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Development of an immunoassay for human epidermal growth factor receptor 2 (HER2)

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Sammendrag

Brystkreft er en av de vanligste kreftformene som rammer kvinner, og omtrent 15-30 % av brystkrefttilfeller består av en svært aggressiv form kalt HER2-positiv brystkreft. Disse pasientene har svulster som overuttrykker human epidermal growth factor receptor 2 (HER2). HER2 er et glykoprotein som finnes på celleoverflaten og den ekstracellulære delen (ECD) av dette proteinet kan bli proteolytisk kløyvd- og frigjort til blodsirkulasjonen. ECDs evne til bli brukt som tumormarkør for oppfølging av pasienter som tidligere har fått diagnosen HER2 positiv brystkreft, er svært interessant. Det finnes få immunologiske analyser for HER2, og målet med denne masteroppgaven var derfor å utvikle en robust og sensitiv immunologisk analyse spesifikk for HER2 ECD.

Seks monoklonale antistoffer (M75, M77, M79, M83, M84 and M89) som gjenkjente både rekombinant og nativt HER2 ble benyttet i utviklingen av en immunometrisk analyse for serum HER2. Antistoffene ble karakterisert, og parkombinasjoner av alle antistoffene ble testet for å finne den antistoffkombinasjonen som ga høyest sensitivitet for nativt HER2. Den beste parkombinasjonen besto av biotinmerket M89 og europiummerket M83, og den ble videre optimalisert og automatisert på et AutoDELFIA instrument. Den nye analysen ble så brukt til å teste 15 serumprøver fra pasienter som var vevs-HER2 positive, og prøveverdiene ble sammenlignet med prøveverdier fra Siemens ADVIA Centaur Serum HER-2/neu immunoassay. Prøveresultatene fra den utviklede analysen ga generelt lavere serum HER2-verdier sammenlignet med prøveresultatene fra Siemensanalysen, men det var en god lineær korrelasjon mellom prøvene. Et sensitivt assay spesifikt for HER2 ECD har blitt utviklet.

Abstract

Breast cancer is one of the most common types of cancer affecting women, and approximately 15-30 % of breast cancer patients have a particularly aggressive type called HER2 positive breast cancer. These patients have tumors that overexpress human epidermal growth factor receptor 2 (HER2). HER2 is a glycoprotein found on the cell surface, and the extracellular domain (ECD) of this protein can be proteolytically cleaved and released into the blood circulation where it could be useful as a tumor marker to monitor the treatment response in HER2 positive patients. However, there are few assays available for HER2. The aim of this master thesis was therefore to develop a robust and sensitive immunoassay specific for HER2 ECD.

Six monoclonal antibodies (M75, M77, M79, M83, M84 and M89) that recognise both recombinant and native HER2 were utilized to develop an immunometric assay for serum HER2. The antibodies were characterized, and antibody pair combinations were evaluated in a two-site assay to find the antibody combination with the highest sensitivity towards native HER2. Biotin labelled M89 and europium labelled M83 was the best combination, and this assay was further optimized and automated using an AutoDELFIA instrument. The final assay was used to test 15 HER2 positive patient samples and the results were compared with patient results obtained in the commercial Siemens ADVIA Centaur Serum HER-2/neu immunoassay. The sample results from the new HER2 assay were generally lower than the sample results from the Siemens assay, but there was a good linear correlation between the assays. Thus, a robust assay with high sensitivity towards HER2 ECD was developed.

Abbreviations:

°C	Degrees in Celsius
Ab	Antibody
cpm	Counts per minute
cps	Counts per second
kDa	kiloDalton
L	Liter
M	Molar (mol/L)
mAb	Monoclonal antibody
MES	Morpholinoethanesulfonic acid
mg	Milligram
ml	Milliliter
mM	Millimolar
nm	Nanometer
PBS	Phosphate-Buffered Saline
rpm	Rotations per minute
smAb	Solid phase monoclonal antibody
µ	Micro
µg	Microgram
µl	Microliter
µm	Micrometer

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1. Introduction

1.1. Background and aim of the study

Breast cancer is the most common type of cancer affecting women, and approximately 10 % of women develop this heterogeneous disease throughout their lifetime [1]. Breast cancer can be divided into three subgroups; hormone receptor positive, human epidermal growth factor receptor 2 positive and triple-negative breast cancer [2]. HER2 positive breast cancer is associated with poor prognosis and relapse [3], and approximately 15-30 % of breast carcinomas are of this aggressive phenotype [4].

There are few useful serum tumor markers for breast cancer, and it is a need for new breast cancer tumor markers. Several studies using serum HER2 as tumor marker has been performed, but the studies show varying results both regarding prognostic value and in monitoring the response to treatment [5-7]. The tumor marker laboratory at the Norwegian Radium Hospital has long experience in developing immunoassays for tumor markers that are as good as, or superior to, existing commercial assays [8;9]. Thus, there was an interest in developing a new immunoassay for serum HER2 at the Norwegian Radium Hospital. The aim of the study was therefore to develop a robust immunometric assay with high sensitivity and specificity for serum HER2.

1.2. HER2

HER2 is a 185 kDa glycoprotein that belongs to the epidermal growth factor receptor (EGFR) family, and is encoded by the ERBB2 gene located on chromosome 17q12 [10]. The HER2 protein consists of an extracellular, a transmembrane and an intracellular domain, where the intracellular domain contains tyrosine kinase activity (figure 1.1) [4]. Other members of the EGFR family, includes the HER1, HER3 and HER4 proteins.

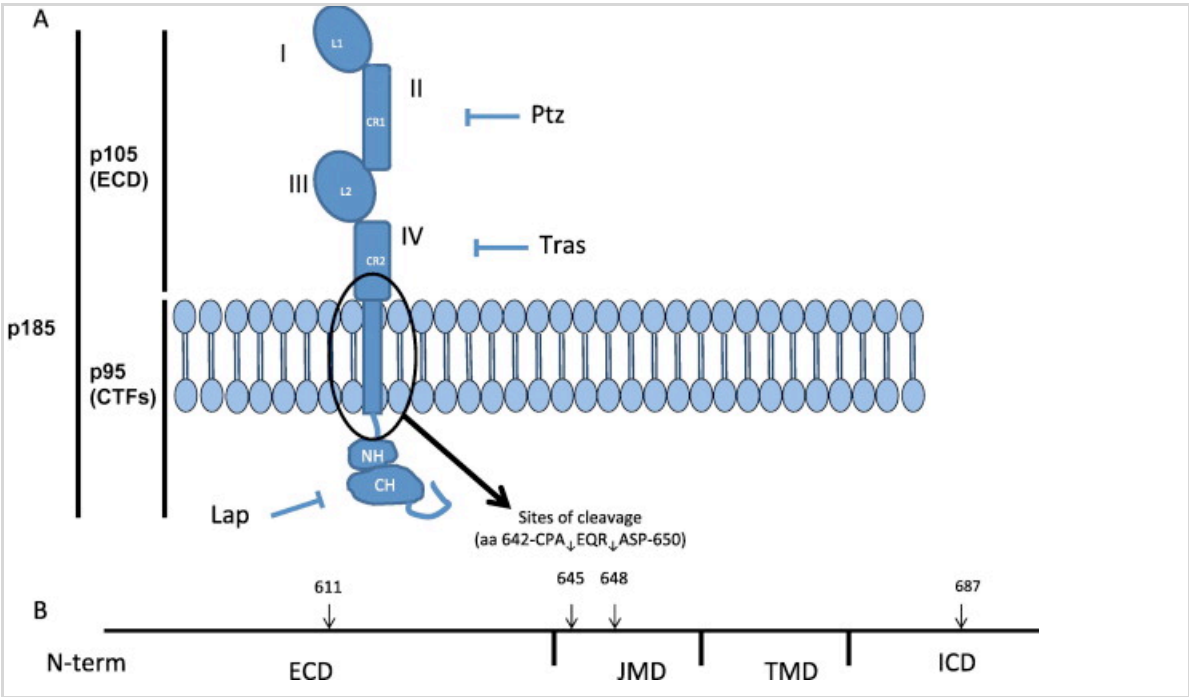

Figure 1.1: An illustration of the HER2 protein, showing the distribution of the extracellular, the transmembrane and the intracellular domain. The extracellular part of the HER2 protein consists of four domains. Two of these domains (I and III) are associated with ligand binding, while the two remaining domains (II and IV) are involved in dimerization of receptors on the cell surface [11]. The monoclonal antibodies pertuzumab and trastuzumab, used for the treatment of HER2 positive breast cancer patients, targets the II and IV extracellular domains of the HER2 protein, respectively [11]. Figure copied from [11].

The tyrosine kinase activity of the HER proteins is activated by binding of a ligand to the extracellular domain, which trigger dimerization of HER proteins on the cell surface [12]. The dimer formed may either be a homodimer, a formation between two similar HER proteins, or a heterodimer, a formation between two different HER proteins [12]. Activation of the tyrosine kinase in the intracellular domain of HER2 induces a cascade of reactions providing intracellular signals to the cell nucleus [12].

In normal cells, the gene expression is strictly regulated. Mutations in the genes encoding the HER receptors may affect this regulation and allow for increased gene amplification. The HER proteins contribute to the regulation of cell growth and survival [13]. If the gene encoding HER2 proteins are subjected to mutations, it may result in increased amplification of the HER2 gene. This will further result in an increased production of HER2 receptors, and when activated, intracellular signals mediated by the receptors to the nucleus may result in uncontrolled and rapid cell growth [14].

Immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) are two common methods for differential diagnosis of breast cancer, and these techniques are used, among others, to evaluate the HER2 status and thus distinguish between HER2-positive and HER2-negative tissue [15]. The techniques may in this way give an indication of the type of therapy the patient will benefit from.

Following surgical removal of tumor tissue, HER2 positive breast cancer patients are treated with conventional chemotherapy and radiotherapy in combination with immunotherapy. In any targeted therapy, the antigen of interest must be significantly higher expressed in tumor cells relative to normal cells. Normally, epithelial cells of breast tissue express low levels of HER2 in healthy humans. In tumor cells that over-express HER2, about 500 000 – 2 000 0000 HER2 receptors are found on the cell surface, while between 25 000 and 185 000 receptors are found on non-over-expressing tumor cells [11]. Trastuzumab (Herceptin) is a humanized recombinant anti-HER2 monoclonal antibody utilized for treating HER2 positive breast cancer by targeting the extracellular domain IV of HER2 [12]. By targeting HER2, trastuzumab may inhibit dimerization of the receptors and thereby inhibit the signal for tumor growth [11]. Pertuzumab is another anti-HER2 monoclonal antibody that can be used in combination with Trastuzumab.

1.3. Tumor markers

Tumor markers are compounds, usually proteins, produced by tumor tissue and released into the blood circulation [16]. These proteins are usually observed at low concentration levels in the blood of healthy people, but in the presence of a tumor, the concentration levels of the markers associated with that particular tumor may be elevated. Immunoassays for tumor markers are normally not sensitive and specific enough to be utilized as diagnostic tests alone. Their main uses are for monitoring the response to treatment and detection of relapse of disease.

The extracellular domain (ECD) of the HER2 protein is proteolytically cleaved by metalloproteases and released into the blood circulation [15]. Using serum measurements of HER2 ECD in blood samples from HER2 positive patients to monitor the tumor response to

therapy is of great interest. Serum testing would be of even greater interest if the HER2 ECD could be used as a marker for recurrence of HER2-positive cancer.

1.4. Monoclonal antibodies

B-lymphocytes play an important role in adaptive immunity by recognizing foreign antigens and secreting specific antibodies. B-lymphocytes are stimulated by the binding of antigens to their antibody receptors on the cell surface, which may lead to the development of specialized plasma cells. These cells protect the host organism from foreign pathogens, by secreting antibodies specific to antigens on the invading pathogen [17].

