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# Development of a reporter gene assay on an automated platform

*Author: Fanny Redin*

Examiner: Lei Ye

University supervisor: Johan Svensson Bonde

Company supervisor: Lone Frier Bovin

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## Abstract

Biological drugs have the potential to trigger unwanted immunogenic responses in patients which might affect the efficacy and safety of the treatment. Evaluating the immunogenicity throughout the drug development process is therefore crucial. For measuring the activity of a biological drug and antibodies against the drug, cell-based reporter gene assays can be used. The iLite reporter gene system developed by Svar Life Science consists of cells expressing receptors which are specific for the target of interest and trigger a signalling cascade. The signalling cascade leads down to a promoter fused to a reporter gene giving a quantifiable readout. The aim of this project was to develop a reporter gene assay on an automated platform using two iLite reporter gene cell lines which are normally ran as manual assays. Automating the assays could reduce the hands-on time and increase the sample throughput. To automate the assay it must be able to be incubated without CO<sub>2</sub>. The hypothesis of replacing the pH buffering of CO<sub>2</sub> incubation with the use of a medium with HEPES was investigated.

Cell lines targeting TNF-alpha have in this project been used as examples of the iLite reporter gene cell lines. One assay measuring the concentration of the anti-TNF-drug infliximab (IFX) have been tested on the automated platform as well as an assay measuring the antibody-dependant cellular cytotoxicity (ADCC) activity of IFX. Assay characteristics such as accuracy and precision have been evaluated.

The study shows that the concentration of IFX can be determined using a reporter gene assay on an automated platform. It was also concluded that for the IFX ADCC assay HEPES can not replace the function of CO<sub>2</sub>. The results indicate that a part of the iLite reporter gene assays can be automated which opens up the possibility of a more high-throughput immunogenicity testing during the drug development process.

## Populärvetenskaplig sammanfattning

Biologiska läkemedel är läkemedel som produceras i eller kommer från levande celler som bakterier och djurceller. De kan användas som behandling mot cancer och andra sjukdomar. Under dessa behandlingar kan immunsystemet hos patienterna försöka angripa läkemedlet genom att till exempel producera proteiner som kallas antikroppar. Dessa binder till läkemedelsmolekylen som då kan förlora sin förmåga att behandla sjukdomen. Reaktionen från immunsystemet kan också orsaka allvarlig skada hos patienten. Det är därför väldigt viktigt att testa att läkemedlet är effektivt och säkert att använda innan det släpps på marknaden. För att öka sannolikheten för en lyckad behandling kan läkemedelsnivån och antikroppar mot läkemedlet mätas hos patienten. En metod för att mäta detta är att använda sig av celler som blivit genetiskt modifierade för att detektera det aktuella biologiska läkemedlet. Dessa kallas rapportörceller. Cellerna svarar på det biologiska läkemedlet och ger ifrån sig ett ljus som går att mäta. Från den uppmätta signalen kan koncentrationen av läkemedlet bestämmas, samt om antikroppar mot läkemedlet finns.

Rapportörögen analyser utförs normalt sett manuellt och tar upp till en timme att göra. I detta projekt har möjligheten att utföra analysen på en automatiserad plattform utvärderats. Ett instrument har använts som automatiskt hanterar reagens och prov. Detta kan minska tiden som behöver spenderas på analysen och möjliggöra en snabbare utveckling och testande av biologiska läkemedel. Analysen har först utförts manuellt för att se om den är möjligt att utföra utan CO<sub>2</sub> vilket man vanligvis använder till celler. Analysen har sen utförts på den automatiserade plattformen som hanterat reagens och inkuberingsstegen. Det har visats att den ena analysen som testats i denna studie kan automatiseras med jämförbara resultat som den vanliga manuella analysen gällande noggrannhet och precision. Den andra analysen som testats visade sämre noggrannhet och precision utan CO<sub>2</sub> jämfört med den vanliga analysen med CO<sub>2</sub>. Resultaten visar också att den kan automatiseras för bara en del av läkemedelsdoserna som testats. Sammanfattningsvis har denna studie visat att en av analyserna som testats kan köras på en automatiserad plattform och den andra analysen kan inte automatiseras fullt ut.

## Popular summary

Biological drugs are drugs that are produced in or derived from living cells, such as bacterial or animal cells. They are used for treating cancer and other diseases. During the treatment, the immune system of the patient might try to fight the drug by for example producing proteins called antibodies that can bind to the drug molecule. The drug might then not be able to treat the disease and the immune response might also cause serious illness to the patient. It is therefore essential to test that the drug is efficient and safe to use before being released to the market. During the treatment, patients can be monitored by measuring the drug level and the antibodies against the drug, which can improve the treatment outcome. One method to measure these levels is to use cells that are genetically modified to detect the specific biological drug of interest. It is called a reporter gene assay. The cells respond to the biological drug and generate a light that can be measured. From the measured signal, the concentration of biological drug and the presence of antibodies against the drug can be determined.

Reporter gene assays are normally performed manually which takes up to an hour to do. In this project it was attempted to perform the assay using an automated system where the liquids are automatically handled. This could reduce the time needed to spend on the assay and enable faster development and testing of biological drugs. The assay has been performed manually to first see if the incubation steps, where you wait for the reagents to react, can be done without CO<sub>2</sub> which is often used when incubating cells. The assay was then setup on the automated platform to automatically handle the liquids and the incubation steps. It was shown that for one of the assays tested, the automated assay is comparable with the standard manual assay when it comes to accuracy and precision. On the other hand, the second assay tested did not show good result when incubated without CO<sub>2</sub>. It was shown that this assay can be automated for only a part of the drug levels tested. In conclusion, one of the assays tested during this study could be transferred to the automated system and the other assay is more problematic to automate.

## **Preface and Acknowledgement**

This project has been performed at the R&D department of Svar Life Science in Malmö. I would like to thank my supervisor Lone Frier Bovin for contributing with her scientific and laboratory knowledge as well as supporting and helping out during the entire project. I am also grateful for the help from the rest of the R&D and iLite team. I would also like to thank Wieslab diagnostic team who taught me how to use their automated platform and managed to find slots for my runs in their busy schedule.

Thank you, Johan Svensson Bonde, for supervising me during the project and Lei Ye for acting as my examiner.

## List of Abbreviations

Ab	Antibody
ADA	Anti-drug antibody
ADCC	Antibody-dependent cellular cytotoxicity
ARC	Assay ready cells
CD	Crohn's disease
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FL	Firefly luciferase
IBD	Inflammatory bowel disease
IFX	Infliximab
NHS	Normal human serum
QC	Quality control
RA	Rheumatoid arthritis
RL	Renilla luciferase
RLU	Relative light unit
RPMI medium	Iscoe Roswell Park Memorial Institute medium
TNFR1	Tumour necrosis factor alpha receptor 1
TNFR2	Tumour necrosis factor alpha receptor 2
TNF-alpha	Tumour necrosis factor alpha

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# 1 Introduction

Biopharmaceuticals or biological drugs are therapeutics whose active component is produced in living cells. It is an expanding group of pharmaceuticals and includes for example hormones, growth factors, vaccines and antibodies [1]. Biopharmaceuticals have improved the treatment of a number of severe and chronic diseases, such as cancer and autoimmune diseases. However, biopharmaceuticals have the potential to trigger unwanted immunogenic responses affecting the efficacy and safety of the treatment. Therefore, it is crucial for companies in the drug development process to evaluate the immunogenicity throughout the different clinical phases [2].

Svar Life Science is a Swedish life science company providing assay solutions for companies in the drug development process and for clinical diagnostic laboratories. The company was established in Malmö, Sweden, in 1992 under the name Euro Diagnostica. Their wide product portfolio includes assay ready cells and enzyme-linked immunosorbent assay (ELISA) kits for detection of a wide variety of antibodies. Wieslab is a part of the Svar Life Science group since 2004 and is providing diagnostic and bioanalytical services.

The cell-based reporter gene system of Svar Life Science, called iLite, allows an easy, accurate and rapid measurement of a wide variety of different pharmaceutical targets. It is a tool useful during the entire drug development process where the biological activity or potency, function, efficiency and safety of the biological drug must be tested. The iLite system uses cells expressing receptors that are specific for the target or ligand of interest. A signalling cascade is triggered when the ligand binds the receptor, leading down to an inducible promoter fused to a reporter gene. The reporter gene, firefly luciferase, gives a quantifiable readout correlated to the amount and activity of ligand [3].

## 1.1 Aim and Objectives

The iLite reporter gene assays are performed manually by plating the cells, adding the sample, incubating the plate in a CO<sub>2</sub> incubator and reading the assay. The aim of this master thesis is to investigate the possibility of transferring the reporter gene assay to an automated platform. It includes being able to incubate the assay without CO<sub>2</sub> and performing the liquid handling steps automatically. This is of great interest for meeting customer demands of assay ready cells on an automated system and not needing a CO<sub>2</sub> incubator. It could reduce the time needed to spend on the assay and increase the sample throughput. Two cell lines will be used as examples of reporter gene cell lines. The tumour necrosis factor alpha (TNF-alpha) assay ready cells will be used to perform an anti-TNF drug concentration determination assay using the anti-TNF drug infliximab. The antibody-dependant cell-mediated cytotoxicity (ADCC) cells will be used to measure the anti-mTNF-alpha ADCC activity. The assays will be run using calibrators and control samples with known infliximab concentrations.

The objectives of the project are to:

- Investigate the possibility of using Iscove Roswell Park Memorial Institute (RPMI) medium with HEPES to replace the pH buffering of CO<sub>2</sub> during incubation of a reporter gene assay.
- Investigate the possibility of automating the manual steps of a reporter gene assay.

## 2 Background

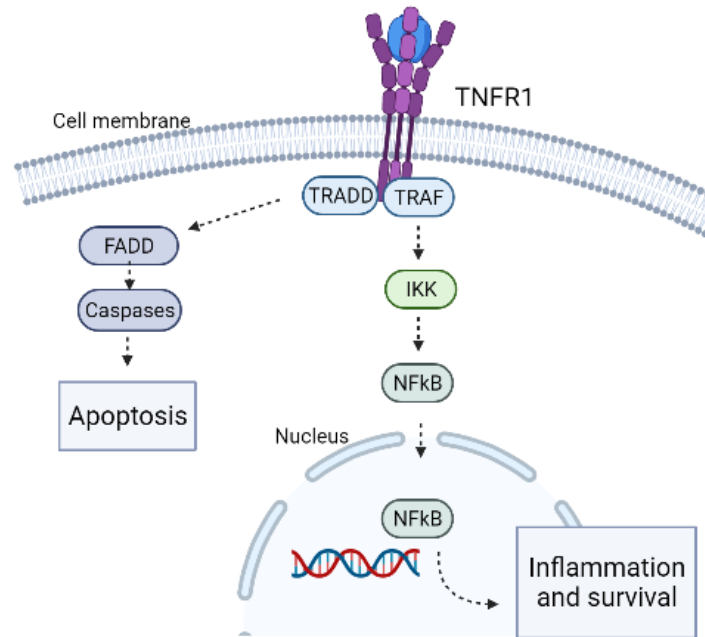
When biological pharmaceuticals are administered to patients they can elicit immune responses that might affect the efficacy and safety of the product. Immunogenicity is characterized by the presence of anti-drug antibodies (ADAs). ADA can be non-neutralizing or neutralizing, where the neutralizing ADA binds to the active site of the drug and thereby neutralizing the drug activity. Non-neutralizing ADAs may still reduce the efficacy of the therapeutic by reducing the bioavailability. ADA formation has been associated with life-threatening adverse events such as hypersensitivity [2].

Many factors can influence the immunogenicity of biological agents and should be considered before starting a treatment. There are both patient-, treatment- and drug-related factors to consider. Patient-related factors include the patient's genotype and immune system activity where patients with a highly active immune system seems to have an increased risk of developing ADA. Treatment-related factors include route of administration, drug dose and treatment duration. The risk of ADA formation is also influenced by concomitant treatments. When it comes to drug-related factors, drug characteristics such as structure, size and presence of T-cell and B-cell epitopes are important. This category also includes the presence of aggregates and particles which depends on the manufacturing and purification process [4]. All these factors make it difficult to predict whether a patient will have an immune response towards a biological pharmaceutical, when it will occur and its clinical significance. Assessment of immunogenicity throughout the drug development process is therefore of big importance and a requirement for the approval of therapeutic proteins [5]. It is also necessary to continue the immunogenicity testing after approval of the biopharmaceutical. By monitoring ADA levels in patients treated with the drug, the long-term immunogenic capacity can be evaluated [2].

For testing the immunogenicity of a product, a screening assay is initially performed to detect ADA in patients treated with the biopharmaceutical. After a confirmatory assay which eliminate false positive samples, the ADA-positive samples are analysed in a characterisation assay to determine the type of ADA and the titer. They should then be tested for neutralizing potential in a neutralizing antibody (NAb) assay [2]. For this, a cell-based assay is recommended by regulatory agencies as it reflect the mechanism of action of the therapeutic [2][5].

