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Affinity and capacity studies in two different test systems for human IgE

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Abstract

The clinical diagnostics company Phadia AB develops and sells two *in vitro* allergy test platforms, ImmunoCAP® System and ImmunoCAP® Rapid. The test systems are principally very similar but differ in many practical aspects. Therefore, the aim of this study was to investigate and compare the capacity of these two test systems to bind specific antibodies and to see how the test results were affected by the antibody's binding affinity to the antigen.

Keywords

Immunoassays, Human Immunoglobulin E, Antibody affinity

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Populärvetenskaplig sammanfattning

Astma och allergi är sjukdomar som har ökat markant i de industrialiserade länderna de senaste årtiondena. Allergi orsakas av att speciella molekyler i kroppens immunförsvar, s.k. antikroppar, reagerar på ämnen (allergen) som egentligen inte är farliga för människor. Det kliniska diagnostikföretaget Phadia AB utvecklar och säljer två olika allergitestsystem, ImmunoCAP® System och ImmunoCAP® Rapid, som båda, via analys av blodprov kan upptäcka om en person har antikroppar som orsakar allergi mot ett givet ämne. De båda systemen är principiellt lika, med allergen fästa på en yta till vilka antikroppar från patientprovet kan binda in om patienten är allergisk mot något av de testade allergenen. Många praktiska detaljer skiljer dock de båda testsystemen åt. Främst kan nämnas att i ImmunoCAP® System interagerar antikropparna med allergenen i en mer eller mindre statisk miljö, medan de i ImmunoCAP® Rapid vandrar i ett flöde förbi de fastsatta allergenen.

Syftet med detta projekt har varit att undersöka och jämföra ImmunoCAP® System och ImmunoCAP® Rapid med avseende på deras förmåga att binda antikroppar och se hur testresultaten påverkas av antikroppens affinitet för allergenet.

Studien visade att även om affinitetsskillnader mellan testsystemen finns är de små. Båda testsystemen har en mycket god förmåga att upptäcka även antikroppar med låg affinitet för allergen.

Examensarbete 20 p
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Table of contents

1. Introduction

1.1 Problem

The prevalence of allergies has increased in the western world during the last decades. Even though effective medication exists, it is always best to try to avoid exposure to the allergen causing the allergic reaction. To be able to do this, these allergens must be identified. This is done through clinical evaluation and diagnostic testing of the patient. The predominant diagnostic tests are skin prick testing (SPT) and *in vitro* blood sample tests, the latter measuring levels of allergen-specific IgE antibodies in the patient's serum or plasma.

The clinical diagnostics company Phadia AB, with headquarters in Uppsala, develops and sells two *in vitro* allergy test platforms, ImmunoCAP® System and ImmunoCAP® Rapid. The ImmunoCAP technology was introduced in 1989 and is today used in more than 3000 laboratories worldwide. ImmunoCAP Rapid was introduced in 2005 and is, as the name implies, a quick-test, giving the results in less than 30 minutes. The test systems are principally very similar but differ in many practical aspects, such as the matrices ability to bind the allergens and the reaction times for antibody-antigen and detection reagent-antibody interactions. In addition, ImmunoCAP System is fully automated, while ImmunoCAP Rapid is performed manually in a device. These differences imply that ImmunoCAP System and ImmunoCAP Rapid might differ in their IgE-binding capacities and therefore yield different results regarding amount of specific IgE antibodies in a test sample.

1.2 Aim of the study

The aim of the study was to investigate the capacity of ImmunoCAP System and ImmunoCAP Rapid to bind specific antibodies and to see how the test results were affected by the antibody's binding affinity to the antigen.

2. Background

2.1 History

The first known case of a possible allergic reaction is that of King Menes of Memphis, the first pharaoh of the first Egyptian dynasty, who might have been killed by a wasp sting followed by anaphylaxis [1]. More recent is the first reported case of pollen-related hay fever; Dr. John Bostock described in 1819 how he during the pollen season suffered from rhinoconjunctivitis and asthma. His observations led to knowledge of the connection between pollen levels in the air and the severity of allergic symptoms. The disease mechanisms though, were not understood for yet another 100 years, when Dr. Ramirez in 1919 found that allergy could be transmitted through blood transfusion. This led to the search for the allergy causing component, called the reagin at the time, in the blood. As with many revolutionary scientific discoveries this was found more or less by chance by a couple of scientists in Uppsala. Hans Bennich and S.G.O. Johansson were studying the biochemical properties of the human immunoglobulins when they found a previously unknown type. Together with findings of other scientists specialized in allergy research (primarily K. and T. Ishizaka, who had purified reagins from serum of allergic patients) they could demonstrate that this immunoglobulin was the factor in human blood, responsible for mediating allergy. They called it immunoglobulin E or IgE for short [2].

2.2 Definition

The term “allergy” is often used to describe any negative reaction to a substance that would normally be tolerated by the body. But there are clinical definitions to what allergy really is. Allergy is defined as a “hypersensitivity caused by exposure to a particular antigen (allergen) resulting in a marked increase in reactivity to that antigen on subsequent exposure, sometimes resulting in harmful immunologic consequences” [3], and is usually classified into four different types. Type 1 allergy is mediated by IgE-molecules. A term often mentioned when talking about type 1 allergy is “atopy”, which can be defined as “a genetically determined state of hypersensitivity to environmental allergens”, i.e. having IgE-antibodies specific for at

least one allergen. Type 2 and 3 allergies are mediated by IgG or sometimes IgM and type 4 allergy is mediated by antigen-specific T_H1-cells and activated macrophages [4]. For this report, the type 1 allergy reaction will be the only one discussed.

2.3 IgE-mediated allergy

The type 1 allergic reaction or immediate hypersensitivity reaction is mediated by the IgE molecule. It is one of the most powerful pathologic reactions of the human immune system and involves many mediators. Upon introduction of an allergen into the body, dendritic cells bind the allergen and migrate to lymph nodes and the spleen. There they present allergen derived peptides to naïve T_H-cells, which directs the T_H-cells to differentiate into T_H2-cells. When interacting with B-cells expressing peptides for the specific allergen on MHC class II receptors, the T_H2-cells start to secret cytokines. This induces immunoglobulin switch, leading to production of IgE antibodies specific for the allergen in question. The newly formed IgE molecules bind to so called Fc-receptors on mast cells. This step is called sensitization. Sensitized mast cells secret mediators of various types when the IgE antibodies on their surface are cross-linked by the allergen. These mediators, including histamines and cytokines, are responsible for many of the clinical manifestations typical of the type 1 allergic reaction. (Figure 1) [5]

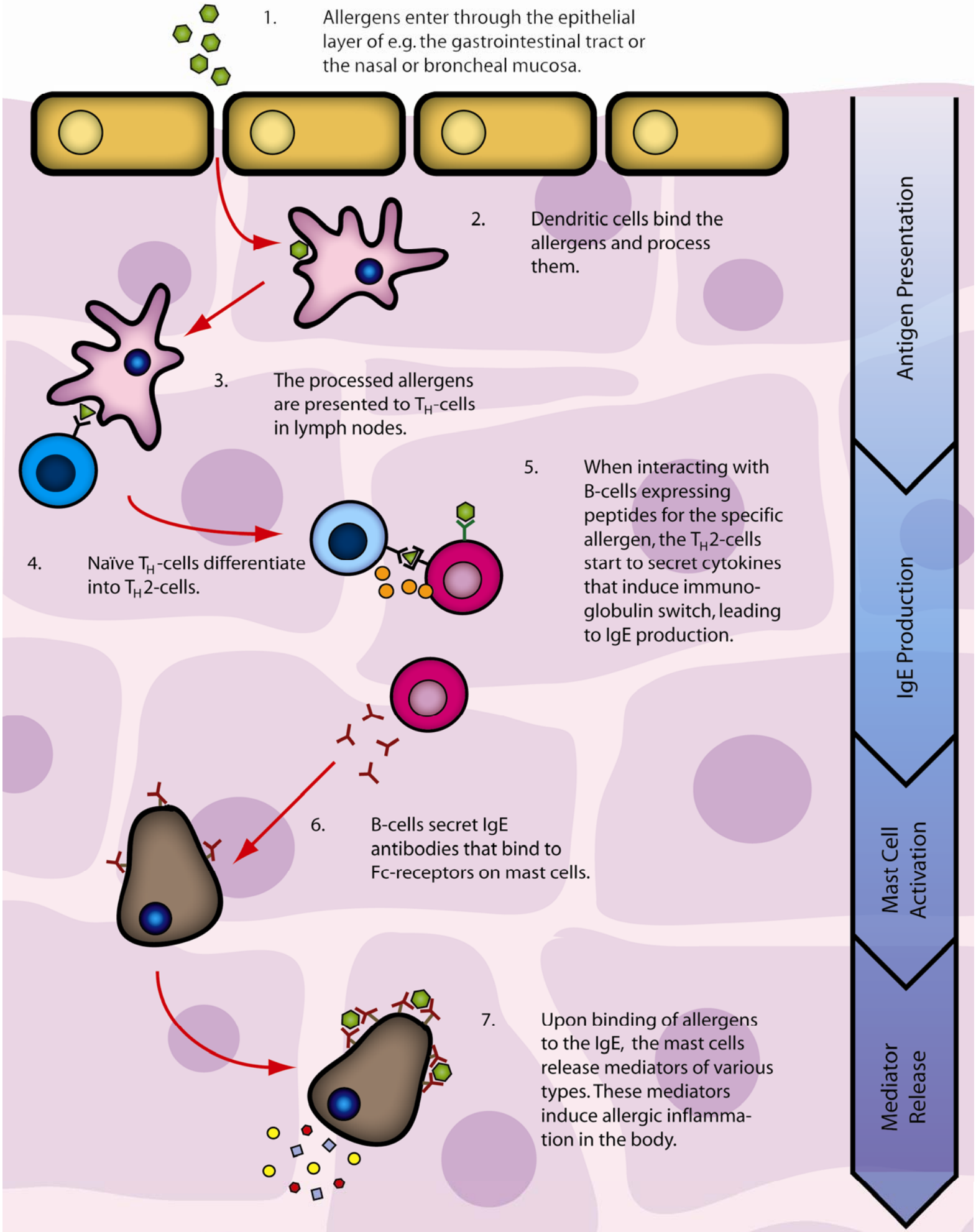

Figure 1. A schematic view of the type 1 IgE-mediated immune response.

2.4 Allergy today

A few decades ago allergy was rather unusual but the prevalence of allergic diseases, especially in children, has tripled or even quadrupled in many industrialized countries in the past 20-30 years [6]. Today 20-40% of the population in western countries develop allergy at some point in life [7, 8]. In 2003 allergic diseases were the most common chronic disorders among children in the member countries of the European Union [9]. In a study of the prevalence of allergic rhinitis in six western European countries, 23% of the subjects had clinically confirmed allergic rhinitis [10]. The prevalence of asthma has increased rapidly and is today one of the most common chronic diseases in the world with around 300 million people affected. If the increase continues as projected there will be another 100 million asthmatics by 2025 [11]. This increase of allergic diseases has in many studies been explained to be dependent on lifestyle and environment, where bad indoor climate, in the form of for example tobacco smoke and dampness, and various outdoors air pollutants, like car exhaust, increase the risk for developing allergies [12-14]. Other studies suggest that the lack of exposure to infections in childhood might lead to increased risk of allergy [15], which could explain why more westernized countries have higher prevalence of allergy [6, 16, 17].

