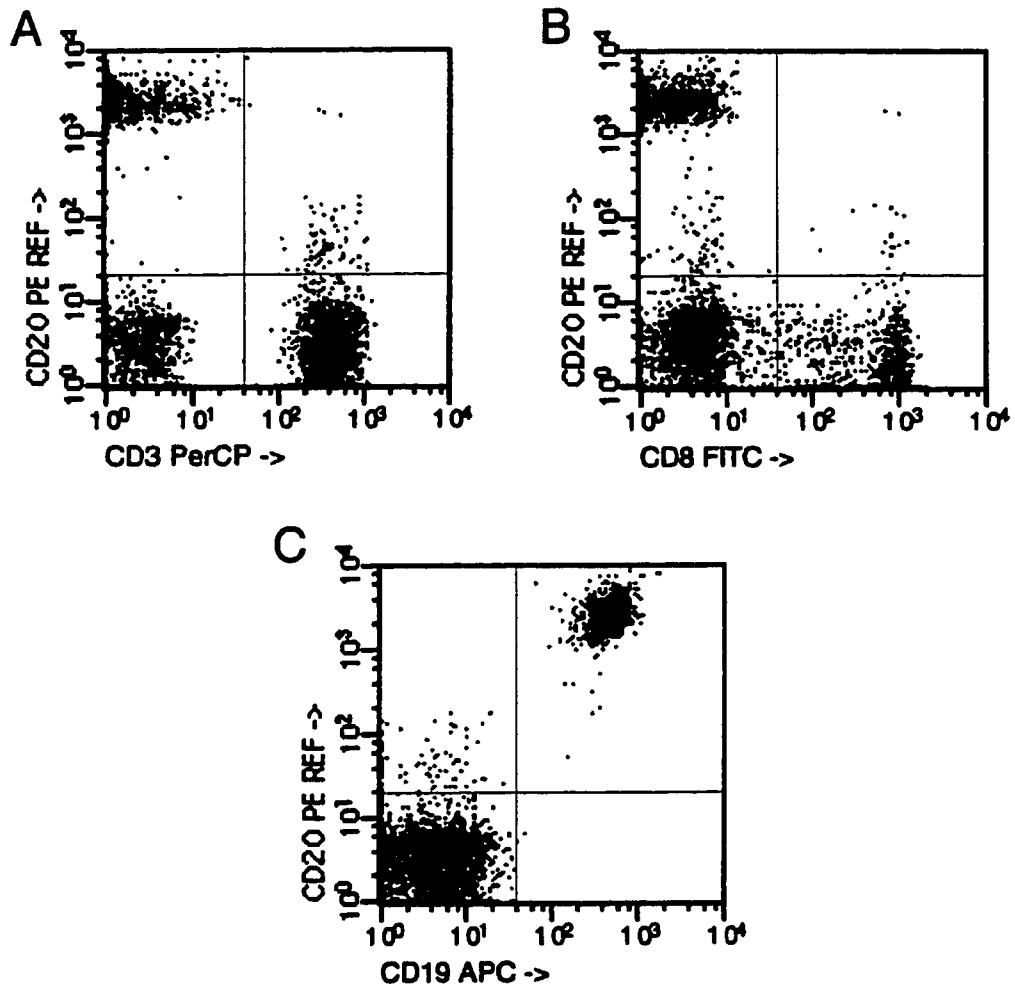
Figure 23: Four color immunofluorescence results of reference CD20-RPE enumerating B cells and T cells of donor 1 using a FACSCalibur. a) Dual color analysis using CD20-RPE and CD3-PerCP. b) Dual color analysis using CD20-RPE and CD8-FITC. c) Dual color analysis using CD20-RPE and CD19-APC.
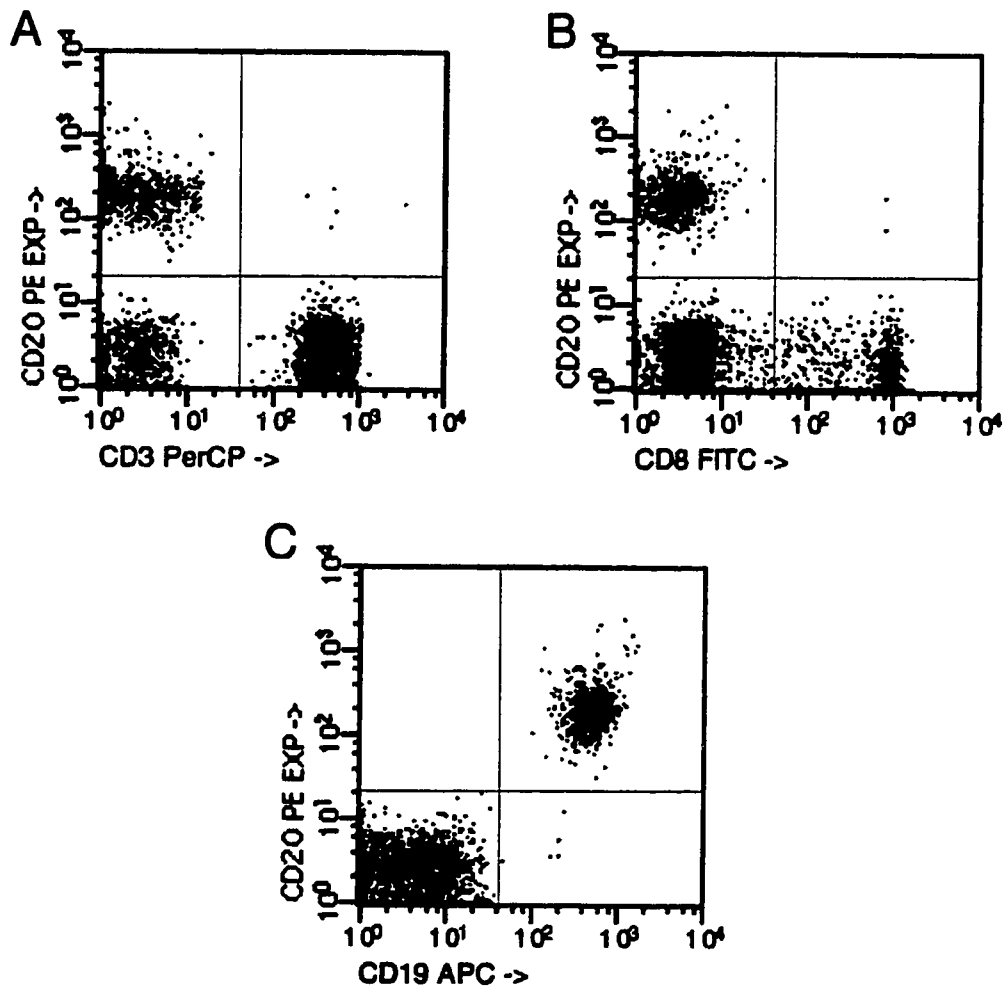
Figure 24: Four color immunofluorescence results of experimental CD20-RPE enumerating B cells and T cells of donor 1 using a FACSCalibur. a) Dual color analysis using CD20-RPE and CD3-PerCP. b) Dual color analysis using CD20-RPE and CD8-FITC. c) Dual color analysis using CD20-RPE and CD19-APC.

Tables 15 and 16 show that the experimental CD20-RPE conjugate and the reference conjugate identified a similar population of B cells. However, when comparing the flow cytometer dot plots (**Figures 23 and 24**) a marked difference is found in the intensity of the fluorescence of the experimental versus the reference CD20-RPE. The reference CD20-RPE identified the B cells with a fluorescence intensity in the 10^3-10^4 range, while the experimental CD20-RPE had a fluorescence intensity in the 10^2-10^3 range. Another difference was that the experimental conjugate did not identify CD20+ T cells. Recall that CD20 epitope is found on B cells and a small population of T cells. The CD20 epitope is clinically used to identify the B cell population, not for the study of the T cell sub-population. Therefore, even though the experimental CD20-RPE failed to identify the CD20+ T cells, the experimental CD20-RPE succeeded as a conjugate since it identified its target epitope.

The dimmer results, acquired by using the experimental CD20-RPE in identifying B cells, can be due to a lower concentration of conjugate than expected. The poor T cell staining by the experimental conjugate can also be explained by concentration, as this population is stained weakly by the reference. It was thought that the experimental CD20-RPE had a concentration of 0.036 mg/ml which was not that different from reference conjugate with a concentration of 0.05 mg/ml. The concentration of experimental CD20-RPE was determined by using **Equation A.3** in **Appendix A** and the relation between A_{280} of peak height of antibody or conjugate acquired by HP1100 Liquid Chromatography System versus antibody concentration. However, the bottled reference used for this test had a measured concentration of 0.18 mg/ml

based on the A_{280} using the spectrophotometer and **Equation A.3**. Note that the spectrophotometer is measuring the A_{280} of all species in sample. Therefore, the concentration of reference conjugate is at most 0.14 mg/ml since 17% of reference is free RPE and cannot target B cells specifically. The reference has 34% of 1:1 conjugate which can be translated to 0.06 mg/ml. But recall that the multiples, those conjugates containing more than three species, can also stain the B cells and thereby drive the concentration of active conjugate to a higher level. More importantly, since some of the multiple species contain more than one RPE molecule they will stain brighter when compared to a stain by a 1:1 conjugate. So the concentration of active conjugate can be approximated as being between 0.06 mg/ml and 0.14 mg/ml for the reference CD20-RPE. Therefore, when comparing experimental vs. reference conjugate in flow cytometry applications, the experimental is expected to perform dimmer since its concentration is less than half of the reference concentration and the experimental contains no multiples. Furthermore, the problem is enhanced by the actual experimental conjugate concentration used which was half the amount of reference conjugate (0.25 µg versus 0.5 µg per test). The concentration of the conjugate affects fluorescence output because B cells contain many CD20 sites for the antibody to bind. The fluorescence output will be brighter as more sites on the B cells are identified by the IgG-RPE conjugate. If one blood sample contains less conjugate, then it is less likely to have brighter B cells when compared to a blood sample with a higher concentration of conjugate. Therefore, the dimness problem could be resolved if more experimental conjugate was used per test.

CONCLUSIONS

This thesis initiates experimentation in determining the applicability of IMAC in IgG and RPE conjugation. The conjugation between RPE and the antibody has been transformed from a liquid-liquid reaction into a liquid-solid phase reaction through the use of IMAC. The nickel ions tested proved positive in binding and immobilizing the antibody and not adsorbing free RPE at the required conjugation reaction pH of 6. The ability to conjugate on the IMAC matrix has been demonstrated. The early benefit from IMAC for the use in the conjugation process has been its ability to make the purification of product cleaner. Yield was not improved by immobilizing the antibody on IMAC under the testing conditions used, but could benefit from further optimization.

The DOE factors tested were reaction time and RPE concentration. The RPE concentration between the range of 5 to 15 mg/ml had a significant effect on several of the responses measured. The reaction time did not cause a significant effect, meaning that this factor is unimportant between the low and high values of 30 to 90 minutes. The formation of conjugate occurred in all of the runs tested in the DOE study. However, the major component on the column after the conjugation reaction was free antibody. Therefore, more experiments are necessary to determine if optimization is possible on IMAC.

Finally, the conjugate proved active in immunofluorescence. The experimental conjugate was equivalent to the reference conjugate in identifying the target B cell population. The variation in ability of the experimental conjugate to enumerate the CD20+ T cells is attributed to concentration.

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Future Studies

Optimization of the DOE used is necessary to further determine the applicability of IMAC for the conjugation reaction. This thesis suggests that further studies will result in a better understanding of how the conjugation reaction occurs on the column. The following testing conditions are good follow-up to this study:

1. The reaction time on the column should be set constant at shorter interval or else tested with a low and high value range of 10 to 60 minutes.

2. The yield of the reaction might be improved by better "mixing" of the reagents during the reaction. The methods used in this thesis were to saturate the column with high RPE concentrations and stopping the flow through the column. Since the antibody is immobilized during the IMAC experiments, an increase in "mixing" can be accomplished by using a continuous flow of RPE through the column for the desired reaction time or reaction in a slurry.

3. This investigation did not test the effect of the antibody loading conditions on the conjugation reaction. The capacity of IMAC to bind the antibody should be examined. The maximum antibody load capacity on the column might improve the number of available reaction sites for the RPE.

4. A method of determining if the reduced antibody has available sulfhydryl sites while bound to the column is crucial for improving the conjugation reaction.

REFERENCES

Asenjo, J. editor, Separation Processes in Biotechnology, Marcel Dekker Inc., New York, 1990.

Becton Dickinson Immunocytometry Systems, Work Instruction #3040: Conjugation of Fluorochrome to Protein, 1997.

Belew, M.; Porath, J.; "Immobilized Metal Ion Affinity Chromatogaphy: Effect of solute structure, ligand density and salt concentration on the retention of peptides." *Journal of Chromatography*, 516 (1990) 333-354.

Boden, V.; Winzerling J.; Vijayalakshmi, M.; Porath, J.; "Rapid one-step purification of goat immunoglobulins by immobilized metal ion affinity chromatography." *Journal of Immunological Methods*, 181 (1995) 225-232.

Box, George E. P.; Hunter, William G.; Hunter, J. Stuart; Statistics for Experimenters, John Wiley and Sons, New York, 1978.

Canaan-Hadden, L.; Ayala, M.; Fernandez-de-Cossio, M. E.; Pedroso, I.; Rodes, L.; Gavilondo, J.V.; "Purification and Application of a Single-Chain Fv Antibody Fragment." *BioTechniques*, 19 (1995) 606-614.

Carlsson, J.; Drevin, H.; Axen, R.; "Protein Thiolation and Reversible Protein-Protein Conjugation." *Biochemical Journal*, 173 (1978) 723-737.

Casey, J; Keep, P.; Chester, K.; Robson, L.; Hawkins, R.; Begent, R.; "Purification of bacterially expressed single chain Fv antibodies for clinical applications using metal chelate chromatography." *Journal of Immunological Methods*, 179 (1995) 105-116.

Davankov, V., Ligand Exchange Chromatography, CRC Press Inc., Boca Raton, Florida, 1988.

Gantt, E., "Phycobilisomes." *Annalytical Reviews in Plant Physiology*, 32 (1981) 327-347.

Glazer, A. N., "Phycobilisomes: Structure and Dynamics." *Annalytical Reviews in Microbiology*, 36 (1982) 173-198.

Goding, J., "Conjugation of antibodies with fluorochromes: modifications to the standard methods." *Journal of Immunological Methods*, 13 (1976) 215-226.

Goldman, M., Fluorescent Antibody Methods, Academic Press, New York, 1968.

Gooding, K.; Regnier, F.; HPLC of Biological Macromolecules, Marcel Dekker, New York, 1990, 179-217 and 513-527.

Hemdan, E.; Porath, J.; "Development of Immobilized Metal Affinity Chromatography: II. Interaction of amino acids with immobilized nickel iminodiacetate and III. Interaction of oligopeptides with immobilized nickel iminodiacetate." *Journal of Chromatography*, 323 (1985) 255-272.

Jonson R.; Todd, R.; Arnold, F.; "Multipoint binding in metal-afinity chromatography: II Effect of pH and imidazole on chromatographic retention of engineered histidine-containing cytochromes c." *Journal of Chromatography A*, 725 (1996) 225-235.

