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"Discovery of Cross-Reactive Antibodies Binding the Major Birch Pollen Allergen Bet v 1 and the Major Apple Allergen Mal d 1 using Yeast Display''

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In loving memory of Anna Mark

Abstract

8-16% of the population in northern and central Europe are affected by seasonal birch pollen allergies. In addition, birch pollen is able to induce complex patterns of immunological cross-reactivity against non-allergenic food allergens, most frequently apples. It is believed that structural similarities between the major birch pollen allergen Bet v 1 and the major apple allergen Mal d 1 allows for immunological cross-reactivity. Recently, it has been demonstrated that pre-selected, allergen-specific monoclonal IgG antibodies (blocking antibodies) can be used for the treatment of allergic disease. Therefore, sufficiently affine cross-reactive antibodies should allow the simultaneous treatment of multiple birch pollen related allergies. Since successful allergen-specific immunotherapy (AIT) is associated with the induction of allergen-specific IgG antibodies we used yeast display to capture and screen the IgG repertoire of a patient who had undergone AIT with recombinant Mal d 1. Heavy and light chain libraries were created in yeast strains of opposite mating type and combined into a combinatorial Fab display library by yeast mating. Using a combination of magnetic and fluorescence activated cell sorting we were able to isolate two cross-reactive antibodies that contained the same heavy chain with two different light chains, and bound Mal d 1 with and EC_{50} value of 97 nM and 130 nM, and Bet v 1 with 62 nM and 110 nM in immobilized antigen ELISA, respectively. Whereas the affinities of the discovered antibodies are well above the affinities necessary to be used as clinical blocking antibodies, they might be useful tools to map cross-reactive epitopes.

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List of Abbreviations

Ac	Acetate
Aga	a-Agglutinin
AIT	Allergen-specific immunotherapy
APC	Antigen presenting cell
Bet v 1	*Betula verrucosa* allergen 1
BSA	Bovine serum albumin
Cγ/ε	γ/ε heavy chain constant domain
$C_{H/L}$	Heavy/Light chain constant domain
CD	Cluster of differentiation
CDLI2T	Christian Doppler Laboratory for Innovative Immunotheraputics
cDNA	Complementary DNA
CDR	Complementarity determining region
DBT	Department of Biotechnology
DNA	Deoxyribonucleic acid
EC_{50}	Half maximum effective concentration
ER	Endoplasmic reticulum
Fab	Antigen binding fragment
FACS	Fluorescence activated cell sorting
Fc	Crystallizable fragment
Fcγ/εR	IgG/IgE receptor
Fel d 1	*Felis catus domesticus* allergen 1
HCDR3	Heavy chain CDR 3
HEK	Human embryonic kidney
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
Ig	Immunoglobulin
IGHV	Ig heavy chain variable domain
IL	Interleukin
ILC	Innate lymphoid cell
MACS	Magnetic cell sorting
Mal d 1	*Malus domesticus* allergen 1
MAT	Mating type
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PEI	Polyethylenimine
PLMB	Patient library Mal/Bet
POI	Protein of interest
PR-10	Pathogenesis-related class 10
pYD	Plasmid yeast display
RNA	Ribonucleic acid
RT	Room temperature
scFav	Single chain Fab
scFv	Single chain variable fragment
SCIT	Subcutaneous immunotherapy
SEC	Size exclusion chromatography
SLIT	Sublingual immunotherapy
TSLP	Thymic stromal lymphopoietin
$V_{H/L}$	Heavy/Light chain variable domain
W2	CARSLTVAGWWAT (HCDR3 sequence)
Y4	CAKRPAVAGGRYYYYGMDV (HCDR3 sequence)

1 Introduction

By current estimates, allergic diseases now affect up to thirty percent of the population in Western countries and the prevalence of allergic disorders is rising globally [1]. Allergic disease results from a maladaptive immune response that promotes the synthesis of antibodies directed at a special class of antigens called allergens. Most allergens are otherwise harmless environmental proteins. In affected individuals, however, exposure to them can cause an array of disorders ranging from allergic dermatitis to anaphylaxis [2]. Diseases that are caused by an exaggerated immune response are called hypersensitivities. Most allergies are characterized by the synthesis of immunoglobulin E (IgE) and therefore classified as type I hypersensitivities [3].

1.1 Development of Allergic Disease

According to current understanding, the development of allergic disease begins with sensitization at barrier epithelia which act as the primary contact site for allergens. Upon encounter, the allergen and other hydrophilic substances are released from the carrier particle (e.g. pollen, dander, etc.), and engage innate pattern recognition receptors on epithelial cells and resident antigen presenting cells (APC). This triggers the production of the pro-allergic epithelial alarmins interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP), and of other more broadly acting pro-inflammatory cytokines. This cytokine milieu polarizes activated T cells and innate lymphoid cells (ILCs) towards a T_H2 phenotype, characterized by the expression of IL-4, IL-5 and IL-13. Collectively, these processes are referred to as allergic inflammation. IL-4, in particular, stimulates B cells to undergo class switching and produce allergen-specific, importantly polyclonal, IgE antibodies. Contrary to other Ig classes, IgE is mostly bound at cellular surfaces, especially on mast cells and basophils. There, it effectively forms “allergen receptors” that sensitize these cell types to an allergen [4]. The effector phase of an allergic response is initiated when cell bound IgE is cross-linked by multivalent allergens. This triggers the degranulation of mast cells and basophils and the release of pre-formed mediators such as histamine, which in turn cause vasodilation, erythema, swelling, contraction of smooth muscle cells and increased production of mucus. Symptoms appear within minutes of antigen exposure and are collectively referred to as the immediate type I hypersensitivity reaction. Mast cells and basophils responding to IgE clustering also secrete a broad range of newly synthesized cytokines, lipid mediators and growth factors, which lead to tissue infiltration by eosinophils, T cells and additional basophils [5]. This can result in the reappearance of symptoms hours after allergen exposure and the subsidence of initial symptoms and is thus called the late phase type I hypersensitivity reaction [3].

1.2 Immunoglobulin E

Like other human Ig classes (with the possible exception of IgG_4), IgE comprises two identical heavy chains and two identical light chains. Both chains fold into 110 amino acid domains of the prototypical Ig-fold, which combine into the characteristic Y-shaped molecule. Unlike IgG, IgD and IgA, that have three heavy chain constant domains, the ε heavy chain comprises four constant domains (Cε1-4) and is therefore more similar to IgM. The IgE specific Cε2 domains are positioned C-terminally of the relatively short hinge between the antigen binding fragments (Fab) and the IgE crystallizable fragment (Fc). They are disulfide-linked and thus take over part of the function of the long hinge region in IgG (Fig. 1A)[6]. Due to Cε2, IgE assumes an asymmetrically bent conformation, in which the Cε2 dimer folds back onto the rest of the Fc (Fig. 1B). This conformation is stabilized by extensive contacts between Cε2 on one chain and Cε3 on the other chain. The

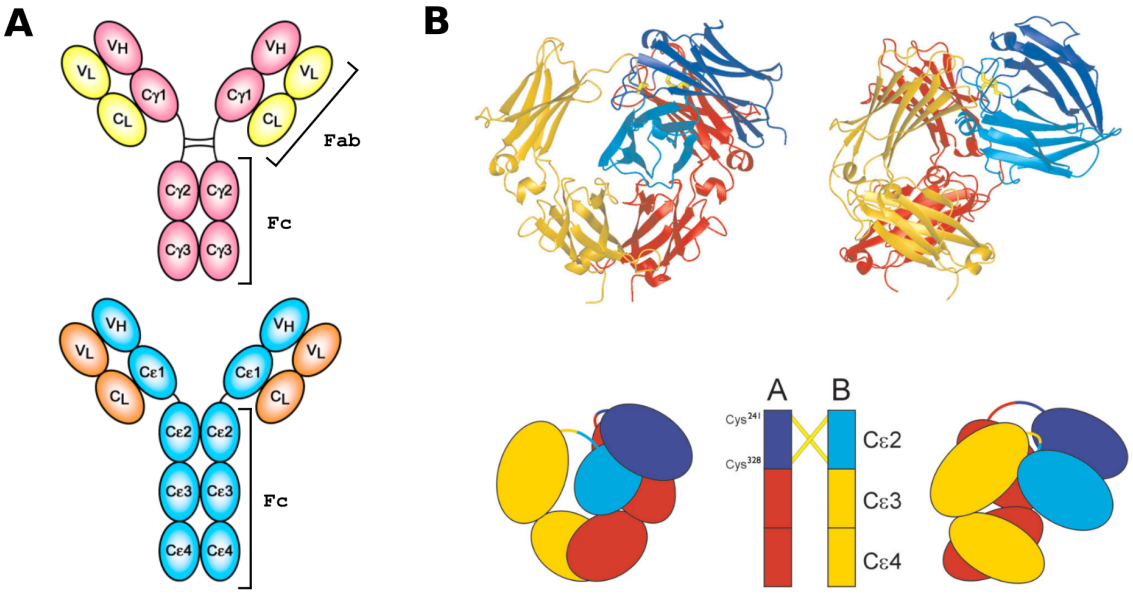

Figure 1: Structure of IgE. **A:** Domain structure of IgG (top) and IgE (bottom). **B:** Crystal structure of IgE-Fc in two orthogonal views (top). Color code corresponds to the schematic representation below. V: Variable domain; C: Constant domain; H: Heavy chain; L: Light chain; γ: γ-heavy chain (IgG); ε: ε-heavy chain (IgE). A adapted from [7], B adapted from [8].

second Cε2 domain points away from the rest of the molecule and interacts very little with it. Cε3 and Cε4 are homologous to Cγ2 and Cγ3 and form a structure that is similar to the Fc of IgG [8]. This, together with the similarity of IgE to IgY, an immunoglobulin in birds, led to the hypothesis that the six-domain Fc represents an ancestral phenotype whereas the hinge region subsequently evolved to allow greater flexibility between Fabs and the Fc [7].

There are currently two known receptors for IgE: The high affinity IgE receptor (FcεRI) is a member of the Ig-superfamily and binds IgE with a K_D of $\sim 10^{-10}$ M, which is uniquely strong among Fc receptors. Binding of IgE-Fc occurs via Cε3 in the bent Cε2-Cε3 linker region. In its tetrameric form, FcεRI is strongly expressed on the surface of mast cells and basophils at densities of up to 200 000 molecules per cell. This leads to the sequestration of most IgE at cell surfaces resulting in IgE having the lowest serum concentration of all Ig classes [6]. Factors that influence allergen mediated FcεRI cross-linking and effector cell release include the total amount of bound IgE, the ratio of allergen-specific to non-specific IgE, the number of epitopes recognized by a patient's repertoire (i.e. clonality) and its affinities [9]. Additionally, a trimeric isoform of FcεRI is expressed by Langerhans cells and peripheral blood dendritic cells, eosinophils and smooth muscle cells. The expression of FcεRI by APCs has been shown to markedly increase the efficiency of antigen presentation to $CD4^+$ T cells [6].

In contrast, the low affinity IgE receptor (FcεRII), also called CD23, is an atypical Ig receptor belonging to the calcium dependent (C-type) lectin superfamily. It binds IgE with a K_D between 10^{-6} and 10^{-7} M and is mainly expressed on B cells. It appears to be involved in the regulation of IgE production, and can facilitate antigen presentation and T cell activation by B cells similar to FcεRI on other APCs. Both mechanisms are believed to enable efficient T cell activation in spite of the very low concentrations at which allergens are typically encountered [6]. Although IgE might be best known for its involvement in allergic disease there is evidence that IgE-mediated immunity is important in the response to parasitic infection and animal venoms [10].

1.3 Birch-Pollinosis and Birch-Pollen Related Food Allergy

Birch pollen allergy is a frequent cause of seasonal allergic rhinoconjunctivitis in northern and central Europe. Sensitization in the general population is estimated to range between 8% and 16% [11, 12, 13]. Allergic rhinoconjunctivitis is characterized by the IgE-mediated inflammation of the membranes lining the nose and eyes. Symptoms include rhinorrhea, nasal obstruction, sneezing and itching. It severely impacts the quality of life of affected individuals through diminished quality of sleep, fatigue, impairment of cognitive function, and reduced physical activity and social life [14]. Its economic impact is often underestimated due to its relatively low direct cost for affected individuals, such as the cost of over-the-counter medications. However, the indirect cost associated with productivity loss is substantial [15]. A study from Sweden estimated the mean productivity loss from allergic rhinitis to be 751€ per individual and year in 2016, summing up to an annual cost of 1.3 billion euros for Swedish society [16].
Sensitization to birch pollen is notably higher in patients diagnosed with other tree pollen allergies, ranging from 50% to 90% [13, 17, 18]. This led to the realization that birch pollen is able to induce complex patterns of immunological cross-reactivity and is actually a major elicitor of such allergies. Subsequently, the birch homologous group was defined including allergens of several other tree species in the order Fagales, notably alder (*Alnus glutinosa*), hazel (*Corylus avellana*), and beech (*Fagus sylvatica*)[13, 19].
Immunological cross-reactivity, however, is not limited to the birch homologous group and aeroallergens. Approximately 70% of birch pollen-allergic patients frequently experience food-related hypersensitivity reactions upon contact with Rosaceae fruits (mainly apple, but also peach), Apiaceae vegetables (e.g. carrots) and nuts (e.g. hazelnuts) [13, 20]. Birch pollen-related food allergies usually cause mild local symptoms such as itching and angioedema of the lips, tongue and throat. More severe symptoms, such as dysphagia or throat swelling, may occur, with some patients also experiencing systemic reactions including urticaria, rhinitis, or anaphylaxis [13].

1.4 Molecular Basis of Cross-Sensitivity

Sensitization most frequently occurs towards the major birch pollen allergen Bet v 1 (*Betula verrucosa* allergen 1) [13, 22]. Bet v 1 is a small ($\sim$ 17 kDa) protein belonging to the PR-10 (pathogenesis-related) family. PR-10 proteins are one of 17 classes of plant proteins that are upregulated in response to biotic and abiotic stress. Their definitive biological function, however, remains elusive. They are present in a wide variety of flowering plants and appear to be strongly conserved [23]. Bet v 1, for example, shares $\sim$ 75% amino acid identity with the pollen allergens of the homologous group and 56-60% identity with the Rosaceae homologs. This high degree of similarity is believed to allow IgE and T cells directed against Bet v 1 to recognize related homologs, which are jointly referred to as PR-10 allergens (Fig. 2). This recognition can then trigger a hypersensitivity reaction in the absence of the sensitizing allergen [24]. However, with repeated exposure, epitope spreading might induce the development of B and T cells reactive exclusively to the new allergens [25, 26]. The degree of cross-reactivity is dependent on the structural conformation of the respective homolog and the epitope repertoire recognized by a patient's IgE and T cells [13]. Some homologs like apple and hazelnut are more frequently recognized than others, like celery or soy, which roughly correlates with the similarity of these allergens to Bet v 1 [24]. Interestingly, most secondary allergens are not allergenic by themselves. For example, the major apple allergen Mal d 1 (*Malus domesticus* allergen 1) is inefficient at inducing IgE antibodies, although almost all major epitopes of Mal d 1 are also present in Bet v 1 and apple allergy without sensitization to Bet v 1 is rare [13, 24].

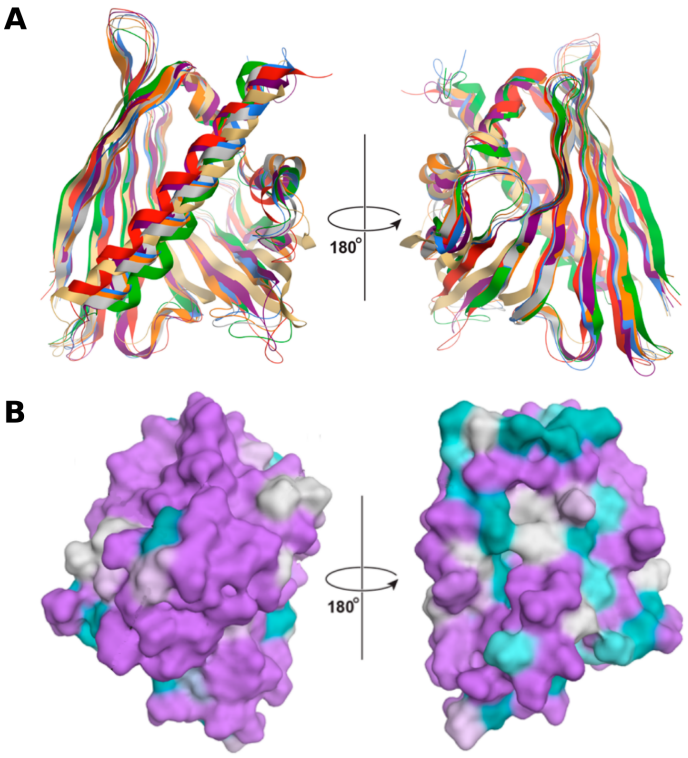

Figure 2: Comparison of PR-10 allergens. Structural similarities between these proteins are believed to enable immunological cross-reactivity. **A:** Overlay of the apple allergen Mal d 1.0101 (green, PDB: 5MMU), the major birch pollen allergen Bet v 1.0101 (blue, PDB: 4A88), the carrot allergen Dau c 1.0103 (orange, PDB: 2WQL), the celery allergen Api g 1.0101 (gray, PDB: 2BK0), the soybean allergen Gly m 4.0101 (yellow, PDB: 2K7H), the strawberry allergen Fra a 1E (red, PDB: 2LPX), and the cherry allergen Pru av 1.0101 (purple, PDB: 1E09). Numbers indicate allergen, decimal points indicate isoform. **B:** Similarity of surface amino acids between Bet v 1.0101 and Mal d 1.0101 using a color gradient from purple (highly similar) to turquoise (highly dissimilar). Figure adapted from [21].