2.5 Diagnostics

The basis for all immunochemical techniques is the existence of the highly sophisticated and sensitive immune system of higher animals. The word immunology is derived from the latin word *immunitas*, meaning “freedom from”, and to combat infectious diseases of ever growing diversity evolution has produced molecules with high specificity for their targets. The most prominent and advanced of these are the immunoglobulins, often referred to as antibodies. The five known classes of immunoglobulins, IgA, IgD, IgG, IgE and IgM, can all bind antigens but have different biological functions [18]. IgG is the most prevalent and important of the immunoglobulins, carrying out numerous tasks in the systemic immune response. The IgA is part of local immune responses and is secreted into saliva, tears and other mucous secretions. IgD is mainly a B-cell receptor that binds antigens as is IgM. IgM is also the first immunoglobulin to appear in an immune response and it is the evolutionary oldest of the immunoglobulins. The IgE is involved in parasitic defence and immediate hypersensitivity [5, 19]. Common to all five classes of immunoglobulins is the molecular structure of the main units, with two heavy and two light chains formed in the shape of the letter Y (Figure 2). The

heavy chains consist of three (for IgA, D, G) or four (IgE, M) constant domains and one variable domain. The light chains consist of one constant and one variable domain. The constant regions assure correct three-dimensional structure for interaction with cells and cell receptors within the body, while the variable domains are responsible for binding antigen. The high sequence variability within the antigen binding parts of the variable domains gives humans the ability to produce a very large number of different antibodies to combat a diverse set of infectious antigens. In fact theoretical calculations have suggested that humans have the ability to produce 10^8 different antibodies. This ability for high diversity in mammalian antibodies gives immunochemists a powerful and sophisticated tool. [5, 18]

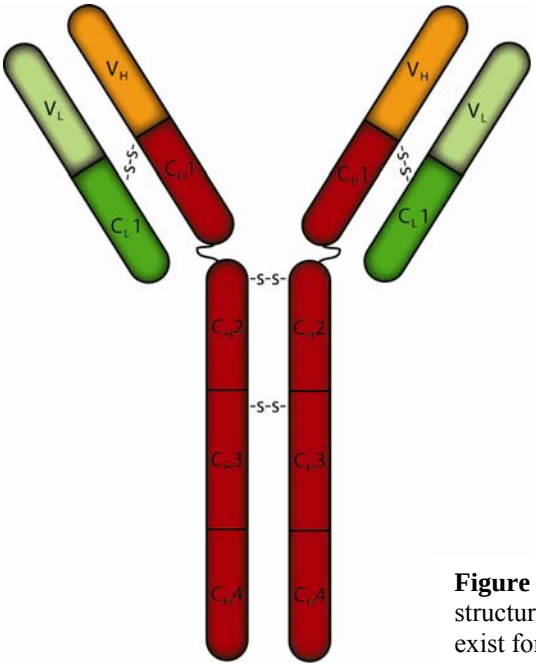

Figure 2. Immunoglobulin structure. The C_H4-regions only exist for IgE and IgM.

2.5.1 Antibody production

Each individual B-cell produces antibodies with a single immunoglobulin sequence. A population of identical B-cells (i.e. clones) will give rise to a set of monoclonal antibodies, i.e. antibodies with identical specificity. If the population of B-cells consists of non-identical B-cells, the resulting antibody yield will be of polyclonal nature, meaning antibodies with different specificities. To produce polyclonal antibodies, animals such as mice, rats, rabbits or sheep etc. are immunized with the antigen in question which makes the host animal produce antibodies against that antigen. When monoclonal antibodies are desired it is common to use so called hybridoma cells to produce them. Hybridoma cells are created by fusing B-

lymphocytes from an immunized animal (in principle mouse, or less common, rat) with *in vitro* grown myeloma cells (not producing antibodies) from the same species. The lymphocytes alone could not survive long *in vitro*, but by fusing the two cell types a cell line with the ability to both produce antibodies and grow continuously is created. The hybridoma cells are then cloned to yield cultures of hybridomas producing monoclonal antibodies.

2.5.2 Antibody labelling

To make antibodies or antibody-bound complexes visible in immunoassays, the molecules are labelled. Labelling can be done in various ways and with various labels. Radiolabelling involves attaching radioactive isotopes to the antibodies and labelling with fluorochromes means that fluorescent molecules are bound to the antibodies. Enzymatic labelling of antibodies is widely used in immunoassays and relies on enzymes' capability to convert substrate molecules into a coloured product. A special high affinity labelling technique called biotinylation is based on the interaction between biotin (vitamin B7) and the bacterial protein streptavidin from *Streptomyces avidinii*.

The preferred label depends on the interaction studied and which technique one wants to employ for detection.

2.5.3 Immunoassays

There are many ways to immunochemically determine the concentration of an analyte in a solution, three general principles can be discerned though; competitive binding immunoassays, immunometric assays and solid-phase immunobinding assays.

Competitive binding immunoassays

In competitive binding immunoassays antigen in the sample solution competes with a known and fixed amount of labelled antigen for binding to a limiting amount of antibody. When equilibrium is reached the bound antigen is separated from the unbound. The amount of labelled antibody-bound antigen is measured and the concentration of antigen in the sample is inversely proportional to this value. By setting up a dilution series of antigen samples of known concentrations, a standard curve can be created from which sample antigen concentrations can be derived.

Immunometric assays

In immunometric assays antigens react with antibodies that have been immobilized on e.g. the wall of the wells of a microtitre plate. In the next step an excess of labelled antibody is allowed to react with the antibody-antigen complexes. Unbound labelled antibody is then washed away and the signal from the bound labelled antibodies is measured, indicating the concentration of antigen in the sample.

Solid-phase immunobinding assays

Solid-phase immunobinding assays use immobilized antigen to measure the antibody concentration of a sample. It is in other words an inverse variant of the immunometric assay principle. Most often the antibody concentration is detected using enzyme-labelled anti-immunoglobulin. The use of enzymes as labels has given the tests (at least those performed using microtitre plates) the name Enzyme-Linked ImmunoSorbent Assays (ELISAs).

2.6 The ImmunoCAP® Technology

2.6.1 ImmunoCAP® System

The ImmunoCAP System is a solid-phase immunoassay, i.e. it uses immobilized antigen to measure the antibody concentration of a sample. The solid phase of ImmunoCAP consists of a three-dimensional cellulose polymer, with the allergens covalently coupled in excess to the polymer. The cellulose structure provides an extremely large inner surface, allowing the coupling of a high amount of allergen material. This cellulose polymer is placed in a plastic cap with openings in the bottom to allow liquid flow-through (Figure 3). When conducting a test run, patient sample is applied to the cellulose polymer. If IgE antibodies specific for the bound allergen exists in the sample, it will bind. A following wash removes unbound IgE. Subsequently, conjugate in the form of enzyme-labelled anti-IgE antibodies is added and forms complexes with the bound IgE. After incubation, the unbound enzyme-antibody conjugate is washed away and the bound complexes are incubated with a developing solution. The enzyme reaction is stopped by addition of a stopping solution and the reaction solution is eluted. The fluorescence of the eluate is then correlated to the specific IgE antibody concentration in the sample by comparison with a calibrator curve [20]. For a schematic view of the procedure, please consult Appendix B. The assays are performed by fully automatic

instruments. There are three different types of ImmunoCAP instruments: ImmunoCAP 100, which has been developed for smaller laboratories, ImmunoCAP 250 for middle-size laboratories and ImmunoCAP 1000 for the largest laboratories.

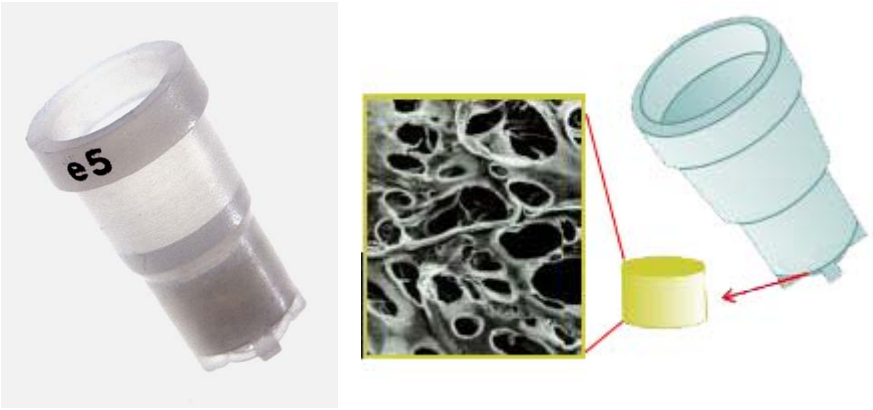

Figure 3.
Left: An e5 ImmunoCAP (dog dander allergen extract)
Right: An ImmunoCAP with the solid phase disc displayed and enlarged. (Figure shown with permission from Phadia AB)

2.6.2 ImmunoCAP® Rapid

ImmunoCAP® Rapid is a lateral flow assay, meaning that the sample and the reagents interact while flowing over the test strip. The allergens are coated as lines on two parallel nitrocellulose membrane strips. When sample is applied at one end it flows towards a sucking membrane (called wick) at the opposite end of the device and specific IgE antibodies in the sample bind to the corresponding allergen. When a developer solution then is added, it releases anti-IgE antibody-conjugated gold particles that bind to the allergen-bound IgE generating a complex that is visualized as red colour on the test strip. Unbound or unspecific bound IgE and gold conjugate are then washed away by the developer solution. Control lines at the end of the strips bind the gold conjugate and assure that the test has worked (Figure 4).

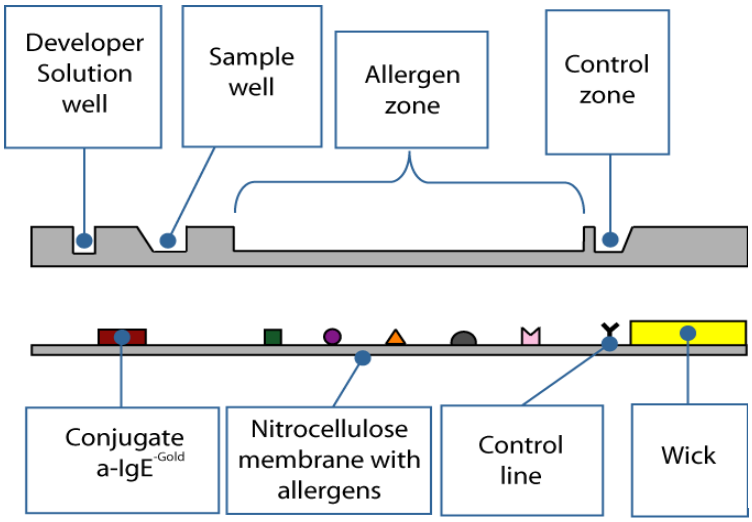

Figure 4. Schematic view of the ImmunoCAP Rapid test device. The upper picture describes the different zones of the test, while the lower picture shows the internal composition and reagents. (Figure shown with permission from Phadia AB)

2.7 The Biacore technology

The Biacore technology is based on the theory of Surface Plasmon Resonance (SPR). This theory is based on the fact that some of the light from a ray of light that passes through a material with relatively high refractive index, such as glass, into a material with lower refractive index, like water, will be reflected from the medium interface. If the angle of incidence, θ_i, (i.e. the angle at which the incident ray of light hits the surface) is greater than a certain critical angle, θ_c, all light will be reflected. This phenomenon is called total internal reflection and the θ_c depends on the refractive indices of the two media involved as given in equation 1,

$$\theta_c = \arcsin\left(\frac{n_2}{n_1}\right) \qquad \textbf{Equation 1}$$

where n_2 is the refractive index of the less dense medium and n_1 is the refractive index of the denser medium. In our case with glass and water, θ_c would be about 62°. No light passes through but light energy can be transferred over the interface and if the glass is coated with a noble metal like gold this energy will enter the electron cloud of the metal. At a certain angle called the surface plasmon resonance angle, θ_{spr}, this energy transfer reaches its maximum hence leading to that the intensity of the internally reflected light reaches a minimum. This angle, which always is greater than the critical angle of total internal reflection ($\theta_{spr} > \theta_c$), depends on the refractive index of the medium. If this gold surface is immersed in an aqueous solution, in which protein is present and can bind to the surface, the refractive index and hence the θ_{spr} will change depending on the amount of bound protein [21, 22].

In the Biacore instrument the scenario just described takes place. A beam of light is sent towards a gold plated sensor chip immersed in a flow channel. A layer of thiolalkane is covalently bound to the gold and coupled to the thiolalkane is a layer of carboxymethylated dextran, which is a polysaccharide consisting of glucose monomers. Ligands are bound to the dextran and as sample molecules bind the ligands the θ_{spr} changes, which a photo sensor detects. The signal is then translated to concentration of bound sample (Figures 5a and 5b).

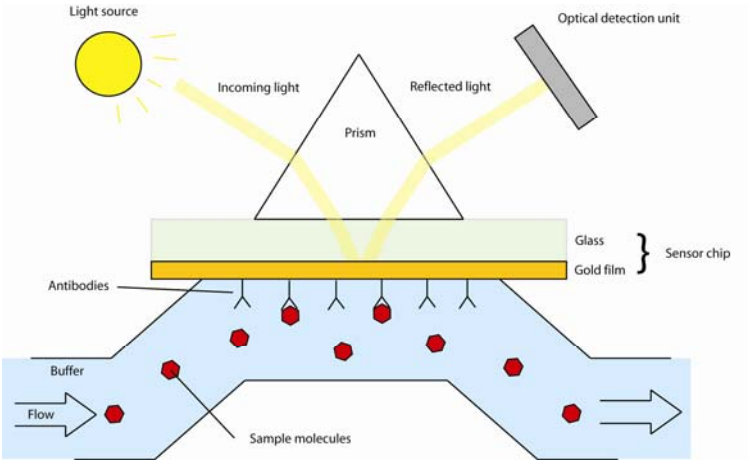

Figure 5a. Biacore sensor cell. Sample molecules travel in the flow channel and affect the beam of reflected light upon binding ligands on the sensor chip surface.