Kronick, M., "The use of phycobiliproteins as fluorescent labels in immunoassay." *Journal of Immunological Methods*, 92 (1986) 1-13.

Kuby, J., Immunology, 2nd ed., W.H. Freeman and Company, New York, 1994, 110-118 and 160-162.

Malik, Vedpal; Lillehoj, Erik; Antibody Techniques, Academic Press, San Diego, California, 1994, 211-214.

Oi, Vernon T.; Glazer, Alexander N.; Stryer, Lubert; "Fluorescent phycobiliprotein conjugates for analyses of cells and molecules." *The Journal of Cell Biology*, 93 (1982) 981-986.

Porath, Jerker, "IMAC-Immobilized metal ion affinity based chromatography." *Trends in Analytical Chemistry*, 7(7) (1988) 254-259.

Porath, J.; Olin, B.; "Immobilized metal ion affinity adsorption and Immobilized Metal Affinity Chromatography of biomaterials. Serum protein affinities for gel-immobilized iron and nickel ions." *Biochemistry*, 22 (1983) 1621-1630.

Sulkowski, E., "Purification of proteins by IMAC." *Trends in Biotechnology*, 3(1) (1985) 1-7.

Weir, D. M.; Herzenberg, L. A.; Blackwell, Caroline; Herzenberg, Leonore; Handbook of Experimental Immunology, Volume 1: Immunochemistry, Blackwell Scientific Publications, Palo Alto, California, 1986, 28.4-28.8.

Wong, J. W.; Albright, R. L.; Wang, N. L.; "Immobilized Metal Ion Affinity Chromatography (IMAC) - chemistry and bioseparation applications." *Separation and Purification Methods*, 20(1) (1991) 49-106.

APPENDIX A

Liquid-Liquid Reaction

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Production of IgG-RPE Conjugate in Liquid Phase

The coupling reaction requires RPE to be modified with a maleimide group and then reacted with the sulfhydryl groups on the hinge region of the reduced antibody. Timing the steps is critical for this reaction. RPE is modified with SMCC and buffer exchanged before the antibody is reduced. The reduction of the antibody creates sulfhydryl groups that will react with each other to reform the disulfide bridge, if not conjugated with the fluorochrome within 30 minutes. Therefore, the modification of RPE is started first and then the reduction of the IgG is timed accordingly. RPE, like many fluorochromes, should be protected from light to prevent photobleaching. Both the antibody and the fluorochrome should be kept on ice unless otherwise specified. Size exclusion chromatography is used to remove reactants from products and to exchange buffers. The columns used were equilibrated prior to each run. Column effluent was monitored at $A_{280 \, nm}$ for each run.

The concentration of RPE is determined by the use of a spectrophotometer. RPE concentration is calculated from absorbance of light at 565 nm as follows:

(Dilution Factor * A_{565}) / 8.3 = concentration (mg/ml)   **Equation A.1**

The 8.3 constant is the absorbance value at 565 nm for a 1 mg/ml RPE solution. The RPE solution is at pH 7.2 in 50 mM sodium phosphate + 1 mM EDTA buffer. The RPE concentration should be in the range of 15 mg/ml before modifying. A 10 mM solution of SMCC in dimethyl sulfoxide is prepared immediately before use. Rapid mixing is required when adding 1.1 μl of SMCC solution per milligram of RPE solution. The reaction is incubated/mixed at room temperature for 60 minutes and covered to protect fluorochrome from light exposure.

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Unreacted reagents were removed through the use of a buffer exchange column. The buffer exchange column, packed with Sephadex G25 media (Pharmacia), was equilibrated with 50 mM MES buffer at pH 6. The SMCC treated RPE reaction mixture did not exceed 15% of the column volume to allow for adequate isolation of the modified RPE. The first band of effluent was easily detected visually, but also monitored at A_{280}, and was collected and kept on ice. Concentration of the modified RPE was determined spectrophotometrically at A_{565} using **Equation A.1**.

Recall that the purpose of modifiyng RPE with SMCC is to provide the fluorochrome with reactive maleimides (see **Figure 3**). To determine the number of available maleimides per RPE molecule, the assay uses 2-mercaptoethylamine hydrochloride (MEA-HCl) as the sulfhydryl group provider. Ellman's reagent is used to quantify the remaining available MEA-HCl molecules in a control tube (total SH) versus a tube with RPE-maleimide. The difference between the A_{412} for total SH and the A_{412} for the unconsumed SH gives the number of maleimides that reacted (**Equation A.2**). Samples containing modified RPE, diluted in 50 mM MES buffer at pH 6, are used to determine unconsumed SH groups. Samples containing only the buffer, 50mM MES buffer pH 6, are used to determine total SH groups. MEA-HCl was added to each of the tubes. While unconsumed SH tubes were incubated for five minutes, the total SH tubes were used to determine total sulfhydryl groups available. Ellman's reagent was added to the total SH tubes. The tubes were vortexed and incubated for 45 seconds then the A_{412} was read in a spectrophotometer. The unconsumed SH tubes require an A_{412} reading before

and after the addition of Ellman's reagent. The absorbance at 412 nm was read again after 1 minute incubation with Ellman's reagent. The number of moles of maleimide per mole of modified RPE was calculated using **Equation A.2**, as seen below. Since the dilution of modified RPE was chosen as 1 mg for unconsummed SH, the denominator in **Equation A.2** is one. The maleimide/RPE value should be between 0.8 and 1.3 in order to continue with conjugation.

Maleimide/RPE = [A_{412}(total SH) - A_{412}(unconsumed SH) X 9] /1 mg RPE   **Equation A.2**

To prepare the antibody for coupling, the disulfide bridges of its hinge area require reduction. The concentration of IgG in buffer 1XPBS pH 7 should be 10 mg/ml for the reduction reaction. The concentration of IgG was determined spectrophotometrically as follows:

(Dilution Factor * A_{280}) / 1.4 = concentration mg/ml   **Equation A.3**

The absorbance value at 280 nm for a 1 mg/ml IgG solution is 1.4. To reduce the antibody, 20 µl of 1 M DTT was added per ml of IgG solution while vortexing. The reaction mixture was incubated for 30 minutes at room temperature without additional mixing. After the incubation time, the reduced antibody was run through a buffer exchange column equilibrated with 50 mM MES buffer pH 6 to remove any DTT. The column (packed with Sephadex G25 media) was monitored at A_{280} and the first eluent was collected and placed on ice. The second, small band which eluted was the unwanted DTT. The concentration and the available sulfhydryl groups were determined via spectrophotometry.

The assay for available sulfhydryl groups is based on the direct reaction of the Ellman's reagent with the reduced sulfhydryl groups on the antibody. The concentration of the IgG solution can be determined in the same assay. Two

blanks are required of 0.49 ml of 50 mM MES buffer pH 6. The first buffer blank was read at A_{412} to zero the spectrophotometer at that wavelength. After the addition of 0.01 ml of 0.01 M Ellman's reagent the A_{412} was read again. This solution represents the Ellman's blank and is saved for one more required reading. Two sample dilutions, 1:10 and 1:14, of IgG solution were made with a final volume of 0.49 ml. The second buffer blank was used to zero the spectrophotometer at A_{280}. The second blank was saved for sequential readings. The 1:14 dilution of reduced antibody was then read at A_{280}. Then a volume of 0.01 ml of 0.01 M Ellman's reagent was added and mixed with a pipette to the cuvette containing 1:14 dilution of IgG. Meanwhile the Ellman's blank was reused to zero the spectrophotometer at A_{412}. After 1 minute of incubation time, the A_{412} was read for the IgG solution and Ellman's reagent mixture. The above steps were repeated for the 1:10 dilution. The concentration of IgG in solution was determined by **Equation A.3**. The available sulfhydryl groups per antibody were determined as follows:

$$\text{moles SH/ moles IgG} = (\Delta A_{412} * 16.5) / A_{280} \qquad \textbf{Equation A.4}$$

The SH/IgG value should be between 8-11 to allow for adequate reaction sites for RPE conjugation.

The conjugation reaction requires both modified RPE and reduced IgG to be cold (on ice) upon mixing. The ratio used for the reaction was 3 mg of modified RPE per 1 mg of reduced IgG (roughly 2:1 molar ratio). The reaction vessel should be at least three times the final reaction volume to allow for adequate mixing. The IgG was added dropwise to the RPE while vortexing. The reaction mixture was then placed in a room temperature water/agitator bath

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and incubated for 60 minutes. The reaction was stopped by mixing in 2.5 µl of 10 mM 2-ME per mg of IgG in the solution. At this point, the reaction mixture was stable for up to five days. Within that time period, the products were purified from the reactants using size exclusion chromatography.

The final sizing column was packed with Toyo_Pearl HW55S media (Tosohaas, Montgomeryville, PA). It was equilibrated with 50 mM sodium phosphate pH 7.2 + 0.15 M NaCl + 2 M Urea + 0.1% NaN$_3$. Since this is the final purification step, the loading volume of the reaction mixture was less than 2.5% of the bed volume. Flow rate through the column was kept under 5 cm/hr. A fraction collector was used to collect product. Once fractionization was completed, electrophoresis was done on appropriate fractions. The fractions containing mostly 1:1 conjugates were pooled together and constitute the product. The product was then buffered exchanged into 1XPBS + 01% NaN$_3$ via buffer exchange chromatography or dialysis.