1.5 Treatment of Allergic Disease

There are currently four approaches to the treatment of allergic diseases: (1) allergen avoidance, (2) symptomatic treatment with antihistamines or glucocorticoids, (3) immune modulation via biologics and (4) allergen-specific immunotherapy. Allergen avoidance, though optimal, is not generally applicable, especially for ubiquitous environmental allergens like pollen. Furthermore, patients that depend on avoidance (e.g. patients allergic to peanuts) constantly have to monitor their surroundings, which greatly impacts their quality of life. Antihistamines are histamine receptor blockers directed at reducing the effects of this mast cell mediator, whereas glucocorticoids are broadly anti-inflammatory substances. Both are effective but may have undesirable side effects and show no lasting effect after discontinuation. Recently, antibody drugs intended to counter the T_H2 response have become available. Targets include the IgE constant domain (Omalizumab), IL-4 receptor subunit alpha (Dupilumab) and TSLP (Tezepelumab). All have been successfully applied in clinical practice but may interfere with other functions of these factors limiting their use to extreme cases. Allergen-specific immunotherapy, on the other hand, can induce long lasting allergen tolerance via immune modulation [27].

1.6 Allergen-Specific Immunotherapy

Allergen-specific immunotherapy (AIT) involves the repeated administration of allergen over several years, either by injection (subcutaneous immunotherapy, SCIT) or sublingually (sublingual

immunotherapy, SLIT). Through a mechanism that is not yet fully understood this can induce a tolerogenic immune response. Such a response is characterized by the production of regulatory T cells expressing anti-inflammatory IL-10, which can inhibit the differentiation of T_H2 cells and stimulate allergen reactive B cells to undergo class switching in favor of IgG and IgA. These antibodies are believed to sterically block IgE from binding allergen and thereby prevent a type I hypersensitivity reaction (Fig. 3) [28].

IgE blocking activity is mostly, but not exclusively, associated with the IgG_4 subclass [29, 30]. Similar to IgE, class switching to IgG_4 can be triggered by IL-4 and IL-13, and IgG_4 has also been implicated in the response against parasites [31, 32]. One important regulator of IgG_4 production, however, is IL-10, which modulates class switching to this subclass and is also required for the differentiation of IgG_4^+ B cells into plasma cells [31]. Structurally, IgG_4 is unique in that the disulfide bonds in the hinge region that connect the two halves of the molecule are weaker than in any other Ig class, which allows reduction and reoxidation *in vivo*. This leads to the random combination of IgG_4 half-molecules of different specificities and to the formation of monovalent bispecific antibodies [32]. As a consequence, IgG_4 cannot cross-link target antigen and form immune complexes. Moreover, IgG_4 has a low affinity for activating IgG receptors (FcγRI, FcγRIIa, etc.), while retaining a relatively high affinity for the inhibitory FcγRIIb. Together this suggests that IgG_4 may have evolved as a way to limit excessive immune responses against non-infectious antigens [31]. Interestingly, Shamji and co-workers demonstrated that functional, rather than total allergen-specific IgG_4 correlated with clinical response in grass pollen AIT [33]. This suggests that apart from allergen recognition, factors like affinity or epitope specificity have an influence on the potency of blocking antibodies.

In addition to the formation of allergen-specific IgG, SLIT is able to induce the production of IgA. IgA is interesting in this context, because it gets transported across mucosal epithelia and is therefore able to bind and neutralize allergen before it enters the body [6]. Moreover, it has been demonstrated that IgA_2 mediated binding of allergen can stimulate IL-10 production in monocytes, hinting at an alternative contribution of IgA to tolerance induction [34]. Suppression of allergic inflammation is particularly interesting in light of data that suggest that the IgE memory compartment is small and that IgE expressing plasma cells mostly derive from IgG^+ memory cells under the stimulation of T_H2 cytokines, at least in the context of food allergy [35].

The clinical efficacy of AIT is generally reported at 30-40% improvement of allergy symptoms or reduction in the use of rescue medication. No significant difference in efficacy between SCIT and SLIT has yet been demonstrated [28, 36]. A 2019 retrospective study determined that 78% of patients that had received AIT met the criterion of at least 30% improvement compared to 23% in placebo treated controls. However, the result of AIT varied between allergens, with about 80% of patients meeting this criterion in birch and grass pollen AIT, but only about 50% in AIT for house cats or pollen of *Artemisia* spp. Moreover, a worse clinical outcome is associated with older age (>35 years), a longer history of allergic disease before the start of therapy, more severe disease (e.g. concomitant atopic dermatitis or allergic asthma) and polysensitization. In such cases, response rates ($\geq$30% improvement) can be as low as 20%, which highlights the need for alternative treatment options [37].

1.7 Passive Immunization with Allergen-Specific Blocking Antibodies

Recently, insights on the mechanism of tolerance induction have inspired a novel therapeutic approach. Ten years ago Flicker *et al.* demonstrated that an allergic response in rats sensitized for two different grass pollen allergens and Bet v 1 could be prevented by supplementation of IgG from rabbits immunized with the respective allergen [38]. Subsequently, Orengo and colleagues utilized mice with humanized antibody loci in combination with *in vitro* display methods to gen-

erate high affinity antibodies against the immunodominant cat allergen Fel d 1 (*Felis catus domesticus* allergen 1). They showed that these pre-selected, high affinity IgG could suppress mast cell degranulation *in vitro* with comparable or greater potency than sera from patients who had undergone AIT. Moreover, they found that only a mixture of two antibodies (REGN1908–1909) with non-overlapping epitopes was able to block mast cell degranulation and prevent reactivity to Fel d 1 in a mouse model for allergy [39]. REGN1908–1909 have undergone further clinical development and recently completed a Phase II trial, with consistently reduced nasal symptoms after challenge with cat hair extract in treated patients [40]. The same approach has since been applied to Bet v 1 which demonstrated that treatment with a cocktail of three monoclonal antibodies (REGN5713-5715) gave similarly promising results in a Phase I trial with birch pollen allergic patients [41, 42]. Both studies emphasized that only a combination of antibodies had full potency, which concurs with the hypothesis of steric blockage of IgE (Fig. 3).
In conclusion, passive immunization with blocking antibodies is a promising avenue in the treatment of allergic disease: Being directed at the allergen rather than the immune system adverse side effects should be minimal. Moreover, since the selected blocking antibodies have defined properties, treatment results are expected to be more consistent than those from AIT. Additionally, this approach is applicable to all allergens for which antibodies of sufficient quality can be engineered. This engineering process, however, is also a major bottleneck. For example, Orengo and colleagues had to screen more than 300 antibodies to find an effective Fel d 1 blocking combination and for the discovery of the anti-Bet v 1 antibody cocktail, 1000 antibodies were screened of which 20 were selected for combinatorial testing [39, 41].

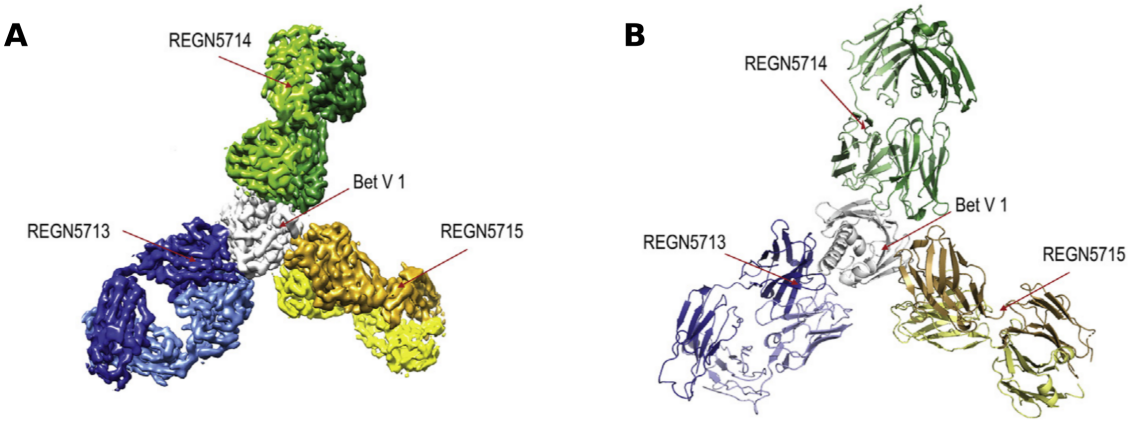

Figure 3: Cryo-electron microscopy (Cryo-EM) structure of Bet v 1 in complex with the Fab fragments of REGN5713-5715. **A:** Cryo-EM map **B:** Model of the same complex in ribbon representation. Binding of REGN5713-5715 to Bet v 1 has been shown to prevent degranulation of IgE sensitized mast cell *in vitro* and *in vivo*. It is believed that REGN5713-5715 sterically block IgE from binding allergen. Figure adapted from [41].

1.8 Yeast Surface Display of Antibodies

Surface display technology has become a powerful tool for the discovery of target-specific antibodies, augmenting the revolutionary hybridoma technology and animal immunization. The principle of all surface display methods is the expression of a protein of interest (POI) on the surface of a carrier particle that physically links it to its genetic information. Then a large number of such particles carrying different variants of the POI is prepared, creating a so-called library. This library can then be screened for desirable properties on the protein level (phenotype), whereas isolated variants can be identified and manipulated on the genetic level (genotype). Many different display platforms have been developed, including phage display, bacterial display, yeast display and even

acellular systems like ribosome display [43].
In yeast display, yeast cells are used as carrier particles. Most frequently, *Saccharomyces cerevisiae* is employed, but display systems for other species have also been developed. Similar to other cellular systems, surface display is achieved by genetically fusing a POI to a cell surface protein [44]. In *S. cerevisiae*, the a-agglutin system is most widely used for surface tethering [43]. A-agglutinin is a cell adhesion protein located in the outer cell wall of *S. cerevisiae*. It is a heterodimer consisting of an anchoring subunit (Aga1), covalently attached to cell wall glycan via a truncated GPI anchor [45], and a receptor subunit (Aga2) that is bound to Aga1 via two disulfide bonds [46]. Due to this arrangement, Aga2 is amenable to both C- and N-terminal fusion, allowing for the flexible orientation of displayed proteins [47, 48].
Surface display of antibodies is complicated by two factors: First, displayed proteins are generally tethered to the carrier particle at a single attachment point, which poses a problem in the display of multimeric proteins. Second, antibodies are large and complex glycoproteins, which might not be highly expressed in all host organisms. These led to the development of smaller and simpler antibody fragments that are still able to recognize antigen. Of these, the most widely used format are single chain variable fragments (scFv), which only consist of the variable domains of both heavy (V_H) and light (V_L) chain, fused into a single polypeptide by a flexible linker [43]. ScFvs are convenient due to their small size, high expression levels and ease of handling as a single polypeptide chain. However, it can happen that the affinity discovered through scFv, is lost upon expression as full-length IgG [49]. The problem is reduced if antibodies are displayed as Fab. This format resembles the natural domain positioning more closely, as Fab fragment has a high degree of structural autonomy [50] and, in comparison with scFv its functional properties are typically better translated upon reformatting [49, 51]. Fab consists of the light chain (V_L-C_L) and the variable and first constant domain of the heavy chain (V_H-C_H1), which are held together by an inter-chain disulfide bond and non-covalent interactions. This assembly is generally stable, however, Fab is less efficiently expressed in prokaryotic systems, due in part to the interchain disulfide bond [49, 52]. In yeast, however, Fab is displayed at densities of about 10^4-10^5 molecules per cell. A side-by-side comparison of scFv, Fab, single chain Fab (scFab) and truncated scFab missing the inter-chain disulfide bond identified Fab as the optimal display format in yeast display [51]. Moreover, the compatibility with fluorescence activated cell sorting (FACS) is one of the greatest advantages of yeast display systems allowing on-line monitoring and fine calibration of clone selection, as well as multi-parameter screening [53].
Another crucial step in any antibody display system is library generation. Recombinant antibody libraries can be grouped into three categories based on the source of antibody diversity: Naïve and immune libraries are constructed from natural sources, like human or animal B cells. Synthetic libraries, on the other hand, are created entirely *in vitro*, whereas semi-synthetic libraries combine these two approaches, for instance by grafting naturally occurring complementarity determining regions (CDR) onto a pre-selected framework with favorable biophysical properties [43]. The functionality of these libraries is critically dependent on the constituent antibody diversity, with larger, more diverse libraries generally yielding higher affinity antibodies [54]. This is a problem in yeast display, because library size is limited by the relatively low transformation efficiency achievable with *S. cerevisiae* [55]. However, there are several strategies to compensate for limited library size: First, yeast display can be used in combination with highly functional immune libraries, which are constructed from one or multiple donors that have either been vaccinated, or recovered from an infection. The resulting antibody repertoire is strongly biased towards the respective antigen, because it has been pre-selected and has undergone affinity maturation *in vivo* [43]. Immune libraries yield on average higher affinity antibodies compared to other library designs, but are limited to non-toxic and non-self targets [56]. The second strategy uses the heterodimeric nature of Fab to its advantage. *S. cerevisiae* can grow vegetatively as both haploid and

diploid cells. Mating exclusively occurs between haploid cells of opposite mating types (MATa and MATα) and is essentially random [57]. If heavy and light chain are cloned into separate vectors, yeast mating offers an elegant way to create large combinatorial libraries from modestly sized sub-libraries [53, 55].

1.9 Aim of this Thesis

This project aimed at the identification of antibodies binding either the major birch pollen allergen Bet v 1, or the major apple allergen Mal d 1 using yeast display. Ideally, a cross-reactive antibody with high affinity for both allergens would be isolated. To this end, a Fab yeast display library was constructed from the IgG repertoire of a patient who had undergone AIT for birch pollen related apple allergy treated in a pilot study with SLIT using recombinant Mal d 1 [58]. Analysis of the sera from patients who had participated in this study demonstrated that AIT was capable of inducing IgE blocking activity, and that this blocking activity was associated with the IgG compartment, specifically IgG_1 and IgG_4 [59]. Here, we describe the construction and screening of an antibody library obtained by combining the patient's heavy chain repertoire with the kappa light chain repertoire through yeast mating.

2 Materials and Methods

2.1 Experimental Outline

Peripheral blood mononuclear cells (PBMCs) isolated from post-SLIT serum were used as the source of rearranged antibody genes. For the capture of the heavy chain repertoire, first, total RNA was extracted and reverse transcribed into cDNA. Then a three step PCR protocol was used to selectively amplify IgG heavy chains and introduce homology sequences necessary for cloning. Initial repertoire capture was carried out by multiplex PCR using a commercially available primer set designed by Larrick and co-workers [60]. These primers bind in the leader sequence upstream of the immunoglobulin heavy chain variable fragment gene (IGHV), which encodes an N-terminal signal peptide that is important for antibody processing and secretion, but gets cleaved off during maturation [61]. Variable fragment genes are diverse, but all are preceded by a leader sequence, which is more strongly conserved than the corresponding IGHV genes [62], and thus allows precise amplification of variable domain families with relatively few oligonucleotides. The primer set comprises three subsets: Subset A is designed to target the IGHV1 family, B is directed at IGHV3, and C at IGHV2 and IGHV4 (Fig. 4). These IGHV families together are present in >95% of IgG heavy chains, whereas IGHV5, IGHV6 and IGHV7 are more rarely used [63]. However, PCR was performed under conditions that were selective for antibody transcripts, but allowed "cross-family" amplification. Heavy chains of the IgG class were selected by using a reverse primer specific for the C-terminus of the IgG-C_H1 domain, which is similar in all sub-classes.
Whereas this project relied on a biologically biased repertoire, technical biases had to be minimized for accurate reproduction. Biased amplification, a well-documented phenomenon in multiplex PCR, can arise from several identified mechanisms [64]. In our system the predominant form of bias is likely selection bias. PCR selection refers to biases caused by properties of the template that influence primer-target interactions. Sequences that closely match a primer will be amplified more readily than their less similar counterparts, as visualized in Fig. 4. Due to the exponential nature of PCR, sequences that are not copied during the first few cycles will be severely under-

	Hu IgVH5'-A	Hu IgVH5'-B	Hu IgVH5'-C
	ATGGACTGGACCTGGAGGRTCYTCTKC	ATGGAGYTTGGGCTGASCTGGSTTTYT	ATGRAMMWACTKTGKWSCWYSCTYCTG
IGHV1-18*01-A|2	----------C-----T---	-----C-GGACCTG----ATC-----C	------TGGACC----------T-C
IGHV1-24*01-A|2	--------C----------	-----C-GCACCTG---GATC--C--C	------TGCACC-----G------T-C
IGHV1-2*02-A|68	--------C----------	-----C-GCACCTG---GATC--C--C	------TGCACC-----G------T-C
IGHV2-26*01-A|98	-------CACTT--CTAC-CA---C-G	-----CACACTTTGCTA-ACA--CC-G	-------C------C-A---A------
IGHV2-5*01-A|57	-------CACTT--CTCC-CG---C-G	-----CACACTTTGCT--ACG--CC-G	-------C------C----------
IGHV2-70*01-A|63	-------TACTT--TTCC-CG---C-G	-----CATACTTTGTT--ACG--CC-G	----------
IGHV3-11*01-A|76	-----G-TTGGGCT---CTGGG-T---	----------C	-----GT-TGG-CT-----G-G--T-C
IGHV3-13*01-A|61	-----G-T-GGGCT---CTGGG-T---	--------G----------C	-----GT-GGG-CT-----G-G--T-C
IGHV3-15*01-A|96	-----G-TTGGGCT---CTGGA-T---	----------A----C	-----GT-TGG-CT-----G-A--T-C
IGHV4-30-2*01-A|3	---A-ACACCTG---TTCT-----C-G	---A-A-ACCT-TG-TT--TC--CC-G	--------C-------T----------
IGHV4-31*01-A|9	---A-ACACCTG---TTCT-----C-G	---A-A-ACCT-TG-TT--TC--CC-G	--------C-------T----------
IGHV4-34*01-A|98	---A-ACACCTG---TTCT-----C--	---A-A-ACCT-TG-TT--TC--CC-C	--------C-------T---------C
IGHV5-10-1*01-A|22	----GG-CA---GCC-TCC-TGG-C--	----G--CAACCGCC-T-CTT-GCC-C	----GGTC-ACCGCC-T-C-TGG---C
IGHV5-51*01-A|87	----GG-CA---GCC-TCC--G--C--	----G--CAACCGCC-T-CTC-CCC-C	----GGTC-ACCGCC-T-C--GC---C
IGHV6-1*01-A|95	---TCTGTCT---TCCTC------C-G	---TCTG-CTCCT-CCT-ATCT-CC-G	---TCTG-CTCC-TCCT----T-----
IGHV7-4-1*01-A|1	----------	-----C-GGACCTG---GATC--C--C	------TGGACC-----G------T-C

Figure 4: Alignment of the primer sets used for initial repertoire capture and IGHV leader sequence genes. Each of the three primer sets shows a varying degree of similarity with the IGHV families, with sequences that are more similar likely being preferentially amplified. However, PCR was purposely performed under low stringency conditions and amplification of lower similarity sequences was frequently observed. Sequence data from [62]. For IGHV1, IGHV2, IGHV3 and IGHV4 only three variants are shown. Degenerate bases are labeled according to IUPAC convention. Alignment was performed manually.