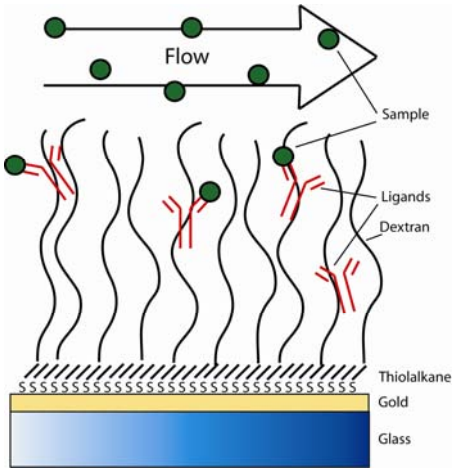

Figure 5b. Biacore sensor chip. Dextran is bound to the sensor chip and sample-binding ligands are coupled to the dextran.

The detected response is measured in resonance units (RU) and a response of 1000 RU corresponds to a change in bound sample of 1 ng/mm^2. Since the matrix is ~100 nm thick, this represents a sample concentration within the matrix of 10 mg/ml [21,22].

In a typical Biacore run analyte, (in this study antigen), is injected and allowed to associate with the ligands, (in this study monoclonal antibodies), of the sensor chip for a preset amount of time. The instrument registers how much antigen has bound and how fast it was bound by the antibodies and from this the association rate constant, k_a, can be calculated by accompanying software. The antigen is then allowed to dissociate from the antibodies. The amount dissociated and the time taken gives the dissociate rate constant, k_d. The sensor chip surface is then regenerated using a regeneration buffer that breaks the bonds between all remaining antigen and the antibodies without affecting the antibodies. The chip is then ready for a new run. The affinity constant, k_A, can be calculated using the association and dissociation rate constants as described by equation 2. The affinity constant gives a measurement of the antibodies' affinity for the antigen.

$$k_A = \frac{k_a}{k_d}$$ **Equation 2**

The appropriate binding level, R_b, for each type of antibodies is calculated using equation 3.

$$R_b = \frac{R_{\max} * m_{mAb}}{m_A}$$ **Equation 3**

where R_{max} is the wanted maximum response level in the kinetics run, m_{mAb} is the antibody mass and m_A is the antigen mass. R_{max} should be in the range 15 to 50 RU in order to give reliable results.

3. Materials and methods

Monoclonal antibodies with three different specificities were used; monoclonal antibodies against human immunoglobulin E (anti-IgE), against the major birch pollen allergen Bet v 1 (anti-Bet v 1) and against the hazel nut allergen Cor a 8 (anti-Cor a 8). An overview of the used monoclonal antibodies and studies made is presented in Appendix A. Used antigens, allergens and allergen extracts are described in table 1.

Table 1. Description of used tests.

Test code	Test reagents
IgE(ND)	Human immunoglobulin E from myeloma patient.
mIgG	Sheep anti-mouse IgG antibodies.
Bet v 1	Major birch pollen allergen (recombinant).
t3	Birch pollen allergen extract, containing Bet v 1.
Cor a 8	Hazel nut allergen (native, lipid transfer protein).
f17	Hazel nut allergen extract, containing Cor a 8.
e1	Cat epithelium and dander allergen extract.

3.1 ImmunoCAP® System

In the present study an in-house ImmunoCAP 100 method was used to measure mouse monoclonal antibodies. The incubation and washing steps were identical to those of the standard method used for measuring human specific IgE antibodies. The sample incubation time was 30 min and the incubation was conducted at a temperature of 37°C. The in-house enzyme conjugate used was composed of β-galactosidase conjugated rabbit anti-mouse IgG antibodies. The response (fluorescence) was compared to a calibration curve, consisting of 5 calibrators with mouse monoclonal antibodies, ranging from 0.1 to 7.0 µg/ml measured on mIgG ImmunoCAP. The same ImmunoCAP 100 instrument was used throughout the study.

3.1.1 Coupling of IgE to ImmunoCAP

IgE ImmunoCAP were made at MIAB, Uppsala, with kind help from Maria Ahlberg, by coupling Phadia in-house myeloma IgE known as IgE(ND) to ImmunoCAP solid phase cellulose polymer at a concentration of 40 µg/ml. Optimization of pH was achieved through mixing IgE(ND) with 0.1 M $NaHCO_3$. The solid phase sheet was left to incubate in the

coupling mixture in a sealed aluminium foil bag over night on shake table at 4°C. The sheet was then washed five times with 100 ml in-house Phadebas buffer. Discs of solid phase were punched out from the sheet and placed within ImmunoCAP plastic holders. The other ImmunoCAP tests used in the work were commercially available, except for Cor a 8 ImmunoCAP, which was a kind gift from Peter Brostedt, Phadia AB, and the ImmunoCAP for total mouse IgG (mIgG), where sheep anti-mouse IgG antibodies had been coupled to the solid phase. The mIgG ImmunoCAP test was used in the study to measure the total antibody concentration in the sample.

3.1.2 Affinity study

Initially, 24 anti-IgE antibodies were run in duplicate against IgE and mIgG ImmunoCAP. By calculating the ratio between the IgE and mIgG response units an estimation of the affinity for each IgE-specific antibody to IgE(ND) was made. The antibodies were diluted in in-house UniCAP mIgG diluent to approximately 2 µg/ml (determined from A_{280} measurements using an approximate ε-coefficient of 1.4). UniCAP mIgG diluent was used as negative control. The same was done for the anti-Bet v 1 and the anti-Cor a 8 antibodies on corresponding ImmunoCAP (Bet v 1, t3 and Cor a 8, f17).

3.1.3 Concentration study

To study the effect of antibody concentration on the ImmunoCAP System study four selected IgE antibodies (mAb164, 209, 216 and 390) were run in seven different concentrations (0.156, 0.313, 0.625, 1.25, 2.5, 5 and 10 µg/ml) against IgE and mIgG ImmunoCAP.

3.1.4 Depletion study

After a manual wash on an AutoCAP instrument (Phadia AB), IgE ImmunoCAP were incubated with either mAb164 or mAb230 at three different concentrations (0.2, 1 and 5 µg/ml) at 37°C for 30 minutes to simulate the sample incubation step of the ImmunoCAP 100 method. Unbound sample was eluted through centrifugation at 2000 rpm for 5 minutes at 4°C using a Beckman TJ-6 centrifuge (Beckman). These eluted samples were then run against IgE ImmunoCAP and compared to non-depleted samples. This study was also performed with the anti-Bet v 1 antibodies and corresponding ImmunoCAP tests (t3), but only with one sample

concentration; 2 µg/ml. Here cat allergen ImmunoCAP tests (e1), for which the anti-Bet v 1 antibodies lacked specificity, were used as negative controls.

3.2 ImmunoCAP® Rapid

When evaluating the capacity of ImmunoCAP Rapid to bind monoclonal mouse antibodies, a laboratory version of the test called Half Dipstick was used. In accordance with the Half Dipstick protocol the membrane strips were placed vertically in 96 well microtitre plate wells. First the strips were put in wells containing 20 µl sample solution for 5 minutes, followed by incubation with 20 µl goat anti-mouse IgG gold conjugate for ten minutes. Finally the test strips went through a two-step wash (First 4 minutes in 20 µl Developer Solution, then 6 minutes in 100 µl Developer Solution). Results were visually read by manual comparison with a so called Rann scale (ranging from 0 to 10) and by using a Fasttrack Reader 3 optical reader, which shows results in the form of top areas, corresponding to the colour intensity of a line. For a schematic view of the procedure, please consult Appendix B.

3.2.1 Coating of nitrocellulose membrane with allergens

IgE(ND) was coupled to particles at a concentration of 67.3 µg IgE(ND)/mg particles, following Phadia standard method. Previously coupled allergen-particles were sonicated for 30 seconds using a Sonics and Materials Vibra Cell VCX-600 (Sonics and Materials) and degassed in an ultrasound bath on ice for 10 minutes before application onto nitrocellulose membrane in 5 (for f17 and t3) or 4 (for IgE[ND]) repetitive lines using a Biodot XYZ 3000 workstation (Biodot) with a Front Line dispenser (Biodot) (Figure 6).

The membranes were dried at 37°C for 1 hour followed by attachment of wick using a Kinematic Matrix 2210 laminator (Kintematic Automation). The glue was left to adhere over night in a silica gel bag containing plastic bag within a sealed aluminium foil bag at 4°C. The membranes were then cut into 4 mm wide strips using a Biodot Cutting Module CM 4000 (Biodot). The strips were henceforth stored at 4°C.

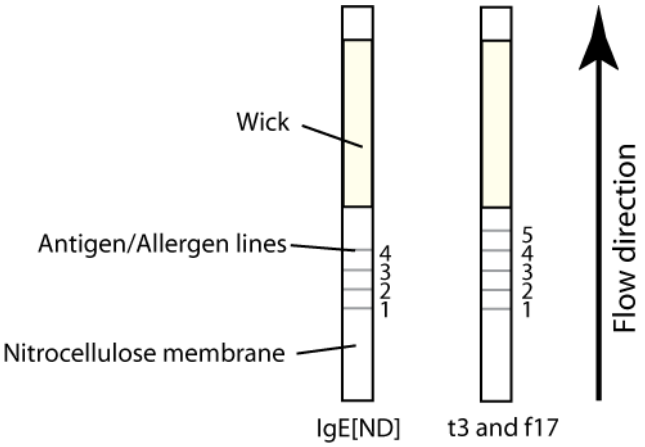

Figure 6. Half dipstick strips in natural size.

3.2.2 Capacity and affinity study

All three sets of antibodies were tested with correspondingly coated strips (IgE[ND] for the anti-IgE experiments, t3 for the anti-Bet v 1 experiments, and f17 for the anti-Cor a 8 experiments). For anti-IgE and anti-Bet v 1, two different concentrations of antibody sample solution (0.1 and 1 µg/ml) and conjugate (optical density [OD] 0.5 and 2) were evaluated. For the anti-Cor a 8 studies, the setup using antibody concentration 1 µg/ml and conjugate with an OD of 0.5 was used. The antibody samples were diluted using UniCAP mIgG diluent and the conjugate was diluted using Developer Solution.

3.2.3 Intervening wash study

An additional 20 µl developer solution washing step was introduced between sample and conjugate incubation. For the anti-Bet v 1 antibodies this step was 2, 6 or 10 minutes. In the case of the anti-IgE antibodies, the intervening wash step was 5 or 10 minutes.

3.2.4 Pre-wetting study

A 20 µl washing step was introduced before sample incubation to wet the membrane. Three different solutions were tested; destilled water, UniCAP mIgG diluent and the Developer Solution. A negative control without pre-wetting was also included. The antibody used in this study was the anti-Bet v 1 antibody mAb894.

3.2.5 Affinity study using radioactively labelled antibodies

Three anti-IgE antibodies (mAb164, 354 and 390) and the two anti-Bet v 1 antibodies (mAb793 and 894) were radioactively labelled with I^{125} (kindly performed by Peter Olsson, Phadia AB) and then run on Half Dipstick according to the same protocol as earlier. Each line was then cut out and put into 55 mm Ellerman test tubes. The zones directly before the first

line and directly after the last line were also cut out for measurement of background. The radioactivity was measured using a 1260 Multigamma II gamma counter (LKB Wallac). To determine the total radioactivity of the sample volume, sample test tubes containing 20 µl of each antibody sample were run in the gamma counter as well. Blank runs with the gamma counter were conducted to determine the background radiation.

3.3 Biacore

3.3.1 Kinetics study

In the study a Biacore T100 instrument (Biacore AB) was used to investigate the kinetic properties of the monoclonal antibodies used. Throughout the study the antibodies were bound to the surface of a Biacore sensor chip of type CM5, while antigen was carried in the aqueous flow.

For each of the three studies (anti-IgE, anti-Bet v 1 and anti-Cor a 8) rabbit anti-mouse ligand (RAM) was amine-coupled to two flow channels of the CM5 sensor chip, one sample channel and one reference channel at 30 µg/ml concentration. The reference channel was used to measure background response levels.

