REPUBLIC OF TURKEY
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THERAPEUTIC MONOCLONAL ANTIBODY (IgG) BINDING PROPERTIES WITH TNFα AND Fcγ RECEPTOR UNDER STRESS CONDITIONS

GÜLİPEK GÜVEN

MASTER THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR

Assoc. Prof. Dr. Özge Can

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DECLARATION

I declare that this thesis study is my own work, that I do not have any unethical behavior in all stages from the planning of the thesis to the writing, that I have obtained all the information in this thesis within the academic and ethical rules, that I refer to all the information and interpretations not obtained by this thesis and that I have listed these resources in the resources list.

Gülipek Güven

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Gülipek Güven

TABLE OF CONTENTS

Page

LIST OF ABBREVIATIONS

CM5	Carboxymethylated dextran
Da	Dalton
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
ELISA	Enzyme-Linked ImmunoSorbent Assay
Fab	Fragment antigen-binding
Fc	Fragment crystallizable region
FC	Flow cell
FcγR	Fc gamma receptor
His	Histidine
IFC	Integrated μ-Fluidic Cartridge
Ig	Immunoglobulin
IgG	Immunoglobulin G
ka	Association rate constant
kd	Dissociation rate constant
KD	Equilibrium dissociation constant
mAb	Monoclonal antibody
MCK	Multi-cycle kinetics
NaOH	Sodium hydroxide
NHS	N-hydroxysuccinimide
PVDF	Polyvinylidene fluoride
Rmax	Maximum response
Ru	Reponse unit
SCK	Single-cycle kineics
SDS	Sodium dodecyl sulfate
SPR	Surface plasmon resonance
t	Time
TNF-α	Tumor necrosis factor alpha

LIST OF FIGURES

Page

LIST OF TABLE

Page

SUMMARY

Understanding of mechanisms of action of monoclonal antibodies in recent years has been the hope for the treatment of many diseases. In addition, with the development of technology every day, the discovery of therapeutics has gained speed and therapeutics market is increasing in the market.

The determination of the binding properties of monoclonal antibodies (mAb) used for therapeutic purposes is of great importance in the field of medical biotechnology. In this study, antibody/antigen and antibody/receptor binding behaviors were examined by surface plasmon resonance technique. Through this technique, binding kinetic analyzes of therapeutic drugs in the in vitro target molecule or receptor were investigated in real time.

This study involves IgG/TNF-α binding on the protein A/G immobilized chip surface and binding of Fcγ receptors (FcγR) to IgG on the chip surface immobilized with anti histidine antibody. Firstly, the methods were optimized and then the Fc region's binding behaviors were investigated by applying chemical deamidation stresses to mAbs (pH 3 and pH 9, 24 hour and 72 hour).

As a result, the ability of the Fc portions of the monoclonal antibodies to bind with the target site decreased at the pH 3 conditions whereas the pH 9 conditions caused a slight change in the Fc binding compared to the stress-free conditions.

In addition, IgG/TNF-α and IgG/FcγRI binding assays, resulting with the KD values of nanomolar levels (E-09) exhibited lower affinity compared to FcγRIIIa experiments which resulted with KD values of (E-06) under the same stress conditions.

Keywords : Monoclonal antibody, Surface plasmon resonance, Biacore, Stress study, FcγR

ÖZET

Terapötik Monoklonal Antikorun (IgG) Stres Koşulları Altında TNF-α'ya ve Fcγ Reseptörüne Bağlanma Özellikleri

Son yıllarda monoklonal antikorların mekanızmalarının anlaşılması bir çok hastalığın tedavisi için umut olmuştur. Ek olarak teknolojinin her geçen gün gelişmesi ile birlikte terapötiklerin keşfi hız kazanmıştır ve piyasada her geçen payı artmaktadır. Terapötik amaçla kullanılan monoklonal antikorların (mAb) bağlanma özelliklerinin belirlenmesi medikal biyoteknoloji alanında büyük önem taşımaktadır. Çalışmada antikor/antijen ve antikor/reseptör bağlanma davranışları yüzey plazmon rezonansı tekniği ile incelenmiştir. Bu teknik sayesinde, in vitro olarak terapötik ilaçların hedef molekül veya reseptör ile bağlanma kinetik analizleri gerçek zamanlı olarak incelenmiştir.

Yapılan çalışma, protein A/G immobilize edilmiş çip yüzeyinde IgG/TNF-α bağlanmasını ve anti histidin antikor ile immobilize edilmiş çip yüzeyinde Fc reseptörlerinin (FcγR) IgG'ye bağlanmasını kapsamaktadır. İlk olarak metodlar optimize edilmiştir, sonrasında mAb'lara kimyasal deamidasyon stresi uygulanarak (pH3 ve pH9, 24 ve 72 saat) bu iki yaklaşım ile Fc bölgesinin bağlanma davranışları incelenmiştir.

Sonuç olarak, monoklonal antikorların Fc kısımlarının hedef bölge ile bağlanma kapasitesi stres uygulanmamış örnekler ile karşılaştırıldığında, pH 3 koşullarında azalma gözlemlenirken pH 9 koşullarında ise hafif bir değişime neden olmuştur. Ek olarak, IgG/TNF-α ve IgG/FcγRI bağlanma deneylerinde, nanomolar (E-09) seviyelerinde KD değerlerinin elde edilmesi ile sonuçlanmışken, aynı stress koşullarında FcγRIIIa , daha düşük afinitede (E-06) KD değerlerinin elde edilmesi ile sonuçlanmıştır.

Anahtar Kelimeler : Monoklonal antikorlar, Yüzey plazmon rezonansı, Biacore, Stres çalışması, FcγR

1. AIM OF STUDY

It is essential to deliver drugs to patients with affordable costs. Therapeutic molecules have been hope in the treatment of important diseases such as rheumatoid arthritis, cancer, cardiovascular and Crohn's diseases. Mostly used theurapeutic molecules in recent years is monoclonal antibodies. Precise analytical characterization of monoclonal antibodies developed as drug in both the process and quality control stage is critical. Physicochemical and functional characterization of the monoclonal antibodies is essential to better understand the structure. Investigation of binding kinetics of monoclonal antibodies is part of this functional characterization.

Fragment antigen-binding (Fab) of antibodies interact with antigen molecules, while Fragment crystallizable region (Fc) are key in activation of cellular pathways. With this activation, various cytokines are released into the environment and a chain of cellular mechanisms occur.

The aim of this study is to investigate the binding properties of anti TNF- α mAb under various stress conditions, such as pH 3 and pH 9. Application of stress studies to the monoclonal antibodies is very important to understand the long term stability of the monoclonal antibodies. Stress conditions can mimic the forced degradation studies to understand the long term stability and the degradation products of the mAb. Degradation can happen both in the Fc and Fab regions of the mAb. In this investigation, we focused on analyzing the change in the binding properties of the Fab and Fc regions of the antibody. The change in the binding characteristics of anti-TNF-α mAB to TNF-α was firstly analyzed by binding Biacore. In addition to this, change in the Fc region binding under stress conditions were observed by binding analysis with FcγRI and FcγRIIIa.

2. INTRODUCTION

2.1 Immune System

The immune system is active in all the body and it has the ability of distinguishing body tissue from foreign tissue or cells and it can remove the dead and faulty cells. There are 2 types of immune responses; one of them is the first line of defense; innate immunity and the second one is the acquired immunity (1).

Many molecular components (eg. complement, cytokines, acute phase proteins) are involved in both innate and acquired immunity. Innate immunity does not necessarily require exposure to a stimulus (eg. antigen) to demonstrate its effectiveness. Thus, innate immunity can produce an immediate response. The acquired immunity requires pre-exposure to an antigen, and therefore takes time to develop after a first encounter with a new foreign molecule. Thanks to the memory cells, the response is faster in subsequent encounters. Acquired immunity in which B and T cells are involved is antigen-specific with the presence of these cells. The acquired immunity is antigen specific, T and B cells are involved (2). In acquired immunity is divided into two. A part of the acquired immunity is cell-mediated immunity. The responses are caused by T cells. The first one is a cell mediated immunity: derived from some T cell responses. The other one is humoral immunity which is derived from B cell responses (B cells secrete a soluble antigen-specific antibody). B and T cells work together to destroy foreign molecules (3). From the natural immune cells, the production and release of cytokines occurs. The production and release of cytokines from innate immune cells. In order for the immune system to function properly, cytokine synthesis and release should be well controlled and sequentially and temporarily regulated (4).

Cytokines are called signal proteins which secreted by cells, are smaller than approximately 80 kDa. These proteins regulate a wide range of biological function with innate and acquired immunity, inflammation and repair, interaction and mostly extracellular signaling. Cytokine is a general classification, which includes lymphokines, monokines, chemokines and interleukin. They exist in broad families that are structurally related but exhibit diverse function (e.g. the Tumor Necrosis

Factor (TNF/TNF) receptor superfamily, interleukin [IL]-1 superfamily and IL-6 superfamily) (5). Cytokine targeting has been found to be effective in therapies that inhibit TNF or IL-6 in most rheumatic diseases (6).

Autoimmune diseases are situations in which an individual's immune system gives an abnormal response to its own cells or tissues. When the immune system is overactive, it attacks and destroys its own tissues. In response to this response, the immune system can produce antibodies against those attacking the body's own tissues (7). The excess levels of Tumor Necrosis Factor – alpha (TNF-α) in the cytokine group have been associated with some autoimmune diseases (8). Considering that elevated levels of TNF-α are detrimental, anti-TNF-α treatments are being developed to block TNF-α activity in patients with autoimmune diseases (9).

2.1.1 Tumor necrosis factor alpha

The first two members of the TNF family are: TNF-α which are known as lymphotoxin-alpha and Tumor Necrosis Factor-beta (TNF-β). TNF-α is a soluble cytokine and is generally active as a homotrimer TNF-α is a non-glycosylated protein with an average molecular weight of 17.5 kDa consisting 157 amino acid resdiues (10). TNFs are a cytokine group which is secreted by macrophages by inflammatory action and may cause cell death of some tumor cell lines. TNF-α is also an important mediator of regulatory processes in autoimmune diseases (11).

Tumor Necrosis Factor-alpha is an effective molecule and has cytotoxic effects on various tumors and other target cells. It is released into the environment by stimulating macrophages, monocytes, neutrophils, T cells and NK cells. Different pathological diseases are related with increased levels of TNF-α production. These are rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), Crohn's disease (CD), ulcerative colitis (UC), cachexia, organ transplant rejections (12).

Tumor Necrosis Factor-alpha has two isoforms after its production. One of them is soluble 17-kDa TNF-α (sTNF-α), the common isoform, and the other is 26-kDa membrane bound form transmembrane (mTNF-α) (13,14). Both of them are involved in the inflammatory response where mTNF-α is a precursor isoform of sTNF-α. Once the expressed TNF has been exposed to the TNF-α converting enzyme (TACE), the

soluble form of TNF-α is separated from the transmembrane TNF-α and mediates biological functions by binding to the Type 1 and 2 TNF receptors (TNF-R1 and -TNF-R2) of distant tissues. Anti-TNF's bind and neutralize the sTNF-α, however shows different effects on mTNF-α producing cells. After binding to TNF receptors, both mTNF-α and sTNF-α mediate many biological functions. After treatment with TACE, mTNF-a interacts with SPPL2b and cytokine production occurs as a result of translocation (Figure 1) (15).

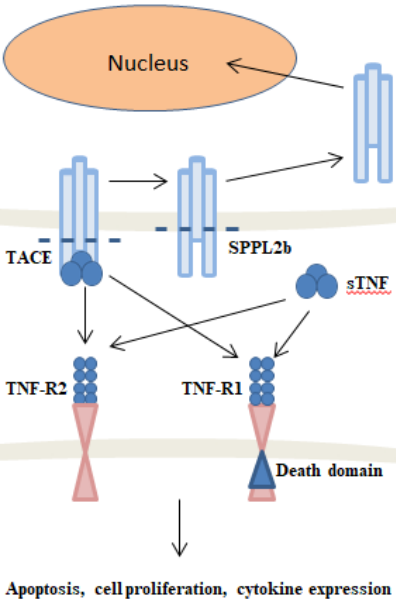


Figure 1. The sTNF-α portion of the expressed TNF-α is separated by TACE and activation of TNF-R1 and TNF-R2 occurs
(Reference : Horiuchi T, Mitoma H, Harashima S, Tsukamoto H, Shimoda T. Transmembrane TNF-alpha: structure, function and interaction with anti-TNF agents. Rheumatology (Oxford). 2010;49(7):1215-28.).

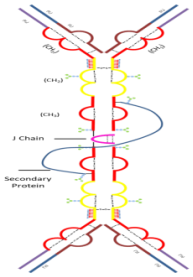
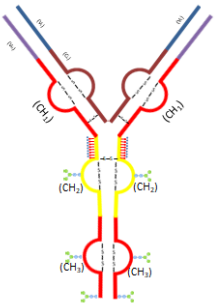
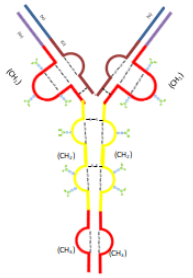
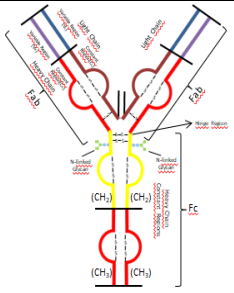
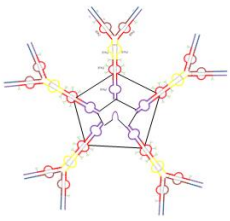
Tumor Necrosis Factor-alpha interacts with TNF-R1 and TNF-R2 receptors and this interaction initiates cellular pathways (15). In many autoimmune diseases, monoclonal antibodies (mAbs) can be used to neutralize the effects of cytokine receptors or cytokines. mAbs are tolerable and specific in the body compared to other therapeutic products (16).

2.1.2 Antibodies

Many body fluids such like tears, respiratory system secretions, salivary, intestinal contents, urine and milk contain antibodies (15). Furthermore, antibodies are again present in high concentrations in the blood serum. Considering, the antibodies are chemically protein structure, they display some features of proteins. For instance, they are described by features such as electrostatic charge and isoelectric point, molecular weight, affinity and their antigenicities. Due to not being familiar with the human body, some substances like infectious agents are called antigens and causes diseases. Antibodies are body's innate defense agents against those kinds of substance, which can find and eliminate them (17).

Antibodies are immunoglobulins (Ig) produced by B-lymphocytes and are part of the immune system. Antibodies are made of glycoprotein which are covalently bonded proteins with sugar residues. These molecules are targeted against a specific antigen and are capable of combining them with the target molecule. They play an important role in the immune system's defense against infections and diseases. According to their stable domain, antibodies are separated into five groups. Each group has an abbreviation of the word immunoglobulin: IgG, IgM, IgA, IgD, and IgE (Table 1) (18-22).

Table 1. Main functions of immunoglobulins and their structures

Ig Class	Function	Structure
IgA	Constitutes 15% of human Igs and is the basic Ig in human secretions. Prevent the microorganisms entering into the organism from binding to the mucosal cells and settle here to form an infection. Two subclasses, IgA1 and IgA2 (18).	
IgD	IgD constitutes 1% of immunoglobulins. Controls lymphocyte activation or suppression. IgD is very sensitive to proteolytic degradation and hence its half-life is short (19). Release in small amounts and is the cell surface receptor for antigen.	
IgE	It mediates hypersensitivity to antigens and takes place in mast activation (20).	
IgG	It facilitates the optimization and bacterial opsonization of bacteria and neutralization of viruses, take part in the complement system. It passes through the placenta (21).	
IgM	IgM is the first antibody produced by mature B cell and released into the blood during the primary response (22).	

IgG is the basic Ig class in the blood and has a four-chain monomer structure. They are produced in excess of the secondary response. In addition to providing complement activation, the tail portion is bound to the specific receptors of macrophages and neutrophils. The phagocytic cells bind to the microorganism surrounded by these receptors and fragments them. IgG is divided into 4 subgroups according to γ-chain sequence (IgG1, IgG2, IgG3, IgG4) (23).

Antibodies are in the subgroup of the protein family which are large group of proteins that conduct to identify and neutralize foreign bodies such as viruses, bacterias and etc. (24). Mono means one in Greek and clone means genetically identical copy. Monoclonal antibodies are identical copies of one type of antibody. Antibodies are divided into two according to their epitopes. The antibody specific to a single epitope is called a monoclonal antibody, when different epitopes are found, it is called polyclonal antibody (25). Epitopes can be defined as the determinant region on the antigen molecule that determines the specificity of the antibody by binding with specific receptors on the antibody. A major milestone in the antibody research was the development of producing monoclonal antibodies via hybridoma technology in 1975 by Köhler and Milstein (26).

2.2 Monoclonal Antibodies

Monoclonal antibodies (mAbs) form a specific site under the group of therapeutic proteins. Antibody molecules include three portions that are similar to Y-shaped, which are connected by a flexible tether (27).

mAbs are group of biotechnological drugs produced by recombinant DNA technology and used in the treatment of many diseases. The therapeutic proteins such as erythropoietins, insulin and mAbs are biotechnological molecules and their demand are increasing day by day all over the world. Nowadays, many of the therapeutic proteins are produced using recombinant DNA technology (28).

mAbs, which have been developed for various target molecules, have recently come to the forefront in the treatment of cancer and many chronic diseases (renal failure, hemophilia and Crohn's disease) with high specificity profiles (29). The antibody consists of two parts. The Fab (fragment of antigen binding) part is made up of light chains (LC) and heavy chains (HC). In the Ig structure, mAbs comprise of two heavy and two light chains, which are bonded to each other with disulfide bonds. The other part is the fragment of crystalisable (Fc) region consisting of only HC (Figure 2). The Fab portion shows variable structure and is the antigen-binding region of the antibody (30).

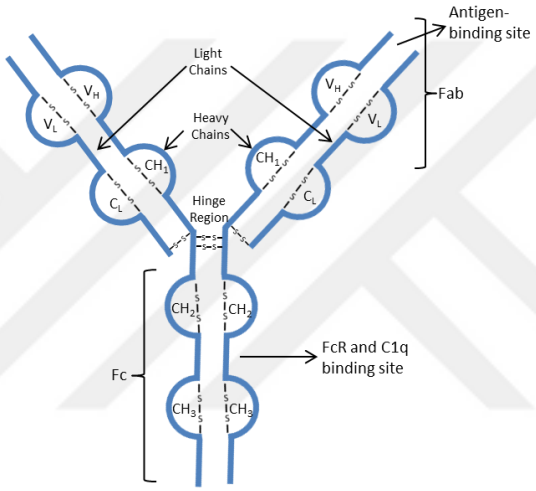


Figure 2. Basic structure of antibody. Top region called Fab and sub region called Fc.
(Reference : Derrick JP, Maiden MC, Feavers IM. Crystal structure of an Fab fragment in complex with a meningococcal serosubtype antigen and a protein G domain. J Mol Biol. 1999;293(1):81-91.)

When the antibody is complexed with the antigen, the binding of the antibody is called paratop, and the binding of the antigen is called the epitope. Antigens contain antigen binding sites, the epitope (Fab), the binding site of the antibodies (Figure 3). The immune system produces a different antibody for each type of epitope. At the other end of the antigen-binding portion of the monoclonal antibody is the Fc region. Various mononuclear cells and leukocytes destroy the antibody cells coated with Fc receptors by carrying out phagocytosis or discharging their lethal granules. Fc region is located in the lower part of “Y”, which interacts with the immune system cells. By performing phagocytosis or discharge of their lethal granules, diverse mononuclear cells and leukocytes demolish the antibody cells coated with Fc receptors (23).

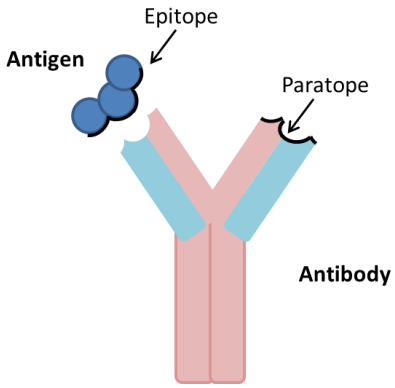


Figure 3. Epitope in the antigen molecule and the paratope fragment present in the antibody.

The other domains are called constant domains, namely C_L, CH_1, CH_2, CH_3. To stimulate inflammation, mAbs in the IgG structure can also activate the classical complement cascade (31). Both light and heavy chains include one C "Constant" and one V "Variable" subunit. The V subunit is responsible for the recognition of the antigen. A whole IgG gene is composed by the union of V and C gene fragments. Each variable region of the heavy and light chains carries three multivariate regions or regions which are known as complementarity determining region (CDR) (32).

Antibodies play a key role in various pathways. Through these mechanisms, it can block ligand and receptor interaction, interact with various Fc receptors on the surface of the effector cell to activate antibody-dependent cellular cytotoxicity by activation of complement-dependent cytotoxicity (CDC), and finally perform opsonization and phagocytosis activity (33).

2.2.1 Monoclonal antibody market

The size of the global mAb market will increase by a compound annual growth rate (CAGR) of approximately 15% between 2017-2022 (Figure 4). The fact that the sector of mAbs has increasingly gained itself in the pharmaceutical market is associated with increased demand for antibodies to treat various diseases and the introduction of biosimilar antibodies. The global sale of monoclonal antibody drugs is about half of all biological drugs, which is $ 75 billion, based on the data of 2013. The total number of approved mAb drugs has reached to 44 today. This number will increase day by day and it is expected to be over 70 in 2020 (34).

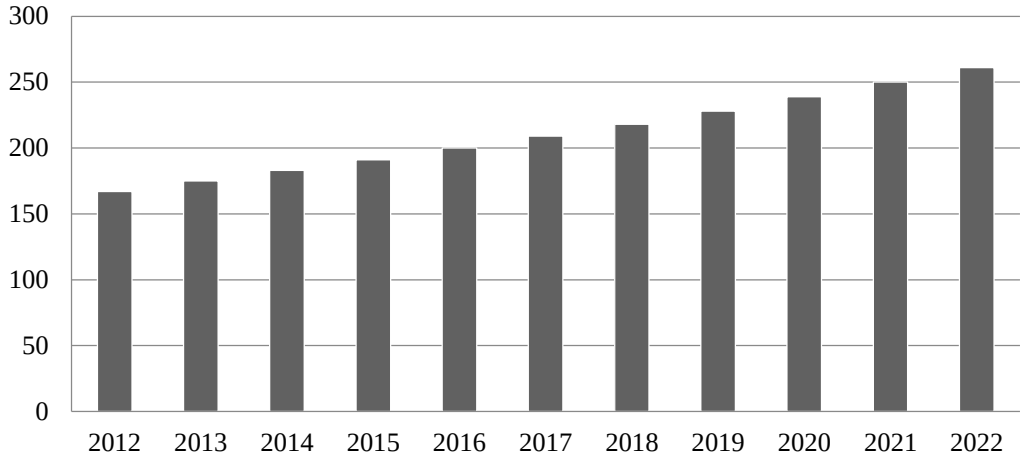


Figure 4. In the table, the x-axis represents years, while the y-axis refers to the global biologic revenue in $ bn. (2012-2022)
(Reference : Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. MAbs. 2015;7(1):9-14.)

2.2.2 Production of mAbs

Upstream process (USP) refers to all the activities needed to gather the materials required in order to create a desired product. The part of USP needs to the initial stage in which cells are grow, e.g. bacterial or mammalian cell lines in bioreactors, Hybridoma technology has emerged to produce monoclonal antibodies used in the treatment of autoimmune diseases led by Kohler and Milstein (Figure 5). With the development of recombinant DNA technology in 1957, Theodore T. Puck developed a Chinese hamster ovary (CHO) cell line (35).

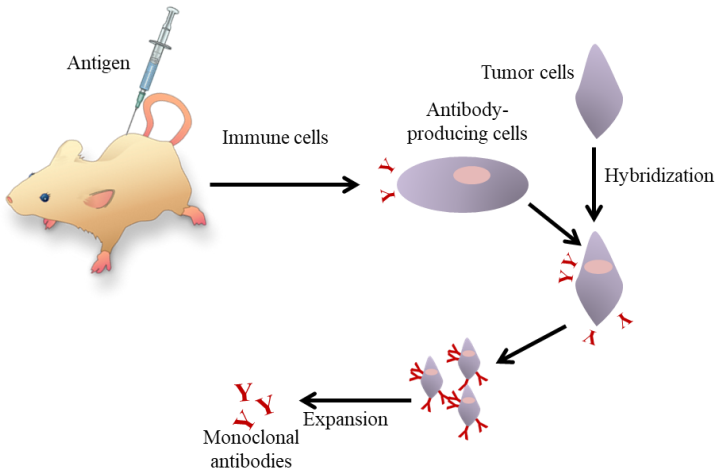


Figure 5. Hybridoma technology allows the fusion of tumor cells with antigen-producing cells. Thus. an unlimited production of antibodies is obtained.

The production of mAbs by the recombinant DNA technology with CHO cell line overcomes hybridoma technology with the advantages of high yield protein production, rapid growth, low cost and safety. High post-translational processing and high productivity is achieved. Also, with the high specific productivity and the right characteristics can be achieved with these cell lines. The other reason for the preferred CHO cells is that it can be stable for long time with the recombinant technologies. USP involves two main steps: media preparation and cell culture with using bioreactors. When the cells reached the desired density (titer), downstream process (DSP) is started (36).

DSP is mainly focused on the purification and recovery of the antibody from coming cell culture. Although, every mAb for DSP represents specific purification or recovery process. Most mAb DSP consists of the same unit operations (37). The traditional purification process is harvesting, followed by main chromatography purification (capture) step with virus inactivation, polishing steps, virus filtration and ultrafiltration or diafiltration, respectively (38). The aim of harvest step is to remove the culture medium and cells from mammalian cell culture. Centrifugation or depth filtrations are used for harvest step.

Protein A chromatography which is the type of affinity system is used for the capture step. The affinity system for mAbs purification in Protein A chromatography is occurred between Protein A ligand and IgG. Following the capture step, some polishing steps can be added to remove contaminants from the solution. These polishing steps can be CEX, AEX or HIC, etc (39).

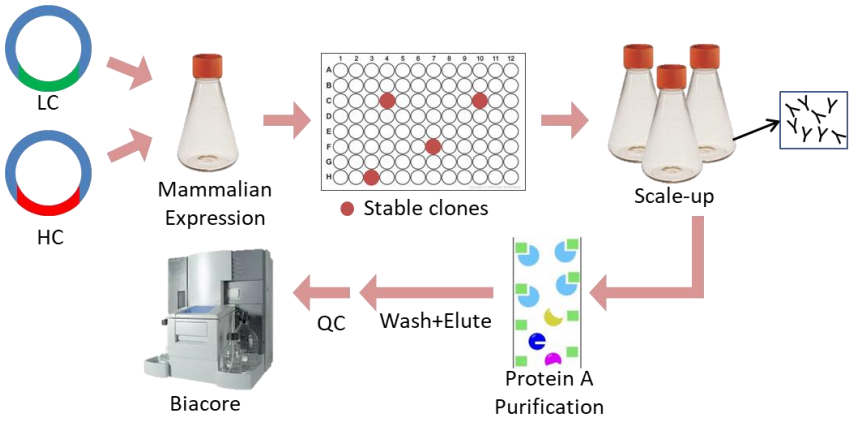


Figure 6. Process development of an antibody and quality control of biological activity

2.2.3 Analytical tools for characterizing biopharmaceuticals

To ensure safety and efficacy of therapeutic proteins, an in-depth physicochemical characterization is required in every stages of process development. Physicochemical properties comprises of intact and reduced molecular mass measurement, primary structure determination, assessment of post-translational modification (PTM), glycopeptide and glycosylation analysis, disulphide bond analysis, higher order structure (HOS) analysis, size and charge-variant analysis. State-of-the-art analytical instruments have been used to monitor the quality of protein from various perspectives. To achieve these, ultra-performance liquid chromatography (UPLC) coupled to quadrupole/time-of-flight mass spectrometer (QToF-MS), capillary electrophoresis, UPLC-based size-exclusion chromatography, circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimeter (DSC) and many other analytical systems have been used (40).

2.2.4 Functional characterization of a mAb

The interaction between the antibody and the antigen is very important for the function of mAb. mAbs can trigger activation or inhibition of a number of cascades by interacting with receptors present in the cell membrane or with soluble cytokines. Due to the different amino acid combinations in the CDR region of the mAbs they can bind to many different antigens. In addition, the Fc portion is important for binding to receptors located on the cell surface so that mAb can function at the cellular and metabolic level. Thanks to Fab and Fc parts, mAb can activate activation of complement and other effector mechanisms. The correlation between cytotoxic effects such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) with the mAb should be demonstrated. In order to understand the properties of mAb at the molecular level and to establish a relationship between its mechanism of action, the mAb must be characterized (41).

In vitro methods such as surface plasmon systems (SPR), Enzyme-Linked Immunobehavior Assay (ELISA) and Kinetic exclusion assay (KinExa) can detect the affinity and KD of the antibody-antigen complex. Potency assays can be carried

out also with an *in vitro* - cell-based bioassays (Figure 7). *In vivo* assays allow the understanding of the mechanism of action of mAbs. These experiments are carried out with molecules specific cell lines. For example, the U937 cell line is used to evaluate the cellular response of TNF and mAbs. Here, a pathway caspase pathway can be used as a marker of TNF-mediated inflammation (42).