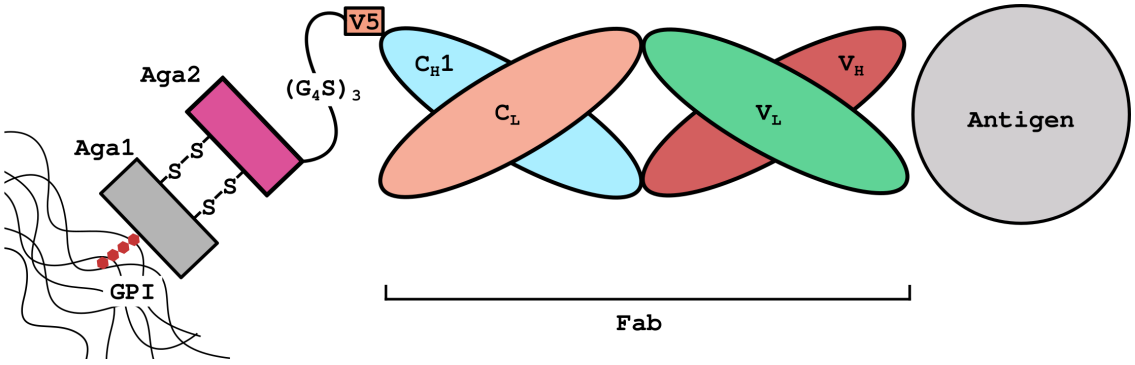

Figure 5: Schematic representation of the Fab display construct used in this study. The heavy chain was expressed as an N-terminal fusion with a-agglutinin receptor subunit (Aga2), which in turn is tethered to the anchoring subunit Aga1. Aga1 is covalently attached to the glycans of the outer cell wall of *Saccharomyces cerevisiae* via a truncated glycophosphatidylinositol (GPI) anchor. Aga2 and the heavy chain constant domain 1 (C_H1) are separated by a flexible linker sequence ($(G_4S)_3$), which serves to further increase the distance of the antigen receptor to the yeast surface, and a V5 tag, which was included for facile detection. The complete light chain (light chain variable (V_L) and constant domain (C_L)) was expressed in its native soluble form. Both chains were targeted to the secretory pathway with Fab assembly taking place in the ER and complete Fab being transported to the yeast surface for display. V_H: Heavy chain variable domain.

represented in the final amplicon [65]. However, rare sequences are just as likely to encode antibodies with desirable properties, but are more likely to be lost by chance in subsequent steps. To counter PCR bias and salvage as many rare sequences as possible, a series of secondary PCRs were performed on each pool using single IGHV gene specific primers. These primers were designed to align with the N-terminal end of the mature V-domain, a region that exhibits little variation between alleles, but enough variation between genes to allow amplification of focused sets of closely related sequences mostly independent of the gene distribution in the template pool. Simultaneously, the human leader sequence was replaced with the vector homology necessary for recombination [66].

Antibody fragments were cloned using gap-driven homologous recombination. In this process, vector and insert are co-transformed into yeast and the plasmid assembled by the cell's DNA repair machinery directed by sequence homologies [67]. While this strategy is generally efficient as a transformation method [68], successful plasmid assembly is still an unlikely event [69] and transformation efficiency is the major bottleneck limiting the size of yeast display libraries [55]. Since most of the DNA initially used is lost unproductively and recombination is limited by insert availability [70], a high copy number of each variant is necessary to achieve high numbers of independent clones. Therefore, the third PCR step was aimed at indiscriminately amplifying all variants.

IGHV family sub-libraries were created by chemical transformation using lithium acetate (LiAc) and a heat shock. Heavy chain fragment was expressed as an N-terminal fusion to Aga2 (Fig.5). N-terminal fusion leaves the V-domains pointing away from the yeast cell wall, increasing the accessibility of the antigen binding pocket [55]. Similarly, a flexible fifteen amino acid linker was placed between the C_H1 domain and Aga2 to further increase the distance to the yeast surface and keep surface interference minimal. Additionally, a V5 tag was inserted at the C-terminus of the C_H1 domain to facilitate detection. The light chain was expressed as its native, soluble form. Both chains were put under the control of the galactose inducible *GAL1* promoter and targeted to the endoplasmic reticulum (ER) by the yeast mating factor α 1 secretion leader. Mating factor α is a pheromone involved in yeast mating, but its secretion signal functions independently of mating status [71]. In the ER chaperones assist in chain folding and assembly [72]. Although the light chains themselves are not tethered to the yeast cell, coupling in the ER is efficient and capture of

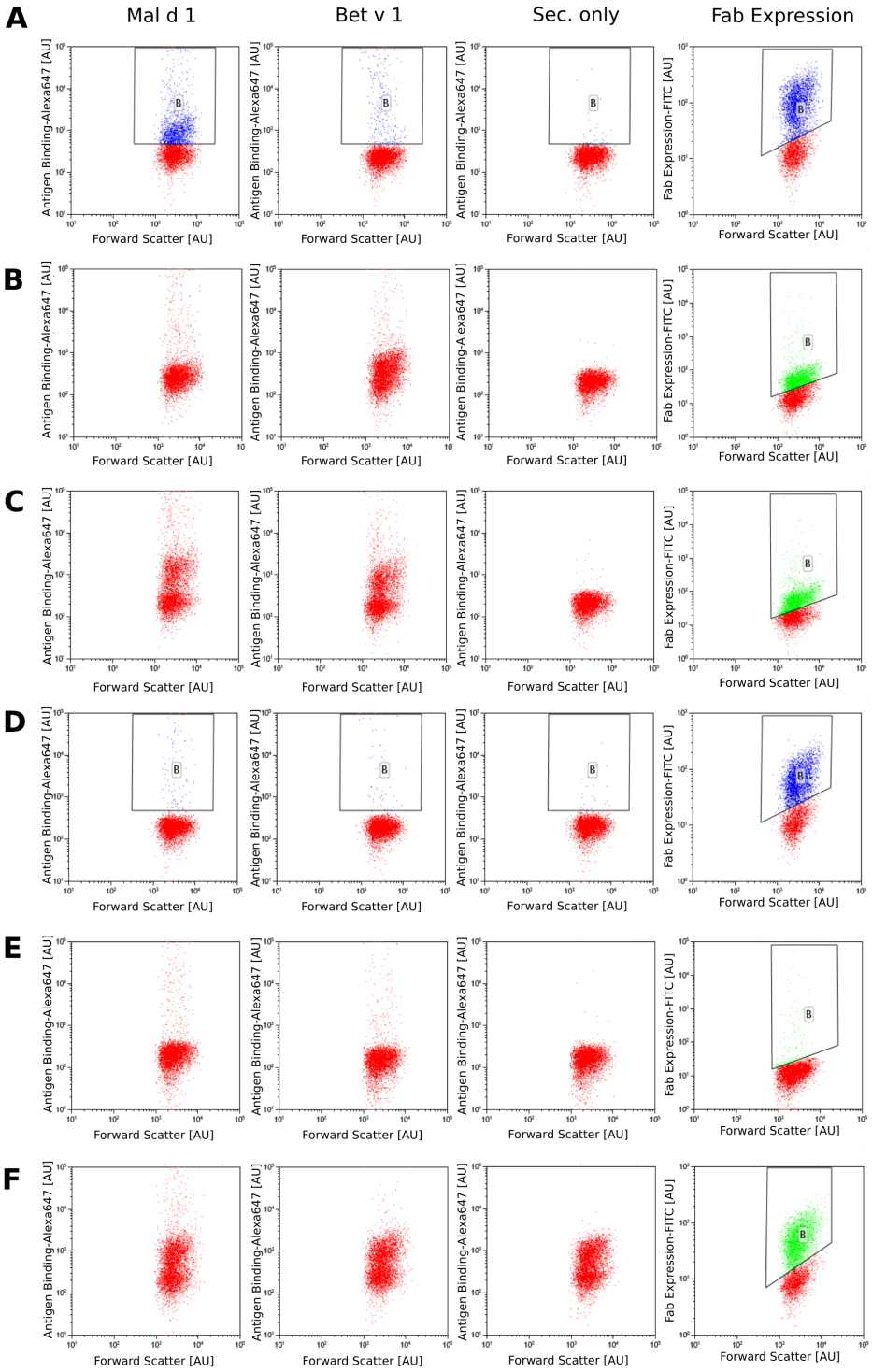

Figure 6: Representative output of flow cytometric screening of individual clones before reformatting. **A**: Mal d 1 binding clone (Mal50_2); **B**: Bet v 1 binding clone (MalBet++_17); **C**: Cross-reactive clone (MalBet++_20); **D**: Non-binding clone (Mal50_20); **E**: Non-functional clone, light chain/Fab negative (Mal100_6). Other clones in this category showed antigen binding, in spite of being light chain/Fab negative; **F**: Clone binding the streptavidin-Alexa647 conjugate secondary (Mal30.2_1). Fab expression was detected either with anti-human κ light chain antibody 3761 (A,D,F) or anti-human Fab antibody 5512 (B,C,E). Percentages of gated populations can be found in Sec. A.8.

secreted light chain by non-carrier yeast (cold capture) is rarely observed [55].
A combination of magnetic cell sorting (MACS) and FACS was used to screen the Fab display library. A MACS step was necessary because the sorting speed of FACS machines places a limit on the diversity that can be reasonably screened. MACS, on the other hand, is able to rapidly sort very large numbers of cells [73] by selectively retaining cells bound to ligand-coated magnetic beads suspended in a static magnetic field [74]. Whereas it does not allow real-time monitoring, even a relatively inefficient MACS selection step can significantly reduce the background of non-binding clones [73]. Thus, in order to reduce sorting time, one round of MACS with biotinylated Mal d 1 and streptavidin coated beads was carried out to pre-enrich binding clones and exclude non-binding or non-functional ones before FACS. An additional factor that had to be considered is the trade-off between stringency and yield. Especially in the first round of selection the library contains a very large number of different clones and desirable clones are rare. Since only very few representatives of each clone can be sampled under these conditions, sorting under too stringent conditions could easily lead to the random loss of desirable clones despite oversampling [75]. To prevent this, the general strategy is to accept lower enrichment factors in early selection rounds, expand the collected population and sort under more stringent conditions in a second selection round [76]. A related problem is variation in expression levels, because a given signal intensity can either stem from a low affinity clone with high expression level or a high affinity clone with low expression level. FACS, however, allows multi-parameter screening, and co-staining for either Fab or light chain expression was used to normalize antigen binding for expression levels [47]. Lastly, gates were chosen to select 0.1% of double positive clones, a standard value in the field if no clear double positive population is visible, but could include as much as 4% based on evaluation of the properties of the respective population during sorting (Fig. 8; [76]; Gordana Wozniak-Knopp, personal communication).
After several rounds of FACS analysis, enriched populations were plated out for subsequent screening of single clones. Since the selected Fab fragments were intended to be used in an IgG format, they had to be recloned and expressed as full length proteins. However, because reformatting and protein production are both labor intensive and time consuming, individual clones were first analyzed for antigen binding and Fab/light chain expression by flow cytometry to exclude non-binding or non-functional clones (Fig. 6 D-F). However, due to multi-copy display on the yeast cell and the resulting avidity effects, this strategy alone could not be used to identify the clones with the highest affinity. Despite that, the assay could qualitatively identify clones reactive with either Mal d 1 or Bet v 1, and cross-reactive ones (Fig. 6 A-C). Additionally all clones picked for individual screening were sequenced. Heavy and light chain genes were isolated form unique clones with correct antibody sequences and subcloned into separate pTT5-based expression vectors using Gibson assembly [77]. V_L-C_H1 and V_L-C_L fragments were PCR amplified directly from yeast lysate, using primers that introduced the necessary 5' and 3' homology sequences. At the constant 3'-end, one primer could be used for all clones, however, the forward primer, binding in the highly variable V-region, had to be selected based on the sequence of each clone individually. Both chains were cloned in frame with secretion signals, a human IGHV3 leader sequence for the heavy chain and the *E. coli* StII leader for the light chain. After the Gibson reaction, the assembly mix was transformed into *Escherichia coli* and correct assembly verified by Sanger sequencing. Correctly, assembled plasmids were amplified and purified. Then heavy and light chain plasmids were combined and used to transiently transfect human embryonic kidney cells (HEK) cells using polyethylenimine (PEI), a widely used transfection agent [78]. Antibodies were then purified from the culture supernatant using preparative affinity chromatography with a protein A column.
To get a first quantitative estimate of the affinities of the recovered antibodies, full length IgG was further characterized using ELISA with immobilized allergen. For this purpose, biotinylated antigen was bound on streptavidin coated microtiter plates and then incubated with the candi-

date antibody at concentrations ranging from 1 μM to 500 pM to determine its half maximum effective concentration (EC_{50}). Antibody binding was detected using an anti-human Fc antibody conjugated to horse radish peroxidase (HRP) as the reporter enzyme. Additionally, each candidate was characterized by Size-Exclusion Chromatography (SEC-HPLC) to verify its molecular size and to examine the potential presence of aggregates and degradation fragments. The propensity for aggregation is a concern for the developability of antibodies, as therapeutic antibodies are often formulated as highly concentrated solutions [54]. Moreover, the presence of aggregates can skew ELISA results by measuring the avidity of the complex instead of the affinity of isolated molecules.

2.2 Methods

The proprietary methodology used for repertoire cloning and display is unspecifically outlined. Primer sequences and vector maps are available at the Christian Doppler Laboratory for Innovative Immunotherapeutics (CDI2T), Department of Biotechnology (DBT), University of Natural Resources and Life Sciences, Vienna (BOKU) upon request. Composition of media used for yeast cultivation are given in [79].

2.2.1 Patient Characteristics

The patient had undergone 16 weeks of sublingual allergen-specific immunotherapy with recombinant Mal d 1 (refer to [59] for more details). PBMCs were isolated from post-SLIT blood and were supplied by Barbara Bohle frozen in liquid nitrogen.

2.2.2 RNA Extraction and cDNA Synthesis

An aliquot of 10^7 cells was thawed, the storage buffer replaced with medium (DMEM, Thermo Fisher Scientific) and the cells transferred to a 1.5 ml Eppendorf™ tube. Total RNA was extracted using TRIzol (Thermo Fisher Scientific) according to the protocol supplied with the Phasemaker Tubes™ (A33248, Thermo Fisher Scientific) but using standard 1.5 ml Eppendorf™ tubes. Yield was determined using a NanoDrop™ spectrophotometer. RNA was used directly for reverse transcription using the SuperScript™ IV First Strand Synthesis kit (Thermo Fisher Scientific) with supplied polyT primers according to the manufacturer's instructions. Complementary DNA was either used immediately or stored at -20 °C.