Before each kinetics run, manual runs were performed at various sample concentrations to find a sample concentration that resulted in appropriate binding levels. In the manual runs sample was injected and then a normal Biacore cycle was conducted. The tested antibody sample concentrations varied from 0.5 µg/ml to 2.2 µg/ml for the anti-IgE antibodies, while the anti-Cor a 8 concentration varied between 10 µg/ml and 15 µg/ml. Both anti-Bet v 1 antibodies were run at 8.5 µg/ml.

The anti-IgE antibodies were run eight at a time with mAb164 as internal control in each run. mAb164 was also run at different sequential positions to investigate if the result was dependent on sample position in the run. The anti-Bet v 1 antibodies were run in a separate run, as the anti-Cor a 8 antibodies. The anti-Cor a 8 antibodies were also run an additional time with an unspecific antibody as negative control. For a detailed description of the method used in the kinetics runs, please consult Appendix C.

4. Results

4.1 ImmunoCAP System

4.1.1 Affinity study

To assess the ratio between specific binding and total antibody concentration, all antibodies were run in duplicate against ImmunoCAP for which they had specificity and against mIgG ImmunoCAP. The anti-IgE antibodies varied between 0.02 (mAb111) and 0.69 (mAb347) in the ratio between specific binding and total antibody concentration (Figure 7a). Most notable was the low ratios on mAb215 and 406. In the anti-Bet v 1 experiment mAb793 showed significantly higher ratio between specific binding and total antibody concentration than mAb894. The ratio between specific t3 binding and total antibody concentration was higher than the ratio between specific Bet v 1 binding and total antibody concentration for both antibodies (Figure 7b). For the anti-Cor a 8 antibodies mAb976 had the highest ratio, while 977 had the lowest. The ratio between specific Cor a 8 binding and total antibody concentration was higher than the ratio between specific f17 binding and total antibody concentration for all antibodies (Figure 7c). See table 2 for a list of all ratios.

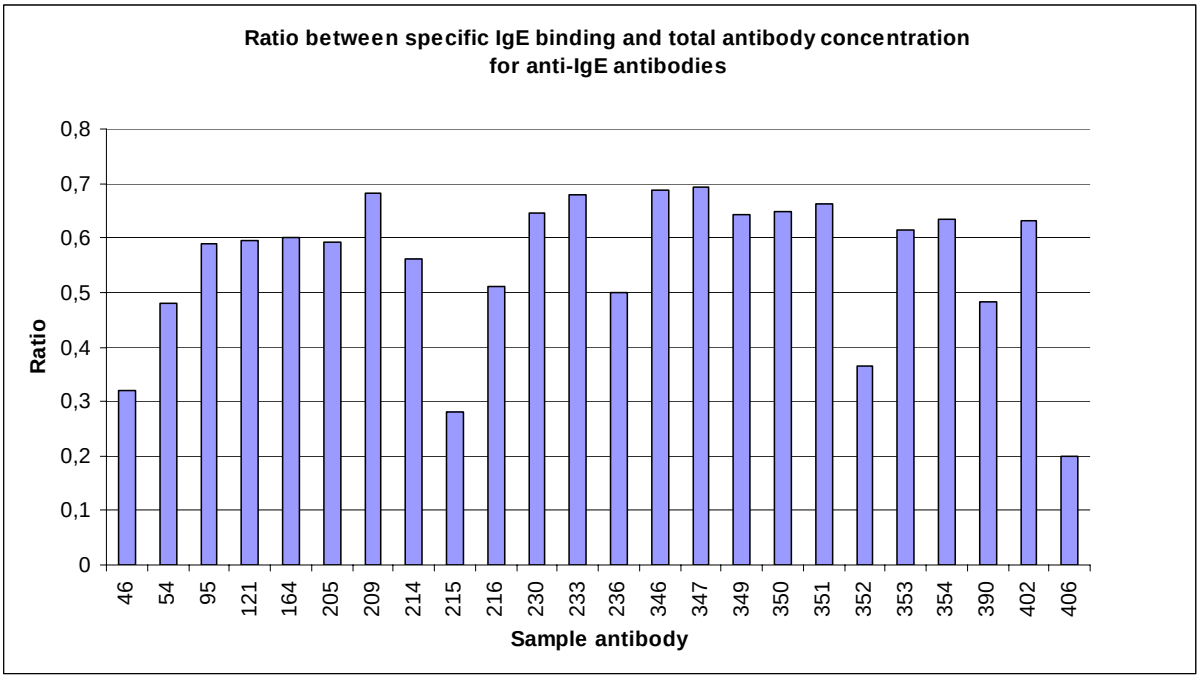

Figure 7a. Ratio between specific IgE binding and total antibody concentration for anti-IgE antibodies.

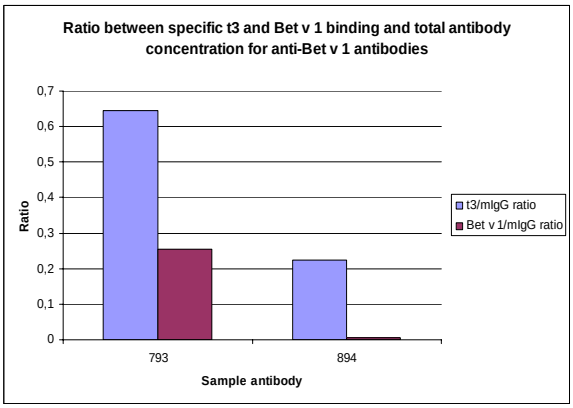

Figure 7b. Ratio between specific t3 and Bet v 1 binding and total antibody concentration for anti-Bet v 1 antibodies.

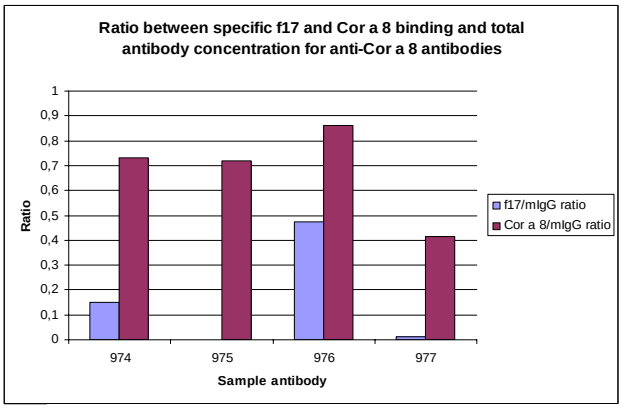

Figure 7c. Ratio between specific f17 and Cor a 8 binding and total antibody concentration for anti-Cor a 8 antibodies.

Table 2. Specific binding/Total antibody concentration ratio sorted in order of increasing ratio. (Green = anti-IgE antibodies, blue = anti-Bet v 1 and yellow = anti-Cor a 8 antibodies, results from both Cor a 8 and f17 ImmunoCAP as indicated.)

Antibody	Specific binding/Total antibody concentration ratio
406	0.20
215	0.28
46	0.32
352	0.36
54	0.48
390	0.48
236	0.50
216	0.51
214	0.56
95	0.59
121	0.59
205	0.59
164	0.60
353	0.61
354	0.63
402	0.63
230	0.64
349	0.64
350	0.65
351	0.66
209	0.68
233	0.68
346	0.69
347	0.69
894 Bet v 1	0.01
793 Bet v1	0.25
894 t3	0.22
793 t3	0.65
977 Cor a 8	0.42
975 Cor a 8	0.72
974 Cor a 8	0.73
976 Cor a 8	0.86
975 f17	-
977 f17	0.01
974 f17	0.15
976 f17	0.47

4.1.2 Concentration study

To investigate if antibody concentration in the sample solution affected the results of the affinity study, four selected IgE antibodies were run in seven different concentrations against IgE and mIgG ImmunoCAP. The sample concentration was shown to affect the ratio between specific binding and total antibody concentration. Higher sample concentrations yielded higher ratios (Figure 8). The increase in response from lowest to highest antibody sample concentration on the mIgG ImmunoCAP was 21-fold on average (not shown).

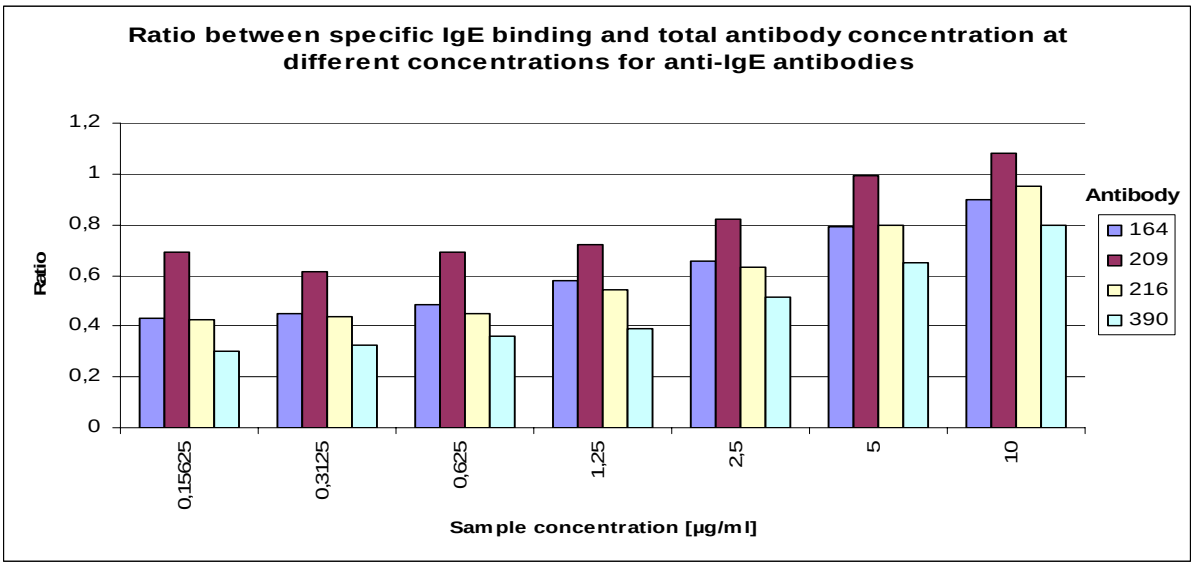

Figure 8. Ratio between specific IgE binding and total antibody concentration at different concentrations for anti-IgE antibodies.

4.1.3 Depletion study

The depletion study was performed to assure that the antigens on the solid phase of the ImmunoCAP were not a limiting factor when binding antibodies. As seen in figure 9a the antigens on the solid phase were not a limiting factor. Regardless of the initial sample concentration, the depleted samples all showed equally low responses. The effect was also proven to result from specific binding. This can be seen in figure 9b where the samples depleted on e1 ImmunoCAP, had responses equal to those of the undepleted samples, while the samples depleted on t3 ImmunoCAP showed low response values.

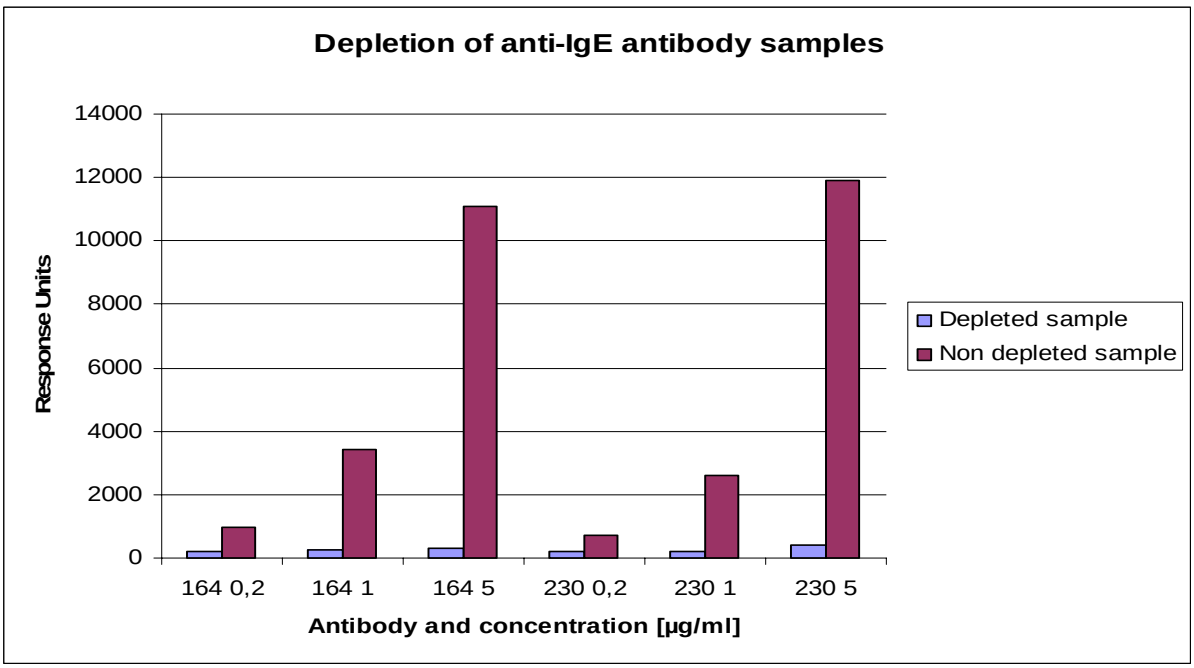

Figure 9a. Depletion of anti-IgE antibody samples analysed on IgE ImmunoCAP.