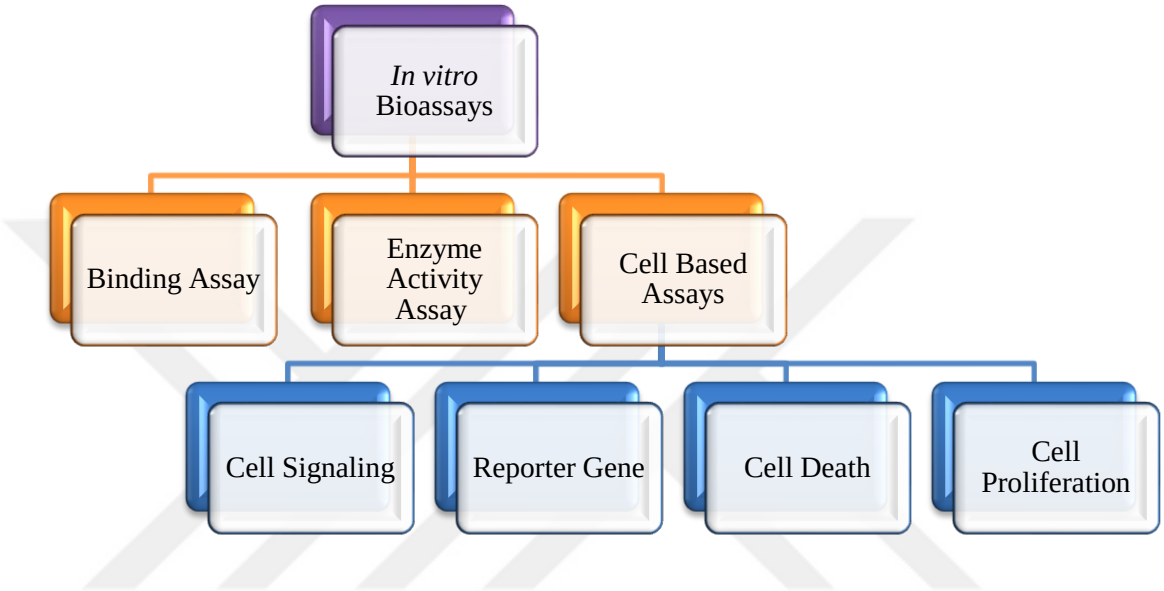


Figure 7. Functional characterization can be performed by in vitro experiments.

2.2.5 Fc receptor family

Fc receptors (FcR) and interactions with immunoglobulin are obvious based on the literature (43). After combining the Fab portion of the antibody with the antigen, they can regulate the activation of the immune system by entering the interaction of the Fc portion and the Fc receptors (Figure 8) (44). These receptors recognize antibodies from the Fc portion as its name suggests. FcR binds to antibodies present in infected cells or pathogens, thereby activating the immune system.

These receptors are glycoproteins and serve as a bridge between natural and adaptive immune systems. Fc receptor is found in the membrane of some immune cells, such as B lymphocytes, NK cells, macrophages, neutrophils. While stimulation of FcRs alone does not primarily produce cytokine production, it interacts with other receptors and leads to activation or inhibition of cytokines by cell and receptor type.

These receptors also play a role in antigen presentation, maturation of dendritic cells and B cell activation. Therefore, Fcs plays a role in the regulation of the natural and adaptive immune response for the development of novel immunological therapeutic approaches (45).

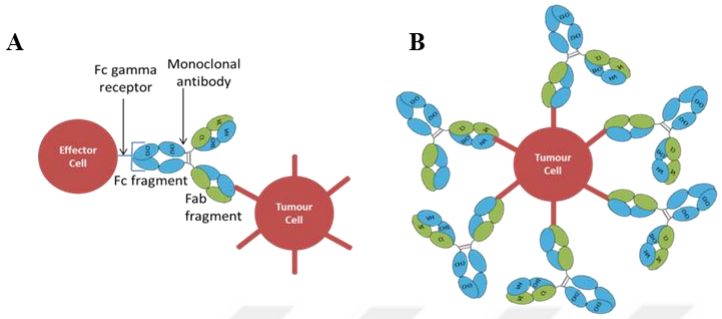


Figure 8. A) Binding of Fc region of the antibody with the Fc receptor on the cell surface. B) The surface of the cells occupies antibodies and opsonization occurs.
(Reference :Rath T, Baker K, Pyzik M, Blumberg RS. Regulation of immune responses by the neonatal fc receptor and its therapeutic implications. Front Immunol. 2014;5:664.)

Fc receptors are divided into groups and subgroups. All Fc receptors are expressed from the same region of the long arm of the chromosome 1 (46). The first classification was made according to Ig class; Fc receptors are divided into groups and subgroups. The first classification was made according to Ig class; FcγR (IgG), FcεRI (IgE), FcαRI (IgA), FcμR (IgM) and FcδR (IgD) (Table 2) (47).

Table 2. Ig groups and Fc groups interacting with them are shown in the table

Ig	Class	Sub-class
IgG	FcγR	CD64 (FcγRI)
		CD32 (FcγRIIa, FcγRIIb, FcγRIIc)
		CD16 (FcγRIIIa, FcγRIIIb)
	FcRn	N/A
IgE	FcεRI	N/A
IgA	FcαRI	
IgM	FcμR	
IgD	FcδR	

Between the Fc receptor subgroups, there are differences between structural, functional, glycosylation patterns and their affinity to IgG (48). Among the FcγR receptors, FcγRI has high affinity, while FcγRII and FcγRIII have low-to-medium affinity (Figure 9). An example of this is the high affinity Fc receptors with dissociation constants ranging from FcγRI and FcεRI, 10^{-8} to 10^{-10} M respectively (49).

FcRs that are part of the immunoglobulin superfamily are of type I transmembrane glycoprotein. Only glycosylphosphatidylinisotol (GPI) anchored FcγRIIIb is not in this structure. Low affinity receptors form two immunoglobulinic domains D1 and D2 located outside the cell. The FcγRI which has a high affinity, also has an area of D3, which is thought to have gained it (50).

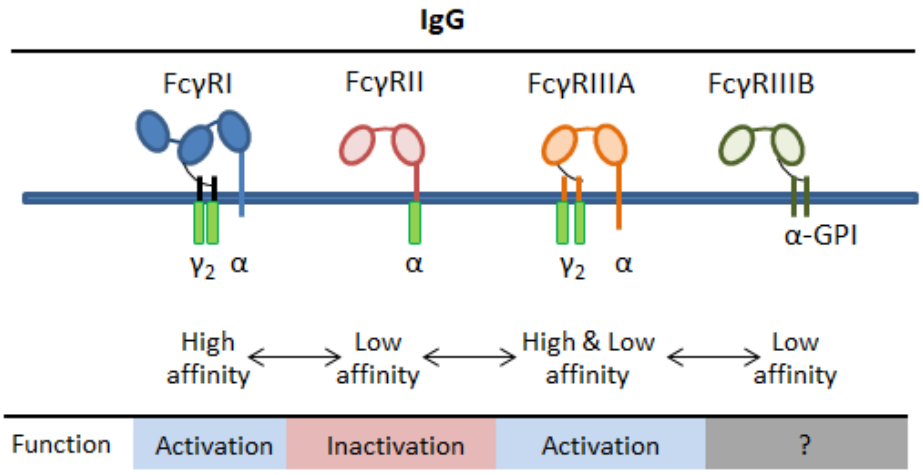


Figure 9. The subgroups, conformation, affinity and primary effects of FcγR that can interact with IgG are shown in the figure.
(References: Hayes JM, Wormald MR, Rudd PM, Davey GP. Fc gamma receptors: glycobiology and therapeutic prospects. J Inflamm Res. 2016;9:209-19.
Lu J, Ellsworth JL, Hamacher N, Oak SW, Sun PD. Crystal structure of Fcgamma receptor I and its implication in high affinity gamma-immunoglobulin binding. J Biol Chem. 2011;286(47):40608-13.)

FcγRI, FcγRIIa, and FcγRIII activate specific receptors and positive excitation occurs in the cells. In contrast, FcγRIIb differs from other receptors and is referred to as an inhibitor receptor. Activator and inhibitor receptors have different phosphorylation motifs. The activation and inhibition ability of the receptors is due to the areas under the membrane. If the receptor has an immunoreceptor tyrosine-

based activation motif (ITAM) in the sub-membrane region, the receptor has the ability to activate signal pathway. Conversely, if the receptor has the immunoreceptor tyrosine-based inhibition motif (ITIM), receptor can inhibit the signal pathway. All these receptors have different phosphorylation patterns (51). FcγRs with activation features; phagocytosis can induce cytokine production and ADCC. FcγRI and FcγRIIa are expressed in myeloid cells such as monocytes, macrophages and dendritic cells. FcγRIIIa is primarily expressed in natural killer (NK) cells, dendritic cells (DCs). FcγRIIIb is not expressed in NK cells. Finally, FcγRIIIb is only found in neutrophils (52).

2.2.5.1 FcγRI

FcγRI (Cluster of Differentiation 64) expression is mainly carried out by mononuclear phagocytes. The extracellular domain of FcγRI contains the Ig domain which allows the antibody to bind with the 1: 1 stoichiometry with the Fc region. In contrast to the determination of the role of FcγRIII in the immune system, the role of FcγRI in the immune system remains unclear (52).

2.2.5.2 FcγRIII

The FcγRIII (Cluster of Differentiation 16) is coded as FcγRIIIa and FcγRIIIb by 2 genes very similar to each other and isoforms of each other. FcγRIIIa is an integral membrane glycoprotein anchored through a transmembrane peptide found on NK cells and initiates a strong signal sequence when stimulated. FcγRIIIb is found in neutrophils. The areas of the receptors outside the membrane are very similar. However, the glycosylphosphatidylinositol (GPI) linkage found in FcγRIIIb reveals the difference. FcγRIIIa has a broader mechanism of action and serves as a facilitator for ADCC (52).

FcγRIIIa is expressed by NK cells. There are two variants of position 158 of FcγRIIIa, V158 and F158. The V158 variant has a higher affinity for IgG1 than F158. Therefore, the V158 variant provides a more effective and high response to therapeutic antibodies (53).

The receptors used for research are usually derived from recombinant cells. Human recombinant FcγRI is obtained from HEK293 cell line and FcγRIIIa is

obtained from E. Coli. Both receptors are combined with a histidine sequence of about 37 amino acids and regains its recombinant protein form after various purification (54).

2.2.6 Antibody-dependent cellular cytotoxicity (ADCC)

ADCC is an immunomodulatory mechanism that allows antibody-mediated lysis and destruction of an infected cell with an Fc receptor-carrying active effector cell (NK cells). In ADCC, FcγR receptors bind to the Fc region of IgG, followed by secretion of various substances such as enzymes with lytic action, granzymes and TNF to destroy the target cell. Thus, various signaling pathways are activated. Various glycosylation patterns occur in the Fc portion of IgG. These patterns induce Fc-mediated activity pathways. IgG-mediated ADCC also includes three Fc receptors (FcyRI, FCγRII and FcyRIIIa). However, FcyRIIIa is often regarded as the main receptor because it is expressed by NK cells (55).

The ADCC mechanism consists of 3 steps:

1. Binding of effector cells containing Fc receptor and infected cells
2. Phosphorylation of the ITAM region under the membrane of the effector cell
3. Induction of the signaling pathway in the effector cell, release of cytotoxic molecules and killing of the target cell (56).

2.3 Surface Plasmon Resonance (SPR)

There are various techniques for screening and measuring protein-ligand interactions. These methods include affinity chromatography, cross-linking, spectroscopic methods, three dimensional conformation detection, surface plasmon resonance, microarray, immunoblotting and ELISA (57). Surface plasmon resonance biosensors have become increasingly popular in the last decade.

In the early 1900s. R.M. Wood has done the first studies on SPR. SPR is a direct measurement technique by measuring the change in refractive index near the metal surface. SPR is an opto-electronic biosensor, based on the light source, prism, sensor chip-gold film, and detector. In SPR application, a thin metal (gold) film is used between two media with permeable and different refractive index. When a beam of

light is emitted in the prism, the gold surface encounters the interface of the film and the solution, and total internal reflection (TIR) occurs. When the incoming light is emitted in an environment with a higher refractive index in a medium having a lower refractive index, the incoming light beam tends to reflect as opposed to refract (58). The light beam with a certain angle of refraction causes the free electrons on the metal surface to form surface plasmons. This phenomenon is called surface plasmon resonance. The SPR angle changes depending on the refractive index of the surface and solution. This angle changes with the mass and density of molecules interfering to the surface of the gold surface. For this reason, angle changes give information about on the surface (59). Plasma waves are recorded with a refractive index of the environment and a shift with the reflected angle of the polarized light. Even 0.0001 degree of gliding in the SPR angle can be detected by the system. The resonance angle can be varied by modifying the surface with various molecules. This allows specific selectivity by modifying the surface relative to the target molecule (60).

The advantage of SPR is that it allows real time study of biomolecular interactions non-invasive conditions and without the need for a marking step. SPR experiments can give information about specificity of an interaction, binding affinity and their levels, association/dissociation rate constants of complexes (61).

2.3.1 Kinetic calculations

The reaction kinetics should be considered in order to evaluate the strength and degree of antigen and antibody interaction in a reaction. This allows an examination of how fast the molecules bind and separated during a cycle and determine the change of the molecule complex over a given time period (62).

2.3.1.1 1:1 Binding kinetics

SPR is used to observe real-time biomolecular interactions between the ligand and the analyte. To determine the kinetic constants of the interaction of an analyte and ligand with SPR, several models can be used, but the most commonly used is the 1:1 and two state binding model. Complex formation of A and B is called "association " and its and separation of this complex is called “dissociation”. k_a is the association and the k_d dissociation rate constant. This complex can be reversible. In

this equation, *A* refers to the first injected and captured molecule of the ligand on the surface of the sensor chip where *B* shows the free analyte in the solution injected to the chip surface after ligand (63).

$$A + B \rightleftharpoons \mathrm{AB}$$

The determination of these kinetic constants is important while detecting mutations on amino acid residues and post-translational -modifications (PTMs) on the IgG or analyze binding to drug targets (64).

During the kinetic evaluation process, the empirical data are processed according to the 1:1 Langmuir interaction model between the analyte and the ligand which is then used to determine the association and dissociation constants. Values for affinity constants are obtained in kinetic measurements, affinity helps find out how powerful the complex of molecules is. When the chip surface reaches a stable baseline in conditioning steps, the analyte is sent to the surface. The associaion and dissociation phases are monitored and the obtained graph is named as a sensorgram. The equilibrium binding constants (KD) are calculated by the concentrations of the analyte sent to the surface. Equilibrium is achieved when ligand binding to analyte and dissociation occur at same rates:

$$K_D = \frac{[A].[B]}{[AB]} = \frac{kd}{ka} = \frac{k-1}{k}$$

KD is abbreviated as the equilibrium dissociation constant. [AB] is the surface concentration of the ligand and analyte formed. [A] refers to the surface concentration of the ligand and the concentration of [B] analyte. The equilibrium dissociation constant describes the non-covalent interaction between the ligand and the analyte. The low KD value indicates the high binding affinity of the ligand and analyte. However, the high KD values represent a weaker interaction between the ligand and the analyte. There is an inverse relationship between KD and affinity (Figure 10) (62).

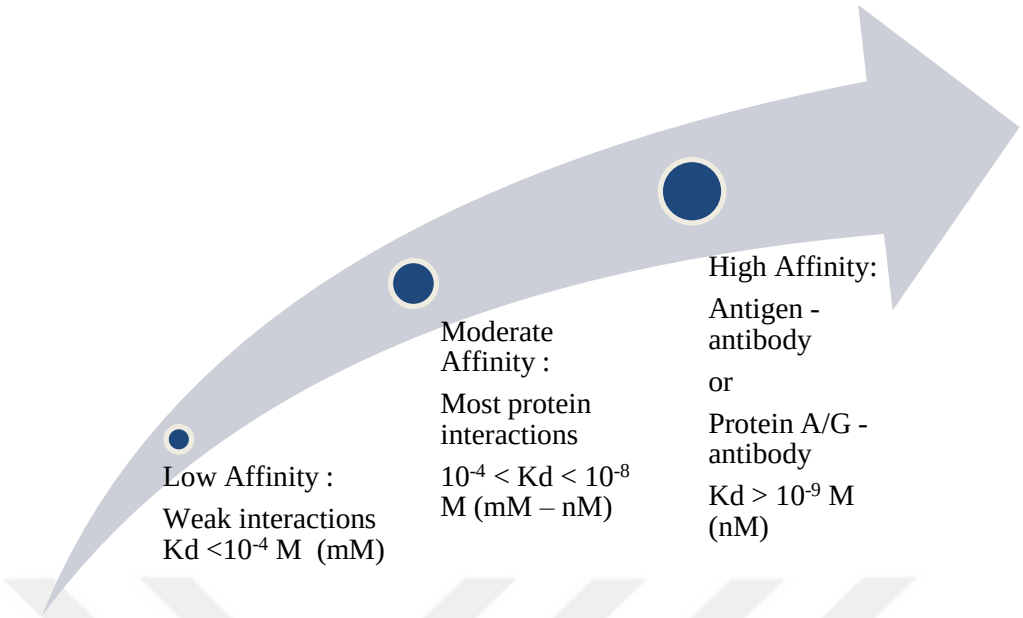


Figure 10. The kd value ranges representing low to high affinity are shown in the figure. (Reference:Lago S, Nadai M, Rossetto M, Richter SN. Surface Plasmon Resonance kinetic analysis of the interaction between G-quadruplex nucleic acids and an anti-G-quadruplex monoclonal antibody. Biochim Biophys Acta Gen Subj. 2018;1862(6):1276-82.)

The U-value and Chi^2 are also important parameters while interpretating the results (63). Chi^2 value is the degree of deviation of the experimental data from the average curve. Low Chi^2 values indicate that the experiment is more successful and appropriate. Under normal conditions, the value of Chi^2 is close to the square of the short-term noise level. On the other hand, U value is a distinctive value for kinetic rate constants. A lower U value means that the results are more reliable (65).

The binding capacity of the analyte and ligand varies according to the immobilization level of the immobilized molecule. The maximum response term (Rmax) defines the binding capacity of the surface. Rmax is the maximum level of binding at which all ligands match the analyte during a cycle. Rmax level may vary according to the experiment. Rmax level is determined according to physical and chemical structure of ligand and analyte. In addition, different Rmax levels are recommended for different type of applications. For kinetic assays, the Rmax level may be desired to be lower, while a higher Rmax level may be required in concentration assay analyzes (66). The Rmax level is calculated by the following formula:

$$Rmax = \frac{analyte\ MW}{ligand\ MW} \times immobilized\ amount\ stoichiometric\ ratio$$

2.3.1.2 Two state binding

Some interactions do not match the 1: 1 binding model. Other binding models may be preferred due to complex interaction status, ligand immobilization or poor experimental conditions. Antibodies other than a stable protein may also be immobilized to the chip surface. These antibodies are not stable because they are not globular.

The two-state kinetic model is a model that allows the analysis of the kinetics at the time of conformational change of the AB structure formed on the sensor chip.

$$A + B \underset{kd_1}{\overset{ka_1}{\rightleftharpoons}} AB \underset{kd_2}{\overset{ka_2}{\rightleftharpoons}} AB^*$$

Two state binding kinetic model equation (Conformational changes)*

This kinetic model defines a conformational change in which a 1: 1 binding of the molecule immobilized to the chip surface will interact with the analyte and then stabilizes the resulting complex. The response of the resulting conformational change is not as mass-dependent as 1:1 binding, it is an effective state. Conformational changes after binding may occur as a result of various receptor-hormone and antibody-antigen interactions (67,68)

Numerical and graphical options are available for the evaluation of the data. Both sensorgrams and mathematical calculations of experimental data are important for the evaluation of results. Thus, differences between experimental and calculated data can be more easily observed. The sensorgrams provides good data with good overlaps. There may be slight differences when the curves are brought to the appropriate position of the sensor, but they are of a size that can be ignored compared to the general. The acceptability of the sensor should be evaluated for testing purposes. Poorly matched sensorgram curves can be clearly identified. If the selected model is not suitable for the purpose of the experiment, both the sensorgram

and the resulting mathematical data do not provide reliable kinetic constants and mathematical data.

2.3.2 Immobilization

The selection of the chip is dependent upon the ligand and its interaction to the molecule to be immobilized, the ligand and the analyte to be studied as well as the purpose of the assay (kinetics, affinity or detection of the analyte concentration). With the Biacore system, which is a product of GE Healthcare, the protein-protein interactions can be examined precisely by the SPR, the Biacore system has many different sensor chips available to users.

Immobilization provides substantial consistency and reproducibility to provide stable protein biosensors. Commonly used solid surfaces include various nanostructured materials such as gold, silicon, glass, plastic and newly developed nanoparticles, nanowires and nanotubes (69). The selective immobilization of proteins as a biorecognition process is an important step in the development of biosensors. Such immobilizations are critical for precise measurements. Immobilization of proteins is important for characterization as well as for detecting biomolecular interactions (70). The immobilization technique has many advantages. It is possible that the immobilized protein can be used again and more than one experiment can be performed with the same protein. The binding of the proteins to the selected suitable surface gives a more stable result and reduces the likelihood of denaturing. This technique minimizes the amount of material spent and saves time and material. Various immobilization methods are available and these include amine binding, thiol coupling, aldehyde binding, high affinity capture and high affinity capture of the biotinylated ligand on immobilized streptavidin.

The capture of the IgG monoclonal antibody on the chip can be accomplished by protein A /G immobilization (Figure 11). Immobilization can be made easily and conveniently with amine capture method. The immobilization of protein A/G and anti histidine antibody was performed on a solid gold glass support and carboxymethyl dextran (Sensor Chip CM5) (71,72).

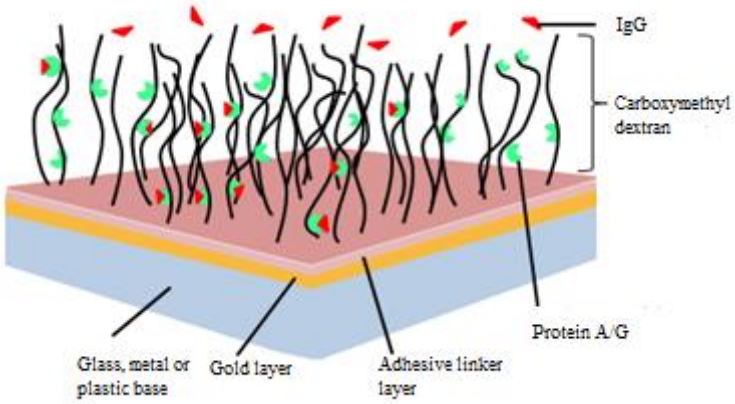


Figure 11. A close-up view of the CM5 chip with a carboxymethyl dextran surface.

Protein A and Protein G which located in the bacterial cell wall (*Streptococcal* strains), have binding sites that recognize high affinity for the Fc region of human and mammalian IgG. In the past, protein A/G was obtained from *Streptococcus* strains (Spa and Spg) (73-75). After further investigation, protein production was started in other host cells (E.coli). The protein used is a fusion protein obtained by fusion of Fc regions of Protein A and G and is of chimeric structure and commercially available. Thus, it has a greater binding capacity than the singular form of both proteins and is less dependent on pH (76). Fused protein A/G is frequently used in the investigation, immobilization and purification of immunoglobulins. Therefore, this chimeric protein was preferred for immobilization. In addition, the affinity of protein A/G to IgG1 is very strong, the kd values of Protein A and G are respectively ~2×10^{-9} M and ~2×10^{-10} M (23). Hence, the fact that it is not affected by the regeneration step is a very important factor in this protein selection.

Protein A/G bind to the Fc region of the antibody so that the correct orientation of the antibody bound to the surface is performed. Thanks to this feature of the protein A/G and Fc region, the antigen binding sites of the antibody will uptake from the surface and the antigen binding activity will remain unaffected (77).

The anti histidine antibody binds to carboxymethydextran from the Fc portion. Thus, the Fab region of the anti histidine antibody remains open and the

antibody and label configuration is provided correctly (Figure 12). With the protein A/G mediated antibody capture and anti histidine antibody immobilization approach, the antibodies to be used in the assay usually do not require any preliminary modification. Thus, captured antibodies do not lose their characteristic and affinity (78).

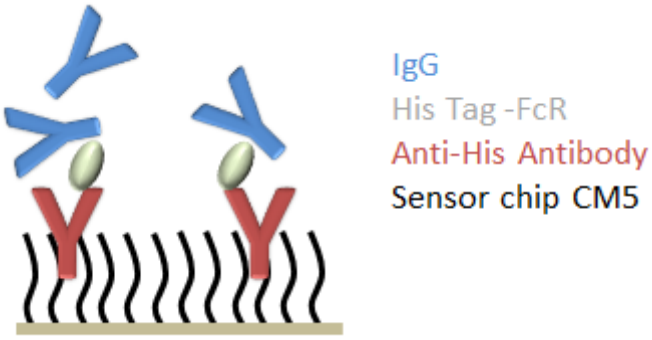


Figure 12. Representation of a classical FcγR assay.

2.3.2.1 Amine coupling

In biological interaction analyzes, the area in which the reaction takes place and the liquid environment are important. Therefore, the surface in the biosensor is usually coated with carboxymethyl dextran specifically for this interaction. The ligand may be covalently attached to the carboxyl group of this dextran and positioned near the surface. The carboxymethyl dextran surface is stable and minimizes the binding of non-specific biomolecules. This binding is due to amine coupling method. To maintain the integrity of the immobilized ligand in the regeneration cycle, the bound analyte should be removed under mild conditions as much as possible. Separating high-precision interactions may not be easy and often require strong reagents. The immobilized ligand should be able to resist these conditions.

The amine coupling method is the most commonly applied method for covalent capture of biological molecules on the sensor surface. By this method, the dextran matrix on the chip surface is first activated with reactive succinimide esters

1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) chemicals (72).

This approach uses the formation of NHS esters from a portion of the carboxyl groups of the carboxymethyl matrix on the chip surface by reaction of the NHS and EDC in the water-containing medium. The ability of proteins (α-amino group, the ε-amino group of lysine, the thiol group of cysteine) and to bind to the matrix surface is formed by nucleophilic displacement of chemical chains on the surface of carboxymethyl matrix with NHS esters (Figure 13) (79). The corresponding protein is passed on the surface in a low ionic strong solution with a lower pH than the isoelectric point. Thus, the protein is concentrated by electrolytic attraction and reacts with esters. Finally, the active esters are converted to amides with ethanolamine.

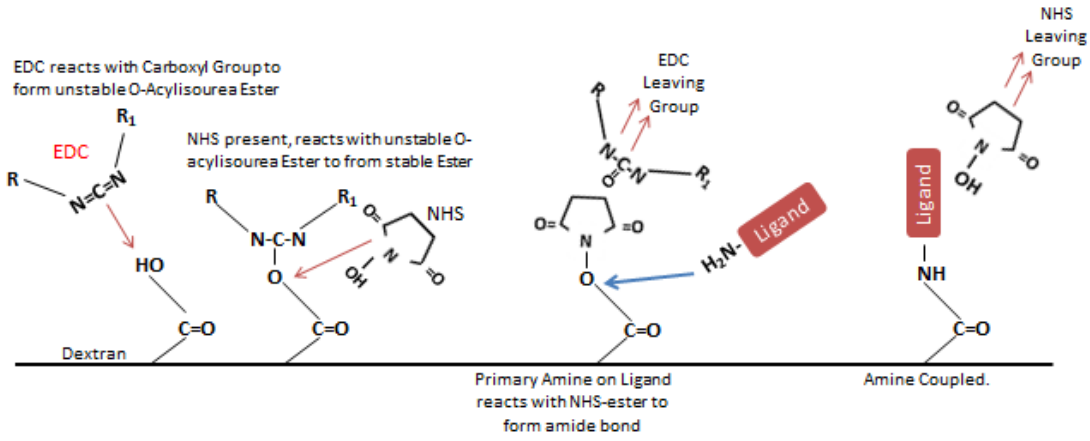


Figure 13. EDC/NHS chemisty.
(Reference : Johnsson B, Lofas S, Lindquist G. Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. Anal Biochem. 1991;198(2):268-77.)

The amount of ligand to be immobilized can be controlled by variables such as concentration, pH, and time. Some proteins can be denatured directly when adsorbed onto the metal surface. Therefore, immobilization is a good way to eliminate this disadvantage. Due to the suitable and flexible hydrophilic properties of the matrix, it minimizes the non-specific adsorption of the ligands in the solution

under ionic strength conditions and ensures the availability of the ligand during analysis (80).

2.3.2.2 Histidine tags

Generally, various labels are used to facilitate purification and purification of recombinant proteins. In addition, SPR systems are used to capture recombinant proteins on the sensor surface. The most widely used labels today include histidine, streptavidin, glutathione-s transferase. Recombinant proteins can be obtained by histidine residues (polyhistidine) added to the C- or N- terminus of the respective protein (76).