### 2.1 TNF-alpha

Tumour necrosis factor alpha (TNF-alpha) is a 17-kDa cytokine that is produced mainly by activated macrophages, T lymphocytes and natural killer cells as a membrane-bound pro-TNF. After cleavage, the TNF-alpha protein is released and is then involved in immunity, apoptosis, cell survival and inflammation. It acts via two receptors called TNF receptor 1, TNFR1, and TNF receptor 2, TNFR2. TNFR1 is expressed on all cell types and regulates most of the biological activities of TNF-alpha. Upon TNFR1 activation two different signalling pathways can be initiated, shown in Figure 1. One is a pathway initiating apoptosis and the other a cell proliferation pathway. The later includes recruitment of TNFR-associated factor-2 (TRAF-2) to the receptor. This leads to activation of I $\kappa$ B kinases (IKK) that in turn activate the transcription factor nuclear factor kappa B (NF- $\kappa$ B). NF- $\kappa$ B enters the nucleus and initiates transcription of proinflammatory and survival genes [6].



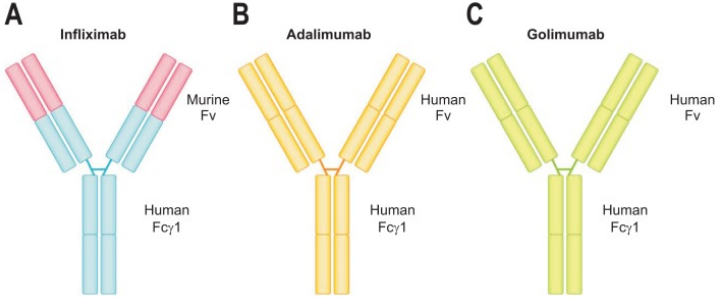
**Figure 1:** The signalling pathways activated by TNF-alpha via TNF receptor 1 (TNFR1). Upon activation of TNFR1 the TNFR-associated factor-2 (TRAF) is recruited leading to activation of the I $\kappa$ B kinases (IKK) and the nuclear factor kappa B (NF- $\kappa$ B). Inflammatory and survival genes are expressed. An apoptosis pathway is also initiated via Fas-associated DD (FADD) and caspases [6]. Created with BioRender.com.

TNF-alpha has a role in oncogenesis by regulating the balance between proliferation and apoptosis signals [6]. Furthermore, it has an important role in the defence to bacteria, virus and parasites for normal response to infections. It is usually not present in detectable levels in healthy individuals. However, during inflammation and infection, the level in serum and tissue increases. Moreover, excessive production of TNF-alpha might be harmful. Several inflammatory diseases are correlated to an overexpression of TNF-alpha, such as the chronic autoimmune inflammatory disease rheumatoid arthritis (RA), the inflammatory bowel disease (IBD) Crohn's disease (CD) and the inflammatory skin disorder psoriasis [7].

### 2.1.1 TNF-inhibitors

Therapeutic monoclonal antibodies (mAb) inhibiting TNF-alpha, so called TNF-alpha antagonists, are used for the treatment of chronic inflammatory or autoimmune diseases such as CD, RA and psoriasis. Amongst the approved therapeutics are infliximab (IFX), which is a chimeric mouse-human immunoglobulin (Ig)G1 $\kappa$  Ab, and the two fully human IgG1 $\kappa$  mAb adalimumab and golimumab [8], see Figure 2. They act primarily by binding to both soluble and transmembrane TNF-alpha, thus neutralizing the TNF-alpha protein and preventing the proinflammatory signal transduction. Another mechanism of action is antibody-dependant cell-mediated cytotoxicity (ADCC). It is a process where effector cells actively induce lysis of cells where antibodies have bound to antigens on the surface [9].

IFX is administered intravenously, whereas adalimumab and golimumab are administered as a subcutaneous injection, i.e. beneath the skin [9]. The use of TNF-alpha antagonists has revolutionized the treatment of many diseases and they are in general safe to use. However, many patients experience treatment failure, either not responding to the drug at all, called primary response failure, or eventually losing the initial response despite frequent drug administration, called secondary response failure [8].



**Figure 2:** Molecular structure of the three TNF-inhibitors infliximab (A), adalimumab (B) and golimumab (C). Fc is the crystalline fragment, Fv the variable fragment and Fc $\gamma$ 1 the human immunoglobulin G1 Fc fragment [10].

### 2.1.2 Immunogenicity of TNF-inhibitors

The secondary response failure seen in some patients treated with TNF-alpha antagonists can be caused by drug immunogenicity where the patient is developing an immune response against the protein [4]. The formation of ADA is caused by the immune system recognising the drug as a foreign substance. As for IFX, the immunogenic component is the nonhuman part of the Ab [11].

Amongst IBD patients treated with IFX, ADA is found in approximately one third [11]. It might lead to reduced clinical efficacy as ADA are associated with reduced drug levels and treatment response [9]. Furthermore, it can put the patient at risk by causing allergic reactions and even severe autoimmune reactions [12].

Therapeutic drug monitoring (TDM) by measuring the anti-TNF drug and ADA levels is a way of having a patient-specific treatment. It optimizes the treatment outcome and reduces the risk of treatment failure and side effects as the therapy is adjusted depending on how the patient is responding [11].

## 2.2 Reporter gene assay

To study the regulation of signal transduction pathways, cell-based reporter gene assays can be used. Typically, the coding region of the reporter gene is cloned downstream of a regulatory promoter sequence of the gene of interest [13]. The reporter gene construct is transferred into cells and the reporter gene will be under the control of the promoter [12]. The ideal reporter gene is not endogenously expressed in the cells and can easily be detected. Bioluminescence reporter genes are commonly used since mammalian cells do not have any background bioluminescence. Firefly (*Photinus pyralis*) luciferase is a bioluminescent protein that catalyses the conversion of its substrate D-luciferin to oxyluciferin while emitting light. The emitted light should be linearly proportional to the concentration of luciferase protein and in that way, the expression of the gene of interest can be monitored. Renilla (*Renilla reniformis*) luciferase is another popular bioluminescent protein which catalyses the oxidation of a different substrate, coelenterazine [13].

The readout from a reporter gene assay using bioluminescence is measured with a luminometer in which the photons are quantified. The signal is amplified in a photomultiplier tube (PMT) in the luminometer and then detected, generating an electrical signal. The instrument reports a numerical value proportional to the electrical signal, called relative light units (RLU) [14].

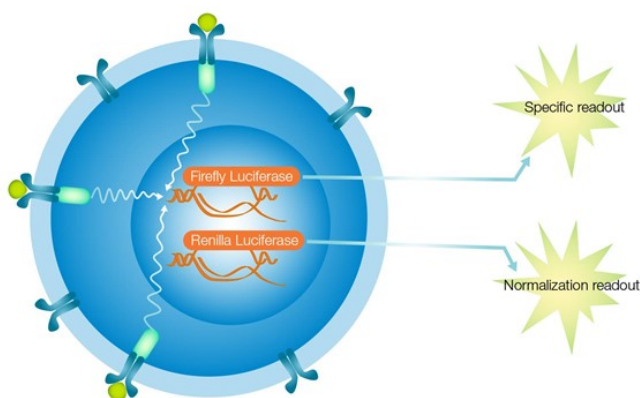
## 2.3 iLite technology

The iLite technology is the base for the iLite cell-based assays of Svar Life Science. The engineered cell-based assay system has a dual reporter gene readout, illustrated in Figure 3. iLite assay ready cells (ARC) can be developed for a wide range of pharmaceutical target compounds and be used for measuring drug activity and immunogenicity. The cells are modified so that when the receptors on the cell surface are activated by the target of the assay, an intra-cellular signalling pathway is triggered. The signalling pathway leads down to a promoter sequence which in turn is fused to a firefly luciferase (FL) reporter gene. When the transcription of FL is triggered and its substrate is added, light will be generated. The luminescence readout correlates to the amount of target compound bound to the receptors [3].

The iLite system also has a second reporter gene, renilla luciferase (RL). This gene is cloned downstream of a constitutive promoter, meaning that it is expressed at a constant level in living cells. The signal generated from the second reporter gene will therefore correlate to the number of living cells. It is used to normalize the results so that differences in cell number as well as serum matrix effects are compensated, and the signal will be target-specific [3].

The iLite technology can be used in the different phases during the drug development process. In the early stages it can be used for drug candidate screening, then for evaluating the function, efficiency and safety of the candidates. After approval of the end product, the iLite technology can be used for immunogenicity studies measuring both the circulating drug level in patients and NAbs against the drug [3]. It is also a good tool for development of biosimilars which are versions of the original drug [2].

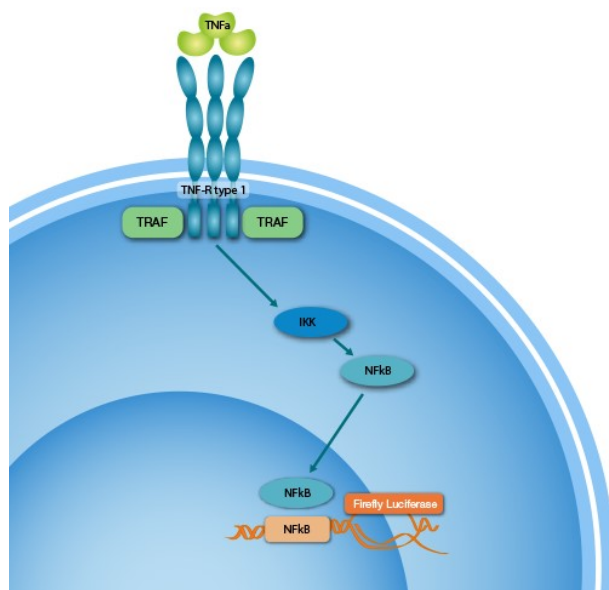
The iLite assays are supplied as ARC that are cryopreserved. The assay is run by thawing the cells, then adding the sample and the cells to a culture plate which is then incubated before reading the assay [3].



**Figure 3:** An illustration of the reporter gene system in iLite cells where the target is activating the receptors on the cell surface. A signalling pathway is triggered leading down to the transcription of firefly luciferase and the specific luminiscence readout. Renilla luciferase is the second reporter gene giving the normalization readout.

### 2.3.1 iLite TNF-alpha

The iLite TNF-alpha ARC is one of the iLite products of Svar Life Science. The genetically engineered reporter gene cell line is of human origin (K562, ATCC# CCL-243) and is designed to respond to TNF-alpha, see Figure 4 [15]. It is a functional bioassay, meaning that it indicates not just the binding of the compound to the receptor, but also the function of the compound [16]. The iLite TNF-alpha ARC can be used for measuring the functional TNF-alpha, the potency of TNF-alpha inhibitors and the presence of NAbs to TNF-alpha inhibitors in test samples such as human serum. The FL signal from the assay is proportional to the functional activity of TNF-alpha in a sample and inversely proportional to the amount of anti-TNF-alpha drug present such as IFX. In turn, the amount of anti-TNF-alpha drug is inversely proportional to the amount of NAbs against the anti-TNF-alpha drug [17].

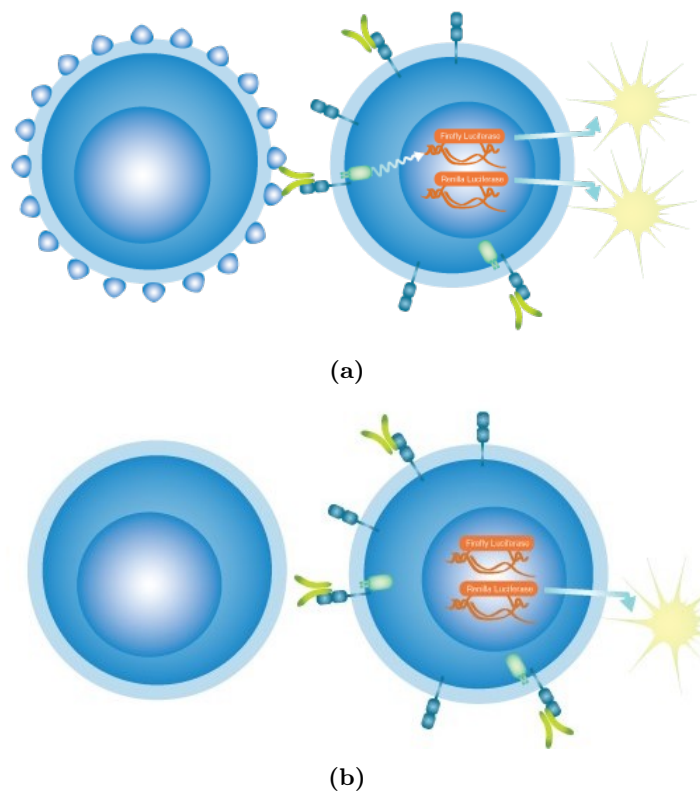


**Figure 4:** The TNF-alpha signaling pathway leading down to the transcription of firefly luciferase. TRAF is the TNFR-associated factor-2, IKK the I $\kappa$ B kinases and NF- $\kappa$ B the nuclear factor kappa B transcription factor.