2.2.3 Amplification of Heavy Chain Fragments

All heavy chain fragments were PCR amplified using 1x Q5™ High-Fidelity Master Mix (New England Biolabs), 0.5 μM primers and sterile deionised water, and the touch-down PCR program: 98 °C, 3 min; 6×(98 °C, 20 s; 61 °C with - 1.5 °C/cycle, (20 s); 72 °C, 20 s); 29×(98 °C, 20 s; 52 °C, 20 s; 72 °C, 20 s); 72 °C, 5 min. An IgG specific reverse primer was used in all the reactions described below. PCR1 was carried out using the Human Ig-Primer Set (Novagen) as forward primers and 1 μl of crude cDNA for template in a total volume of 25 μl. The resulting amplicons (A, B, C) were analyzed by agarose gel electrophoresis, purified and the DNA concentration measured using NanoDrop™. In PCR2 the IGHV gene specific primers were used as forward primers and 40 ng per reaction of the PCR1 amplicons were used as templates in a total volume of 25 μl. PCR products were first analyzed by agarose gel electrophoresis. Then amplicons were purified and DNA concentration determined spectrophotometrically. Reactions that did not produce pure amplicons were excluded. PCR3 was carried out in a total volume of 100 μl using ∼80 ng per

reaction of step 2 amplicon as template. To facilitate pipetting the necessary template volume of all samples processed in one batch was averaged and this volume added to all PCR3 reactions, as long as there were no clear outliers (±10%). Each amplicon was then analyzed by agarose gel electrophoresis. Reactions that did not result in the expected product size and amount were excluded. The remaining PCR products were purified using preparative agarose gel electrophoresis and stored at -20 °C until further use.

2.2.4 Purification of PCR Products

PCR products were purified using the Illustra™ GFX™ PCR, DNA and Gel Band Purification Kit with a modified protocol: First MicroSpin™ columns and collection tubes were assembled. If DNA was purified from gel, 500 µl Capture Buffer were added to the gel slice in a 1.5 ml Eppendorf™ tube and incubated at 55 °C until gel slice was dissolved. The resulting mixture was then added onto the column. Otherwise 500 µl Capture Buffer were added to the column, followed by the PCR reaction mix. DNA was loaded onto the column by centrifugation at 11000 g for 1 min at room temperature (RT). Flow-through was discarded and 500 µl Wash Buffer added to the column followed by centrifugation (11000 g, 1 min, RT). Next, the column was transferred to a fresh 1.5 ml Eppendorf™ tube and 40 µl of sterile deionised water were delivered onto the filter. The wet column was then incubated for 5 min at RT. Product was eluted by centrifugation (11000 g, 1 min, RT).

2.2.5 Agarose Gel Electrophoresis

Gel electrophoresis was performed on 1.5% agarose TAE (40 mM tris-base, 20 mM acetic acid, 1 mM EDTA, *p*H 8) gels, stained with ethidium bromide (1.5 nM) according to standard molecular biology protocols.

2.2.6 Sanger Sequencing

Heavy and light chain fragments were PCR amplified under the following conditions: 1x MyTaq™ Red Mix (VWR), 0.5 µM primer; total volume: 10 µl; cycling: 95 °C, 2 min; 29×(95 °C, 15 s; 55 °C, 15 s; 72 °C, 15 s); 72 °C, 5 min. For display plasmid (pYD) sequencing, 2 µl of lysate, pipetted off the top of the sample to prevent transfer of cell debris were added on top of the reaction volume (total volume of 12 µl). If colonies of *E. coli* were sequenced, colonies were picked off the plate and transferred directly into the reaction mixture. PCR products in 4 µl reaction mixture were analysed by agarose gel electrophoresis. Positive reactions were prepared for sequencing by digestion of leftover primers and dNTPs with Exo I (NEB, 20 U/µl) and rSAP (NEB, 1 U/µl), respectively. Per reaction 0.25 µl each of ExoI and rSAP were each diluted in 10 µl of deionised water and 10 µl of this mixture pipetted to the remaining PCR product. These mixtures were incubated at 37 °C for 20 min and enzymes inactivated at 80 °C for 20 min. Then 10 µl of the digest was mixed with 4 µl of 10 µM sequencing primer and sent to be sequenced by Microsynth's sequencing service.

2.2.7 Chemical Transformation of Yeast

Chemical transformation was performed as described in [79]. In brief, pYD was assembled out of insert produced in PCR3 (see above), linearized vector and a promotor fragment. Ready to use vector, prepared previously, was linearized by restriction digest with AleIv2 (New England Biolabs) and PvuII (New England Biolabs) (15 µg plasmid DNA, 1x SmartCut™ buffer (New England Biolabs), 0.2 U/µl of each enzyme); total volume: 100 µl; incubated 90 min at 37 °C). Digest was purified by preparative agarose gel electrophoresis and the concentration determined

spectrophotometrically. Promotor fragment was PCR amplified (1x Q5™ High-Fidelity Master Mix (New England Biolabs), 0.5 µM primer, 25 ng template; total volume: 100 µl; Cycling: 95.0 °C, 3 min; 6×(95 °C, 15 s; 64 °C, 15 s; 72 °C, 15 s); 29×(95 °C, 15 s; 55 °C, 15 s; 72 °C, 15 s); 72 °C, 5 min), purified by preparative agarose gel electrophoresis and the concentration determined spectrophotometrically. Insert was prepared by pooling all products of PCR3 by IGHV family of the primer used in PCR2 and the reaction pool of PCR1. The total amount of insert was calculated assuming a volume of 30 µl per purified amplicon. For transformation, on the first day, a pre-culture of EBY100 (heavy chain, Trp-auxotrophic) was prepared in YPD supplemented with 400 mg/l adenine (YPAD) and incubated overnight at 30 °C with shaking. On the next morning, it was used to inoculate an appropriate volume of YPAD (cells in 5 ml correspond to one transformation) to an initial OD_{600} of 0.4, which was then incubated for approximately 5 h with shaking at 30 °C to reach an OD_{600} of 2-3. This culture was then distributed to 50 ml Falcon™ tubes and cells collected by centrifugation at 1000 g for 5 min at RT. Cells were washed once with 25 ml of deionised water and pelleted again (1000 g, 5 min, RT). Next, cells were resuspended in 3 ml of 200 mM LiAc and incubated at 30 °C for 15 min with shaking. In the meantime the transformation mix was prepared: 33.3% (v/v) PEG 3350, 100 mM LiAc, 278 µg/ml of heat-shocked salmon sperm DNA (95 °C, 5 min; Carl Roth), plasmid assembly mix and water to a total volume of 360 µl/per 1 µg of linearized backbone. If more backbone was required to maintain the vector-to-insert ratio for a given sub-library, the volume of transformation mix was scaled up as whole multiples ($n \times 360$ µl, $n \in \mathbb{N}$), transforming at a lower plasmid concentration. The same was done if the volume necessary was larger than 360 µl. Then 300 µl of liAc treated cells per 360 µl of transformation mix were distributed to 2 ml Eppendorf™ tubes and cells collected by centrifugation (1000 g, 5 min, RT). Excess LiAc was poured off, and cell pellets resuspended in the remaining liquid. Then transformation mix was added and mixed vigorously by vortexing. The mixture was then incubated at 30 °C for 30 min with shaking, followed by the heat-shock at 42 °C for 45 min without shaking. After the heat-shock, cells were collected by centrifugation (1000 g for 5 min), the supernatant discarded and cells resuspended in 1 ml of SD medium supplemented with 2% (w/v) glucose without tryptophan (SD-trp). To determine the size of the created library a sample was plated onto SD-trp agar plates to get ∼100 colonies per plate (expected transformation efficiency: 10^6 transformants per µg vector) and incubated at 30 °C for 48 h. The remaining cells were used to inoculate 1 ml of SD-trp medium per µg vector DNA, rounded up to the nearest five milliliters (e.g. 3 µg vector corresponds to 5 ml culture volume). The culture was grown for 36-48 h at 30 °C, passaged once 1:20, and incubated for another 24 h. Then 15% glycerol stocks were prepared and sub-libraries stored at -80 °C.

2.2.8 Yeast Mating

Yeast mating was performed as previously described [79]. In brief, on the first day the respective heavy and light chain sub-libraries were combined into one heavy chain and one light chain pre-culture by inoculating 100 ml of SD-trp and SD-leu supplemented with 2% (w/v) glucose with 20× the respective sub-library size from the glycerol stock (assuming 10^6 cells per µl). Pre-cultures were incubated over night at 30 °C with shaking. The next morning, 200 ml of each medium were inoculated to an OD_{600} of two and grown at 30 °C for 5 h. Then 700 OD units (assuming an OD_{600} of 1 corresponds to 10^7 haploid cells/ml) were transferred to 50 ml Falcon™ tubes and cells collected by centrifugation (1000 g, 5 min, RT), the supernatant discarded and the cells of each culture transferred to 100 ml of YPD, *p*H 4.5, and incubated for 1 h at 30 °C with shaking. After that cells were collected (1000 g, 5 min, RT), each culture was resuspended in 2.5 ml of YPD, *p*H 4.5, combined and mixed well. This mixture was then plated onto a large agar plate (YPD, *p*H 4.5; 245 x 245mm; 600 mm^2) and yeast cells were allowed to mate for 6 h at 30 °C. To harvest diploid cells, 10 ml of SD-/- medium + 2% (w/v) glucose were pipetted onto

the agar plate and cells were collected using a cell-scraper. The suspension was transferred to a Falcon™ tube, cells collected by centrifugation (1000 g, 5 min, RT), washed once with 45 ml of SD medium lacking both tryptophan and leucine (SD-/-) + 2% (w/v) glucose and collected again by centrifugation. Then the cell pellet was resuspended in 35 ml of fresh SD -/- medium + 2% (w/v) glucose (volume including the cell pellet) and the resulting suspension used to prepare dilutions for enumeration. Assuming 7×10^9 cells in 35 ml, the original suspension was diluted $1{:}10^3$, $1{:}10^4$, $1{:}10^5$ and $1{:}10^6$ and 100 µl of each dilution were plated onto SD-leu, SD-trp and SD-/- plates. The rest was used to inoculate 2×500 ml of SD-/-, which were incubated at 30 °C for 48 h, passaged once 1:20, and incubated for another 24 h. Then 15% glycerol stocks were prepared and the mated library stored at -80 °C.

2.2.9 Flow Cytometry

Yeast was grown in SD-/- supplemented with 2% (w/v) glucose in round-bottom 24 well plates (Whatman). Per well, 2 ml of medium were inoculated. If a library was measured, wells were inoculated with the number of cells corresponding to 20 × the library size from glycerol stocks (assuming 10^6 cells per µl). If single clones were analyzed, wells were inoculated by transferring a single colony from an agar plate to the liquid medium. Cultures were grown for 24 h at 30 °C with shaking. This culture was then used to inoculate 2 ml per culture of induction medium (SD-/- supplemented with 2% (w/v) galactose and 1% (w/v) raffinose) to an OD_{600} of $\sim$1 in a fresh 24 well plate. The resulting cultures were incubated at 20 °C for 2 days with shaking. After that, yeast cells were diluted to an OD_{600} of $\sim$1 in 2% (w/v) bovine serum albumin in 1x phosphate buffered saline (2% BSA/PBS) and blocked for 15-30 min at room temperature with shaking. Then, blocked yeast cells were distributed into the wells of a round bottom 96-well microtiter plates (100 µl/well), centrifuged for 5 min, at 1000 g and at RT, and the supernatant discarded. Biotinylated antigens (obtained as unlabeled proteins for the laboratory of Barbara Bohle and biotinylated in-house) were diluted in 2% (w/v) BSA/PBS, added to the cells (100 µl/well) and allowed to bind 30 min at RT with shaking. After binding, cells were collected by centrifugation (5 min, 1000 g, 4 °C) and the supernatant discarded. Then labeled conjugate in 2% (w/v) BSA/PBS was added (100 µl/well) and incubated on ice, protected from light, for 15-30 min (Streptavidin-AlexaFluor™ 647 (S32357, Invitrogen): 1:1000; anti(α)-V5-tag-Fluorescein isothiocyanate (FITC)(MA1-80281, Thermo Fisher Scientific): 1:100; α-human Fab-FITC (F5512, Sigma Aldrich): 1:200; α-human κ light chain-allophycocyanin (MH1051, Life Technologies™): 1:50; α-human κ light chain-phycoerythrin (MA1-10389, Invitrogen): 1:50; α-human κ light chain-FITC (F3671-2ML, Sigma Aldrich): 1:100). Stained cells were again collected by centrifugation (5 min, 1000 g, 4 °C), resuspended in 200 µl/well of cold PBS and analyzed on a Guava™ easyCyte (Merck). Typically 5000 events, in a maximum analysis time of 180 s were analyzed. Flow cytometry data were analyzed using the Kaluza Analysis Software (Beckman Coulter GmbH). Gates were set to include 1% of events in secondary-only controls.

2.2.10 Magnetic Cell Sorting™

First, 250 ml of SD-/- supplemented with 2% (w/v) glucose were inoculated with the amount of yeast cells corresponding to 20x the library size from glycerol stock (assuming 10^6 cells/µl). Then, 250 ml of induction medium (Sec. 2.2.9) were inoculated at an OD_{600} of 1 and incubated at 20 °C with shaking for 48 h. MACS™ was performed with 4×10^9 cells. Cells were collected by centrifugation (1000 g, 5 min, RT). For blocking, the pellet was resuspended in 5 ml of 10% (w/v) BSA/PBS and rotated at RT for 30 min. After blocking, cells were collected by centrifugation (1000 g, 5 min, RT). Then 10% (w/v) BSA/PBS and biotinylated antigen was added to a final volume of 4 ml. The resulting mixture was rotated at RT for 1 h. Binding was quenched

by the addition of 8 ml of cold μMACS buffer (0.25% (w/v) BSA, 1xPBS, 2mM EDTA) and the mixture was placed on ice. Cells were collected (1000 g, 5 min, RT) and resuspended in 5 ml of cold μMACS buffer. Then 200 μl of streptavidin coated paramagnetic microbeads (Miltenyi Biotec) were added and mixed vigorously, followed by 15 min of incubation on ice. The mixture was agitated every 2-5 min to prevent excessive sedimentation. During incubation, a cold MACS column (Miltenyi Biotec) was placed in the magnetic separator (Miltenyi Biotec) and equilibrated with 5 ml of μMACS buffer. Next, cells were transferred to 20 ml of cold μMACS buffer and strained through a 40-μm-filter to remove aggregates. The resulting suspension was loaded onto the column in $\sim$7 ml aliquots. Between aliquots, once dripping had stopped, the column was reoriented in the magnetic field and washed with 5 ml of μMACS buffer. Then the column was washed 3$\times$ with 5 ml of μMACS buffer, again reorienting the column in the magnetic field between aliquots. After washing, the column was removed from the magnetic field and a plunger was used to push bound cells into a sterile 15 ml Falcon™ tube (the beads were co-eluted and then lost during cultivation). Cells were collected (1000 g, 5 min, RT) and resuspended in 10 ml of SD-/- medium supplemented with 2% (w/v) glucose and added to 40 ml of medium prepared in a 100 ml Erlenmeyer flask. From this culture, serial dilutions were plated directly to determine the library size. Culture was grown for 48 h at 30 °C with shaking, passaged once 1:20 and grown for another 24 h. Then 15% (w/v) glycerol stocks were prepared and stored at -80 °C.

2.2.11 Fluorescence Activated Cell Sorting

An appropriate volume (maximum 8×10^5 cells/ml) of SD-/- supplemented with 2% (w/v) glucose were inoculated with 20$\times$ the size of the desired population and grown for 24 h at 30 °C with shaking. Then, the same volume of induction medium (Sec. 2.2.9) was inoculated to an initial OD_{600} of 1 and incubated at 20 °C for 48 h. After that, cells from 2 ml of induced yeast culture (OD_{600} of approximately 20) were collected by centrifugation (1000 g, 5 min, RT), resuspended in 10% (w/v) BSA/PBS for blocking and rotated for 30 min at RT. In the meantime, biotinylated antigens were diluted at the desired concentrations in 10% (w/v) BSA/PBS. After blocking, cells were collected by centrifugation (1000 g, 5 min, RT), resuspended in 1 ml of antigen solution and allowed to bind while rotating for 30 min at RT. Binding was quenched by the addition of 1 ml of ice-cold PBS. Cells were collected (1000 g, 5 min, 4 °C) and resuspended in 1 ml of fresh 10% (w/v) BSA/PBS to which staining conjugate was added (conjugates were used at the same dilutions as described in Sec. 2.2.9). After addition, the mixture was vortexed and incubated for 15-30 min on ice. Cells were collected (1000 g, 5 min, 4 °C) and resuspended in 500 μl of ice-cold PBS and kept on ice until sorting began. Sorting was performed by Gordana Wozniak-Knopp on the Sony SH800S cell sorter. Cells were collected into SD-/- medium supplemented with 2% (w/v) glucose. Samples were plated onto double negative agar to obtain single clones. The culture was grown for 24 h at 30 °C. Then 15% (w/v) glycerol stocks were prepared and stored at -80 °C.

2.2.12 Preparation of Yeast Lysates

For each clone, 31 μl of digestion mix was prepared, consisting of 30 μl of Digestion buffer and 1 μl of Zymolyase (5 U/μl), in a 1.5 ml Eppendorf™ tube (both reagents are supplied with the Zymoprep™ Yeast Plasmid Miniprep II kit, Zymo Research). Zymolyase is a bacterial enzyme mix that can digest the yeast cell wall. Approximately half of a yeast colony (grown on SD-/- agar at 30 °C for 48 h) was picked and transferred into the digestion mix and incubated at 37 °C to 45 min. Cells were then ruptured by heating to 95 °C for 5 min and freezing at -80 °C for 20 min. Then samples were thawed, and cellular debris removed by centrifugation (8000 g, 1 min, RT). Lysates were stored at -20 °C.