APPENDIX B
BioCAD Output for IMAC Runs

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Figure B.1: BioCAD fluorometer and spectrophotometer recordings of Run 1 (begins after RPE loading). The factors were RPE concentration of 5 mg/ml and reaction time of 90 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.
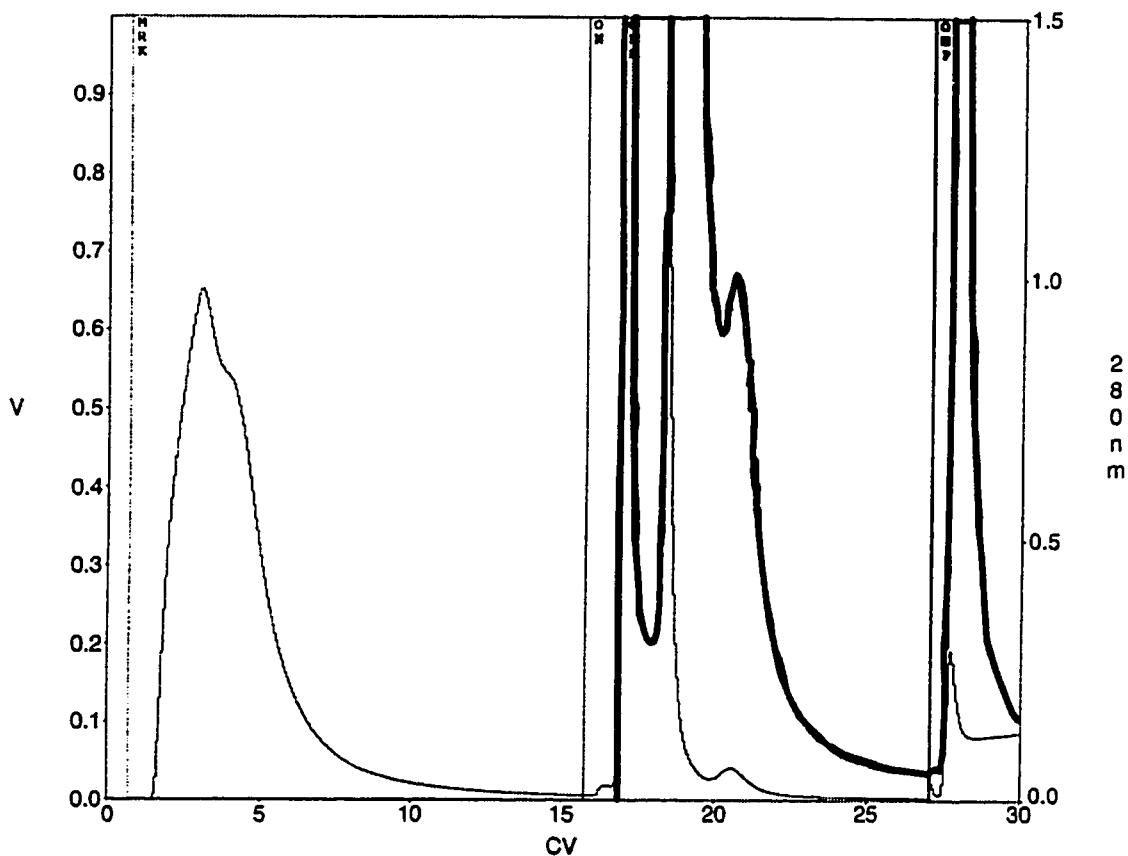
Figure B.2: BioCAD fluorometer and spectrophotometer recordings of Run 2. The factors were RPE concentration of 15 mg/ml and reaction time of 90 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.
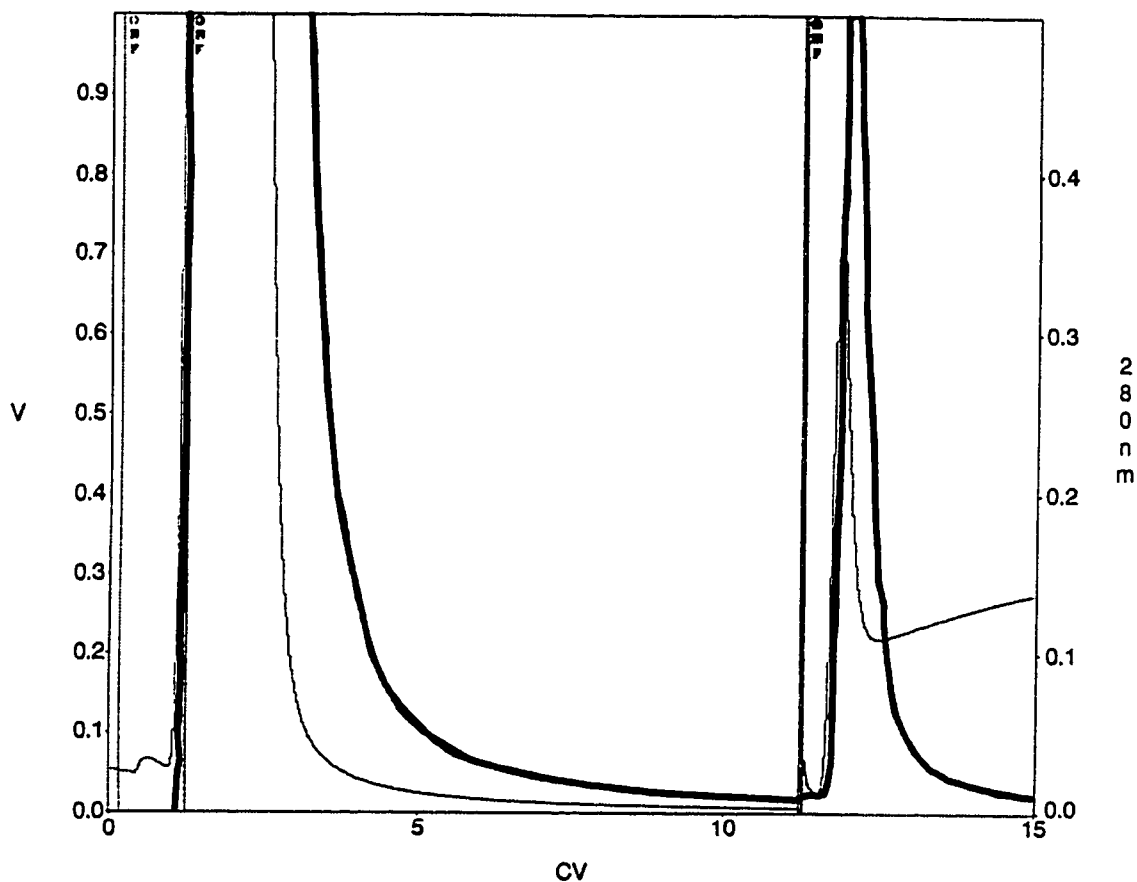
Figure B.3: BioCAD fluorometer and spectrophotometer recordings of Run 3 (begins after RPE loading). The factors were RPE concentration of 5 mg/ml and reaction time of 30 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.
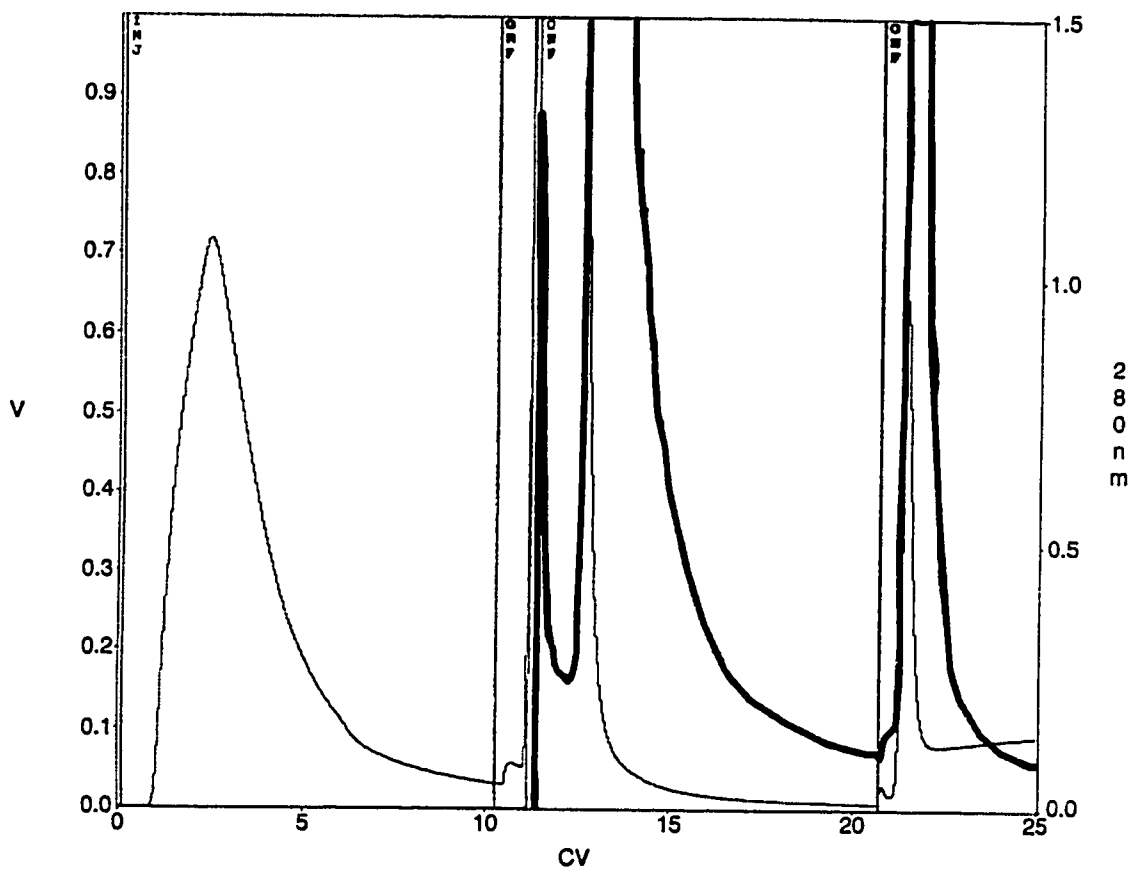
Figure B.4: BioCAD fluorometer and spectrophotometer recordings of Run 4. The factors were RPE concentration of 15 mg/ml and reaction time of 30 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.
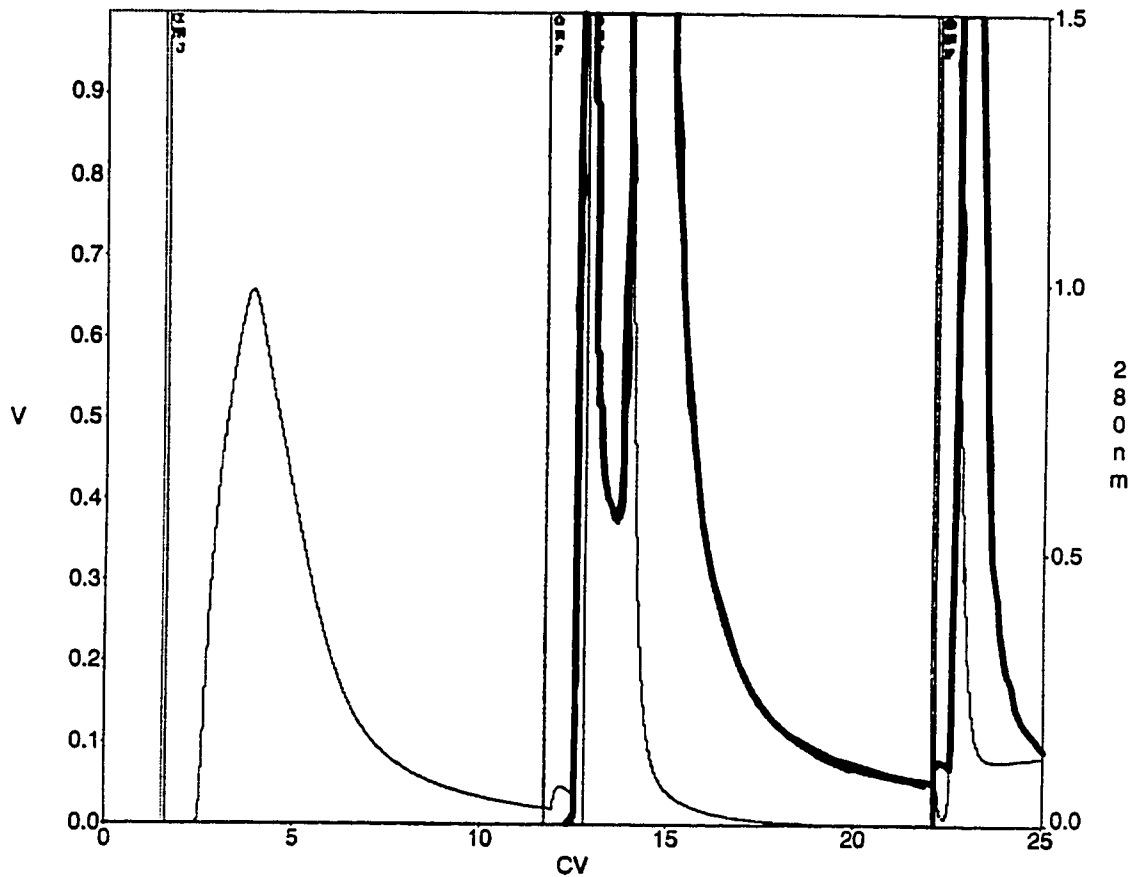
Figure B.5: BioCAD fluorometer and spectrophotometer recordings of Run 5. The factors were RPE concentration of 10 mg/ml and reaction time of 60 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.
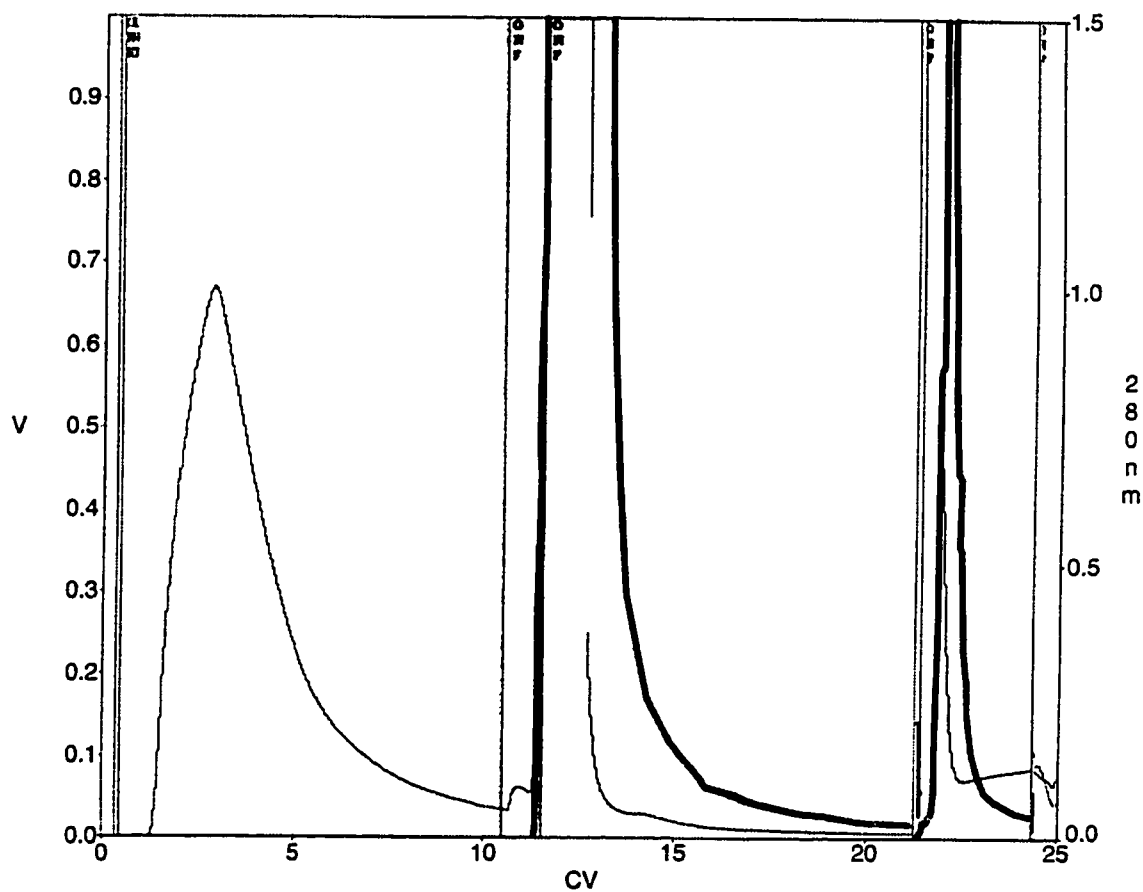
Figure B.6: BioCAD fluorometer and spectrophotometer recordings of Run 6. The factors were RPE concentration of 5 mg/ml and reaction time of 90 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.

