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Antibodies

Expression, Purification and Application

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Abstract

Today antibodies are an important part in the treatment of diseases such as cancer and Guillain–Barré syndrome. It is of course important to be able to regulate the levels of the antibodies in the treatment. In this study a general method for antibody production were investigated. The antibody construct was made for transportation out of the cell. Four different tagged variants of an antibody were studied in order to find the variant best regulate by the addition of a ligand; Antibody with Tag 1, Antibody with Tag 1 and cleavage site in between, Antibody with Tag 2, and Antibody with Tag 2 and cleavage site in between.

The Antibody with Tag 2 and cleavage site in between was found to be best regulated by the addition of the ligand. Despite the fact that the expression rate of this construct was not the highest, the ratio between with and without ligand gave the best dynamic range. The antibodies inside the cell weren't regulated by the ligand and the concentration of antibodies inside the cell was higher than the concentration of antibodies outside the cell.

Abbreviations

Antibody	Ab
Western blot	WB
Deoxyribonucleic acid	DNA
Human embryonic kidney	HEK
horseradish peroxidase	HRP
Enzyme-Linked Immunosorbent Assay	ELISA
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
polyethylenimine	PEI
poly-L-Lysine	PLL
Ethylenediaminetetraacetic acid	EDTA

Antikroppar

En människa exponeras och angrips varje dag av virus och bakterier och immunförsvaret skyddar oss från dessa angrepp. Immunförsvaret utgörs av vita blodkroppar som bildas i benmärgen och sprids vidare ut i kroppen. De vita blodkropparna finns i många olika varianter och varje variant har en specifik uppgift. Lymfocyter är en typ av vita blodkroppar och kan dela in i två huvudgrupper: B-lymfocyter och T-lymfocyter.

När kroppen infekteras av en patogen (virus, bakterie, svamp eller parasit) aktiveras B-lymfocyter och börjar producerar antikroppar. Antikropparna binder till ytan på de främmande partiklarna (patogenerna) och guidar andra celler till platsen för infektionen. Antikropparna dödar alltså inte den främmande partiklen utan fungerar mer som signal för de celler som utför själva attacken och dödar patogenen.

Antikroppar är Y formade proteiner och har förmågan att binda till ytan på främmande partiklar, så kallade antigen. Antikropparna delas upp i klasser och människan har fem olika klasser av antikroppar: IgG, IgA, IgM, IgE och IgD. Antikropparna som tillhör klasserna IgM och IgG spelar en viktig roll i immunförsvaret medan antikropparna som tillhör klassen IgE hjälper till vid allergiska reaktioner. Antikroppar tillhörande klassen IgA finns i saliv och håller på så sätt munnen relativit fri från patogener. Funktionen för antikropparna i klassen IgD är inte känd men man tror att antikropparna i denna klass hjälper till vid aktiveringen av B-celler.

Idag används antikroppar i olika behandlingar av sjukdomar t.ex. av behandling av Guillain-Barré syndrom. Celler kan stimuleras att produceras antikroppar och en vanlig metod för detta är transfektion, det vill säga inducerande av genetiskt material till cellerna.

I denna studie producerades olika varianter av en antikropp genom transfektion. Syftet var bland annat att hitta ett sätt att reglera antikropparnas aktivitet. En Antikropps aktivitet avgörs bland annat av hur stabil antikroppens struktur är. Om antikroppen faller i sönder eller påverkas på ett sätt som ändrar dess struktur kommer också antikroppens förmåga att fungera och uträtta sitt jobb förändras. En molekyl som stabiliserar antikroppens struktur kan därför tillsättas och på så sätt kan antikroppens aktivitet regleras.

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Introduction

Ab:s

All antibodies (Ab:s) are members of a protein family called immunoglobulins and are composed of four polypeptides: two heavy chains and two light chains that are held together with disulfide bonds. In mammals are there five different classes of Ab:s: IgM, IgG, IgA, IgE and igD. The constant region of an Ab is a given constant amino acid sequence that is identical for all the Ab:s in a class of a species. The variable region of an Ab binds to the antigen with a high specificity and the interacting part of the antigen is called the epitope. (Brooker, Widmaier et al. 2014)

IgG as well as IgE and IgD are monomers. IgG, see Figure 1, also known as immunoglobulin G, plays an important part in the immune system and consists of 12 domains. (Harris, Skaletsky et al. 1998) Each light chain consists of 2 homologues domains and each heavy chain has four domains. These domains all adopt a structure called the immunoglobulin fold. The immunoglobulin structure consists of beta-sheets held together by a disulfide bond. Three loops at one end of the fold create a potential binding site for antigens.(Berg, Tymoczko et al. 2012)

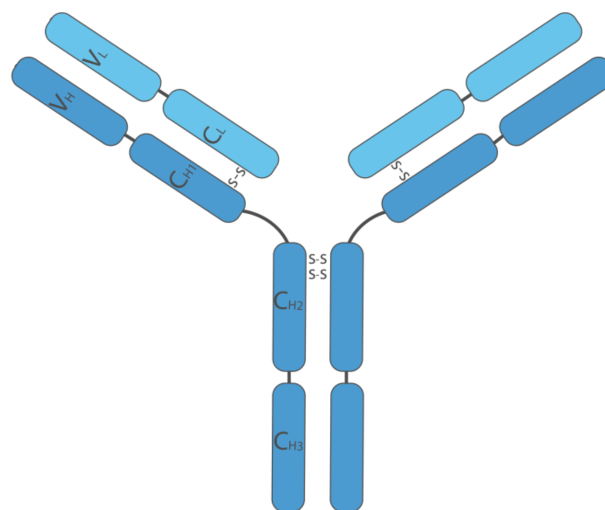

Figure 1. IgG plays an important part in the immune system and consists of 12 domains that together forms 4 polypeptide chains; two light chains and two heavy chains. The polypeptide chains are held together by disulfide bonds. Each light chain (in light blue) consists of 2 domains and each heavy chain (in dark blue) consists of 4 domains. One domain on each light chain is variable (V_L). There is a variable domain on each heavy chain (C_L) too. The rest of the domains are constant (C_L or C_H).