### 2.3.2 iLite ADCC

The iLite product portfolio also includes iLite ADCC bioassays for measuring the efficacy of antibodies to induce ADCC. These assays combine effector cells with positive and negative target cells for the specific antibody, see Figure 5 [18]. The effector cells originate from a human T lymphocyte cell line, Jurkat (ATCC #TIB-152). They are genetically engineered to over express the Fc receptor Fc $\gamma$ RIIIa (CD16) [19]. For measuring the ADCC activity of anti-TNF-alpha antibodies, the target cells to be used are the iLite ADCC Target mTNF-alpha (+). They are based on a human embryonic kidney cell line, HEK293 (ATCC# CRL-1573). The cells have been genetically engineered to express high levels of the surface antigen mTNF-alpha [20]. The effector cell and the target cell interact by binding of the Fc $\gamma$ RIIIa (CD16) receptor to the Fc region of the antibody bound to the target cell. The ADCC process is triggered and instead of inducing lysis of the target cell as in the in vivo process, luciferase is produced by the intracellular pathway activation in the engineered effector cell. The luminescence signal correlates to the efficacy of the antibody drug to elicit ADCC [19].

The iLite ADCC cells also have the secondary luciferase readout for normalizing the signal. The system also includes target negative cells that originate from the same cells as the positive target cells but do not express the target molecule. These enable the detection of nonspecific effects [19].



**Figure 5:** Illustrations of the iLite ADCC system where a positive target cell is activating the effector cell (a) and a negative target cell is not interacting with the effector cell (b)

## 2.4 Automation

The iLite assays are performed manually by preparing all reagents and adding them to each well of the plate. This takes up to an hour for a laboratory technician to perform. For some of the iLite assays, the analyte is first incubated together with its inhibitor before adding the ARC and again incubating the plate in a CO<sub>2</sub> incubator.

Automation for life science laboratories is of big importance today as the volumes that are being handled are usually very small and therefore both time-consuming and tedious to handle by hand. In drug discovery experiments a huge number of compounds are usually being screened to find one drug candidate and there is a need for high throughput screening (HTS) [21].

### 2.4.1 Pipetting

Automated liquid handling systems are used in many life science laboratories for an efficient drug discovery process. Automated systems can free laboratory technicians from repetitive work and increase the sample throughput by performing the assay more rapidly than is possible manually [22].

Dynex DSX® Automated Enzyme-Linked Immunosorbent Assay (ELISA) system, shown in Figure 6, is used at Wieslab to perform ELISAs. The instrument is in this project used for evaluating to possibility of automating the pipetting and incubation steps of the iLite reporter gene assays. Using a liquid handler for parts of the iLite assays could make it more automated and reduce the



time needed to spend on each assay. The Dynex DSX instrument has a pipette module with one pipette from which pipetting of samples, standards, controls, and reagents to 96-well plates is performed. It uses disposable pipette tips. The instrument has two temperature-controlled plate incubators, and the plate is automatically moved between the loading and incubation stations. The assay run is controlled by an assay file that is set up in the Dynex Revelation DSX software, reducing the time needed to spend on the assay for the operator, after just loading the reagents and samples into the instrument [23].

The use of an automated liquid handling system also opens up the possibility of running the assay in a 384 well plate which would give higher throughput. This format can be difficult to work with manually due to small volumes and wells.



**Figure 6:** Dynex DSX® Automated ELISA instrument [24].

### 2.4.2 Incubation

One objective of this study is to explore the possibility of incubating the assay without CO<sub>2</sub>. This would meet customer demands of not needing a CO<sub>2</sub> incubator. Moreover, it could simplify the assay and reduce the hands-on time by being able to leave the plate in the automation platform for the incubation steps.

The iLite ARC are suspended in RPMI 1640 medium and are normally incubated in 5% CO<sub>2</sub>. The function of CO<sub>2</sub> is to buffer the system to ensure stable pH [25]. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is an organic buffering agent present in some cell culture media [26]. It can be used to maintain stable pH in the absence of CO<sub>2</sub> and the hypothesis is that using media containing HEPES will allow the iLite ARC to be incubated without CO<sub>2</sub>.

CO<sub>2</sub> incubators also control the temperature and the humidity around the cells which ideally should be kept constant. Opening of the incubator door can cause fluctuations of these levels, creating stress to the cells and potentially add variations to the experiment [27].

Another factor to consider is the evaporation from the culture plate during incubation. The evaporation will be larger from the peripheral wells than from the central ones, due to being more exposed to the surroundings which is not saturated with water, called edge effect. The corner wells are most exposed to the surrounding and therefore prone to a large evaporation. As a consequence of the evaporation, components in the medium might reach concentrations that are harmful to the cells. Also, the concentrations of the reagents are altered which might additionally

affect the assay performance. The rate of evaporation is affected by many factors, such as the area of the space between the plate edge and the lid and the environmental humidity. Using a plate with a lid and a humidity close to 100% is preferred for reducing evaporation problems. Another way to avoid that the edge effect is causing problem to the assay is to exclude the peripheral wells and instead fill these with medium or water as a reservoir for evaporation [28].

## 2.5 Statistical analysis

The luminescence readout of the calibrators will be plotted against the logarithmic of the IFX concentration, generating dose response curves. The response is ideally described by a sigmoidal curve. For the TNF-alpha cells this will be an inhibition curve where the response is high at low drug concentrations forming an upper plateau and then having a negative slope as well as a lower plateau of minimal response. The readout from the ADCC assay will instead be dose response curves with positive slopes as the signal increases with IFX concentration. The concentration of the quality control (QC) samples are calculated from interpolation with the dose response curves.

Some important characteristics used to evaluate the performance of a bioanalytical method are the accuracy and the precision. The accuracy describes how close the value determined by the method is to the true value. It should be assessed using QC samples with known amount of the analyte. The QC samples should be analysed against a calibration curve that is independently prepared. The obtained value is compared to the true value and the accuracy is reported as the percentage of the true value. Both the accuracy within an assay run and between different runs should be evaluated and should be 85-115% for a general bioanalytical method [29].

The precision, or percent coefficient of variation (CV), describes the closeness of repeated measures [29]. CV% is calculated according to equation (1)

$$CV\% = \frac{\sigma}{\mu} \times 100 \quad (1)$$

where  $\sigma$  is the standard deviation and  $\mu$  is the mean value of the measurements. Precision should be determined by analysing the QC samples within each run as well as between three runs [29]. The inter-assay and intra-assay precision for an assay used for immunogenicity testing of therapeutic proteins are expected to be below 20%. However, higher CV% is accepted in cell-based assay formats since a biological response can be more variable than binding studies [30].

The  $R^2$  value is used to describe the goodness of fit, meaning how well-fitted the data is to the model. Another critical assay characteristic is the signal fold induction upon treatment with the drug, calculated from the ratio between the signal of two different drug concentrations. It can be used to describe the dynamic range of the assay.

## 2.6 Characteristics and capacity of current assays

The TNF-alpha cells are QC tested by Svar Life Science where the acceptance criteria for the current IFX drug concentration assay includes an  $R^2$ -value above 0.98 and a signal fold induction between the 5 and 50 ng/mL IFX above 3.0.

When Wieslab, the bioanalytical and diagnosis service of Svar Life Science, is using the iLite TNF-alpha cells to run the IFX drug concentration assay a standard curve is run in duplicate as well as a positive control and a negative control in duplicates. 10 samples are run in four dilutions and duplicates on each plate. If the outer wells are excluded and only the inner 70 wells, including the top row are being used, 7 samples can be run.

The QC acceptance criteria for the ADCC cells targeting mTNF-alpha are an  $R^2$ -value above 0.95 and a signal fold induction between the 167 ng/mL and 0 ng/mL anti-TNF-drug above 25.

### 3 Method

The method used during this study is presented below and the material and equipment are shown in Table 2.

**Table 2:** Material and equipment used to evaluate the possibility of automating reporter gene assays.

Material and equipment	Supplier
iLite TNF-alpha assay ready cells (ARC)	Svar Life Science
iLite ADCC Effector (V) cells	Svar Life Science
iLite mTNF-alpha (+) ARC	Svar Life Science
TNF-alpha	Svar Life Science
RPMI	Fischer Scientific
RPMI + 25 mM HEPES	Fischer Scientific
Heat inactivated fetal bovine serum (FBS)	Gibco
Normal human serum (NHS)	CELLect
Infliximab (IFX)	Svar Life Science
White-walled 96-well culture plate	Perkin Elmer
Dual-Glo Luciferase reagent	Promega
Dual-Glo Stop & Glo Renilla substrate	Promega
Dual-Glo Stop & Glo Buffer	Promega
GloMax Explorer Luminometer	Promega
Automated ELISA system	Dynex DSX

#### 3.1 TNF-alpha assay

To evaluate the possibility of transferring a reporter gene TNF-alpha assay to an automated platform, the performance of the assay when including HEPES in the medium was first tested as well as when incubating the assay without CO<sub>2</sub>. The performance of iLite TNF-alpha ARC in RPMI with HEPES and incubation in a heat cabinet was evaluated by doing an anti-TNF drug concentration determination assay using IFX and testing parameters such as precision, accuracy and plate homogeneity. The assay was then set up on the Dynex DSX® Automated ELISA system.

##### 3.1.1 Reagent preparation

The reagents used for the current standard TNF-alpha assay is shown in Table 3 as well as the reagents for the assay tested in this study. A serial dilution of IFX was performed to have the concentrations shown in Table 4. IFX control solutions called QC1, QC2, QC3 and QC4, were

prepared in IFX concentrations ending up on the sloping part of the dose response curves.

**Table 3:** The reagents used in the standard current IFX concentration assay using the TNF-alpha cells and the reagents to be tested during this study.

Current	Tested
16 ng/mL TNF-alpha in RPMI + 10% FBS	16 ng/mL TNF-alpha in RPMI + 10% FBS + 25 mM HEPES
IFX in RPMI + 8% NHS	IFX in RPMI + 8% NHS + 25 mM HEPES
TNF-alpha cells in RPMI	TNF-alpha cells in RPMI + 25 mM HEPES

**Table 4:** Concentration of IFX for the dose response curves where the solution is diluted four times during the assay

Concentration of IFX (ng/mL)	
Solution	In assay
10	2.5
20	5.0
40	10
80	20
120	30
160	40
200	50

### 3.1.2 Manual TNF-alpha assay

To evaluate the accuracy and the precision of the manual assay, the plate layout showed in Figure 7 was used. RPMI was used for half of the plate and RPMI with HEPES for the other half. Two set of dose response curves were run in duplicates at each half of the plate, as well as four QC samples in quadruplicates.

One assay run was performed by adding 25  $\mu$ L of IFX reference dilutions and control solutions to the assigned wells of a white-walled 96-well culture plate. 25  $\mu$ L of 16 ng/mL TNF-alpha solution were added to all wells. The plate with lid on was placed in a plate shaker for approximately 10 seconds at 900 rpm after which it was incubated for 30 minutes at 37 °C with 5% CO<sub>2</sub>. A vial of iLite TNF-alpha ARC with approximately  $2.5 \times 10^7$  cells/mL was thawed in a 37 °C water bath. The vial was diluted in 6 mL RPMI and 50  $\mu$ L of the cell solution was added to each well (equivalent to approximately 200 000 – 300 000 cells/well). The plate was again mixed on the

plate shaker for approximately 10 seconds at 900 rpm and then incubated for 3h at 37 °C with 5% CO<sub>2</sub>. The plate was equilibrated to room temperature for 10 minutes. 80 µL of Dual-Glo Luciferase reagent was added to each well and the plate was protected from light while incubating on a plate shaker for 10 minutes. It was then read in a luminometer. 90 µL of Dual-Glo® Stop & Glo® Substrate was diluted in 9 mL Dual-Glo® Stop & Glo® Buffer and 80 µL of the solution was added to each well. The plate was protected from light while incubating on a plate shaker for 10 minutes and then read in a luminometer.

Three replicate assay runs were performed where the halves of the plate with and without HEPES were flipped each time. The assay was then repeated three times with the modification of incubating the plate in a 37 °C heat cabinet.

	Dose-response curve		QC1/QC2	Dose-response curve		QC3/QC4	Dose-response curve		QC1/QC2	Dose-response curve		QC3/QC4	
	1	2	3	4	5	6	7	8	9	10	11	12	
A	50	50	40	50	50	20	50	50	40	50	50	20	
B	40	40		40	40		40	40		40	40		40
C	30	30		30	30		30	30		30	30		30
D	20	20		20	20		20	20		20	20		20
E	10	10	30	10	10	10	10	10	30	10	10	10	
F	5	5		5	5		5	5		5	5		5
G	2.5	2.5		2.5	2.5		2.5	2.5		2.5	2.5		2.5
H													

**Figure 7:** The 96-well plate layout used for evaluating the accuracy and precision of the manual reporter gene assay where the number is corresponding to the concentration of IFX in ng/mL. Two dose response curves are run in duplicate on each half of the plate, ranging from 2.5 to 50 ng/mL IFX, as well as four IFX control solutions, QC1, QC2, QC3 and QC4, in concentrations of 40, 30, 20 and 10 ng/mL IFX respectively.

Assay plate homogeneity was explored using a final assay concentration of 50 ng/mL IFX in all 96 wells of a plate. The assay was performed as described previously with either RPMI medium and a CO<sub>2</sub> incubator (37 °C 5% CO<sub>2</sub>) or RPMI medium with HEPES and a heat cabinet at 37 °C.