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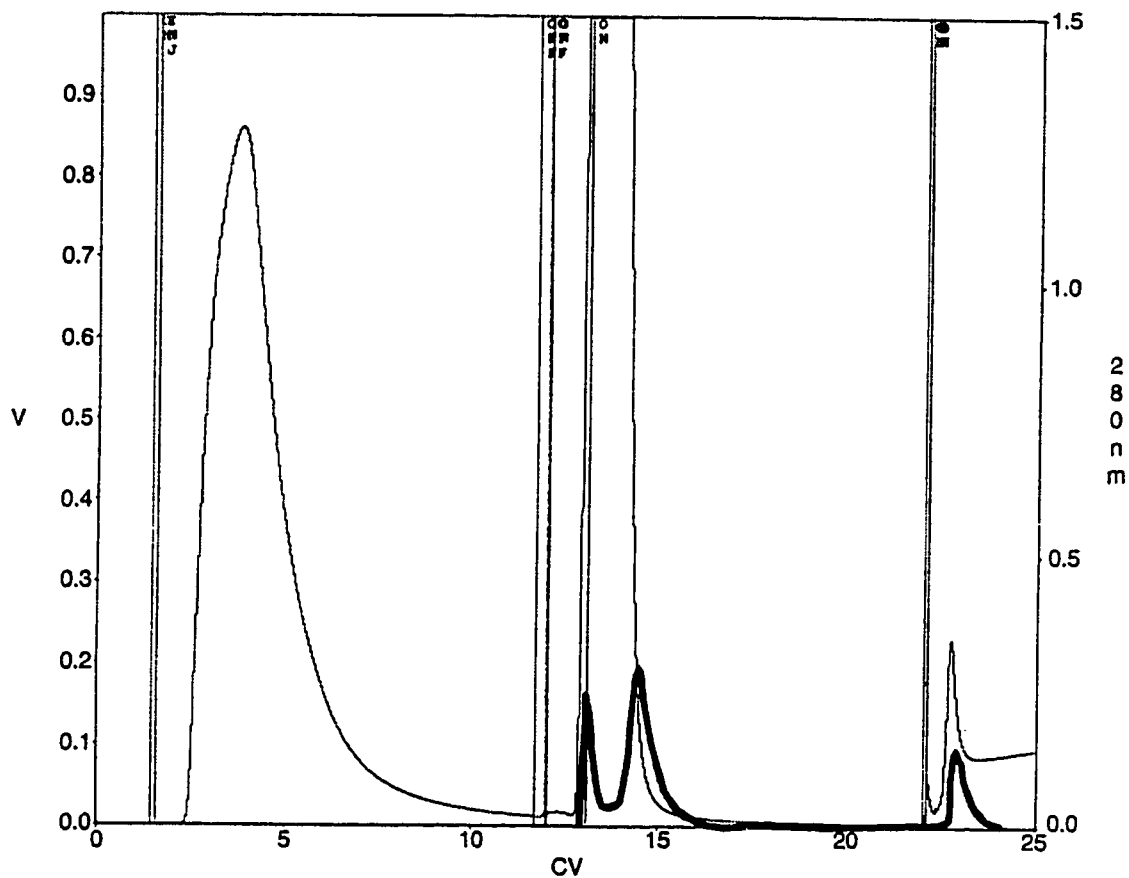
Figure B.7: BioCAD fluorometer and spectrophotometer recordings of Run 8. The factors were RPE concentration of 10 mg/ml and reaction time of 60 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.
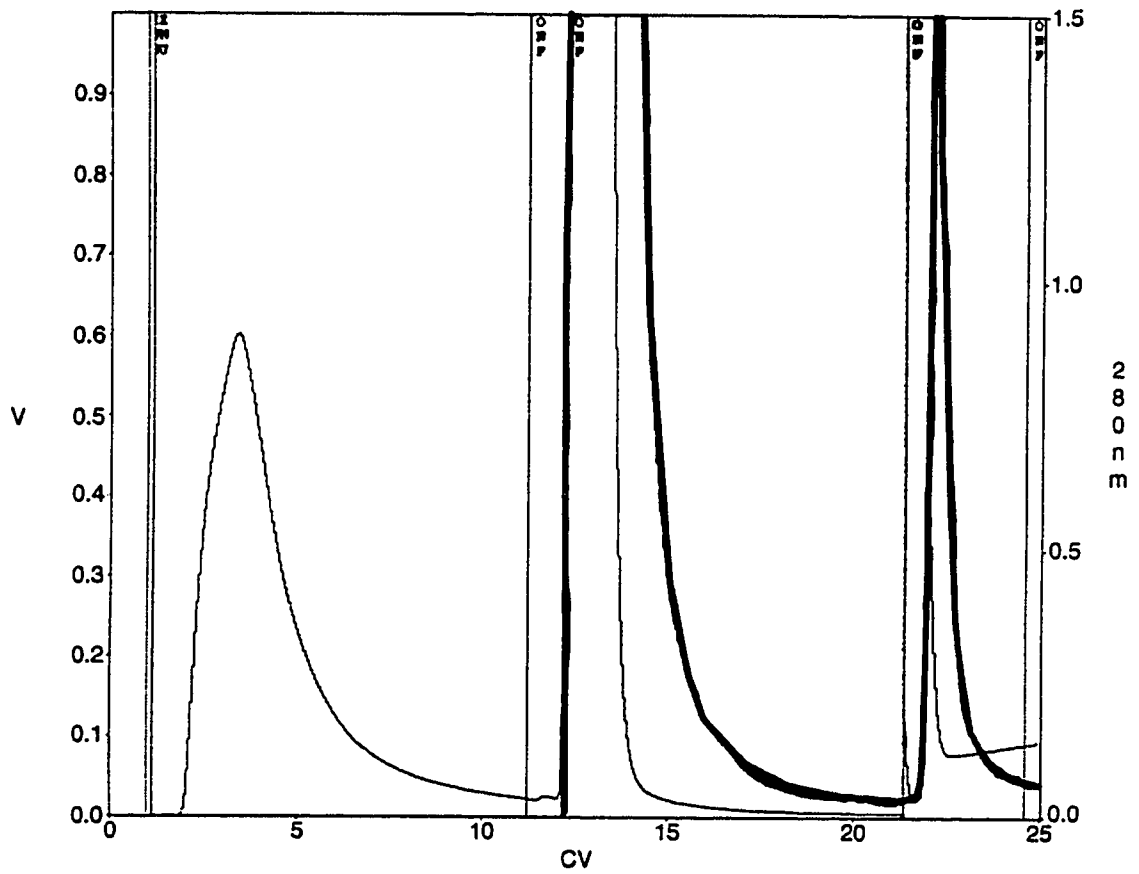
Figure B.8: BioCAD fluorometer and spectrophotometer recordings of Run 9. The factors were RPE concentration of 5 mg/ml and reaction time of 30 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.
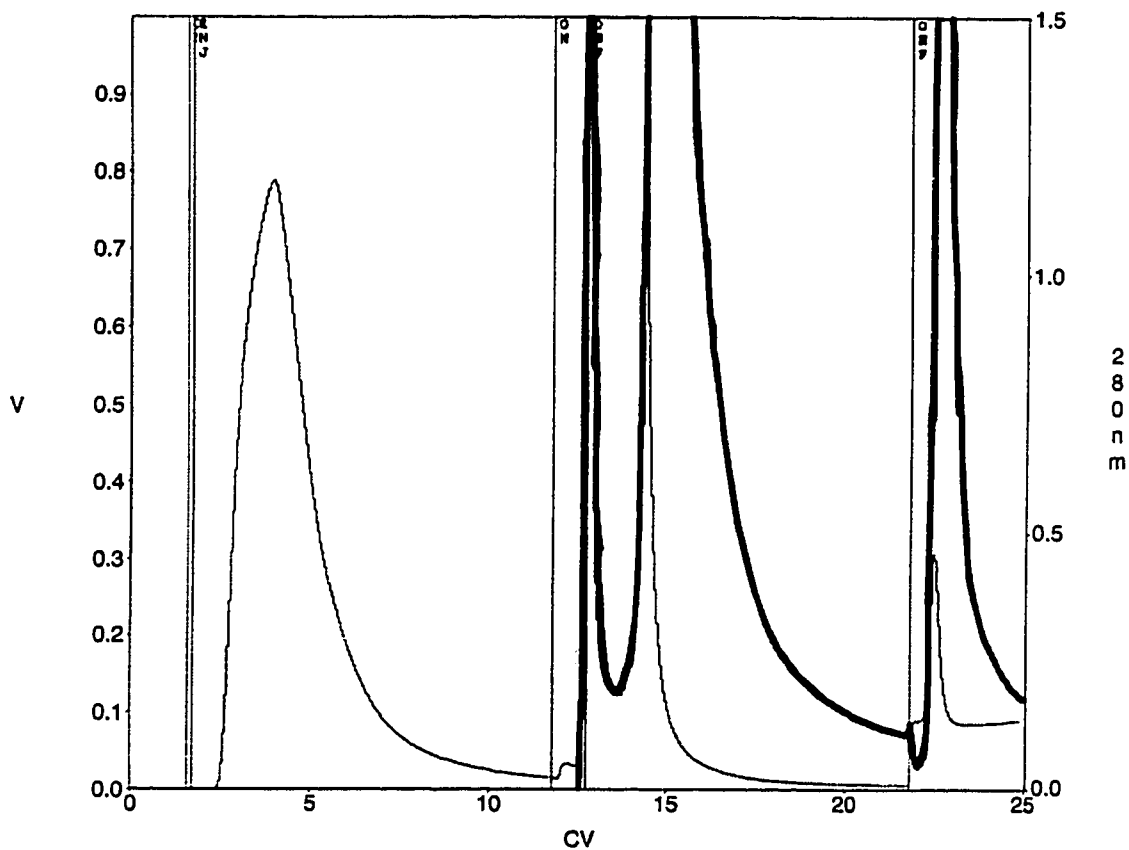
Figure B.9: BioCAD fluorometer and spectrophotometer recordings of Run 10. The factors were RPE concentration of 15 mg/ml and reaction time of 30 minutes. Absorbance was measured with units of 280 nm (right Y axis). The fluorescence was measured with units of volts (left Y axis). The X axis measures flow through the column in Column Volumes.

APPENDIX C
HP1100 Liquid Chromatography Results
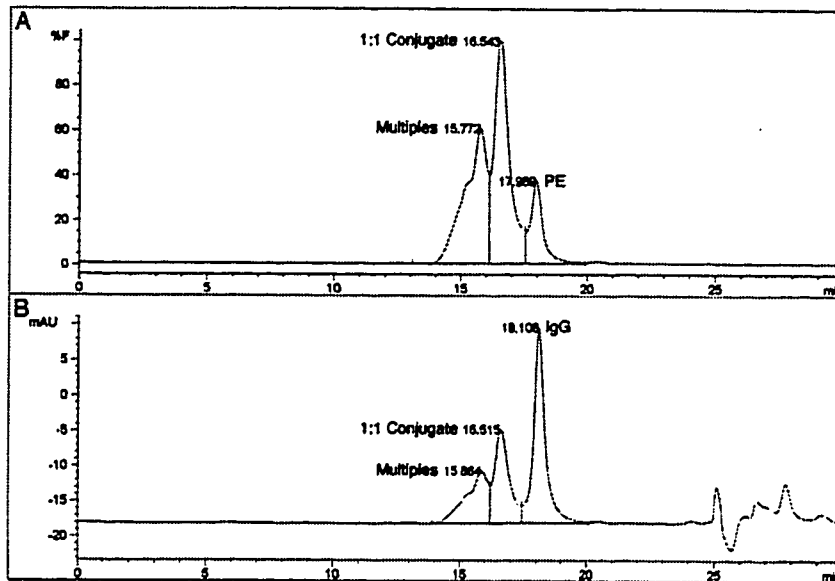
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Figure C.1: HP1100 liquid chromatography analysis of Run 1. The factors tested for Run 1 were 5 mg/ml PE and 90 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A$_{280}$.

Table C.1: Fluorometer signal readings for run 1.

Species	Time (min.)	Width (min.)	Area (%F*s)	Height (%F)	Area %
Multiples	15.799	0.7173	652.38861	12.41002	28.8025
1:1 Conjugate	16.556	0.6371	1147.2838	25.88669	50.6517
Free RPE	17.993	0.479	457.09357	13.92258	20.1803

Table C.2: Spectrophotometer signal reading for run 1.

| Species	Time (min.)	Width (min.)	Area (mAU*s)	Height (mAU)	Area %
Multiples	15.881	0.7033	76.43053	1.48991	20.2853
1:1 Conjugate	16.627	0.6229	137.37318	3.20152	36.46
IgG	18.081	0.612	162.97403	3.79246	43.2547
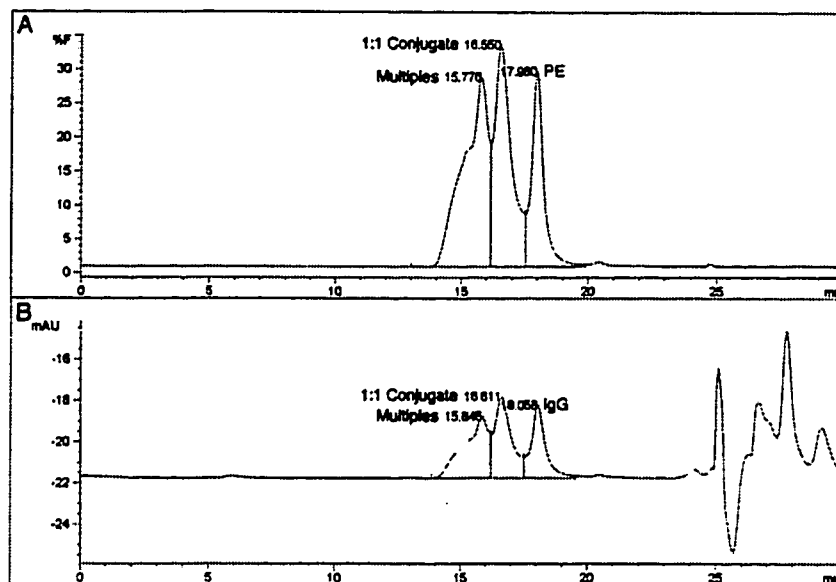
Figure C.2: HP1100 liquid chromatography analysis of Run 2. The factors tested for Run 2 were 15 mg/ml PE and 90 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A280.

Table C.3: Fluorometer signal readings for run 2.

Species	Time (min.)	Width (min.)	Area (%F*s)	Height (%F)	Area %
Multiples	15.776	0.901	1905.5899	27.89381	42.8767
1:1 Conjugate	16.55	0.6684	1559.2295	32.69152	35.0835
Free RPE	17.98	0.4931	979.52405	28.77618	22.0398

Table C.4: Spectrophotometer signal reading for run 2.

| Species	Time (min.)	Width (min.)	Area (mAU*s)	Height (mAU)	Area %
Multiples	15.845	0.8984	203.27484	2.98836	38.441
1:1 Conjugate	16.611	0.6694	184.49944	3.93445	34.8904
IgG	18.058	0.5648	141.02319	3.5319	26.6687

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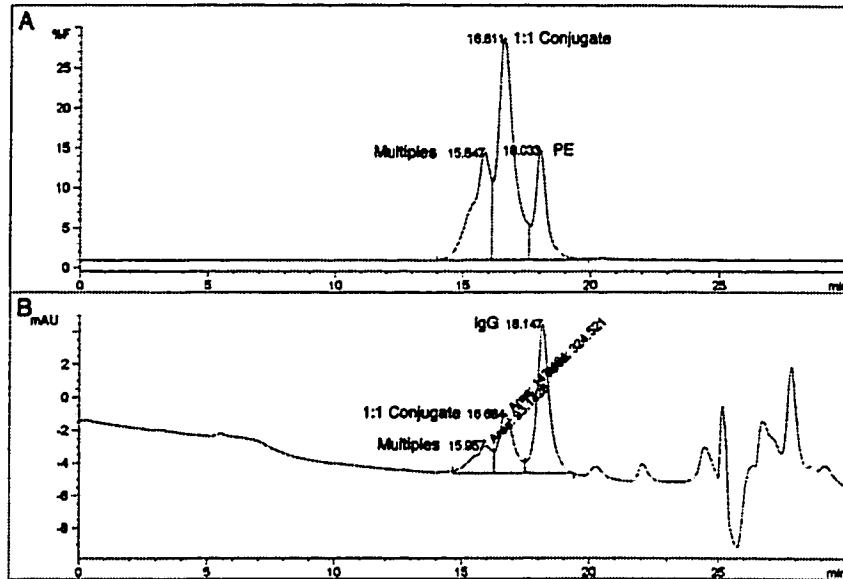
Figure C.3: HP1100 liquid chromatography analysis of Run 3. The factors tested for Run 3 were 5 mg/ml PE and 30 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A_{280}.

Table C.5: Fluorometer signal readings for run 3.

Species	Time (min.)	Width (min.)	Area (%F*s)	Height (%F)	Area %
Multiples	15.847	0.6981	671.93848	13.37547	28.3112
1:1 Conjugate	16.611	0.6646	1232.2105	27.61513	51.9174
Free RPE	18.033	0.5138	469.2551	13.62585	19.7714

Table C.6: Spectrophotometer signal reading for run 3.

| Species	Time (min.)	Width (min.)	Area (mAU*s)	Height (mAU)	Area %
Multiples	15.957	0.9236	93.14283	1.68086	16.4809
1:1 Conjugate	16.684	0.6795	147.49408	3.61771	26.0979
IgG	18.147	0.5942	324.52094	9.10314	57.4213

103
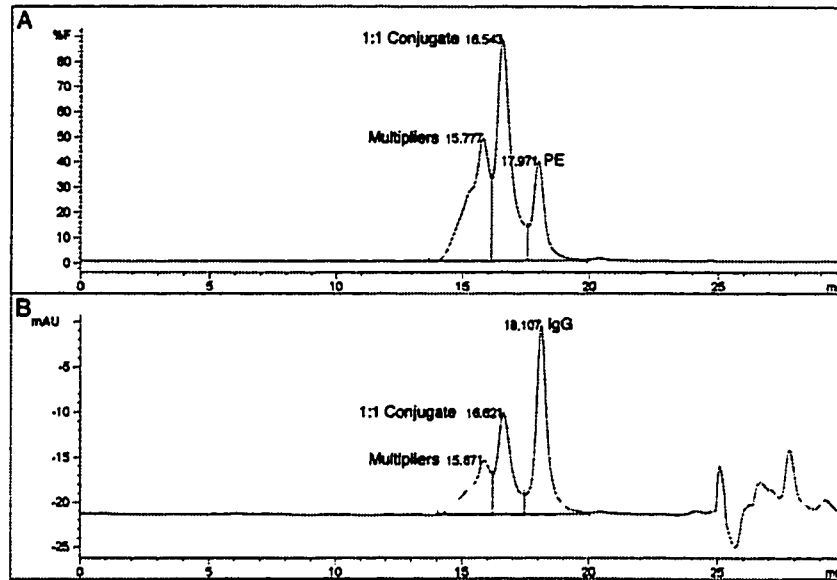
Figure C.4: HP1100 liquid chromatography analysis of Run 4. The factors tested for Run 4 were 15 mg/ml PE and 30 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A_{280}.

