LABORATORY EXERCISE 1

Characterization of a human recombinant antibody fragment to be used in a diagnostic test

Evaluation by SDS-PAGE, NanoDrop™, ELISA, Biacore™ and Antibody microarray.

Supervisors:

Fredrika Axelsson (fredrika.axelsson@immun.lth.se; 046-22 29832)
Anders Carlsson (anders.carlsson@immun.lth.se; 046-22 24479)

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Flow chart

Bacterial culture

Expression of scFv antibody fragment
(clone F3)

Purification of scFv (F3)

Analysis of purified scFv (F3)

LAB DAY 1 (Wednesday)

PURITY
SDS-PAGE

CONCENTRATION
NanoDrop™

CONCENTRATION
ELISA
Coating

APPLICATION
Ab Microarray
Spotting

LAB DAY 2 (Thursday)

BINDING CHARACTERISTICS
Biacore™

Assay
ELISA analysis

Assay
Microarray analysis

LAB DAY 3 (Friday)

EVALUATION OF RESULTS AND DISCUSSION
INTRODUCTION

The purpose of these laboratory experiments is to characterize a human recombinant single-chain Fv (scFv) antibody fragment by using several different analytical methods and to illustrate one of the differences between a scFv antibody fragment and an intact antibody of the isotype IgG. The methods we will use for characterization are SDS-PAGE, NanoDrop™, ELISA and Biacore™. As an application, an Antibody Microarray will be used for identification of viral antigen in complex samples.

Cytomegalovirus (CMV) is a herpes virus with high prevalence (>80 %) in the human population. The virus does not pose any health problem to healthy individuals, but for immuno-compromised individuals such as AIDS- and transplantation patients and congenitally infected babies the disease can be devastating. CMV is the most common congenital infection and cause of birth defects (e.g. hearing loss and mental retardation). Antibodies with the ability to bind to pathogens (e.g. CMV) and neutralize them have a great therapeutical interest. Several pharmaceuticals today are based on antibodies.

The scFv antibody fragment, F3, we will examine during the study binds to an envelope protein of CMV called glycoprotein B (gB). The scFv F3 is an affinity-maturated variant of the intact IgG antibody, ITC88. ITC88 was isolated from peripheral blood lymphocytes of an individual infected with CMV. With he variable domains of ITC88 as a template, and small library of scFv was created. From this library, clone F3 was selected through phage display. Due to its potential to bind and neutralize CMV, there is a possibility to design a vaccine or a diagnostic test based on this antibody.

In the experiments, a short biotinylated peptide, AD-2, of the envelope protein gB of CMV will be used. The peptide AD-2 has the sequence biotin-ANETIYNTTLKYGDV, which includes the linear epitope that F3 binds to.

Recombinant proteins, such as scFv antibody fragments, are often produced in modified bacteria or yeast cells. A plasmid with the gene coding for the protein is transformed into the bacteria and the bacteria are then cultured in media. When the culture has reached exponential phase with a suitable cell density the production of the recombinant protein is induced through the activation of a promotor.

Due to time limitations the scFv F3 has already been produced and purified in a culture of E. coli. The antibody fragment has been constructed with an affinity tag, which consist of six histidines. The his-tag binds to Ni²⁺-NTA, which was used for purification by affinity chromatography. The affinity tag is also used as a "handle" for detection, which is what you are going to take advantage of in the ELISA.
Laboratory Instructions

Remember that you are working with proteins that are sensitive to degradation by proteases that may be present in all samples. Low temperatures will lower the activity of any proteases. Because of this, always try to work with protein containing solutions in cold rooms or on ice.

Day 1. SDS-PAGE, NanoDrop™, Antibody Microarray, and coating of ELISA plates

SDS-PAGE

Purpose: Check purity of produced scFv antibody fragments.

By using the SDS-PAGE analysis, we will evaluate the purity of the scFv antibody fragment sample and illustrate the difference between an intact antibody (IgG) and an antibody fragment. Intact antibodies and scFvs will be reduced and denatured when boiled in the presence of a denaturing agent (mercaptoethanol, which is toxic and smells bad). The antibody samples will then be applied on a precasted acryl amide gel. When an electric current is applied the proteins migrate through the gel due to the charges applied to the proteins by SDS molecules. The rate of migration is dependent on the size of the protein.

Protocol

1. Prepare your antibody sample according to the table. One group will also prepare the IgG and one group will prepare a positive control. One group will also handle the molecular weight marker.

   | Protein sample (diluted in PBS) | 6,5 ul |
   | Reducing agent (10x)           | 1 ul   |
   | SDS loading buffer (4x)        | 2,5 ul |
   | Total volume                   | 10 ul  |

2. Incubate the samples at 95°C for 5 minutes. Let cool for a minute before opening the tube lid. Prepare the gel equipment while you wait.