High affinity antibodies which are complementary to these labels may be immobilized on the surface to capture recombinant proteins. Immobilization of anti histidine antibodies to the chip surface can be carried out under suitable physiological conditions. The covalent binding of the anti-histidine antibody with the carboxymethyl dextran renders this interaction irreversible. Thus, the anti histidine antibody cannot be removed from the surface (80).

Thanks to the labeled proteins, a directed orientation occurs on the chip surface and an optimum area is provided for the experiments. The anti histidine antibody recognizes regions containing consecutive histidine residues at the amino or carboxyl-terminus of the opposite molecule. Histidine tags are common because of their small complexity and are unlikely to be involved in the function or structure of proteins (81).

2.3.3 Kinetic analysis

A kinetic binding analysis consists of three main steps namely, association, dissociation and regeneration (Figure 14). SPR experiments can be carried out with 2 different approaches. Experiments are setting up according to single cycle kinetics (SCK) or multi cycle kinetics (MCK) approach.

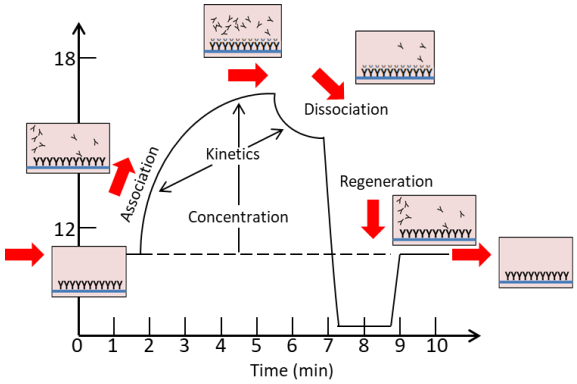


Figure 14. Representation of SCK in an experiment.

In MCK, each analyte concentration is injected into the surface in separate cycles and the surface regenerates after each injection of analytes. In SCK, the injection of analytes with different concentrations is carried out in a single cycle without regeneration. Regeneration is carried out after the analyte having the highest concentration is sent to the surface (Figure 15). To the surface of the sensor chip, the analytes are injected either from the lowest to the highest, or can be injected from the short separation time to the longest separation time (82). Single cycle kinetics are based on 1:1 interaction. This approach shortens the duration of the experiment and provides more sensitive data. The analyte concentration and kinetic range can be determined by SCK.

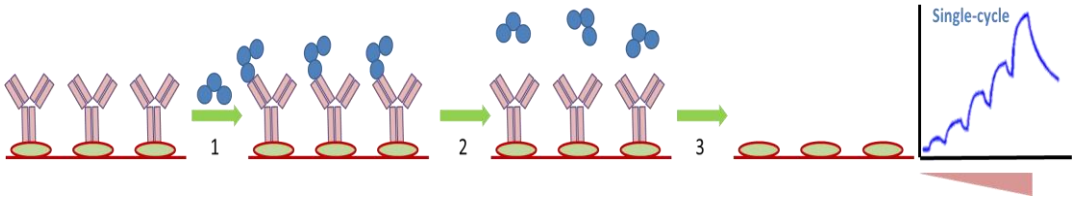


Figure 15. Representation of SCK during the experiment. (1) Association , (2) Dissociation and (3) Regeneration

2.3.4 Forced degradation (stress study) on biopharmaceuticals

The activities of IgG molecules are highly sensitive to unfolding state of protein as well as its aggregation amount. The deterioration or aggregation of secondary, tertiary or quaternary structures of these proteins may result in loss of activity, as well as alteration of their toxicity and immunogenicity profile. It is possible to monitor these changes that may occur in the IgG by stress studies and to

predict the results. It also gives us information about the behavior of IgG in the long term.

The non-enzymatic deamidation of asparagine (Asn,N) and glutamine (Gln,Q) residues can alter the function of the therapeutic molecule and potentially lead to a decrease in bioactivity (83). During deamidation, an amide functional group is separated from these amino acids. Deamidation can cause structural and biologically significant changes as a result of unfavorable negative charge in IgG. The sensitivity of asparagine (Asn) to deamidation depends on the structure of the adjacent amino acids, the tertiary structure and ionic strength of the protein, the pH of the solution and the temperature in the storage condition. Deamidation stress can be carried out in a chemical way (acidic or basic pH treatment).

3. MATERIALS AND METHODS

The cell culture process consists of seed train expansion of CHO cells in shake flasks, wave bags, followed by growth and mAb expression in a production bioreactor. Expansion of the CHO cell culture from a single vial of the Working Cell Bank (WCB) is accomplished by sub-culturing into shake flasks of increasing size. The culture is further expanded using a series of cell bags; the number of which is dependent upon the process scale. The culture is expanded through the seed bioreactors until sufficient cells are available to inoculate the production bioreactor at the appropriate cell density. Cell growth and mAb expression continue for approximately 12-15 days, at which time the culture is harvested. mAb was produced from CHO-M cell line and purified by using consecutive different chromatography techniques and filtration methods clarification conducted with by using Millistak PODs (Merck Millipore). As a capture step, Protein A chromatography was performed which is the most known capture step for monoclonal antibody purification. Following capture step, viral inactivation was performed for 60 minutes. After viral inactivation, cation exchange chromatography, viral filtration and hydrophobic interaction chromatography were performed respectively for ensuring that impurity levels were below the limits. As a final step, Tangential flow filtration were performed in order to adjust final concentration and buffer exchange. At the end of the process, excipients were added and final filtration was performed. The concentration of the monoclonal antibody was determined by using Nanodrop One (Thermo Fisher) with the extinction coefficient value, 1.39.

3.1 Sample Description

DRS (Drug Reference Standard), a biosimilar (IgG1), has been accepted as a reference in all experiments. This molecule is the drug substance, obtained from the 3 runs from the 3-liter bioreactors. This substance is manufactured under GMP conditions. Stress conditions were applied to all samples except DRS for comparison.

The 200L GMP (DS 1) substance is a biosimilar product of the same innovator obtained from the 200-liter bioreactor under GMP conditions. DP 200L nGMP (DP

1) is a biosimilar product of the same innovator obtained from a 200 liter bioreactor under non-GMP conditions. DP 200L GMP (DP 2) is a biosimilar product of the same innovator obtained from 200 liter bioreactor under GMP conditions. Both DP 1 and DP 2 are in final form in the formulation buffer in the syringe form. Innovator 1 and 2 are commercially available IgG1 molecules from two different batches, both in the final form and in the syringe. While all products except Innovator 2 are in the form of 40 mg / 0.8 mL. The Innovator 2 was supplied in the form of 40 mg / 0.4 mL (Table 3). Initial concentrations have been measured for each sample by spectrophotometry and not taken as they were stated on the drug formulation.

Table 3. Information of IgG samples used in the experiments.

	Sample Description		Lot No	Initial Concentration	Expiry Date
1	Biosimilar	DS 3x3L Bioreactor	DRS	48.41 mg/mL	04/2020
2	Biosimilar	DS 200L GMP	DS 1	48 mg/mL	12/2019
3	Biosimilar	DP 200L nGMP	DP 1	47.6 mg/mL	07/2020
4	Biosimilar	DP 200L GMP	DP 2	49.9 mg/mL	09/2020
5	Innovator	Innovator Product	Innovator 1	50.2 mg/mL	11/2019
6	Innovator	Innovator Product	Innovator 2	98.6 mg/mL	03/2020

SPR binding experiments were conducted using with Biacore T200 instrument (GE Healthcare, Uppsala Sweden). Before each experiment the equipment was checked to be clean and in good working condition. For the maintenance of Biacore T200 once a week desorb procedure and once a month desorb and sanitize procedure were performed according to the Biacore T200 Instrument Handbook. All running buffer solutions used in the experiment were prepared at room temperature and using ultra-pure water (Milli-Q). Then the running buffers were filtered with a 0.22 μm polyethersulfone membrane (Sartolab RF Vacuum Filter, Cat# 180C3E) and the final form of running buffers can be stored 1 week in room temperature. All experiments were performed by SCK method.

Table 4. Summary of experiments

	Without Stress	With Stress			
		pH 3		pH 9	
		24-h	72-h	24-h	72-h
IgG – TNF-α binding	☒	☒	☒	☒	☒
IgG – FcγRI binding	☒	☒	☒	☒	☒
IgG – FcγRIIIa binding	☒	☒	☒	☒	☒

3.2 Binding Kinetics Analysis with Protein A/G Immobilized Chip

3.2.1 Immobilization of protein A/G onto CM5 chip surface

All assays were performed at a same temperature, sample compartment set to 12°C and analysis temperature is 25 °C, using fresh prepared HBS-EP + 1X pH 7.4 (0.1 M HEPES, 1.5 M NaCl, 0.03 M EDTA and 0.5% v/v Surfactant P20, GE Healthcare, Cat# BR100669) as running buffer. Protein A/G was immobilized with amine coupling method to the CM5 sensor chip surface with the approach of NHS/EDC chemistry as previously described.

EDC and NHS (GE Healthcare, Amine Coupling Kit, Cat# BR 100050) are dissolved separately with 10 mL of ultra-pure water (Milli-Q) and aliquoted in Biacore vials and stored at -18°C or below. 1 M ethanolamine-HCl pH 8.5 (GE Healthcare, Amine Coupling Kit, Cat# BR 100050) stored at 4°C. Lyophilized protein A/G (Thermo Scientific™ Pierce™ Recombinant Protein A/G Cat# 21186) was dissolved with sterile PBS (Thermo Fisher, Cat# 14190-094) and equal volume of UltraPure™ Glycerol (Invitrogen, Cat # 15514011) was added for a final concentration of 0.5mg/ml and the aliquoted were stored at -80°C. CM5 sensor chip

(GE Healthcare, Series S Sensor Chip CM5, Cat # BR100530) was stored at 4°C, but it was brought to room temperature for 30 min and docked into device.

For ligand immobilization, protein A\G, was diluted to 50µg/mL in 10 mM sodium acetate pH 4.0 (GE Healthcare, Cat# BR100349). This sodium acetate pH level determined by pH scouting wizard. For kinetic analysis 2 flow cells were chosen (one reference and one active flow cell) and immobilized with an aim for 1200 RU level.

For surface activation NHS/EDC were mixed with 1:1 (v/v) ratio and injected to chip surface with 10 µl/min flow rate and for 450 seconds. When the surface activation step is completed protein A/G was injected with the aim of 1200 RU. To calculate the concentration of protein A/G a pre-concentration the system performs concentration determination to protein A/G itself before immobilization. This step takes place before surface activation with NHS/EDC. Thus, the system decides how much protein A/G sodium acetate solution to inject. After this determination, the protein A/G which is temporarily retained on the surface is removed from the chip surface by washing with NaOH (GE Healthcare, Amine Coupling Kit, Cat# BR 100050). With the pre-concentration step the system calculates the contact time which is required for 1200 RU immobilization level.

After protein A/G immobilization, 1 M ethanolamine-HCl pH 8.5 was injected into the sensor surface for 450 seconds with a 5 µl/min flow rate to block the residuary free ester groups in both the sample and reference flow channels. Adequate EDC, NHS, NaOH and ethanolamine volumes are calculated by the Biacore T200 Control Software (GE Healthcare, Version 2.0.1). The total amount of immobilized total protein A/G is determined by the difference between the final response unit and the initial buffer baseline.

After immobilization process, verification step applied. This procedure confirms whether immobilization is achieved based on a short binding assay. This verification procedure consists of 3 main steps; conditioning, startup and wash. Data collection rate is 10Hz and HBS-EP+ 1X is used as the running buffer. During conditioning

step, HBS-EP+ 1X buffer was injected with 60 seconds of contact and dissociation time with a 30µl/min flow rate.

The protein content of IgG molecule is measured by optical density (OD) at 280 nm via Nanodrop. After concentration determination reference was diluted to 2.5 µg/mL using HBS-EP+ 1X buffer. IgG molecule to be used as a reference, 300 µl was injected over the immobilized CM5 sensor surface at a flow rate of 5 µl/min in the startup step for 5 seconds. TNF-α (Recombinant Human TNF-α Protein, CF Cat # 210-TA-100/CF) concentrations were established following the preparation of the test material samples. TNF-α is pre-diluted from 5714 nM to 200 nM. After this pre-dilution step, 4 sequentially folded dilutions of TNF-α from 200nM to 2.5 mM (20, 10, 5 and 2.5) were prepared in running buffer. The prepared TNF-α dilutions are consecutively injected to the chip surface for 300 seconds at a flow rate of 50 µl/min from the lowest to the highest concentration. Following accomplish of the injection of TNF-α, dissociation was trace in HBS-EP+ 1X buffer for 1200 second at the 50 µl/min flow rate. After this dissociation time, two consecutive regeneration steps were performed with glycine-HCl pH 1.5 (GE Healthcare, Cat # BR100354) 30 second and flow rate of 30 µl/min (84). Normalization of the detector is applied in this step using BIAnormalizing solution (GE Healthcare, Biacore Maintenance Kit, Cat #BR100651). In addition, the prime of the system is user preference, but it was done in every experiment. Once the immobilization and verification steps have been completed successfully, the chip is ready for use and can be used for 1 month. The immobilized chip was stored at 4°C in HBS-EP+ 1X buffer.

3.2.2 Capture of mAb to the chip surface and TNF-α Binding Kinetics

Immobilized chip was docked into instrument and immobilized flow cells were chosen. Test material (IgG) will be captured onto a single flow cell through the binding of the antibody to the Protein A/G surface (test flow channel), whilst a second flow cell remains protein A/G alone (control flow channel). Data collection rate is 10Hz and HBS-EP+ 1X is used as the running buffer. The method was configured according to the single cycle kinetics model. The conditioning cycle was repeated 5 times to ensure the surface stabilization of the chip prior to binding kinetic

analysis. At this step the running buffer was carried out at the 30 µl/min flow rate for 60 seconds over the both test and control surface.

After concentration determination reference was diluted to 2.5 µg/mL using HBS-EP+ 1X buffer. Then, TNF-α concentrations were established following the preparation of the test material samples. TNF-α is pre-diluted from 5714 nM to 200 nM. Based on this pre-dilution, 4 sequentially folded dilutions of TNF-α from 200nM to 2.5 mM (20, 10, 5 and 2.5) were prepared in running buffer. Vortex step was applied to ensure good mixing of the samples during each dilution. A startup step was taken between the conditioning and the analysis step. This startup cycle same as chip verification step but 50 µl IgG solution injected over the surface as reference. After startup cycle, a wash step was applied with regeneration with glycine-HCl pH 1.5. IgG molecules to be analyzed as a test material, 90 µl was injected over the immobilized CM5 sensor surface at a flow rate of 30 µl/min in the analysis step for 5 seconds. This sample preparation same as reference preparation/dilution. This flow is only injected into the test flow channel.

The prepared serial TNF-α dilutions are consecutively injected to the both immobilized chip surface for 300 seconds at a flow rate of 50 µl/min from the lowest to the highest concentration without regeneration solution. Following accomplish of the injection of TNF-α, dissociation was trace in HBS-EP+ 1X buffer for 2400 second at the 50 µl/min flow rate. After this dissociation time, two consecutive regeneration steps were performed with glycine-HCl pH 1.5 30 second and flow rate of 30 µl/min. Both normalization of detector and prime were performed before injections.

Interaction of the captured material with increasing concentrations of TNF-α will be recorded as changes in Response Units (RU) by the Biacore Control Software. The data will be double referenced i.e, 1) the RU response from the control flow channel will be subtracted from the test flow channel, and 2) each series of injections will be preceded by blank (running buffer) injections which will be subtracted from the following TNF-α injections.

3.3 Binding Kinetics Analysis by FcγRI and FcγRIII

3.3.1 Immobilization of anti histidine antibody onto CM5 chip surface

All assays (including immobilization, FcγRI and FcγRIIIa) were performed at a same temperature, sample compartment set to 12°C and analysis temperature is 25 °C, using fresh prepared PBS-P+ 1X pH 7.4 (0.2 M phosphate buffer with 27 mM KCl, 1.37 M NaCl and 0.5% Surfactant P20, GE Healthcare, Cat# 28995084) as running buffer. Anti histidine antibody (His Capture Kit, GE Healthcare, Cat# 29234602) was immobilized with amine coupling method to the CM5 sensor chip (Series S Sensor Chip CM5, GE Healthcare, Cat# BR100530) surface with the approach of NHS/EDC chemistry as previously described.

EDC and NHS are dissolved separately with 10 mL of ultra-pure water (Milli-Q) and aliquoted in Biacore vials and stored at -18°C or below. 1 M ethanolamine-HCl pH 8.5 stored at 4°C. CM5 sensor chip was stored at 4°C, but it was brought to room temperature for 30 min and docked into device.

For kinetic analysis 2 flow cells were chosen (one reference and one active flow cell) and immobilized with an aim for 7000 RU level. For surface activation NHS/EDC were mixed with 1:1 (v/v) ratio and injected to chip surface with 10 µl/min flow rate and for 420 seconds. When the surface activation step is completed anti histidine antibody was injected with the aim of 7000 RU. His Capture kit provided by GE healthcare also contains a buffer for immobilization of the anti histidine antibody. The solution prepared by adding 5 µl antibody to 95 µl immobilization buffer is recomended by GE Healthcare. To calculate the concentration of anti histidine antibody, a pre-concentration the system performs concentration determination to anti histidine antibody itself before immobilization. This step takes place before surface activation with NHS/EDC. Thus, the system decides how much anti histidine antibody solution to inject. After this determination, the anti histidine antibody which is temporarily retained on the surface is removed from the chip surface by washing with NaOH. With the pre-concentration step the system calculates the contact time which is required for 7000 RU immobilization level.

After immobilization, 1 M ethanolamine-HCl pH 8.5 was injected into the sensor surface for 420 seconds with a 5 µl/min flow rate to block the residuary free ester groups in both the sample and reference flow channels. Adequate EDC, NHS, NaOH and ethanolamine volumes are calculated by the Biacore T200 Control Software (GE Healthcare, Version 2.0.1). The total amount of immobilized total anti histidine antibody is determined by the difference between the final response unit and the initial buffer baseline.

3.3.2 Capture of His-tag FcγRI and FcγRIII to the chip surface and mAb Binding Kinetics

3.3.2.1 FcγRI and IgG1 binding kinetics:

FcγRI (CD64 Human Recombinant Protein with His-Tag, Sino Biological, Cat# 10256-H08H) was dissolved and aliquoted to a concentration of 0.250 mg/ml in sterile double distilled (MilliQ) water. Aliquotes can be stored between -20 and -70 ℃ for 1 year.

Anti histidine antibody immobilized CM5 chip was docked into instrument and immobilized flow cells were chosen. Test materials will be captured onto a single flow cell through the binding of the his-tag FcγRI to the anti histidine antibody surface (test flow channel), whilst a second flow cell remains anti histidine antibody alone (control flow channel). Data collection rate is 1 Hz and PBS-P + 1X pH 7.4 was used as the running buffer. The method was configured according to the single cycle kinetics model. The conditioning cycle was repeated 10 times to ensure the surface stabilization of the chip prior to binding kinetic analysis. At this step the running buffer was injected at the 30 µl/min flow rate for 60 seconds over the both test and control surface. 600 seconds for dissociation was found suitable. For the regeneration cycle in the conditioning step was performed in both flow cells with glycine-HCl pH 1.5 30 µl/min flow rate for 30 seconds.

In the start-up step, the 1 mg/mL his-tag FcγRI was injected into the test channel on the chip surface at a rate of 5 µl/min for 45 seconds. After capture of his-tag FcγRI, a stabilization period of 600 seconds was applied. Regeneration in the

start-up step was performed in both flow cells with glycine-HCl pH 1.5 30 µl/min flow rate for 30 seconds in both 2 immobilized flow cells.

In sample cycles, his-tag FcγRI is captured by the anti histidine antibody on the chip surface, at a rate of 5 µl/min for 45 seconds in test flow cell. Concentration determination of IgG reference was determined with KD value in manual runs.

The molecular weight of IgG (monomer) = 148000 Da. IgG is pre-diluted to 3378 nM. Based on this pre-dilution, 4 sequentially folded dilutions of IgG from 3378 nM to 2 nM (128, 32, 8 and 2 nM) were prepared in running buffer. Vortex step was applied to ensure good mixing of the samples during each dilution. The IgG concentrations are sent to the chip surface with 30 µl/min for 60 seconds and a time of 600 seconds were allowed to dissociation in both flow cells. Regeneration in the sample step was carried out with glycine-HCl pH 1.5 30 µl/min flow rate for 30 seconds in both flow cells.

3.3.2.2 FcγRIIIa and IgG1 binding kinetics

FcγRIIIa (CD16a Human Recombinant Protein (176 Val) with histidine-tag, Sino Biologicals, Cat# 10389-H08H1) was dissolved and aliquoted to a concentration of 0.250 mg/ml in sterile double distilled (MilliQ) water. Aliquotes can be stored between -20 and -70 ℃ for 1 year.

Anti histidine antibody immobilized CM5 chip was docked into instrument and immobilized flow cells were chosen. Test materials will be captured onto a single flow cell through the binding of the his-tag FcγRIIIa to the anti histidine antibody surface (test flow channel), whilst a second flow cell remains anti histidine antibody alone (control flow channel). Data collection rate is 10 Hz and PBS-P + 1X pH 7.4 was used as the running buffer. The method was configured according to the single cycle kinetics model.

The conditioning cycle was repeated 10 times to ensure the surface stabilization of the chip prior to binding kinetic analysis. At this step the running buffer was carried out at the 30 µl/min flow rate for 60 seconds over the both test and control surface. 150 seconds for dissociation was found suitable. For the regeneration cycle

in the conditioning step was performed in both flow cells with glycine-HCl pH 1.5 30 µl/min flow rate for 30 seconds.

In the start-up step, the his-tag FcγRIIIa was injected into the test channel on the chip surface at a rate of 5 µl/min for 30 seconds. After capture of his-tag FcγRI, a stabilization period of 300 seconds was applied. Regeneration in the start-up step was performed in both flow cells with glycine-HCl pH 1.5 30 µl/min flow rate for 30 seconds in both 2 immobilized channels.

In sample cycles, his-tag FcγRIIIa is captured by the anti histidine antibody on the chip surface , at a rate of 5 µl/min for 30 seconds in test flow cell. After capture of His-FcγRIIIa, a stabilization period of 600 seconds was applied. Concentration determination of IgG reference was determined with KD value in manual runs. IgG is diluted to 2000 nM and based on this dilution, 4 sequentially folded dilutions of IgG from 2000 nM to 31.5 nM (2000, 500, 125 and 31.5 nM) were prepared in running buffer. Vortex step was applied to ensure good mixing of the samples during each dilution. The IgG concentrations are sent to the chip surface with 30 µl/min for 60 seconds and a time of 600 seconds were allowed to dissociation in both flow cells. Regeneration in the sample step was carried out with glycine-HCl pH 1.5 30 µl/min flow rate for 30 seconds in both flow cells.

3.4 Stress Studies

In stress studies, chemical deamidation was applied. Prepared buffers and IgG samples were mixed in a 1: 1 ratio and incubated at 37 ℃, 5 % CO_2 for 24 and 72 hours. After stress conditions are applied to the samples (DS 1, DP 1, DP 2, Innovator 1 and Innovator 2) they were aliquoted and stored at -80 °C.

3.4.1 Chemical Deamidation

Chemical deamidation was applied at 2 different pH levels for 24 and 72 hours. Two different buffer solutions were formed for the chemical deamidation. These were prepared with citric acid for pH 3 and sodium bicarbonate for pH 9.

3.4.1.1 Preparation of 100 mM citric acid pH 3

For citric acid pH 3, 0.21 g of monohydrate citric acid (Merck, Cat # 1370035000) was weighed. Dissolved monohydrate citric acid in 9 mL ultra-pure water. The pH was adjusted with NaOH 5M at pH 3.0 ± 0.1. The volume was completed at 10 mL. The prepared buffer was filtered with 0.45 µm PVDF filter and stored no more than one week.

3.4.1.2 Preparation of 200 mM sodium bicarbonate pH 9

For sodium bicarbonate pH 9, 0.168 g of sodium bicarbonate (Merck, Cat # 1063291000) was weighed. Dissolved sodium bicarbonate in 9 mL ultra-pure water. The pH was adjusted with NaOH 5M at pH 9.0 ± 0.1. The volume is then completed at 10 mL. The prepared buffer was filtered with 0.45 µm PVDF filter and stored no more than one week.

4. RESULTS

All experiments were performed as described in the materials and method chapter. The information of the samples used is described in the materials and methods. DRS (Drug Reference Standart) is an IgG product produced from a 3x3 liter bioreactor. The DRS was used as a control sample throughout all the study to ensure that the system operation is at optimum conditions. For this reason, the first and last sample injection was used as a DRS control sample which was from the same preparation without stress application.

To check the suitability of the optimized method, the relative % KD value of the DRS 2 (last) sample is expected to be between 80-120% when the relative %KD of the DRS 1 is accepted as 100%.

CM5 sensor chip was used in the all experiments. This sensor chip type is preferred because it offers a high surface capacity for immobilization of many different ligands (such as organic molecules, protein and nucleic acid). Since IgG-TNF tests are common in general use, HBS-EP+ 1X was used as working buffer. In FcγR experiments, it was decided to use PBS as a working buffer after a search on the literature and GE publications (86).

In IgG-TNF binding assays, the concentration of both molecules was determined by manual experiments (data not shown). In the FcγR experiments, concentration levels have been obtained from published data as a reference and optimized using our system (87). Then, the optimum concentrations both FcγRs and IgG samples were determined by manual experiments (data not shown). Experiments were repeated several times with control samples. This ensures that the method and the concentrations are suitable for testing. In all experiments, each sample is measured twice in consecutive runs and the tabulated results are average of two duplicate injections.

It was concluded that after all experiments, Innovator 1 yielded ambiguous results compared to biosimilars. Sensorgrams of all experimental sets are given in appendix section.

For assays related to primary mechanism action of anti TNF-α monoclonal antibody, binding kinetics to sTNF-α assay, similarity was concluded when the ± standard deviation (SD) for the difference in means between the products was contained within an equivalence acceptance criterion (EAC) of ± 2 SD of the dataset for the reference product batches tested. For the remaining functional assays measuring other mechanisms of action (FcγRs) which have low risk of affecting clinical outcome, the quality range was defined as the mean of the reference product batches tested ± 3 SD.

4.1 IgG-TNF-α Binding

4.1.1 pH scouting

Prior to IgG-TNF binding assays, the optimum sodium acetate pH for immobilization of protein A/G to the CM5 chip was determined by pH scouting method. In this method, sodium acetate with varying pH values have been used (pH 4.0, pH 4.5, pH 5.0 and pH 5.5). Sodium acetate pH 4.0 was determined to be the optimal pH value for maximum protein immobilization (Figure 16).

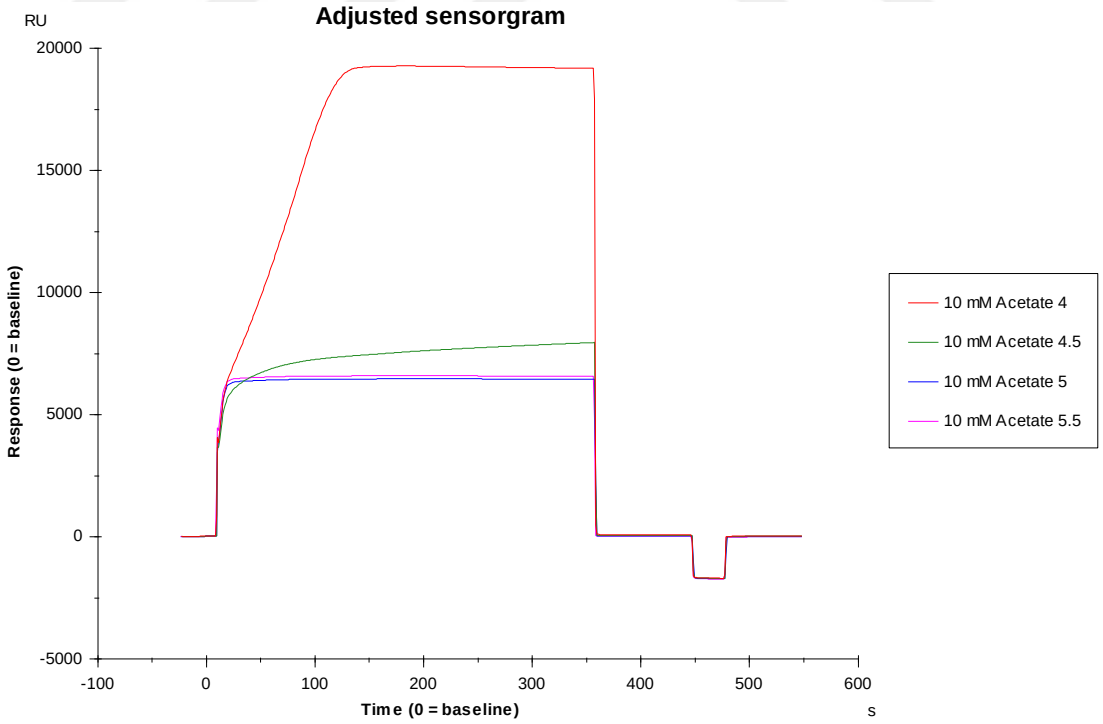


Figure 16. pH scouting sensorgram for protein A/G immobilization.