Table C.7: Fluorometer signal readings for run 4.

Species	Time (min.)	Width (min.)	Area (%F*s)	Height (%F)	Area %
Multiples	15.777	0.7828	2821.0432	48.48237	35.8416
1:1 Conjugate	16.543	0.5918	3675.4395	87.91371	46.6968
Free RPE	17.971	0.5026	1374.3709	39.43135	17.4615

Table C.8: Spectrophotometer signal reading for run 4.

Species	Time (min.)	Width (min.)	Area (mAU*s)	Height (mAU)	Area %
Multiples	15.871	0.7809	344.93835	6.00445	22.5822
1:1 Conjugate	16.621	0.592	463.6358	11.23658	30.353
IgG	18.107	0.4994	718.9043	20.94637	47.0648

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Figure C.5: HP1100 liquid chromatography analysis of Run 5. The factors tested for Run 5 were 10 mg/ml PE and 60 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A$_{280}$.

Table C.9: Fluorometer signal readings for run 5.

Species	Time (min.)	Width (min.)	Area (%F*s)	Height (%F)	Area %
Multiples	15.772	0.8044	3642.3079	59.98811	39.4663
1:1 Conjugate	16.543	0.6061	4263.1494	99.05193	46.1935
Free RPE	17.969	0.5178	1323.4377	36.60918	14.3402

Table C.10: Spectrophotometer signal reading for run 5.

Species	Time (min.)	Width (min.)	Area (mAU*s)	Height (mAU)	Area %
Multiples	15.864	0.8076	442.87805	7.41364	22.8469
1:1 Conjugate	16.615	0.5918	550.24066	13.34148	28.3854
IgG	18.108	0.4977	945.34271	27.66598	48.7677
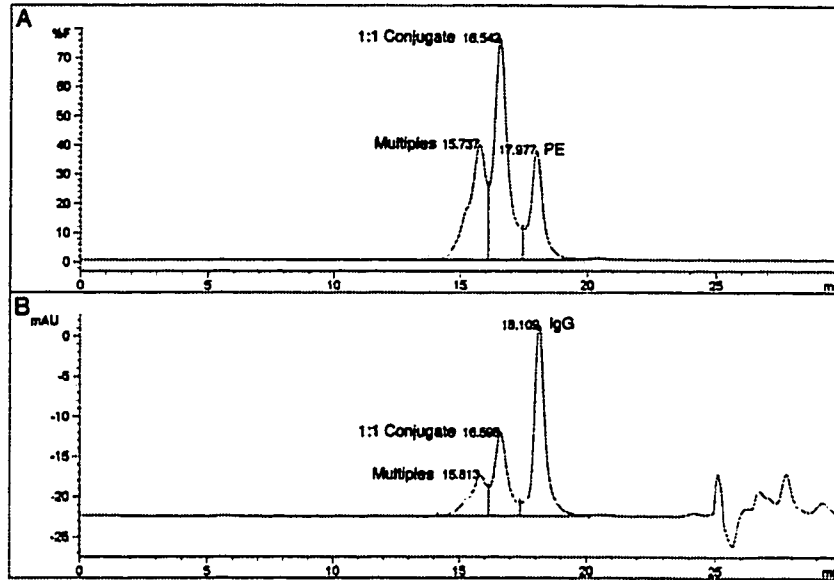
Figure C.6: HP1100 liquid chromatography analysis of Run 7. The factors tested for Run 7 were 15 mg/ml PE and 90 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A_{280}.

Table C.11: Fluorometer signal readings for run 7.

Species	Time (min.)	Width (min.)	Area (%F*s)	Height (%F)	Area %
Multiples	15.737	0.6606	1883.0515	39.46833	30.6383
1:1 Conjugate	16.542	0.5541	2932.9673	76.0169	47.721
Free RPE	17.977	0.5101	1330.0563	37.47018	21.6407

Table C.12: Spectrophotometer signal reading for run 7.

Species	Time (min.)	Width (min.)	Area (mAU*s)	Height (mAU)	Area %
Multiples	15.813	0.6632	245.93115	5.13848	17.5138
1:1 Conjugate	16.598	0.5568	398.82346	10.42841	28.4018
IgG	18.109	0.465	759.46204	23.82703	54.0844

106
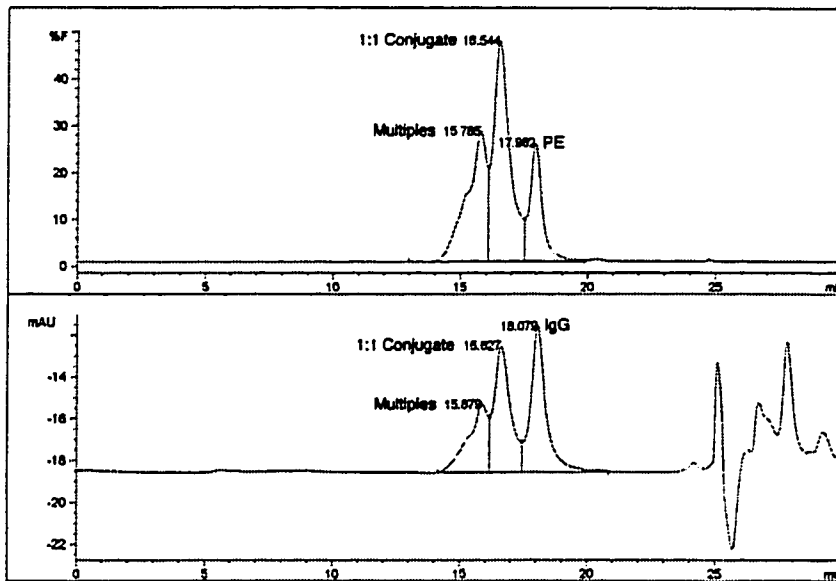
Figure C.7: HP1100 liquid chromatography analysis of Run 8. The factors tested for Run 8 were 10 mg/ml PE and 60 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A_{280}.

Table C.13: Fluorometer signal readings for run 8.

Species	Time (min.)	Width (min.)	Area (%F*s)	Height (%F)	Area %
Multiples	15.785	0.7386	1534.9238	27.86532	33.421
1:1 Conjugate	16.544	0.6594	2151.1641	47.22632	46.8388
Free RPE	17.962	0.5045	906.60742	25.39289	19.7402

Table C.14: Spectrophotometer signal reading for run 8.

Species	Time (min.)	Width (min.)	Area (mAU*s)	Height (mAU)	Area %
Multiples	15.879	0.7438	178.20389	3.25384	23.6862
1:1 Conjugate	16.627	0.6417	270.68274	6.07989	35.9781
IgG	18.079	0.6171	303.46683	6.99052	40.3357
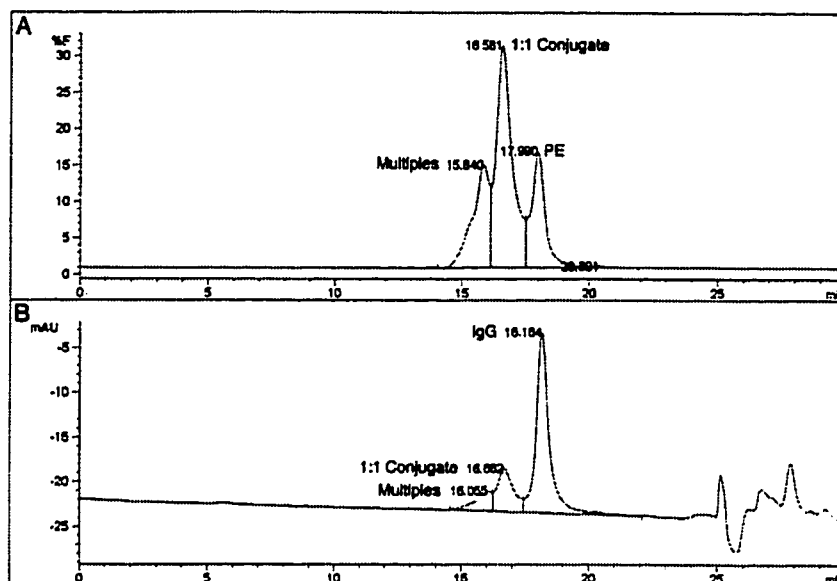
Figure C.8: HP1100 liquid chromatography analysis of Run 9. The factors tested for Run 9 were 5 mg/ml PE and 30 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A$_{280}$.

Table C.15: Fluorometer signal readings for run 9.

Species	Time (min.)	Width (min.)	Area (%F*s)	Height (%F)	Area %
Multiples	15.84	0.6526	661.63416	14.07076	25.1285
1:1 Conjugate	16.581	0.6297	1353.9745	30.51218	51.4232
Free RPE	17.99	0.542	609.77124	15.94951	23.1588

Table C.16: Spectrophotometer signal reading for run 9.

Species	Time (min.)	Width (min.)	Area (mAU*s)	Height (mAU)	Area %
Multiples	16.055	0.6746	104.22265	2.22516	9.7498
1:1 Conjugate	16.682	0.6467	215.07829	4.83908	20.1201
IgG	18.164	0.5316	749.67096	20.21888	70.1301

108
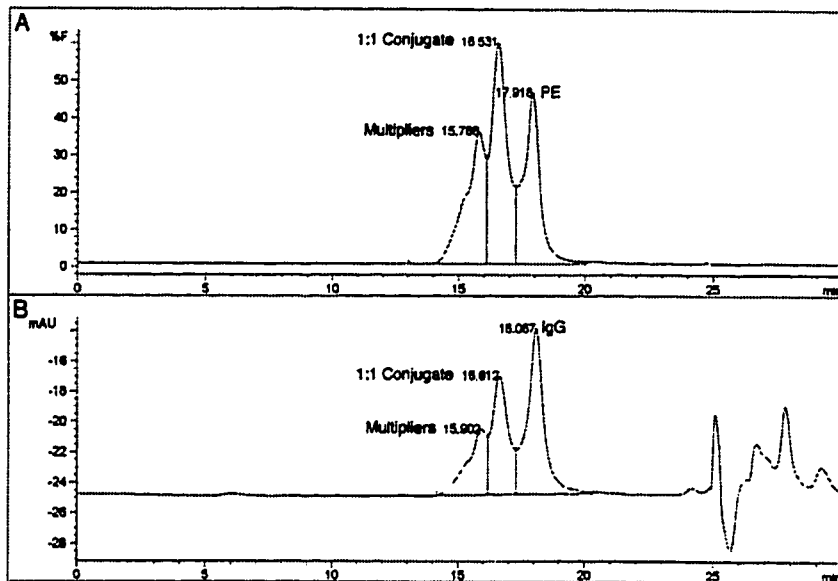
Figure C.9: HP1100 liquid chromatography analysis of Run 10. The factors tested for Run 10 were 15 mg/ml PE and 30 minute reaction time on IMAC. a) Fluorescence output (EX 488 nm, EM 573 nm). b) Spectrophotometer output read at A_{280}.

Table C.17: Fluorometer signal readings for run 10.

Species	Time (min.)	Width (min.)	Area (%F*s)	Height (%F)	Area %
Multiples	15.786	0.7417	1951.5729	35.70546	29.0316
1:1 Conjugate	16.531	0.6226	2649.1231	59.59455	39.4084
Free RPE	17.918	0.6337	2121.5359	46.03442	31.56

Table C.18: Spectrophotometer signal reading for run 10.

Species	Time (min.)	Width (min.)	Area (mAU*s)	Height (mAU)	Area %
Multiples	15.902	0.7412	233.61043	4.32208	21.2261
1:1 Conjugate	16.612	0.6408	348.70038	7.84637	31.6833
IgG	18.067	0.6582	518.27191	10.92669	47.0907

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APPENDIX D
Design-Ease Statistical Reports

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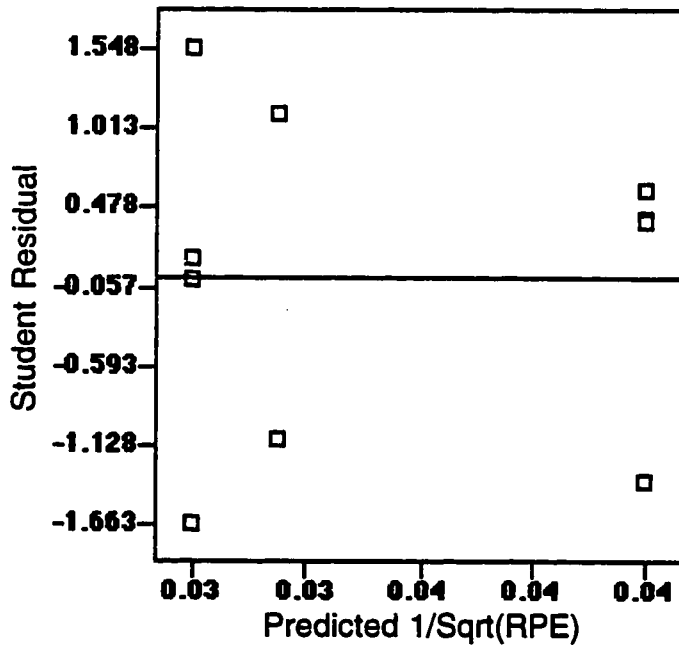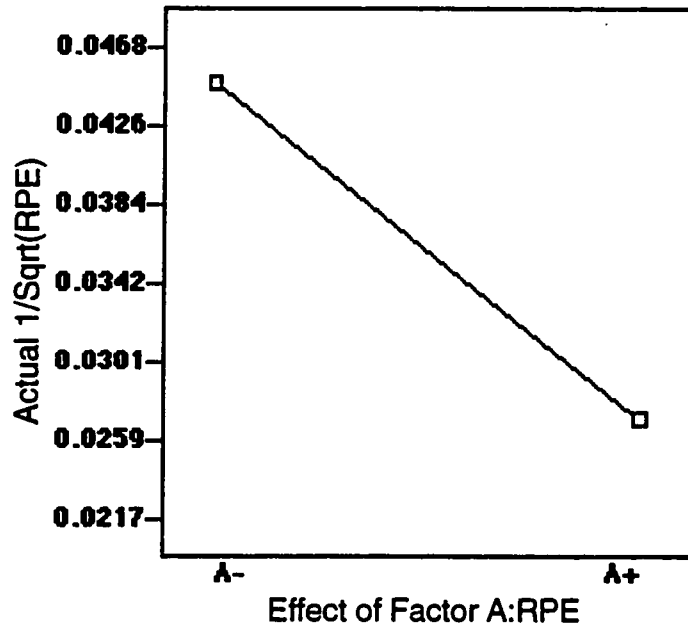**Report D.1:** Statistical analysis for the Yield (mAU) response with the data transformed by the inverse function. The yield response was calculated by measuring the absorbance peak area of the conjugate and antibody species from the HP1100 analysis of the fractions collected from IMAC.
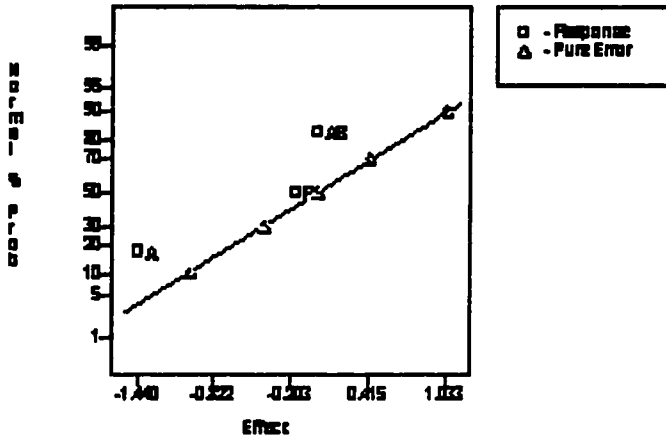
PROB>F = 0.4373
PROB>|t| = 0.1341 for A
PROB>|t| = 0.8376 for B