### 3.1.3 Automated TNF-alpha assay

Dynex DSX® Automated ELISA system was used for evaluating the possibility of automating the pipetting and incubation steps of the reporter gene assay. Revelation DSX software was used to set up the assay according to the protocol described in *3.1.2 Manual TNF-alpha assay*.

The reagents were prepared manually according to *3.1.1 Reagent preparation*. The IFX control solutions and calibrator solutions were placed in assigned positions in the Dynex instrument, as well as the 16 ng/mL TNF-alpha solution. The assay was setup so that the IFX solutions were first added to the assigned wells of the white-walled 96-well culture plate and then the TNF-alpha solution was pipetted to all wells being used. The instrument automatically placed the plate in an incubator set to 37 °C, where the plate was being mixed for 10 seconds at the low shaking setting. The TNF-alpha ARC were manually thawed and diluted as previously described in *3.1.2 Manual TNF-alpha assay*, and then placed in the assigned position of the Dynex instrument when the 30 minutes incubation time was up. The cells were added to the wells and the plate was incubated in the instrument for 3h at 37 °C with an initial 10 seconds mixing. The plate was read manually as previously described in *3.1.2 Manual TNF-alpha assay*.

To evaluate the accuracy and the precision of the automated assay the plate layout shown in

Figure 8 was initially used. After analysing the results, see 4.1.3 *Effect of automatisation*, it was decided to exclude the bottom row and the outer columns of the plate as well as to increase the concentration of QC sample 4. The adapted plate layout is shown in Figure 9, where 100  $\mu$ L medium was added to the wells not being used. The adapted plate layout was used to test different ways of combining the manual assay and the use of the automated platform to perform the assay. The pipetting steps were performed manually and the incubation was done in the automated platform as well as the other way around with automated pipetting and incubating in a heat cabinet.

Plate homogeneity was explored using a final assay concentration of 50 ng/mL IFX in all 96 wells of a plate.

	Dose-response curve		QC1	Dose-response curve		QC2	Dose-response curve		QC3	Dose-response curve		QC4
	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	40	50	50	30	50	50	20	50	50	10
B	40	40		40	40		40	40				
C	30	30		30	30		30	30				
D	20	20		20	20		20	20				
E	10	10		10	10		10	10				
F	5	5		5	5		5	5				
G	2.5	2.5		2.5	2.5		2.5	2.5				
H												

**Figure 8:** The 96-well plate layout used for evaluating the accuracy and precision of the automated reporter gene assay where the number is corresponding to the in assay concentration of IFX in ng/mL. Four dose response curves are run in duplicate, ranging from 2.5 to 50 ng/mL IFX, as well as four IFX control solutions, QC1, QC2, QC3 and QC4, in concentrations of 40, 30, 20 and 10 ng/mL IFX.

	Dose-response curve		QC1	Dose-response curve		QC2	Dose-response curve		QC3	QC4	12
	1	2	3	4	5	6	7	8	9	10	11
A		50	50	40	50	50	30	50	50	20	15
B		40	40		40	40					
C		30	30		30	30					
D		20	20		20	20					
E		10	10		10	10					
F		5	5		5	5					
G		2.5	2.5		2.5	2.5					
H											

**Figure 9:** The 96-well plate layout used for evaluating the accuracy and precision of the automated reporter gene assay. Three dose response curves are run in duplicate, ranging from 2.5 to 50 ng/mL IFX, as well as four IFX control solutions, QC1, QC2, QC3 and QC4, in concentrations of 40, 30, 20 and 15 ng/mL IFX.

### 3.2 ADCC assay

To evaluate the possibility of transferring a reporter gene ADCC assay to the automated platform, the assay was first performed manually with incubation in a heat cabinet before setting it up on the Dynex DSX® Automated ELISA system. The ADCC assay was performed using the positive mTNF-alpha target cells. The negative target cells were not included in the study since only the specific signal is needed to evaluate the possibility of automating the assay. These assays were performed using RPMI medium with HEPES.

### 3.2.1 Reagent preparation

An infliximab stock solution was prepared using RPMI with HEPES and 4% NHS for a final in assay concentration of 2% HS. An IFX serial dilution was performed according to Table 5. IFX control solutions called QC1, QC2, QC3 and QC4, were prepared in IFX concentrations ending up on the sloping part of the dose response curves.

**Table 5:** Concentration of IFX for the dose response curves where the solution is diluted two times during the assay

Concentration of IFX (ng/mL)	
Solution	In assay
1200	600
400	200
133	67
44	22
15	7.4
4.9	2.5
1.2	0.6

### 3.2.2 Manual ADCC assay

The plate layout shown in Figure 10 was used for evaluating the performance of the manual ADCC reporter gene assay in the heat cabinet and in the CO<sub>2</sub> incubator. RPMI medium was added to the wells not being used for the assay.

40  $\mu$ L of IFX reference dilutions and control solutions were added to the assigned wells of a white-walled 96-well culture plate. A vial of iLite ADCC Effector (V) ARC and a vial of mTNF-alpha (+) were thawed in a 37 °C water bath. 200  $\mu$ L of each vial was diluted in 3.44 mL RPMI + 25 mM HEPES and 40  $\mu$ L of the cell solution was added to each well. The plate was mixed on a plate shaker for approximately 10 seconds at 900 rpm and then incubated for 4h at 37 °C. The plate was equilibrated to room temperature for 10 minutes. 80  $\mu$ L of Dual-Glo Luciferase reagent was added to each well and the plate was protected from light while incubating on a plate shaker for 10 minutes and then read in a luminometer. 90  $\mu$ L of Dual-Glo® Stop & Glo® Substrate was diluted in 9 mL Dual-Glo® Stop & Glo® Buffer and 80  $\mu$ L of the solution was added to each well. The plate was protected from light while incubating on a plate shaker for 10 minutes and then read in a luminometer.

Three assay replicates were run with incubation in the CO<sub>2</sub> incubator and three assay replicates in the heat cabinet. Assay plate homogeneity was explored using a final assay concentration of 10 ng/mL IFX in all 96 wells.



	Dose-response curve		QC1	Dose-response curve		QC2	Dose-response curve		QC3	QC4		
	1	2	3	4	5	6	7	8	9	10	11	12
A		0.6	0.6	50	0.6	0.6	40	0.6	0.6	30	10	
B		2.5	2.5		2.5	2.5						
C		7.4	7.4		7.4	7.4						
D		22	22		22	22						
E		67	67		67	67						
F		200	200		200	200						
G		600	600		600	600						
H												

**Figure 10:** The 96-well plate layout used for evaluating the accuracy and precision of the ADCC reporter gene assay where the number is corresponding to the concentration of IFX in ng/mL. Three dose response curves are run in duplicate, ranging from 0.6 to 600 ng/mL IFX, as well as four IFX control solutions, QC1, QC2, QC3 and QC4, in concentrations of 50, 40, 30 and 10 ng/mL IFX.

### 3.2.3 Automated ADCC assay

Dynex DSX® Automated ELISA system was used for evaluating the possibility of automating the pipetting and incubation steps of the ADCC reporter gene assay. The reagents were prepared manually according to *3.2.1 Reagent preparation*. The IFX control solutions and calibrator solutions were placed in assigned positions in the Dynex instrument. The assay was setup to first add the IFX solutions to the assigned wells of the white-walled 96-well culture plate and then the TNF-alpha solution was pipetted to all wells being used. The instrument automatically placed the plate in an incubator set to 37 °C, where the plate was being mixed for 10 seconds at the low shaking setting. The ADCC Effector (V) cells and the mTNF-alpha (+) cells were manually thawed and diluted according to *3.2.2 Manual ADCC assay*, and then placed in the assigned position of the Dynex instrument. The cells were added to the wells and the plate was incubated in the instrument for 4h at 37 °C with an initial 10 seconds mixing. The plate was read manually as described in *3.2.2 Manual ADCC assay*.

Plate homogeneity was explored using a final assay concentration of 10 ng/mL IFX in all 96 wells of a plate.

### 3.3 Data analysis

GraphPad Prism 9 was used to draw dose response curves by plotting the FL signal in RLU or FL signal normalized relative to the RL activity against the log concentration of IFX. The dose response curves were analysed using the "sigmoidal dose-response (variable slope)" model in GraphPad Prism, which is described by equation (2)

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(LogIC50 - x) \times HillSlope}} \quad (2)$$

where Bottom and Top are the Y values at the bottom and top plateaus. LogIC50 is the X value corresponding to the Y value halfway between Bottom and Top where LogEC50 instead is used for dose response curves with positive slope. HillSlope describes the steepness of the curve.

The responses of the QC samples were interpolated with the dose response curves to generate an IFX concentration and compare with the true value of the QC sample. Microsoft Excel was used to do statistical analyses and draw homogeneity plots.

## 4 Results

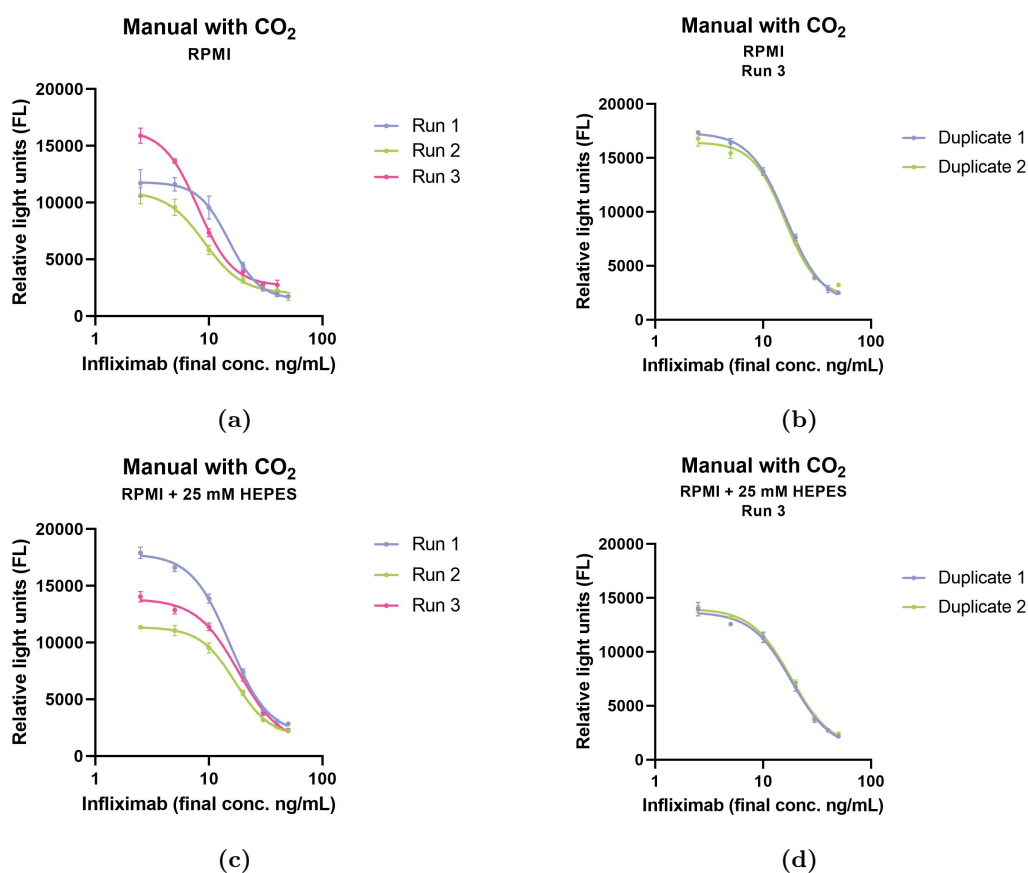
### 4.1 TNF-alpha assay

The results from the TNF-alpha assay is presented in this section where the performance of the assay is evaluated when introducing HEPES in the media, when performing the incubation without CO<sub>2</sub> and when automating the manual steps of the assay.

#### 4.1.1 Effect of HEPES

To see the effect of using RPMI with HEPES for the IFX concentration determination assay, the assay was performed using either RPMI or RPMI with 25 mM HEPES. The dose response curves are shown in Figure 11. Both the repeatability between runs (a and c) and within run (b and d) are shown for the two different media.

The FL signal induction and the R<sup>2</sup>-values of the dose response curves are presented in Table 6 as average values of the three runs. The fold induction, i.e the ratio between the readout of the wells with 5 ng/mL IFX and 50 ng/mL IFX, are approximately the same for the two different conditions. The R<sup>2</sup>-value for the dose response curves should be above 0.98 according to the QC criteria which is fulfilled for both assays.



**Figure 11:** Infiximab dose response curves from the manual TNF-alpha reporter gene assay incubated with CO<sub>2</sub> and using RPMI medium (a and b) or RPMI with HEPES (c and d).

**Table 6:** Average FL induction and R<sup>2</sup>-values for the TNF-alpha reporter gene assay performed manually with either RPMI or RPMI + 25 mM HEPES and incubation in CO<sub>2</sub>.