3. Carefully apply the samples on the gel.

4. Run the gel at 115 V for 45-60 minutes.

5. Take out the gel from the plastic and wash it with water for 5 minutes in a tray.

6. Stain the gel by adding 20 ml of Simply Blue Safe Stain and incubate for 1 hour.

7. Destain with water over night.
**NanoDrop™**

**PURPOSE:** Check **PROTEIN CONCENTRATION** of produced scFv antibody fragments.

The production yield of recombinant proteins (e.g. scFv antibody fragments) is hard to predict and highly dependent on the production protocol used and variation in expression efficiency between different proteins. After each production, the researcher must check the concentration in order to be able to plan the following steps in the project. If the produced protein will be used in an application (e.g., in a diagnostic test), knowledge of correct concentration is essential. There are different ways to determine protein concentration and the choice of method usually depends on protein purity (do you only have one protein or a mixture of different proteins), expected concentration range, and knowledge of the protein sequence. One of the most widely used methods to determine concentration of a pure protein sample is to measure the absorbance at 280 nm using a spectrophotometer. Amino acids with aromatic side chains absorb light of 280 nm. The value obtained in absorbance units (AU) is then used to calculate the concentration using *Beer’s law*:

\[ A = E \cdot b \cdot c \]

where \( A \) is absorbance, \( E \) is a protein specific extinction coefficient, \( b \) is the path length of the light and \( c \) is the concentration.

The NanoDrop™ instrument calculates the protein concentration using this formula from measuring only a microliter amount of protein sample added to the instrument pedestals.

**Protocol**

1. Start the ND-1000 software.
2. Choose method type: “Protein A280”.
3. Clean the pedestals using some water and a tissue.
4. Lift the sampling arm and add 1,5 \( \mu l \) of dH2O onto the lower pedestal. Gently, lower the sampling arm again. Press “OK” and wait for the instrument to start up.
5. Lift the sampling arm and wipe off the liquid (both lower and upper pedestals) with a tissue.
6. Place 1,5 \( \mu l \) of sample buffer (PBS) onto the pedestal and lower the arm again. Press “Blank”.
7. Wipe off and add 1,5 \( \mu l \) of your protein sample. Choose sample type: Abs = 1 mg/ml.
8. Type in the sample name in the “Sample ID” box.
9. Press “Measure”.
10. Repeat step 6-8 two more times.
11. Press “Show report” to see your results in a list or as a plotted spectrum.
12. Wipe off the pedestal with a tissue and dH2O.
Antibody Microarray (Spotting)

PURPOSE: Parallel sensitive DETECTION of a number of targets in complex protein mixtures (e.g. serum samples). The method is used to detect differential expression patterns of proteins in healthy vs. disease patients.

Theoretical background
Antibody Microarray is a new technology that enable thousands of proteins to be analyzed simultaneously in a single experiment by using different antibodies towards a wide variety of targets. This technology plays an important role within proteomics, where the long term goal is to analyze all proteins expressed in a sample (e.g. a blood sample or a tissue sample). Today, antibody microarrays are used in cancer research, trying to identify proteins that are associated to the disease. Identification of such disease specific proteins is useful for development of diagnostic tools or new therapeutics.

Antibody microarrays are fabricated by dispensing antibodies, or in our case antibody fragments, in a defined pattern (an array) on a glass or plastic slide using a robot. The antibody fragments are dispensed in very small droplets (300 pL per droplet, 1600 spots/cm²). There are different ways of immobilizing the antibodies to the surface, including covalent binding, adsorption or affinity binding. In these experiments, the antibody fragments will be adsorbed to a polymer surface. After immobilization of the antibodies, different samples will be analyzed using the fabricated microarrays. The first step is to block (usually with milk) the naked surface between the arrayed antibodies to prevent unspecific binding of the sample to the surface. After a washing procedure, fluorescently labeled (e.g. with Cy3 or Cy5) samples are added to the microrarray and the analytes are allowed to bind to the antibodies during an incubation step. After additional washing steps, bound analytes are detected using a fluorescence scanner.

In this laboratory experiment your scFv sample together with a negative and a positive control scFv antibody fragment will be spotted onto a slide (see picture in the protocol). The positive control scFv (pure F3 of known concentration) will be titrated to generate a standard curve needed to determine the concentration of your antibody sample.