Lymphocytes, B cells and T cells, play an important part in the immune system. The B cells are activated by cytokines secreted from helper T cells. The B cells start to secret Ab:s when activated and the secreted Ab:s bind to the antigens and guide other cells and molecules to the place of the infection. (Brooker, Widmaier et al. 2014)

Artificial Ab production

Ab:s can be industrial synthesized in animals. The animal are injected with an immunogen, a carrier protein (often keyhole limpet hemocyanin) linked to the antigen. This foreign molecule starts an immune response and Ab:s for the antigen are produced. The blood contains a mixture of different Ab:s specific for all the antigens that the animal has been exposed to. Polyclonal Ab:s recognize multiple epitopes while monoclonal Ab:s recognize a single specific epitope. (Berg, Tymoczko et al. 2012)

Human embryonic kidney cells 293 (HEK293) is a cell line derived from embryonic kidney cells combined with adenovirus type 5 DNA that has been integrated in chromosome 19. (Graham, 1977)(Louis, 1997). HEK293 cells are commonly used for transfection experiments i.e. the process of introducing genetic material into a host cell for protein production for example production of Ab:s (Eliyahu, 2005).

The genetic material can be introduced into the cell through either a viral delivery system or a non-viral delivery system. A recombinant virus lacking the ability to replicate is used in the viral delivery system. The virus infects the host cell followed by production of the protein of interest. Retroviruses were among the first viruses to be used in this method (Rosenberg , 1990). The non-viral delivery system uses gene delivery agents that are capable to form complexes with the DNA. The complex enters the cell via endocytosis. There are different types of delivery agents: cationic lipids and cationic polymers (Eliyahu, 2005).

Cationic lipids are a commonly used delivery agent. A cationic lipid is able to condense and form a complex with the DNA. Cationic polymers are another delivery agent including polyethylenimine (PEI) and poly-L-Lysine (PLL). The cationic polymers have usually a lower toxicity compared to cationic lipids but also lower transfection efficiency. (Eliyahu, 2005)

Aim

V5 tagged Ab:s was produced in HEK293 cells and secreted out into the cell media. The cells were stimulated by non-viral transfection to produce the Ab of interest.

Four different variants of the Ab were studied in order to find the variant best regulated by the addition of a specific ligand; Ab with Tag 1, Ab with Tag 1 and cleavage site in between, Ab with Tag 2, and Ab with Tag 2 and cleavage site in between. The non-modified variant of the Ab was purified to serve as positive control and concentration standard in all experiments.

Method

Unless specifically states all chemical used in all experiments were of biochemical grade or better from Sigma Aldrich (Darmstadt, Germany).

Purification of the Ab

V5 tagged Ab 1 was produced in HEK293 cells and secreted out into the cell media. The V5 tag contains 14 amino acids: GKPIPNPLLGLDST (Southern, 1991 #18). Two batches of purified Ab 1 were produced from the same starting material. In both the batches the purification kit MBL#3317 (MBL ink, Täby, Sweden) were used.

V5 tagged protein purification procedure

An overview of the purification can be seen in Figure 2.The cell media was collected and the cell debris was removed by centrifugation. The supernatant was collected and stored at 4° C until further processing. The Anti-V5 tag beads were added to the supernatant and incubated over night at 4° C. The sample was centrifuged at 400 x g for 5 minutes and 1 ml of the supernatant was transferred to a spin column. The rest of the supernatant was stored in the fridge and late analyzed with SDS-PAGE.

The spin column was placed in a sampling tube and centrifuged 10 sec at 15000 x g. The flow through was stored and the spin column was placed in a new sampling tube. 0.6 ml washing solution was added to the column followed by centrifugation 10 seconds at 15000 xg. This step was repeated two additional times. The flow trough was saved and stored in the fridge. An elution peptide solution were added to the column and incubated for 5 minutes to elute the protein followed by centrifugation 10 seconds at 15000 x g to collect it. This step was repeated twice

Two batches of purified Ab 1 were produced with following modifications: In the incubation step 60 µl Anti-V5 tag beads were added in the first batch and 120 µl were added in the second batch. In the first batch the incubation was done with sideways rotation along the axis of the tube. For the second batch the incubation was done with end over end rotation perpendicular to the tube axis. The volume of the elution peptide solution was 60 µl in the first batch and 120 µl for the second batch.