Table 5. pH scouting data for protein A/G immobilization.

Cycle	Relative response (RU)
1 (sodium acetate pH 5.5)	6436.0
2 (sodium acetate pH 5.0)	6559.3
3 (sodium acetate pH 4.5)	7930.1
4 (sodium acetate pH 4.0)	19158.1

4.1.2 Protein A/G CM5 chip immobilization

The immobilization data of the protein A/G on the CM5 chip is given in the table. Immobilization was targeted at 1200 RU levels and this value was provided in immobilization (Table 6).

Table 6. Protein A/G immobilization levels

Flow Cell	Procedure	Method	Ligand	Response Bound (RU)	Response Final (RU)	Target Reached
1	Target level	Amine Protein AG	Protein A/G 50 µg/mL pH 4.0	1259.2	1425.1	Yes
2	Target level	Amine Protein AG	Protein A/G 50 µg/mL pH 4.0	1244.5	1379.9	Yes

After immobilization, a single-sample verification experiment was performed to make sure that the binding levels were appropriate (Figure 17).

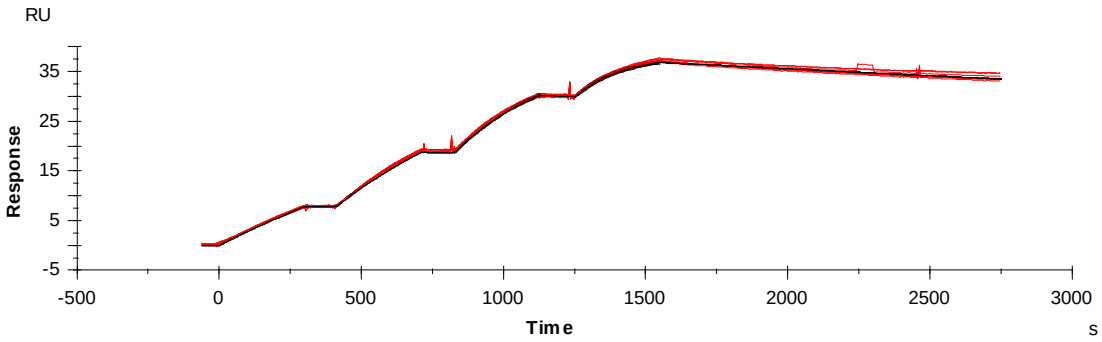


Figure 17. Sensorgram of the post-immobilization verification run.

4.1.3 IgG - TNF-α binding results

IgG-TNF-α binding is described in the method section. IgG-TNF-α binding of non-stressed samples was measured by several different assay sets. Each experiment set fulfilled the system acceptance criteria. A single KD value has been assigned to each replicate. Then, mean values of the KD's were used for comparison (Figure 18). The red bars show ± 2 standard deviation window. DRS is considered %100. Relative response values and all corresponding data are given in Table 7.

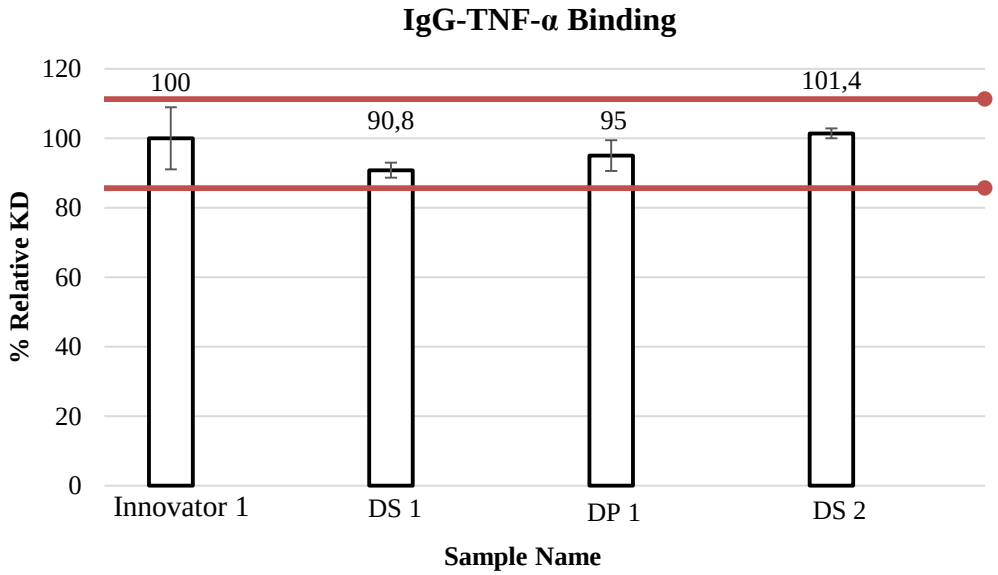


Figure 18. Average relative KD values of the stress-free IgG samples.

In the test of pH 3 24-h samples, the system acceptance criteria were fulfilled. (DRS 1 and DRS 2) The Innovator 1 gave a different relative KD value to other biosimilars (Figure 19). When look into mean capture levels, the Innovator 1 is not captured to the chip surface (-0.87 RU) (Table 8). When the mean capture levels of the stress-free samples and the stress samples were compared, a decrease in the level of stressed samples was observed. (Table 8). While the affinities of all samples, including DRS, were at E-10 level under stressed and stress-free conditions, however Innovator 1 pH 3 24-h showed higher affinity compared to other samples (E-14) (Table 8).

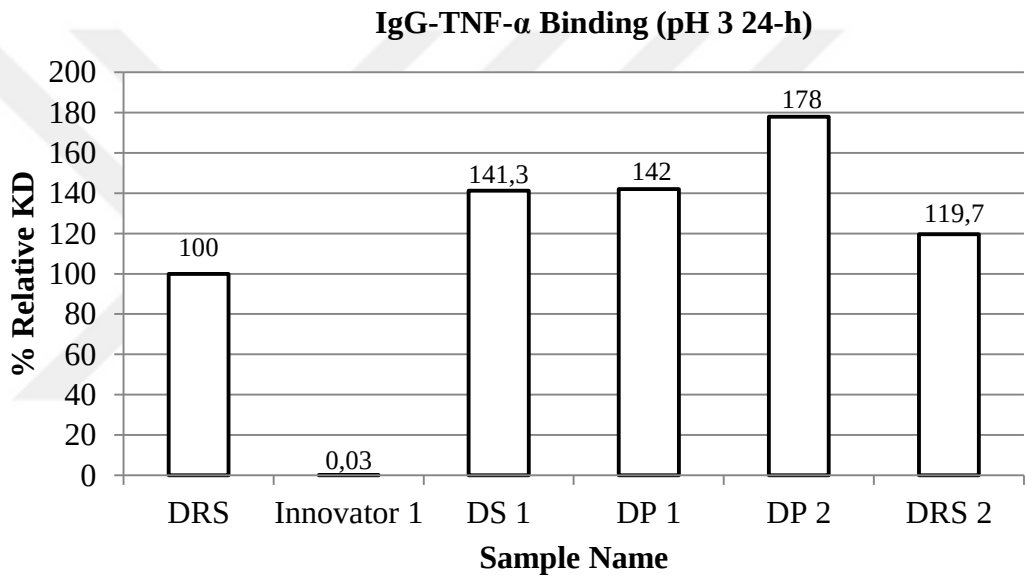


Figure 19. Relative KD values of stressed IgGs binding to TNF-α (pH 3 for 24-h).

Innovator 1's sensorgram show that there's no capture of IgG and TNF-α onto the chip surface (Figure 20).

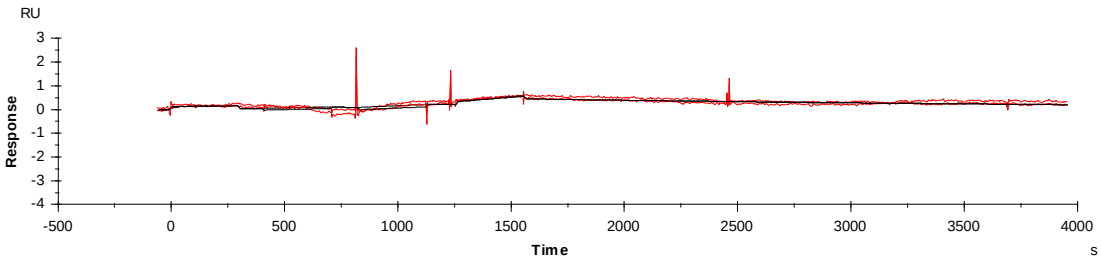


Figure 20. The IgG-TNF-α binding sensorgram of Innovator 1 (pH 3 for 24-h).

In the test of pH 3 72- h samples, the system acceptance criteria were fulfilled (DRS 1 and DRS 2). Since the Innovator 1 has a Relative KD value of %279685 relative to the DRS (%100), the data is not shown in the Figure 21. Mean capture level of all stressed samples decreased according to pH 3 24-h condition (Table 8). While the affinities of the samples in the experimental set were at "E-10", the Innovator 1 had "E-07" affinity. As a result, it was observed that Innovator 1 increased affinity in pH 3 conditions at 24-h and its affinity decreased in 72 hours.

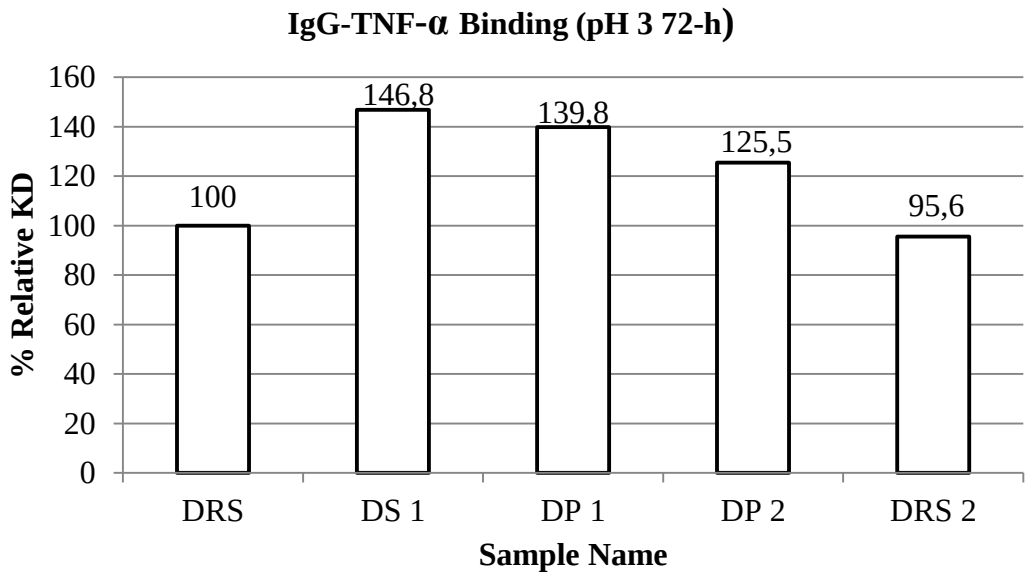


Figure 21. Relative KD values of stressed IgGs binding to TNF-α (pH 3 for 72-h)

Innovator 1's sensorgram show that there's no capture of IgG and TNF-α onto the chip surface (Figure 22).

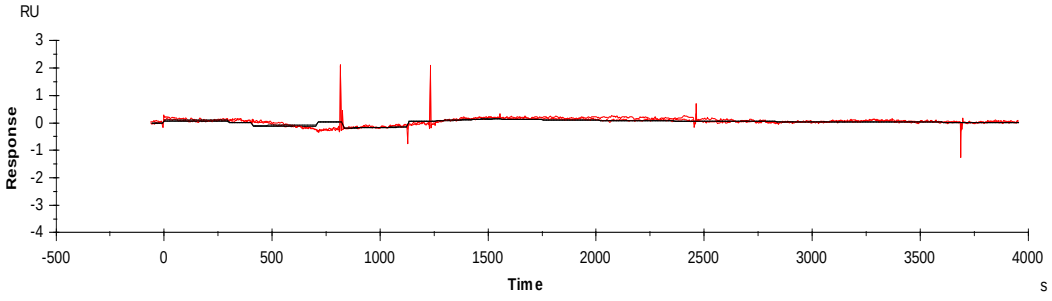


Figure 22. The IgG-TNF binding sensorgram of Innovator 1 (pH 3 for 72-h).

In the test of pH 9 24-h samples, the system acceptance criteria were fulfilled (DRS 1 and DRS 2). All samples gave a similar and acceptable relative KD value to DRS (Figure 23). The IgG samples exposed to pH 9 24-h stress did not show any extraordinary binding properties. No significant changes were observed at the mean capture level. Compared to pH 3 stress conditions, more IgG capture was achieved on the chip surface (Table 8). All samples in the experimental set showed similar levels of affinity.

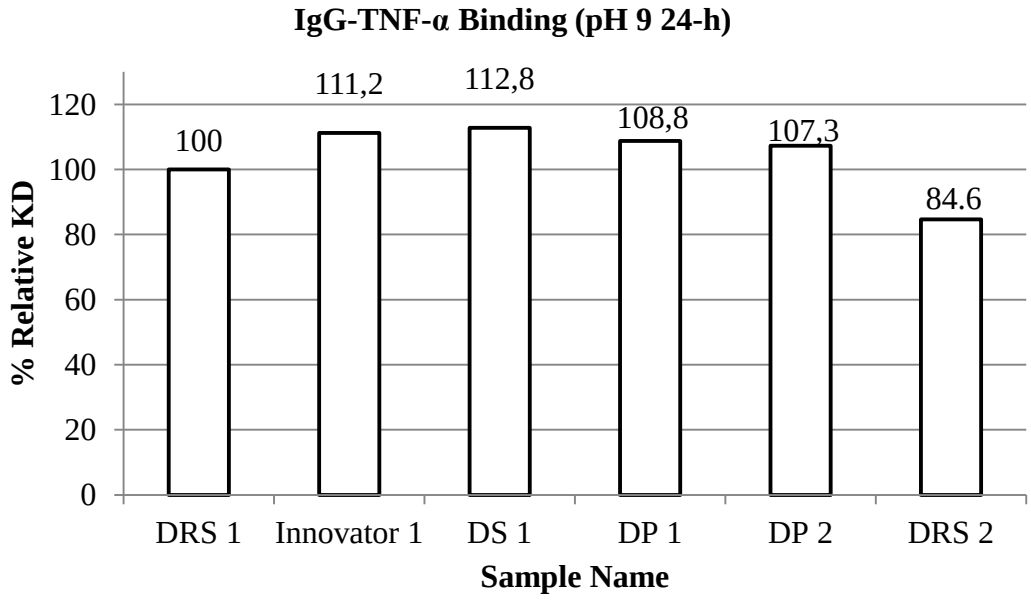


Figure 23. Relative KD values of stressed IgGs binding to TNF-α (pH 9 for 24-h)

In the test of pH 9 72-h samples, the system acceptance criteria were fulfilled (DRS 1 and DRS 2). All samples have a different relative KD value to DRS (Figure 24). While DP 2 was not affected by the stress condition too much, Innovator 1, DS 1 and DP 1 differed slightly. Considering the mean capture level of DRS, mean capture levels similar to the results of pH 9 24-h were obtained (Table 8).

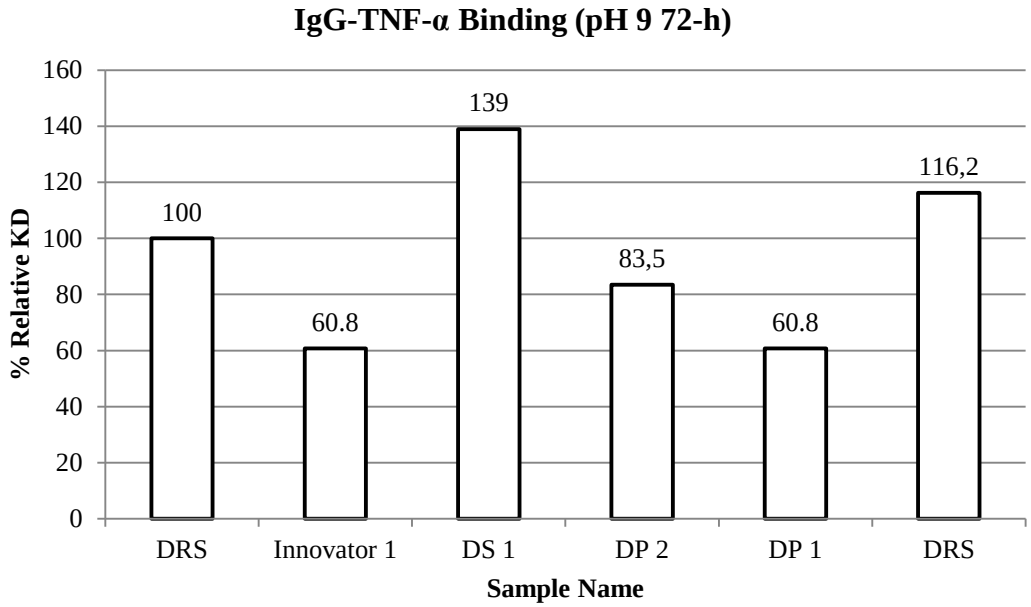


Figure 24. Relative KD values of stressed IgGs binding to TNF-α (pH 9 for 72-h)

In the test of Innovator 2 samples, the system acceptance criteria were fulfilled (DRS 1 and DRS 2). While Innovator 1 cannot achieve a good binding at pH 3 (24-h and 72-h) and pH 9 72-h. Innovator 2 has shown a better binding similarity under these conditions. When looking at pH 9 relative KD data, no specific change was observed (Figure 25). When the mean capture level is examined, it is seen that the antibody capture on the chip under the conditions of pH 3 has a sudden decrease. At the pH 9 conditions, almost half-reduction in antibody capture on the chip surface was observed (Table 9).

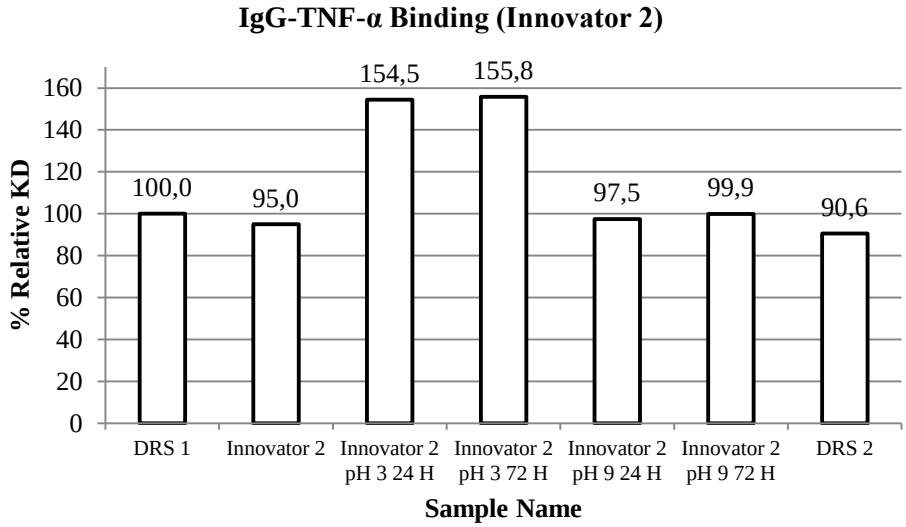


Figure 25. Relative KD values of Innovator 2 binding to TNF-α at different conditions

Table 7. The results of the stress-free IgG-TNF-α experiments are shown below.

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	tc	Chi² (RU²)	U-value	% Relative KD	Mean Capture Level
DRS 1	4.41E+05	6.94E-05	1.58E-10	28.63	9.04E+19	0.0683	1	100.0	65.5
DP 1	4.49E+05	6.62E-05	1.48E-10	31.91	6.07E+19	0.141	1	93.8	74.3
DRS 2	4.45E+05	6.82E-05	1.53E-10	28.8	3.30E+14	0.0745	1	97.2	65.0
DRS 1	5.35E+05	5.80E-05	1.08E-10	27.2	7.27E+21	0.0838	1	100.0	69.2
DP 1	5.38E+05	5.60E-05	1.04E-10	27.82	4.58E+14	0.0799	1	96.0	71.5
DRS 2	5.29E+05	5.58E-05	1.06E-10	28.6	4.89E+14	0.0962	1	97.3	69.7
DRS 1	5.79E+08	6.19E-02	1.07E-10	25.58	2.61E+07	0.0443	1	100.0	72.0
DP 1	5.44E+08	5.81E-02	1.07E-10	24.63	6.14E+07	0.0351	1	99.9	67.3
DRS 2	5.55E+08	5.89E-02	1.06E-10	25.67	3.78E+07	0.141	2	99.3	72.0
DRS 1	6.86E+05	1.04E-04	1.52E-10	15.49	3.42E+06	0.0745	2	100.0	77.5
Innovator 1	7.69E+05	1.15E-04	1.50E-10	12.96	2.81E+06	0.0332	1	98.4	65.5
DP 2	7.56E+05	1.27E-04	1.68E-10	15.36	2.96E+06	0.0977	2	110.5	81.7
DS 1	1.06E+06	1.45E-04	1.37E-10	12.38	1.89E+06	0.0235	1	90.1	66.9
DRS 2	1.34E+06	1.63E-04	1.22E-10	13.42	1.63E+06	0.151	3	80.3	79.1

Table 7: The results of the stress-free IgG-TNF-α experiments are shown below. (cont')

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	tc	Chi² (RU²)	U-value	% Relative KD	Mean Capture level
DRS 1	3.104E+05	6.548E-05	2.109E-10	30.87	4.51E+17	0.0393	1	100.0	85.3
Innovator 1	3.083E+05	7.162E-05	2.323E-10	29.26	2.22E+17	0.0414	1	94.8	86.8
DP 2	3.095E+05	6.832E-05	2.207E-10	28.43	6.85E+15	0.111	1	92.1	86.1
DRS 2	3.118E+05	6.535E-05	2.096E-10	32.06	1.49E+14	0.0415	1	103.9	76.7
DRS 1	3.48E+05	8.40E-05	2.42E-10	18.81	1.03E+08	0.0782	2	100.0	79.9
Innovator 1	3.67E+05	9.02E-05	2.46E-10	18.27	1.49E+19	0.0206	1	101.6	76.5
DS 1	3.86E+05	8.48E-05	2.20E-10	19.47	1.62E+19	0.0261	1	92.5	81.2
DRS 2	3.84E+05	8.30E-05	2.17E-10	20.1	5.32E+13	0.0224	1	82.8	83.4
DRS 1	2.87E+05	7.01E-05	2.44E-10	24.53	3.44E+18	0.0602	1	100.0	85.3
Innovator 1	2.86E+05	7.35E-05	2.57E-10	23.38	8.30E+15	0.0619	1	105.2	86.8
DP 2	2.86E+05	7.54E-05	2.64E-10	22.69	1.35E+14	0.055	1	108.1	86.1
DRS 2	2.85E+05	7.38E-05	2.59E-10	24.41	6.93E+16	0.0401	1	106.1	76.7
DRS 1	2.84E+05	8.32E-05	2.93E-10	19.52	7.48E+06	0.0638	1	100.0	51.6
DP 2	2.82E+05	7.84E-05	2.78E-10	23.39	1.46E+15	0.0569	1	94.9	60.1
DS 1	2.87E+05	7.56E-05	2.64E-10	25.18	4.74E+07	0.0402	1	90.0	67.7
DRS 2	2.73E+05	8.00E-05	2.93E-10	20.68	8.28E+07	0.0577	1	100.1	51.2

Table 8. The results of IgG-TNF-α binding assays of stressed IgG samples are shown below.

Stress Condition	Sample Name	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	tc	Chi² (RU²)	U-value	% Relative KD	Mean Capture Level
pH 3 - 24H	DRS-1	3.13E+05	5.25E-05	1.68E-10	29.10	1.71E+20	3.97E-02	1	100	101
	Innovator 1	1.66E+05	1.03E-08	6.18E-14	0.55	1.10E+08	2.15E-02	95	0.368	-0.875
	DS 1	2.76E+05	6.55E-05	2.37E-10	13.90	5.03E+17	7.73E-02	2	141	41.5
	DP 1	2.76E+05	6.58E-05	2.38E-10	18	1.22E+16	1.97E-02	1	178	55.9
	DP 2	2.65E+05	7.92E-05	2.99E-10	13.10	1.89E+17	1.99E-02	1	142	39.3
	DRS-2	3.08E+05	6.18E-05	2.01E-10	30.60	1.96E+16	6.48E-02	1	119	91.8
pH 3 - 72H	DRS-1	2.85E+05	6.53E-05	2.29E-10	25.30	1.08E+18	1.85E-02	1	100	86.1
	Innovator 1	1.28E+03	8.17E-04	6.40E-07	13.50	9.99E+07	1.35E-02	43	280000	-0.275
	DS 1	2.67E+05	8.97E-05	3.36E-10	7.77	3.45E+14	3.14E-02	2	147	25
	DP 1	2.67E+05	8.56E-05	3.20E-10	9.83	2.33E+14	2.33E-02	1	140	30
	DP 2	2.68E+05	7.71E-05	2.87E-10	9.51	7.56E+15	1.93E-02	1	126	29.9
	DRS-2	3.02E+05	6.60E-05	2.19E-10	26.30	4.49E+15	3.38E-02	1	95.6	71.6
pH 9 - 24H	DRS-1	2.72E+05	8.31E-05	3.06E-10	25.30	1.06E+07	8.21E-02	1	100	86.5
	Innovator 1	2.47E+05	8.41E-05	3.40E-10	21.90	1.76E+07	6.60E-02	1	111	68.8
	DS 1	2.34E+05	8.06E-05	3.45E-10	20.50	1.96E+17	2.69E-02	1	113	60.2
	DP 1	2.38E+05	7.92E-05	3.33E-10	22.90	5.77E+13	3.54E-02	1	109	71.6
	DP 2	2.64E+05	8.68E-05	3.28E-10	19.10	8.19E+06	3.38E-02	1	107	59.2
	DRS-2	2.67E+05	6.92E-05	2.59E-10	26.00	1.79E+07	3.18E-02	1	84.6	82.6
pH 9 - 72H	DRS-1	3.20E+05	3.10E-05	9.69E-11	32.80	3.51E+14	3.05E-02	2	100	72.6
	Innovator 1	3.05E+05	1.80E-05	5.89E-11	27.70	8.11E+19	1.78E-01	7	60.8	51.8
	DS 1	3.24E+05	4.37E-05	1.35E-10	26.40	3.16E+14	9.71E-02	2	139	42.7
	DP 1	3.43E+05	2.02E-05	5.89E-11	28.80	2.67E+14	4.31E-02	3	60.8	44.4
	DP 2	3.01E+05	2.44E-05	8.10E-11	30.50	4.01E+14	1.94E-01	5	83.6	47.7
	DRS-2	3.49E+05	3.93E-05	1.13E-10	28.90	6.40E+18	8.90E-02	2	116	66.2

Table 9. The results of IgG-TNF-α binding assays of Innovator 2 samples are shown below.

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	tc	Chi² (RU²)	U-value	% Relative KD	Mean Capture level
DRS 1	2.540E+05	9.960E-05	3.930E-10	32.67	3.39E+15	3.48E-01	1	100.0	116.4
Innovator 2	2.737E+05	1.020E-04	3.727E-10	30.94	3.81E+15	8.33E-02	1	95.0	115.6
Innovator 2 pH 3 24-h	2.304E+05	1.397E-04	6.063E-10	4.69	1.35E+09	1.66E-02	2	154.5	10.8
Innovator 2 pH 3 72-h	2.741E+05	1.676E-04	6.114E-10	3.062	8.57E+05	1.62E-02	3	155.8	7.2
Innovator 2 pH 9 24-h	2.692E+05	1.030E-04	3.827E-10	19.34	1.68E+17	4.47E-02	1	97.5	68.5
Innovator 2 pH 9 72-h	2.692E+05	1.056E-04	3.922E-10	18.19	4.40E+07	3.06E-02	1	99.9	65.2
DRS 2	3.050E+05	1.085E-04	3.555E-10	29.58	1.67E+07	6.60E-02	1	90.6	118.5

4.2 IgG - FcγR Binding

4.2.1 Anti histidine antibody immobilization onto CM5 chip

Before the Fc gamma assays, the anti histidine antibody was immobilized on the CM5 sensor chip with carboxymethyl dextran. Immobilization was targeted at 7000 RU levels and this value was provided in immobilization. As a result, immobilization is successfully achieved (Table 10).

Table 10. Anti histidine antibody immobilization informations

Flow Cell	Procedure	Method	Ligand	Response Bound (RU)	Response Final (RU)	Target Reached
1	Target level	Anti His Antibody (Custom)	Anti His Antibody	7009.9	7028.7	Yes
2	Target level	Anti His Antibody (Custom)	Anti His Antibody	7048.7	7019.6	Yes

4.2.2 IgG - FcγRI binding

IgG- FcγRI binding is described in the method section. Each experiment set fulfilled the assay acceptance criteria (DRS 1 and DRS 2). Mean values of the stress free samples obtained values were taken and samples were compared (Figure 26). The red bars show the ± 3 standard deviation window. DRS is considered %100.