Predicted 1/Yield

Effect of Factor A:RPE

Effect of Factor B:Reaction Time

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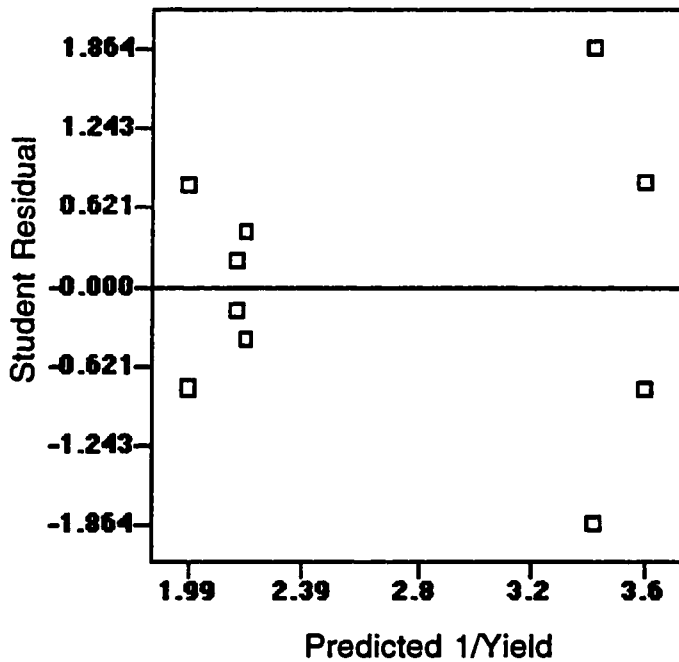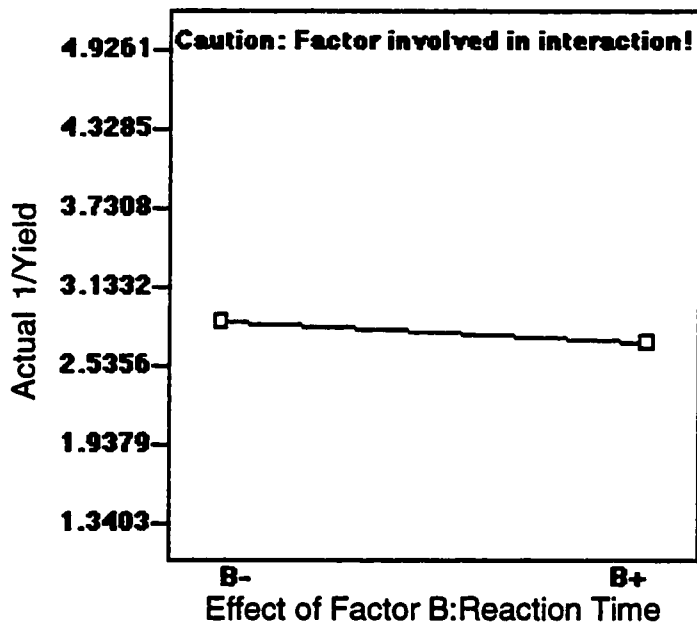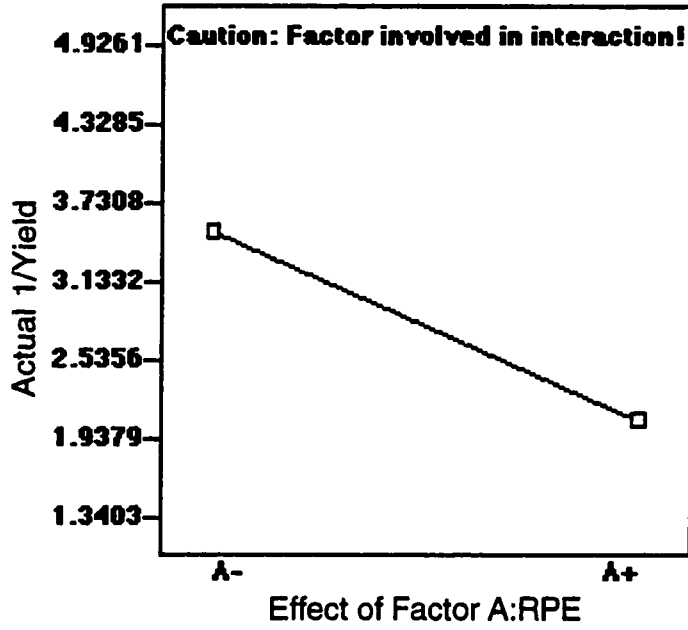
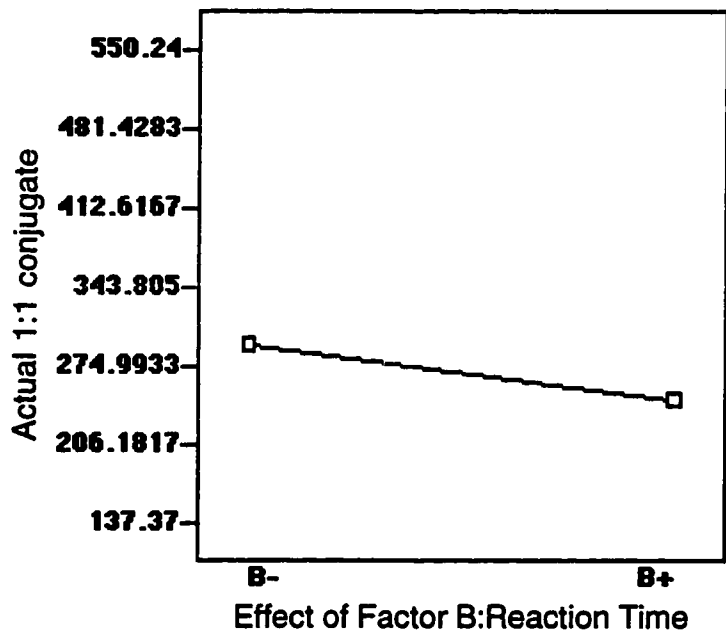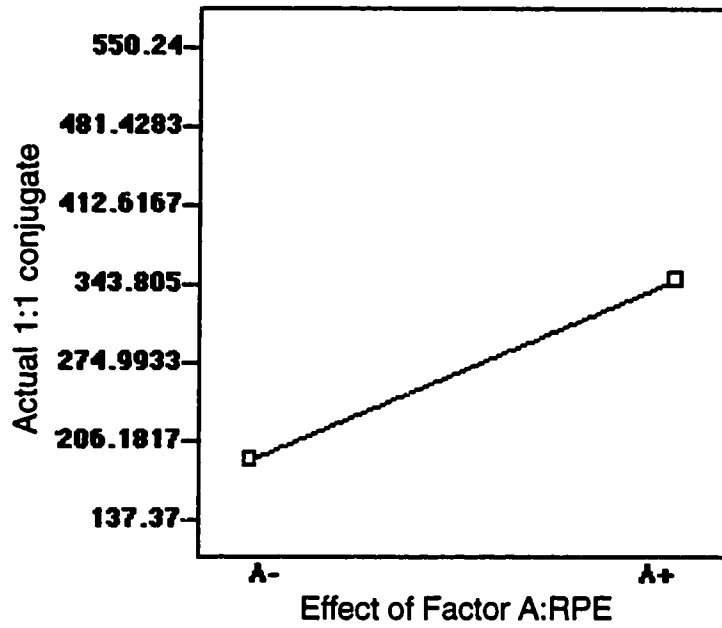**Report D.2:** Statistical Analysis for the IgG (mAU) response with the data not transformed. The IgG response was calculated by measuring the absorbance peak area of the antibody from the HP1100 analysis of the fractions collected from IMAC.
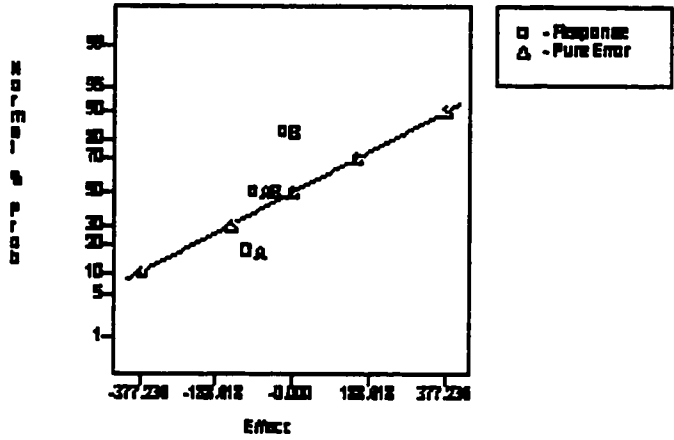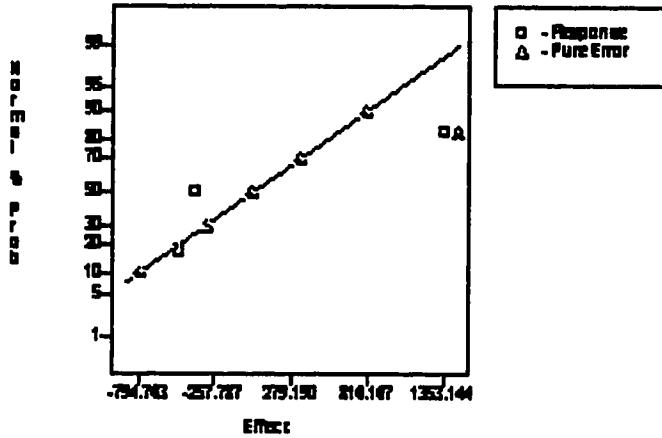

PROB>F = 0.9645
PROB>|t| = 0.7198 for A
PROB>|t| = 0.9375 for B
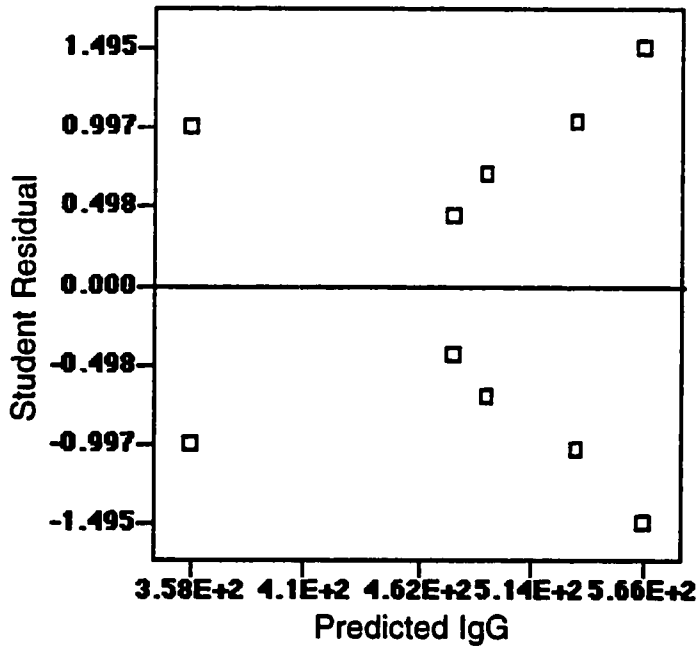
Report D.3: Statistical Analysis for the IgG (mAU) response with the data transformed by the inverse function. The IgG response was calculated by measuring the absorbance peak area of the antibody from the HP1100 analysis of the fractions collected from IMAC.
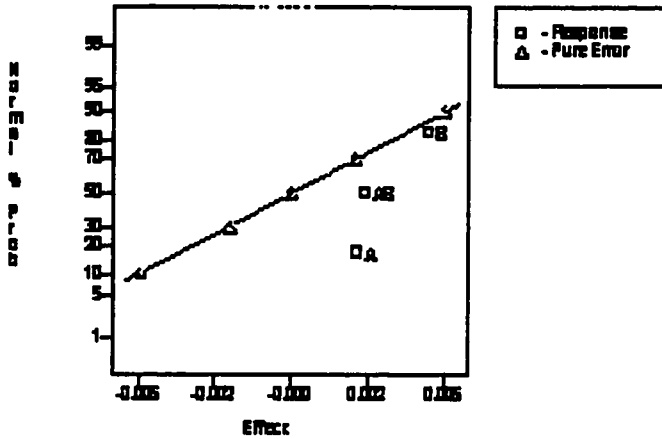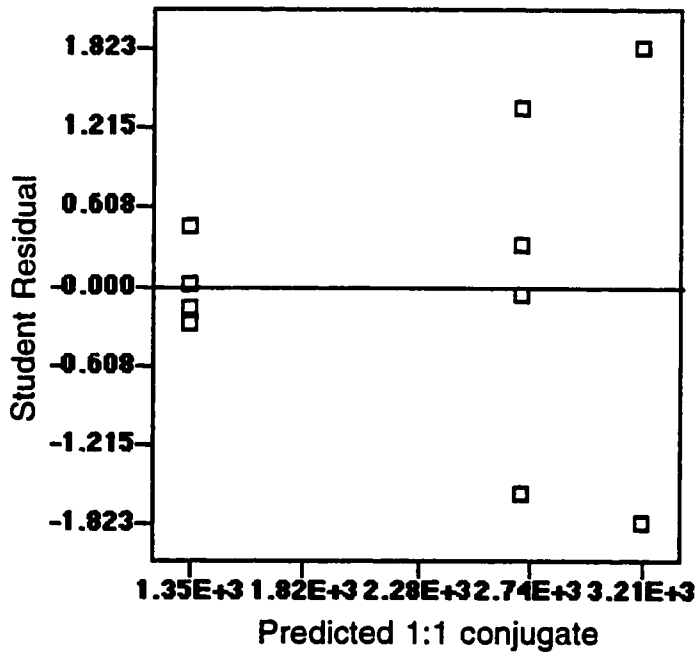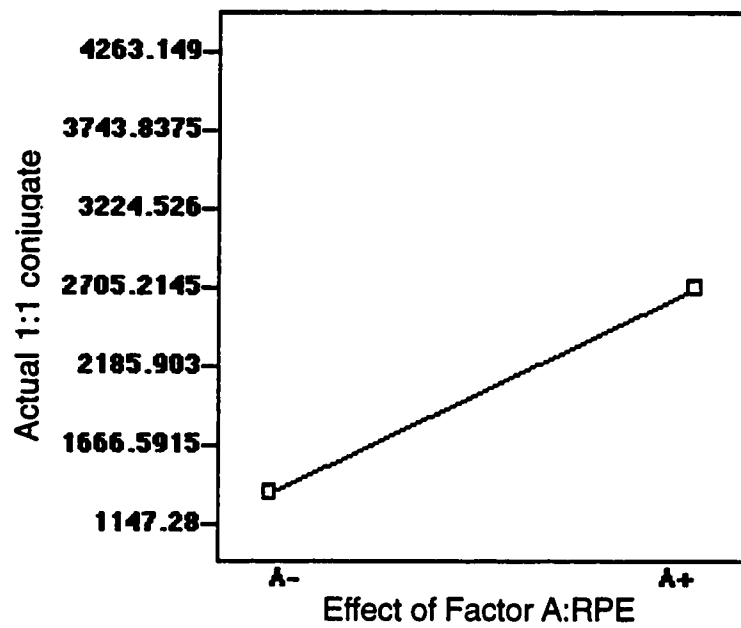