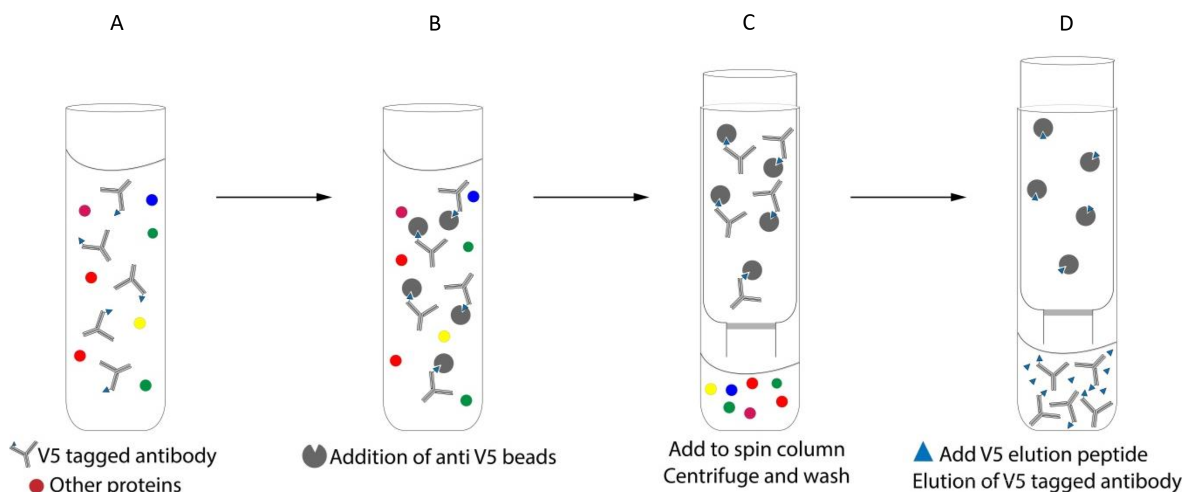

Figure 2. The media containing the Ab of interest was harvested (A). Anti v5 tag beads were added to the cell media and incubated overnight. The beads were bound to the V5 tag of the Ab (B). The solution was transferred to a spin column. The Ab:s bound to the beads stayed in the column and unwanted proteins were removed (C). V5 elution peptide was added to elute the Ab of interest (D).

Removing the V5 elution peptide

The following steps were done to remove the V5 elution peptide using a 3 kDa cut off Amicon ultra spin column filter device (UFC500324, Solna, Sweden) and an overview can be seen in Figure 3. 500 µl Phosphate buffered saline (PBS, product#: 14190094, sigma Aldrich) was added to a spin column followed by centrifugation 2 minutes at 15000 x g. This was done to wash the column before adding the sample. For the first batch was 110 µl of the Ab 1 added to the column. For the second batch was 230 µl of the Ab 1 added to the column. 400 µl PBS was added followed by centrifugation 5 minutes at 15000 x g. This step was repeated 9 times. The purified Ab 1 was collected by turning the column upside down and centrifuge 5 minutes at 20817 x g. The eluted Ab 1 concentration was measured

using nanodrop at wavelength 280 nm with PBS as blank and the eluted Ab 1 was analyzed with SDS-PAGE and western blot (WB). The purified Ab 1 was aliquoted and stored at -80˚C. The purified Ab was then used as quality control and concentration standard for all the following experiments.

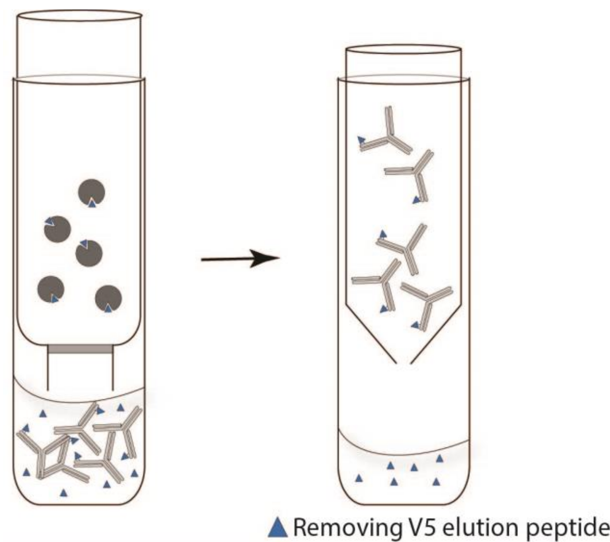

Figure 3. This step was done in order to remove the free V5 elution peptides. The solution with purified Ab:s and free V5 elution peptides were transferred to a new column. The free V5 elution peptides were removed and the Ab:s were collected by turning the column upside down and centrifuge.

Tagged Ab test for HEK293 cells

HEK293 cells were trough transfection stimulated to produce the Ab. Six different plasmid vectors were used, five of these encoding an Ab construct (see Figure 4) and one serving as transfection control encoding for a florescent protein. The rest of the plasmid vectors encoded five different variant of the Ab: unmodified Ab (Ab 1), Ab with Tag 1 (Ab 2), Ab with Tag 1 and cleavage site in between (Ab 3), Ab with Tag 2 (Ab 4), and Ab with Tag 2 and cleavage site in between (Ab 5).