2.2.13 Reformatting of Heavy and Light Chains

For subcloning, heavy and light chain sequences were PCR amplified from yeast lysates using clone specific forward primers and chain specific reverse primers. PCR was performed under the following conditions: 1x Q5™ High-Fidelity Master Mix (New England Biolabs), 0.5 µM primers, 2 µl of lysate pipetted off the top of the sample to prevent transfer of cell debris, and sterile deionised water to a total volume of 25 µl; Cycling: 98 °C, 3 min; 6×(98 °C, 20 s; 64 °C with -1.5 °C/cycle, 20 s; 72 °C, 20 s); 29×(98 °C, 20 s; 55 °C, 20 s; 72 °C, 20 s); 72 °C, 5 min. Amplicons were analyzed by agarose gel electrophoresis. For Gibson assembly, 9 µl of crude PCR product were mixed with 1 µl of linearized pTT5-based heavy or light chain vector (50 ng/µl, prepared in house) and thoroughly mixed. Then 5 µl of the mixture were discarded and replaced by 5 µl of NEBuilder™ HiFi DNA Assembly Master Mix (New England Biolabs). Reactions were incubated at 55 °C for 1 h and directly used for transformation or stored at -20 °C until use.

2.2.14 Transformation of *E. coli*

For the transformation of electrocompetent *E. coli* TOP10 cells (prepared in-house), 100 µl of deionised water, 20 µl of freshly thawed cells and 1 µl of DNA solution were combined in a cold electroporation cuvette (1 mm gap, VWR or Sigma Aldrich). Electroporation was performed at 2.5 kV, 400 Ω and 25 µF aiming for a pulse length of 5 ms. If arching occurred, the amount of DNA was reduced to 0.5 µl or the water volume increased up to 200 µl. After electroporation, cells were transferred into 500 µl of warm SOC (Super Optimal Broth with Catabolite Represion) medium (2% (w/v) peptone (casein), 0.5% (w/v) yeast extract, 10 mM NaCl, 25 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM (w/v) glucose) and left to recover at 37 °C for 1 h. After that, cells were collected by centrifugation (2000 g, 2 min, RT) and medium down to 100 µl was removed. Cells were resuspended in the remaining 100 µl medium, plated onto dry low salt LB (Sec. 2.2.15) agar plates supplemented with 100 µg/ml ampicillin and incubated at 37 °C overnight.

2.2.15 Preparation of Plasmid DNA from *E. coli*

For the preparation of plasmid, a colony of transformed *E. coli* was picked and used to inoculate 4 ml of low salt LB (lysogeny broth) medium (10 g/l peptone, 5 g/l yeast extract, 5 g/l NaCl) supplemented with 100 µg/ml of ampicillin. The culture was then shaken at 37 °C overnight. Plasmid DNA was purified using the NucleoSpin™ Plasmid Easy Pure Kit (Macherey-Nagel) according to the manufacturer's instructions except that the lysis step was extended to 15 min, and for elution, loaded and washed columns were wet with 100 µl of deionised water and incubated for 5 min before collection of purified DNA. Then DNA concentration was determined spectrophotometrically and plasmid preparations stored at -20 °C.

2.2.16 Transient Transfection of HEK Cells

25 µg of each heavy and light chain expression vector (pTT5) were mixed and added to 2.5 ml of FreeStyle™ F17 Expression Medium (Thermo Fisher Scientific). This mixture was then added to 100 µg of PEIpro™ (VWR) transfection reagent in another 2.5 ml of F17 medium (mass ratio of DNA to PEI 1:2 in 10% (w/v) culture volume), mixed and incubated at RT for 15 min. The DNA:PEI mixture was then transferred dropwise to 50 ml of HEK 293-6E cells, which were at a density of 1.5 to 2×10^6 cells/ml in F17, while shaking gently. The culture was then incubated at 37 °C under 5% CO_2 and humidified atmosphere for 5 days. Cells were fed with tryptone TN-1 (Organotechnie) to a final concentration of 0.5% (w/v) on the second day post-transfection. Then, supernatant was harvested as described below.

2.2.17 Purification of Antibodies by Affinity Chromatography

HEK production culture was transferred to a 50 ml Falcon™ tube and cells pelleted by centrifugation at 2200 g for 20 min at RT. Supernatant was buffered with 0.1 M sodium phosphate, *p*H 7.0. The sample was filtered using a 0.45 µm mountable syringe filter and stored on ice. Before starting purification, all necessary buffers were degassed. For fast protein liquid chromatography the ÄKTA™ system (Cytiva) was first purged with 30 ml of 20% (v/v) EtOH followed by 30 ml of 0.1 M Na_2HPO_4/NaH_2PO_4, *p*H 7, at 25 ml/min. Then the flow rate was reduced to 1 ml/min and a 1 ml protein A column (HiTrap™ Protein A HP, Cytiva) was inserted under flow. The column was equilibrated with approximately 5 column volumes of 0.1 M Na_2HPO_4/NaH_2PO_4. Once the column was equilibrated (A_{280} and conductivity stable), buffered HEK supernatant could be loaded. After loading was complete, the tubing was flushed with 0.1 M glycine, *p*H 2.5, at 5 ml/min. Then the flow rate was reduced back to 1 ml/min, antibody eluted with 0.1 M glycine, *p*H 3.5, and collected in fractions of 1 ml, which were immediately neutralized with 6 µl of 2 M Tris *p*H 9.0. Then, the column was first regenerated with 0.1 M glycine, *p*H 2.5, and then the matrix was equilibrated with 0.1 M Na_2HPO_4/NaH_2PO_4, *p*H 7. Eventually the column was rinsed with 20% (v/v) EtOH and stored in 20% (v/v) EtOH at 4 °C. The concentration of the collected and neutralized fractions was determined by measuring the absorbance at 280 nm using NanoDrop™. Protein-containing fractions with an antibody concentration >0.5 mg/ml were transferred to dialysis tubing with a retention mass of 10 kDa (Snake Skin Dialyse Tubing, Thermo Fisher Scientific) and placed into 100x the sample volume 1x PBS. Dialysis proceeded with gentle stirring overnight at 4 °C. Then the samples were transferred into fresh tubes, the antibody concentration was measured again and the preparations were stored at -80 °C until further use.

2.2.18 Immobilized Allergen ELISA

First, biotinylated antigen was bound to a streptavidin coated, flat bottom 96-well microtiter plate (Nunc™ Immobilizer™, Thermo Fisher Scientific) by adding 100 µl/well of biotinylated allergen in PBS (Mal d 1: 2.5 µg/ml; Bet v 1: 5 µg/ml) and incubating for 15-60 min at RT with shaking. Then excess antigen was washed off (3x 200 µl/well PBS). For blocking, 200 µl/well of 4% BSA/PBS were added and incubated for 1 h at RT with shaking. During blocking, an 8-step 1:3 dilution series starting at 2 µM sample antibody in PBS was prepared in an uncoated, non-binding flat-bottom 96-well microtiter plate (Nunc™ MicroWell 96-Well Microplates, Thermo Fisher Scientific). If sample antibody could not be recovered at high enough concentrations, it was added at the highest possible concentration. BIP-1 ([80], supplied by Barbara Bohle), a Mal d 1/Bet v 1 cross-reactive hybridoma antibody, served as a positive control and was used at dilutions starting from 5 µg/ml. After blocking, the allergen coated plate was washed (3x 200µl/well PBS) followed by the addition of 50 µl/well 4% BSA/PBS. Then 50 µl/well of the antibody dilution series was transferred onto the allergen coated plate, resulting in a 1:2 dilution of antibody and BSA (highest conc. 1 µM, 2% (w/v) BSA). The mixture was incubated 1 h at RT with shaking. After washing (3x 200µl/well PBS) secondary antibodies in 2% (w/v) BSA/PBS (α-human Fc-HRP (A0170, Sigma Aldrich): 1:4000; α-mouse Fc-HRP (A2554, Sigma Aldrich): 1:2500) were added (100 µl/well) and left to bind for 1 h at RT with shaking. Excess secondary antibodies were washed off (3x 200µl/well PBS) and then 100 µl/well of HRP-substrate 3,3',5,5'-tetramethylbenzidin (RT, applied as liquid Substrate System for ElISA, Sigma Aldrich) were added for color development. Once enough color had developed (assessed visually) enzymatic color formation was quenched with 100 µl/well of 30% H_2SO_4. Color development was quantified by measuring absorbance at 450 and 620 nm using a Spark™ Microplate reader (Tecan).

2.2.19 SEC-HPLC

Antibodies were thawed and diluted with PBS, so that after addition of 2 M NaCl in one tenth of the volume of the initial protein solution, 20 µg of protein could be injected in a maximum volume of 100 µl. If antibody concentration was insufficient to reach this target amount, 100 µl of crude solution with added NaCl were loaded directly and the amount of injected protein noted. Samples were analyzed on the LC-20A Prominence HPLC system (Shimadzu) equipped with a Superdex™ 200 Increase 10/300 GL column (Cytiva). SEC-HPLC was carried out by Gerhard Stadlmayr or Gordana Wozniak-Knopp.

3 Results

3.1 Construction of the Fab Display Library PLMB4

Transformation of ∼69 µg insert DNA yielded 2.5×10^7 independent clones in 16 sub-libraries at a transformation efficiency of $1.07 \pm 0.45 \times 10^6$ cfu/µg vector as determined by plating a sample of each sub-library onto selective agar (Tab. A.2.1; Fig. A.1.1). To validate the quality of this library, a total of 112 clones were analyzed by Sanger sequencing. Inserts were PCR-detectable in 72% of clones and 57 readable sequences could be obtained. Frameshifts in heavy chain inserts or secretion signal (recombination homology) were only found in three clones, which suggests an overall library correctness of 95% (Tab. A.3.1) in accordance with previously published results [81]. Additionally, the data was analyzed for duplicate V-domain and CDR3 aa-sequences, because duplicates at this small sample size could indicate low library diversity. Indeed, both kinds of duplicates were found, but were rare and restricted to sub-libraries deriving from the A pool (data not shown). To create the most diverse library possible one full set of sub-libraries (A: VH1[1],3,5,7; B: VH1,3; C: VH2,4) with as little sequence overlap as possible were combined resulting in the PLMB4 (Patient Library Mal/Bet No. 4) heavy chain sub-library with a size of 1.95×10^7 clones (Tab. 1).

Table 1: Sub-libraries constituting the heavy chain sub-library.

Name	Size
2A_VH1	4.5×10^6
1A_VH3	2.6×10^6
2A_VH5	1.2×10^6
2A_VH7	1.6×10^6
1B_VH1	3.4×10^6
1B_VH3	5.7×10^6
1C1_VH2_1L	$2,3 \times 10^5$
1C1_VH2_2L	1.3×10^5
1C1_VH4_1L	9.5×10^4
Total:	1.95×10^7

Table 2: Sub-libraries constituting the kappa light chain sub-library.

Name	Size
K1	2.2×10^6
K2	2.6×10^6
K3	2.5×10^6
K4	1.7×10^6
K5	5.2×10^6
KSB1	1.4×10^6
KSB2	1.9×10^6
KSB3	3.0×10^6
KSB4	1.7×10^6
Total:	2.2×10^7

For the construction of the PLMB4 Fab display library, the heavy chain sub-library was combined with a kappa light chain sub-library prepared by Gordana Wozniak-Knopp using an analogous methodology to the construction of the heavy chain sub-library (Tab. 2). Since previous projects had shown that lambda light chains are less likely to produce binding antibodies due to random heavy/light chain pairing, only kappa light chains were used ([82], Gordana Wozniak-Knopp, personal communication). The heavy chain sub-library (EBY100, MATa) was mated with the light chain sub-library (BJ5464, MATα) on solid, low *p*H agar. Both of these strains are auxotrophic for tryptophan and leucine and diploids carrying both plasmids could thus be selected by growing mated cells in medium lacking both (haploids carrying the heavy chain plasmid had been selected in medium supplemented with leu, but lacking trp, and vice versa). Mating efficiency was at 70% as determined by plating samples of the mating mix onto leu-, trp- and double negative agar. The mated Fab library contained 1.4×10^8 independent clones based on

[1] A: VH1 refers to the sub-library which was created from transcripts amplified from amplicon A (PCR1) using IGHV1 gene specific primers (PCR2).

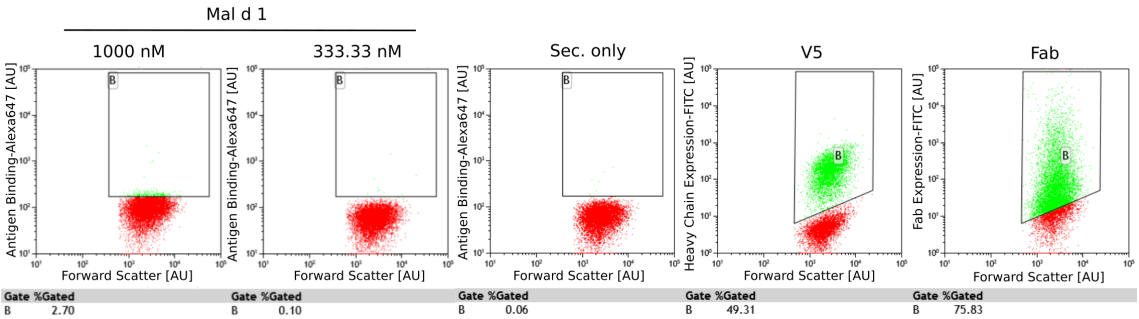

Figure 7: Quality control of mated Fab library using flow cytometry. Antigen binding was tested by incubating induced yeast with a 1:3 dilution series of biotinylated Mal d 1 and detected with streptavidin-Alexa647 conjugate. Binding was only recorded at the highest concentration of 1 µM (only first two steps shown, complete series given in Fig. A.4.1). Heavy chain expression was analyzed using labeled αV5 antibody. Light chain expression was inferred from the binding of labeled polyclonal anti-human Fab antibody.

the number of colonies on one countable double negative plate.
At this point the mated library was induced for the first time and a series of tests were performed to validate the proper function of the display system (Fig. 7). To determine the appropriate antigen concentration at which to begin sorting, the library was incubated with a dilution series of biotinylated Mal d 1. Cells were stained with labeled streptavidin and analyzed by flow cytometry. Slight antigen binding reactivity was detected at the highest concentration of 1 µM Mal d 1 (for full dilution series see Fig. A.4.1). This signal appeared to be shifted upwards due to random fluctuations in the detector, but was recorded in all replicates and therefore interpreted as genuine. Heavy chain expression was analyzed using labeled anti-V5 (MA1-80281) antibody and detected in about 50% of induced cells, consistent with previously published results [72]. The presence of a non-expressing population is a known phenomenon and does not interfere with selection of the library [83]. Detection of the light chain is critically dependent on correct Fab assembly and therefore a convenient proxy for Fab expression. However, due to the COVID-19 pandemic, our preferred anti-human κ light chain antibody (F3761) was not available at the time of analysis and light chain expression was tested using a polyclonal anti-human IgG Fab antibody (5512). Unfortunately, we could not observe a well-defined light chain positive cell population as detected with the preferred anti-human kappa chain antibody. Validation with two other anti-human kappa light chain antibodies (MH10515 and MA1-10389) yielded similar results, but these were known to be less effective in this system (Fig. A.4.1B) (Gordana Wozniak-Knopp, personal communication). Nonetheless, we argued that the fact that a positive signal was consistently reported by all three antibodies, was enough evidence for proper light chain expression and proceeded with library selection.