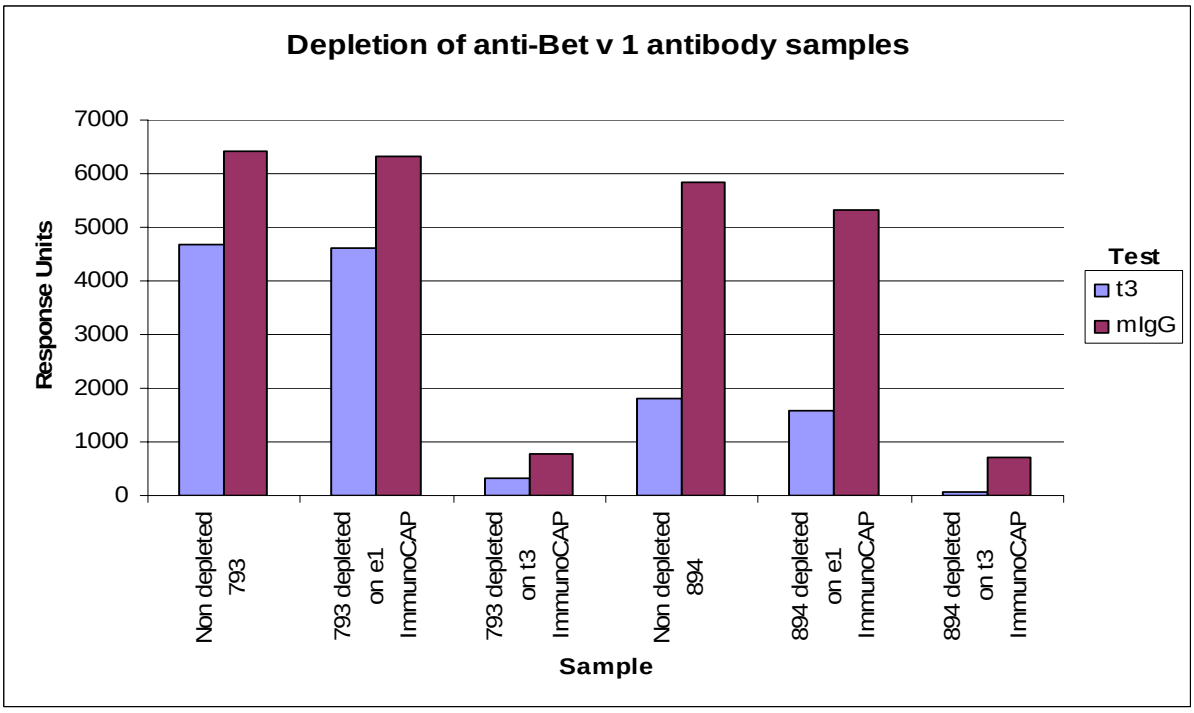

Figure 9b. Depletion of anti-Bet v 1 antibody samples.

4.2 ImmunoCAP Rapid

4.2.1 Capacity and affinity study

The combination of antibody concentration 1 µg/ml and conjugate with an OD of 0.5 was found to give the clearest results and the presented data is from using that setup. As in the

ImmunoCAP System study, there was some variation between the anti-IgE antibodies (Figure 10a). Most of the antibodies with low responses in the ImmunoCAP System study showed low responses here as well. In the anti-Bet v 1 experiment mAb793 again showed a higher response than 894 (Figure 10b). Notable here is the tendency of mAb894 to show increasingly higher responses from line 1 to 5, which is in contrast to all other antibodies of the study. For the anti-Cor a 8 antibodies the responses for line 1 was nearly the same for all four antibodies, but in lines 3 to 5 mAb975 had the highest responses, while mAb976 had the lowest (Figure 10c). The ratio between the response of the first and last line is showed in table 3. A high ratio can be interpreted as indicating that most of the antibody has bound to the first lines (i.e. the higher the ratio the higher the antibody affinity for the antigen)

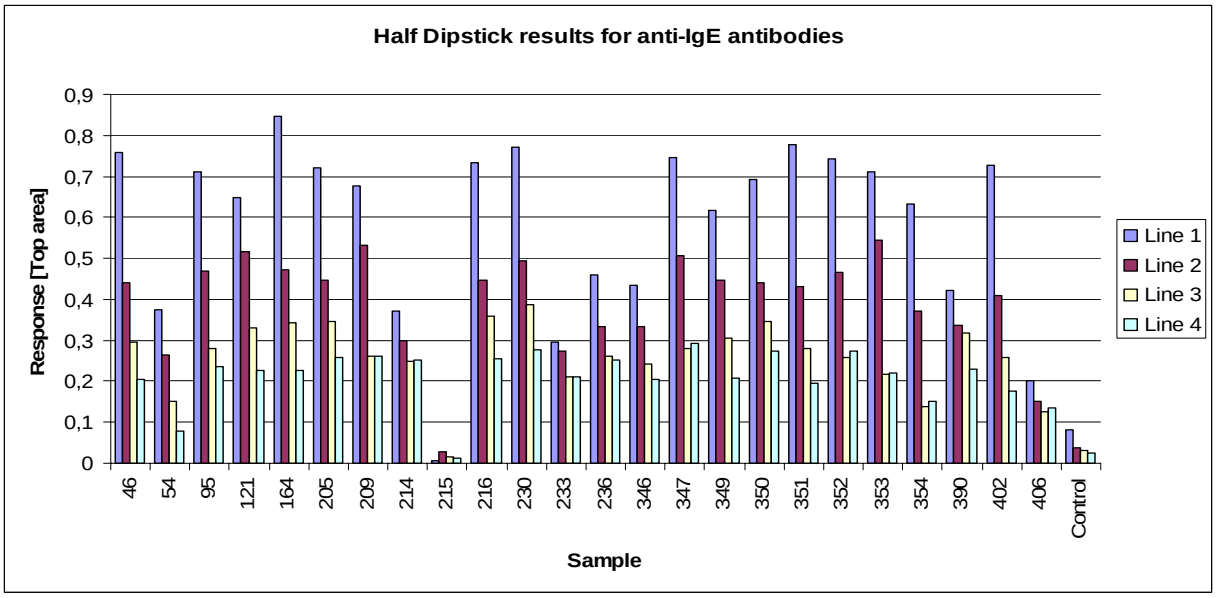

Figure 10a. Half Dipstick results for anti-IgE antibodies.

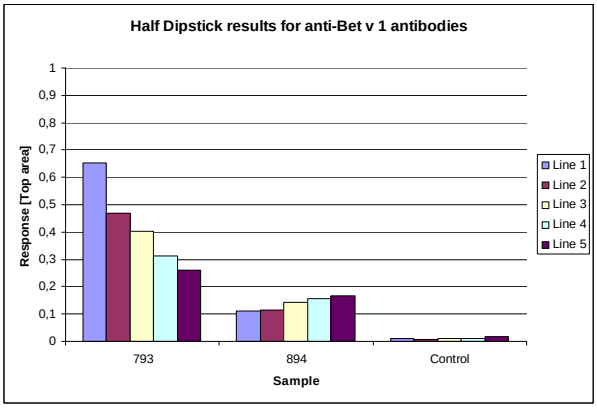

Figure 10b. Half Dipstick results for anti-Bet v 1 antibodies.

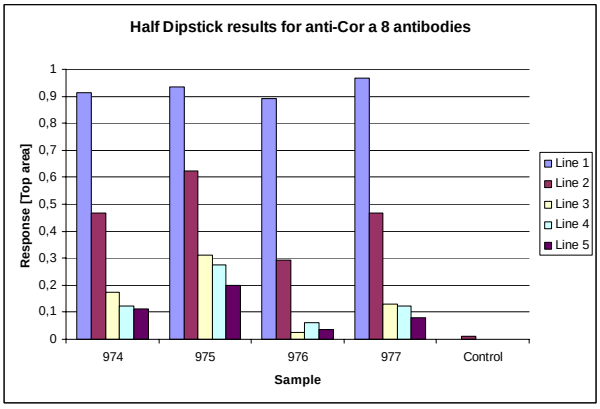

Figure 10c. Half Dipstick results for anti-Cor a 8 antibodies.

Table 3. First/last line ratio sorted in order of increasing ratio. (Green = anti-IgE antibodies, blue = anti-Bet v 1 and yellow = anti-Cor a 8 antibodies.)

Antibody	First/Last line ratio
215	0.43
233	1.40
214	1.47
406	1.49
236	1.82
390	1.85
346	2.12
350	2.52
347	2.56
209	2.61
352	2.70
230	2.78
205	2.79
121	2.84
216	2.90
349	2.95
95	3.03
353	3.22
46	3.72
164	3.74
351	4.00
402	4.13
354	4.24
54	4.72
894	0.66
793	2.52
975	4.37
974	11.17
977	13.07
976	29.84

4.2.2 Intervening wash study

The intervening wash step was introduced to investigate if the gold conjugate possibly could bind to unbound antibodies lingering in the membrane, but there was no apparent effect of intervening wash on the results. There was some variation between samples with different length of intervening wash, but there was no trend indicating that an intervening wash gave lower responses. For the anti-Bet v 1 antibodies there was a slight decrease in responses for the samples with intervening wash (Figure 11a,b). Unfortunately, an error in the scanning procedure led to the absence of values for line 5 for several of the samples.

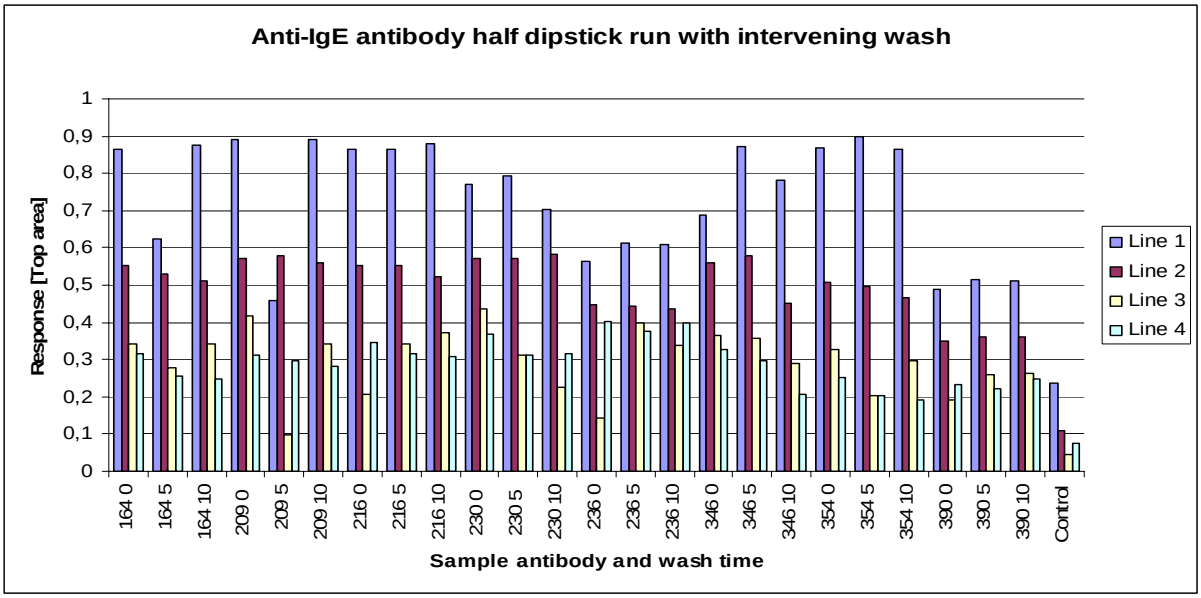

Figure 11a. Anti-IgE antibody half dipstick run with intervening wash.