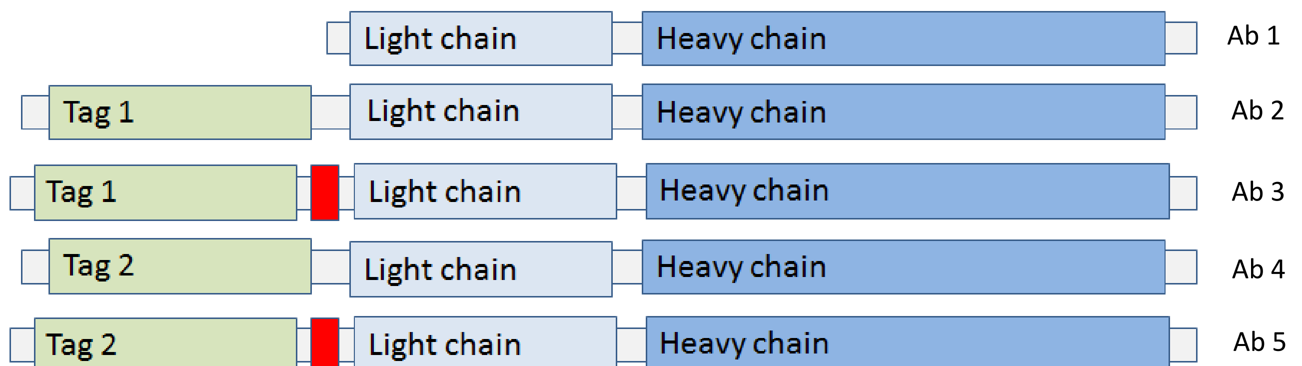

Figure 4. Six different plasmid vectors were used; five of these can be seen in the figure above all encoding an Ab construct. The last one served as transfection control encoding for a florescent protein. Five different variants of the Ab were studied: Unmodified Ab (Ab 1), Ab with Tag 1 (Ab 2), Ab with Tag 1 and cleavage site (Ab 3), Ab with Tag 2 (Ab 4) and Ab with Tag 2 and cleavage site (Ab 5). The red rectangle in the picture indicates the cleavage site.

Cell splitting

HEK293 cells were grown on a plate at 37° C. The plate was washed with 5 ml PBS before the addition of trypsin. 2.5 ml trypsin was added and distributed over the plate followed by two minutes

incubation. 7.5 ml media was added and the trypsin-media solution was pipetted up and down three times before added to a new tube and centrifuge 5 minutes at 300 xg. The supernatant was discarded and new media (Dulbecco's Modified Eagle Medium PN 10938025, Waltham, Massachusetts, USA) with 5 % Fetal Bovine Serum, (PN 10270-106, Waltham, Massachusetts, USA)and 2 mM L-glutamine(PN 25030024, Waltham, Massachusetts, USA)) was added to the pellet. For the determination of the concentration 100 µl sample and 100 µl trypan blue were mixed and analyzed in a Biorad TC20 cell counter (Biorad, Hercules, California, USA). The cells were distributed in 6-well plates with 25 ml cells per well ($1.1*10^6$ cells per well) and Incubate in 37° C for about 24 h.

Transfection

6 different plasmid vectors were used for the transfection. For the plasmid vectors encoded for the florescent protein and the unmodified variant of the Ab 3 replicates were done and 6 replicates for the rest of the plasmid vectors. A solution containing the plasmid vector and TransIT-2020 (MIR 5400, Madison, USA) was prepared and incubated 15 minutes before transferred to the wells with cells. The solution was prepared so that each well contained 2.5 µg plasmid vector and 7.5 µl heated TransIT-2020. To reach a total volume of 250 µl per well the plasmid vector was diluted in heated optiMEM (PN 31985062, Waltham, Massachusetts, USA). The media was exchanged 6 hours after the transfection.

A ligand was added to some of the wells at the same time as the media was changed. The ligand was added to 3 of the 6 replicates. No ligand was added to the cells that produced the florescent protein and Ab 1. The ligand was first dissolved in DMSO to a concentration of 24 mM and then diluted further with heated media into a final concentration of 10 µM. 1.5 ml ligand containing media or 1.5 ml media (DMEM (PN 10938025, Waltham, Massachusetts, USA) with 5 % FBS, 2 mM L-glutamine (PN 25030024, Waltham, Massachusetts, USA)) were added per well.

Harvest

The Ab are transported out of the cell and for the harvest 1 ml supernatant from each well was transferred to new tubes and centrifuged at 168 x g for 5 minutes. The supernatant was transferred to new tubes after the centrifugation and stored in the fridge.

The cells were also collected to be able to analyze the concentration of the Ab inside the cell. The remaining media in each well was removed and the wells were washed using 0.4 ml PBS per well. After the washing 0.4 ml Trypsin+ Ethylenediaminetetraacetic acid (EDTA) (PN 25300054, Waltham, Massachusetts, USA) was added to each well followed by 5 minutes incubation. 0.4 ml/well media was added and the cell solution was pipetted up and down 5 times before transferred to a new tube and centrifuged at 168 x g for 5 minutes. The supernatant was discarded and the cells were stored on ice for further analysis. For the viability test 100 µl of each sample was added to a second tube and mixed with trypan blue before centrifugation.

Sample preparation

The media samples were analysed without any preparation and the cell samples were homogenized before further analysis. A 50x Tris acetic acid EDTA buffer (TAE buffer) stock was made, to a final concentration of 0.005 M EDTA, 2 M Tris and 5.72% acetic acid. Water was added up to the final volume and the pH was adjusted to 7.4. The stock was diluted 100 times to a final working concentration of 0.5x TAE buffer with the addition of protease and phosphatase inhibitors.

To the cell samples 100 µL 0.5x TAE buffer was added and the samples were homogenized on ice with a Vibra cell (Sonics materials INC, Danbury, CT USA), amplitude set to 40 and frequency to 25.

The protein Assay Kit I (cat#5000111) was used for protein determination of the cell samples. The cell sample was diluted 20 times in PBS. 5 µl homogenised and diluted sample was added per well followed by addition of 25 µL reagents A and 200 µL reagents B per well. The plate was incubated 30 minutes and was read at 750 nm.