3.2 Screening of PLMB4

The first sorting campaign began with one round of MACS with 1 µM Mal d 1, to capture the affinity recorded in the titration experiment (Fig. 7). The resulting library, PLMB4.1, had a size of $8.8 \pm 1.4 \times 10^6$ clones, based on the results of a dilution series across two plates, representing ∼6% of the original library. After expansion this population was co-stained for antigen binding and Fab expression. It was then further sorted using FACS (Fig. 8A): The first branch of the sorting tree was designed to enrich Mal d 1 binding clones and began with selection with 1 µM Mal d 1, which already led to some enrichment (“Mal1x”). To optimize the stringency of the second selection round three parallel sorts with 1 µM, 100 nM and 10 nM were carried out, which led

to dose-dependent enrichment. “Mal2x” and “Mal10” were excluded due to too much and too little enrichment, respectively. The sorting step leading to the “Mal2x” population was additionally performed with neutravidin-phycoerythrin to exclude the possibility of enriching clones binding the streptavidin conjugate (data not shown). The other branch was designed to select for cross-reactive

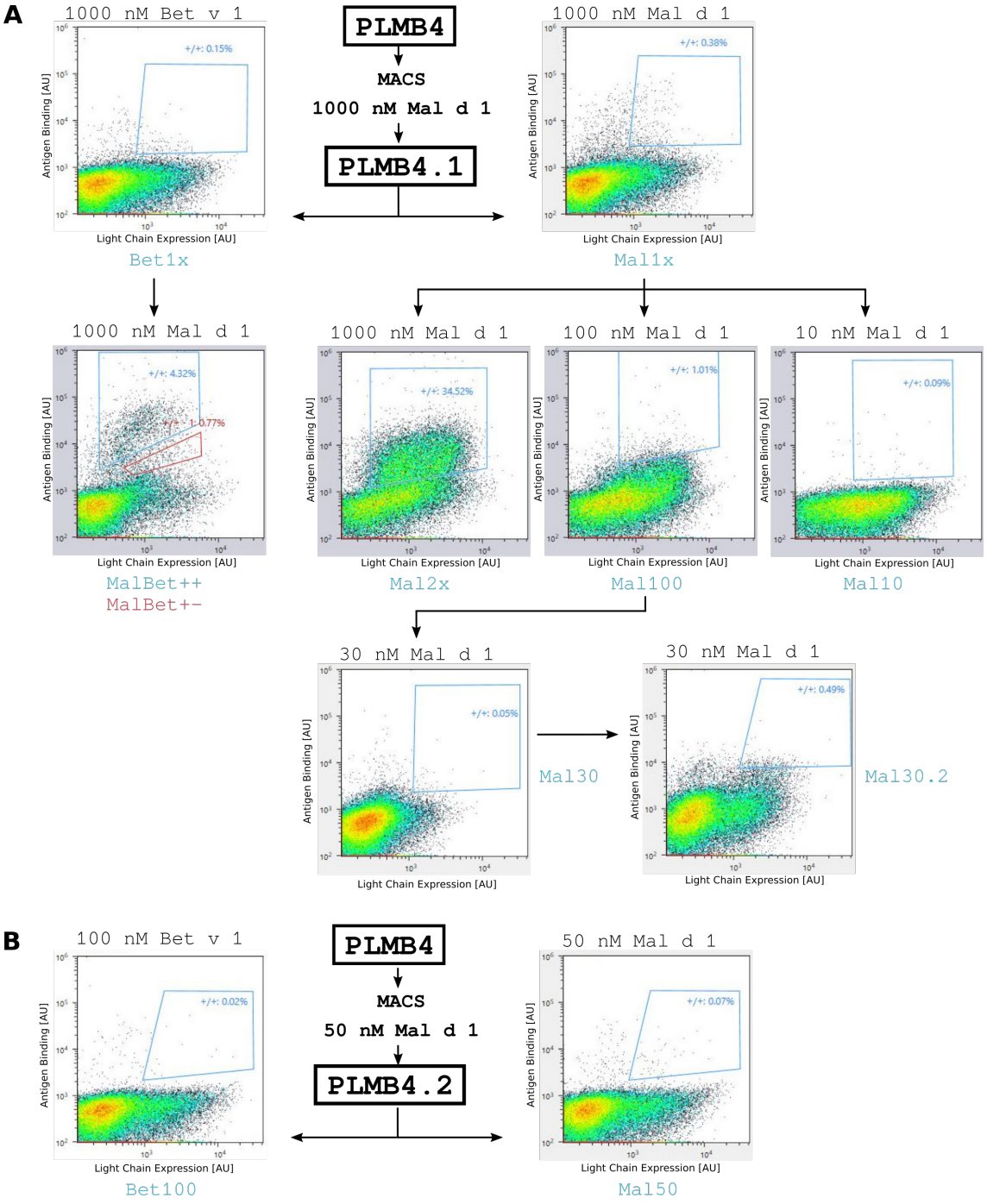

Figure 8: Fluorescence activated cell sorting (FACS) of PLMB4 (patient library mal/bet No. 4). **A** First sorting campaign. **B** Second sorting campaign. Colored labels represent the name given to the population selected in the gate of the same color. Clones expressing intact Fab and bind antigen (double positive) fall in the upper right quadrant of the plot. Double negative cells together with non-expressing cells are located at the bottom left (50% are expected to be non-expressing cells and likely make up the bulk of this signal [83]). The rest either do not bind antigen (bottom right) or do not express correctly folded Fab (top left).

clones and sufficient enrichment was achieved after one sort with 1 μM Bet v 1 followed by one sort with 1 μM Mal d 1. Two populations, "MalBet++" and "MalBet+-", were collected for further testing.

First, we investigated the pool "Mal1x" for Mal d 1 binders. Of 24 colonies initially picked, one did not grow and three were excluded during flow cytometric pre-screening (two non-functional, one non-binder; Sec. A.8.1). The remaining clones all showed affinity for Mal d 1 and sequencing revealed no duplicate antibodies, however, four heavy chain CDR3 sequences (HCDR3) were present in 13/20 clones (sequence data is given in Sec. A.9). Based on these data 15 clones were chosen for recloning, eleven of which could be expressed as IgG. Four clones bound either Mal d 1 or both Mal d 1 and Bet v 1, but with affinities well above the target EC_{50} range (ELISA data is given in Sec. A.10, see Tab. A.7.1 for an overview of the screening process).

To identify the most promising sort for the isolation of cross-reactive clones, the sorts "MalBet++", "MalBet+-" and "Mal100" were tested using flow cytometry (Fig. A.5.1). "Mal100" was analyzed based on the premise that enrichment of high affinity Mal d 1 binders also includes high affinity cross-reactive antibodies. Based on these data "MalBet++" was initially chosen for individual screening. Of 24 selected clones, nine were found to be non-functional (Fab negative) and three additional clones were excluded for exhibiting no cross-reactivity or being only weakly reactive to either antigen. The remaining eleven clones were either cross-reactive (6), predominantly Mal d 1 binders that indicated some degree of cross-reactivity (4) and one Bet v 1 binder (Sec. A.8.2). However, while attempting to amplify inserts from these clones for sequencing, we found that only four carried a detectable light chain plasmid in spite of showing a medium to strong light chain signal in flow cytometry. After additional PCR based attempts to recover the light chain sequences, plasmid DNA was directly purified from yeast cells, transformed into *E. coli* and grown on kanamycin containing selective medium (additional selection marker of light chain display vector). The very few colonies that could be isolated, all contained seemingly the same, very large plasmids (>10 kb), based on restriction digest and gel electrophoresis (data not shown). We therefore hypothesized that recombination between the light and heavy chain plasmids had occurred, which contain vast regions of homology. This likely led to the display of Fab epitopes that were sufficient for selection during FACS. The remaining four antibodies could be successfully reformatted and expressed, with two showing some affinity to one or both allergens, but neither with an EC_{50} below 1 μM. Interestingly, two of the HCDR3 sequences found in multiple Mal1x clones, CAKRPAVAGGRYYYYGMDV (Y4) and CARSLTVAGWWAT (W2), were also present in multiple "MalBet++" clones (Y4: 5/8 "MalBet++", 4/20 "Mal1x"; W2: 1/8 "MalBet++", 6/20 "Mal1x")[2].

Next, ten clones of the "Mal100" population were screened. Two clones did not show Fab expression in flow cytometry and one clone gave a Fab signal but the light chain coding sequence could again not be PCR amplified (Sec. A.8.3). Sequencing of the remaining clones revealed that the screen included one duplicate heavy chain and all but one clone contained either Y4 or W2, which at that point were believed to be low affinity binders. The unique clone, Mal100_5, however, was reformatted into full-length IgG and exhibited increased affinity to both antigens (Fig. 9A). To test whether the biotinylation of the antigen interfered with binding of Mal100_5 the immobilized allergen ELISA was repeated using unmodified antigen that was adsorbed directly onto hydrophilic polystyrene based 96-well plates. Indeed, the binding curve was shifted to lower concentration and began to show signs of saturation, which allowed the determination of its EC_{50}. Under these conditions Mal100_5 bound Mal d 1 and Bet v 1 with an EC_{50} of 37 nM and 43 nM, respectively (model parameters are given in Tab. A.11.1; Note that EC_{50} values are not directly comparable because plates were coated with 2.5 μg/ml Mal d 1 but 5 μg/ml Bet v 1).

[2] Additional data up to this point can be found in the bachelor theses of Laura Payer and Johanna Schrotter, available in the CDLI2T, DBT, BOKU, Muthgasse 18, 1190 Vienna.

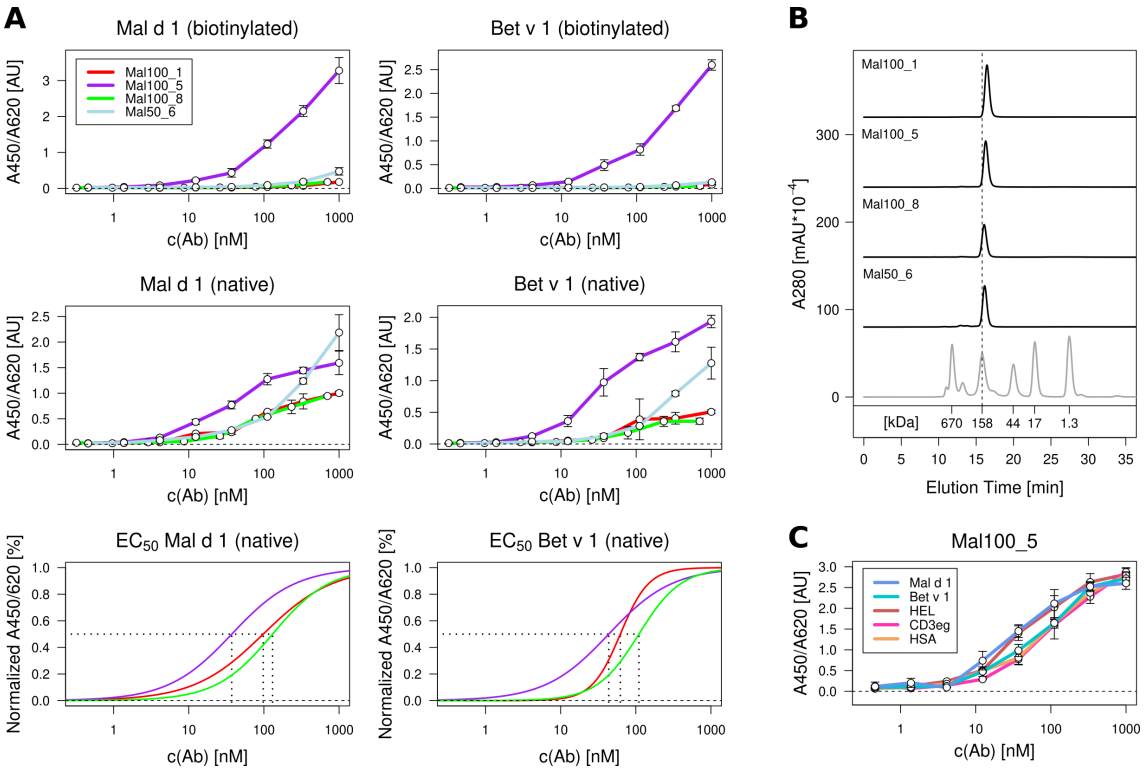

Figure 9: Characterization of the highest affinity antibodies identified as part of this thesis. **A:** Binding curves of Mal100_1, 5 and 8 and Mal50_6 in immobilized allergen ELISA. Top: biotinylated allergens; Middle: native allergens. The data suggest that biotinylation (lysine coupled) interferes with epitope recognition. Data represent two experiments, errors are given as standard deviation. Bottom: Comparison of modeled binding curves derived from native allergen data used for the calculation of EC_{50} values. EC_{50} for Mal d 1 and Bet v 1, respectively: Mal100_1: 97 nM and 62 nM, Mal100_5: 37 nM and 43 nM, Mal100_8: 130 nM and 110 nM. Parameters for model quality are given in (Tab. A.11.1). The binding curve of Mal50_6 did not approach saturation and no EC_{50} could be determined. Note that signal intensity varied between experiments and analysis is based on the shape of the binding curve, and that EC_{50} values are not directly comparable because Mal d 1 wells were coated with half the amount of allergen compared to Bet v 1. **B**: SEC-HPLC profiles of the presented antibodies that were used to verify the identity and integrity of the expressed IgG. All antibodies eluted at a time typical for IgG and show very little signs of aggregation and no fragmentation. Small differences in the elution time might be due to differences in glycosylation or hydrophobicity. **C**: Reactivity of Mal100_5 with the target allergens Mal d 1 and Bet v 1 as well as the unrelated antigens HEL (hen egg lysozyme), CD3eg (CD3 epsilon/gamma fusion protein) and HSA (human serum albumin) using the same ELISA assay as in A. Data courtesy of Gordana Wozniak-Knopp.

The discovery of Mal100_5 seemed to prove that enrichment had been successful and that PLMB4 contained binders in the target EC_{50} range, however, "Mal100" only contained one novel sequence in ten screened clones. Thus, to keep the screening effort minimal, we decided to further sort "Mal100" using FACS. The first selection round with 30 nM Mal d 1 yielded very few clones, but after expansion, a second sort with 30 nM Mal d 1 suggested successful enrichment. Unfortunately, flow cytometric analysis in bulk (Fig. A.6.1) and of individual clones (data not shown) revealed that this population ("Mal30.2") showed affinity for the streptavidin-conjugate and sequencing revealed that both "Mal30" and "Mal30.2" only contained one heavy chain, paired to one of three different light chains (based on 24 clones each). Throughout the part of the selection campaign presented here, Mal100_5 remained the highest affinity antibody isolated from PLMB4 for both allergens. It was therefore selected to be tested with other PR-10 allergens after screening had concluded and preliminary data suggested broad cross-reactivity. However, further testing revealed, that Mal100_5 also bound unrelated proteins (Fig. 9C) and thus lacked the desired se-

lectivity.
Concurrently to the further sorting of "Mal100", we decided to begin a second selection campaign starting from PLMB4 (Fig. 8B). This sorting campaign was designed to only select the highest affinity clones present in the library. One round of MACS with 50 nM Mal d 1 yielded PLMB4.2 with a size of $3.4 \pm 0.5 \times 10^6$ clones (based on a dilution series across three plates), representing $\sim$ 2.5% of PLMB4. PLMB4.2 was further selected by FACS: Selection with 100 nM Bet v 1 yielded almost no double positive cells that were not tested further. The sort with 50 nM Mal d 1 yielded more clones and was plated for further testing. Since only about 10% of plated cells manage to form colonies (Gordana Wozniak-Knopp, personal communication), the sort was grown on two plates to make sure sufficient colonies were available. From the initially selected 24 clones ("Mal50"), 8 were excluded after flow cytometric pre-screening (Sec. A.8.4). Sequencing of the remaining 16 clones revealed two novel HCDR3 sequences, which were successfully reformatted, but were not expressed. Additionally, eleven clones were found to carry Y4 containing heavy chains of which five, Mal50_3,6,10,14 and 19, had the same aa-sequence, albeit with different light chains. Interestingly, this heavy chain had also been present in Mal100_1 and Mal100_8, again with different light chains. This over-representation suggested higher than average affinity and three clones, Mal100_1, Mal100_8 and Mal50_6, were reformatted and expressed. Neither Mal100_1 nor 8 showed any affinity for the biotinylated version of either antigen, but bound native allergen (Fig. 9). The EC_{50} of Mal100_1 for Mal d 1 and Bet v 1 were determined at 97 nM and 62 nM and at 130 nM and 110 nM for Mal100_8. Mal50_6 exhibited weak affinity for biotinylated Mal d 1 and no affinity for biotinylated Bet v 1, but was cross-reactive with native antigen, albeit with lower affinity (Fig. 9). This analysis highlighted the potential of this clonotype and indicated that enrichment had been successful. Therefore, we argued that clones with unique HCDR3 sequences might have special reactivity. To identify more novel HDR3 sequences, 24 additional clones ("Mal50B") were screened (Sec. A.8.5). Four novel HCDR3 sequences could be identified (Mal50B_1,13,14 and 19) and were reformatted. Additionally, Mal50_17 had the same HCDR3 as Mal1x_5, a clone that had not been expressed, and was also reformatted. However, none of these antibodies bound to either antigen with particularly high affinity. Testing with native antigen was carried out by Gordana Wozniak-Knopp and Jonas Klammsteiner[3] and identified Mal50B_14 as a binder with a reactivity comparable to Mal50_6.

[3]Refer to the bachelor thesis of Jonas Klammsteiner for these data and the characterization of more "Mal50" and "Mal50B" clones (Tab. A.7.1), available in the CDLI2T, BOKU, DBT, Muthgasse 18, 1190 Vienna. Additional data on the selection and analysis of PLMB4 will be available in the bachelor thesis of Kira Markovitsch, once it is handed in.

4 Discussion

Herein we report the construction of a combinatorial Fab yeast display library representing the IgG repertoire of a patient that had undergone SLIT for birch pollen related apple allergy with recombinant Mal d 1 with a size of 1.95×10^7 independent clones. This library was used to search for cross-reactive antibodies binding both the major birch pollen allergen, Bet v 1, and the major apple allergen, Mal d 1. After selection using a combination of MACS and FACS, 24 clones could be expressed as full length IgG, of which four were cross-reactive and had affinities within the target EC_{50} range of 1 µM to 500 pM in immobilized antigen ELISA. The highest affinity clone, Mal100_5, bound both Mal d 1 and Bet v 1 with an EC_{50} of approximately 40 nM but was later shown to promiscuously bind unrelated allergens. The three remaining antibodies contained the same heavy chains but coupled with different light chains. EC_{50} values could be determined for two of these clones, Mal100_1 and Mal100_8, and were measured at 97 nM and 130 nM for Mal d 1, and 62 nM and 110 nM for Bet v 1, respectively.

Whereas the isolated antibodies exhibit the desired reactivity profile, their affinities are still well above the pico- to single-digit nanomolar affinity range necessary to be used as (pre-)clinical blocking antibodies [41]. It is estimated that naïve yeast display libraries with a size of 10^7 to 10^8 clones typically yield antibodies with affinities in the 1000-100 nM range [84]. On the other hand, Ferrara *et al.* demonstrated that immune libraries yield on average antibodies with affinities one order of magnitude below the affinities isolated from naïve libraries over a range of different platforms [56]. Thus the isolated antibodies are at the top of the expected affinity range of 100-1 nM. This suggests that the library should contain clones in the mid-to-low affinity range as well, which poses the question, why we were unable to isolate higher affinity antibodies.