	RPMI	RPMI + 25 mM HEPES
<b>FL induction</b>	5.8	5.5
<b>R<sup>2</sup></b>	0.990	1.00

The sample accuracy and precision between runs and within a run is shown in Table 7 for the TNF-alpha assay with RPMI or RPMI + HEPES. The accuracy of the QC concentrations determined from the three runs are presented for the two different conditions and each control sample, as well as the precision. The QC criteria for the accuracy of the iLite TNF-alpha ARC is 80-120% of expected concentration. The between-run accuracy for both assays are close to 100% for each QC solution. The variation between runs, or the inter-assay precision CV% is below 9% for the assay with RPMI and below 11% for the one with RPMI + HEPES. The within-run accuracy and precision is presented for run 3 which were chosen to be representative. The within-run precision is below 5% and 3% respectively.

These data indicate that the accuracy, precision, fold induction and goodness of fit of the TNF-alpha reporter gene assay with RPMI + HEPES is comparable to the standard assay with RPMI.

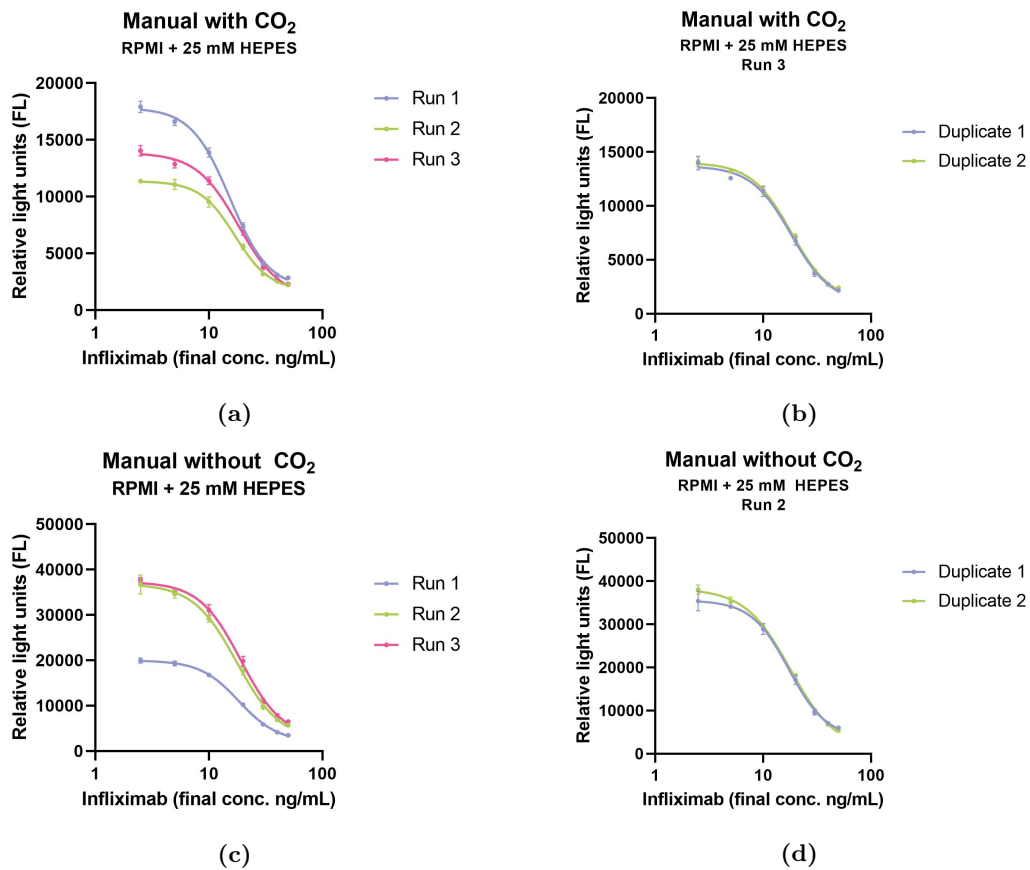
**Table 7:** The between-run and within-run sample accuracy (acc%) and precision (CV%) of the manual TNF-alpha reporter gene assay with RPMI or RPMI + 25 mM HEPES and incubation with CO<sub>2</sub>.

	RPMI				RPMI + 25 mM HEPES			
	Between-run		Within-run		Between-run		Within-run	
	acc%	CV%	acc%	CV%	acc%	CV%	acc%	CV%
<b>QC1</b>	97%	8.8%	106%	4.8%	99%	7.5%	92%	0.5%
<b>QC2</b>	100%	3.9%	105%	2.1%	98%	3.1%	101%	2.2%
<b>QC3</b>	95%	1.0%	96%	1.9%	95%	11%	101%	3.4%
<b>QC4</b>	99%	5.8%	101%	4.1%	105%	7.7%	110%	2.9%

#### 4.1.2 Effect of CO<sub>2</sub>

To see the effect of incubating the assay without CO<sub>2</sub>, the assay was performed using RPMI + 25 mM HEPES and incubating the plate either in a CO<sub>2</sub> incubator or in a heat cabinet. The dose response curves are shown in Figure 12. Both the repeatability between runs (a and c) and within run (b and d) are shown for the two different conditions.

The FL signal induction and the R<sup>2</sup>-values of the dose response curves are presented in Table 8. The fold inductions are similar for the two different conditions and the R<sup>2</sup>-values are 1.00.



**Figure 12:** Infliximab dose response curves from the manual reporter gene assay incubated with CO<sub>2</sub> (a and b) and without CO<sub>2</sub> (c and d).

**Table 8:** Average FL induction and R<sup>2</sup>-values for the TNF-alpha reporter gene assay performed manually with RPMI + 25 mM HEPES and incubation with or without CO<sub>2</sub>.

	CO <sub>2</sub>	No CO <sub>2</sub>
<b>FL induction</b>	5.5	5.7
<b>R<sup>2</sup></b>	1.00	1.00

The sample accuracy and precision between runs and within a run is shown in Table 9. The between-run accuracy for both assays are close to 100% for each QC solution. The between-run CV% is below 11% for the assay with CO<sub>2</sub> and below 14% for the one without CO<sub>2</sub>. A representative run was chosen for the within-run analysis, run 3 for the assay with CO<sub>2</sub> and run 2 for the assay without CO<sub>2</sub>. The within-run accuracy and precision is similar for the two assays where the within-run precision is below 3% and 4% respectively.

These data indicate that when RPMI + HEPES is used for the TNF-alpha reporter gene assay, the effect of CO<sub>2</sub> is not significant.

**Table 9:** The between-run and within-run sample accuracy (acc%) and precision (CV%) of the manual TNF-alpha reporter gene assay with RPMI + 25 mM HEPES and incubation with or without CO<sub>2</sub>.

	CO <sub>2</sub>				No CO <sub>2</sub>			
	Between-run		Within-run		Between-run		Within-run	
	acc%	CV%	acc%	CV%	acc%	CV%	acc%	CV%
<b>QC1</b>	99%	7.5%	92%	0.5%	98%	4.0%	95%	4.4%
<b>QC2</b>	98%	3.1%	101%	2.2%	95%	0.5%	95%	3.1%
<b>QC3</b>	95%	11%	101%	3.4%	94%	3.1%	94%	2.3%
<b>QC4</b>	105%	7.7%	110%	2.9%	105%	14%	111%	2.8%

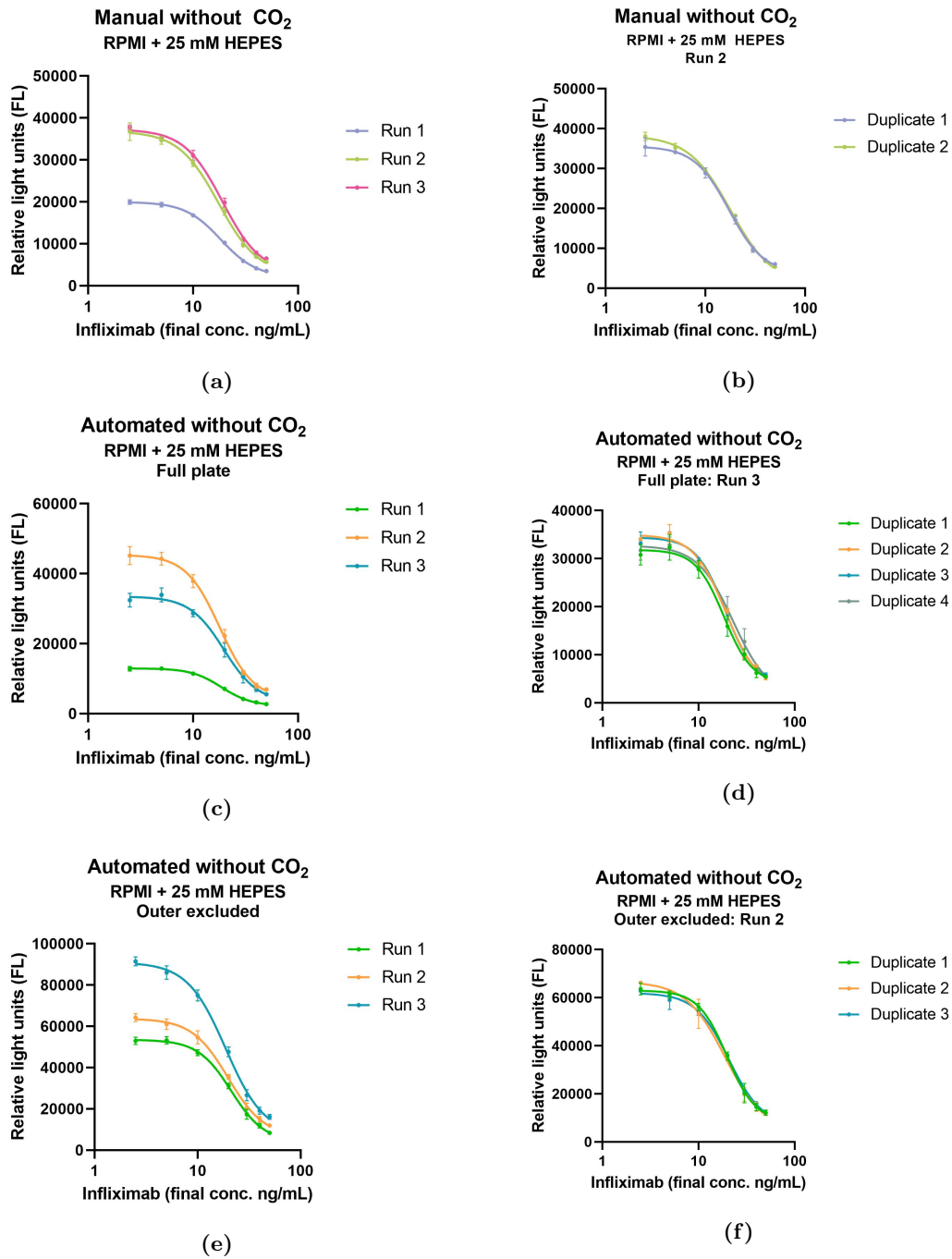
### 4.1.3 Effect of automatisation

To see the effect of automating the manual steps of the TNF-alpha reporter gene assay, the assay was performed either manually or on an automated platform where either the full plate was used or the center 70 wells. The dose response curves are shown in Figure 13. Both the repeatability between runs (a, c and e) and within a run (b, d and f) are shown for the three different assays.

The FL signal induction and the  $R^2$ -values of the dose response curves are presented in Table 10. The fold inductions are similar for the three different conditions and the  $R^2$ -values are close to 1.

**Table 10:** Average FL induction and  $R^2$ -values for the TNF-alpha reporter gene assay with RPMI + 25 mM HEPES and incubation without  $CO_2$  performed manually or on the automated platform using the full plate or excluding the outer wells.

	Manual	Automated full plate	Automated outer wells excluded
<b>FL induction</b>	5.7	5.8	5.6
<b><math>R^2</math></b>	1.00	0.991	1.00



**Figure 13:** Infliximab dose response curves from the manually performed reporter gene assay (a and b), the automated assay (c and d) and the automated assay where the outer wells have been excluded (e and f).

The sample accuracy and precision between runs are shown in Table 11. The between-run accuracy for the automated assay when using the full plate is too high, 130%, for QC sample 4. When excluding the outer wells and increasing the concentration of QC sample 4, the accuracy is improved to 95%. The between-run CV% is also improved for each QC sample.

**Table 11:** The between-run sample accuracy (acc%) and inter-assay precision (CV%) of the TNF-alpha reporter gene assay with RPMI + 25 mM HEPES and incubation without CO<sub>2</sub> performed manually or on the automated platform using the full plate or excluding the outer wells.

	Manual		Automated full plate		Automated outer wells excluded	
	acc%	CV%	acc%	CV%	acc%	CV%
<b>QC1</b>	98%	4.0%	102%	6.3%	96%	2.3%
<b>QC2</b>	95%	0.5%	104%	1.7%	97%	3.1%
<b>QC3</b>	94%	3.1%	103%	3.7%	93%	1.7%
<b>QC4</b>	105%	14%	133%	4.9%	95%	4.3%

The within-run accuracy and precision is presented in Table 12. The accuracy and precision is similar for the manual assay and the automated one when the outer wells are excluded. These data indicate that the automated assay where the outer wells have been excluded is comparable to the manual assay.