MSD immunoassay

An immunoassay measures the presence of a molecule based on the molecule's interaction with Ab:s. The first immunoassay was introduced in the 1960s and where then used to analyze hormones. Berson and Yalow (Berson and Yalow.1959) first introduced the immunoassay in their work with insulin and Ekins (Ekins 1960)used it in his work with thyroxine. The development of monoclonal Ab production improved both the sensitivity and specificity of the immunoassay. (Christopoulos, Diamandis et al. 1996)

All type of immunoassays is based on the interaction between a specific Ab and an antigen. In Enzyme-linked immunosorbent assay (ELISA) an enzyme are covalently linked to an Ab and the enzyme produces a detectable product upon addition of substrate. Horseradish peroxidase is a commonly used enzyme and oxidizes a substrate using hydrogen peroxide.(Hnasko 2015) There are several types of ELISA, two of them are called sandwich ELISA and indirect ELISA. In sandwich ELISA the antigen are trapped between two Ab:s. (Berg, Tymoczko et al. 2012) In this study a type of indirect ELISA was used, se Figure 5. In indirect ELISA binds a primary Ab to the analyte followed by binding of the secondary Ab. The whole experiment was carried out twice and the concentration of the samples was estimated against a standard (purified Ab 1) with a concentration of 1 µg/ml. A plate coated with streptavidin was used. The streptavidin binds to the biotin in the biotin-antigen complex and the Ab of interest binds to the antigen. The detection Ab binds to the Ab of interest and is labeled with a detectable sulfo tag.

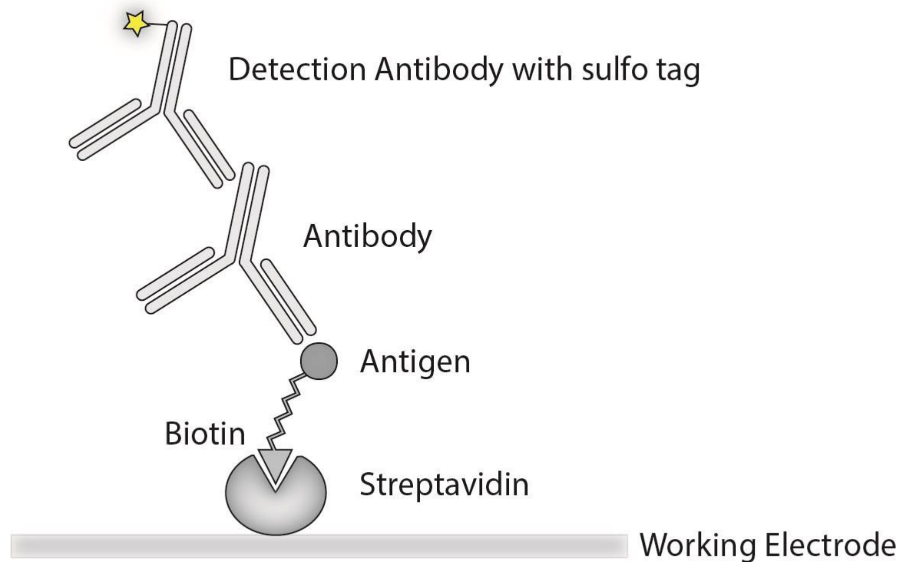

Figure 5. A plate coated with streptavidin was used. The streptavidin binds to the biotin in the biotin-antigen complex and the Ab of interest binds to the antigen. The detection Ab binds to the Ab of interest and is labeled with a detectable sulfo tag.

A plate coated with streptavidin was used (MSD#L15SA-1, Rockville, Maryland, USA).

- 30 µl biotin-Antigen ($5*10^{-3}$ µg/ml) was added per well.
- 25 µl sample with expressed Ab was added per well.
  Media samples were analyzed undiluted and cell samples were diluted 5 times in 1 % Blocker A.
  1 % blocker A (MSD#R93BA-2, Rockville, Maryland, USA) was used as blank.
- 25 µl Goat Ab antimouse with sulfo-tag (MSD#R32AC-5, Rockville, Maryland, USA) was added per well.
- 150 µl 2x Read buffer (MSD#R92TC-2, Rockville, Maryland, USA) in milliQ water was added per well and the signal was read in MESO QuickPLEX SQ 120.

The plate was washed with 3x300 µl 0.05 % Tween 20 in PBS before each new addition. After each new addition the plate was incubated 1 hour at 750 rpm. The concentration of the Ab was normalized against the total protein amount of the sample (measured with protein Assay Kit I (cat#5000111)). The total protein amount in each sample was assumed to be directly proportional to the number of viable cells during protein production.

SDS-PAGE and Western blot

In western blot the samples are separated by SDS-PAGE followed by transfer of the protein to a membrane. An Ab specific for the protein of interest is added followed by the addition of a secondary Ab. The secondary Ab is today often labeled with the enzyme horseradish peroxidase. Horseradish peroxidase oxidizes a substrate using hydrogen peroxide. The product can then be detected by luminescence. (Christopoulos, Diamandis et al. 1996)

SDS-PAGE analyze was performed at 90 V in 15 minutes followed by 200 V in 45 minutes and the gel CriterionTGX stain free 4-15 %(Cat# 5678084,Kaliforna, USA) was used. For WB the membrane Trans-Blot turbo was used (Cat # 1704157, California, USA) and the membrane was blocked in 5 % milk in TBS-T20 for 1 hour after the transfer.

For the purified Ab (Ab 1) the detection Ab (goat anti mouse IgG-HRP, SC-2005, diluted 1000 times in 2 % milk in TBS-T20) was added followed by incubation over nigh before visualization. Both a primary Ab (rabbit anti-V5, Ab206566, diluted 1000 times in 2 % milk in TBS-T20) and a detection Ab (Goat anti rabbit,SC-2004, diluted 10000 times in 2 % milk in TBS-T20 used) was used for the antibodies inside and outside the cell. The membrane was washed three times in 10 ml TBS-T20 before the addition of the detection Ab. The membrane was stained with Clarity Western ECL Substrate before visualized in ChemiDOC Imaging System (BioRad).