An antigen can be almost any type of biological molecule that can be recognised by antibodies [17]. An epitope is a specific site on the antigen where antibodies can bind. One B-cell produces monoclonal antibodies that bind to one specific epitope on the antigen. During the lifetime of a human being, about 10^{10} different antibodies are synthesized in the body [18]. While monoclonal antibodies are homogeneous, polyclonal antibodies are collections of many monoclonal antibodies and do not recognize one specific epitope, but may detect several epitopes on the antigen of interest.

Immunoglobulin G (IgG) is a 150 kDa glycoprotein and is the most common class of antibodies used in immunoassays. The IgG molecule consists of two identical heavy - and two identical light polypeptide chains, which are bound by disulphide bonds, see figure 1.2. The heavy chains contain four domains, while the light chains contain two domains. The polypeptide chains are composed of two types of domains, constant -and variable domains. Hypervariable regions are found in the variable domains of both the light and the heavy chains and is where the antibody binds the antigen.

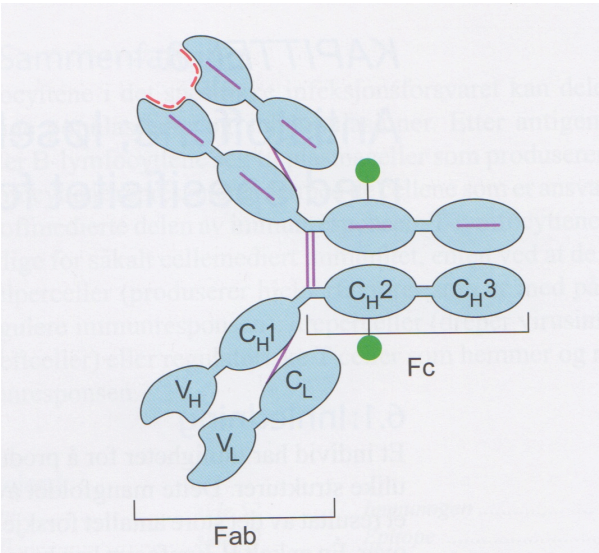

Figure 1.2: A simplified figure of the structure of an IgG molecule, showing the distribution of the light-and heavy polypeptide chains and the constant- and variable domains. At the V_H (variable heavy chain) and V_L (variable light chain) domains in the Fab part of the IgG molecule is the antigen-binding site. Figure copied from Lea T. [17].

The antigen binding sites on the IgG molecule is significantly smaller than the ECD of the HER2 protein. The HER2 ECD contains multiple epitopes which may be either overlapping or non-overlapping, and several IgG molecules can bind to epitopes on a single HER2 ECD. Overlapping epitopes may lead to competition between the antibodies that try to bind, while non-overlapping epitopes may allow antibodies with different specificity towards the epitopes to bind independently of each other.

1.5. Immunological analysis

Immunoassay is a technique used to detect and quantify the amount of a certain antigen, based on the specificity of the reaction between an antibody and its antigen. The use of antibodies in immunological analysis has great benefits due to the vast variation, specificity and high affinity of the antigen-antibody reaction [19].

Immunoassays can be divided into competitive and non-competitive (immunometric) assays. Competitive assays usually utilize polyclonal antibodies and use antibodies in deficit, while immunometric assays normally use monoclonal antibodies (mAbs) in excess, and does often have the benefit of being more sensitive than competitive assays [17].

One type of immunometric method referred to as a sandwich assay, utilizes antibodies that bind to different epitopes on the antigen, hence, antibodies with different specificities. A monoclonal antibody is immobilized to a solid phase (e.g. a bead or the wall of a well), which is incubated with the sample containing the specific analyte, see figure 1.3. Addition of a second antibody labelled with a reporter molecule will then bind to the immobilized antigen and make it possible to measure the amount of antigen present.

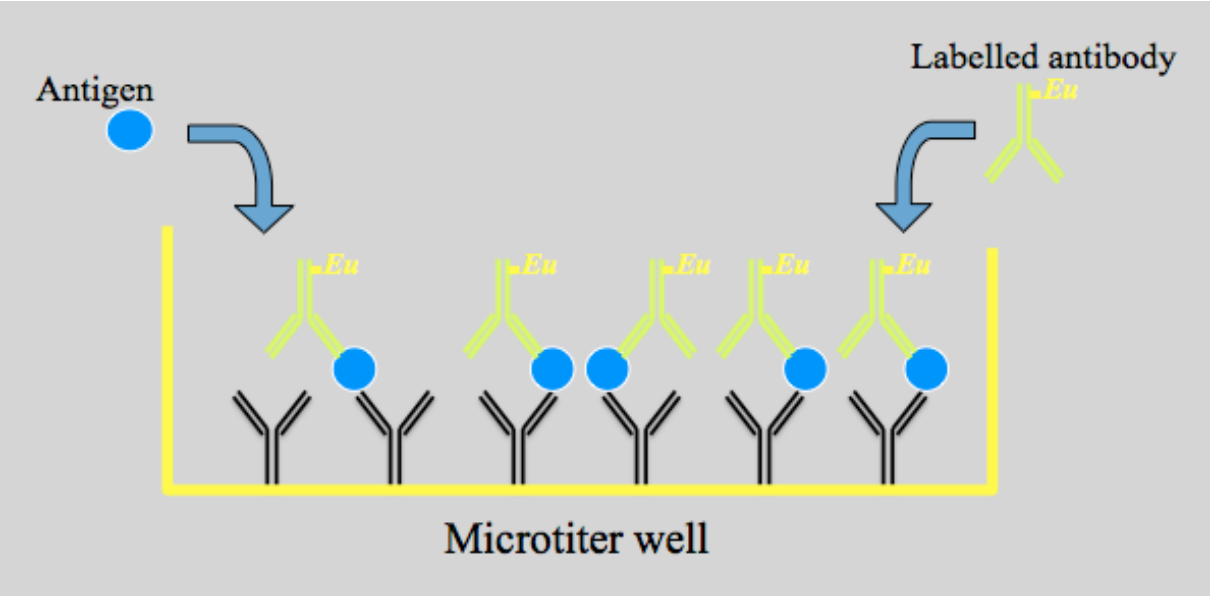

Figure 1.3: A simplified illustration of a two-site assay. 1. Antibodies (black) are immobilized on the walls of a microtitre well. 2. Then the antigen of interest is added to the well and is bound to the antibody. 3. Europium labelled antibodies (light green) are added as tracer.

Interference by heterophilic antibodies can be a problem in immunoassays [20]. Heterophilic antibodies are found in the blood of some patients, and it is crucial that the assays are protected against these antibodies to avoid false high values and possible misdiagnosis. To prevent this problem, several proteases such as papain, pepsin or bromelain can be utilized to specifically fragment IgG into biologically active fragments. Papain fragments IgG into Fab- and F_c fragments (figure 1.4), and the proteases bromelain and pepsin cut the IgG molecule into $F(ab')_2$ and F_c fragments, where the $F(ab')_2$ fragments contain two antigen specific binding sites, called paratopes [19]. Bromelain has been found to cleave mouse IgG1 better than papain [21]. When $F(ab')_2$ or Fab fragments are used in an immunoassay, the interference due to heterophilic antibodies that bind to the F_c part of the IgG molecule is eliminated [22]. The majority of heterophilic antibodies bind to the F_c part of the antibody, but some are able to bind to the $F(ab')_2$ fragments [22]. An additional way to prevent

interference in these assays is to add a blocking antibody or blocking reagent to the reagents/buffers used in the assay [23]. Aggregated antibodies used as blocking antibodies in the assay are more potent to block heterophilic antibodies than antibodies that are not aggregated, and it is important that the blocking antibody is from the same species and of the same isotype as the reagent antibodies [23].

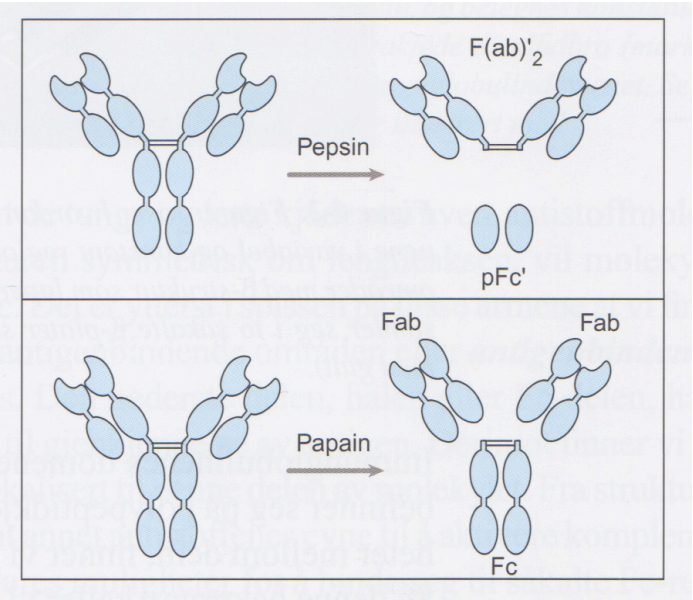

Figure 1.4: A simplified figure of how pepsin and papain fragment IgG molecules, and the distribution of the resulting fragments. Bromelain reacts similar to pepsin. Figure copied from Lea T. [17].

The monoclonal antibodies utilized in this thesis were produced before the thesis began, by the department of medical biochemistry at the Norwegian Radium Hospital. The mAbs were produced after immunization of BALB/c mice with recombinant HER2. Initial tests identified monoclonal antibodies produced by 19 cell lines, where the antibodies recognized both recombinant and native HER2. The six most promising candidates (M75, M77, M79, M83, M84 and M89) were subcloned and further tested.

In this master thesis, the six monoclonal antibodies specific for HER2 ECD were characterized and evaluated in two-site assays utilizing different combinations of the antibodies to identify the best assay combination that recognized native HER2. Monoclonal antibodies labelled with biotin were used as solid phase antibodies on streptavidin-coated plates in order to exploit the strong affinity between streptavidin and biotin, with a dissociation constant, $K_D = 4 \times 10^{-14}$ M [24]. The second (tracer) antibody was labelled with

europium (III) chelates as reporter molecules. The final assay was optimized and automated using an AutoDELFIA instrument.

There are several methods that can be used to label antibodies. One method is radioisotopic labelling, were iodine-125 (^{125}I) is the most common radioisotope utilized in immunoassays [19]. It emits gamma rays that are detected by solid scintillation counting that measure ionizing radiation [19]. Time-resolved fluorescence is another method where Europium (Eu^{3+})-chelate is commonly used as tracer molecule. This method is based on counting the fluorescence emission after a certain time delay, to ensure that it is the signal from the specific substance that is counted [25]. Eu^{3+}-chelate has a long fluorescence decay time that makes it easier to distinguish the fluorescence emission from that specific substance than from background interference [26].

2. Materials and methods

More detailed information about the chemicals and proteins are found in appendix 1.

2.1. Determination of protein concentrations

In a protein or a peptide, it is primarily the aromatic amino acids tryptophan and tyrosine that are able to absorb light at 280 nm. The absorbed light was measured at 280 nm using a spectrophotometer. The extinction coefficient for a whole mouse IgG is 1,43. This number was used after measuring the absorbance at 280 nm to calculate the antibody concentration in mg/ml by dividing the absorbance at 280 nm value with the extinction coefficient.