PROB>F = 0.6071
PROB>ltl = 0.6033 for A
PROB>ltl = 0.3017 for B
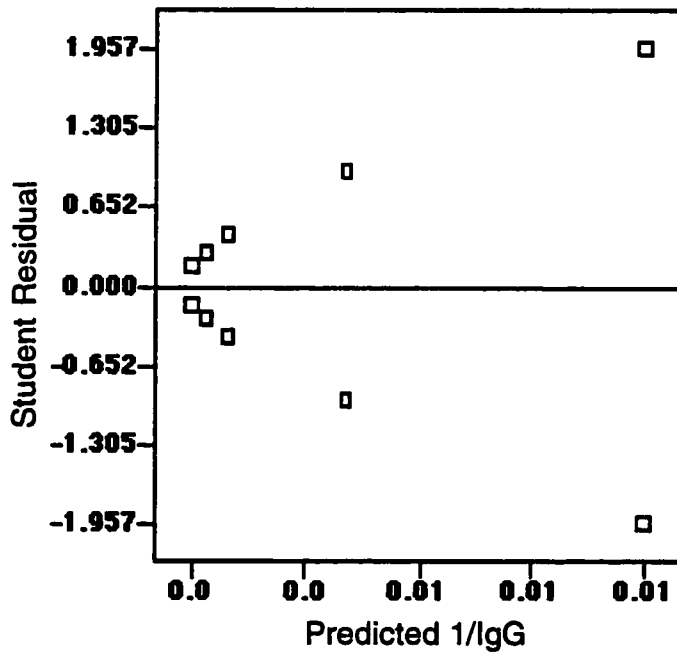
Report D.4: Statistical analysis for the RPE (%F) response with the data not transformed. The RPE response was calculated by measuring the fluorescence peak area of the RPE from the HP1100 analysis of the fractions collected from IMAC.
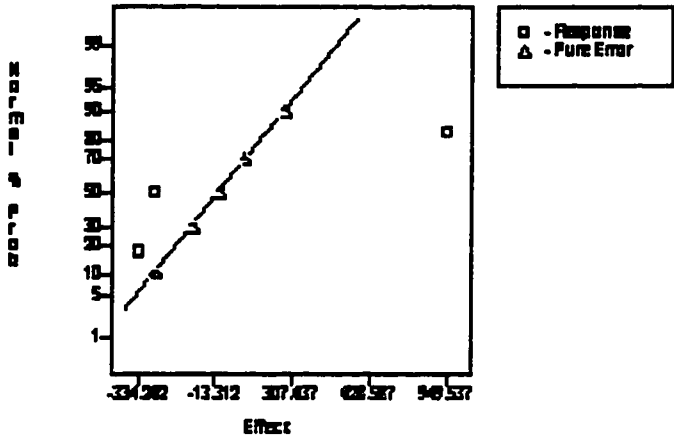
PROB>F = 0.0222
PROB>|t| = 0.0062 for A
PROB>|t| = 0.1709 for B

Effect of Factor A:RPE

Effect of Factor B:Reaction Time

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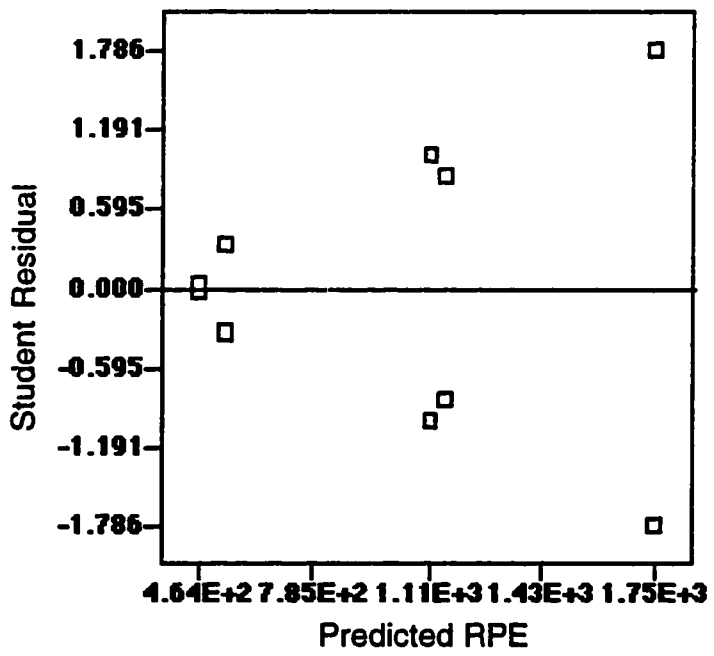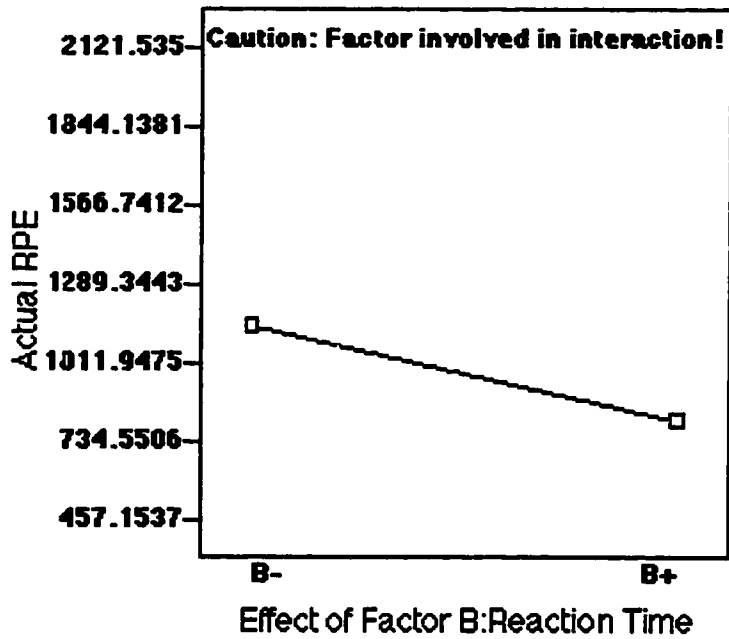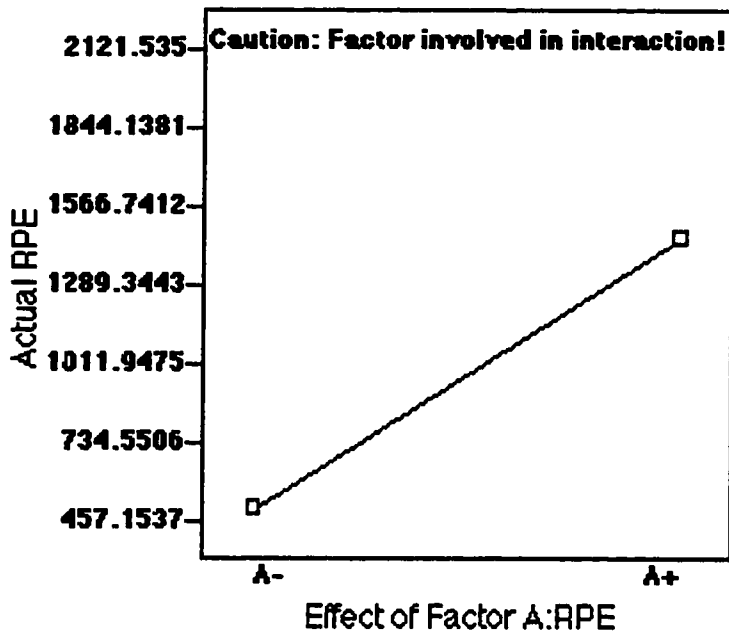
Report D.5: Statistical analysis for the RPE (%F) response with the data transformed by the reciprocal square root function. The RPE response was calculated by measuring the fluorescence peak area of the RPE from the HP1100 analysis of the fractions collected from IMAC.


PROB>F = 0.0002
PROB>ltl = 0.0002 for A

Predicted 1/Sqrt(RPE)
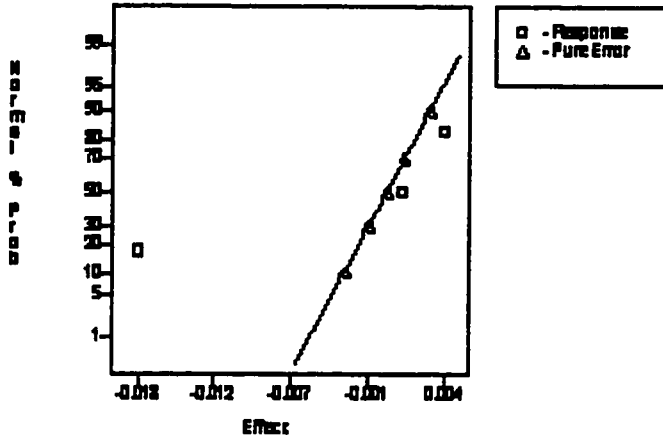
Report D.6: Statistical analysis for the 1:1 Conjugate (mAU) response with the data not transformed. The 1:1 conjugate response was calculated by measuring the absorbance peak area of the 1:1 conjugate from the HP1100 analysis of the fractions collected from IMAC.
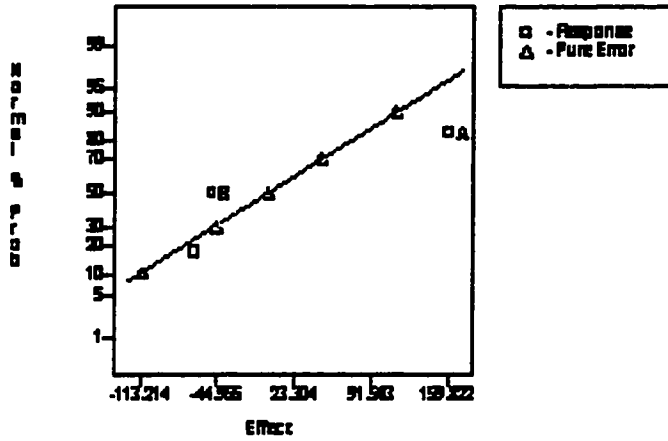
PROB>F = 0.2237
PROB>|t| = 0.1088 for A
PROB>|t| = 0.5815 for B

Effect of Factor A:RPE

Effect of Factor B:Reaction Time

120
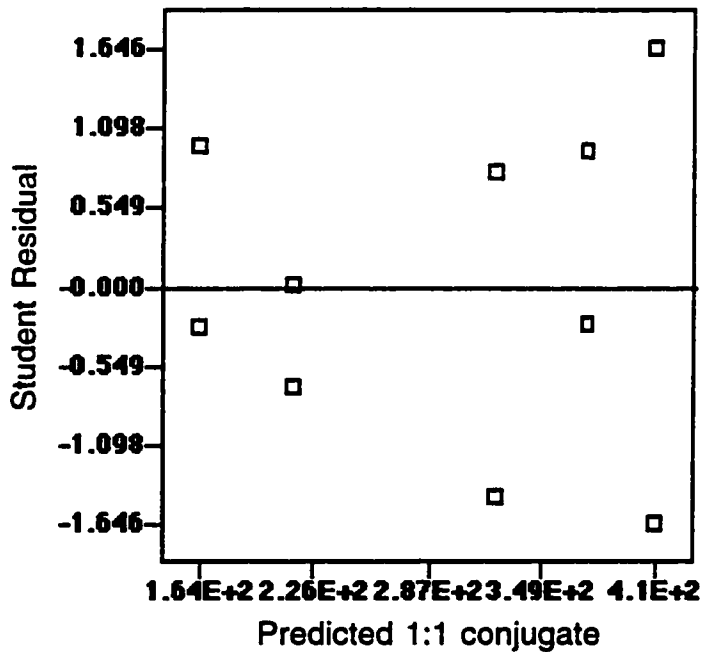
Report D.7: Statistical analysis for the 1:1 conjugate (%F) response with the data not transformed. The 1:1 conjugate response was calculated by measuring the fluorescence peak area of the 1:1 conjugate from the HP1100 analysis of the fractions collected from IMAC.