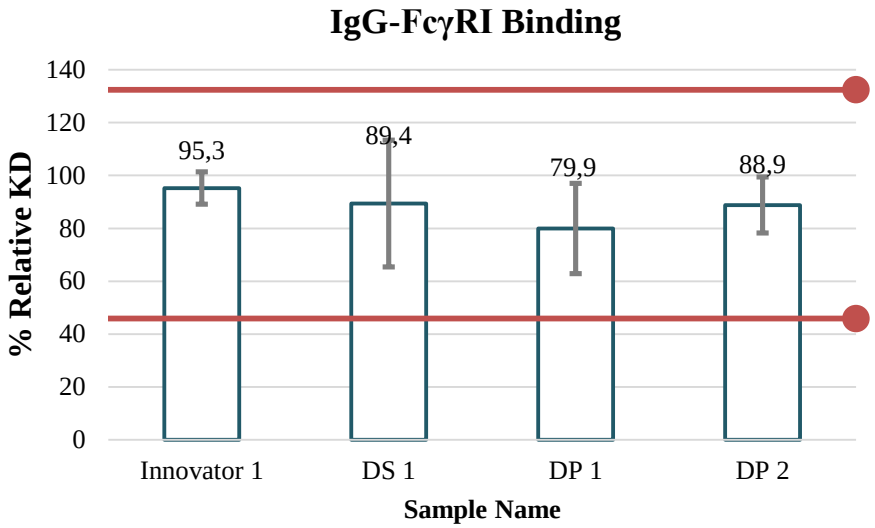


Figure 26. Average relative KD values of the stress-free IgG samples.

In the test of pH 3 24-h samples, the system acceptance criteria were fulfilled (DRS 1 and DRS 2). The Innovator 1 gave a different relative KD value to other samples (12792.2 %) (Figure 27). Therefore the relative KD value it is not shown in the figure. When the results were examined, Innovator 1 gave a lower RMax level compared to other samples (Table 11). Other samples differed slightly from the DRS samples. While the affinity of other samples except for the Innovator 1 is similar and at the E-09 level, the affinity of Innovator 1 is lower and it is at the E-07 level.

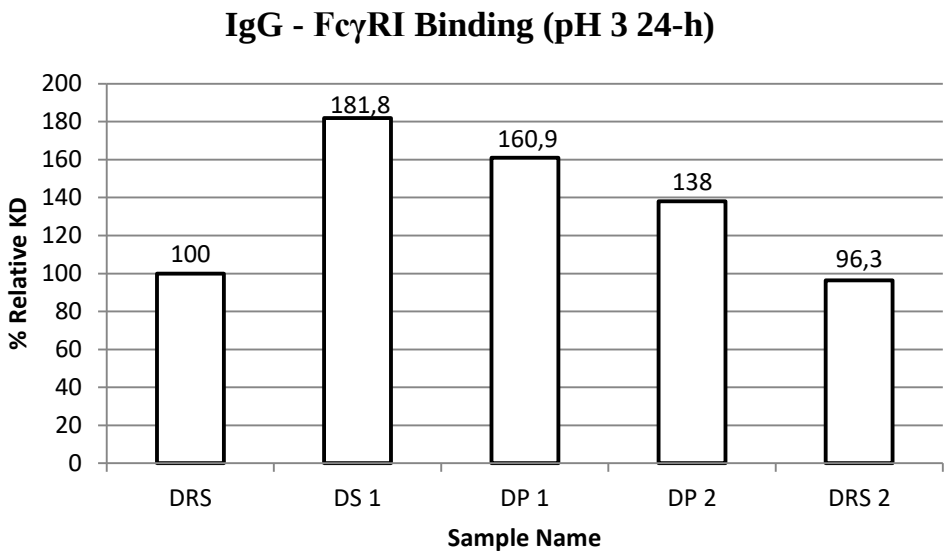


Figure 27. Relative KD values of stressed IgGs binding to FcγRI (pH 3 for 24-h).

Innovator 1's sensorgram show that there's decreased capture of IgG onto the chip surface (Figure 28).

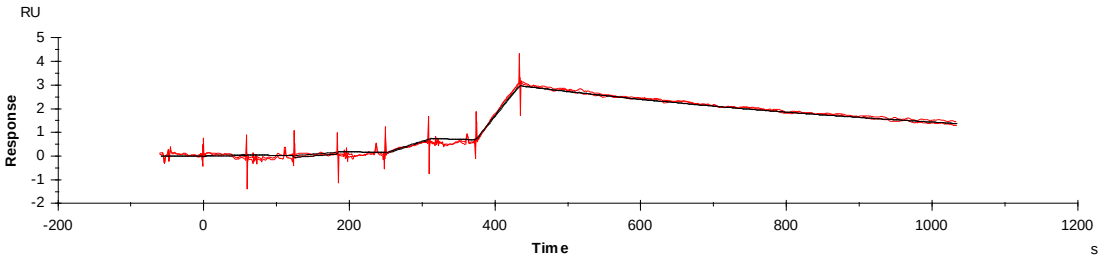


Figure 28. The IgG- FcγRI binding sensorgram of Innovator 1 at pH 3 24-h stress condition.

In the test of pH 3 72-h samples, the system acceptance criteria were fulfilled (DRS 1 and DRS 2). The Innovator 1 gave a different relative KD value to other biosimilars (Table 12). When the results were analyzed, Innovator 1 gave a lower Rmax level compared to the other samples (Table 12). In addition, Rmax levels decreased for all samples according to pH 3 24-h stress conditions. While the affinity of other samples except for the Innovator 1 was similar and at the E-09 level, the affinity of Innovator 1 was higher and it was at the E-10 level.

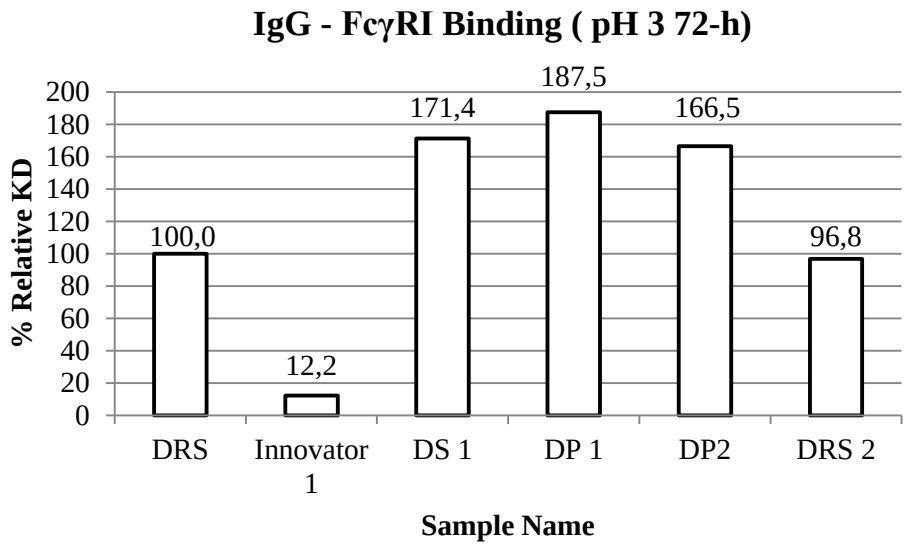


Figure 29. Relative KD values of stressed IgGs binding to FcγRI (pH 3 for 72-h).

The sensorgram of the innovator 1 shows that the IgG capture on the chip surface is reduced (Figure 30).

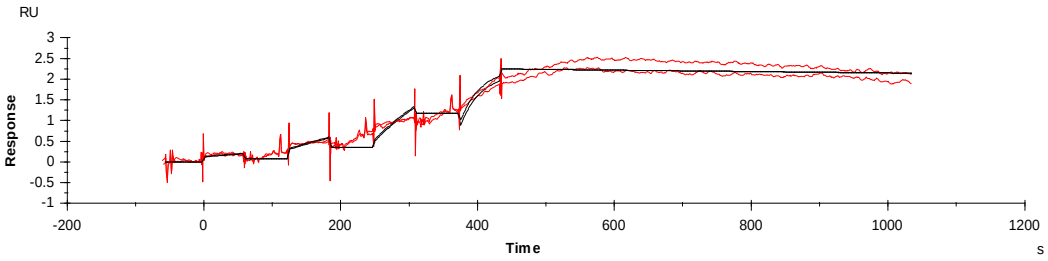


Figure 30. The IgG- FcγRI binding sensorgram of Innovator 1 at pH 3 72-h stress condition.

In the test of pH 9 24-h samples, the system acceptance criteria were fulfilled (DRS 1 and DRS 2). All samples gave a similar and acceptable relative KD value to DRS (Figure 31). The IgG samples exposed to pH 9 24-h stress did not show any extraordinary binding properties. No significant changes were observed at the Rmax level (Table 12). The affinity of all samples is at E-09 level.

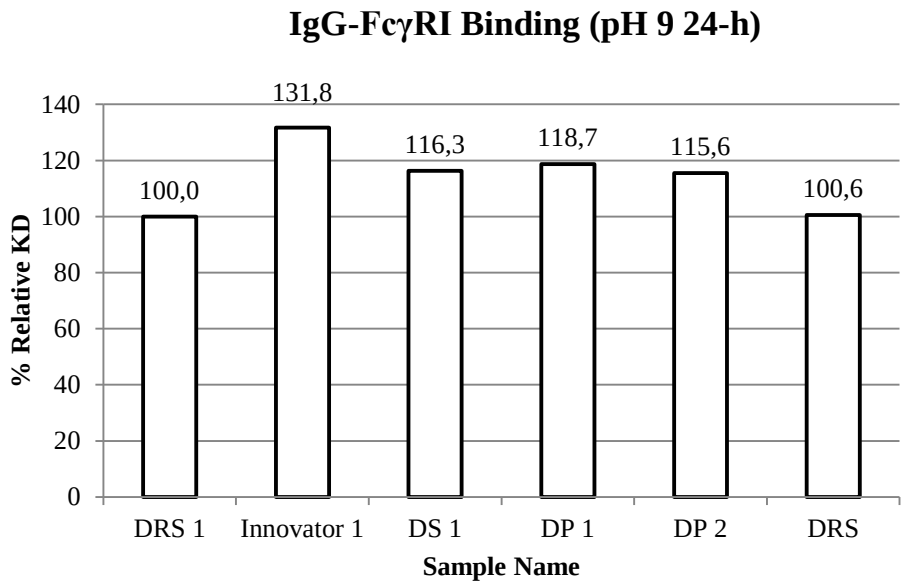


Figure 31. Relative KD values of stressed IgGs binding to FcγRI (pH 9 for 24-h).

In the test of pH 9-72 h samples. the system acceptance criteria were fulfilled (DRS 1 and DRS 2). All samples gave a different relative KD value to DRS (Figure 32). No significant kinetic differences were observed between pH 9 24-h and pH 9 72-h stress conditions. The Rmax levels of these two conditions are very similar to each other (Table 11). The affinity of all samples is at E-09 value.

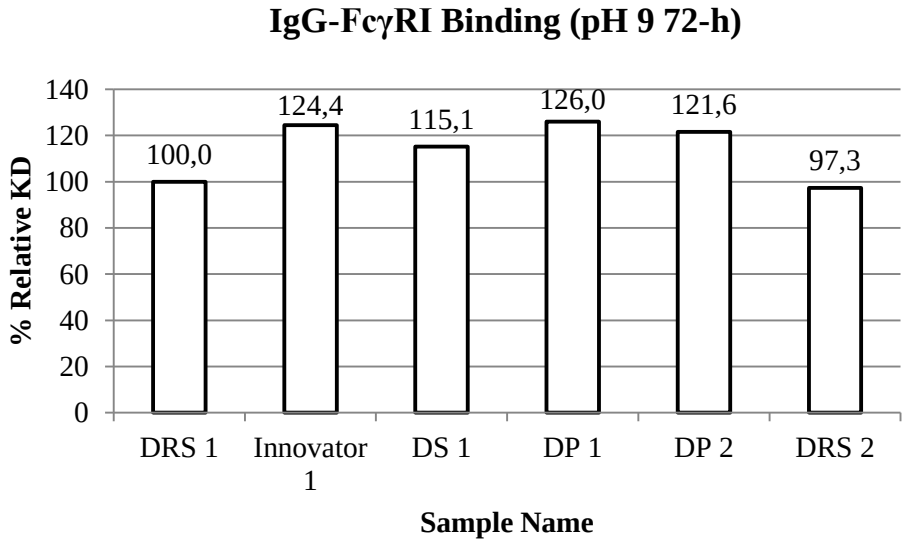


Figure 32. Relative KD values of stressed IgGs binding to FcγRI (pH 9 for 72-h).

While Innovator 1 cannot achieve a good binding at pH 3 24 & 72 hours. Innovator 2 has shown a better binding similarity under these conditions. In the test of Innovator 2 samples. the system acceptance criteria were fulfilled (DRS 1 and DRS 2). When looking at pH 9 relative KD data, no specific change was observed (Figure 33). When the Rmax is examined, it is seen that the antibody capture on the chip under the conditions of pH 3 has a decrease. (Table 13).

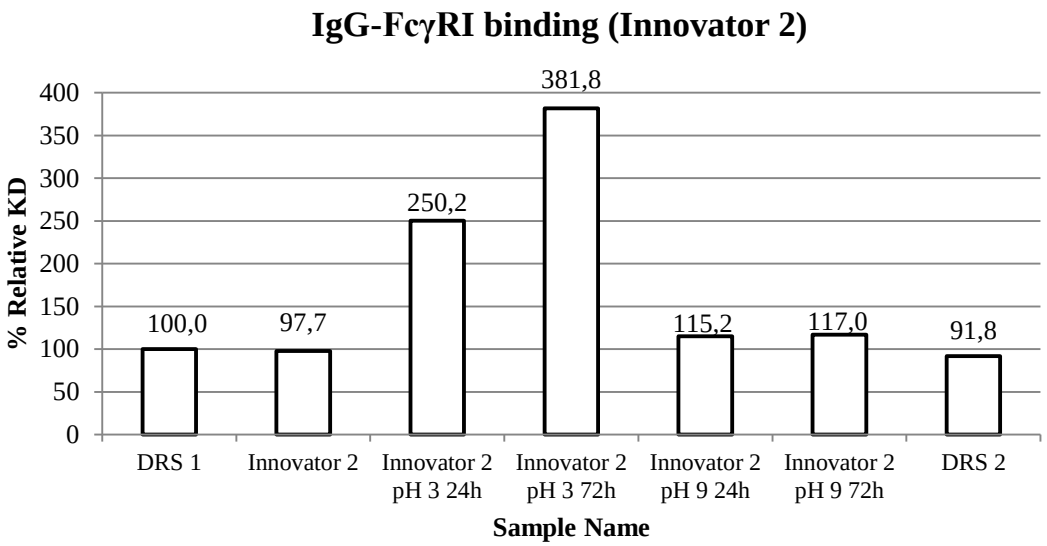


Figure 33. Relative KD values of Innovator 2 binding to FcγRI at different conditions

Table 11. The results of the stress-free IgG- FcγRI experiments are shown in the table.

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	tc	Chi² (RU²)	U-value	% Relative KD	Mean Capture level
DRS 1	2.39E+05	1.48E-03	6.19E-09	252.4	8.49E+17	6.91	1	100.0	189.13
Innovator 1	2.82E+05	1.46E-03	5.18E-09	255	3.93E+18	3.31	1	83.7	253.55
DS 1	2.61E+05	1.48E-03	5.66E-09	240.1	5.55E+17	3.09	1	91.5	249.35
DP 1	3.11E+05	1.44E-03	4.62E-09	256.7	2.92E+18	3.87	1	74.7	247.00
DP 2	2.81E+05	1.46E-03	5.18E-09	253.1	5.56E+18	3.4	1	83.7	250.78
DRS 2	2.50E+05	1.35E-03	5.41E-09	225.8	2.36E+08	7.5	1	87.5	243.39
DRS 1	2.72E+05	8.55E-04	3.14E-09	41.51	7.19E+19	0.124	1	100.0	69.925
Innovator 1	2.74E+05	9.00E-04	3.29E-09	43.83	9.22E+15	1.2	1	104.6	74.525
DS 1	2.83E+05	8.52E-04	3.01E-09	35.47	8.54E+15	0.432	1	95.9	70.15
DP 2	2.88E+05	8.66E-04	3.01E-09	40.77	3.76E+19	0.785	1	95.9	72.65
DRS 2	3.08E+05	8.77E-04	2.84E-09	28.51	3.82E+15	0.331	1	90.5	65.875
DRS 1	2.87E+05	1.19E-03	4.14E-09	55.12	7.50E+15	0.321	1	100	80.55
Innovator 1	2.89E+05	1.17E-03	4.03E-09	48.59	3.40E+15	0.887	1	97.5	78.25
DS 1	3.23E+05	1.08E-03	3.34E-09	50.07	5.71E+19	0.753	1	80.8	74
DP 1	3.02E+05	1.07E-03	3.52E-09	45.46	3.84E+15	0.485	1	85.2	73.025
DP 2	2.82E+05	1.02E-03	3.60E-09	47.18	5.50E+15	1.11	1	87.0	75.475
DRS 2	2.97E+05	1.06E-03	3.57E-09	44.29	7.24E+15	0.495	1	86.3	72.225

Table 12. The results of IgG- FcγRI binding assays of stressed IgG samples are shown in the table.

Stress Condition	Sample Name	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	tc	Chi² (RU²)	U-value	% Relative KD	Mean Capture Level
pH 3 – 24h	DRS-1	4.31E+05	1.11E-03	2.575E-09	297.4	4.64E+17	31.9	1	100.0	242.1
	Innovator 1	3.89E+03	1.28E-03	3.294E-07	82.95	9.79E+07	0.0173	9	12792.2	238.8
	DS 1	2.22E+05	1.04E-03	4.682E-09	194.3	1.07E+16	0.963	1	181.8	236.3
	DP 1	2.46E+05	1.02E-03	4.144E-09	202.2	9.56E+15	2.02	1	160.9	233.5
	DP 2	2.91E+05	1.04E-03	3.556E-09	217.6	1.66E+16	3.93	1	138.1	230.5
	DRS-2	4.38E+05	1.09E-03	2.48E-09	271	7.51E+16	27.3	1	96.3	228.1
pH 3 – 72h	DRS-1	4.46E+05	1.05E-03	2.361E-09	269	4.13E+17	28	1	100.0	231.0
	Innovator 1	2.68E+05	7.74E-05	2.888E-10	2.409	1.25E+13	0.0256	26	12.2	228.8
	DS 1	2.82E+05	1.14E-03	4.046E-09	127.8	1.41E+07	0.247	1	171.4	226.7
	DP 1	2.25E+05	9.97E-04	4.428E-09	146.5	4.64E+07	0.401	1	187.5	223.9
	DP 2	2.70E+05	1.06E-03	3.93E-09	130.8	1.88E+07	0.348	1	166.5	221.8
	DRS-2	4.52E+05	1.03E-03	2.29E-09	243.7	5.55E+17	24.8	1	96.8	220.1
pH 9 – 24h	DRS-1	4.29E+05	1.06E-03	2.46E-09	209.5	2.31E+16	16.4	1	100.0	194.2
	Innovator 1	3.42E+05	1.11E-03	3.24E-09	177.4	1.15E+16	5.03	1	131.8	192.4
	DS 1	3.70E+05	1.06E-03	2.86E-09	184.9	3.03E+15	7.39	1	116.3	190.4
	DP 1	3.67E+05	1.07E-03	2.92E-09	179.8	1.77E+15	6.56	1	118.7	188.6
	DP 2	3.77E+05	1.07E-03	2.85E-09	179.4	1.12E+16	7.49	1	115.6	186.8
	DRS-2	4.32E+05	1.07E-03	2.48E-09	193.5	1.75E+18	13.3	1	100.6	185.5
pH 9 – 72h	DRS-1	4.57E+05	1.09E-03	2.39E-09	202	4.00E+17	19.7	1	100.0	189.3
	Innovator 1	3.65E+05	1.09E-03	2.98E-09	171	1.27E+16	6.16	1	124.4	187.1
	DS 1	3.83E+05	1.05E-03	2.75E-09	172.2	1.80E+16	8.27	1	115.1	184.9
	DP 1	3.54E+05	1.07E-03	3.01E-09	163.1	1.26E+16	4.73	1	126.0	184.6
	DP 2	3.72E+05	1.08E-03	2.91E-09	166.7	1.08E+16	6.43	1	121.6	183.7
	DRS-2	4.61E+05	1.07E-03	2.33E-09	186.6	5.61E+17	16.2	1	97.3	182.9

Table 13. The results of IgG- FcγRI binding assays of Innovator 2 samples are shown in the table.

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	tc	Chi² (RU²)	U-value	% Relative KD	Mean Capture level
DRS 1	4.11E+05	1.15E-03	2.78E-09	218.6	2.17E+16	16.4	1	100.0	219.125
Innovator 2	4.19E+05	1.14E-03	2.72E-09	217	1.08E+21	16.4	1	97.7	218.8
Innovator 2 pH 3 24-h	1.86E+05	1.29E-03	6.97E-09	92.97	1.36E+07	0.266	1	250.2	218.275
Innovator 2 pH 3 72-h	9.98E+04	1.06E-03	1.06E-08	84.36	5.03E+07	0.0642	1	381.8	217.75
Innovator 2 pH 9 24-h	3.48E+05	1.12E-03	3.21E-09	180.7	2.22E+15	6.97	1	115.2	217.45
Innovator 2 pH 9 72-h	3.60E+05	1.17E-03	3.26E-09	182.6	1.17E+16	4.87	1	117.0	217.075
DRS 2	4.17E+05	1.07E-03	2.56E-09	194	3.66E+17	14.9	1	91.8	216.35

4.2.3 IgG - FcγRIIIa Binding

IgG - FcγRIIIa binding is described in the method section. Mean values of the stress free samples obtained values were taken and samples were compared (Table 14) The horizontal bars show the ± 3 standard deviation corridor.

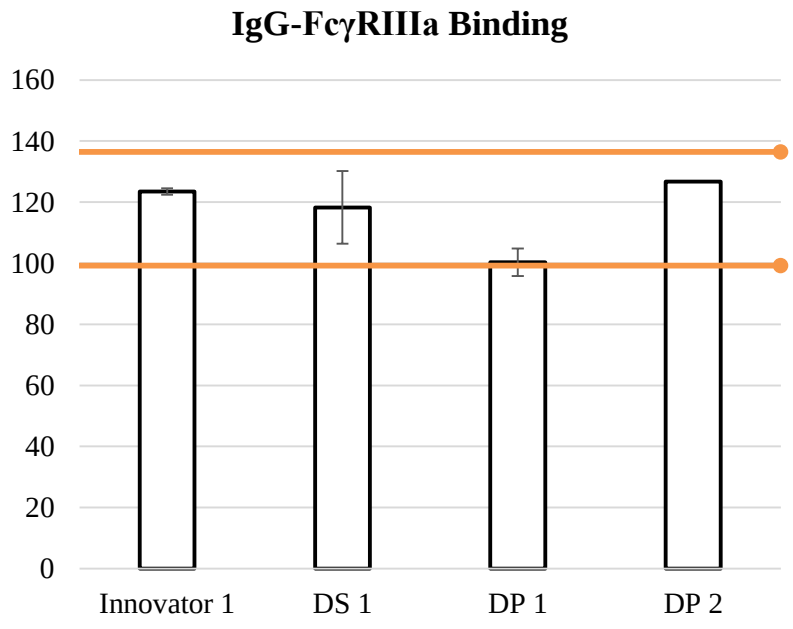


Figure 34. Average relative KD values of the stress-free IgG samples.

In the test of pH 3 24-h samples. the assay acceptance criteria were fulfilled (DRS 1 and DRS 2). The Innovator 1 and DP 2 gave a different relative KD value to other samples (Figure 35). Although the relative KD's of these two samples are similar, but their sensorgrams are different (Figure 36 and Figure 37). When the results were examined, Innovator 1 gave a lower RMax level compared to other samples (Table 15) The affinity in the stress-free experiments of the samples was about E-07 for all samples. The affinity of Innovator 1 and DP 2 was increased at pH 3 24-h (E-08).

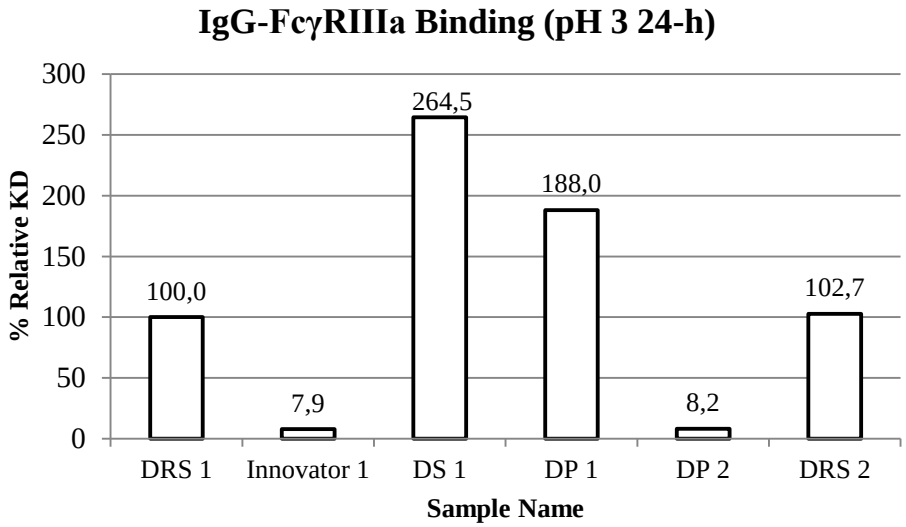


Figure 35. Relative KD values of stressed IgGs binding to FcγRIIIa (pH 3 for 24-h).

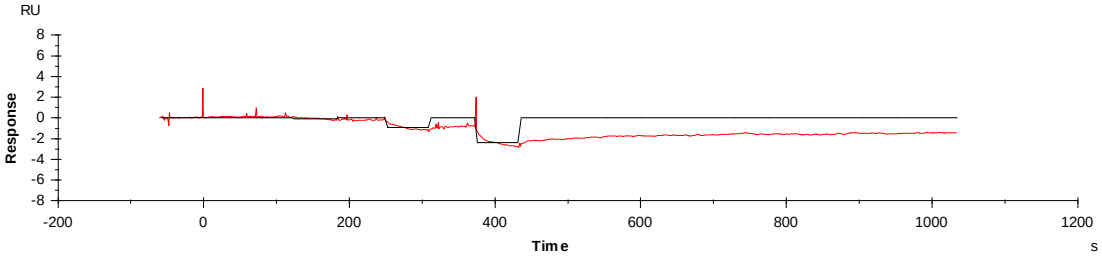


Figure 36. The IgG- FcγRIIIa binding sensorgram of Innovator 1 at pH 3 24-h stress condition

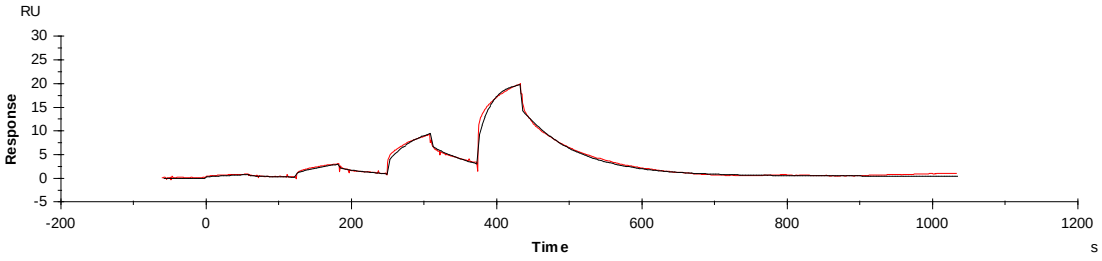


Figure 37. The IgG- FcγRIIIa binding sensorgram of DP 2 at pH 3 24-h stress condition

In the test of pH 3-72 h samples, the system acceptance criteria were fulfilled (DRS 1 and DRS 2). All stressed samples gave a different relative KD value to DRS (Figure 38). When the results were analyzed, Innovator 1 and DS 1 gave a lower Rmax level compared to the other samples (Table 15). At pH 3 72-h, the affinity of Innovator 1, DP 1 and DP 2 increased to E-06 levels from E-07 level.

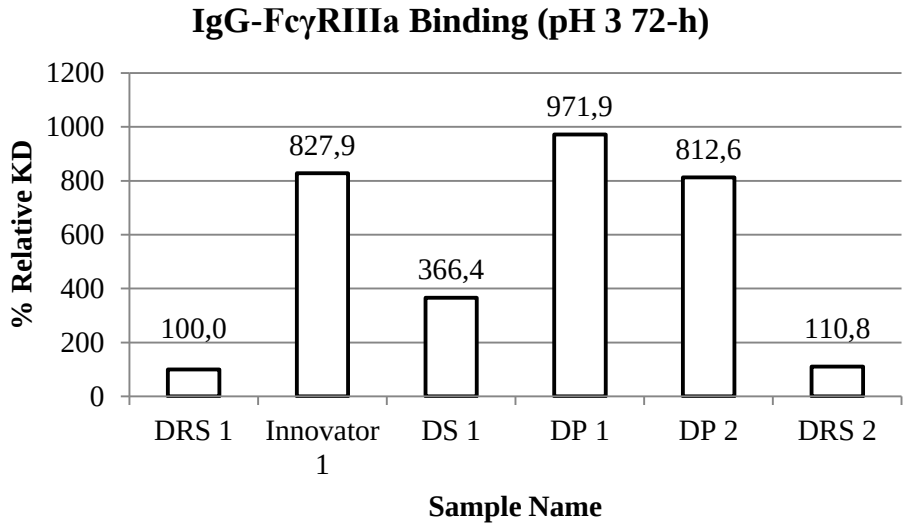


Figure 38. Relative KD values of stressed IgGs binding to FcγRIIIa (pH 3 for 72-h).