The trivial explanation would be that an insufficient number of clones was screened. However, one has to consider the economics of screening. In the last sorts ("Mal100", "Mal50") there was about one novel HCDR3 sequence (used as a proxy for antibody diversity) in six clones as compared to every second clone in, for example, "Mal1x". This indicates that the species-richness curve was leveling off and that ever greater effort would have been necessary for the discovery of new variants. On the other hand, this also suggests that enough of the population had been sampled to be able to make assumptions about its properties, which would further suggest that our initial assumption was wrong and that the average affinity of the captured repertoire was lower than expected. This could be due to the limited success of the AIT treatment to induce high quality IgG blocking antibodies or a humoral immune response that is biased towards producing high concentrations of low affinity antibodies. Both factors, however, should have had an effect on treatment success. As mentioned previously, the clinical response of AIT correlates better with the amount of functional (presumably higher affinity) IgG_4 rather than total allergen-specific IgG_4 titers [33] and for SARS-CoV2 infections it has been demonstrated that the induction of high concentration of low affinity antibodies was associated with a more severe course of disease [85]. Thus, both failure to induce high-quality blocking antibodies as well as a less ideal antibody response should have been recognized during the initial trial and subsequent quantification of IgG [59, 58]. Another potential problem related to the patient's natural repertoire is the combinatorial nature of the system used, that results in the non-native pairing of heavy and light chains. Adler and coworkers directly compared native and randomly paired scFv libraries generated from immunized mice with humanized antibody loci and found that the random library had both a higher false positive rate (antibodies with native pairing were more likely to bind target antigen) and higher false negative rate (binders present in the native library were not identified in the randomly paired approach) [82]. However, 30% of binders isolated from the random library were also identified in the native library and 33% of binders only found in the random library were actually natively paired as they were present in the pre-sort native library. Furthermore, the highest affinity binder

actually had a pairing not found in the native library. Additionally, large scale sequencing data suggest that pairing of heavy and light chain is approximately random *in vivo*, especially in the naïve (IgM) repertoire and that light chains exhibit considerable promiscuity even after antigen exposure [82] (and sources therein), [86]. Another possibility would be that these clones had been lost during repertoire cloning. Repertoire cloning has an effect on library diversity, however, it has been demonstrated that this effect is moderate between the method used here and RACE (rapid amplification of cDNA ends) based approaches [87]. Furthermore, studies have shown that highly functional antibody display libraries can be created from a single patient and that high affinity antibodies can be found using the presented system [81, 88]. Additionally, loss of variants during cloning is independent of the properties of the encoded protein and should therefore be random in regard to affinity.

Whereas neither of these possibilities can be excluded, our data suggest that biotinylation of the allergens is the dominant factor interfering with antibody selection in this particular library. There is, to the authors knowledge, no data on the IgG epitope landscape of Mal d 1 following AIT, however, epitopes for Bet v 1 have been mapped with some accuracy. These data show that, similar to other humoral immune responses, Bet v 1 is recognized via several distinct epitopes and although some common epitopes exist, immunodominance varies between individuals [89, 90]. Assuming that this is also true for Mal d 1, it is possible that this patient's response is directed predominantly against an epitope that is destroyed or at least partially obstructed by biotinylation. This is corroborated by the frequent occurrence of the Y4 HCDR3 in all selected populations (41/75 heavy chain sequences) that was shown to be unreactive with biotinylated, but bound native allergens to some degree. Allergens were conjugated to biotin by lysine coupling at a molar ratio, biotin to protein, of 5:1 (Gordana Wozniak-Knopp, personal communication). Mal d 1 has several surface accessible lysine residues [91, 92] and we hypothesized that incompletely biotinylated allergen present in the allergen preparation used for MACS and FACS allowed us the selection of these variants in the first place. Therefore, the simplest way to address this problem would be the generally lower the degree of biotinylation, however, the coupling ratio was already chosen to be quite low (Gordana Wozniak-Knopp, personal communication). This suggests that biotinylation preferentially occurs at a particular lysine residue, that is activated by its molecular environment. If this is the case, selection would need to be carried out with a mixture of two allergen versions that had been biotinylated using different coupling chemistries (reviewed in[93]).

Independent of this, the discovered antibodies could be affinity-improved. Several techniques exist for *in vitro* affinity maturation (reviewed in [94]), however, this display system is particularly well suited for chain shuffling. It is believed that the heavy chain and in particular HCDR3, the most diverse of the heavy chain CDRs, forms the basis of antibody-antigen interaction (selectivity) which is augmented and modulated by the combination with a light chain (affinity), as demonstrated by Mal100_1, Mal100_8 and Mal50_6 in this work [95]. A selected heavy chain could therefore be mated back to the light chain sub-library and improved variants isolated with the same methodology used for discovery [55]. It would be interesting to see, if different light chains would change the affinity to both allergens simultaneously (one cross-reactive epitope) or bias it towards either allergen (possible recognition of two similar, but distinct epitopes).

Even without affinity improvement, the isolated antibodies might be a useful as a research tool, especially for epitope mapping. It has become increasingly clear that not all epitopes on a given allergen have the same potential for sensitization and IgE induction. For example, recent data demonstrated that, strikingly, IgE, and $IgG_{1/4}$ induced by AIT tend to recognize differential epitope patterns on Bet v 1 [90]. Similar findings had previously inspired research towards engineering novel allergen vaccines with reduced potential for anaphylaxis, IgE engagement and induction, and an increased potency for inducing protective IgG [28]. Furthermore, knowledge on dominant IgE and IgG epitopes could help in the selection of the most potent blocking antibodies [96].

5 References

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A Supplementary Data

A.1 Derivation of Sub-Libraries

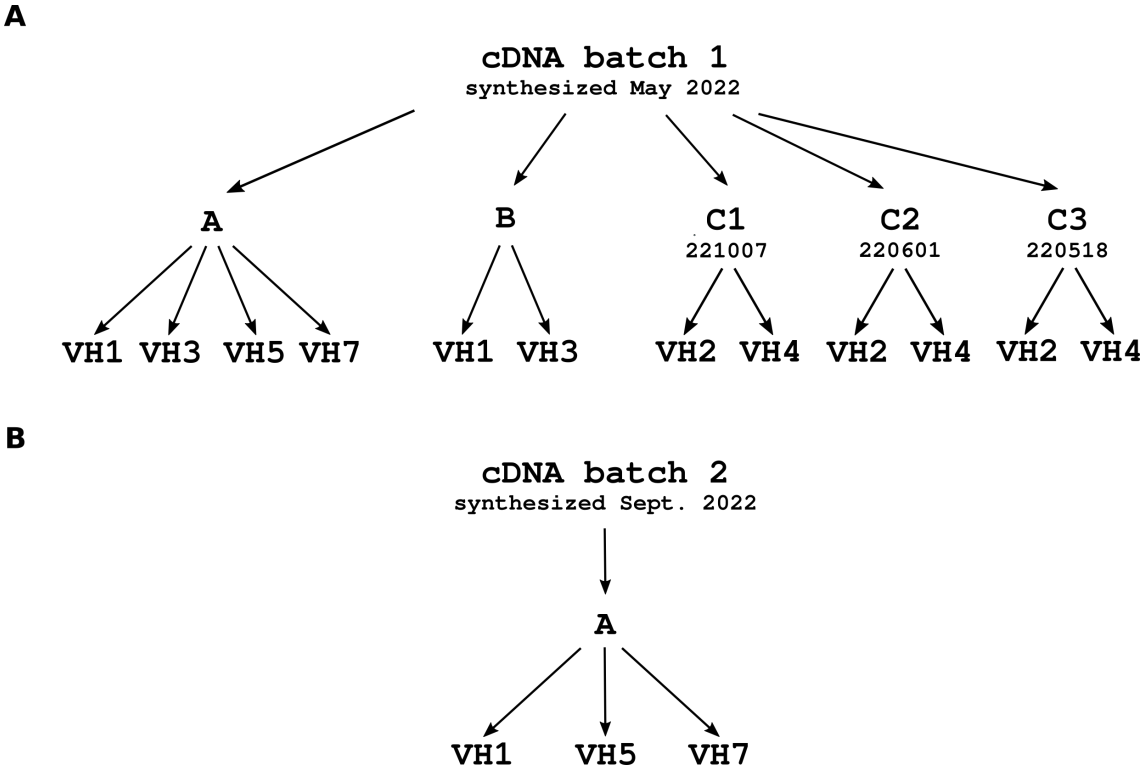


Figure A.1.1: Derivation tree of amplicons used to create IGHV family sub-libraries. Two batches of patient PBMCs were used for the extraction of total RNA and cDNA synthesis. From the first batch of cDNA (**A**), synthesized in May 2022, the A and B pool were synthesized only once, whereas the C pool was synthesized multiple times (batches indicated by date of synthesis). VHx in the third level denotes transformed reaction pools, that were amplified in the second PCR step with primers belonging to the corresponding IGHV family. Primers of VH5 and VH7 were applied to pool 1B, but yielded no detectable amplification product (data not shown). Similarly, PCRs on any C pool using the VH6 primer did not produce any usable product (data not shown). From the second batch of cDNA (**B**) all pools were synthesized but only the A pool was transformed, because the previously synthesized pools yielded better amplicons, as evaluated by agarose gel electrophoresis. The VH3 primers were also applied to pool 2A, but were eventually not transformed for the same reason.

A.2 Size of Heavy Chain Sub-Libraries

Table A.2.1: Size of the PLMB4 heavy chain sub-libraries and the mass of insert used for transformation. See Fig. A.1.1 for naming convention.

Name	**Insert** [μg]	**Size** [cfu]
1A_VH1	10.13	2.6×10^6
1A_VH3	12.22	2.6×10^6
1A_VH5	2.42	2.5×10^5
1A_VH7	1.55	2.8×10^5
2A_VH1	10.43	4.5×10^6
2A_VH5	2.82	1.2×10^6
2A_VH7	3.60	1.6×10^6
1B_VH1	4.76	3.4×10^6
1B_VH3	11.71	5.7×10^6
1C1_VH2_1L	0.63	2.3×10^5
1C1_VH2_2L	0.55	1.3×10^5
1C1_VH4_1L	0.39	9.5×10^4
1C2_VH2	1.81	8.9×10^5
1C2_VH4	2.61	9.7×10^5
1C3_VH2	1.23	5.7×10^5
1C3_VH4	1.74	6.5×10^5

A.3 Quality Control of Heavy Chain Sub-Libraries

Table A.3.1: Quality control of heavy chain sub-libraries. Per sub-library, four to twelve colonies grown on selective agar were analyzed by Sanger sequencing. Clones were sequenced to exclude the possibility of systematic errors during repertoire capture and assure the fidelity of plasmid assembly. The readable sequences (about half the clones sent to sequencing) suggest a library correctness of about 95%. Sub-libraries are given in the order in which they were analyzed to illustrate that unreadable sequences are rarer in later analyses, suggesting that improved skills had reduced operator's errors.

Sub-library	Analyzed	Detectable Insert	Correct	Frameshift	Seq. issue
2A_VH1	8	7	4	0	3
1B_VH1	8	5	2	1	2
1A_VH3	8	8	4	0	4
1B_VH3	8	4	3	0	1
2A_VH5 (first)	8	5	1	1	3
2A_VH7	8	7	4	0	3
1C_221007_VH2_1L	4	2	1	0	1
1C_221007_VH2_2L	4	2	2	0	0
1C_221007_VH4_1L	4	2	1	0	1
2A_VH5 (second)	12	8	8	0	0
1A_VH1	8	6	5	0	1
1A_VH5	8	7	6	0	1
1A_VH7	8	7	6	1	0
1C_220518_VH2	4	2	1	0	1
1C_220518_VH4	4	2	2	0	0
1C_220601_VH2	4	3	3	0	0
1C_220601_VH4	4	4	4	0	0
Total	112	81	57	3	21

A.4 Quality Control of Fab Library

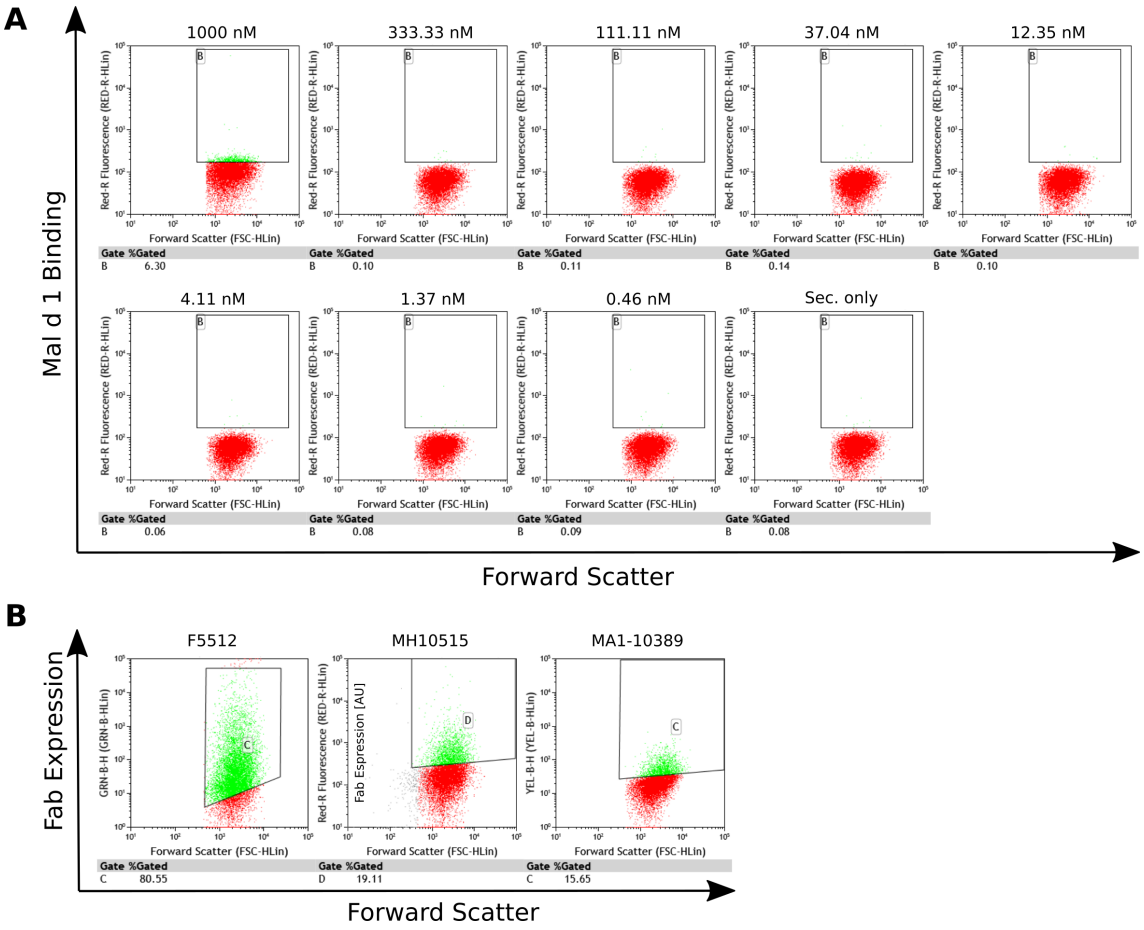


Figure A.4.1: A: Flow cytometric analysis of the antigen affinity of the unsorted Fab library after mating. Induced cells were incubated with a dilution series of biotinylated Mal d 1 and stained with streptavidin-Alexa647 conjugate. **B:** Analysis of Fab expression with different, directly labeled, antibodies. F5512: Goat anti-human IgG (Fab specific)-fluorescein isothiocyanate; MH10515: Mouse anti-human kappa light chain-allophycocyanin; MA1-10389: Mouse anti-human kappa light chain-phycoerythrin. Gates were set to capture 0.1% of signal in cells-only controls. The data represents one of two replicas.