PROB>F = 0.0522
PROB>ItI = 0.0522 for A

Predicted 1:1 conjugate

Effect of Factor A:RPE

Report D.8: Statistical analysis for the multiples (%F) response with the data not transformed. The multiples response was calculated by measuring the fluorescence peak area of the multiples from the HP1100 analysis of the fractions collected from IMAC.
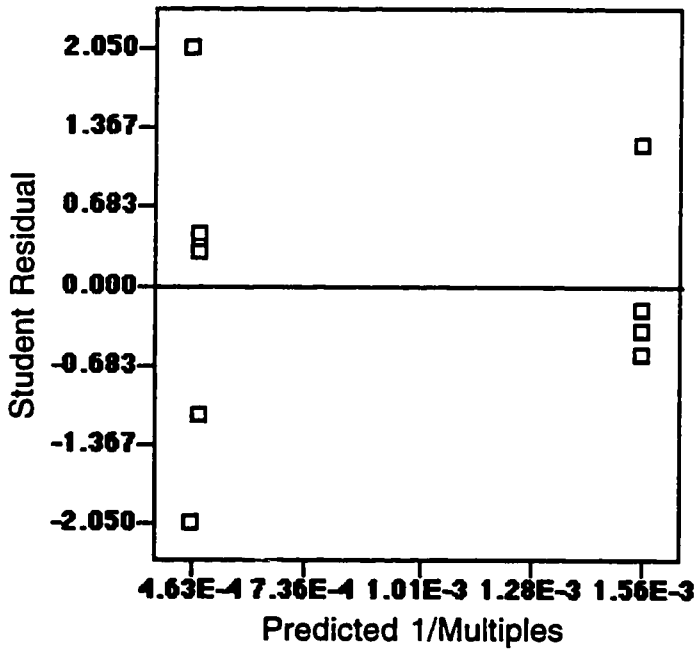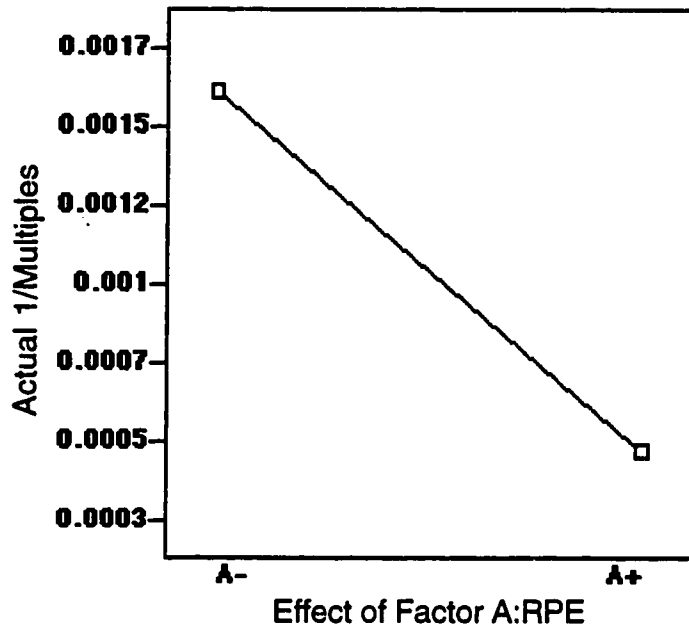

PROB>F = 0.0128
PROB>ItI = 0.0128 for A

Effect of Factor A:RPE
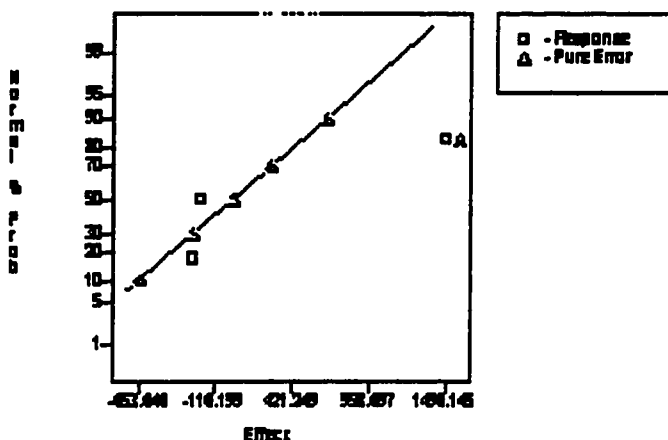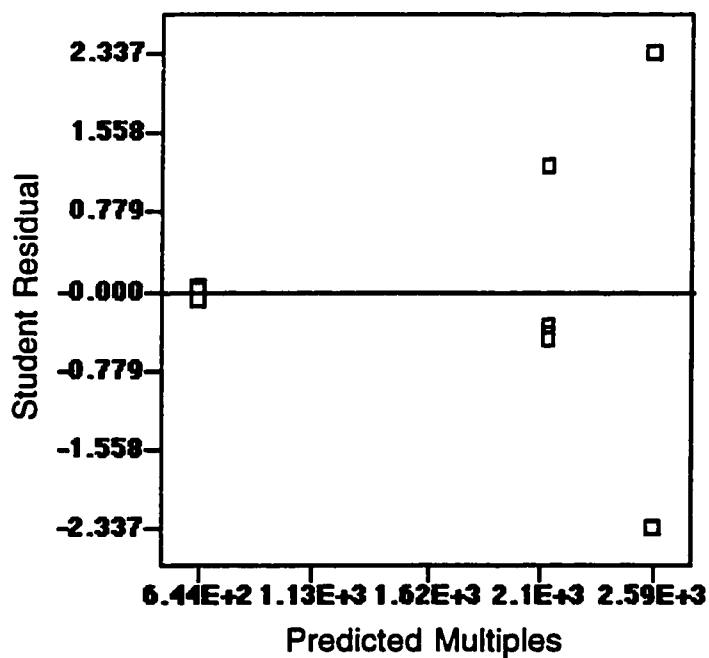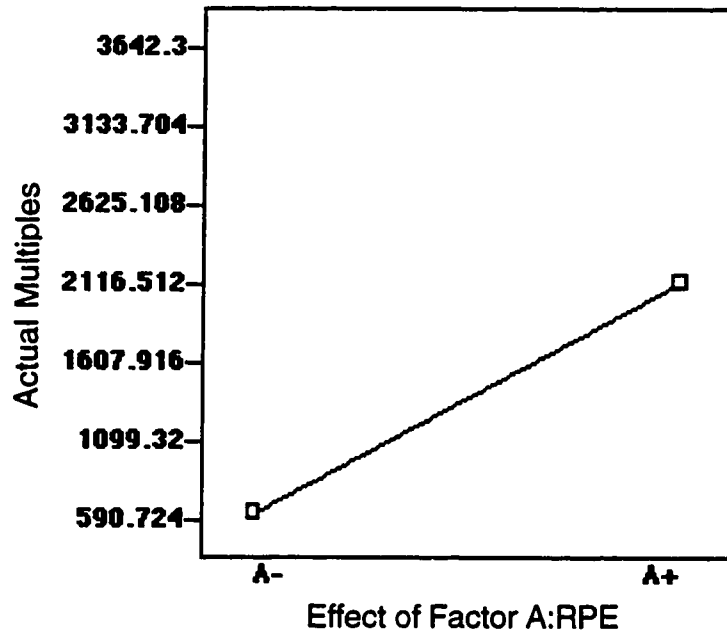
Report D.9: Statistical analysis for the Multiples (%F) response with the data transformed by the inverse function. The multiples response was calculated by measuring the fluorescence peak area of the multiples from the HP1100 analysis of the fractions collected from IMAC.
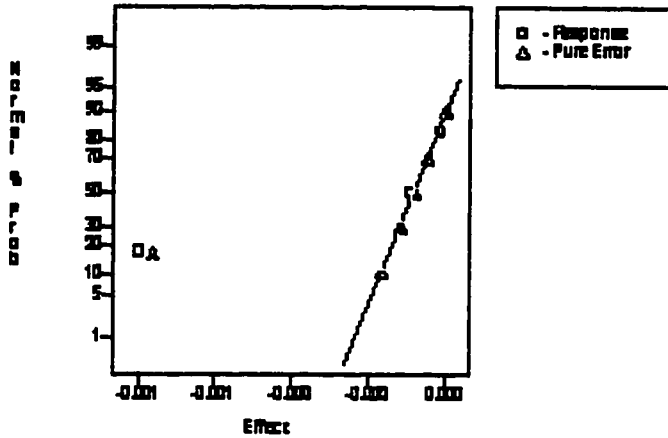
PROB>F = 0.0001
PROB>|t| = 0.0001 for A

Method For Antibody Absorbance Correction

The absorbance retention time for the antibody and the RPE are very close when analyzed by size exclusion chromatography on the HP1100 system. This became a problem when measuring the amount of free antibody acquired from each IMAC run. The fluorometer was used to detect RPE in the fractions since IgG does not fluoresce. However, the spectrophotometer detected both the RPE and antibody by one peak. IgG was the main component in the fractions collected, but the peak measured by the HP1100 was inflated due to the RPE being detected at the same time as the antibody by the spectrophotometer. To correct for the RPE absorbance, the following equation was used:

$$\left(\begin{array}{c} \text{fluorescence} \\ \text{peak area} \\ \text{RPE} \end{array}\right) * \frac{65.90}{875.09} = \left(\begin{array}{c} \text{absorbance} \\ \text{peak area} \\ \text{RPE} \end{array}\right)$$

$$\left(\begin{array}{c} \text{absorbance} \\ \text{peak area} \\ \text{at about 18 min.} \end{array}\right) - \left(\begin{array}{c} \text{absorbance} \\ \text{peak area} \\ \text{RPE} \end{array}\right) = \left(\begin{array}{c} \text{absorbance} \\ \text{peak area} \\ \text{IgG} \end{array}\right) \qquad \textbf{Equation D.1}$$

The ratio used to convert RPE fluorescence to an absorbance value was determined by dividing the peak absorbance area by peak fluorescence area of a known concentration of RPE analyzed on the HP1100 system. The value obtained for absorbance peak area of IgG is thereby corrected by using this method.

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APPENDIX E
FACSCalibur Results of Donor 2
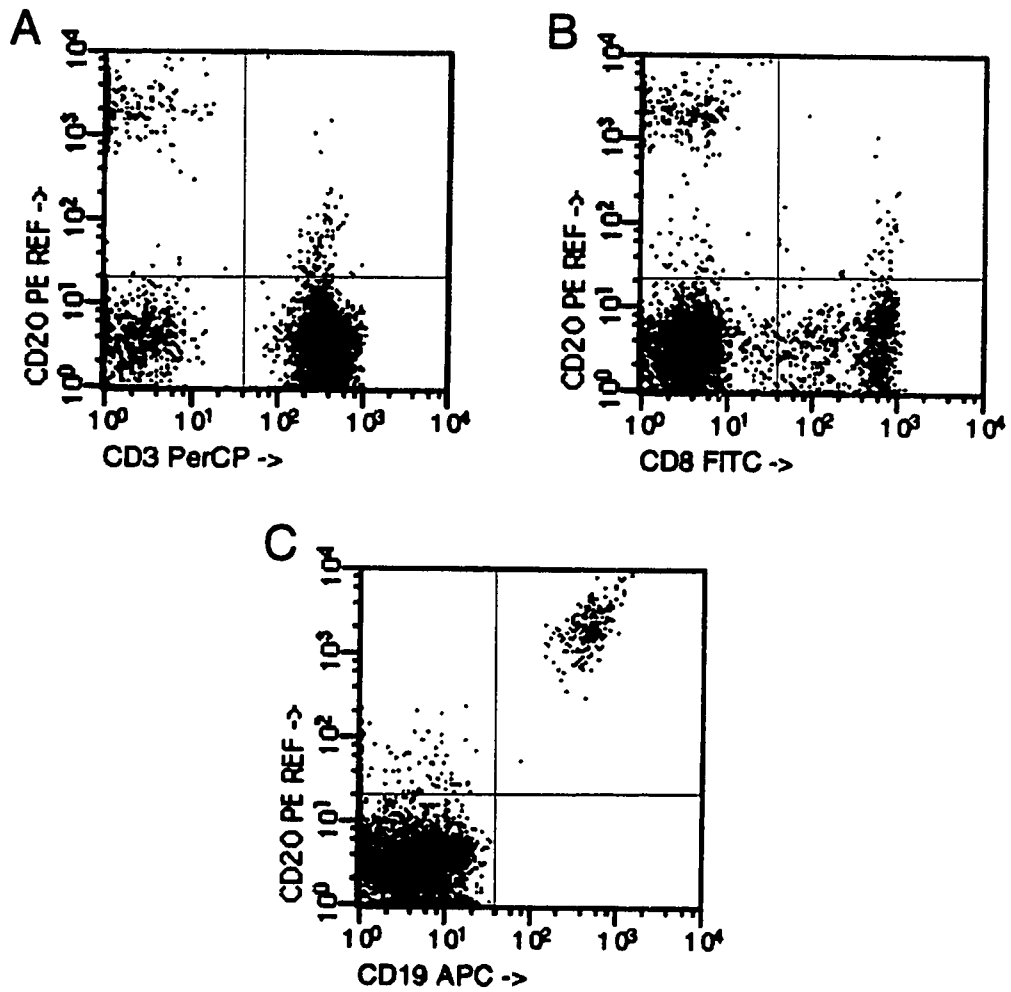
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Figure E.1: Four color immunofluorescence of reference CD20-RPE results enumerating B cells and T cells of donor 2 using a FACSCalibur. a) Dual color analysis using CD20-RPE and CD3-PerCP. b) Dual color analysis using CD20-RPE and CD8-FITC. c) Dual color analysis using CD20-RPE and CD19-APC.
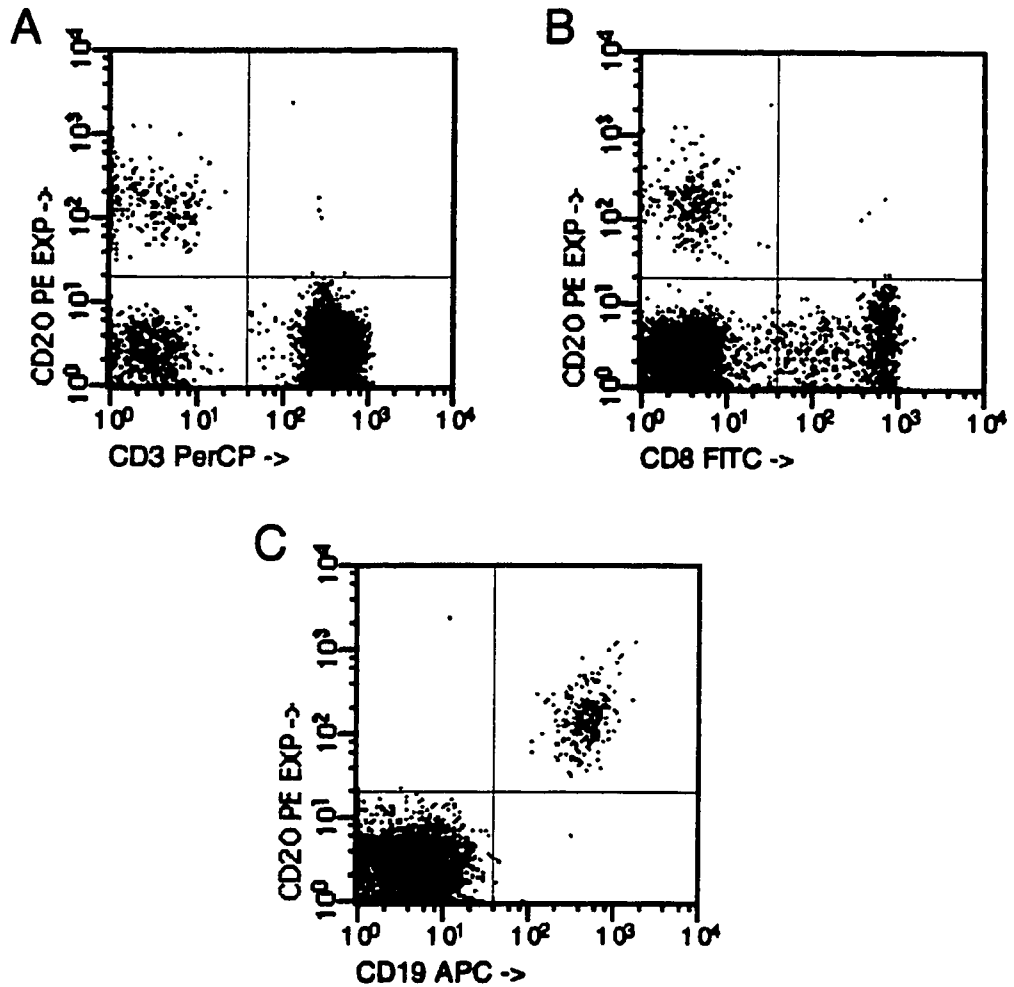
Figure E.2: Four color immunofluorescence of experimental CD20-RPE results enumerating B cells and T cells of donor 2 using a FACSCalibur. Part a) Dual color analysis using CD20-RPE and CD3-PerCP. Part b) Dual color analysis using CD20-RPE and CD8-FITC. c) Dual color analysis using CD20-RPE and CD19-APC.