**Table 12:** The within-run sample accuracy (acc%) and intra-assay precision (CV%) of the TNF-alpha reporter gene assay with RPMI + 25 mM HEPES and incubation without CO<sub>2</sub> performed manually or on the automated platform using the full plate or excluding the outer wells.

	Manual		Automated full plate		Automated outer wells excluded	
	acc%	CV%	acc%	CV%	acc%	CV%
<b>QC1</b>	95%	4.4%	100%	5.5%	96%	3.1%
<b>QC2</b>	95%	3.1%	106%	7.4%	95%	2.7%
<b>QC3</b>	94%	2.3%	106%	7.0%	93%	3.7%
<b>QC4</b>	111%	2.8%	136%	6.4%	92%	5.6%

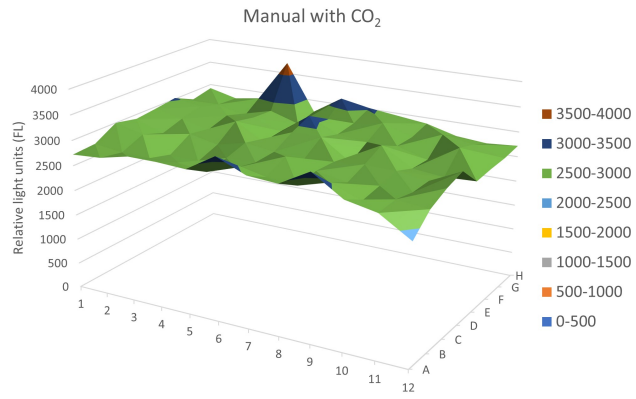


#### 4.1.4 Homogeneity

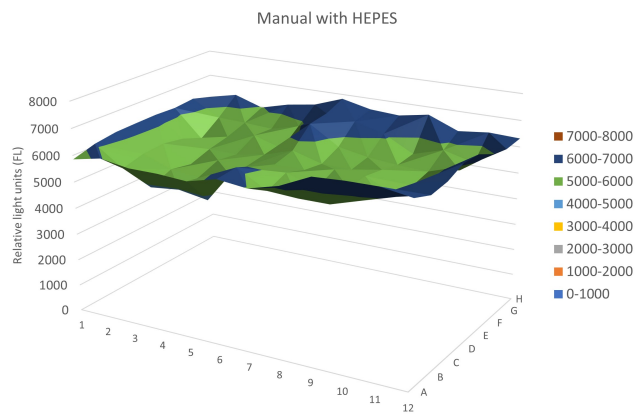
The firefly signal for all 96 wells are plotted in Figure 14 showing the plate homogeneity for three TNF-alpha reporter gene assays, (a) being the manual assay with RPMI and CO<sub>2</sub>, (b) the manual assay with RPMI + HEPES and (c) the automated assay.

The CV% calculated from the homogeneity plates are presented in Table 13. The CV% for the rows and columns is referring to the row or column with the highest variance and the plate CV% is calculated from all 96 wells. The plate variance for the manual assay with HEPES is higher than for the manual assay with CO<sub>2</sub>, 9% compared to 6%. The highest variation within a row is equal between the two conditions, 10%, and the highest variation within a column is higher for the plate incubated in CO<sub>2</sub>, 13% compared to 10%. The edge effect is evaluated from the CV% between top and bottom rows, outer columns and the center and edge parts of the plate. The variation between center and edge is larger for the plate with HEPES.

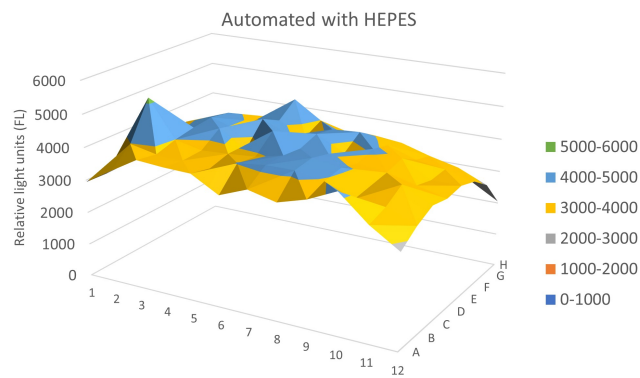
For the automated assay the calculations are based on if the full plate are being used or if the outer columns and bottom row are being excluded. The variance is lower if excluding the outer parts, where the plate CV% is reduced from 15% to 8%. The edge effect is overall also reduced, where the variance between the top and bottom rows and between the center and edge of the plate is reduced from 19% to 0.3% and from 17% to 6% respectively. The variance between the outer columns is higher for the case where the outer parts have been excluded. The results from these plate indicate that the firefly signal is more homogeneous when excluding the outer parts of the plate.



(a)



(b)



(c)

**Figure 14:** The FL signal plotted for each well of the plate for the manual assay with CO<sub>2</sub> (a), the manual assay with HEPES (b) and the automated assay (c).

**Table 13:** FL RLU homogeneity for the manual assay with CO<sub>2</sub> and with HEPES as well as the automated assay when analysing the entire plate or when the bottom row and outer columns are excluded. The results are presented as the highest CV% seen across a row and a column as well as the CV% between all wells of the plate. The edge effect is evaluated from the CV% between the outer rows and columns, and center and edge of the plate.

<b>CV%</b>	<b>Manual with CO<sub>2</sub></b>	<b>Manual with HEPES</b>	<b>Automated Full plate</b>	<b>Automated Outer wells excluded</b>
Row	<10%	<10%	<14%	<11%
Column	<13%	<10%	<19%	<17%
Plate	6%	9%	15%	8%
<b>Edge effect</b>				
Top and bottom rows	2%	1%	19%	0.3%
Outer columns	5%	4%	2%	7%
Center and edge	2%	9%	17%	6%

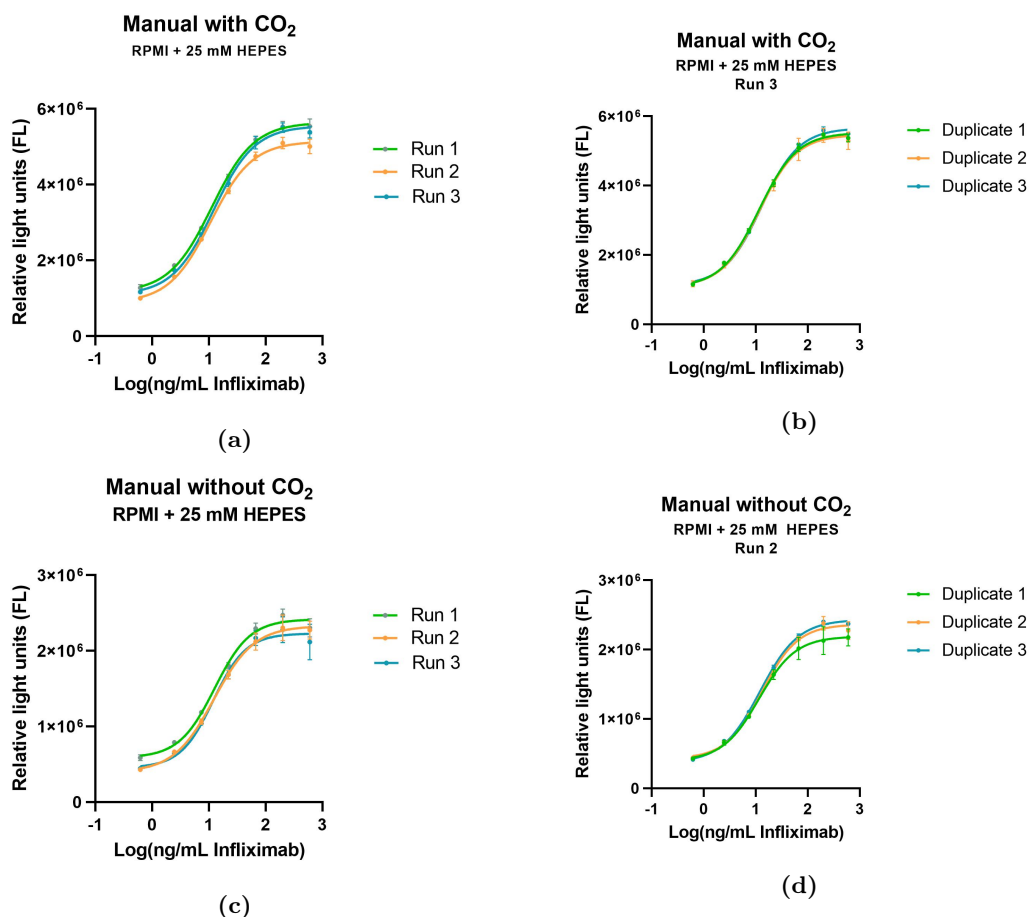
## 4.2 ADCC assay

The results from the ADCC assay is presented in this section where the performance of the assay is evaluated when performing the incubation without CO<sub>2</sub> and when automating the manual steps of the assay.

### 4.2.1 Effect of CO<sub>2</sub>

To see the effect of incubating the ADCC assay without CO<sub>2</sub>, the assay was performed using RPMI + 25 mM HEPES and incubating the plate either in a CO<sub>2</sub> incubator or in a heat cabinet. The dose response curves are shown in Figure 15, illustrating the repeatability between runs (a and c) and within run (b and d) for the two different conditions.

The FL signal induction and the R<sup>2</sup>-values of the dose response curves are presented in Table 14 as average values of the three runs. The fold induction, i.e the ratio between the readout of the wells with 200 ng/mL IFX and 0.6 ng/mL IFX, are similar for the two different conditions. the R<sup>2</sup>-value should be above 0.95 according to the QC criteria which is fulfilled for both assays. However, the R<sup>2</sup>-value is higher when incubating with CO<sub>2</sub>.



**Figure 15:** Infiximab dose response curves from the manual ADCC reporter gene assay incubated with CO<sub>2</sub> (a and b) and without CO<sub>2</sub> (c and d).

**Table 14:** Average FL induction and R<sup>2</sup>-values for the IFX ADCC reporter gene assay performed manually with RPMI + 25 mM HEPES and incubation with or without CO<sub>2</sub>.

	CO <sub>2</sub>	No CO <sub>2</sub>
<b>FL induction</b>	4.7	4.9
<b>R<sup>2</sup></b>	1.00	0.987

The sample accuracy and precision between runs and within a run is shown in Table 15 for the IFX ADCC assay incubated with or without CO<sub>2</sub>. For the within-run analysis, run 3 was chosen as a representative run for the assay with CO<sub>2</sub> and run 2 for the assay without CO<sub>2</sub>. The accuracy and precision for the assay with CO<sub>2</sub> are within the generally acceptable range of 85-115 % and below 15 %. For the assay without CO<sub>2</sub>, on the other hand, both accuracy and precision is too low for QC samples 1 and 2.

These data indicate that in the absence of CO<sub>2</sub> the accuracy and precision of the IFX ADCC assay is outside of the acceptable ranges.

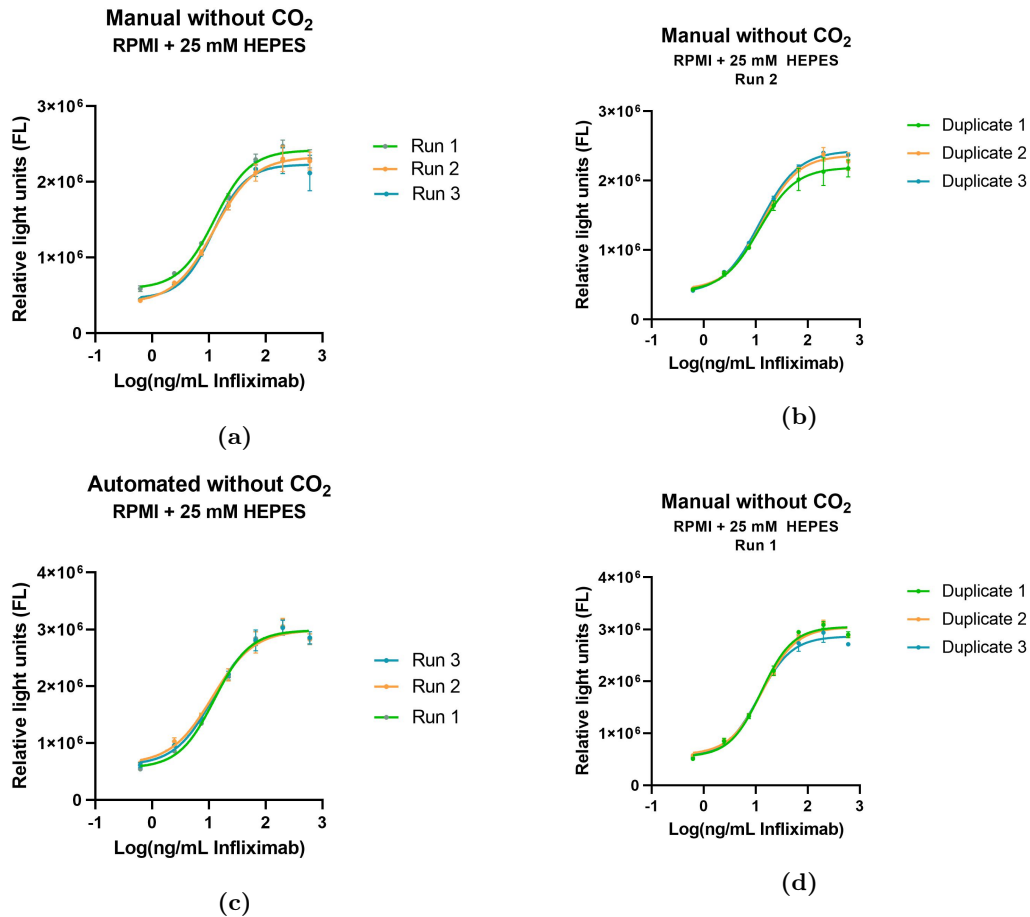
**Table 15:** The between-run and within-run sample accuracy (acc%) and precision (CV%) of the manual ADCC reporter gene assay with RPMI + 25 mM HEPES and incubation with or without CO<sub>2</sub>.