2.2. Purification of antibodies on protein A column

Materials:

- Supernatant containing the protein
- Binding buffer (1x: 0,1 M Na_2HPO_4 and 0,01 % NaN_3 (pH 8,2))
- 0,25 M EDTA (pH 8,2)
- Protein A elution buffer (1x: 25 mM citric acid, 25 mM Na_2HPO_4, 25 mM NaCl, 0,01 % NaN_3 (pH 3,1))
- 1 M NaOH
- rProtein A Sepharose (Fast Flow, Pharmacia, GE Healthcare Life Sciences)
- Whatman sterile filter, 0,22 µm (GE Healthcare Life Sciences)
- Beckman Coulter Avanti J-25I Centrifuge (Nerliens Meszansky AS)
- PHM 92 LAB pH METER (Radiometer Copenhagen)
- UV-1800 Spectrophotometer (Shimadzu)
- Econo Pump, system controller and fraction collector (Bio Rad)
- 20 % ethanol

Preparation of sample:

The sample was spun for 30 minutes at 10 000 rpm at 4 °C. Added 0,25 M EDTA to final ≈ 7,7 mM and binding buffer 5x to final 1x. pH was adjusted to 8,2 using 1 M NaOH, and the sample was filtered with 0,22 µm filter to remove any protein precipitates.

Procedure preformed at 4 °C:

The column was first equilibrated with 5-10 times the column volumes of binding buffer, and pH and absorbance at 280 nm (A_{280}) was measured. Then the sample was added, and the column was washed with 5-10 column volumes of binding buffer or until the A_{280} of the flow through had reached the baseline. The antibody was then eluted by adding 3-4 column volumes of Protein A elution buffer. pH and A_{280} were measured in the fractions during the whole elution to find out when the protein was eluted. The column was then re-equilibrated with binding buffer until A_{280} reached 0. The fractions with high absorbance were collected and pooled. pH was adjusted to 7 with 1 M NaOH and A_{280} was measured, and the sample was then filtered through a 0,22 µm filter. Finally, the antibody concentration was calculated by dividing the A_{280} value by the extinction coefficient for immunoglobulin G.

Wash and storing of the column:

The column was washed with five column volumes of ultra-pure water. The column was then washed with 5 column volumes of 20 % ethanol and stored in the ethanol solution at 4 ºC.

The purification was performed as described earlier [21].

2.3. Isotyping of antibodies

Materials:

- Antibodies
- PBS (0,01 M NaH_2PO_4, 0,15 M NaCl (pH 7,2))
- Vortex-Genie 2 (Scientific industries)
- IsoStrip Mouse Monoclonal Antibody Isotyping Kit including capped development tubes containing lyophilized latex beds and isotyping strips (Roche)

Procedure:

The antibodies were diluted to 0,5 µg/ml in PBS. 150 µl of each diluted antibody sample was dispensed into different capped development tubes containing lyophilized latex beads and incubated for 30 seconds at room temperature. The solutions were then mixed with a vortex mixer until all the beads were dissolved. One isotyping strip were placed in each development tube and incubated for 5 – 10 minutes. Then a positive control band appeared, and the results could be read.

2.4. Fragmentation of IgG to $F(ab')_2$

Materials:

- Antibodies
- Bromelain
- Protease inhibitor E-64
- Buffer with pH 7 (1x: 0,05 M Tris, 0,1 M NaCl and 5 mM EDTA)
- Binding buffer (5x)
- rProtein A Sepharose
- Whatman sterile filter, 0,22 µm
- PHM 92 LAB pH METER
- UV-1800 UV Spectrophotometer
- Water bath (Grant)

Procedure:

pH of the antibody sample was adjusted to 7,5 with binding buffer, to obtain an optimal fragmentation. The protein A column was equilibrated with binding buffer until pH 8,2 was reached. The antibody sample was filtered with 0,22 µm filter to remove any aggregates. A_{280} was measured and the antibody concentration was calculated using the extinction coefficient. The sample was added 1:10 volume ratio of buffer with pH 7, and bromelain was added to get a 1:20 ratio between the antibody and bromelain. The solution was incubated in a water bath at 37 °C for 2 hours, and the fragmentation reaction was stopped by adding 10 µg/ml of the protease inhibitor E-64. Finally, the $F(ab')_2$ and F_c fragments were separated using a protein A column. This procedure is described in "2.2 Purification of antibodies on protein A

column". Calculations are shown in appendix 2. The fragmentation was performed as described earlier [21].

2.5. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis is a method utilized to separate proteins based on their size. Using the anionic detergent SDS, a reducing agent and heat, the proteins become denatured and the negatively charged SDS give the proteins a negative charge. The proteins will then migrate to the positive pole of an electric field based on the protein size.

Materials:

- Antibodies
- NuPAGE 4-12 % Bis-Tris Protein Gel (Novex, Life Technologies)
- Mes-SDS Running buffer NuPAGE 20x (1x: 50 mM MES, 50 mM Tris Base, 0,1 % SDS, 1 mM EDTA (pH 7,3)) (Novex, Life Technologies)
- Sodium dodecyl sulphate loading buffer (SDS Lb) (1x: 50 mM Tris, 2 % SDS, 0,1 % bromophenol blue, 10 % glycerol)
- Imperial Protein Stain (Thermo Scientific)
- SeeBlue Plus2 Prestained Standard (Novex, Life Technologies)
- Water bath

Sample treatment:

The sample was boiled in a water bath for 3 minutes to denature the proteins.

Procedure:

The Mes-SDS Running buffer NuPAGE 20 was diluted to 1x with deionized water. The NuPAGE 4 – 12 % Bis-Tris Protein Gel was placed in the electrophoresis chamber, and the chamber was filled with the diluted Mes-SDS Running buffer. Then the samples could be loaded. 10 µl SeeBlue standard was added to the first well. 15 µl sample containing the $F(ab)_2$ –fragment and 5 µl SDS Lb four times concentrated was mixed and 10 µl of the mix was added to the second well. 5 µl of the sample containing the F_c - part and possibly some remaining non-fragmented antibodies was mixed with 5 µl SDS Lb 2 times concentrated and

added to the third well. 5 µl of a sample containing a whole IgG antibody and 5 µl SDS Lb 2 times concentrated was mixed and added to the fourth well. The proteins and fragments in the gel were separated at 200 V.

Procedure for staining the gel:
Ultra-pure water was used to wash the gel for 5 minutes and the wash was repeated three times. Then, the gel was stained with imperial protein staining on a shaker for fifteen minutes. Finally, the gel was washed with ultra-pure water for one hour on a shaker.

2.6. Biotin labelling of antibodies and $F(ab')_2$ fragments

Materials:

- Antibodies
- EZ-link Sulfo –NHS-LC Biotin
- PD 10 Desalting Columns (GE Healthcare Life Sciences)
- Pyro-mag stir (Cenco Instrumenten B. V.) and a magnet
- 1 M glycine
- Whatman sterile filter, 0,22 µm
- UV-1800 Spectrophotometer
- PHM 92 LAB pH METER
- Elution buffer (0,05 M Tris, 0,15 M NaCl, 0,05 % NaN_3 (pH 7,8))

Procedure:
The antibodies must be in a buffer absent of amino groups. To determine the protein concentration, A_{280} was measured and the protein concentration was calculated. The pH was adjusted to 7,5 and 10 mM NHS-biotin (5x molar excess of biotin; 3,33 µl/mg monoclonal antibody) was added while the sample was stirred. The solution was then incubated at room temperature for 30 minutes, and 1 M glycine (100 µl/ml solution) was added to stop the reaction. PD-10 columns with elution buffer were used to remove the free biotin in each sample. The sample was then filtered with 0,22 µm filter. A_{280} was measured and the protein concentration was calculated. The biotin labelling was performed as described earlier [21].

2.7. Iodine labelling of antibodies

I only observed during this procedure. The procedure was performed in a separate room with lead cover and equipment used only during experiments with radioactivity.

Materials:

- Antibodies
- NAP-5 columns (GE Healthcare Life Sciences)
- Iodine-125
- IODO-GEN Pre-Coated Iodination Tubes (Pierce, Thermo Scientific)
- Iodination buffer (0,025 M Tris and 0,4 M NaCl (pH 7,4))
- Iodination elution buffer (0,01 M $NaPO_4$, 0,3 M NaCl, 0,01 % Thimerosal and 0,1 % BSA (pH 7,3))
- Saturated tyrosine solution
- Ethylene glycol
- Soft Nitrile Examination Gloves (Klinion Protection)
- Wallac WIZARD 1470 Automatic Gamma Counter (Wallac, Perkin Elmer life sciences)
- Extra lab coat

Procedure:

A NAP-5 column was equilibrated with 10 ml iodination elution buffer and the iodogen tube was prewashed with 1 ml iodination buffer. 100 µl iodination buffer with 2,5 µl ^{125}I (0,125 nmol) was added. The tube was incubated for 5 minutes at room temperature while shaking the tube a few times a minute. The ^{125}I-solution was added to a tube containing 100 µl of protein solution in iodination buffer. The tube was incubated at room temperature for 5 minutes. 50 µl tyrosine solution (10 mg/ml) was then added to the tube and the tube was incubated for 5 minutes in room temperature. To separate the free iodine in the solution from iodine bound to the protein, the solution was separated on a NAP-5 column. The protein was eluted in 0,2 ml fractions of iodination elution buffer, and radioactivity were measured using Wallac WIZARD 1470 Automatic Gamma Counter. The appropriate fractions were mixed and 1:2 ratio of ethylene glycol was added, and the radioactivity in 10 ml of the solution was measured.

2.8. Europium labelling of antibodies

Materials:

- Antibodies
- Europium p-isothiocyanatophenyl-EDTA complex
- Molecularporous membrane tubing (MWCO: 12-14000) (Spectral Medical Industries, Inc.)
- Whatman sterile filter, 0,22 µm
- Borate buffer (0,02 M borate, 0,15 NaCl, pH 8.0)
- PD 10 Desalting Columns
- Amicon ultra, 50 K (Millipore)
- Elution buffer
- DELFIA®/AutoDELFIA® Enhancement solution (Wallac, Perkin Elmer life sciences)
- UV-1800 UV Spectrophotometer
- Distillate water
- Victor™ 1420 Multilabel Counter (Wallac, Perkin Elmer life sciences)

Eu-isothiocyanat pre-treatment:
0,2 mg europium^{3+}-chelate was dissolved in 0,1 ml water.

Antibody pre-treatment:
The antibody samples were dialysed or added to a PD-10 column to change the buffer to borate buffer. The antibody solution was concentrated to 1-2 mg/ml on an Amicon ultra, 50 K. The samples were then filtered with 0,22 µm filters.

Procedure:
Antibody and europium^{3+}-chelate were mixed with 12X molar excess of europium^{3+}-chelate. 1/10 volume of 0,1 M borate buffer was added to the reaction mix, and the solutions were incubated for 72 hours at room temperature. Gel filtration by PD-10 columns was utilized to separate free- and bound Eu^{3+}-chelate in the solutions. The columns were first equilibrated with 25 ml of elution buffer, and then the samples were added to the columns and eluted with elution buffer in 0,5 ml fractions. 5 µl of each fraction was first diluted 1:100 in 0,5 ml elution buffer. 5 µl of the diluted solution was further diluted 1:40 in 0,2 ml enhancement solution

and fluorescence was measured. The peak fractions were collected, and fluorescence was measured in dilutions of 1:10 000 and 1:100 000 against a 1:100 diluted europium standard (100 nmol/L) in enhancement solution, to calculate the concentrations of the incorporated europium molecules. A_{280} was measured in the collected samples and the protein concentration was calculated. The calculations for the number of Eu^{3+} per IgG and recovery (%) are shown in appendix 3. The europium labelling was performed as described earlier [21].

2.9. Coating of plates with different antibodies

Materials:

- Antibodies
- Blocking buffer (1x: 0,05 M Tris, 6 % D-sorbitol, 0,1 % BSA, 0,05 % NaN_3 and 0,2 mM DTPA (pH 7,0))
- Coating buffer (1x: 27,6 g 0,2 M NaH_2PO_4 in 1 L distilled water)
- AutoDELFIA wash buffer (0,05 M Tris, 0,15 M NaCl, 0,1 % Germall and 0,05 % Tween 20) (Wallac, Perkin Elmer life sciences)
- DELFIA Platewash (Wallac, Perkin Elmer life sciences)
- Pyro-mag stir and a magnet
- C8 maxi break apart nunc-immuno module (Nunc A/S)
- Soft absorbent paper

Procedure:

Day 1: Each well should contain 1 µg antibody with a total volume of 200 µl per well. The antibodies were diluted to 5 µg/ml in coating buffer, mixed and 200 µl of the dilution was added to each well. The plates were put in a moist box at 37 °C and incubated over night.