Result

Purification of the Ab

Two batches of purified Ab:s (Ab 1) were produced from the same starting material. The WB for the second purification of Ab 1 is shown in 0. The supernatant was removed from the starting material after the overnight incubation. The band intensity of the starting material and the supernatant were similar for the first purification. After the second batch has the band intensity for the supernatant significant decreased.

The band intensity before and after removing the V5 elution peptide (0, lane 11 and 12) indicates a loss of Ab:s in the last purification step. This loss occurred in both first and second purification.

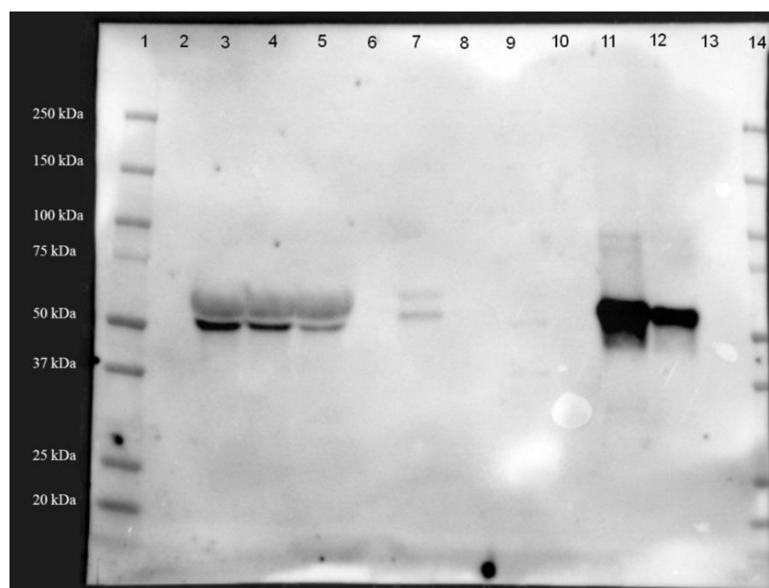

Figure 6. WB for the second purification. The dual color marker was loaded in lane 1 and 14 (5 µl loaded) and lane 9 (5 µl, 10 times diluted). The starting material of the first purification and second purification were loaded in lane 3 and lane 4.The supernatant were removed from the starting material after the overnight incubation and was loaded in lane 5. The wash and the flow through were loaded in lane 7 and 8. Sample with V5 elution peptide and the final product were loaded in lane 11 and 12. No samples were loaded to lane 2, 6, 10 and 13.

Tagged Ab test for HEK293 cells

Assay

The assay was performed twice and the result can be seen in Figure 7, Figure 8 and Figure 9. The data from the first experiment are shown in blue and the data from the second experiment are shown in pink. The concentration of the Ab was calculated from the assay signal. The concentration was then normalized against the total protein amount of the sample. The total protein amount in each sample was assumed to be directly proportional to the number of cell. In Figure 7 and Figure 8 the data are compared to a baseline (the concentration of the non-modified Ab, Ab 1).

The data for the Ab:s exported out of the cells are shown in Figure 7. The expression level was higher in the presence of the ligand for all the Ab:s. Ab 2 and Ab 3 had a very low expression level. The data for the Ab:s inside the cell are shown in Figure 8. The expression level was similar for all the Ab:s i.e the expression levels were not affected by the addition of the ligand.

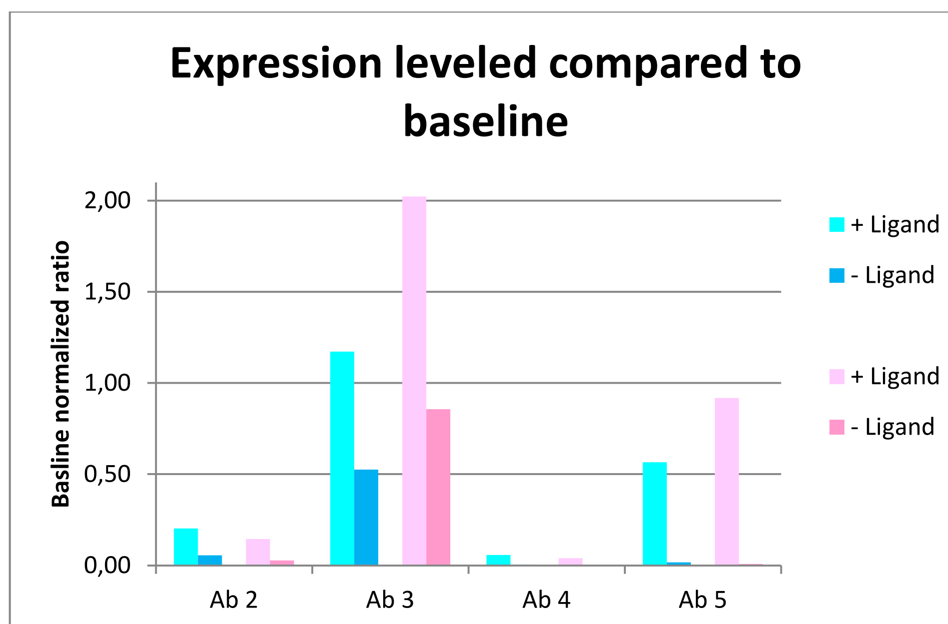

Figure 7. Assay result for the Ab:s exported out to the media. The assay was performed twice. The data from the first experiment are shown in blue and the data from the second experiment are shown in pink. The concentration of the Ab was normalized against the total protein concentration. The data were also normalized against the signal from unmodified Ab. The cell expression levels were compared to baseline levels of the media samples.