	CO <sub>2</sub>				No CO <sub>2</sub>			
	Between-run		Within-run		Between-run		Within-run	
	acc%	CV%	acc%	CV%	acc%	CV%	acc%	CV%
<b>QC1</b>	114%	13%	103%	7.5%	235%	51%	245%	94%
<b>QC2</b>	90%	5.6%	85%	3.8%	110%	20%	134%	37%
<b>QC3</b>	96%	2.0%	97%	2.9%	94%	5.8%	95%	16%
<b>QC4</b>	93%	5.5%	98%	2.8%	90%	2.0%	98%	2.8%

#### 4.2.2 Effect of automatisation

To see the effect of automating the manual steps of the IFX ADCC reporter gene assay, the assay was performed either manually or on an automated platform where the center 70 wells were used. The dose response curves are shown in Figure 16, illustrating the repeatability between runs (a and c) and within a run (b and d).

The FL signal induction and the  $R^2$ -values of the dose response curves are presented in Table 16. The fold inductions and the  $R^2$ -values are similar for the two different conditions.



**Figure 16:** Infliximab dose response curves for the ADCC reporter gene assay performed either manually (a and b) or on an automated platform (c and d).

**Table 16:** Average FL induction and  $R^2$ -values for the IFX ADCC reporter gene assay performed either manually or automated.

	Manual	Automated
<b>FL induction</b>	4.9	5.1
<b><math>R^2</math></b>	0.987	0.988

The sample accuracy and precision between runs and within a run is shown in Table 17 for the IFX ADCC assay performed manually or automated. Run 1 was chosen as a representative run for the automated assay for calculations of the within-run parameters. The automated assay has higher accuracy and precision than the manual one. However, for QC sample 1 the values are still outside the acceptable ranges.

These results indicate that the assay performance is improved by automating the manual steps. However the accuracy and precision for parts of the tested IFX range are lower than acceptable.

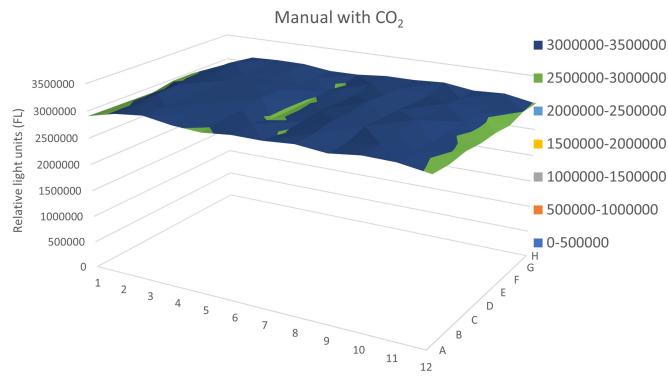
**Table 17:** The between-run and within-run sample accuracy (acc%) and precision (CV%) of the ADCC reporter gene assay performed manually or automated.

	Manual				Automated			
	Between-run		Within-run		Between-run		Within-run	
	acc%	CV%	acc%	CV%	acc%	CV%	acc%	CV%
<b>QC1</b>	235%	51%	245%	94%	128%	22%	151%	15%
<b>QC2</b>	110%	20%	134%	37%	97%	21%	104%	18%
<b>QC3</b>	94%	5.8%	95%	16%	95%	1.4%	93%	9.1%
<b>QC4</b>	90%	2.0%	98%	2.8%	87%	5.3%	92%	1.4%

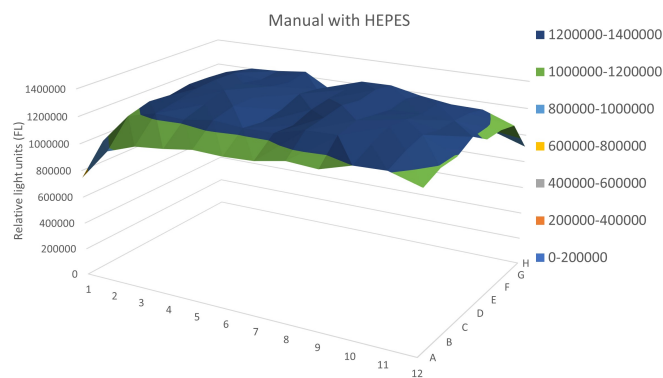
### 4.2.3 Homogeneity

The firefly signal for all 96 wells are plotted in Figure 17 showing the plate homogeneity for all three ADCC assays. The signal from the manual assay with CO<sub>2</sub> is more homogenous across the plate.

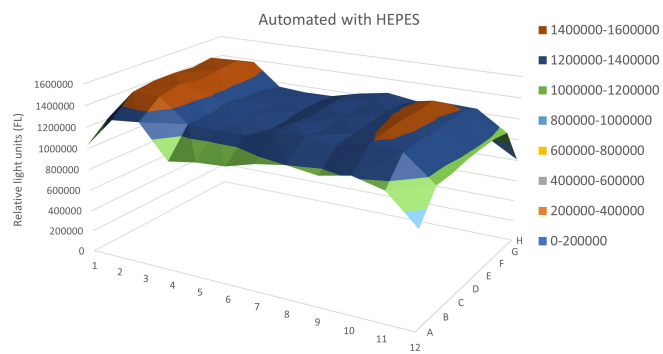
The CV% calculated from the homogeneity plates are presented in Table 18 as both the variation across the rows, columns and the plate as well as between different parts of the plate. For the manual ADCC assay, the variation is higher when the incubation is done with HEPES in the heat cabinet than when it is incubated in an incubator with CO<sub>2</sub>. The automated ADCC assay has a lower plate homogeneity and larger edge effect than the manual assays. However, when excluding the outer columns and the bottom row from the calculations the CV% is lowered, where the plate CV% is reduced from 13% to 9% and the variation between the center and edge of the plate is reduced from 13% to 0.5%.



(a)



(b)



(c)

**Figure 17:** The FL signal plotted for each well of the plate for the manual ADCC assay with HEPES and CO<sub>2</sub> (a), the manual assay with HEPES (b) and the automated assay with HEPES (c).



**Table 18:** FL RLU homogeneity for the manual ADCC assay with CO<sub>2</sub> and with HEPES as well as the automated assay when analysing the entire plate or when the bottom row and outer columns are excluded. The results are presented as the highest CV% seen across a row and a column as well as the CV% between all wells of the plate. The edge effect is evaluated from the CV% between the outer rows and columns, and center and edge of the plate.

<b>CV%</b>	<b>Manual with CO<sub>2</sub></b>	<b>Manual with HEPES</b>	<b>Automated Full plate</b>	<b>Automated Outer wells excluded</b>
Row	<3%	<12%	<10%	<9%
Column	<2%	<11%	<15%	<8%
Plate	2%	10%	13%	9%
<b>Edge effect</b>				
Top and bottom rows	1%	4%	12%	9%
Outer columns	1%	11%	5%	7%
Center and edge	2%	13%	13%	0.5%

## 5 Discussion

The aim of this degree project was to develop a reporter gene assay on an automated platform. One objective was also to evaluate the possibility of incubating the assay without CO<sub>2</sub>. Two cell lines were included in the study, where the first one was used for determining the concentration of an anti-TNF-drug, infliximab (IFX), and the second one to determine the ADCC activity of the same anti-TNF-drug. Assay characteristics that were evaluated and compared between the manually performed assays and the automated assays include sample accuracy, precision, plate homogeneity and signal fold induction.

For all results presented, the firefly signal has been used instead of the normalized signal. It is due to the fact that the results showed that for these assay runs, the renilla signal added variation rather than reducing it. Raw data from one run is shown in *Appendix A* to demonstrate this.

### 5.1 TNF-alpha assay

For the objective of evaluating the possibility of incubating the TNF-alpha reporter gene assay without CO<sub>2</sub>, it was first evaluated if RPMI with HEPES could be used as medium. HEPES does not have much effect on the accuracy and the precision of the assay. The fold induction is reduced from 5.8 to 5.5 when using RPMI + HEPES compared to RPMI. As the acceptance criteria is 3.0 for the fold induction, this reduction is negligible. It can be concluded that it is possible to use RPMI + HEPES for this assay.

The effect of CO<sub>2</sub> during incubation was then evaluated. The dose response curves show that the repeatability within a run is similar when incubating with and without CO<sub>2</sub>. The dose response curves from different runs, however, show that the signal varies more between runs in the absence of CO<sub>2</sub>. From studying the accuracy, precision, fold induction and goodness of fit it can be concluded that the assay without CO<sub>2</sub> is comparable to the one with CO<sub>2</sub>.

The effect of automatising was evaluated using the Dynex DSX automated platform. When using the same plate layout as was done for the manual assay, the accuracy of QC sample 4 was too low. As this QC sample was placed in column 12, the low accuracy could depend on an edge effect. A new plate layout was tested where column 1, 12 and row H were excluded. The concentration of QC sample 4 was also increased in order to end up on the sloping part of the curve rather than on the lower plateau. The automated assay with the outer parts excluded are having accuracy and precision comparable to the manual assay. The automated assay even has a higher between-run precision.

For evaluating the homogeneity of the automated assay, homogeneity plots have been drawn. The homogeneity plots show that the signal is more homogeneous when incubating with CO<sub>2</sub>, except for one well with an increased signal. The homogeneity plate from the manual assay with HEPES shows an edge effect which is also confirmed by the high calculated variation between the center and edge of the plate. This suggests that the heat cabinet is not providing an environment as stable as the CO<sub>2</sub> incubator. In the CO<sub>2</sub> incubator the humidity is controlled and the air is circulating, providing as uniform conditions as possible.

The homogeneity plot for the automated assay show both a low homogeneity of the signal and an edge effect. The firefly signal is lower in the corner wells and in row H which is the row closest to the door to the incubation compartment. The variation across the plate was evaluated when using the full plate and when excluding the outer columns and the bottom row from the calculations. The CV% calculated is drastically reduced when the outer parts are excluded and

the edge effect is not evident. The results indicate that the incubator is not able to keep the temperature at 37 °C in the entire space and that the plate is not fully isolated from the outer temperature. The incubation is performed without a lid on the plate, which could increase the edge effect. Moreover, the humidity is not controlled. However one advantage of the automated incubation is that the door is not opened during the incubation time which is common when using a CO<sub>2</sub> incubator in a shared lab and can cause fluctuating temperatures.

The automated assay was setup to have as few manual steps as possible. The samples, reagents and cells were added to the plate by the instrument and the incubation steps were also performed in the automated platform. As the automated platform does not have a luminescence reader, the firefly and renilla luciferase substrates were added by manual pipetting. If having a luminescence reader near the automated platform, these pipetting steps could most likely also be automated. However, due to the fact that the pipetting module only has one pipette, there is a delay between when the substrate is added in the first well until it is added in the last. This might affect the signal.

From studying the raw firefly and renilla luciferase signal of the automated assay a high variation was observed between some wells with dose response duplicates, see Appendix B. To understand if this variation is from the automated pipetting or the incubation steps, different runs were performed to combine the manual pipetting, incubation in heat cabinet, automated pipetting and incubation in the automated platform. The data showed that when doing the pipetting to the plate manually the raw FL signal variation between duplicates is low and when the pipetting steps are automated some of the calibrator duplicates on each plate have high variance, up to approximately 20%. The automated platform used should have a pipetting precision of  $\leq 3\%$  CV. However, the instrument does only have one pipette and as a consequence there is a delay between when the reagents are added in the first one well and the last well, hence the reaction times will differ. When performing the pipetting steps manually, the cell solution and the calibrator solutions are added using a multichannel pipette which reduces the handling time. However, multichannel pipettes can add tip-to-tip variance. The use of an automated platform with an eight-channel pipetting head could reduce the time for the pipetting to be performed and likely reduce the variation between dose response duplicates if all tips are adding the same volumes.

The plate layout with bottom row and outer columns excluded means that 70 of 96 wells are being used. It reduces the efficiency of the automated assay as a lower number of samples can be run on each plate. However, it is only a decrease of three samples per plate. Moreover, the automated pipetting seems to give an increased variance of the FL signal between duplicates. However, when using the dose response curves to calculate the accuracy and precision of the assay, the automated assay is comparable to the manual one and even has a higher between-run precision. If the automated or the manual assay is the preferred one will depend on how much variance can be accepted in relation to how high-throughput sampling is needed.