Day 2: The plates were washed two times with AutoDELFIA wash buffer on DELFIA plate washer and 300 µl blocking buffer was added to each well. The plates were incubated at room temperature over night in a moist box.

Day 3: The blocking buffer was removed using the DELFIA platewash and the remaining liquid in the wells were “hit” out on soft absorbent paper. The plates were dried at room temperature for 3 hours and were then ready for use.

Calculations are shown in appendix 4. The coating of plates were performed as described earlier [27].

2.10. Cross-inhibition

Materials:

- Recombinant human HER2 labelled with iodine-125
- MAbs: M75, M77, M79, M83, M84 and M89
- PBS with 0,3 % BSA
- C8 maxi break apart nunc-immuno module coated with mAbs (1 μg/well)
- DELFIA Platewash
- DELFIA Plateshake (Wallac, Perkin Elmer life sciences)
- AutoDELFIA wash buffer
- Wallac WIZARD 1470 Automatic Gamma Counter

Procedure:

50 μl iodine labelled recombinant HER2, 0,07 μg/ml, was mixed with 50 μl mAb 10 μg/ml in PBS with 0,3 % BSA for each of the six antibodies. As a negative control, 50 μl iodine labelled recombinant HER2 mixed with 50 μl PBS with 0,3 % BSA was used. The reaction solutions were incubated for 1 hour at room temperature. Calculations are shown in appendix 5. 100 μl of the samples were added to mAb-coated plates in such a way that all antibody combinations were tested. The plates were incubated for 1 hour at room temperature on DELFIA plateshaker, and were then washed three times with AutoDELFIA wash buffer using the DELFIA platewash. Finally, radioactivity was counted in each well and percent binding calculated using the Wallac WIZARD 1470 Automatic Gamma Counter. The cross-inhibition test was performed as described earlier [28].

2.11. Testing antibody combinations

Materials:

- C8 maxi break apart nunc-immuno module plates coated with mAbs: M75, M77, M79, M83, M84 and M89
- M75, M77, M79, M83, M84 and M89 labelled with iodine-125
- PBS with 0,3 % BSA
- 100 µg/L HER2
- DELFIA Platewash
- DELFIA Plateshake
- Wallac WIZARD 1470 Automatic Gamma Counter
- AutoDELFIA wash buffer

Procedure:

100 µl of 100 µg/L recombinant human HER2 was added to the mAb plates. 100 µl PBS with 0,3 % BSA was used as negative control. The plates were incubated for 1 hour at room temperature on a DELFIA plateshaker, and were then washed three times with AutoDELFIA wash buffer using the DELFIA platewasher. 100 µl of ≈ 50 000 cpm iodine labelled mAbs were added to the wells in such a way that all antibody combinations were tested. The plates were incubated for 1 hour at room temperature on a DELFIA plateshaker, and were then washed six times with AutoDELFIA wash buffer. Finally, radioactivity was counted in each well and percent binding was calculated.

2.12. Preparation of standards A, B, C, D, E and F

The standards consisted of different concentrations of recombinant human ErB2/HER2 and standard matrix (50 mM Tris, 150 mM NaCl, 0,1 % Germall and 6 % BSA).

First 1 ml of HER2 was diluted 1:100 in 99 ml of standard matrix.

Then the standards were prepared as follows:

Standard F: 12 ml 1:100 dilution of HER2 and 48 ml standard matrix (1:5)

Standard E: 15 ml standard F and 45 ml standard matrix (1:4)

Standard D: 15 ml standard E and 45 ml standard matrix (1:4)

Standard C: 15 ml standard D and 45 ml standard matrix (1:4)

Standard B: 15 ml standard C and 45 ml standard matrix (1:4)

Standard A: Standard matrix

Then the calibrators were aliquoted and stored at – 30 °C. Finally they were calibrated against the Siemens Healthcare Diagnostics ADVIA Centaur Serum HER-2/neu immunoassay and the concentrations of standard A-F ranged from 0 to 350 µg/L.

2.13. Optimization of the immunoassay

The optimization of the assay included testing of different incubation times for the second and third incubation period, and testing several concentrations for the biotin labelled antibody and the europium labelled antibody.

Standard A-F and a control reference material, breast cancer cell line SK-BR3, was used in the optimization. This reference material has an overexpression of the HER2 gene product, namely the HER2 protein. Pooled patient sera from patients at the Norwegian Radium Hospital were also used as control samples. These two in-house control samples, DNR control 1 and DNR control 2, had concentration 18,7 µg/l and 165 µg/l respectively. Two control samples from Siemens were also included in the tests, Siemens control 1 and Siemens control 2, which contain 12.8 µg/l and 98.2 µg/l of recombinant HER2 respectively.

Materials:

- M89- and M89 $F(ab')_2$ fragment labelled with biotin
- M83 labelled with europium
- AutoDELFIA buffer (0,05 M Tris, 0,15 M NaCl, 0,02 mM DTPA, 0,1 % Germall, 0,01 % Tween 20, 5 % BSA, 0,5 % bovine IgG, 15 mg/L MAK 33 and 3 mg/L tartrazine)

- AutoDELFIA wash buffer
- AutoDELFIA Streptavidin Microtitration Strips, 8 x 12 wells (Wallac, Perkin Elmer life sciences)
- DELFIA Plateshake
- DELFIA Platewash
- Standard A-F
- Control samples
- Cell line SK-BR3 (American Type Culture Collection ATCC)
- AutoDELFIA Enhancement solution
- Disposable plastic pipette (Nunc Brand Products)
- Victor^{TM} 1420 Multilabel Counter
- AutoDELFIA^{TM} 1235 Automatic immunoassay system (Wallac, Perkin Elmer life sciences)

The first step in the optimization was to determine the optimal incubation times. Started by testing different incubation times for the second incubation period, after the samples were added. All of the optimization tests were performed manually. However, due to poor parallel values for some of the standard curves, parts of the optimization testing were performed again using the AutoDELFIA instrument.

Procedure:
200 µl of 2 µg/ml biotin labelled M89 in AutoDELFIA buffer was added to each well in a streptavidin plate, and incubated for 30 minutes at room temperature on the DELFIA plateshaker. The wells were washed 3 times with AutoDELFIA wash buffer on the DELFIA platewasher. 150 µl AutoDELFIA buffer and 50 µl of the standards and samples were added to the wells, and the plates were incubated at room temperature for either 30, 60, 90 and 120 minutes on the DELFIA plateshaker and washed 6 times with AutoDELFIA wash buffer. 200 µl of 1 µg/ml europium labelled M83 in AutoDELFIA buffer was added to the wells. The plates were incubated for 60 minutes at room temperature on the DELFIA plateshaker and then washed 6 times. Finally, 200 µl enhancement solution was added and the plates were incubated for 5 minutes at room temperature on the DELFIA plateshaker. Europium cps was then measured on Victor^{TM} 1420 Multilabel Counter.

The procedure for testing incubation times for the third incubation period, after adding europium labelled M83 antibody, was identical, except for the second incubation period. The second incubation period was 60 minutes, and the incubation times tested for the third incubation period were 30, 60, 90 and 120 minutes.

After testing incubation times, different concentrations of M89 labelled with biotin and M83 labelled with europium were tested. Instead of using the whole antibody, $F(ab')_2$ fragment of M89 was utilized. This procedure was identical with the procedure for testing the incubation times, except that the third incubation period was 60 minutes. First, biotin labelled M89 $F(ab')_2$ fragment with 1, 2, 3 and 4 μg/ml concentration in AutoDELFIA buffer was tested. Then europium labelled M83 with 0,5 μg/ml, 1,0 μg/ml and 2,0 μg/ml concentration in AutoDELFIA buffer was tested.

The assay was ultimately used to test serum samples from HER2 positive breast cancer patients using the AutoDELFIA instrument and compared with a commercially available immunoassay for HER-2/neu (ADVIA Centaur, Siemens, Norway). All participants had given written informed consent to participate in the study.

3. Results

3.1. Purification and characterization of the monoclonal antibodies

The monoclonal antibodies were produced at the Department of Medical Biochemistry at the Norwegian Radium Hospital prior to the start of this project. The monoclonal antibodies were produced in cell cultures.

All of the antibodies were purified on protein A columns. Characterization of the antibodies was done with two independent methods. Cross-inhibition is a method to characterize the antibodies towards each other and was performed to identify whether the antibodies had overlapping or non-overlapping epitopes. Isotyping is a method to determine the specific isotype of an antibody.

3.1.1. Cross-inhibition

Cross-inhibition was performed to separate the six different antibodies towards HER2 into groups related to the epitope specificities. Based on the similarity and differences in the inhibition patterns, it was possible to separate the antibodies into different groups. The differentiation was based on complete inhibition (> 80 %) and the inhibition pattern towards the other antibodies. The six antibodies were divided into three groups; A, B and C. Group A was further divided into the two subgroups A1 and A2. Group A1 consisted of M75 and M89, group A2 consisted of M77, group B included M79 and M83, while group C only comprised M84 (table 3.1). Complete inhibition (> 80 %) is marked as bold type in the table below. The cross-inhibition results from all the antibody combinations with two identical mAbs show almost 100 % inhibition.

Table 3.1: The results of the cross-inhibition test as the percent inhibition (100 % - % binding) between the HER2 antibodies.

smAb	Groups	Inhibiting monoclonal antibody					
		M75	M89	M77	M79	M83	M84
		A1		A2	B		C
M75	A1	**91**	**95**	17	55	56	-2
M89		**83**	**100**	**92**	-1	3	5
M77	A2	**82**	**96**	**97**	-42	4	-4
M79	B	36	65	-5	**99**	**98**	-1
M83		48	72	-1	**99**	**100**	6
M84	C	-66	-9	-28	-3	30	**98**

3.1.2. Isotyping of antibodies

The isotyping method provides information about what isotype the antibody consist of and the type of light chain. Table 3.2 shows that all of the six mAbs have kappa light chains. M83 has IgG2a isotype, while the remaining mAbs have IgG1 as their isotype.

Table 3.2: An overview of the results from the isotyping test of M75, M77, M79, M83, M84 and M89.

mAb	**M75**	**M77**	**M79**	**M83**	**M84**	**M89**
Isotype	IgG1	IgG1	IgG1	IgG2a	IgG1	IgG1
Type of light chain	κ	κ	κ	κ	κ	κ

3.2. Antibody combinations

To identify the best antibody combinations, M75, M77, M79, M83, M84 and M89 antibodies were tested against each other using mAb coated plates and ^{125}I-labelled mAbs as tracer antibodies. The values from the negative control were subtracted from the resulting values and the results are shown in figure 3.2. The three best antibody combinations all had M89 as solid phase, with M79, M83 and M84 as tracer mAbs. A table with the exact values are shown in Appendix 6.