In the test of pH 9 24-h samples, the system acceptance criteria were fulfilled (DRS 1 and DRS 2). All samples gave a similar and acceptable relative KD value to DRS (Figure 39). The IgG samples exposed to pH 9 24-h stress did not show any extraordinary binding properties. No significant changes were observed at the Rmax level (Table 15). The affinity of all samples was approximately at E-07 level.

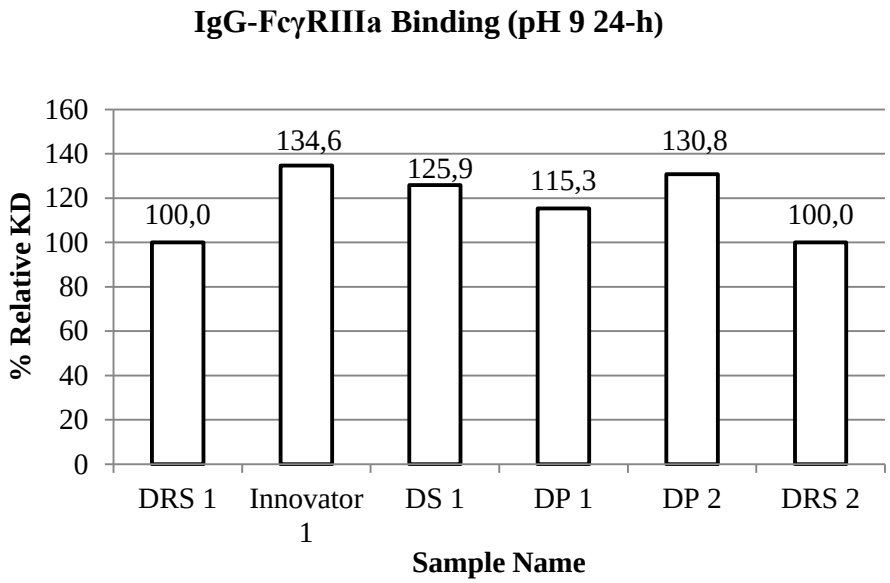


Figure 39. Relative KD values of stressed IgGs binding to FcγRIIIa (pH 9 for 24-h).

In the test of pH 9 72-h samples. the system acceptance criteria were fulfilled (DRS 1 and DRS 2). All samples gave a different relative KD value to DRS (Figure 40). No significant kinetic differences were observed between pH 9 24-h and pH 9 72-h stress conditions. The Rmax levels of these two conditions are very similar to each other. Although the pH stress time increases. the binding of stressed samples is better than the data obtained in 24 hours (Table 15).

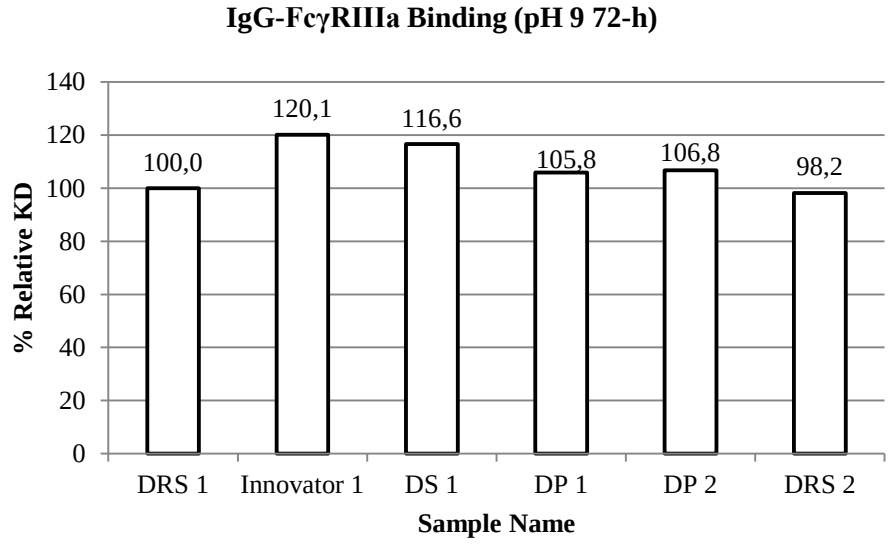


Figure 40. Relative KD values of stressed IgGs binding to FcγRIIIa (pH 9 for 72-h).

As in previous experiments, all stress conditions were performed in another innovator (Innovator 2) and examined in the same experimental set. In the test of Innovator 2 sample, the system acceptance criteria were fulfilled (DRS and DRS 2). As it is apparent from the same set of experiments, Innovator 2 could not show a similar binding to DRS under conditions of pH 3 (Figure 41). In pH 9 conditions, the binding properties are much more similar than those of DRS. Furthermore, when the Rmax values are examined, it is seen that the pH 3 values are lower than the others (Table 16). It has been observed that the affinity of innovator 2 in pH 3 24-h increased compared to the stress-free conditions and decreased to 24 hours in 72 hours, but still higher than in stress-free samples.

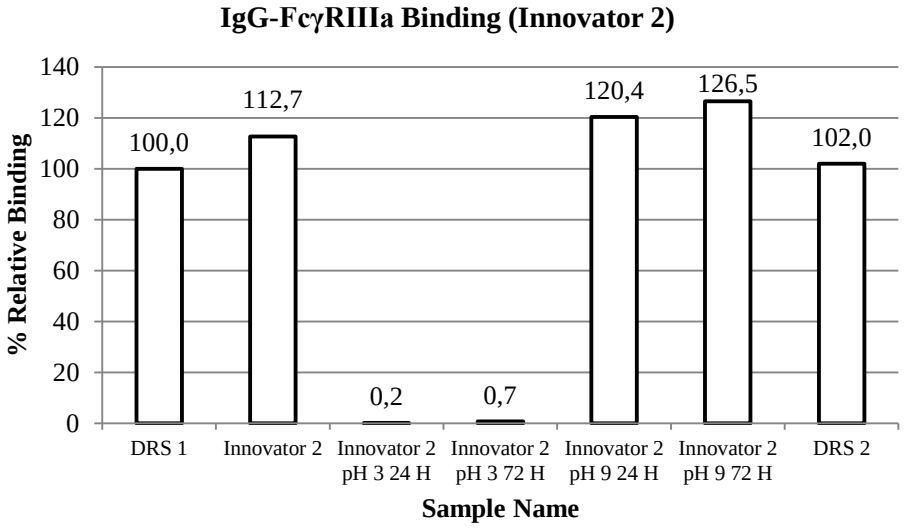


Figure 41. Relative KD values of Innovator 2 binding to FcγRIIIa at different conditions.

Table 14. The results of the stress-free IgG- FcγRIIIa experiments are shown in the table.

Sample ID	ka1 (1/Ms)	kd1 (1/s)	ka2 (1/s)	kd2 (1/s)	KD (M)	Rmax (RU)	tc	Chi² (RU²)	U-value	% Relative KD	Mean Capture Level
DRS 1	1.09E+05	6.75E-02	1.91E-02	1.09E-02	2.26E-07	31.84	1.04E+20	0.277	N/A	100	29.55
Innovator 1	7.21E+04	2.51E-02	2.22E-03	8.46E-03	2.76E-07	30.87	1.62E+21	0.274	N/A	122.3	30.65
DS 1	1.95E+05	1.94E-01	2.57E-02	1.08E-02	2.94E-07	32.12	4.86E+20	0.185	N/A	130.2	29.1
DP 2	1.65E+05	1.44E-01	2.18E-02	1.00E-02	2.75E-07	32.46	1.19E+20	0.269	N/A	121.9	29.65
DRS 2	1.74E+05	1.32E-01	2.62E-02	1.11E-02	2.26E-07	31.32	9.91E+20	0.235	N/A	100.2	28.1
DRS 1	4.83E+04	1.22E-02	9.30E-06	2.22E-02	2.53E-07	13.09	7.44E+08	0.179	N/A	100	15.2
Innovator 1	1.44E+05	1.27E-01	4.01E-02	2.23E-02	3.16E-07	16.73	6.56E+19	0.122	N/A	124.8	15.55
DS 1	9.37E+04	6.87E-02	2.17E-02	1.26E-02	2.70E-07	14.14	3.32E+12	0.0814	N/A	106.4	15.2
DP 2	2.04E+05	2.35E-01	3.01E-02	1.21E-02	3.29E-07	16.39	1.38E+20	0.0522	N/A	129.9	15.35
DRS 2	1.50E+05	1.30E-01	3.13E-02	1.27E-02	2.51E-07	15.48	9.39E+20	0.0991	N/A	99.1	15.05
DRS 1	2.82E+05	2.80E-01	3.28E-02	1.24E-02	2.73E-07	20.18	1.62E+15	0.0881	N/A	100	18.8
Innovator 1	2.43E+05	2.70E-01	4.59E-02	2.00E-02	3.37E-07	20.59	1.30E+17	0.16	N/A	123.31	19
DP 2	3.39E+05	3.87E-01	2.72E-02	1.20E-02	3.51E-07	20.86	9.76E+11	0.0644	N/A	128.47	18.8
DRS 2	2.86E+05	2.80E-01	3.51E-02	1.26E-02	2.59E-07	18.96	3.77E+15	0.0875	N/A	94.87	18.3

Table 15. The results of the stressed IgG- FcγRIIIa experiments are shown in the table.

Stress Condition	Sample ID	ka_1 (1/Ms)	kd_1 (1/s)	ka_2 (1/s)	kd_2 (1/s)	KD (M)	Rmax (RU)	tc	Chi^2 (RU^2)	U-value	%Relative KD	Mean Capture Level
pH 3 – 24-h	DRS-1	2.46E+05	2.15E-01	3.19E-02	1.14E-02	2.30E-07	37.56	1.68E+17	0.281	N/A	100.0	25.9
	Innovator 1	2.25E+06	1.27E-01	1.37E-02	6.38E-03	1.81E-08	0.00289	1.00E+08	1.76	N/A	7.9	25.4
	DS 1	1.32E+05	2.93E-01	2.53E-02	9.53E-03	6.08E-07	27.96	1.50E+21	0.118	N/A	264.5	25.5
	DP 1	1.32E+05	2.39E-01	3.08E-02	9.70E-03	4.32E-07	27.13	9.77E+08	0.157	N/A	188.0	25.7
	DP 2	2.17E+04	1.32E-02	1.83E-04	5.86E-06	1.89E-08	19.41	1.30E+08	0.166	N/A	8.2	25.7
	DRS-2	3.70E+05	3.65E-01	3.91E-02	1.23E-02	2.36E-07	38.1	1.85E+07	0.189	N/A	102.7	25.7
pH 3 – 72-h	DRS-1	5.02E+04	1.37E-02	7.76E-04	1.96E-03	1.96E-07	30.31	3.65E+20	0.475	N/A	100.0	26.7
	Innovator 1	4.42E+05	7.35E-01	3.31E-03	1.21E-01	1.62E-06	0.01494	1.01E+08	1.53	N/A	827.9	26.6
	DS 1	6.34E+04	4.61E-02	4.12E-05	3.00E-03	7.17E-07	3.346	1.00E+08	5.49	N/A	366.4	26.6
	DP 1	1.29E+04	9.62E-02	8.16E-03	2.80E-03	1.90E-06	28.92	4.46E+14	0.112	N/A	971.9	26.9
	DP 2	1.38E+04	2.19E-02	2.73E-06	4.56E-03	1.59E-06	14.64	1.00E+08	1.17	N/A	812.6	27.0
	DRS-2	2.41E+05	1.95E-01	3.04E-02	1.12E-02	2.17E-07	37.5	1.22E+19	0.317	N/A	110.8	27.2
pH 9 – 24-h	DRS-1	2.58E+05	2.25E-01	3.42E-02	1.14E-02	2.18E-07	43.94	1.00E+20	0.332	N/A	100.0	30.9
	Innovator 1	1.91E+05	1.81E-01	4.21E-02	1.88E-02	2.93E-07	40.92	2.90E+13	0.276	N/A	134.6	31.0
	DS 1	2.53E+05	2.57E-01	2.84E-02	1.05E-02	2.74E-07	40.54	2.93E+19	0.285	N/A	125.9	31.0
	DP 1	2.41E+05	2.31E-01	3.01E-02	1.07E-02	2.51E-07	40.71	1.73E+19	0.318	N/A	115.3	31.1
	DP 2	2.40E+05	2.51E-01	2.86E-02	1.07E-02	2.85E-07	39.63	4.32E+13	0.252	N/A	130.8	31.0
	DRS-2	2.53E+05	2.18E-01	3.31E-02	1.13E-02	2.18E-07	43.28	3.77E+20	0.374	N/A	100.0	31.0
pH 9 – 72-h	DRS-1	2.53E+05	2.10E-01	3.39E-02	1.14E-02	2.09E-07	50.16	9.68E+11	0.476	N/A	100.0	36.2
	Innovator 1	2.33E+05	1.94E-01	4.52E-02	1.95E-02	2.51E-07	47.92	3.21E+11	0.372	N/A	120.1	36.2
	DS 1	2.68E+05	2.45E-01	2.87E-02	1.04E-02	2.44E-07	45.17	2.46E+19	0.4	N/A	116.6	36.1
	DP 1	2.20E+05	1.83E-01	2.91E-02	1.05E-02	2.22E-07	44.5	1.32E+20	0.492	N/A	105.8	35.8
	DP 2	2.97E+05	2.48E-01	2.81E-02	1.03E-02	2.24E-07	45.09	6.97E+18	0.433	N/A	106.8	35.9
	DRS-2	2.53E+05	2.08E-01	3.37E-02	1.12E-02	2.06E-07	49.06	3.65E+09	0.467	N/A	98.2	35.7

Table 16. The results of IgG- FcγRIIIa binding assays of Innovator 2 samples are shown in the table.

Sample ID	ka1 (1/Ms)	kd1 (1/s)	ka2 (1/s)	kd2 (1/s)	KD (M)	Rmax (RU)	tc	Chi² (RU²)	U-value	% Relative KD	Mean Capture Level
DRS 1	2.19E+05	1.90E-01	2.88E-02	1.10E-02	2.39E-07	29.78	1.37E+21	0.214	N/A	100.00	26.25
Innovator 2	2.60E+05	2.32E-01	4.48E-02	1.94E-02	2.69E-07	31.16	6.57E+08	0.152	N/A	112.67	26.25
Innovator 2 pH 3 24-h	2.34E+05	6.84E-03	5.84E-03	7.79E-05	3.85E-10	13.57	8.43E+13	0.182	N/A	0.16	26.1
Innovator 2 pH 3 72-h	4.31E+05	9.51E-02	2.60E-02	1.94E-04	1.63E-09	18.4	3.20E+22	0.25	N/A	0.68	26.0
Innovator 2 pH 9 24-h	2.03E+05	1.88E-01	4.15E-02	1.87E-02	2.88E-07	28.39	7.56E+18	0.165	N/A	120.41	25.9
Innovator 2 pH 9 72-h	1.69E+05	1.50E-01	3.55E-02	1.83E-02	3.03E-07	27.54	1.32E+14	0.155	N/A	126.52	25.7
DRS 2	2.16E+05	1.98E-01	3.07E-02	1.12E-02	2.44E-07	28.44	4.21E+16	0.192	N/A	101.97	256

5. DISCUSSION AND CONCLUSION

Monoclonal antibodies are one of the most important class of biological molecules that attracted significant attention especially in recent years. In the discovery phase of mAbs, firstly the mechanisms of action (MoA) of antibodies are identified which helps the emergence of biotherapeutic drugs. Biotherapeutic drugs have been the hope for most diseases such as cancer, immunological disorders, and inflammatory diseases and their share is increasing day by day in the pharmaceutical market. Functional characterization of the mAbs is an important step in both process development and drug release. Binding analysis is one of the most important functional characterization which needs to be optimized.

Biopharmaceuticals are complex molecules with a wide variety of functional groups. These molecules may be exposed to different environmental conditions and stresses during the process, transportation and storage. For this reason, different types of degradation may occur in the functional groups or the molecules themselves. These degradations can cause imbalances and may pose a risk to the effects of the therapeutics in the final form. Forced degradation studies can be helpful to understand the stability of a molecule. Different stress conditions are frequently used for this purpose. Among them, pH-dependent stress is one of the forced degradation study. Primary amino acid sequences and secondary structures of monoclonal antibodies may vary or degrade depending on the stress conditions. Studies have shown that the degradation of proteins in the liquid medium is pH dependent process. In connection with this, determination of the pH range where proteins are stable is important to ensure stability (88).

Anti-TNF-α antibody in IgG1 structure is a biosimilar therapeutic product which is developed by Turgut İlaçları A.Ş. DRS, DS 1, DP 1 and DP 2 was produced by Turgut İlaçları A.Ş, is the biosimilar of the commercial product (Innovator 1 and Innovator 2) which has an IgG1 subtype. A wide range of results were obtained using two different commercial products and three different biosimilars candidates.

This study aimed in understanding the binding behaviors of an IgG1 subtype therapeutic monoclonal antibody under different pH stress conditions.

The experiments were performed to compare the binding kinetics of the antibody with an antigen (TNF-α) and the receptors (FcγRs). To analyze the binding kinetics of biomolecular interactions, an optical biosensor-SPR (Biacore T200, GE Healthcare) was used. The experiments were divided into two groups, namely stressed samples and stress-free samples. Firstly, IgG-TNF-α, IgG-FcγRI and IgG-FcγRIIIa binding studies have been optimized. Then, chemical deamidation stress was applied to the IgG samples except DRS. The binding assays were carried out with IgG samples subjected to stress without any change in the optimized method.

The study revealed that pH-dependent and time-dependent stress conditions resulted in various structural changes in the antibody. There is no official source and protocol for the selection and duration of stress conditions for therapeutic antibodies (89). Based on the previous published data, pH 6 solution is accepted as the best condition for a stable IgG1 (90). With this fact in mind, pH studies were planned to be performed at a pH 6±3. In order to be able to observe meaningful time-dependent changes in acidic and basic pH conditions, temperature kept at 37 °C when determining the degradation of biopharmaceutical molecules (90).

In the first step, the capture of IgG on the sensor chip surface was achieved by protein A/G. CM5 sensor chip has carboxymethyl dextran matrix and it carries a negative charge at values above pH 3.5 (85). Therefore, the pH of the sodium acetate used as immobilization solution should be higher than this level. In pH scouting, it was decided that the best level for protein A/G immobilization in CM5 chip was sodium acetate pH 4. For anti histidine antibody immobilization, pH-scouting was not performed. The immobilization was carried out with the sodium acetate pH 4.5 recommended by the His Capture Kit.

The binding kinetic analysis of IgG-TNF-α at pH 3 and after 24-h and 72-h stress conditions have shown that there was no significant changes in the relative binding of the antigen. However, the capture level of the mAb was slightly decreased at pH 3 compared to all other conditions except for Innovator 1. In the case of

Innovator 1, after incubation with stress condition this mAb could not bind by Protein A/G. These capture level decreases resulted in a Rmax level decrease. According to the results obtained from pH 9 72-h stress conditions, slight changes in KD values were observed. When the results were analyzed in detail, it was observed that this change was caused by the dissociation rate in correlation with the incubation time to pH solution but the association rate was not affected. It is believed that the change in conditions resulting from stress at pH 9 72-h does not affect the binding behavior between the antibody and the antigen, however, the dissociation behavior is affected by these conditions as shown in Table 8. Normally, the U-values of the results are expected to be less than 10, but this value is expected to be 1 to 2 for the quality of the data. When the U-value of the pH 9 72-h result was examined, it was seen that the values were accepted for the experiment but not at the desired level.

To understand this dramatic change in capture and Rmax level of Innovator 1, we have analyzed this sample with a size exclusion ultra-performance liquid chromatography (SE-UPLC). The molecular sizes can be determined by comparison of the retention time to standards of known molecular weights [91]. Incubation of Innovator 1 at pH 3 resulted in increased % low molecular weight (LMW) peak area and decreased % monomer peak area. The monomer peak was entirely degradaded. The incubation of Innovator 1 with pH 9 increased the formation of high molecular weight (HMW) HMW2, LMW1, and LMW2. The monomer peak area % was decreased.

For further investigation, LMW of Innovator 1 was analyzed by both non-reduced and reduced CE-SDS (capillary electrophoresis sodium dodecyl sulfate) that involves heat denaturing of protein in the presence of SDS. The denatured sample is separated by size in a capillary, which provides the sieving selectivity for the separation. For non-reduced, incubation of Innovator 1 at pH 3 increased fragment peaks area %. Incubation of Innovator 1 at pH 3 dramatically decreases % IgG peak area and increased in % HH peak area. At end of the chemical deamidation stress, almost the entire IgG peak was degraded. In pH 9 stress condition indicated that a slight decreasing was observed in % IgG peak area.

For reduced condition, heavy chain (HC) ratio of Innovator 1 was dramatically decreased with increasing incubation time where other peaks that are corresponding to middle molecular weight (MMW) fragments were increased. % light chain (LC) peak area of Innovator 1 was dramatically decreased with increasing pH 3 treatment and total degradation was observed at the end of the treatment. In pH 9 stress condition indicated slight decreasing in % LC and HC peak area.

The effect of any stress on biological mechanisms depends on the amino acid sequence motif and levels of modifications. A change in the Fc region may not directly affect the binding affinity of Fab region. Based on this fact, changes in the Fab region may not make a difference in the functions of the Fc region (92). For this reason, an antigen – antibody binding analysis methods were designed and the FcγR receptor binding were evaluated too.

There are differences between the variability of FcγRIIIa to IgG binding, FcγRIIIa-158V and FcγRIIIa-158F. *in vitro* studies on the ADCC anaylsis have shown that the cells with homozygous FcγRIIIa-158V have higher yields than those with homozygous FcγRIIIa-158F or heterozygous FcγRIIIa-158V/F. Therefore, we have chosen FcγRIIIa-158V variant in our experiments (93-95). In the interaction of FcγRIIIa-IgG, there are actively two binding sites on IgG. Studies have shown that the CH_3 region affects the binding while CH_2 region affects both the binding and signaling pathway (96).

The binding kinetic analysis with IgG–FcγRI of pH 3 24-h and 72-h stress conditions has shown that the KD values were not significantly changed but the Rmax level changed after the incubation time of the pH 3 was changed. IgG and FcγRI binding affinities were not affected in pH 9 stress condition. In IgG – FcγRIIIa pH 3 stress conditions has shown that the affinities and the Rmax levels were decreased in a correlation with the exposure time to the stress condition. FcγRIIIa binding affinities were not affected in pH 9 stress condition.

In the pH 3 experiments of all the experimental sets, although the software provided a KD value, when the results were examined it was seen that Innovator 1 was not bound to Protein A/G or FcγR on chip surface. These outcomes were

obtained according to the mean capture level and Rmax levels of these assays. In the pH 9 experiments of all the experimental sets, there were a slight change in the KD values but there was not a dramatically change in the capture level or Rmax level. For this reason Innovator 2 stress analysis were added to the sample sets and these results have demonstrated that it has a similar behavior as the biosimilar product batches.

When the results were examined, at pH 3 level, U-value and Rmax levels of Innovator 1 and FcγRI binding has shown similar properties with IgG-TNF-α binding results. Depending on the pH incubation time, U-value of Innovator 1 was increased and the Rmax level was decreased. For Innovator 2, after stress conditions the Rmax level showed a similar behavior but there was no change on U-value.

For FcγRIIIa binding, since U-value score is a property of 1: 1 binding, it is not possible to compare U-value in this section. Two-state binding model was used for FcγRIIIa binding analysis. For this reason, only KD values, Rmax and Chi^2 scores could compared. When Chi^2 values were considered at pH 3, Innovator 1 was determined outside of the desired range where Innovator 2 Chi^2 values were accepted.

As a further study of this thesis might be to analyze deamidation amount and sites under pH stress conditions. Deamidation is the most observed modification among the all others. Simply, deamidation is a chemical reaction where the amide functional group on the side chains of asparagine (Asn) and glutamine (Gln) residues is converted to the carboxylic acid groups (103). Due to this reaction; changes in the structure, function, stability and conformation of the protein/peptide may occur which causes negative effects and breakdown of the structure. The degree of deamidation is dependent upon a number of factors, such as the primary sequence of the protein and high order structure, pH, temperature and the chemicals in the solution (98). According to the literature, both Protein A/G and FcγRIIIa interact with the CH_2 and CH_3 domain of the Fc portion of the antibody whereas FcγRI interacts only with the CH_2 domain (95,96,99,100).

To understand the molecular binding properties of the antibodies to antigen and FcγR's different pH stress conditions were applied as a part of forced degradation studies. Our results show that due to the different binding sites on the antibodies these analysis showed different results. For all experiments, a remarkeble deterioration in pH 3 conditions was observed. In the conditions of pH 9, a dramatic change did not generally occur, but only a significant change was observed in IgG-TNF-α binding in after 72 hours. The relative KD values of IgG-TNF-α and IgG-FcγR bindings are similar in the same stress conditions, whereas the change of the relative KD values of IgG- FcγRIIIa binding is striking. In further studies, it can be understood that the change in amino acids affects binding properties.

According to these results we have seen that these stress conditions have different effects on antigen, FcγRI and FcγRIIIa binding affinities. This can be further investigated with a deamidation analysis on these stressed samples by peptide mapping. It can be speculated that possible demiadion on the molecule may have affect the high order structure of the monoclonal antibody under various stress conditions.