Protocol
1. Fabrication of the scFv arrays will be performed using a spotter.
2. Create incubation-chambers by drawing barriers around the arrays using a hydrophobic pen.
3. Block the arrays with 50 µl 5% (w/v) milk powder in PBS o/n at room temperature.

ELISA (the theory can be found on page 8)
1. Dilute the streptavidin (SA) 1/1000 in PBS and add 100 µl to at least 48 wells in a microtiter plate. Cover the plate with glad wrap (yes, this is actually the leading brand name) and put in a refrigerator over night.
Day 2. Biacore™ studies, ELISA and Antibody Microarray.

**Biacore™**

PURPOSE: Real time monitoring of the characteristics of protein-protein interaction (e.g. antibody binding to its antigen).

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**Theoretical background**

Biacore™ is a biosensor with which you can study the interaction between two biomolecules. BIA stands for Biospecific Interaction Analysis (in real time), and you measure the interaction as it happens without the need for any labelling of reagents.

The technique is based on that one of the molecules is immobilized to a gold-dextran surface while the other molecule is flowed past that surface. A sensogram is generated during the experiment and it shows if binding occur and to what extent. From the sensogram it is possible to determine the binding association and dissociation constants from which the affinity constant can be calculated. It is also possible to e.g. determine concentrations, specificities (cross reactions) and perform epitope mapping with a Biacore™ instrument.

**Immobilization**

The heart of the Biacore™ is the sensor chip. It consists of a glass surface covered with a thin layer of gold on one side. Gold is the metal of choice since it is inert and gives a good and stable signal. The layer of gold is covered with a covalently bound hydrogel consisting of carboxymethylated dextran. It is possible to immobilize biomolecules covalently to the dextran through a well-defined chemistry. Immobilization can be performed by different principles depending on which biomolecule that is to be bound. Examples of such immobilization chemistries are amine coupling, thiol coupling, aldehyde coupling and streptavidin-biotin coupling. Amine coupling is the most common and will be described more thoroughly.

The dextran is initially activated by reacting with N-hydroxysuccinimid (NHS), a reaction that is catalyzed by N-ethyl-N’-(dimethylaminopropyl)-karbodiimid (EDC). Approximately 30-40% of the carboxyl groups on the dextran will be converted into reactive N-hydroxysuccinimid esters. These NHS esters will then react with neutral primary amino groups on the biomolecules and form a covalent bond. After coupling the biomolecules, the remaining NHS esters will be deactivated by ethanolamine. Since the dextran surface net charge is negative it is important that the biomolecules are positively...
charged so that they will be attracted by the surface and make binding possible. The buffer in which the biomolecules are diluted should therefore have a pH lower than the isoelectric point of the biomolecules. The amount of immobilized molecules can be regulated by the choice of buffer, concentration and contact time.

**Principle of detection**
Surface plasmon resonance (SPR), which is the basis of analysis with the Biacore™ instrument, is an optical phenomenon that arises in a thin metal film at total internal reflection. This phenomenon creates a sharp dip in the intensity of the reflected light at a certain angle. This resonance angle depends on several factors, but mostly it is dependent of the refractive index of medium that is in contact with the sensor chip. The refractive index is directly proportional to the concentration of solubilised material in that medium. By keeping other factors constant, it is possible to measure the changes in concentration of macromolecules in the thin film of solution in contact with the dextran covered gold film. The optical system uses several detectors to determine the resonance angle, and the resonance signal is acquired as a response in resonance units (RU). A response (i.e. a change in resonance signal) of 1000 RU corresponds to a change in the surface concentration on the chip of 1 ng/mm². As one of the interacting molecules is immobilized, the other is passed over the surface in a continuous flow. As the soluble molecule binds to the immobilized molecule, the resonance angle will change and the response is registered. The response is acquired in the form of a sensogram in real time, i.e. as it happens.

**The sensogram**
The sensogram is a graph that depicts the changes in resonance signal as a function of time and is acquired on a computer screen continuously as the interaction occurs. Absolute values are usually irrelevant since it is the change in resonance signal, which corresponds to the change in surface concentration, that is the relevant parameter. The amount of bound protein is appreciated from the increase in resonance signal compared to the baseline. Kinetic data is obtained from the sensogram as the rate at which the signal changes.

Using a Biacore™ instrument we will here look at the interaction between the antibody fragment F3 and a peptide AD2 of its corresponding antigen (gB).