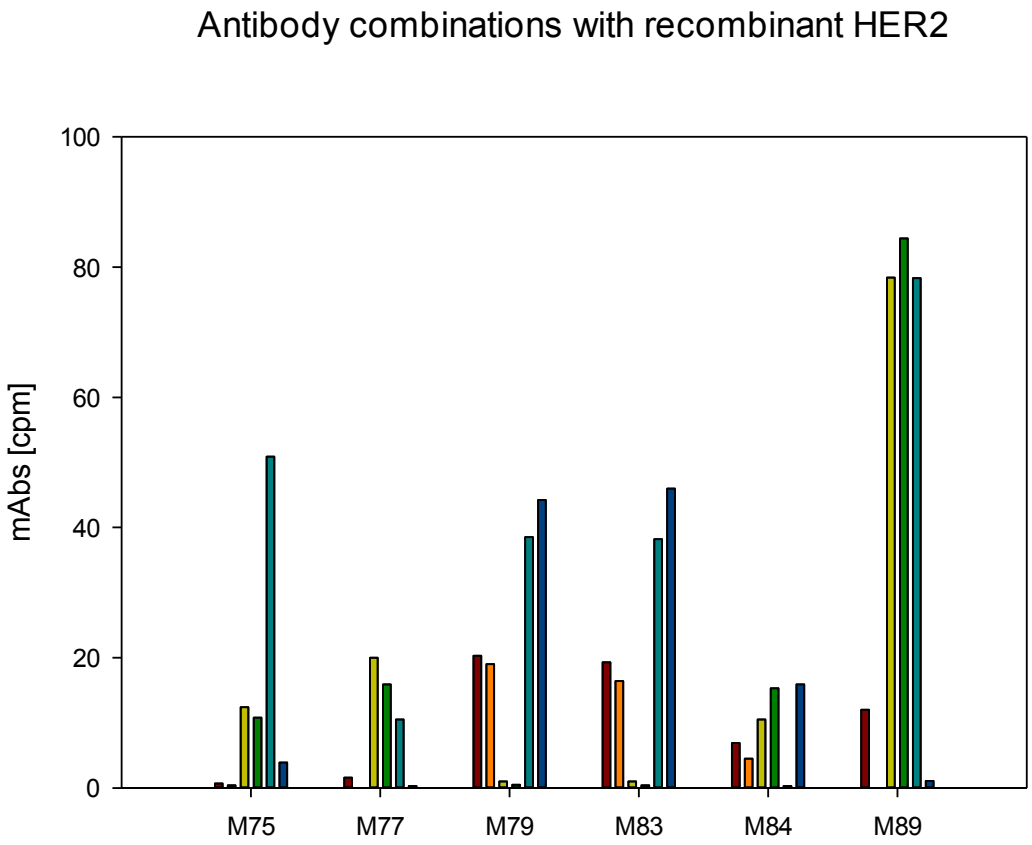

Figure 3.2: The coloured bars show the binding of the different mAbs labelled with ^{125}I in a sandwich assay with recombinant HER2 immobilized to plates coated with the different mAbs. Red = ^{125}I -M75, orange = ^{125}I -M77, yellow = ^{125}I -M79, green = ^{125}I -M83, turquoise = ^{125}I -M84 and blue = ^{125}I -M89.

3.3. Fragmentation of solid phase antibody

The solid phase antibody was fragmented into F(ab')$_2$ and F$_c$ fragments using bromelain, followed by separation of the fragments on protein A column. SDS-polyacrylamide gel electrophoresis was performed to evaluate the fragmentation. Figure 3.1 is a picture of the gel after fragmentation and separation of the M89 antibody, where well number 2 contain the F(ab')$_2$ fragment. Well number 3 contains the F$_c$ part and some remaining unfragmented M89 indicating that the fragmentation was not complete. However, the F(ab')$_2$ fragment used further in the studies was pure.

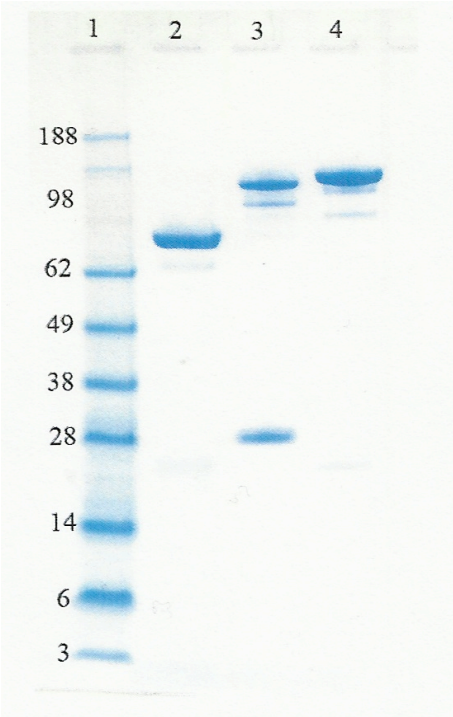

Figure 3.1: Picture of a SDS-polyacrylamide gel. Well 1: SeeBlue Plus2 Prestained Standard. Well 2: $F(ab')_2$ fragment of the M89 antibody. Well 3: Unfragmented M89 antibody and the F_c fragment of M89. Well 4: The whole antibody.

3.4. Europium labelling of the antibodies

The antibodies were labelled with europium as tracer molecule, and the table below shows the number of europium molecules per antibody and percent recovery. Calculations are shown in appendix 3. The incorporation of Eu^{3+} per monoclonal antibody molecule varied from 5,9 to 8,2.

Table 3.3: An overview of the number of labelled europium molecules per antibody and the percent recovery.

mAb	M79	M83	M84	M89
Number of Eu^{3+} per mAb	5,9	6,2	7,5	8,2
Recovery (%)	78 %	70 %	99 %	84 %

3.5. The three best antibody combinations

The three best antibody combinations where further tested manually in immunofluorometric assays using $F(ab')_2$ fragments of the M89 solid phase antibody and Eu-labelled tracer mAbs. Calibrators containing 0 to 350 µg/l of recombinant HER2 (standard A-F) were used in the evaluation, see figure 3.3. Siemens control 1 containing recombinant HER2, two in-house control samples from pooled patient sera and the SK-BR-3 Breast Adenocarcinoma human cell line were also used in the evaluation, see table 3.4.

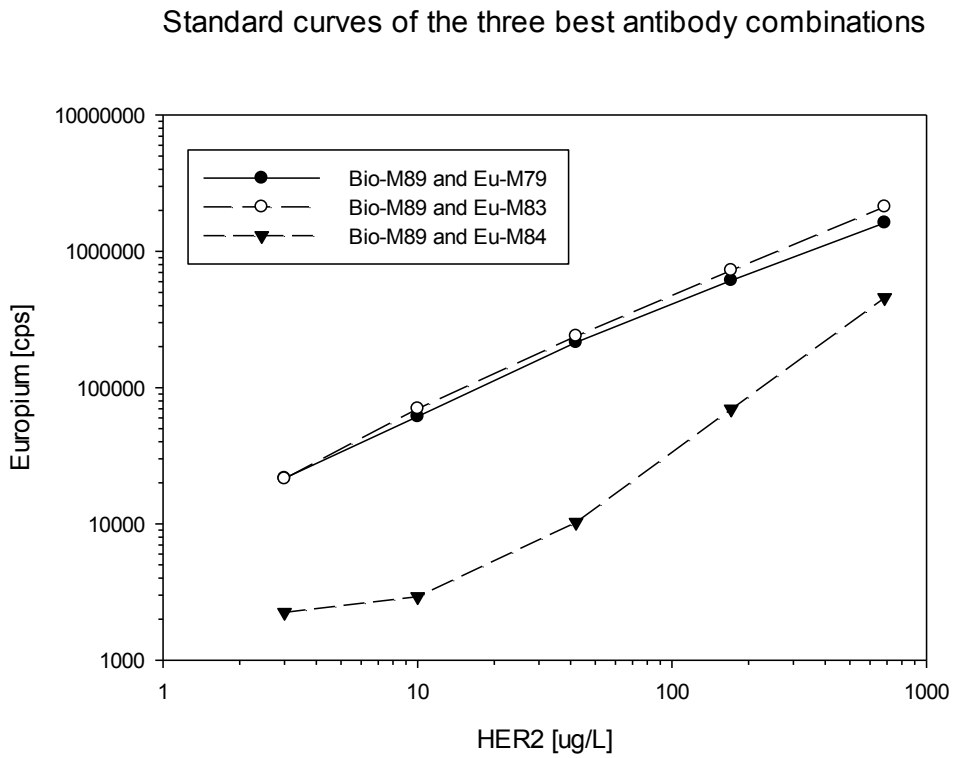

Figure 3.3: Standard curves for the three antibody combinations. Both axes of the figure are shown as logarithmic values.

The antibody combinations bio-M89 and Eu-E79, and bio-M89 and Eu-M83 were superior compared to bio-M89 and Eu-M84. Sample concentrations determined by the standard curves are shown in table 3.4. Reference values defined by measuring the samples in the Siemens ADVIA Centaur serum HER-2/neu immunoassay were performed prior to this thesis and are given in the table.

Table 3.4: A list of the sample concentrations (µg/L) for the samples tested with the three antibody combinations. Reference values from the Siemens assay are also included in the table.

Samples	HER2 [µg/L]			
	Bio-M89 and Eu-M79	Bio-M89 and Eu-M83	Bio-M89 and Eu-M84	Siemens reference values
Siemens Control 1	11,271	10,414	12,262	12,75
DNR Control 1	17,021	15,127	16,037	24,2
DNR Control 2	98,240	83,507	148,948	204,9
Cell line SK-BR3	14,040	6,178	11,947	11,4

To ensure that the best antibody combinations were chosen, the four next best antibody combinations from the results in figure 3.2 were also evaluated manually in immunofluorometric assays, with antibodies labelled with Eu^{3+} as reporter molecule. These results are only shown in Appendix 7.

3.6. The final antibody combination

The two different combinations bio-M89 and Eu-M79, and bio-M89 and Eu-M83 were re-tested using the AutoDELFIA instrument (figure 3.4), to ensure that the best antibody combination was chosen.

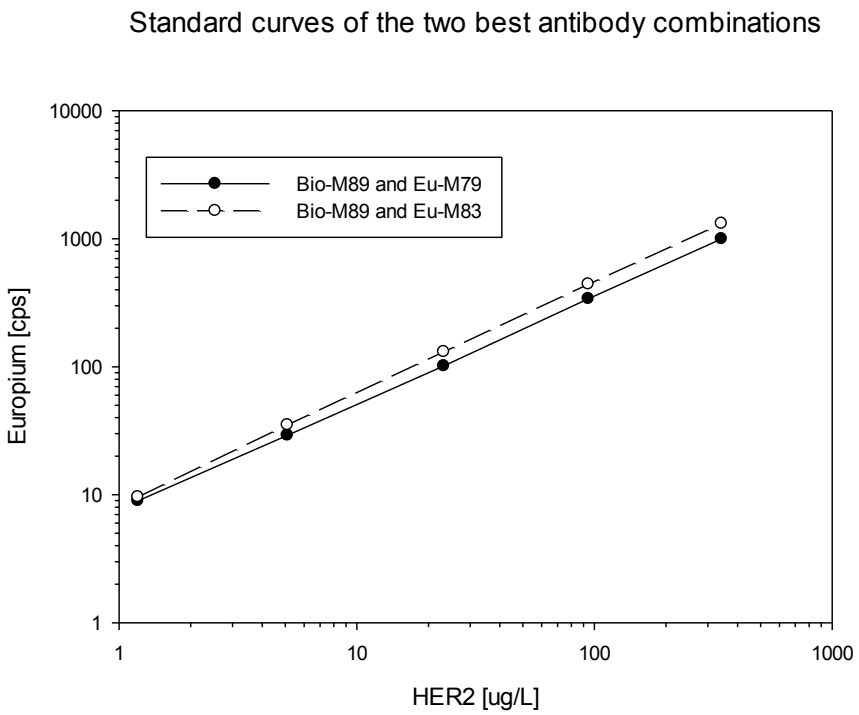

Figure 3.4: Standard curves of the two best antibody combinations. Both axes of the figure are shown as logarithmic values.

The assay combination bio-M89 and Eu-M83 gave generally higher europium signals than bio-M89 and Eu-M79. The sample concentrations determined by the standard curves from figure 3.4 are shown in table 3.5.

Table 3.5: The sample concentrations for the two best antibody combinations. Siemens reference values are also included in the table.