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APPENDIX :

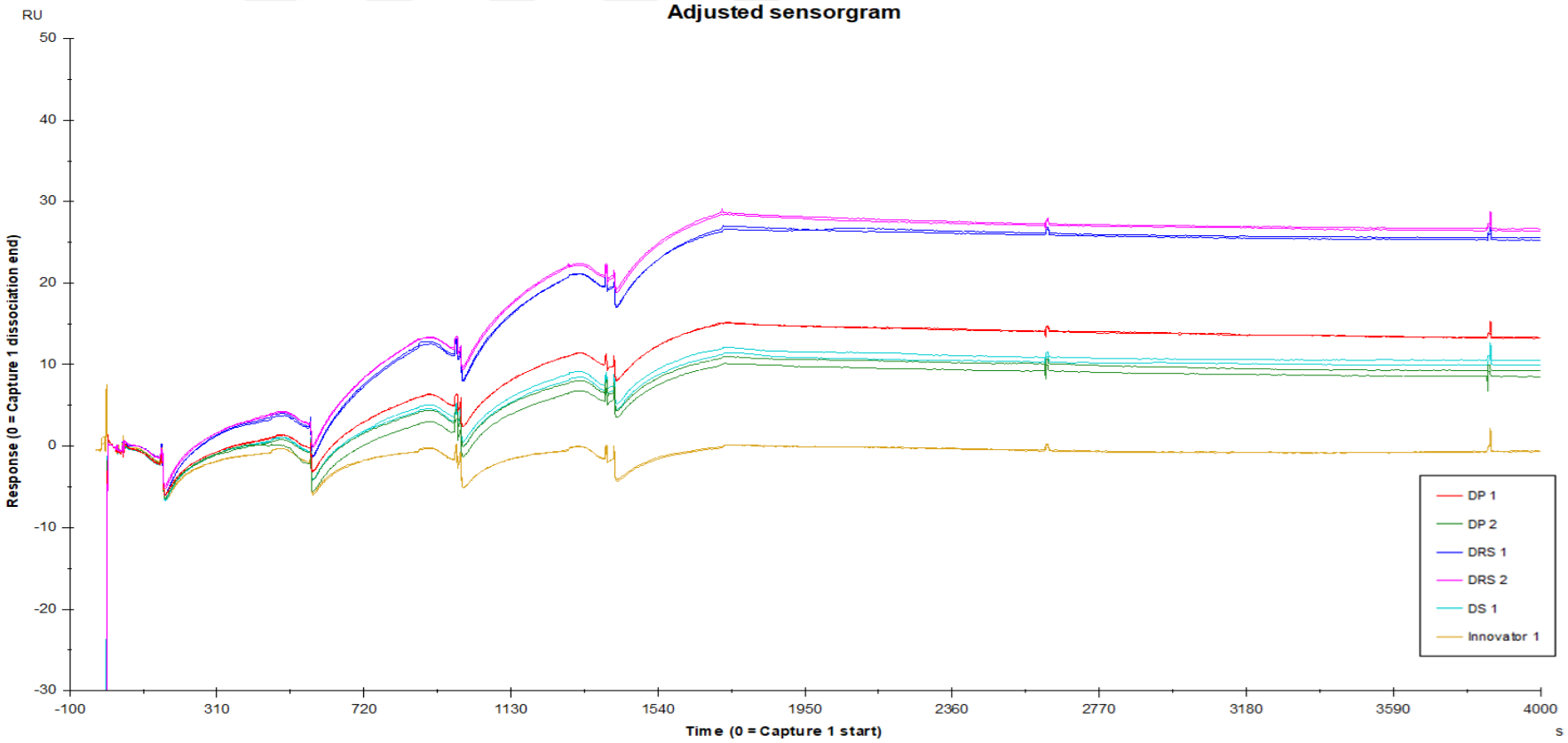

Figure 42. IgG-TNF-α binding sensorgram. Samples under pH 3 24-h stress condition

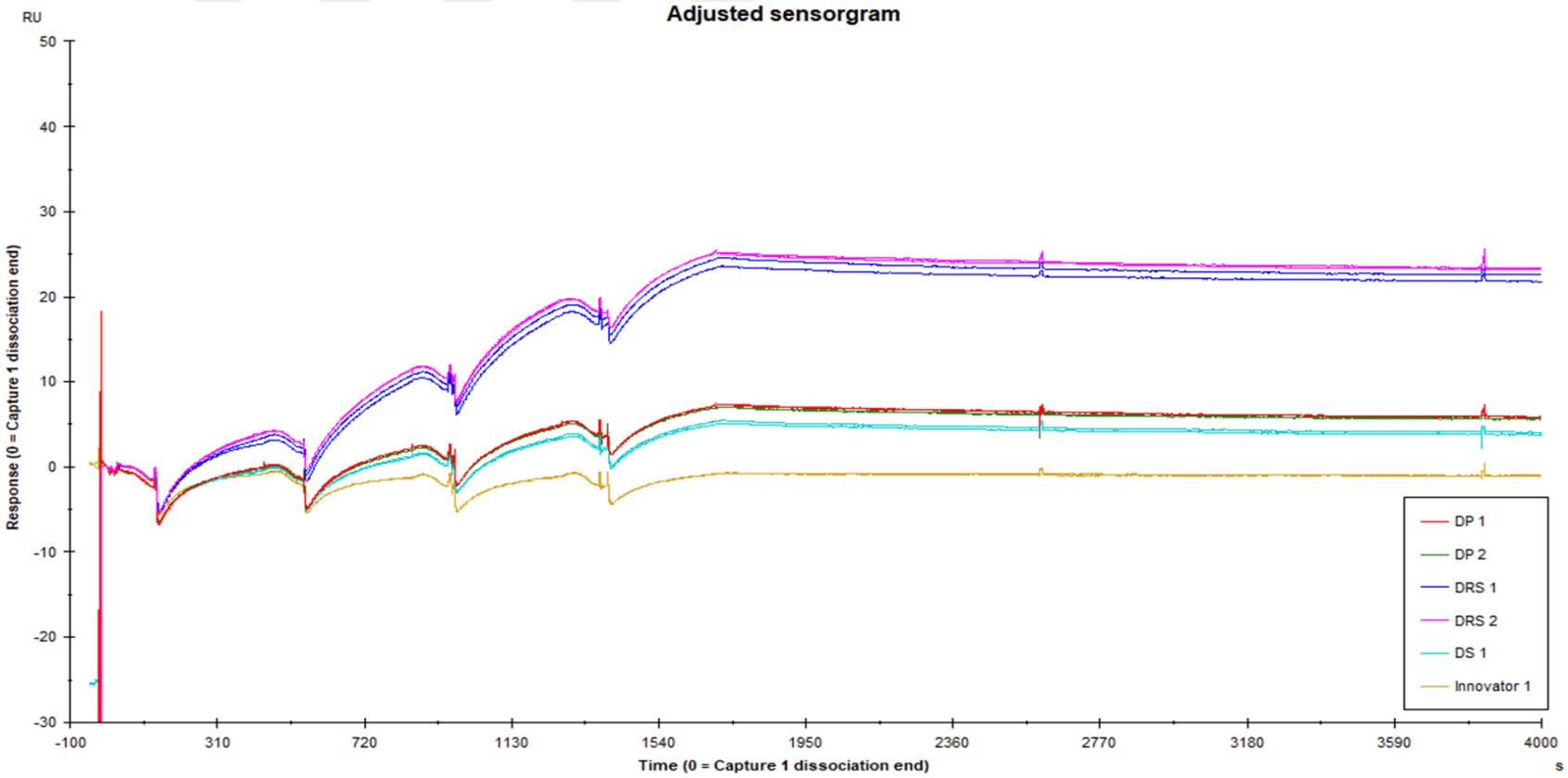

Figure 43. IgG-TNF-α binding sensorgram. Samples under pH 3 72-h stress condition

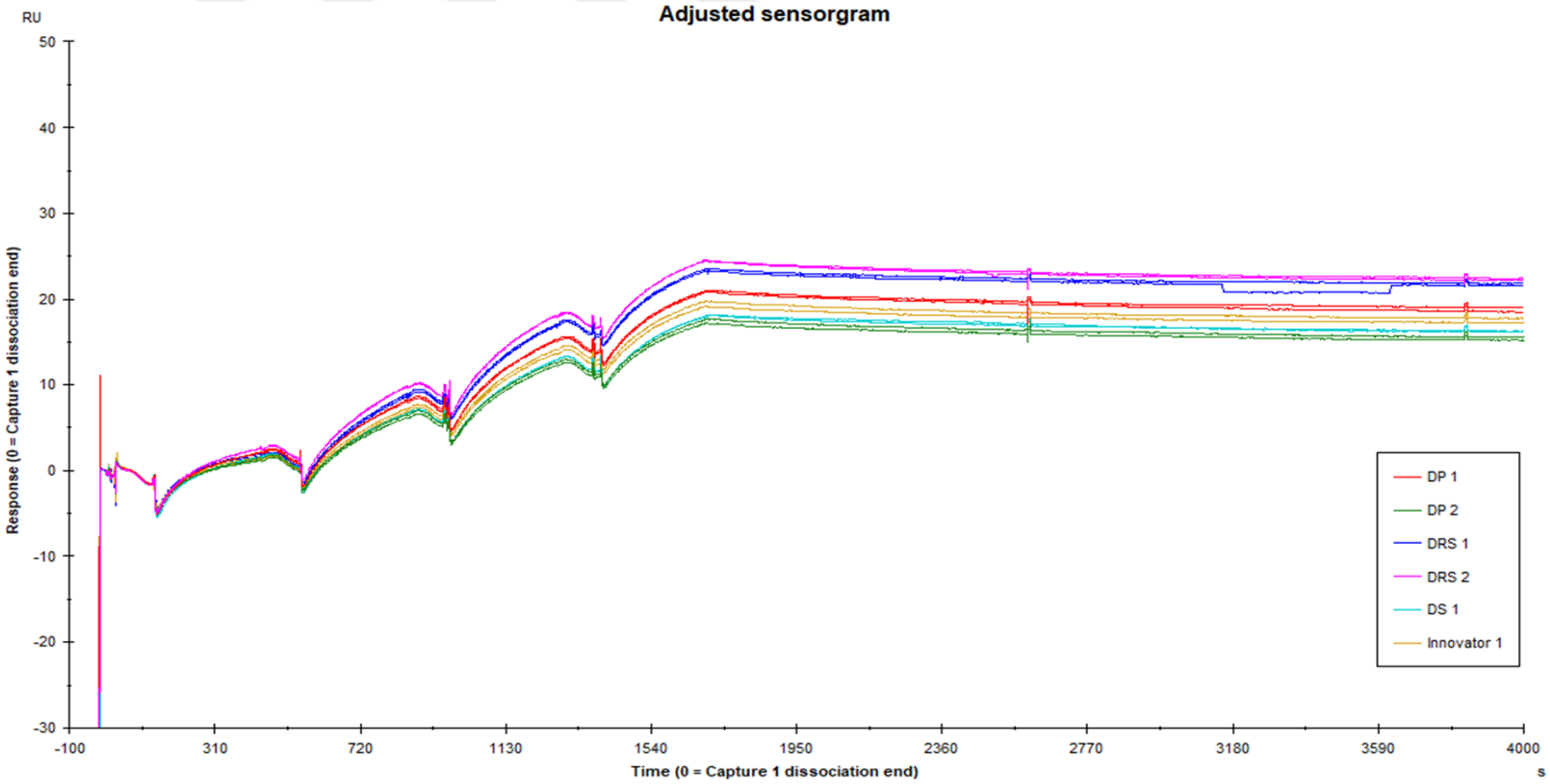

Figure 44. IgG-TNF-α binding sensorgram. Samples under pH 9 24-h stress condition

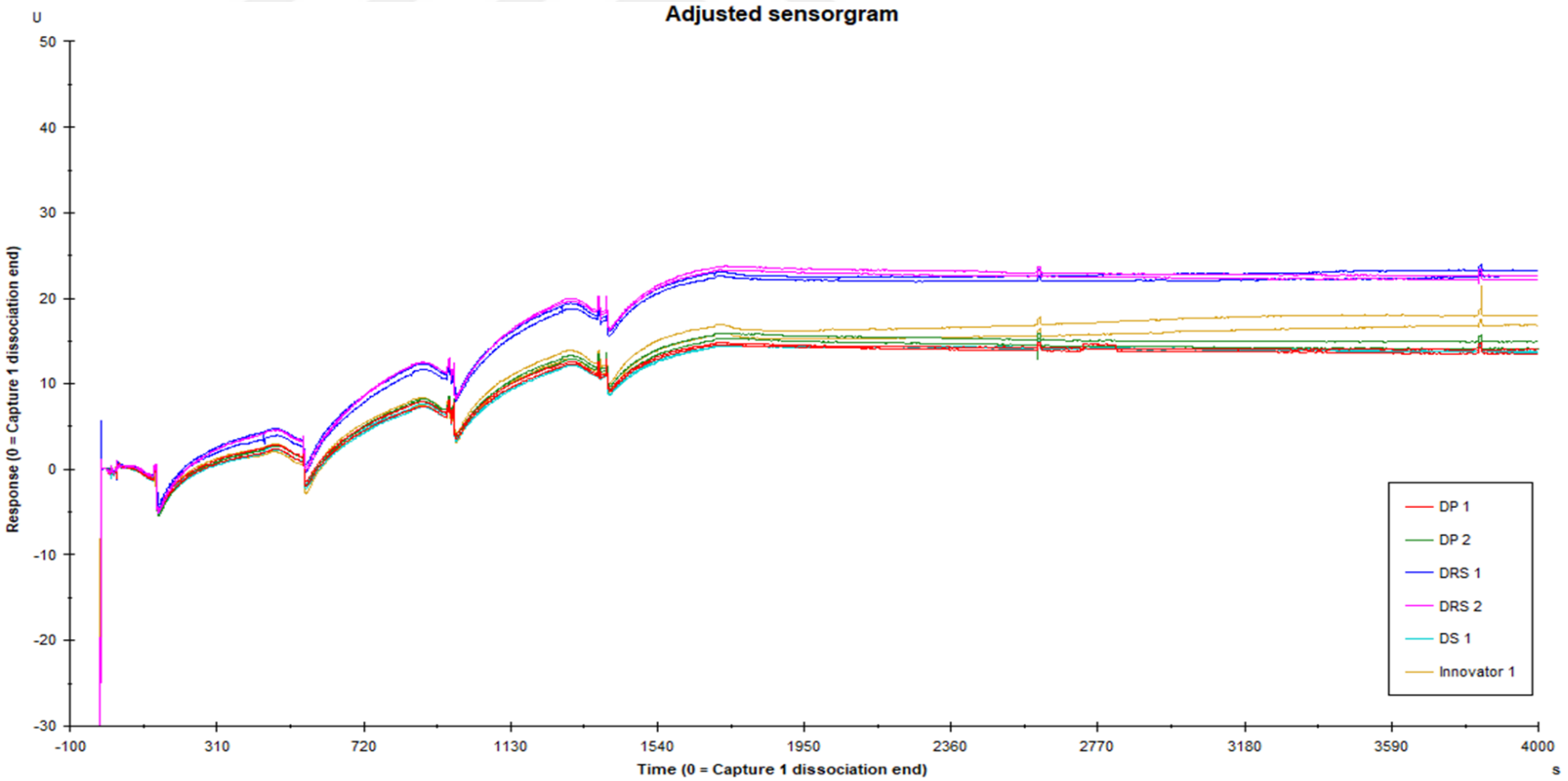

Figure 45. IgG-TNF-α binding sensorgram. Samples under pH 9 72-h stress condition

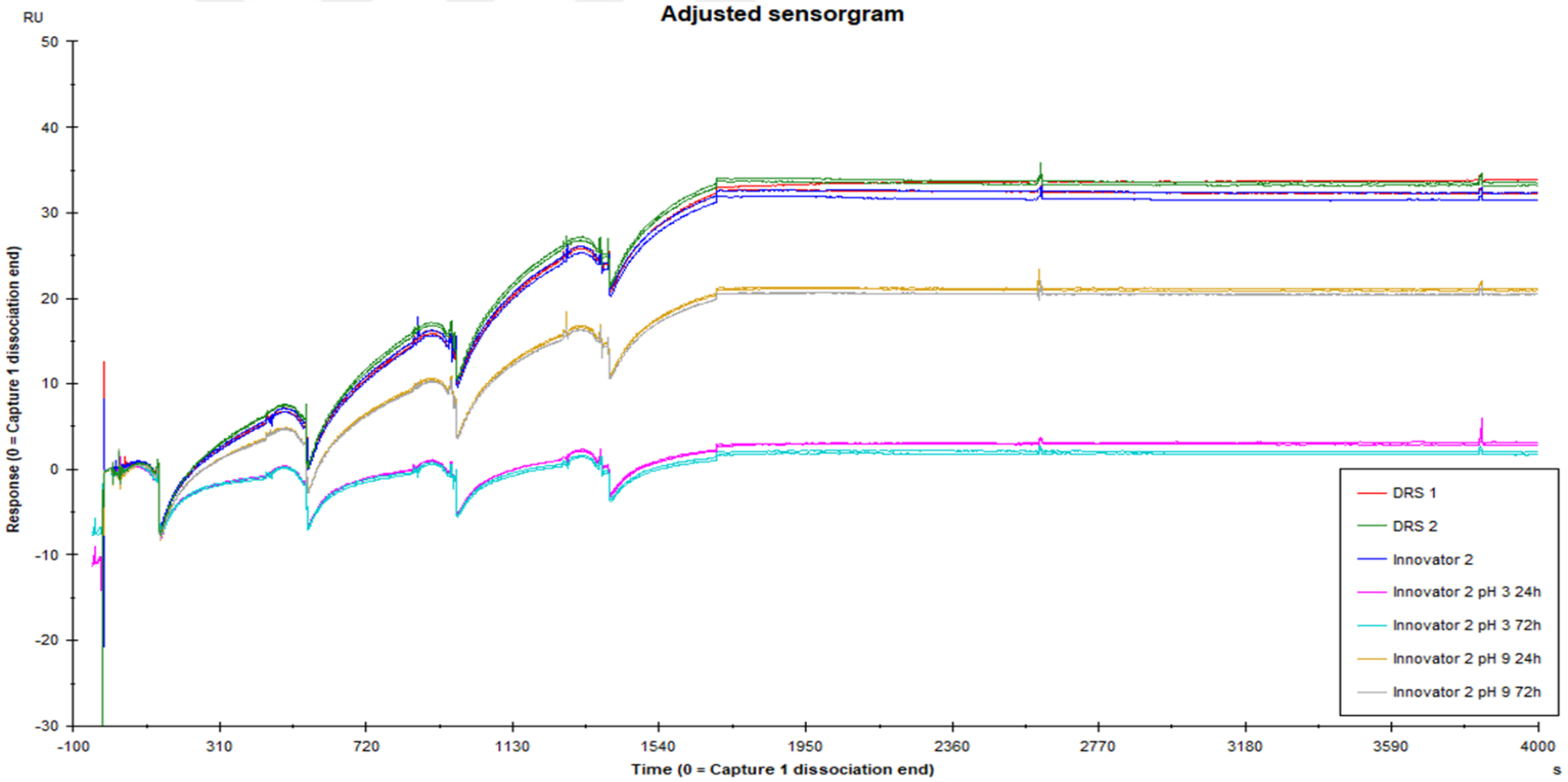

Figure 46. IgG-TNF-α binding sensorgram of Innovator 2 (stress-free and stressed samples)

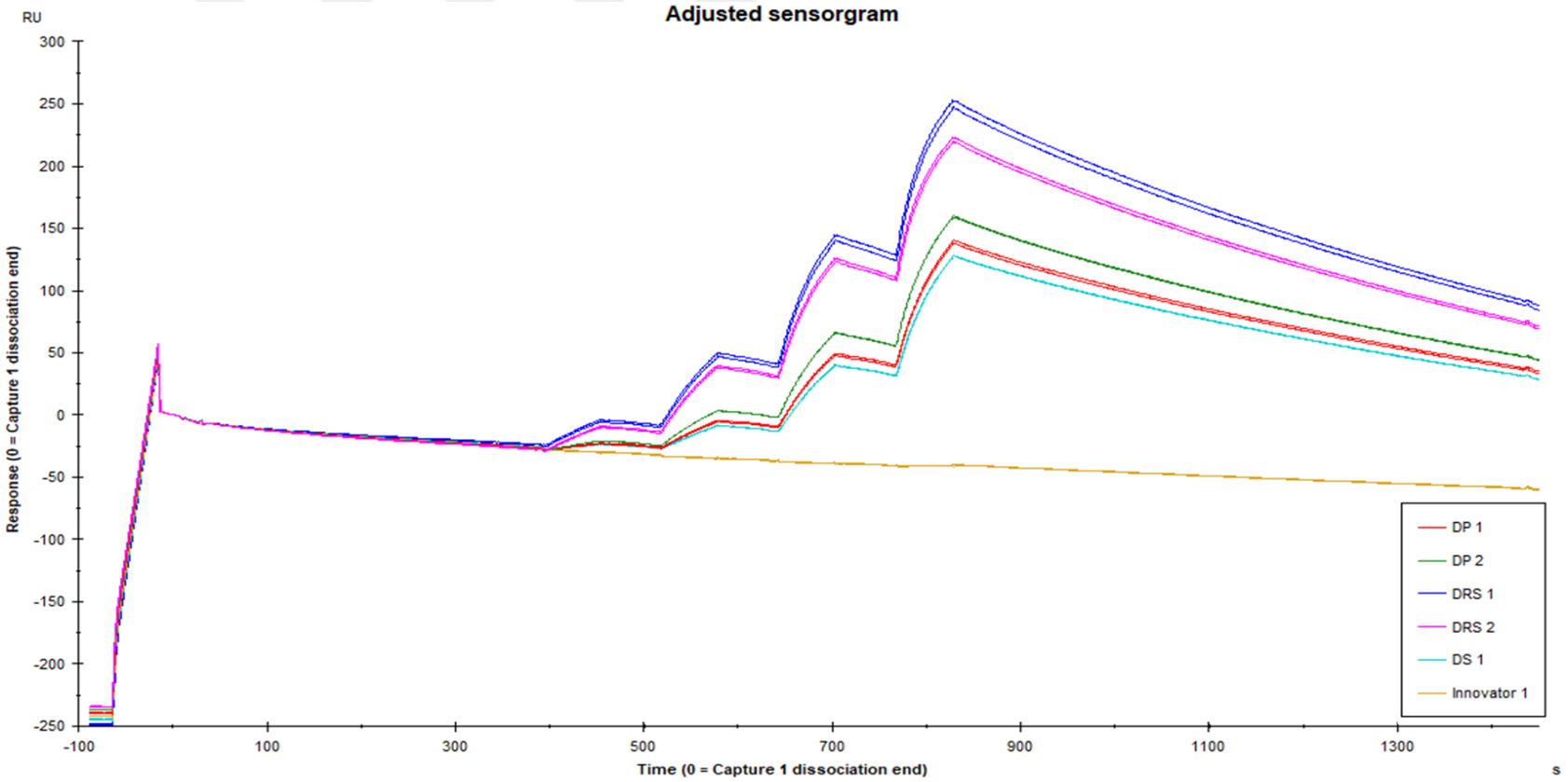

Figure 47. IgG-FcγRI binding sensorgram. Samples under pH 3 24-h stress condition

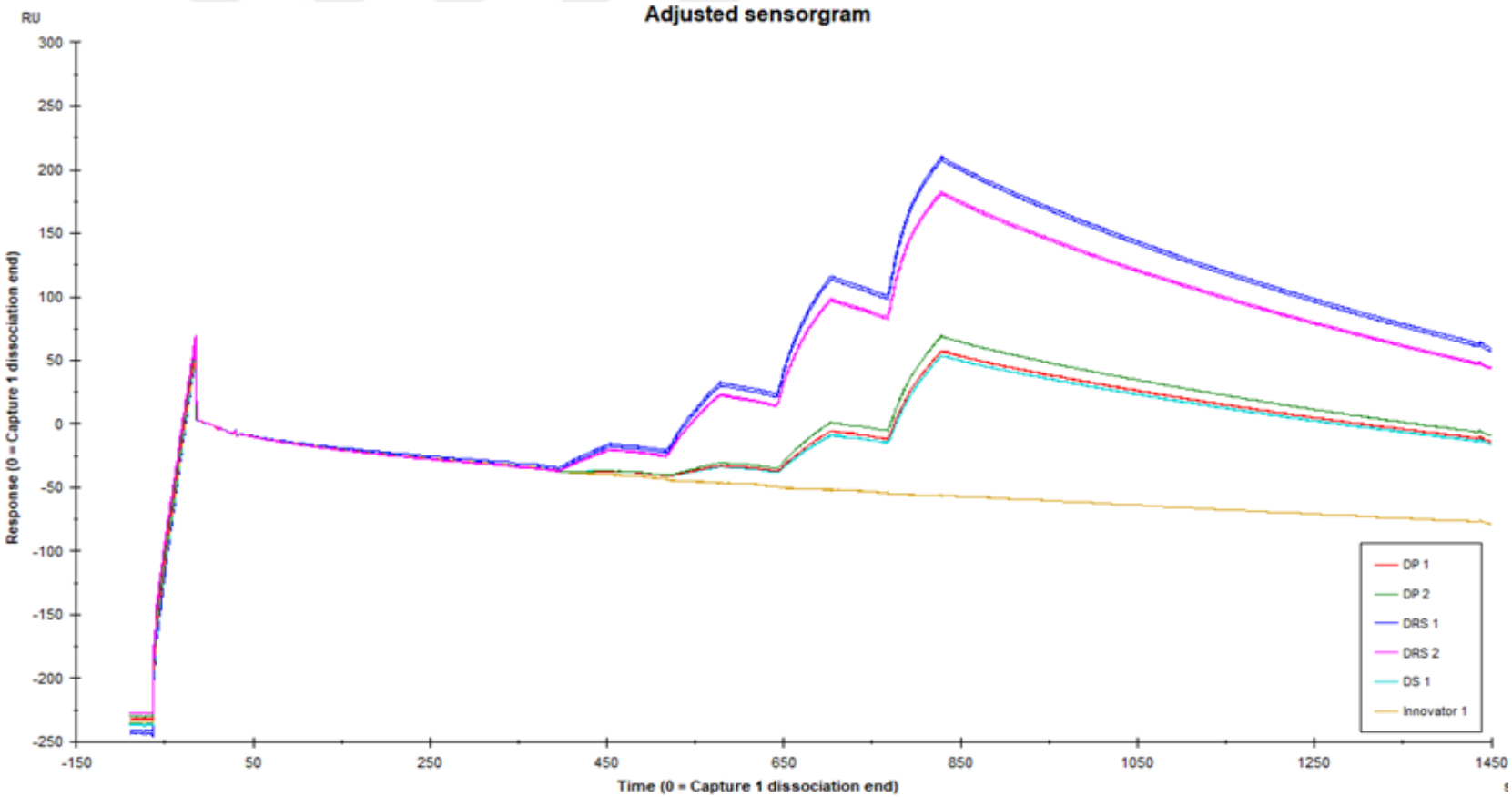

Figure 48. IgG-FcγRI binding sensorgram. Samples under pH 3 72-h stress condition

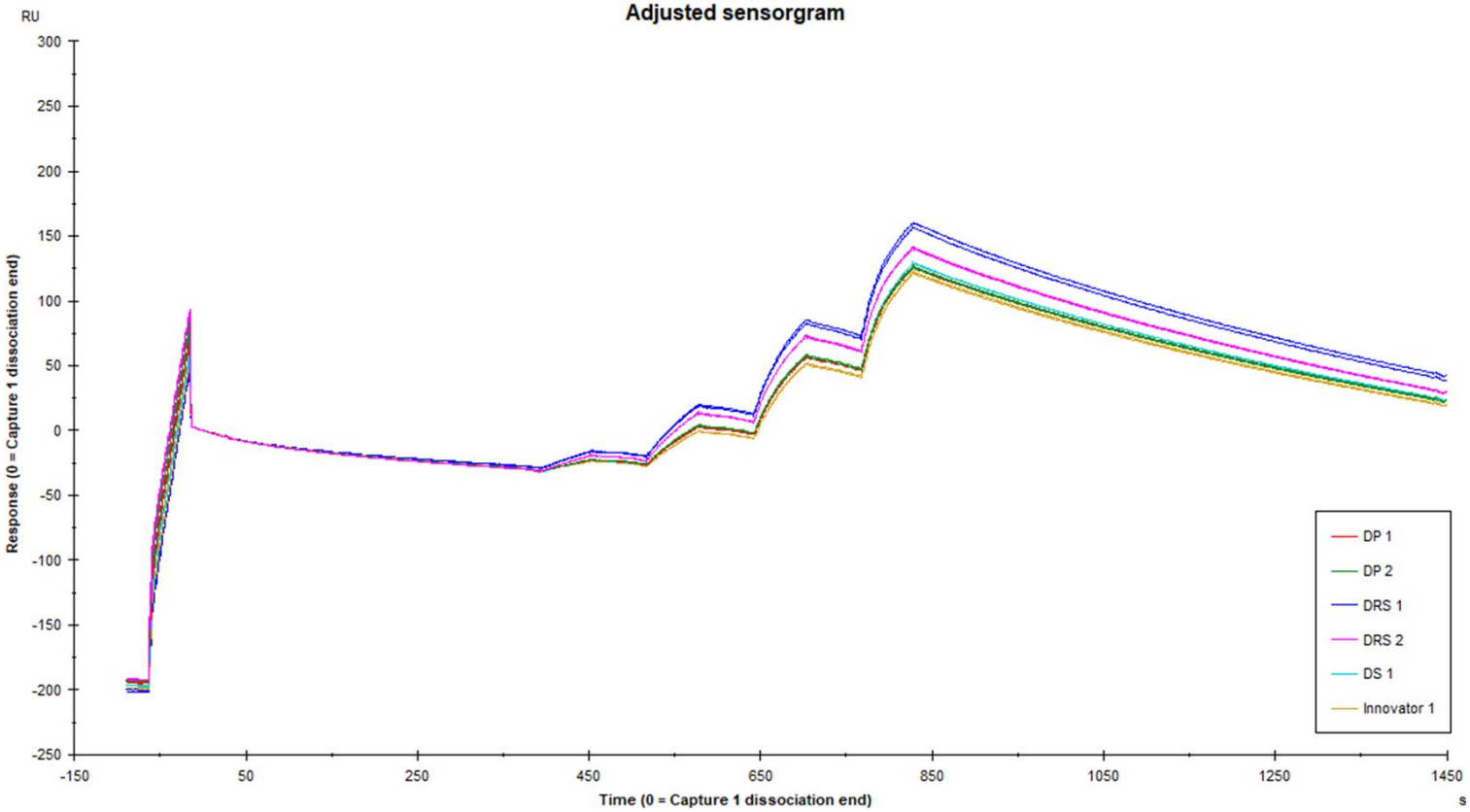

Figure 49. IgG-FcγRI binding sensorgram. Samples under pH 9 24-h stress condition