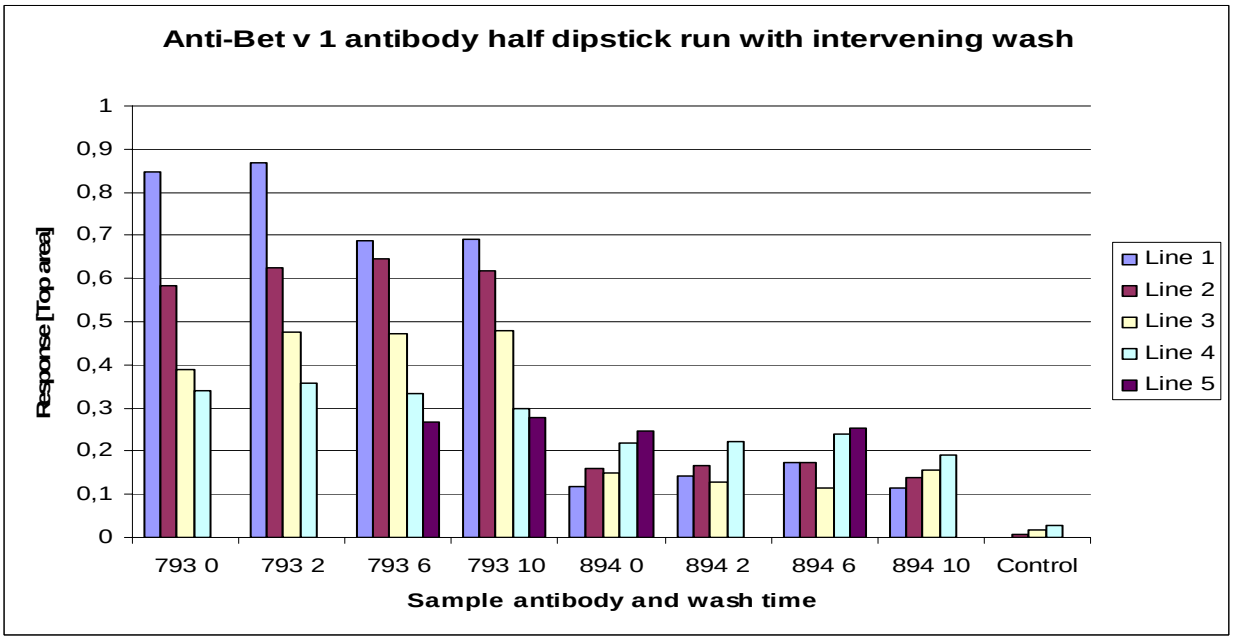

Figure 11b. Anti-Bet v 1 antibody half dipstick run with intervening wash. Error in the scanning procedure led to the absence of values for line 5 for several of the samples.

4.2.3 Pre-wetting study

There was no apparent effect of pre-wetting of the nitrocellulose membrane on the results of mAb894 run on the t3 test strips. The test strip that had been pre-wet with developer solution showed slightly lower responses for all lines, but the increasing trend from line 1 to 5 persisted (Figure 12).

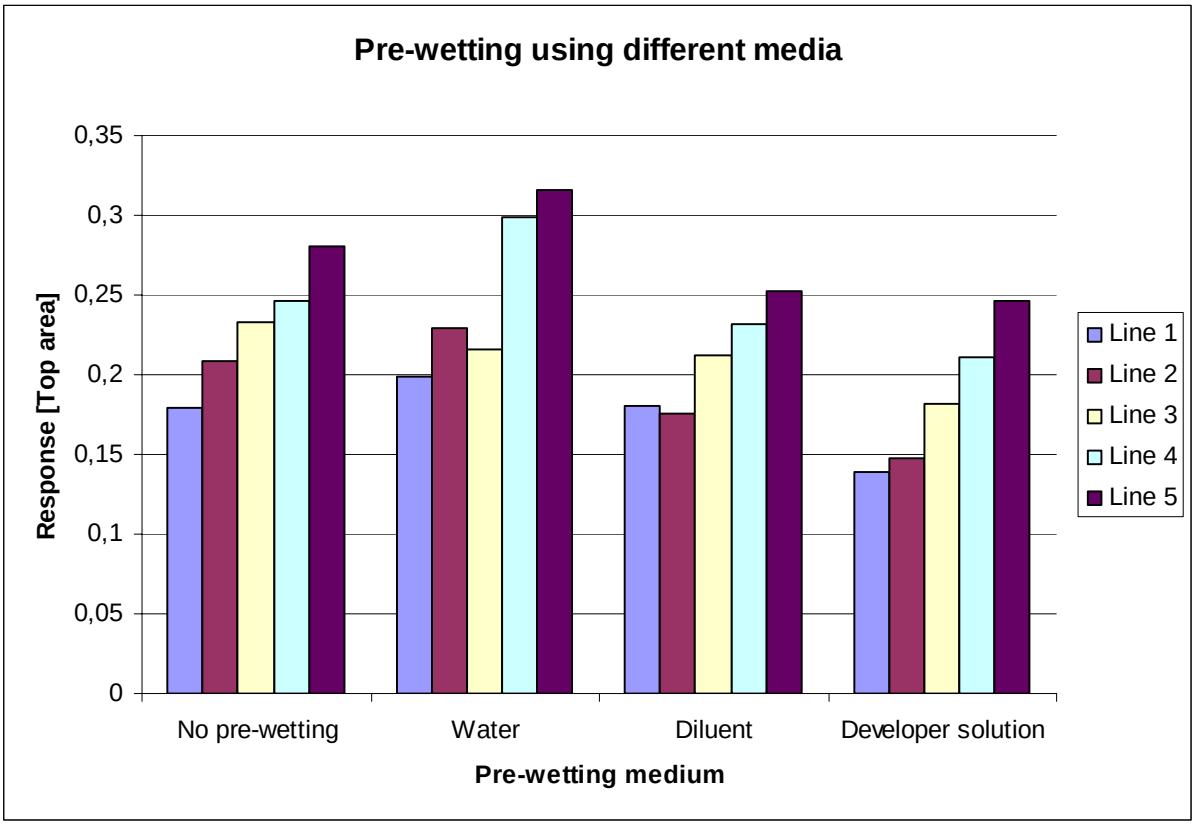

Figure 12. Pre-wetting using different media. Sample antibody mAb 894 was run on t3-coated test strips.

4.2.4 Affinity study using radioactively labelled antibodies

The results for the three I^{125}-labelled anti-IgE antibodies (mAb164, 354 and 390) correspond quite well with the results from the previous half dipstick experiments (Figure 13a). The actual response levels are not as interesting as the change from line 1 to 4, since the amount of radioactivity differed in the different samples, as can be seen in figure 13b. The 1 to 4 ratios were 6.31 (mAb164), 7.74 (mAb354) and 1.95 (mAb390). Figure 13b also shows that almost all mAb354 in the sample bound to the test strip, while only a fraction of the mAb164 and 390 bound. In the case of the anti-Bet v 1 antibodies, the trend of increasing response from line 1 to 5 for mAb894 showed to be connected with the monoclonal antibody and not the gold conjugate. Interesting here is the trend from line 1 to 5 for mAb793, which is now

increasing, just like it is for mAb894 (Figure 13c). Only a fraction of the sample bound for both antibodies (Figure 13d).

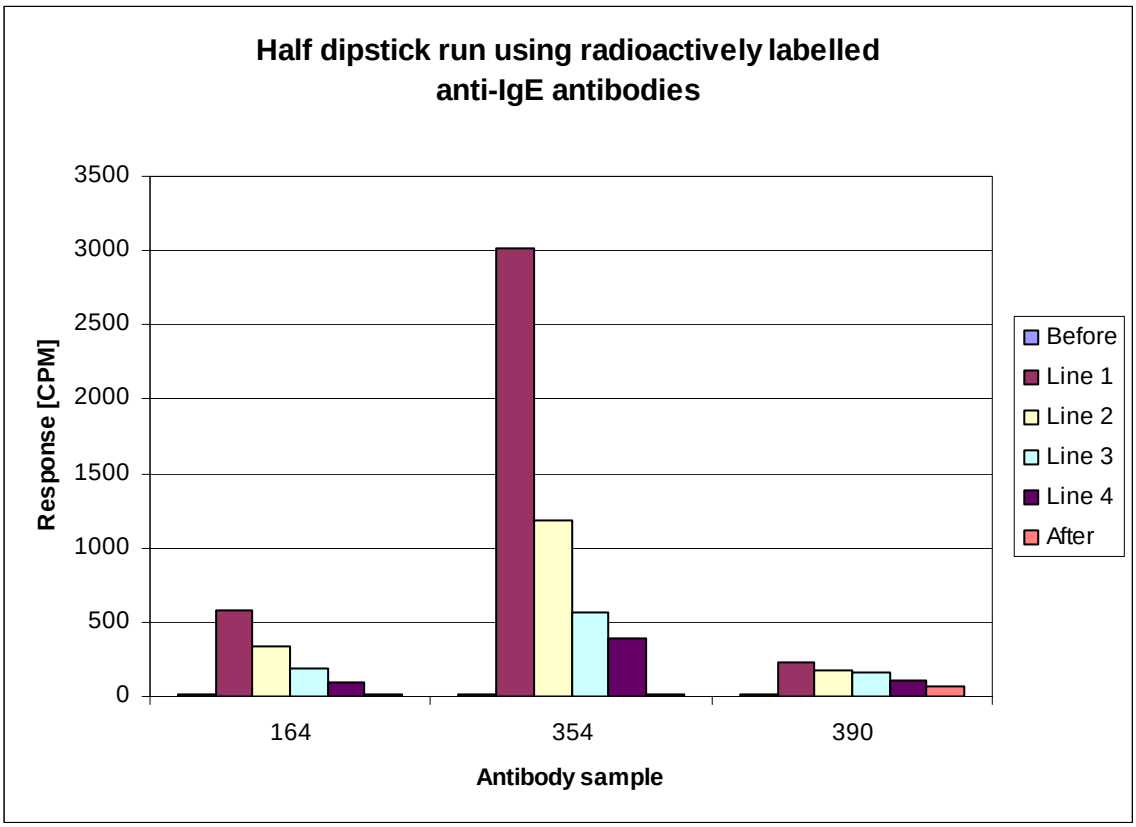

Figure 13a. Half dipstick run using radioactively labelled anti-IgE antibodies.

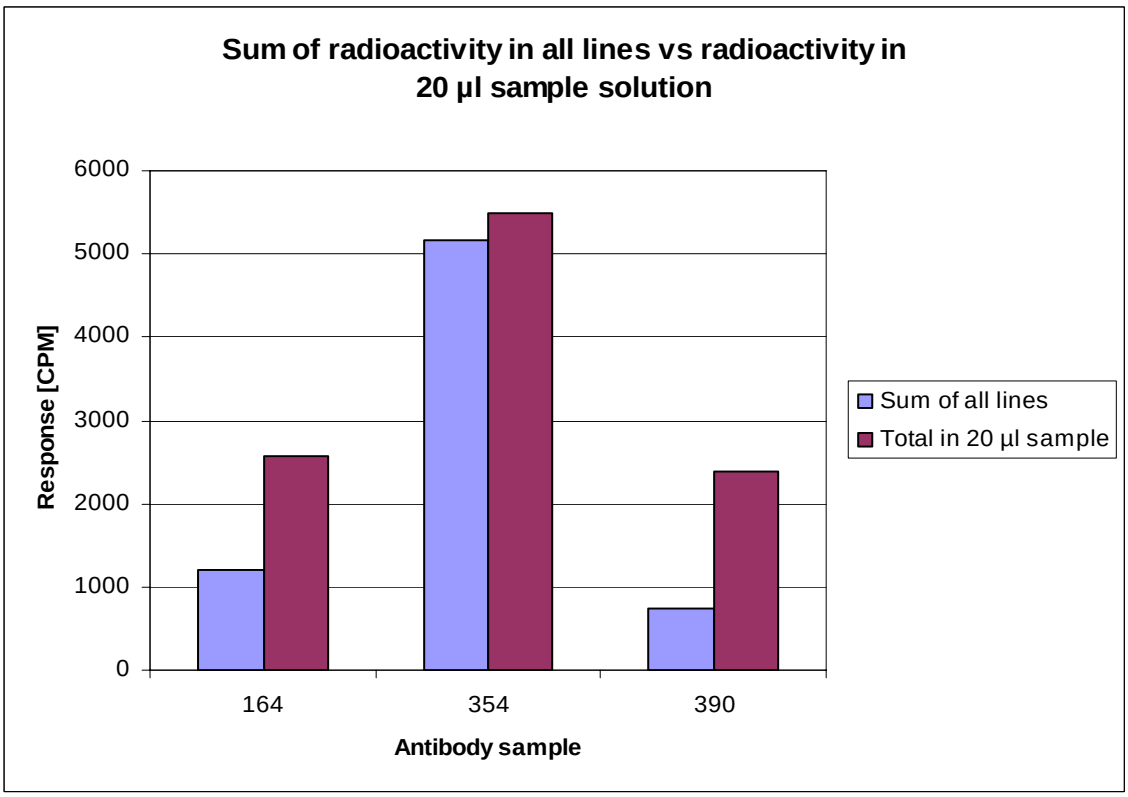

Figure 13b. Sum of radioactivity in all lines vs radioactivity in 20 µl sample solution.