In conclusion it is possible to determine the concentration of an anti-TNF drug using a reporter gene assay on an automated platform with accuracy and precision comparable to the manual assay. The automated assay requires less hands-on time meaning that more patient samples can be analysed and more drug candidates can be screened in one day.

## 5.2 ADCC assay

To evaluate the possibility of running the IFX ADCC assay on the automated platform without CO<sub>2</sub>, the assay was first performed manually with RPMI + HEPES and incubation in either a CO<sub>2</sub> incubator or in a heat cabinet. The center 70 wells of the plate were used based on previous results from the automated platform indicating that the temperature is not kept in column 1, 12 and row H. From the dose response curves it can be seen that variation is added in the absence of CO<sub>2</sub>, especially towards the upper plateau of the curves. It results in that the concentration of the QC sample with the highest IFX concentration can not be determined accurately. The accuracy and precision of QC sample 2 is also lower than what is acceptable for a general bioanalytical method. This indicates that the ADCC assay is not able to be incubated without CO<sub>2</sub>.

The effect of automatising the manual steps of the ADCC assay was studied by comparing the manual runs with the automated runs. From the dose response curves as well as the calculated parameters it can be seen that the automated assay has higher accuracy and precision both between-run and within-run. However, there is still large variations between the measurements with higher concentrations resulting in that QC sample 1 has low accuracy and precision.

When it comes to plate homogeneity, the homogeneity plots show that the signal is more homogeneous when incubating the assay with CO<sub>2</sub>. The homogeneity plate from the manual assay without CO<sub>2</sub>, with HEPES shows an edge effect with reduced signal in the outer wells and especially in the corner wells. The edge effect is also confirmed by the CV% calculated between the center and edge of the plate. This agrees with the results from the TNF-alpha assay and again suggests the CO<sub>2</sub> incubator is providing a more stable environment.

The homogeneity plot for the automated assay shows an edge effect as well as high variation between the columns. The firefly signal is reduced primarily in column 1, 12 and row H. The variation across the plate was evaluated when using the full plate and when excluding the outer columns and the bottom row from the calculations. The plate homogeneity is improved when the outer parts are excluded and the edge effect is reduced.

The study has shown that for the IFX concentration assay, RPMI with HEPES can replace the pH buffering of CO<sub>2</sub>. It is also possible to automate the manual steps of the assay. However, for the IFX ADCC assay, RPMI with HEPES can not replace the use of CO<sub>2</sub>. These two different reporter gene assays are using different cell types. In the ADCC assay, effector cells and target cells are interacting, whilst the TNF-alpha assay include only one cell line. The metabolism of the cells might influence how much pH buffering is needed. Furthermore, the ADCC assay is incubated during one more hour.

## 6 Conclusion

The overall aim of this project was to develop a reporter gene assay on an automated platform. In conclusion, one of the two reporter gene assays tested in this study could be transferred to the automated platform. It was also shown that the pH buffering of CO<sub>2</sub> during incubation of the assay could be replaced with a medium with HEPES. The other assay included in the study did on the other hand show poor performance when run without CO<sub>2</sub>. The automated assay showed higher accuracy and precision than the manual assay without CO<sub>2</sub>. However the automated assay is not able to be used for the upper part of the tested drug range.

### 6.1 Future work

In this study only two reporter gene cell lines have been included with the conclusion that one of them could be transferred to an automated platform and the other one could not. For future work other ARC could be tested in the heat cabinet to see if HEPES can replace CO<sub>2</sub> which would open up the possibility of automating the assay. Moreover, other anti-TNF-drugs should be tested with the automated TNF-alpha assay as only infliximab (IFX) has been tested in this study.

When knowing that the TNF-alpha assay can be run outside a CO<sub>2</sub> incubator and using a liquid handler, it can also be interesting to study the possibility of running the assay in a 384 well plate format to further increase the efficacy.

The automated ADCC assay could also be further optimised by evaluating what IFX range is possible to use in this format. Finally, the automated assays could be further automated by also pipetting the FL and RL substrates with the liquid handler. The plate would then only needed to be manually placed in a luminometer preferably positioned next to the automated platform.

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# Appendices

## A Normalized data

Raw data for one of the TNF-alpha reporter gene assay runs are shown in Figure 18, 19 and 20 to show that the renilla signal is in this case adding variance and hence the normalized FL/RL ratio was not used for the calculations during this study.

Raw data: FireFly Luciferase												
	1	2	3	4	5	6	7	8	9	10	11	12
A	2275	2521	3040	2763	2495	4279	2470	2786	6849	3208	3102	9237
B	2892	3178	3100	3351	3546	5106	3483	3220	7124	3187	2955	10560
C	4109	3854	3307	4143	4034	3994	4198	4079	6953	4811	4063	10830
D	6914	7418	3053	6877	7593	4063	7199	6944	6205	7177	6489	9893
E	11460	11250	3096	10930	11510	3808	11620	12110	7254	11600	10940	10520
F	12380	13300	3399	12390	13800	4568	12450	13380	7034	12720	12600	10460
G	12320	12480	3110	13960	12800	4051	13020	12230	6262	13470	12210	10420
H			2585			3404			5893			7053

Raw data: Renilla Luciferase												
	1	2	3	4	5	6	7	8	9	10	11	12
A	474700	497600	503400	369200	444400	429500	428000	436900	449500	477500	512200	522500
B	473300	503300	478500	378000	442100	398300	430700	433700	453800	475900	502100	534400
C	522100	494400	469800	369700	408400	371000	401600	398300	424600	456200	487300	511000
D	506500	499700	479000	371000	418300	364200	395400	389300	451300	441800	494800	511500
E	537300	524400	506700	394700	438600	412000	417100	415700	423200	473200	489200	513300
F	572100	563000	502600	438700	469700	454000	468200	461600	456300	515700	520700	539800
G	594800	598800	588500	457600	487000	500000	474900	439000	533000	507800	553100	590200
H			568300			478200			516000			593200

**Figure 18:** The firefly luciferase and renilla luciferase raw signals in all wells from one of the IFX concentration assays

Processed data from GraphPad (based upon FL values)												
FL	Log <sub>10</sub> (ng/ml infliximab)				ng/ml infliximab				ng/ml infliximab			
	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
Calculation based upon Dose-response 1 (column 1-2)	1.58461726	1.46349333	1.31306289	1.18709692	38	29	21	15				
	1.57657079	1.40810433	1.29031967	1.09484372	38	26	20	12				
	1.55099955	1.48607277	1.30796306	1.0778239	36	31	20	12				
	1.58324978	1.48041485	1.34624292	1.14680227	38	30	22	14				
	1.57657079	1.50133467	1.29272308	1.10288016	38	32	20	13				
	1.54105852	1.4428481	1.30389223	1.10288016	35	28	20	13				
	1.57526538	1.4812144	1.34360358	1.11064207	38	30	22	13				
	1.66203642	1.54105852	1.36340644		46	35	23	20				
		Average		38	30	21	14					

Processed data from GraphPad (based upon FL/RL values)												
FL/RL	Log <sub>10</sub> (ng/ml infliximab)				ng/ml infliximab				ng/ml infliximab			
	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
Calculation based upon Dose-response 1 (column 1-2)	1.58619077	1.40666409	1.28033314	1.21199129	39	26	19	16				
	1.54618605	1.33697375	1.26790543	1.12596832					22	19	13	
	1.51618243	1.38542946	1.24970888	0.99630425	33	24	18	10				
	1.55317226	1.37533435	1.31598888	1.15146588	36	24	21	14				
	1.57701042	1.42989333	1.23019827	1.07854493	38	27	17	12				
	1.52734241	1.40391752	1.27540876	1.14673838	34	25	19	14				
	1.68996166	1.46717516	1.3630692	1.21199129	49	29	23	16				
			1.51094202	1.37038671	1.3562502					32	23	23
		Average		38	26	20	15					

Processed data from GraphPad (based upon FL values)												
FL	Log <sub>10</sub> (ng/ml infliximab)				ng/ml infliximab				ng/ml infliximab			
	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
Calculation based upon Dose-response 2 (column 4-5)	1.63049013	1.49089958	1.31006214	1.16353151					31	20	15	
	1.62160045	1.42450694	1.29364883	1.06597465					27	20	12	
	1.59290931	1.51765932	1.30396398	1.04936471	39	33	20	11				
	1.62898435	1.51097532	1.34990537	1.11953217	43	32	22	13				
	1.62160045	1.53560081	1.28579516	1.07398815	42	34	19	12				
	1.58159127	1.46625182	1.29910248	1.07398815	38	29	20	12				
	1.62015167	1.5119217	1.34672762	1.08182706	42	33	22	12				
	1.7117932	1.58159127	1.37059209	1.29788922	51	38	23	20				
		Average		42	32	21	13					

Processed data from GraphPad (based upon FL/RL values)												
FL/RL	Log <sub>10</sub> (ng/ml infliximab)				ng/ml infliximab				ng/ml infliximab			
	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
Calculation based upon Dose-response 2 (column 4-5)	1.89986223	1.50629498	1.36684153	1.3124241	79	32	23	21				
	1.74689619	1.42338788	1.35578601	1.2661209	56	27	23	18				
	1.67836575	1.47944563	1.34051617	1.23311183	48	30	22	17				
	1.76616605	1.46718311	1.40128877	1.27738653	58	29	25	19				
	1.85127822	1.53744964	1.32537963	1.24993505	71	34	21	18				
	1.70176945	1.50273861	1.36240078	1.27514966	50	32	23	19				
		1.59223554	1.45270241	1.3124241					39	28	21	
		1.66800723	1.46128712	1.44713646					47	29	28	
		Average		57	34	24	20					

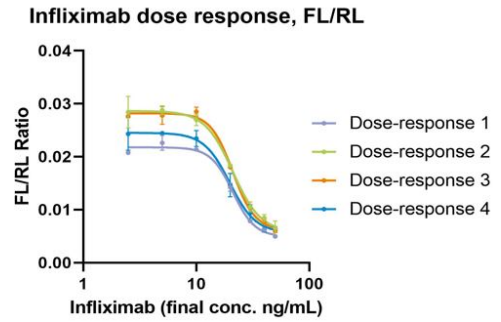
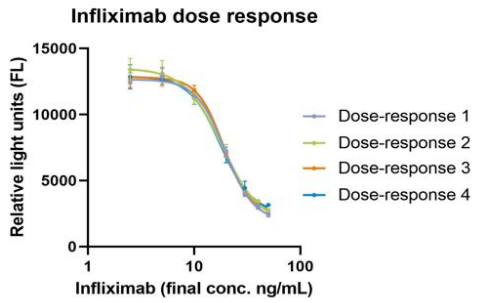
**Figure 19:** Data processed from the signals in Figure 16 by interpolation with dose response curves 1 and 2 as examples. The FL signal has been used in the left section and the normalized FL/RL signal in the right one.



FL	QC1	QC2	QC3	QC4
Dose-resp 1	38	30	21	14
Dose-resp 2	42	32	21	13
Dose-resp 3	44	30	21	15
Dose-resp 4	50	32	20	13
Average	44	31	21	14
Expected	40	30	20	10
Accuracy %	109%	104%	104%	137%
CV%	10.7%	3.43%	1.4%	4.6%

FL/RL	QC1	QC2	QC3	QC4
Dose-resp 1	38	26	20	15
Dose-resp 2	57	34	24	20
Dose-resp 3	51	31	24	20
Dose-resp 4	46	28	21	16
Average	48	30	22	18
Expected	40	30	20	10
Accuracy %	120%	100%	110%	178%
CV%	16.6%	11.15%	10.1%	14.9%



**Figure 20:** Results based on the data and calculations in Figure 18 and 19, showing that using the FL signal (left) give higher accuracy and precision than when using the FL/RL ratio (right).

## B Variation of raw signal

The firefly signal from one run of the automated TNF-alpha assay is shown in Figure 21 as well as the CV% calculated from the data. As an example the variation between column 2 and 3 in row B and between column 5 and 6 in row C are more than 15%.

Raw data: FireFly Luciferase												
	1	2	3	4	5	6	7	8	9	10	11	12
A		8430	7730	14500	8730	8640	17700	8560	8390	28200	35500	
B		9650	12100	13300	12300	12800	23300	12600	11700	32800	37400	
C		14400	17100	13100	19900	14800	16900	19200	19000	33400	42600	
D		28900	33100	12900	31200	30700	27900	31200	31600	34000	43000	
E		45600	48300	12000	49300	46200	19300	47600	46100	33200	41200	
F		51600	54700	12300	54700	54700	16500	52500	50800	32900	40300	
G		50300	52400	11500	54000	55500	15700	52600	53000	32000	37000	
H												
Variance (CV%) for FireFly Luciferase												
	1	2	3	4	5	6	7	8	9	10	11	12
A		6.13%		7.69%	0.73%		22.65%	1.42%		5.97%	7.44%	
B		15.93%			2.82%			5.24%				
C		12.12%			20.79%			0.74%				
D		9.58%			1.14%			0.90%				
E		4.07%			4.59%			2.26%				
F		4.12%			0.00%			2.33%				
G		2.89%			1.94%			0.54%				
H												

**Figure 21:** The firefly signal from one run of the automated assay and the CV% calculated from the dose response duplicates and the QC sample replicates.