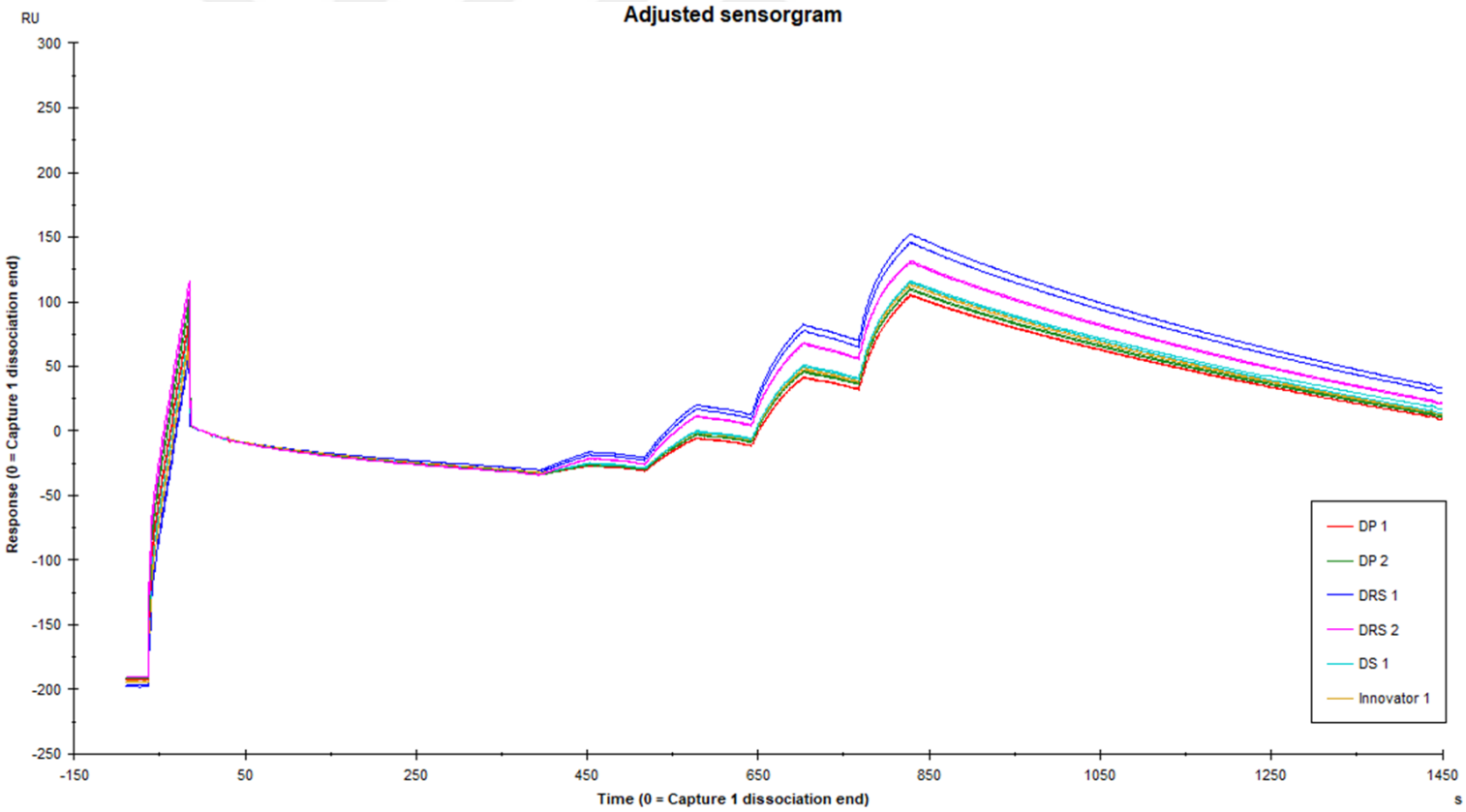

Figure 50. IgG-FcγRI binding sensorgram. Samples under pH 9 72-h stress condition

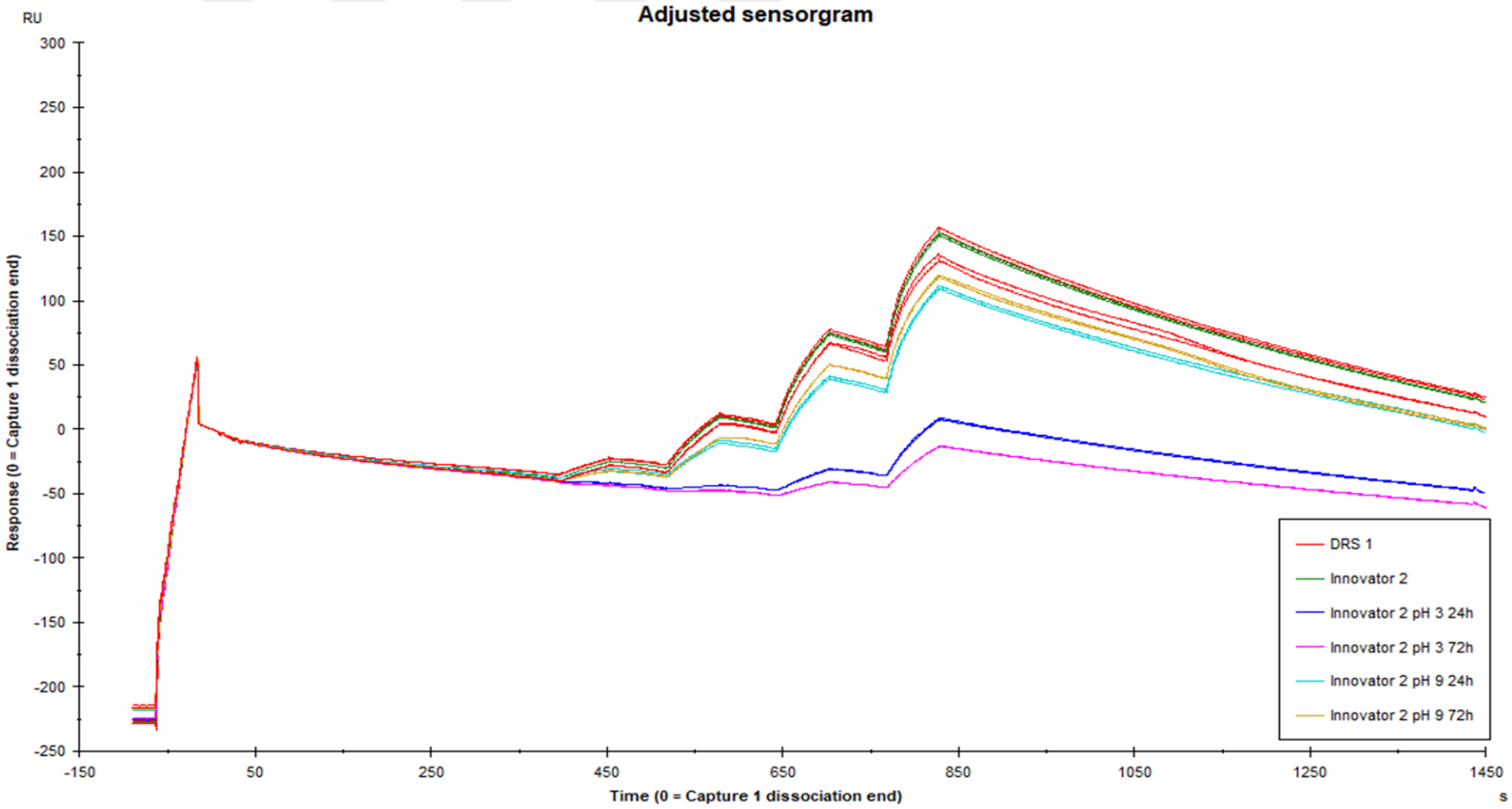

Figure 51. IgG-FcγRI binding sensorgram of Innovator 2 (stress-free and stressed samples)

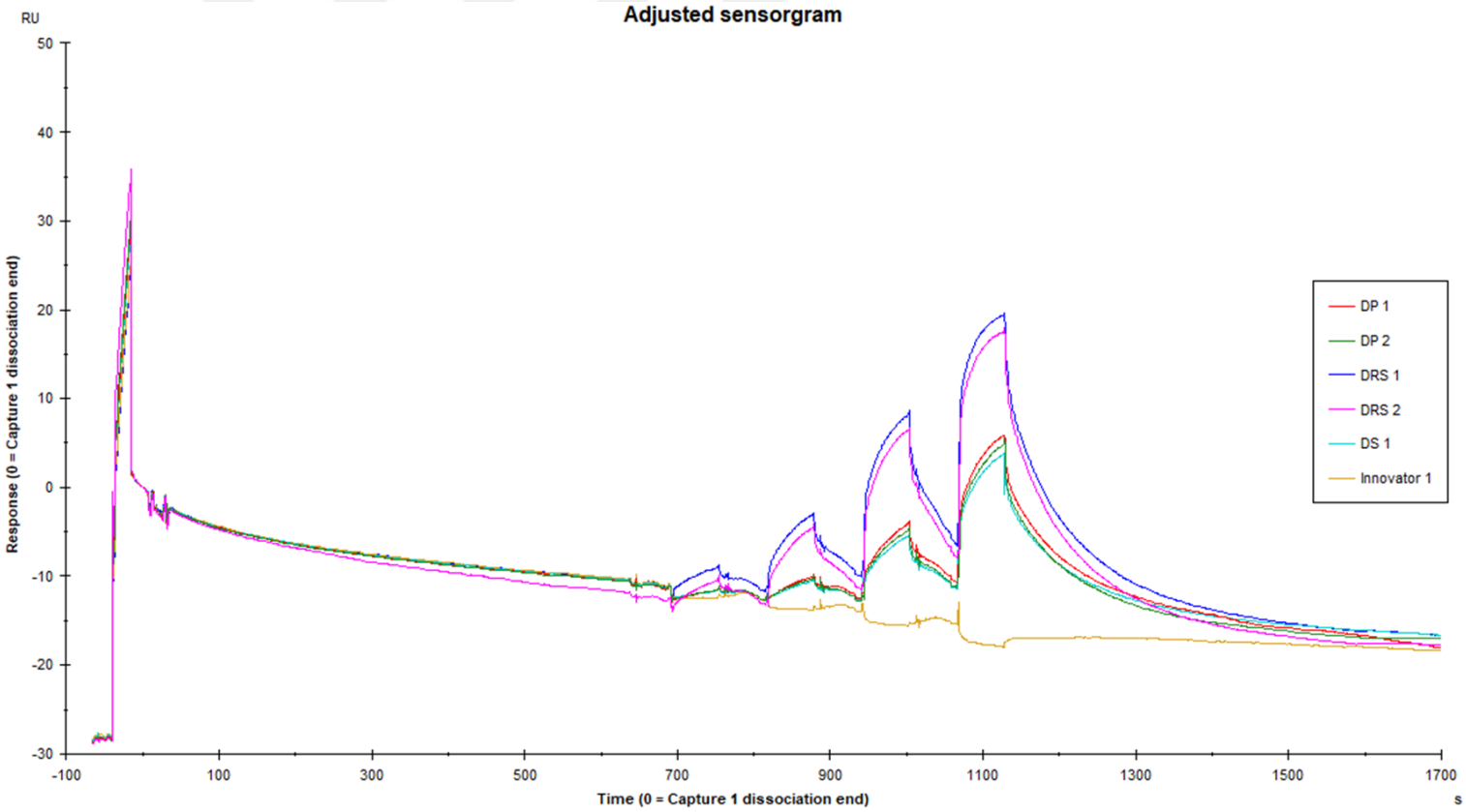

Figure 52. IgG-FcγRIIIa binding sensorgram. Samples under pH 3 24-h stress condition

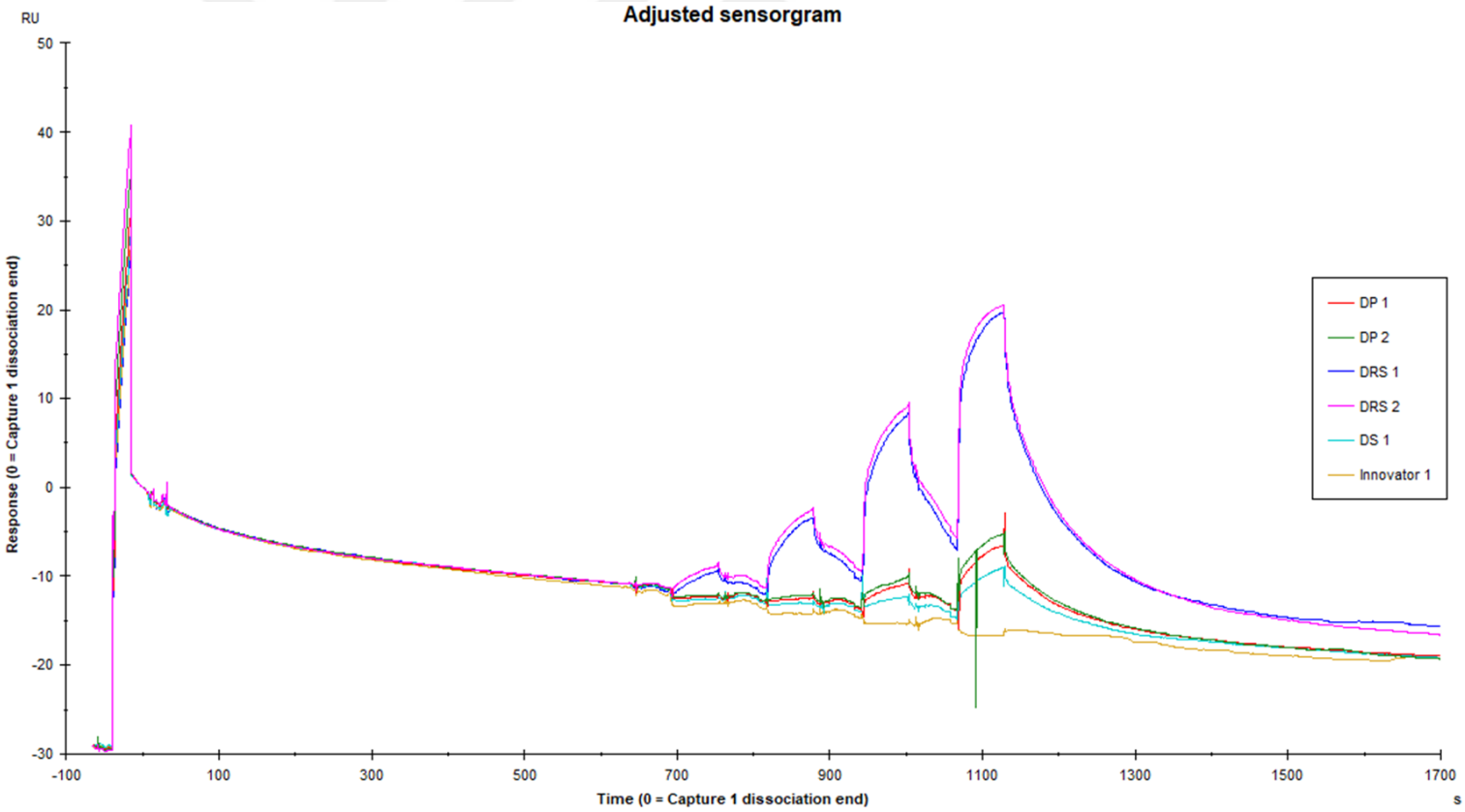

Figure 53. IgG-FcγRIIIa binding sensorgram. Samples under pH 3 72-h stress condition

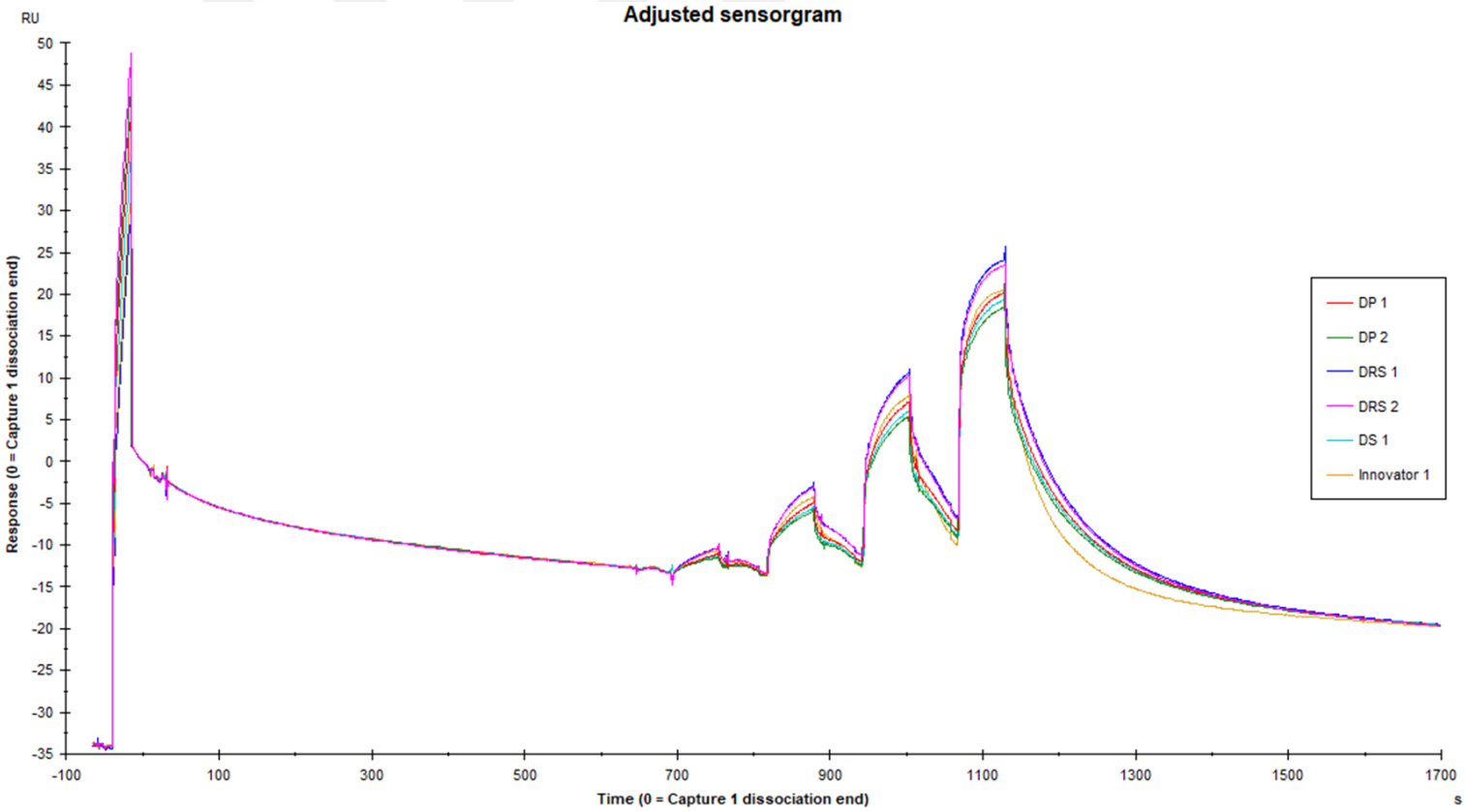

Figure 54. IgG-FcγRIIIa binding sensorgram. Samples under pH 9 24-h stress condition

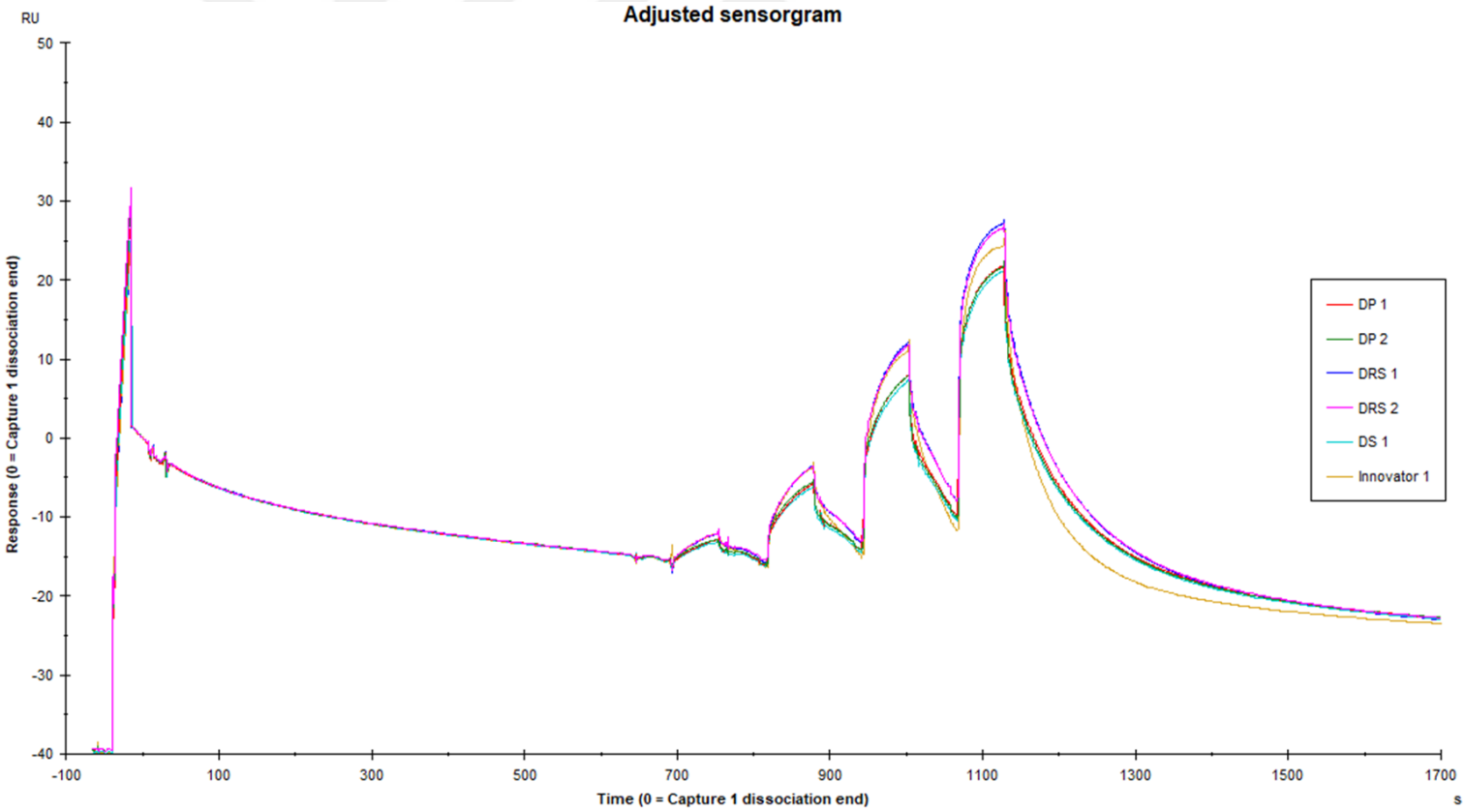

Figure 55. IgG- FcγRIIIa binding sensorgram. Samples under pH 9 72-h stress condition

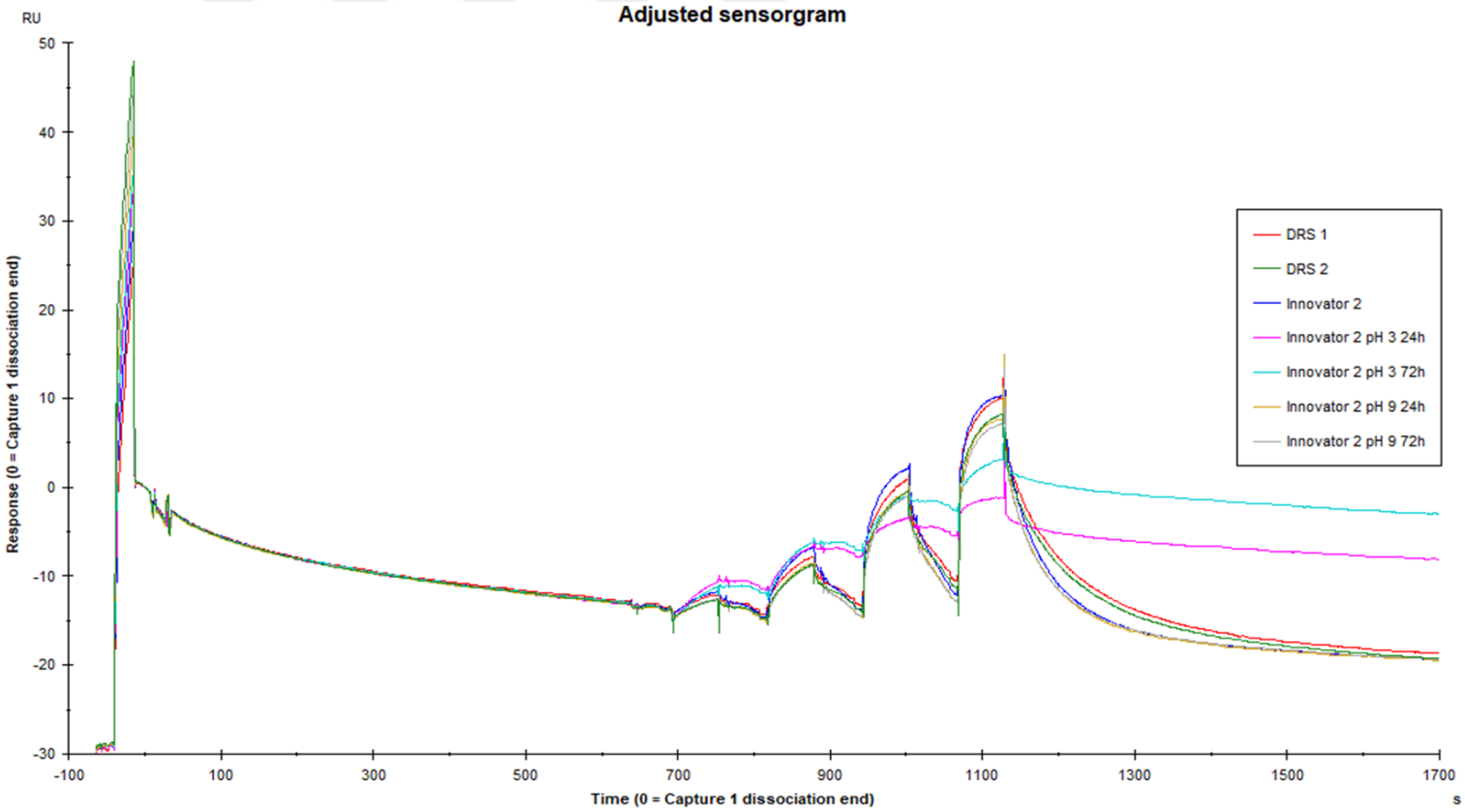

Figure 56. IgG-FcγRIIIa binding sensorgram of Innovator 2 (stress-free and stressed samples)

CURRICULUM VITAE

Personal Information

Name	Gülipek	**Surname**	Güven
Place of Birth	Eskişehir / Turkey	**Date of Birth**	06.11.1995
Nationality	TC	**Telephone number**	05388823900
E-mail	gulipekguven@gmail.com		

Education

Level	**Institution Name**	**Graduation Year**
Master of Science	Acıbadem Mehmet Ali Aydınlar University (Full scholarship) – Medical Biotechnology	2019
Undergraduate	Acıbadem Mehmet Ali Aydınlar University – Molecular Biology and Genetics (Minor : Healthcare Management)	2017
High School	Tan Anatolian High School	2013

Work Experience

Position	**Corporation**	**Duration**
Protein Functional Analysis Junior Specialist	Turgut Pharmaceuticals	07.2018 - …

Foreign Languages

Language	**Reading***	**Speaking***	**Writing***
English	Advanced	Advanced	Advanced

***** Evaluated as advanced, good, intermediate, beginner

Foreign Language Exam Results [#]

YDS	YÖK-DİL	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE
63,75								

[#] All successful exams should be enrolled.

[#] YDS: Yabancı Dil Sınavı; YÖK-Dil: Yükseköğretim Kurumları Yabancı Dil Sınavı; IELTS: International English Language Testing System; TOEFL IBT: Test of English as a Foreign Language-Internet-Based Test TOEFL PBT: Test of English as a Foreign Language-Paper-Based Test; TOEFL CBT: Test of English as a Foreign Language-Computer-Based Test; FCE: First Certificate in English; CAE: Certificate in Advanced English; CPE: Certificate of Proficiency in English

Other Exams

Name of the Exam	Quantitative	Equally Weighted	Verbal
ALES**	75,16	71,40	77,71

**ALES: Akademik Personel ve Lisansüstü Eğitimi Giriş Sınavı

Computer Skills

Program	Ability to Use
Microsoft Office	Advanced
Mac OS	Advanced
Biacore Evaluation Software	Advanced

* Evaluated as advanced, good, intermediate, beginner

Projects

Name of the Project	Institution	Position	Years
TÜBİTAK 2209/B - Production of human cell lines carrying green fluorescent protein by using pseudotyped ecotropic retroviruses	Acıbadem Mehmet Ali Aydınlar University	Coordinator	2016
TÜBİTAK 1002- Development of validated surface plasmon resonance methods for determination of therapeutic monoclonal antibody (IgG)/antigen binding properties	Acıbadem Mehmet Ali Aydınlar University	Researcher	2019

Conferences

Name of Conference	Position	Institution/Place	Duration/Year
Certificate in Occupational Health and Safety	Attendee	Acıbadem Mehmet Ali Aydınlar University	2016
II. KUGEN MBG Scientific Meeting	Attendee	Koç University	2016
GenoFuture'14	Attendee	Acıbadem Mehmet Ali Aydınlar University	2014
Genomics in Reproductive Medicine	Attendee	Acıbadem Mehmet Ali Aydınlar University	2013

International and National Courses and Certificates

Name of Certificate	Institution	Year
Certificate in Molecular Biology	New England Biolabs	2015
Certificate of Experimental Animal Use	Acıbadem Mehmet Ali Aydınlar University	2015

Proceedings

Name	Conference	Year
Comparative Binding Characteristics of Biosimilar and Innovator Monoclonal Antibodies Under Stress Conditions	Dipia	2018
Comparison Of Methionine Oxidation Profiles Of Biosimilar Tur01 Monoclonal Antibody To Its Reference Under Oxidative Stress	IMSC	2018

GÜLİPEK GÜVEN

TC ACIBADEM MEHMET ALİ AYDINLAR
UNIVERSITY INSTITUTE OF HEALTH SCIENCES

MASTER THESIS ISTANBUL 2019