Samples	HER2 [µg/L]		
	Bio-M89 and Eu-M79	Bio-M89 and Eu-M83	Siemens reference values
Siemens Control 1	12,43	12,08	12,75
DNR Control 1	20,63	17,43	24,2
DNR Control 2	101,65	85,86	204,9
Cell line SK-BR3	7,03	7,38	11,4

Bio-M89 and Eu-M83 were chosen as the final antibody combination. The final immunoassay was further optimized.

3.7. Optimization of the immunoassay

The immunoassay optimization was divided into two parts, incubation periods and reagent concentrations. The different incubation periods were tested and determined first, and then the reagent concentrations were tested and determined. Standards A-F, Siemens controls, in-house controls and the cell line SK-BR3 were used in the optimization. Some of the control samples were not included in all of the optimization steps due to limited access.

Two incubation periods were evaluated. The first period was the incubation after the sample was added to the wells, which was the second incubation period in the assay. This period was tested for 30, 60 and 90 minutes incubations (figure 3.5a). The other incubation period was the incubation after the tracer antibody was added to the wells, which was the third incubation period in the assay. This period was tested for 30, 60, 90 and 120 minutes incubations (figure 3.5b).

Further optimization of the immunoassay for HER2 consisted of testing the reagent concentrations of biotin labelled $F(ab')_2$ fragment of the M89 antibody and europium labelled tracer antibody M83. 1 μg/ml, 2 μg/ml, 3 μg/ml and 4 μg/ml concentrations were tested for the solid phase $F(ab')_2$ fragments of M89, while 0,5 μg/ml, 1 μg/ml and 2 μg/ml concentrations were tested for the tracer antibody M83, see figure 3.5c and 3.5d respectively.

Several control samples were tested during the optimization and their concentrations were determined using the individual standard curves. These results, including reference values of the samples from the Siemens assay are presented in figure 3.6. Exact values of the sample concentrations are shown in table 6.6 – 6.9 in appendix 8.

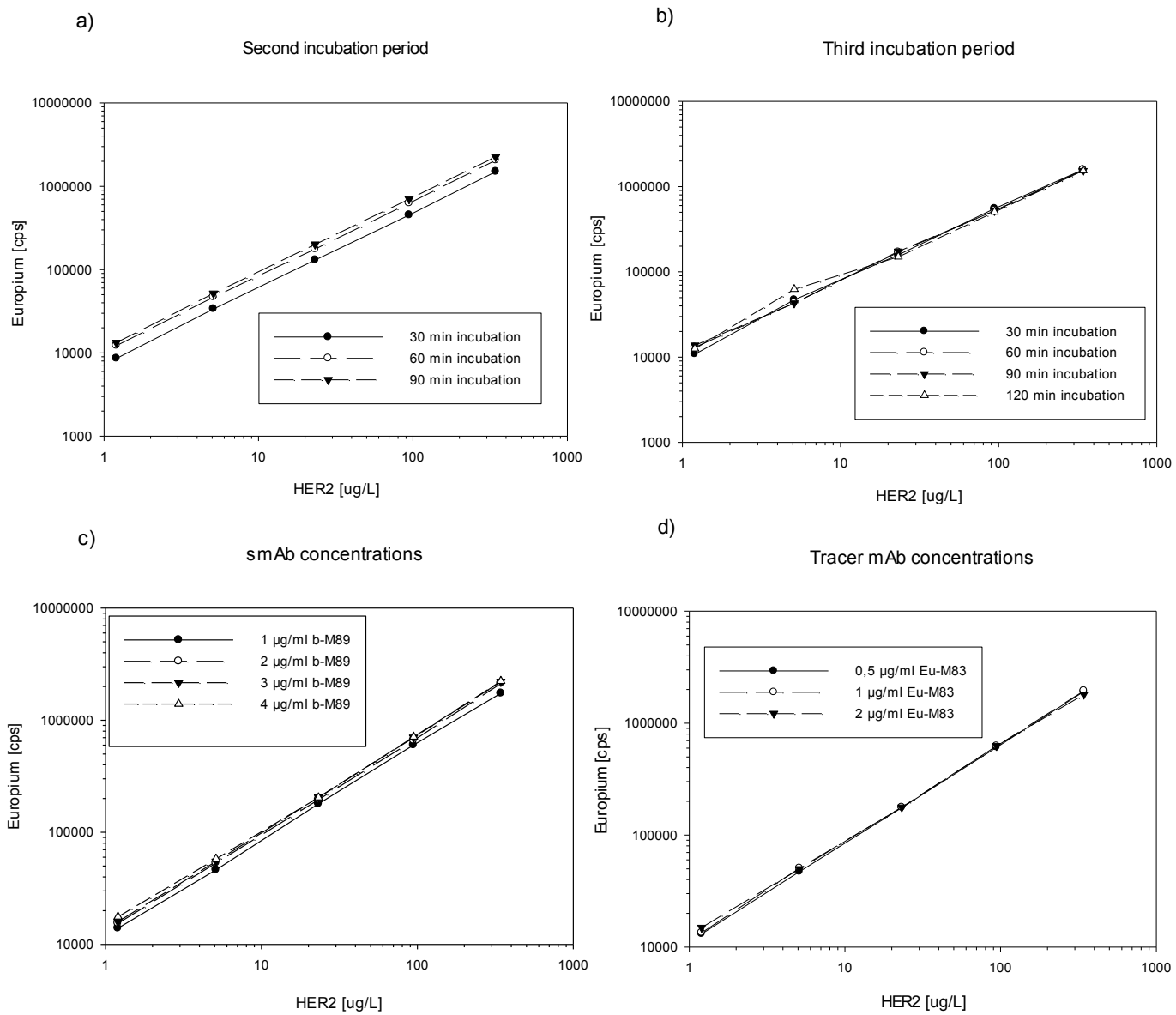

Figure 3.5: A presentation of the standard curves of the incubation periods and reagent concentrations tested during the optimization. All axes are presented as logarithmic values. The figures show a) the three standard curves for 30, 60 and 90 minutes incubations for the second incubation period, b) the standard curves for 30, 60, 90 and 120 minutes incubations for the third incubation period, c) the standard curves for 1 µg/ml, 2 µg/ml, 3 µg/ml and 4 µg/ml concentrations of the biotin labelled $F(ab')_2$ fragment of M89, and d) the standard curves for 0,5 µg/ml, 1 µg/ml and 2 µg/ml Eu-M83 tracer antibody.

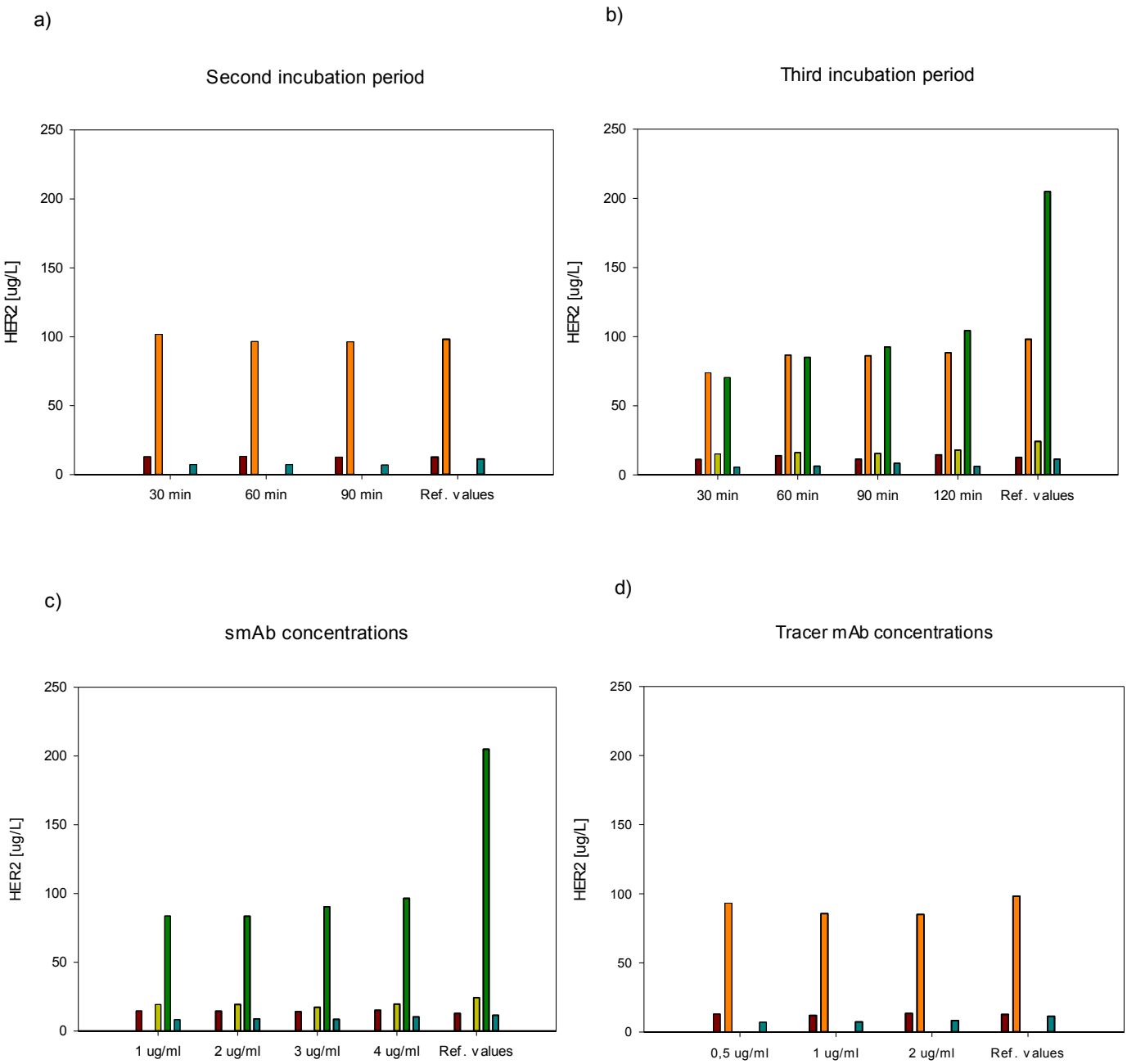

Figure 3.6: A presentation of the sample concentration results from the optimization of both incubation periods and reagent concentrations. The coloured bars show the different control samples; Red = Siemens control 1, orange = Siemens control 2, yellow = DNR control 1, green = DNR control 2, turquoise = SK-BR3. Siemens reference values of the samples are included to the right in each figure. The figures show a) control sample concentrations from the optimization of the second incubation period, b) control sample concentrations from the optimization of the third incubation period, c) control sample concentrations from the optimization of smAb concentrations, and d) control sample concentrations from the optimization of tracer mAb concentrations.

3.8. HER2 ECD in patient samples

The assay was standardized towards the Siemens ADVIA Centaur serum HER-2/neu immunoassay, meaning that the concentrations of the calibrators (standard A-F) were determined by measuring them as samples in the Siemens assay. This made it possible to compare the results from the final HER2 assay with values from the same patient samples tested on the Siemens assay.

15 patient samples were tested in the new HER2 assay and the commercial Siemens assay. The samples were from tissue HER2 positive breast cancer patients tested by immunohistochemistry. The results are shown in table 3.6. The table also includes the individual coefficients of variation (CV) of the samples in the new HER2 assay. A correlation curve of the 15 patient samples tested in the two assays is shown in figure 3.7.

Table 3.6: 15 patient samples tested in the Siemens assay and in the new HER2 assay, including the CV (%) of each sample tested in the new assay.