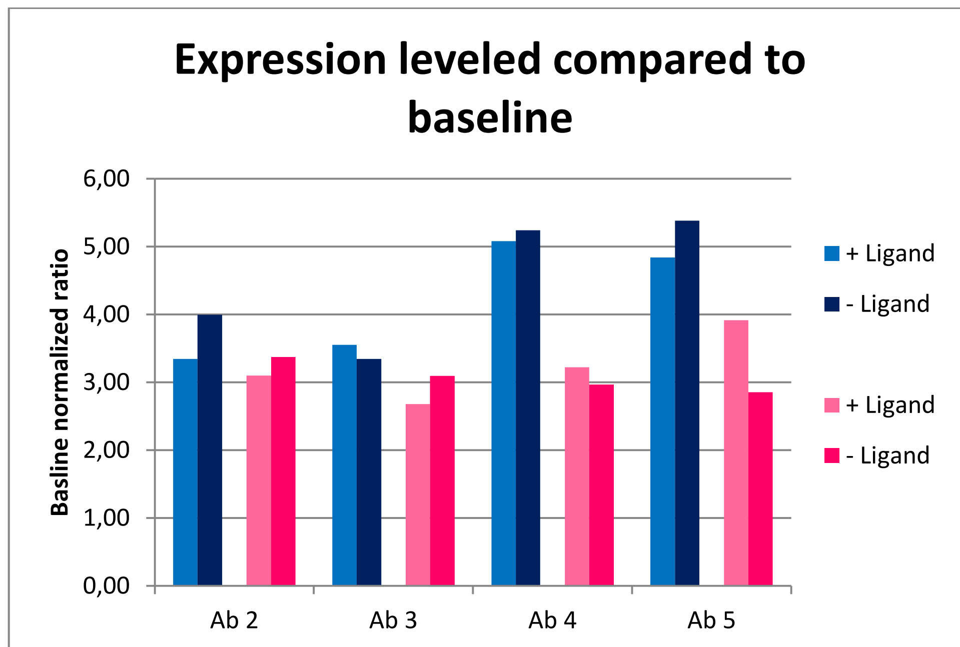

Figure 8. Assay result for the Ab:s inside the cell. The assay was performed twice. The data from the first experiment are shown in blue and the data from the second experiment are shown in pink. The concentration of the Ab was normalized against the total protein concentration. The data was also normalized against the signal from unmodified Ab. The cell expression levels were compared to baseline levels of the cell samples.

In Figure 9 is the signal ratio between the samples with and without ligand shown for both the Ab.S inside and outside the cells. The Ab:s inside the cells had a very low ratio. Ab 5 had the highest ratio of the Ab:s outside the cells followed by Ab 4.

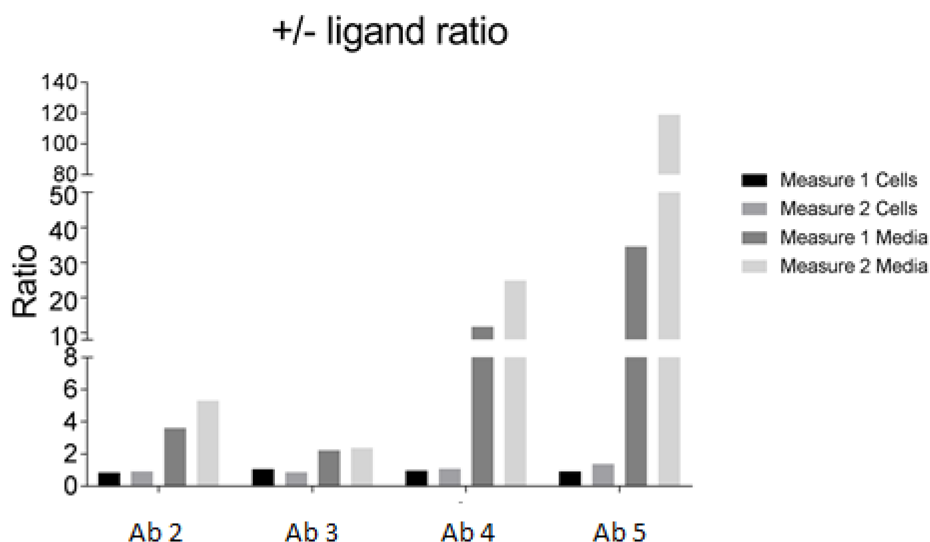

Figure 9. The graph shows the signal ratio between samples with and without ligand for both the Ab:s exported out of the cells and the Ab:s inside the cells. The assay was performed twice.

SDS-PAGE and WB

The WB for the Ab:s inside the cells is shown in Figure 10. No band is visible for the negative control expressing fluorescent protein (lane 3).Two bands are visible for all other samples: one at approximately 50 kDa and one around 25 kDa. The band intensity for Ab 3 (lane 7) is lower than the

rest of the bands on the blot. The most intense band corresponds to the Ab 5 with ligand treatment (lane 12).

The WB for the Ab:s exported out of the cells is shown in Figure 11. Three samples have higher band intensity than the rest of the samples; Ab 1 (lane 4) and Ab 3 with and without ligand treatment (lane 7 and 8). For most of the samples only one band is visible.