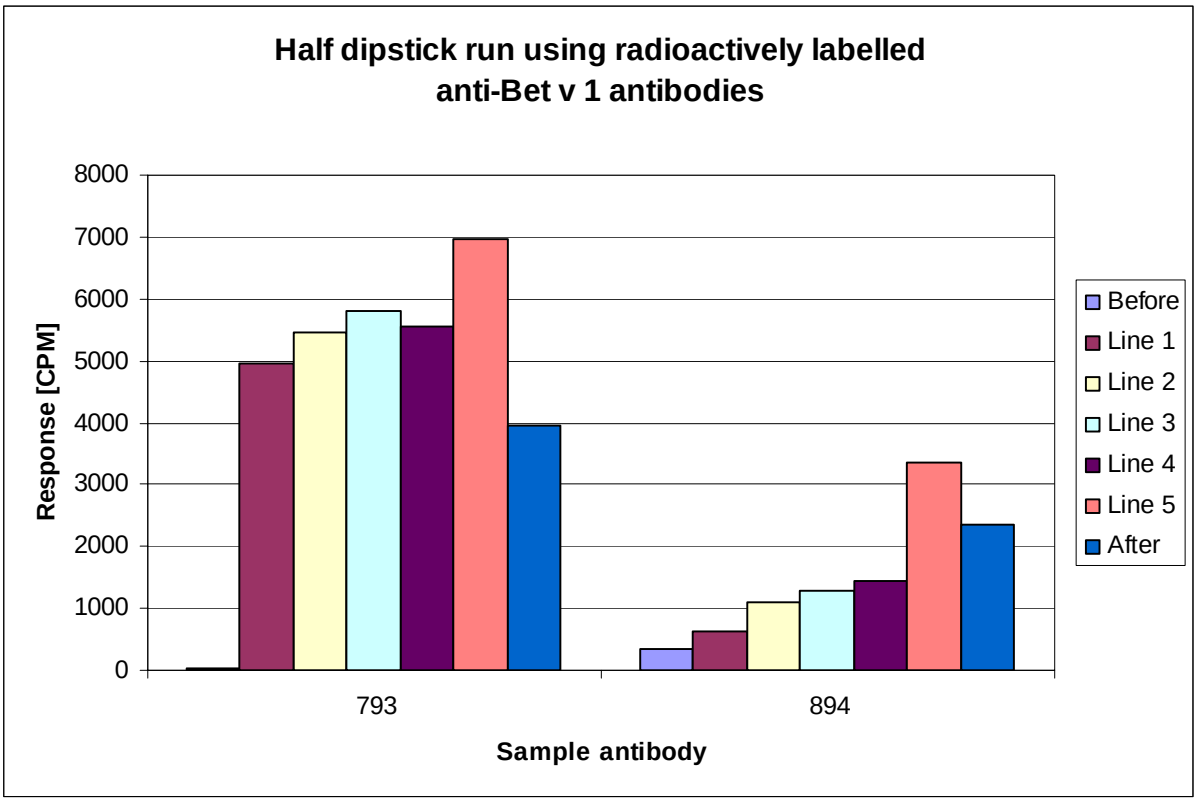

Figure 13c. Half dipstick run using radioactively labelled anti-Bet v 1 antibodies.

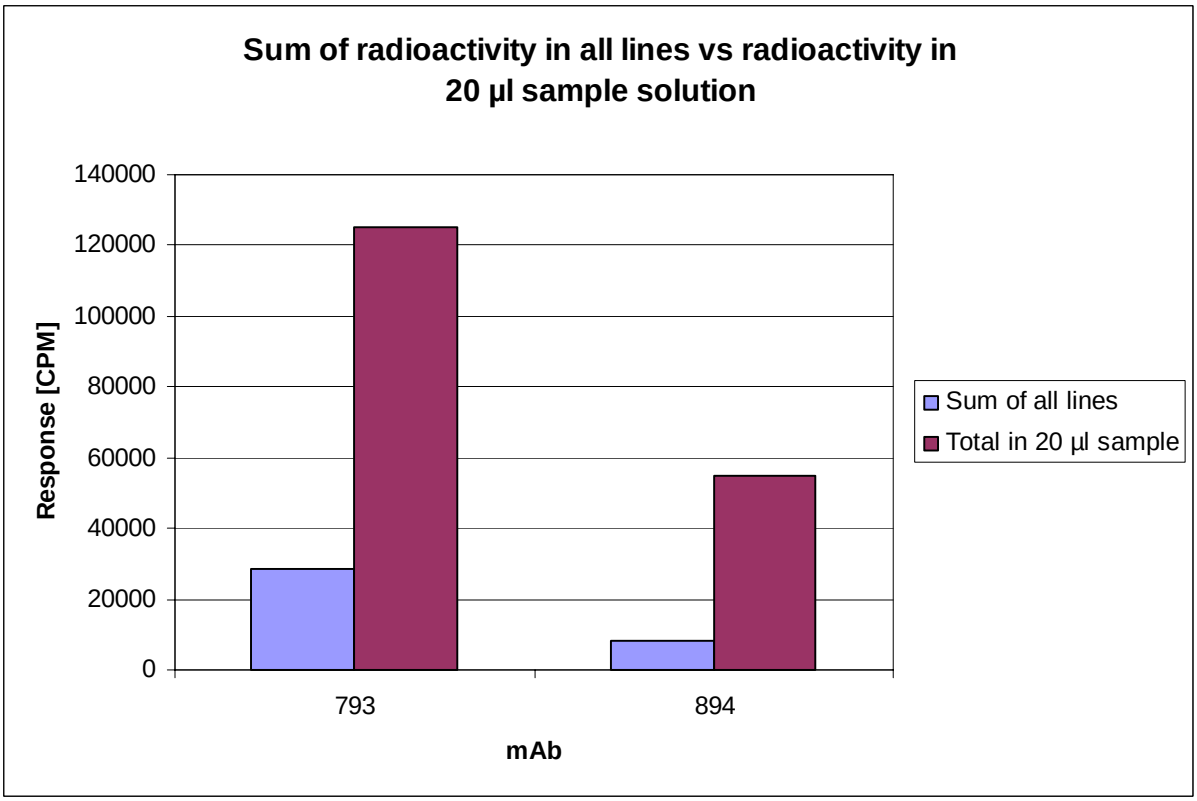

Figure 13d. Sum of radioactivity in all lines vs radioactivity in 20 µl sample solution.

4.3 Biacore

The aim of getting R_{max} values in the 15-50 RU range was achieved for all antibodies except mAb214, 215, 406 and 894 (data not shown). The kinetics data for these clones was therefore regarded as unreliable and has been disregarded. The kinetics data for the remaining antibodies can be seen in table 4. In the anti-Cor a 8 antibody experiment, the negative control showed higher response values than expected (data not shown) and possible reasons to this are discussed in the next section. Even if the exact kinetics data values for the anti-Cor a 8 antibodies therefore could be questioned, the results are still interesting for comparison between the four of them.

Table 4. Kinetics data from Biacore experiments sorted in order of increasing affinity. (Green = anti-IgE antibodies, blue = anti-Bet v 1 and yellow = anti-Cor a 8 antibodies.)

Antibody	k_a	k_d	k_A
236	2,06E+04	3,43E-03	6,01E+06
352	1,07E+04	1,54E-03	6,94E+06
230	1,45E+04	6,64E-04	2,19E+07
216	1,46E+04	5,70E-04	2,57E+07
233	9,99E+03	3,78E-04	2,64E+07
121	2,02E+04	4,67E-04	4,32E+07
390	4,89E+04	1,12E-03	4,37E+07
347	2,29E+04	4,35E-04	5,28E+07
346	1,84E+04	3,22E-04	5,72E+07
205	1,08E+04	1,28E-04	8,48E+07
46	2,56E+04	2,39E-04	1,07E+08
95	4,24E+04	2,83E-04	1,50E+08
54	1,76E+04	9,85E-05	1,79E+08
350	2,11E+04	1,08E-04	1,95E+08
402	3,89E+04	1,76E-04	2,22E+08
351	2,63E+04	9,38E-05	2,80E+08
353	2,44E+04	6,62E-05	3,68E+08
209	1,15E+04	2,69E-05	4,27E+08
349	2,60E+04	5,88E-05	4,41E+08
164	3,69E+04	4,95E-05	7,45E+08
354	3,01E+04	3,03E-05	9,93E+08
793	8,01E+04	6,10E-05	1,31E+09
975	6,80E+05	4,73E-04	1,44E+09
977	1,37E+07	6,13E-03	2,23E+09
976	1,56E+06	5,07E-04	3,08E+09
974	1,27E+06	3,49E-04	3,64E+09

5. Discussion

5.1 ImmunoCAP System

5.1.1 Affinity study

There was some variation among the anti-IgE antibodies in the ratio between specific binding and total antibody concentration. Still, 17 out of 24 antibodies had ratios in the range 0.5-0.7 and 11 antibodies had a ratio above 0.6. Therefore no antibodies could be seen upon as clearly superior than average. The ratios for mAb46, 215, 352 and 406 on the other hand were clearly lower than for the average antibody and therefore their affinity was expected to be low. No satisfactory explanation to the low ratios of mAb46 and 406 was found. The explanation to the low ratio of mAb215 and 352 was found when searching Phadia antibody registers; all of the anti-IgE antibodies were IgG antibodies, however, mAb352 and 353 were of subclass G2a and mAb215 was of subclass G2b, while the rest were of subclass G1. Actually mAb352 had the fourth lowest ratio between specific binding and total antibody concentration and even though mAb353 had the relatively high ratio 0.61, the matter of subclass could be the explanation to the low ratios of mAb215 and 352. The reason for this could be that the mIgG ImmunoCAP is binding mouse IgG antibodies of subclass G2a and G2b better than those of subclass G1 and thus leading to lower ratios between specific binding and total antibody concentration for the antibodies of subclass G2a and G2b.

In the case of the anti-Bet v 1 monoclonal antibodies, mAb793 showed significantly higher ratio between specific binding and total antibody concentration than mAb894. It seems as though mAb973 simply has superior affinity. The ratio between specific t3 binding and total antibody concentration was higher than the ratio between specific Bet v 1 binding and total antibody concentration for both antibodies. The reason for the ratio between specific t3 binding and total antibody concentration being higher than the ratio between specific Bet v 1 binding and total antibody concentration for both antibodies could possibly be explained by that the antibodies bind better to the native Bet v 1 in the t3 extract than to the recombinant Bet v 1 on the Bet v 1 ImmunoCAP.

For the anti-Cor a 8 antibodies, mAb976 had the highest ratio, while 977 had the lowest. In the case of the Cor a 8/mIgG ratio mAb974, 975 and 976 had ratios of similar magnitudes

while that of mAb977 was significantly lower and appeared to have clearly lower affinity than the others. The ratio between specific Cor a 8 binding and total antibody concentration was higher than the ratio between specific f17 binding and total antibody concentration for all antibodies. The reason for the ratio between specific Cor a 8 binding and total antibody concentration being higher than the ratio between specific f17 binding and total antibody concentration for all antibodies could probably be that the amount of Cor a 8 on the f17 ImmunoCAP, based on hazel nut extract, is lower than on the pure Cor a 8 ImmunoCAP.

5.1.2 Concentration study

Trying to explain why higher concentrations of antibody sample give higher ratio between specific binding and total antibody concentration could initially lead one to assume that the mIgG ImmunoCAP are occupied by antibodies to such a great extent already at low concentrations, that an increase in sample concentration would not lead to an increase of bound antibody of the same magnitude as it would in IgE ImmunoCAP. The increase in response on the mIgG ImmunoCAP (21 fold on average) following the 64 fold increase in sample concentration of the experiment, taken together with the fact that the increasing ratio can be seen even at the low concentrations contradicts this assumption though. An explanation could be that the sample antibodies have lower affinity for the IgE(ND) than for the mIgG and an increase in concentration favours the interaction of lower affinity more than that of higher affinity. In other words; the anti-IgE-IgE(ND) interaction needs higher concentrations of antibody more than the anti-IgE-mIgG interaction does. A third possibility could be that the enzyme conjugate is able to bind IgE-bound monoclonal antibodies at higher concentration better than monoclonal antibodies bound to the sheep antibodies of the mIgG ImmunoCAP, due to sterical hindrance in the latter case.