Siemens serum HER2 [µg/ml]	**DNR HER2 [µg/ml]**	**CV (%)**
12,1	4,83	0,9
15,1	6,27	1,3
30,7	26,46	2,4
17,3	13,96	0,1
2350,3	1116,41	0,4
48,7	15,99	3,5
10,5	3,67	2,2
74,8	78,98	1,5
38,4	33,32	5,1
145,3	128,53	0,4
18,5	14,85	5,3
280,6	223,99	1,3
19,7	16,09	2,2
15,7	7,29	1,9
649,8	395,18	1,5

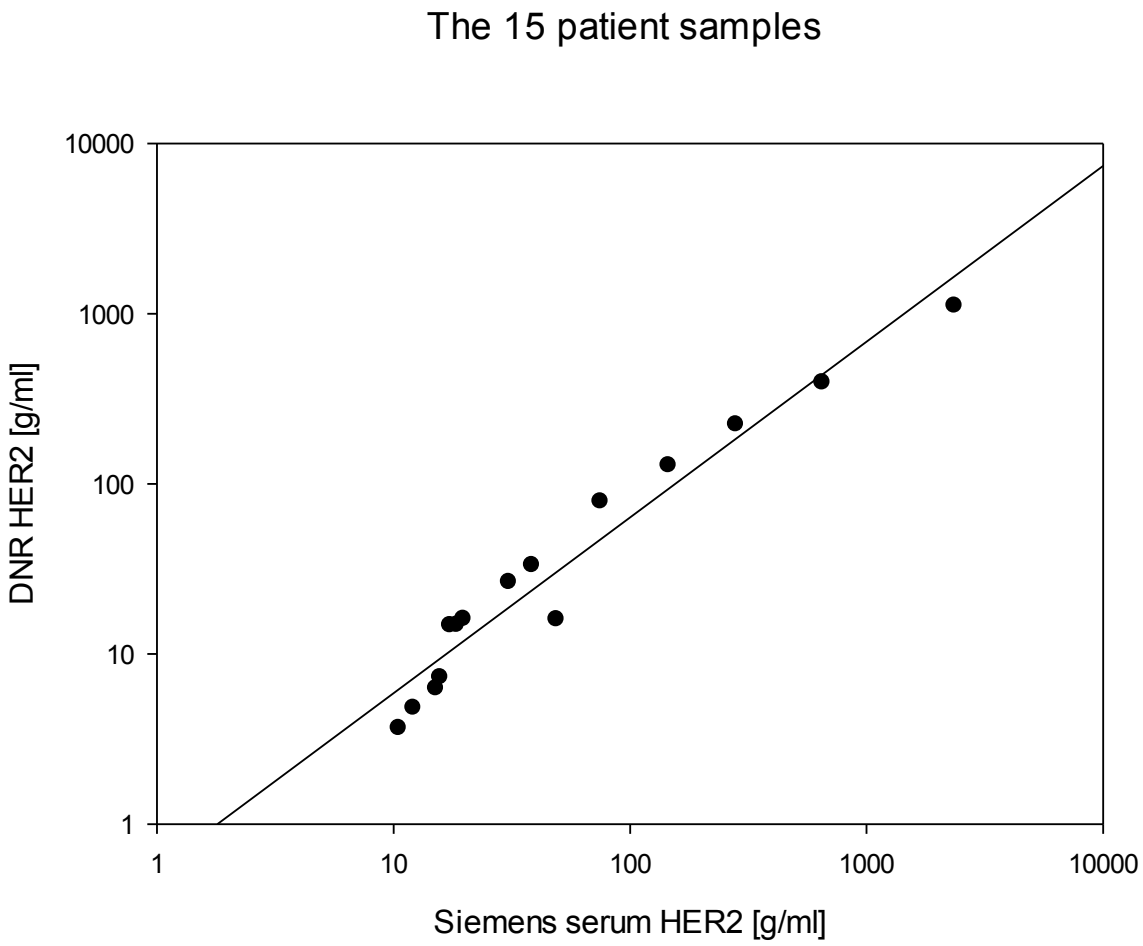

Figure 3.7: The correlation curve of the 15 patient samples, shown as the Siemens assay vs. the new HER2 assay.

4. Discussion

4.1. Characterization and evaluation of the antibodies for the development of an immunoassay for HER2 ECD

In this master thesis, the aim was to develop an immunometric assay using monoclonal antibodies specific for the extracellular domain of HER2. An advantage of immunometric assays is the use of mAbs in excess, favouring the formation of the antigen-antibody complex. This enhances the sensitivity and improves the assay kinetics. However, interference from heterophilic antibodies can be a challenge in immunometric assays, and it is critical that the final assay is protected against interference [23].

The six different antibodies towards HER2 were characterized by cross-inhibition and isotyping. Based on the cross-inhibition results, the antibodies were divided into three different groups (A, B and C), where group A was further subdivided into A1 and A2 (table 3.1). Antibody combinations that showed complete inhibition in the cross-inhibition test indicate a poor combination, where the antibodies most likely bind to the same epitope or neighbour epitopes on the HER2 antigen. The cross-inhibition tests also resulted in several antibody combinations with low percent inhibition, and thus make a good basis for constructing a sandwich assay. The isotyping of the mAbs resulted in a majority of IgG1 isotypes, except for mAb M83 that possessed IgG2a. All of the mAbs comprised kappa light chains. The isotyping result may be explained by the genetic background of the BALB/c mice for favouring the IgG1 isotype during the immune response towards the immunogen.

Combining the antibodies using recombinant HER2 as antigen was performed to find the best antibody combination. There were three antibody combinations that were significantly better than the others, and had almost twice as high binding to HER2 than the four next best combinations (figure 3.2). These three antibody combinations were therefore expected to have a low percent inhibition result from the cross-inhibition test. The three best mAb combinations, bio-M89 and ^{125}I-M79, bio-M89 and ^{125}I-M83, and bio-M89 and ^{125}I-M84, had only -1 %, 3 % and 5 % inhibition in the cross-inhibition test, respectively. Thus, the low percent inhibition result correlated with the results from the antibody combination test. When testing antibody combinations with two identical mAbs, a poor combination outcome is expected, since the antibodies contain similar paratopes that will bind to the same epitope on

the antigen. This corresponds to the results from the cross-inhibition testing, where every combination test with identical antibodies got almost 100 %. Antibodies in the other combinations that got almost 100 % inhibition, bind to the same epitope or to nearby epitopes. When the first (solid phase) antibody binds to the antigen, the antigen may undergo a conformational change due to the binding, which may result in non-overlapping binding sites for the second antibody and result in a lower percent inhibition value. This may explain the negative percent inhibition values.

The three best mAb combinations were further tested, using Eu^{3+} as tracer molecule, against a calibration curve with recombinant HER2 and against several control samples. The standard curves for the mAb combinations bio-M89 and Eu-M79, and bio-M89 and Eu-M83 were linear with high sensitivity, while the standard curve for bio-M89 and Eu-M84 was far from linear and had a lower sensitivity (figure 3.3). Bio-M89 and Eu-M84 was therefore excluded as a suitable antibody combination. The standard curve for bio-M89 and Eu-M83 had generally higher Eu^{3+} cps-values than bio-M89 and Eu-M79, and was therefore more sensitive. The control samples and the breast cancer cell line gave similar values in both combinations (Table 3.5). Thus, the combination of bio-M89 and Eu-M83 was chosen as the final assay combination because it had the highest sensitivity.

Europium was used as tracer molecule because of its superior sensitivity. The number of labelled europium molecules per antibody affects the sensitivity of the assay, where a good incorporation will increase the sensitivity. However, incorporation of too many europium molecules may change the protein structure and potentially affect the antibody's ability to bind to the antigen. An incorporation of 4-15 europium molecules per antibody is considered to be optimal (DELFIA Eu-labelling Reagent 1244-301 protocol, Wallac, Perkin Elmer life sciences). The antibodies labelled with Eu^{3+} had between 5,9 and 8,2 europium molecules per antibody (table 3.3), thus there were a good and comparable europium incorporation of the antibodies that enhances the sensitivity of the assay. The percent recovery for the europium labelled antibodies was between 70 – 99 %, which was as expected.

4.2. Optimization of the immunoassay for HER2 ECD

It was important to do an optimization of incubation periods and reagent concentrations for the immunometric assay for several reasons. The incubation lengths affect the efficiency of the assay. The incubation periods have to be long enough for complete reactions to occur, but too long incubations will make the assay less efficient. The antibody and reagent concentrations also affect the sensitivity of the assay. It is important that the assay has a high sensitivity, however, using more antibodies and reagents than necessary will only increase the costs.

The assay consisted of three incubation periods. The first incubation period was not optimized due to the instant binding between streptavidin and biotin ($K_D = 4 \times 10^{-14}$ M) and 30 minutes is enough time for the biotin and streptavidin to bind. The second incubation period was tested for 30, 60 and 90 minutes incubations. The resulting standard curves were good and linear for the three incubation tests (figure 3.5a). The standard curve for the 30 minutes incubation had considerably lower Eu^{3+} cps-values than incubation for 60 and 90 minutes, and was therefore excluded as an option. Incubation for 60 and 90 minutes gave almost similar Eu^{3+} cps values, where the standard curve for 90 minutes incubation had slightly higher Eu^{3+} cps. Although 90 minutes incubation time gave slightly better sensitivity than 60 minutes, 60 minutes incubation was chosen because the time saved was considered more valuable.

The third incubation period was tested for 30, 60, 90 and 120 minutes. The four resulting standard curves were rather similar and the signals for the highest calibrator were quite similar (figure 3.5b). The sample concentrations for the 30 minutes incubation were lower than for the other three. The incubations for 60 and 90 minutes resulted in somewhat similar concentrations, while 120 minutes incubation gave the highest results. However, even though 120 minutes incubations resulted in higher concentration values, the increase was not worth the longer assay duration, and thus 60 minutes was chosen as the best incubation time for the third incubation period.

Biotin labelled M89 $F(ab')_2$ fragment with 1, 2, 3 and 4 μg/ml concentrations were tested during the optimization of reagent concentrations (figure 3.5c). Due to the low Eu^{3+} -counts for the highest calibrator when using 1 μg/ml bio-M89, and no significant increase in Eu^{3+} -

counts using 3 μg/ml and 4 μg/ml concentrations, 2 μg/ml of biotin labelled M89 $F(ab')_2$ was chosen as the concentration of the solid phase antibody.

Concentrations of 0,5 μg/ml, 1,0 μg/ml and 2,0 μg/ml were tested for M83 tracer antibody and the respective standard curves appear almost as one joint line (figure 3.5d), indicating no difference between the tested concentrations. The control samples and cell line SK-BR3 concentrations were rather similar as well (figure 3.6d). 1 μg/ml europium labelled M83 is usually utilized [8], and 1 μg/ml tracer concentration was therefore chosen, since neither of the other two concentrations gave better results.

4.3. Protection of the assay against interference

There were several ways that the final assay was protected against interference. The M89 antibody utilized as solid phase in the final assay was fragmented using bromelain, and $F(ab)_2$ - fragments were used in the final assay. Using $F(ab)_2$ – fragments when testing patient samples is important, because it eliminates interference from heterophilic antibodies that are able to cross react with the assay by binding to the F_c – part of the antibodies. However, when testing antibody pair combinations and optimizing the best combination, it was not necessary to utilize $F(ab)_2$ –fragments, and the whole antibody was utilized during these tests. Aggregated immunoglobulin was added to the buffer to block interference from potential heterophilic antibodies that bind to other parts of the antibody, to protect the final assay when testing patient samples.

4.4. HER2 ECD in patient samples

The final developed assay had a high sensitivity and specificity towards HER2 ECD. The assay was further used to test 15 patient samples from patients that were HER2 positive by immunohistochemistry. It is very important that the assay is reproducible and stable. The individual CV values for the assay after testing the patient samples were between 0,1 and 5,3 %, indicating a good reproducibility within the assay.

The 15 patient samples were also tested in the Siemens assay. The values of the patient samples from the final HER2 assay was generally lower than for the Siemens assay (table 3.6), however, there was a good linear correlation between the sample values from the two assays (figure 3.7). From the patient results, it can be expected that the new assay will give lower values in serum samples from normal individuals compared to the Siemens assay. Thus, the discrepancy between the two assays may probably be explained by a lower cut-off value for the new assay compared to the cut-off value of 15 ng/ml for the Siemens assay. A sensitive immunoassay specific for the extracellular domain of HER2 was successfully developed.