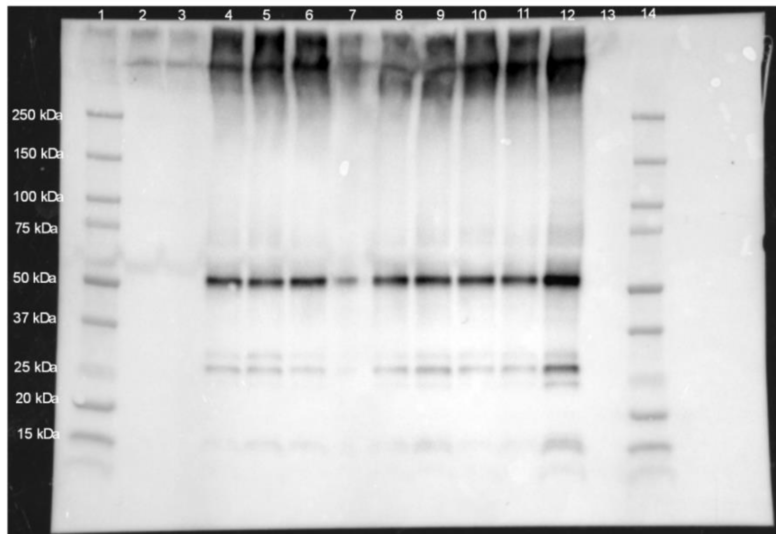

Figure 10. WB for the Ab:s inside the cell. The dual color marker are loaded in lane 1 and lane 14 (5 µl loaded). The samples are loaded in the following order starting from lane 3: cells expressing fluorescent protein, Ab 1, Ab 2, Ab 2 with ligand treatment, Ab 3, Ab 3 with ligand treatment, Ab 4, Ab 4 with ligand treatment, Ab 5 and Ab 5 with ligand treatment. An Anti V5 Ab (ab206566) was used as primary Ab and goat anti rabbit Ab (sc-2004) was used as secondary Ab.

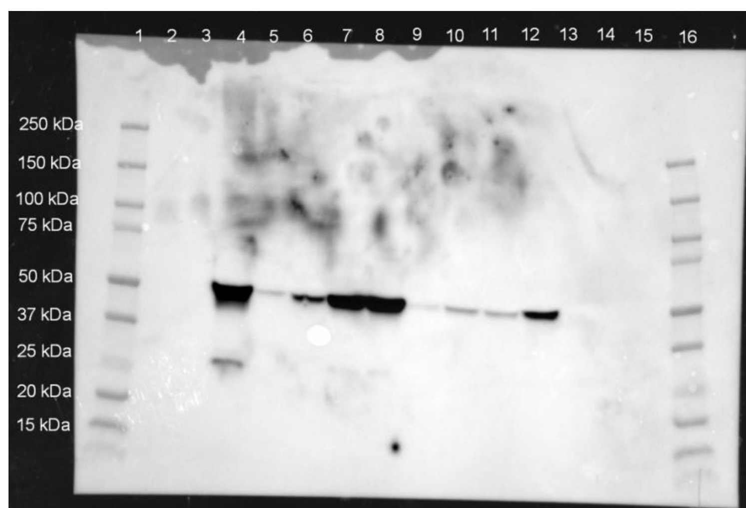

Figure 11. WB for the Ab:s exported out of the cell. The dual color marker are loaded in lane 1 and lane 16 (5 µl loaded). An unstained marker (bio rad) is loaded in lane 14 (5 µl loaded).The samples are loaded in the following order starting from lane 3: fluorescent protein, Ab 1, Ab 2, Ab 2 with ligand treatment, Ab 3, Ab 3 with ligand treatment, Ab 4, Ab 4 with ligand treatment , Ab 5 and Ab 5 with ligand treatment. An Anti V5 Ab (ab206566) was used as primary Ab and goat anti rabbit Ab (sc-2004) was used as secondary Ab.

Discussion

Purification of Ab

By comparing the band intensity in 0 of the samples before and after removal of the V5 elution peptide (lane 11 and 12) it is concluded that there was a loss of the Ab in the removal step. The flow trough was sampled and tested to make sure that no Ab was washed out (lane 8). The Ab:s that are lost in this step must be absorbed in the filter as it can't it be found in the flow through.

The WB for the second purification is shown in 0. The band intensity for the supernatant are stronger than for the starting material, indicating that more of the Ab was bound to the V5 beads in the second purification. The band intensity for the supernatant and the starting material were similar in the first purification, indicating that there was a lot of the Ab left in the starting material. From the WB, 0, it is concluded that the best way of shaking the sample during the incubation was with end over end rotation as done in the second purification.

Tagged Ab test for HEK293 cells

The expression level for Ab 3 was higher than the expression level of Ab 5 as seen in Figure 7. The ligand was added to stabilize and regulate the activity of the Ab. From the assay result, Figure 9, it is concluded that the Ab 5 are best regulated with the ligand as the +\ - ligand ratio is high. Ab 3 wasn't as efficiently regulated. The two Ab:s without cleavage site have very low concentration both in the presence and absence of ligand (Figure 7) and from this it is concluded that the cleavage site is important for the expression level.

The concentration of the Ab was higher inside than outside the cell. As seen in Figure 8 wasn't the Ab:s inside the cell regulated by the ligand. The WB for the Ab:s inside the cells is shown in Figure 10. Two bands are visible for all the samples: one at approximately 50 kDa and one around 25 kDa. The heavy chain of the Ab has a weight of approximately 50 kDa and the light chain has a weight of 27 kDa. The V5 tag is attached to the heavy chain and only the heavy chain was predicted to be visible in the WB as an Ab specific for the V5 tag was used as primary Ab. The band at 27 kDa can't be the light chain and it is therefore concluded that the Ab is degraded inside the cell.

Conclusion

From the assay result, Figure 9, it is concluded that Ab 5 are best regulated with the ligand as the +\ - ligand ratio is high. The Ab:s without cleavage site aren't regulated by the ligand as the concentration is low both in the presence and absent of ligand. Inside the cell was the concentration of the Ab high and they weren't regulated by the ligand.

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