**Protocol**
1. Immobilization of the antigen to the chip.
   a) Dilute streptavidin 1/10 in 10 mM Na-acetate buffer pH 4.4. You will need 120 µl of this solution. Dilute the AD2 peptide 1/100 in the running buffer, hepes complete (HC). You will need 120 µl of this solution.
   b) Place the tubes in their correct position according to the instructions of the instrument (as shown by the supervisor).
   c) Start the biosensor.
2. Analysis of the binding between F3 and its antigen (the AD2 peptide).
   a) Dilute your antibody sample 1/10 and 1/100 in HC. One group will dilute the positive control antibody (pure F3 of known concentration) 1/10 and 1/100 in HC. 120 µl of each dilution will be sufficient.
b) Place the tubes in their correct position according to instructions in the program file on the instrument computer.

c) Start the biosensor. The chip will be regenerated between samples with 20 mM HCl, 150 mM NaCl.

ELISA

**PURPOSE**: Determine **CONCENTRATION** or **SCREEN** large number of proteins (e.g. antibody candidates for antigen binding).

Here, we will perform an immunoassay to determine the scFv concentration of the purified antibody sample.

In an ELISA (Enzyme Linked Immunosorbent Assay), either an antigen or an antibody is immobilized on a solid phase such as the polystyrene surface of a microtiter plate. In this experiment it is the biotinylated antigen that will be immobilized via the protein streptavidin. We will therefore start by immobilizing streptavidin to the surfaces of the microtiter wells. Since the affinity between streptavidin and biotin is very high the streptavidin will catch the biotinylated peptide when it is added to the wells. After this, you will add dilution series of both your antibody sample and a positive control scFv (pure F3 of known concentration). By generating a standard curve from known concentrations of scFv F3, it is possible to determine the concentration of your purified antibody sample. Bound scFvs will be detected by addition of an enzyme-labeled (horse radish peroxidase, HRP) anti-His<sub>6</sub>-antibody, hydrogen peroxide and the compound OPD. Due to its aromatic benzene ring, OPD absorbs light in the UV region of the electromagnetic spectrum. The enzyme, HRP, will reduce H<sub>2</sub>O<sub>2</sub> and the two amine groups on OPD will be oxidized into nitro groups. This oxidization will create a larger conjugated system, which absorbs light in the visible part of the electromagnetic spectrum.

**Protocol (cont.)**

2. Dilute the antigen AD2-biotin. You will need 100 µl per well with a concentration of 1 µg/ml (dilute it 1/1000 in 1% BSA in PBS).

3. Wash the plate 3 times with NaCl/Tween solution.

4. Add the antigen AD2-biotin. Incubate for 1h at 37°C.

5. Make serial dilutions of your antibody sample and the positive control antibody (1/1000, 1/2000, 1/4000, 1/8000 and 1/16000) in 1% BSA in PBS. You will need at least 220 µl of each dilution.

6. Wash the plate 3 times with NaCl/Tween solution.

7. Add your serial diluted antibody samples and positive control antibody. Add 100 µl 1% BSA/PBS to two of the remaining wells (blank). Cover the plate with glad wrap and incubate at 37°C for 1 h.

8. Wash the plate 3 times with NaCl/Tween solution.

9. Dilute HRP-anti-His<sub>6</sub> 1/500 in 1% BSA in PBS. Cover the plate with glad wrap and incubate at 37°C for 1 h.

10. Prepare the substrate solution just before washing the plate.
11. Wash the plate 3 times with NaCl/Tween solution.
12. Add 100 µl substrate solution (30 ml 34.7 mM citric acid, 66.7 mM Na-phosphate, pH 5.0 + 1 tablet of ortophenylenediamine (20 mg) + 12.5 µl 30% H2O2) to every well, incubate at room temperature for 10 minutes. Stop the reaction by adding 150 µl 1 M H2SO4 to each well. NB! The citrate buffer should be at approximately room temperature prior to the addition of the OPD tablet and the H2O2. The substrate solution should not be left standing for extended periods of time prior to its use.
13. Measure the absorbance at 490 nm.
14. Plot a standard curve of the absorbance as a function of the concentration of the positive control antibody.

**Antibody Microarray (Assay and Microarray and analysis)**

**Protocol (continuation from Day 1)**
4. Wash the arrays 4 times with 0.05 % Tween 20 (v/v) in PBS (PBS-T).
5. Add 50 µl of sample solution, one sample per array, and incubate for 1h at room temperature.
6. Wash the arrays 4 times with PBS-T.
7. Add 50 µl Streptavidin-AlexaFluor647, 1 µg/ml in PBS, and incubate for 1h at room temperature.
8. Wash the arrays 4 times with PBS-T.
9. Dry the arrays under a stream of nitrogen.
10. Scan the arrays and quantify the signals. Determine the concentration of your purified scFv sample.

**The END.**