4.5. Future perspectives

Before the new HER2 assay potentially can be used for follow-up of HER2 positive patients, a cut-off value for the assay must be determined using an appropriate number of samples from healthy people. In addition, a large study with HER2 positive patient samples must be performed to determine the clinical usefulness of the assay.

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6. Appendix

6.1. Appendix 1: Chemicals and proteins

Table 6.1 A list of the different chemicals utilized in the thesis, including the manufacturer with address, and catalogue number and degree of purity.

Chemical	Manufacturer	Product/catalogue number	Purity
Borate	Merck, 64271 Darmstadt Germany	Art. 165	99,8 %
Bovine serum albumin (BSA)	Sigma-Aldrich, St. Louis, USA	A4503	≥ 96 %
Bromelain, ID-Diluent 1	Bio Rad Laboratories, (DiaMed-ID), 1785 Cressier FR Switzerland		
Bromophenol blue	Chroma-gesellschaft, Münster, Germany	4F093	
Citric acid	Riedel-de Haën AG seelze hannover	33114	99,5 %
D-sorbitol	Sigma-Aldrich, St. Louis, USA	S1876	≥ 98 %
Diethylenetriamine pentaacetic acid (DTPA)	Sigma-Aldrich, St. Louis MO 6378 U.S.A.	D6518	≥ 99 %
Ethanol 96 %, Rektifisert sprit	Kemetyl Norge AS, Delitoppen 3, N-1540 Vestby	200-578-6	
Ethylene diamine tetra-acetate (EDTA)	Sigma-Aldrich Co, P.O. Box 14508 St. Louis, MO 63178 St. Louis, USA	E-5134	99 – 101 %

Ethylene glycol	Sigma-Aldrich, Co. 3050 Spruce Street, St. Louis, MO 63103 USA	03750	≥ 99,5 %
Europium p-isothiocyanatophenyl-EDTA complex	Kaivogen OY, Turku, Finland		
EZ-link Sulfo –NHS-LC Biotin	Thermo scientific	21327	
Germall	Allan Sjøstrand A/B		
Glycerol	Merck, 64271 Darmstadt Germany	K31116193 236	99-100 %
Glycine	Sigma-Aldrich Co, P.O. Box 14508 St. Louis, MO 63178 USA	G7126	≥ 99 %
Iodine-125	Hartmann analytic	185299	
Methiolat-Thimerosal	Sigma-Aldrich, St. Louis, USA	T5125	
Na_2HPO_4	Merck, 64271 Darmstadt Germany	A0110846 003	99 – 102 %
NaCl	Merck, 64271 Darmstadt Germany	K42436404 142	99,5 %
NaN3	Merck, 64271 Darmstadt Germany	106688 0250	> 99 %
NaOH	Merck, 64271 Darmstadt Germany	B676398 522	99 %
Sodium dodecyl sulphate (SDS)	Bethesda Research Laboratories, Life Technologies Inc. Gaithersburg, MD 20877 U.S.A.	5525UA	99,5 %

Tartrazine	Aldrich Chemical Co.Ltd. Gillingham, Dorset, England		
Tris	Sigma Life Technologies, Inc. Gaithersburg, MD 20898 USA	T1378	99 %
Tween 20	Serva, D-69115 Heidelberg Carl-Benz		
Tyrosine	Sigma-Aldrich, St. Louis, USA	T3754	≥ 98 %

6.2. Table with recombinant and native proteins.

Protein	**Manufacturer**	**Catalogue number**	**Purity**
Bovine IgG	Sigma-Aldrich, St. Louis, USA	G7516	≥ 97 %
E-64 protease inhibitor	Roche, Indianapolis, USA	10874523001	
HER2	Sino Biological Inc, B-212 Zhonghe Street, BDA Beijing 100176	10004-H08H	97 %
MAK 33	Roche, Indianapolis, USA	11939661	

6.2. Appendix 2: Fragmentation of antibodies to $F(ab')_2$

Calculations for the fragmentation of the M89 antibody sample:

Calculating the concentration and amount of antibody:

Volume of sample with antibody= 11,77 ml.

$A_{280\ nm}$ = 2,53

$A_{280\ nm}$/the extinction coefficient = 2,53/1,43 = 1,8 mg/ml

1,8 mg/ml * 11,77 ml = 20,8 mg

Calculation of the bromelain volume added:

mg monoclonal antibody = ml bromelain

3 * 20

Since the bromelain concentration is 3 mg/ml, and the ratio between antibody and bromelain should be 1:20 in terms of weight, 20 needed to be multiplied with 3.

20,8 mg = 0,347 ml

3*20

6.3. Appendix 3: Calculation of the number of labelled Eu^{3+} per antibody and percent recovery

A calculation example for antibody M79:

Eu^{3+} (µmol/l) = (Eu^{3+} signal for M79)/ (Eu^{3+} signal for standard (1nmol/l) * 1000)

Eu^{3+} (µmol/l) = (2118966 * 10^4)/ (1,06 * 10^6 *10^3)

Eu^{3+} (µmol/l) = 20

M79 (mg/ml) = (A_{280} – 0,008 * Eu^{3+} (µmol/l)) / extinction coefficient

M79 (mg/ml) = (0,944 – 0,16) / 1,43

M79 (mg/ml) = 0,548

M79 (µmol/l) = (M79 (mg/ml) * 10^6) / 160 000 (g/l)

M79 (µmol/l) = (0,548 * 10^6) / 160 000

M79 (µmol/l) = 3,4

Eu^{3+} per M79 = Eu^{3+} (µmol/l) / M79 (µmol/l)

Eu^{3+} per M79 = 20 / 3,4

Eu^{3+} per M79 = 5,9

Recovery (%) = M79 recovered (mg) * 100 % / M79 added (mg)

Recovery (%) = (1,096 * 100 %) / 1,4

Recovery (%) = 78 %

6.4. Appendix 4: Calculations during the coating of plates with different antibodies

Table 6.3: The table gives an overview of the antibodies with their respective concentrations.

	Antibodies					
	M75	M77	M79	M83	M84	M89
Concentration mg/ml	1,44	1,06	0,92	1,25	1,56	1,8

Amount and volume of the antibody per well: 1 µg/well and 200 µl/well.

Total volume per plate:

0,2 ml/well * 100 wells/plate * 1 plate = 20 ml

There were 96 wells per plate and this number was rounded up to 100.

Total amount of antibody per plate:

1 µg/well * 100 wells/plate * 1 plate = 100 µg = 0,1 mg

Calculation example for the dilution of the M75 antibody:

$\frac{0{,}1 \text{ mg}}{1{,}44 \text{ mg/ml}}$ = 0,069 ml = 69 µl

69 µl M75 was diluted in 20 ml buffer.

6.5. Appendix 5: Cross-inhibition of HER2 mAbs

50 µl of iodine labelled recombinant HER2 measured 11 047 cpm which is ~ 10 000 cpm. The antibodies were diluted to 100 µg/ml in PBS with 0,3 % BSA. A total volume of 1 ml = 1000 µl.

A calculation example of this dilution:

M75: 1,44 mg/ml = 1440 µg/ml

1440 µg/ml / 100 µg/ml = 14,4

1000 µl / 14,4 = 69,4 µl M75

69,4 µl M75 was added to PBS with 0,3 % BSA to a total volume of 1 ml.

6.6. Appendix 6: Table of all the antibody combinations

Table 6.4: The table presents the results from the antibody combination test, where the three best antibody combinations are marked in bold. PBS with 0,3 % BSA was used as a control, while HER2 100 µg/L indicates how good the antibody combination was.

smAb		Iodine labelled monoclonal antibodies					
		M75	M77	M79	M83	M84	M89
M75	PBS with 0,3 % BSA	0,1	0,1	1,3	0,7	1,9	0,1
	HER2 100 µg/L	0,8	0,5	13,7	11,5	52,8	4,0
M77	PBS with 0,3 % BSA	0,1	0,1	1,0	0,5	0,2	0,1
	HER2 100 µg/L	1,7	0,1	21,0	16,4	10,7	0,4
M79	PBS with 0,3 % BSA	0,3	0,2	0,6	0,2	1,7	0,1
	HER2 100 µg/L	20,6	19,2	1,6	0,7	40,2	44,3
M83	PBS with 0,3 % BSA	0,3	0,1	0,7	0,4	1,3	0,1
	HER2 100 µg/L	19,6	16,5	1,7	0,8	39,5	46,1
M84	PBS with 0,3 % BSA	0,2	0,1	3,6	2,2	0,1	0,1
	HER2 100 µg/L	7,1	4,6	14,1	17,5	0,4	16,0
M89	PBS with 0,3 % BSA	0,1	0,1	**0,5**	**0,3**	**0,1**	0,1
	HER2 100 µg/L	12,1	0,1	**78,9**	**84,7**	**78,4**	1,2

6.7. Appendix 7: A table with the results from the evaluation of the four next best antibody combinations

Table 6.5: The four next best antibody combinations. The results are shown as Eu^{3+} [cps].

Standard	B-M75 and Eu-M84	B-M79 and Eu-M84	B-M79 and Eu-M89	B-M83 and Eu-M84
A	3579	1830	2637	3328
B	2685	2295	17352	1775
C	4299	2809	71610	3184
D	16688	11254	208754	16059
E	106282	82146	652956	91956
F	303501	444164	1532629	638524

6.8. Appendix 8: Tables with the exact values of the results from the optimization of the final assay

Table 6.6: The table presents the sample concentration results after testing 30, 60 and 90 minutes incubations for the second incubation period and reference values from the Siemens assay.

Samples	Concentration (µg/L)			
	30 minutes incubation	60 minutes incubation	90 minutes incubation	Siemens reference values
Siemens Control 1	13,04	13,29	12,80	12,75
Siemens Control 2	101,78	96,61	96,32	98,2
Cell line SK-BR3	7,39	7,31	7,04	11,4

Table 6.7: An overview of the concentrations for the control samples tested during the optimization of the third incubation period. Reference values from the Siemens assay are also given in the table.

Samples	Concentration (µg/L)				
	30 minutes incubation	60 minutes incubation	90 minutes incubation	120 minutes incubation	Siemens reference values
DNR Control 1	15,21	16,11	15,56	18,00	24,2
DNR Control 2	70,40	85,07	92,59	104,37	204,9
Siemens Control 1	11,41	14,00	11,52	14,61	12,75
Siemens Control 2	73,92	86,78	86,27	88,38	98,2
Cell line SK-BR3	5,66	6,35	8,49	6,24	11,4

Table 6.8: The table gives an overview of the sample concentrations after testing different concentrations of the solid phase, and reference values from the Siemens assay.

Samples	Concentration (µg/L)				
	1 µg/ml solid phase Ab	2 µg/ml solid phase Ab	3 µg/ml solid phase Ab	4 µg/ml solid phase Ab	Siemens reference values
Siemens Control 1	14,66	14,43	14,04	15,09	12,75
DNR Control 1	19,24	19,26	17,24	19,61	24,2
DNR Control 2	83,71	83,45	90,35	96,55	204,9
Cell line SK-BR3	8,32	8,86	8,48	10,24	11,4

Table 6.9: An overview of the sample concentrations after testing 0,5 µg/ml, 1 µg/ml and 2 µg/ml concentrations of europium labelled M83 and Siemens reference values.

Samples	Concentration (µg/L)			
	0,5 µg/ml tracer Ab	1 µg/ml tracer Ab	2 µg/ml tracer Ab	Siemens reference values
Siemens Control 1	13,01	12,00	13,41	12,75
Siemens Control 2	93,25	85,62	85,00	98,2
Cell line SK-BR3	7,11	7,29	8,30	11,4

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