5.1.3 Depletion study

The depletion study clearly showed that the antigens on the solid phase of the ImmunoCAP were not a limiting factor for the responses, since nearly all antibodies bound to the solid phase during the sample incubation step. It also showed that the depletion effect was specific. These are important prerequisites for many of the assumptions made in the discussion.

5.2 ImmunoCAP Rapid

5.2.1 Capacity and affinity study

The anti-IgE antibodies displaying low ratio between specific binding and total antibody concentration in the ImmunoCAP System study, showed low responses in the ImmunoCAP Rapid capacity and affinity study as well (mAb352 excluded). The ranking of the first/last line ratio corresponded quite well with the specific binding and total antibody concentration ratio ranking in the ImmunoCAP System study, with mAb354 as the antibody with clearly highest ratio, followed by mAb164. Antibodies with a quite high ratio in the ImmunoCAP System study, but ranking in the bottom half in the ImmunoCAP Rapid study, (mAb209, 233, 346, 347 and 350), showed the most deviating values. Deviating values were found with two antibodies, (mAb46 and 54), with the opposite relationship, i.e. low ranking in the ImmunoCAP System study, but ranking in the top half in the ImmunoCAP Rapid study. The background was higher than desired, an issue that never was solved but does not affect the internal ranking of the anti-IgE antibodies.

In the anti-Bet v 1 experiment, mAb793 behaved much like the anti-IgE antibodies, while mAb894 displayed an odd trend with increasing responses from the first to the last line. The reason for this could be that the antigen-antibody interaction is weak, leading to that part of the bound antibodies detach from each line. This would lead to an increasingly longer time each line is exposed to the sample solution, with the last line getting the longest time of exposure. This could explain the trend of mAb894.

Although all four anti-Cor a 8 antibodies displayed similar response patterns, there were still obvious differences among them, especially between mAb975 and 976. With a response ratio of approximately 30 between first and last line, mAb976 appears to have the highest affinity for the f17 allergen extract. This is a result that is well in agreement with the results from the ImmunoCAP System study, where mAb976 clearly has the highest ratio between specific binding and total antibody concentration on the f17 ImmunoCAP. In contrast to the ImmunoCAP System study, mAb975 was now the clearly inferior antibody, while mAb977 had the second highest ratio. The reasons for this would be of interest for future study.

5.2.2 Intervening wash study

For the anti-IgE antibodies an intervening wash step between sample and conjugate incubation did not affect the results, but for the anti-Bet v 1 antibodies there was a hint of lowered responses for the longer wash times. Even if the effect is real, it is still too small to be of greater significance. This means that the antibodies apparently have bound their antigens and the response shown is that of antigen-bound antibody-conjugate complexes.

5.2.3 Pre-wetting study

The primary reason for the pre-wetting study was to see if the trend of increasing response from line one to five for mAb894 could depend on the fact that the rate of liquid flow in the dry nitrocellulose membrane could be different from when the nitrocellulose is already wet. The results were not in any way affected by this treatment and the increasing response trend sustained.

5.2.4 Affinity study using radioactively labelled antibodies

The affinity study using radioactively labelled antibodies was performed because when reading and interpreting the red colour lines of the test, it is in fact the conjugate that is seen, not the antibodies directly. So, to investigate what really happens to the antibodies and to confirm that the gold conjugate readings are representative images of what actually happens with the monoclonal antibodies, radioactive I^{125} labels were attached to selected antibodies. The previous half dipstick results were confirmed by the radioactive study. It was surprising that less than half of the total radioactivity of the mAb164 sample could be seen in the lines. Still the ratio between line 1 and line 4 is significantly higher than that of mAb390. It is also only mAb390 among the anti-IgE antibodies that shows any radioactivity in the zone after the last line. These results confirm the earlier findings regarding the affinity ranking among the three of them. In the anti-Bet v 1 study the most remarkable finding was the reversed trend from first to last line for mAb793. This was unexpected but could be explained by conjugate depletion in the normal study, which would lead to falsely low values in the last lines. This seems unlikely though, since the amount of conjugate in the anti-Bet v 1 study was equal to that of the anti-IgE study. Still, mAb793 had a lower response on line 1 than for example mAb164 and 354 and the response of the second line of mAb793 was still lower than that of the first, in contrast to what was seen in the radioactivity study. Taken together with the fact

that the anti-IgE results were confirmed in the radioactivity study, this contradicts the assumption of conjugate depletion.

5.3 Biacore

The results from the Biacore study corresponded well with the findings in the ImmunoCAP System and ImmunoCAP Rapid affinity studies. The affinity ranking among the anti-IgE antibodies corresponded very well with the first/last line ratio ranking of the ImmunoCAP Rapid study, but also corresponded quite well with the ratio between specific binding and total antibody concentration in the ImmunoCAP System study. Among the anti-Cor a 8 antibodies, mAb975 still had the lowest affinity in the Biacore, while mAb974 had the highest affinity, followed by mAb976. It is still consistent though with mAb975 with low affinity and mAb976 with high affinity, as it also was seen in the ImmunoCAP Rapid study. As expected, mAb215 did not work well in the Biacore kinetics run. Also the earlier low-response antibodies mAb406 and 894 were unable to reach sufficient response levels, as for some reason mAb214 as well. The rather unexpected problem with the anti-Cor a 8 antibodies could be connected with the fact that Cor a 8 is a lipid transfer protein (LTP). LTPs are proteins with high isoelectric point that readily bind hydrocarbons, primarily fatty acids and phospholipids, where the LTPs bind the hydrophobic parts of these molecules [23, 24, 25]. This means that Cor a 8 could bind non-paratopic, hydrophobic areas on the antibodies, if such exist. The fact that the negative control displayed significant responses supports this. In fact, IgG molecules have been proven to have hydrophobic areas in the Fab-region that can bind non-specifically through hydrophobic interaction [26, 27]. This further supports this hypothesis. One could also suspect the possibility that Cor a 8 could bind the dextran on the Biacore chip surface, but then it should do so in the reference channel as well and this would be subtracted by the software. There is also the possibility of self aggregation among the Cor a 8 molecules, which would lead to that binding of one Cor a 8 might actually mean binding to a whole aggregate of Cor a 8 molecules, hence leading to a greater accumulation of sample near the chip surface and thus resulting in a falsely high response.

6. Conclusions

The aim of the study was to investigate the capacity of ImmunoCAP System and ImmunoCAP Rapid to bind specific antibodies and to see how the test results were affected by the antibody's binding strength to the antigen. The internal affinity ranking of antibodies of each specificity corresponded well between the two test systems. The sample concentration affected the ratio between specific binding and total antibody concentration in the ImmunoCAP System study. The sensitivity for low-affinity antibodies of both tests was good, since even the antibodies of lowest affinity gave satisfactory response levels. The fact that the antibodies with unacceptably low binding response in both tests (mAb215, 406 and 894) also failed to work in the Biacore kinetics runs speaks to the test systems' advantage and clearly indicates that it was the antibodies that were non-functional. All in all it can be stated that the higher Biacore-rendered affinity value an antibody has, the stronger response it will yield in both ImmunoCAP Rapid and ImmunoCAP System. Based on the findings of this study the author would advise the usage of mAb354 as human IgE antibody conjugate for the ImmunoCAP Rapid test.

In conclusion, no major difference between ImmunoCAP System and ImmunoCAP Rapid was observed, regarding their ability to detect antibodies of lower affinity.

Future research

As mentioned earlier, investigating the reasons to why mAb977 appeared to be of lowest affinity among the anti-Cor a 8 antibodies in the ImmunoCAP System study, while mAb975 seemed to be of clearly lowest affinity in the ImmunoCAP Rapid study would be of interest for future study. Also, further investigation concerning the results of mAb894 on ImmunoCAP Rapid would need to be done. Most interestingly would be to investigate the reasons to why mAb793 has an increasing trend from first to last line when using radioactively labelled antibodies, while the trend is opposite when using the normal conjugate.

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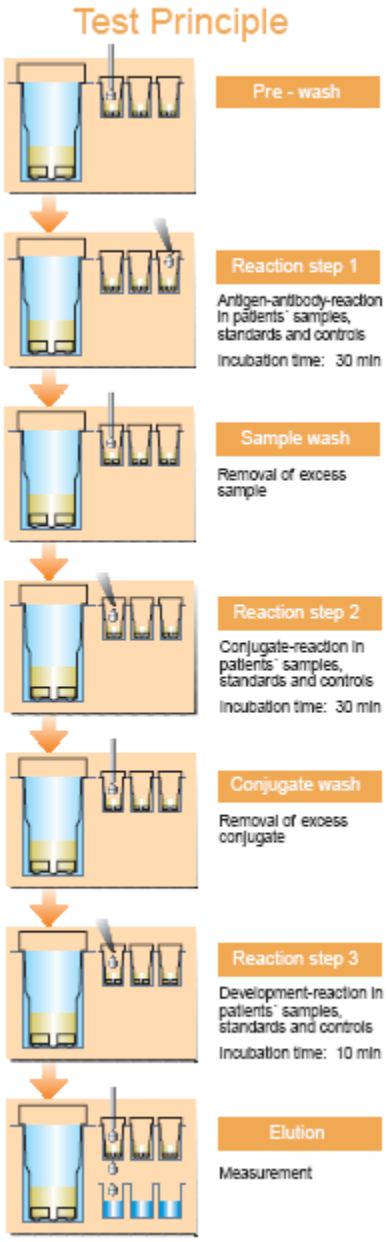
Appendix A

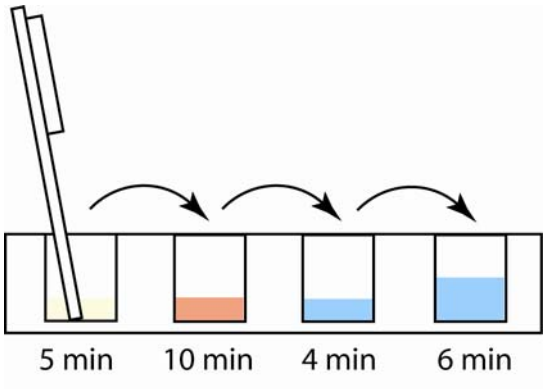
Overview table of the used monoclonal antibodies and studies made. (Green = anti-IgE antibodies, blue = anti-Bet v 1 and yellow = anti-Cor a 8 antibodies.)

	ImmunoCAP System			ImmunoCAP Rapid				Biacore
mAb	**Affinity**	**Concentration**	**Depletion**	**Capacity and affinity**	**Intervening wash**	**Pre-wetting**	**Radioactivity**	**Kinetics**
46	X			X				X
54	X			X				X
95	X			X				X
121	X			X				X
164	X	X	X	X	X		X	X
205	X			X				X
209	X	X		X	X			X
214	X			X				X
215	X			X				X
216	X	X		X	X			X
230	X		X	X	X			X
233	X			X				X
236	X			X	X			X
346	X			X	X			X
347	X			X				X
349	X			X				X
350	X			X				X
351	X			X				X
352	X			X				X
353	X			X				X
354	X			X	X		X	X
390	X	X		X	X		X	X
402	X			X				X
406	X			X				X
793	X		X	X	X		X	X
894	X		X	X	X	X	X	X
974	X			X				X
975	X			X				X
976	X			X				X
977	X			X				X

Appendix B



Typical ImmunoCAP system test run procedure. (Figure shown with permission from Phadia AB)



Typical half dipstick test run procedure.

Appendix C

Biacore test method setup.

General settings

Sample compartment temperature	25°C

Cycle types

Start-up
Test cycles

Start-up cycle

Sample

Solution	Selected sample antibody
Contact time [s]	180
Dissociation time [s]	60
Flow rate [µl/min]	10

Regeneration

Solution	Glycine pH 1.7
Contact time [s]	180
Flow rate [µl/min]	30

Test cycle

Capture

Solution	Sample antibody
Contact time [s]	15
Flow rate [µl/min]	10
Stabilization period [s]	60

Sample

Solution	Sample analyte (antigen)
Contact time [s]	360
Dissociation time [s]	1200
Flow rate [µl/min]	30
Concentration	Variable

Regeneration

Solution	Glycine pH 1.7
Contact time [s]	180
Flow rate [µl/min]	30