A.5 Cross-reactivity Test, First Sorting Campaign

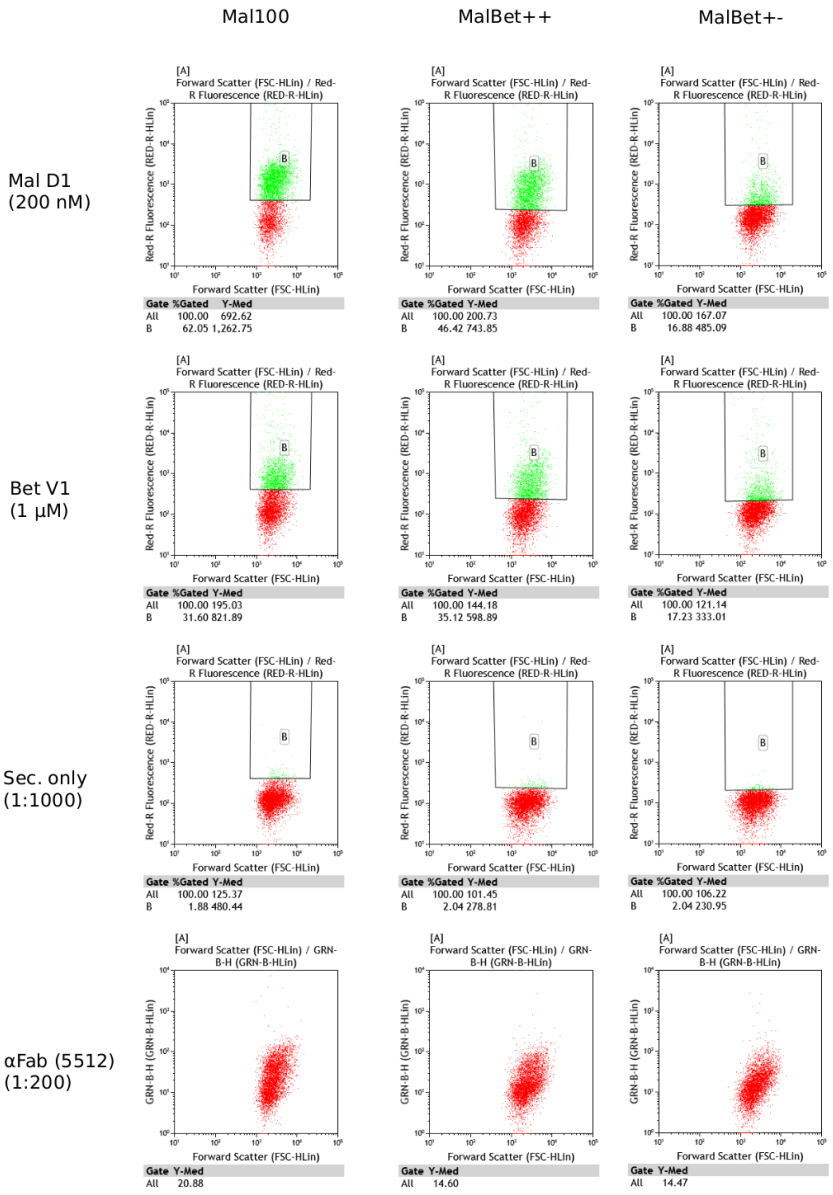


Figure A.5.1: Flow Cytometric Test of Sorts Isolated in the First Sorting Campaign (Fig. 8). Mal100 selected by: 1× MACS 1 µM Mal d 1, 2× FACS: 1 μM and 1× 100 nM Mal d 1, MalBet++/MalBet+- selected by: 1× MACS 1 µM Mal d 1, 2× FACS: 1 µM Bet v 1 and 1 µM Mal d 1. Test was performed on sorts in bulk. Based on this data MalBet++ was selected as the most promising sort for the isolation of cross-reactive clones.

A.6 Cross-Reactivity Test, Second Sorting Campaign

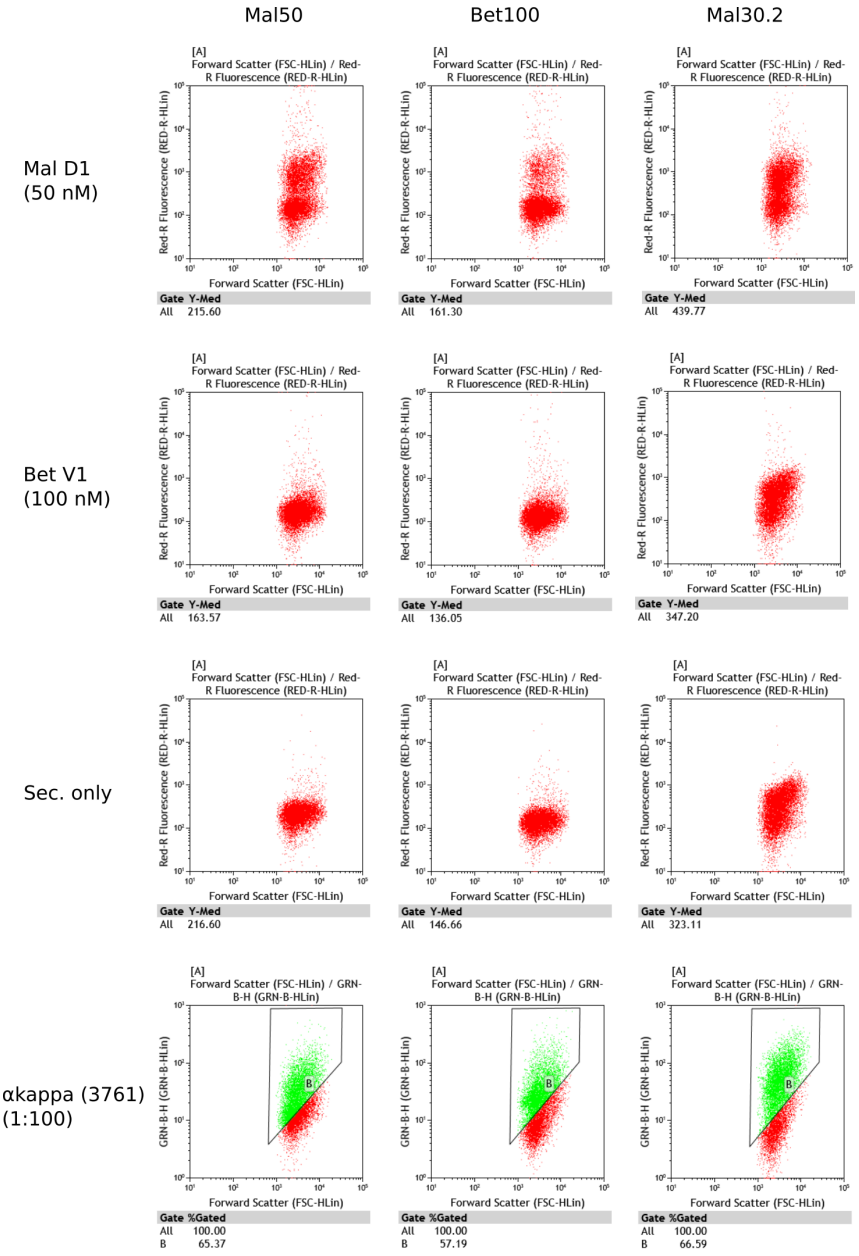


Figure A.6.1: Flow cytometric test of sorts isolated in the second sorting campaign (Fig. 8). Mal50: 1× MACS 50 nM Mal d 1 and 1× FACS 50 nM Mal d 1; Bet100: 1× MACS 50 nM Mal d 1 and 1× FACS 100 nM Bet v 1; Mal30.2: MACS 1 µM Mal d 1, FACS: 1× 1 µM, 1× 100 nM and 2× 30 nM Mal d 1. Mal50 showed considerable enrichment of Mal d 1 binders and was further analyzed. Bet100 did not seem to contain any cross-reactive clones and was excluded. Mal30.2 contained clones with an affinity for the streptavidin-based secondary labeling reagent, was later shown to only contain one antibody and was also excluded.

A.7 Screening Overview

Table A.7.1: Overview of clones that were assessed individually and at which point they were excluded from further analysis. MsP: Master plate (could the clone be grown as pure culture); Flow. Cyt.: Flow cytometric pre-screening (see Sec. A.8; HC/LC: Both heavy and light chains were present, free of frameshifts and could be sequenced (NR: None readable); Primer: Recloning primers where available or ordered; pTT5: Recloning; Expression: Protein production by transient transfection in HEK cells.

Clone	MsP	Flow. Cyt.	HC	LC	Primer	pTT5	Expression
Mal1x_1	yes	no					
Mal1x_2	yes	yes	yes	yes	no		
Mal1x_3	yes	yes	yes	yes	no		
Mal1x_4	yes	no					
Mal1x_5	yes	yes	yes	yes	yes	yes	no
Mal1x_6	yes	yes	yes	yes	yes	yes	no
Mal1x_7	yes	yes	yes	yes	yes	yes	yes
Mal1x_8	yes	yes	yes	yes	yes	yes	yes
Mal1x_9	yes	yes	yes	yes	yes	yes	no
Mal1x_10	yes	yes	yes	yes	yes	yes	yes
Mal1x_11	yes	yes	yes	yes	yes	yes	yes
Mal1x_12	yes	yes	yes	yes	yes	yes	yes
Mal1x_13	yes	yes	yes	yes	no		
Mal1x_14	yes	no					
Mal1x_15	yes	yes	yes	yes	yes	yes	no
Mal1x_16	yes	yes	yes	yes	yes	yes	yes
Mal1x_17	yes	yes	yes	yes	yes	yes	yes
Mal1x_18	yes	yes	yes	yes	yes	yes	yes
Mal1x_19	yes	yes	yes	yes	yes	yes	yes
Mal1x_20	yes	yes	yes	yes	yes	yes	yes
Mal1x_21	yes	yes	yes	yes	yes	yes	yes
Mal1x_22	no	yes					
Mal1x_23	yes	yes	yes	yes	yes	no	
Mal1x_24	yes	yes	no	yes			
MalBet++_1	yes	no					
MalBet++_2	yes	yes	yes	no			
MalBet++_3	yes	no					
MalBet++_4	yes	no					
MalBet++_5	yes	no					
MalBet++_6	yes	no					
MalBet++_7	yes	no					
MalBet++_8	yes	yes	yes	no			
MalBet++_9	yes	no					
MalBet++_10	yes	yes	yes	no			
MalBet++_11	yes	yes	yes	no			
MalBet++_12	yes	no					
MalBet++_13	yes	yes	yes	no			
MalBet++_14	yes	yes	yes	yes	yes	yes	yes
MalBet++_15	yes	no					
MalBet++_16	yes	no					

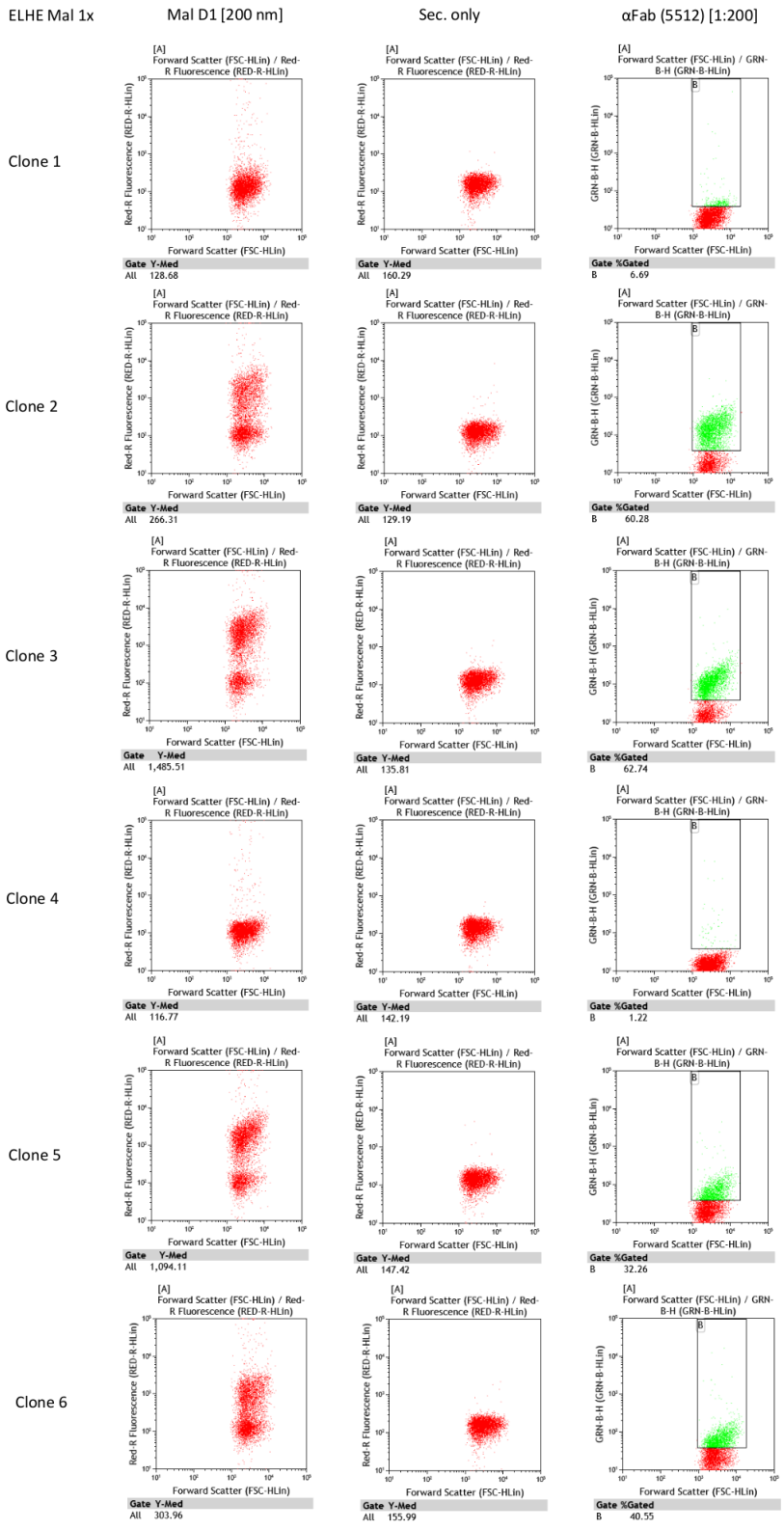
Clone	MsP	Flow. Cyt.	HC	LC	Primer	pTT5	Expression
MalBet++_17	yes	yes	yes	yes	yes	yes	yes
MalBet++_18	yes	no					
MalBet++_19	yes	no					
MalBet++_20	yes	yes	yes	yes	yes	yes	yes
MalBet++_21	yes	no					
MalBet++_22	yes	yes	yes	no			
MalBet++_23	yes	yes	yes	no			
MalBet++_24	yes	yes	yes	yes	yes	yes	yes
Mal100_1	yes	yes	yes**	yes	yes	yes	yes
Mal100_2	yes	no	yes	no			
Mal100_3	yes	yes	yes	no			
Mal100_4	yes	yes	yes	yes			
Mal100_5	yes	yes	yes	yes	yes	yes	yes
Mal100_6	yes	no	yes	no			
Mal100_7	yes	yes	yes	yes			
Mal100_8	yes	yes	yes**	yes	yes	yes	yes
Mal100_9	yes	yes	yes	yes			
Mal100_10	yes	yes	yes	yes			
Mal50_1	yes	no					
Mal50_2*	yes	yes	yes	yes	yes		
Mal50_3*	yes	yes	yes**	yes	yes***		
Mal50_4	yes	no					
Mal50_5	yes	no					
Mal50_6	yes	yes	yes**	yes	yes	yes	yes
Mal50_7	yes	yes	yes	yes	no		
Mal50_8	yes	no					
Mal50_9	yes	yes	yes	yes	no		
Mal50_10*	yes	yes	yes**	yes	yes***		
Mal50_11*	yes	yes	yes	yes	yes		
Mal50_12	yes	yes	yes	yes	yes		
Mal50_13	yes	no					
Mal50_14*	yes	yes	yes**	yes	no		
Mal50_15	yes	yes	yes	yes	yes	yes	no
Mal50_16	yes	yes	yes	yes	yes***		
Mal50_17	yes	yes	yes	yes	no		
Mal50_18	yes	no					
Mal50_19*	yes	yes	yes**	yes	yes		
Mal50_20	yes	no					
Mal50_21	yes	yes	yes	yes	yes		
Mal50_22	yes	yes	yes	yes	yes		
Mal50_23	yes	yes	yes	yes	yes***	yes	no
Mal50_24	yes	no					
Mal50B_1	yes	yes	yes	yes	yes	yes	yes
Mal50B_2*	yes	yes	yes	yes			
Mal50B_3	yes	no					

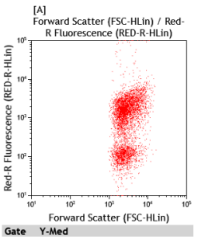
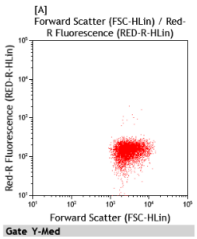
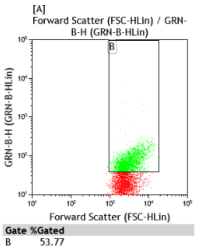
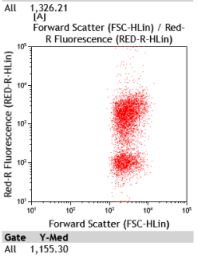
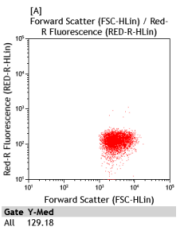
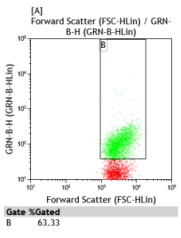
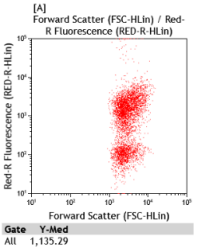
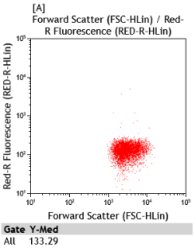
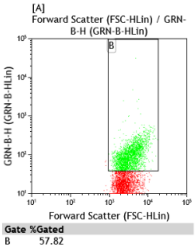
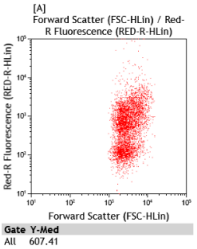
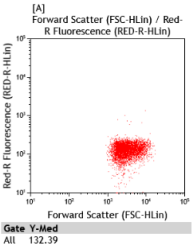
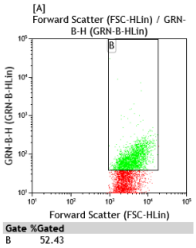
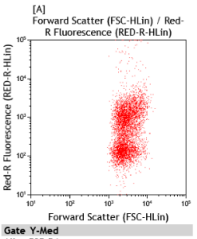
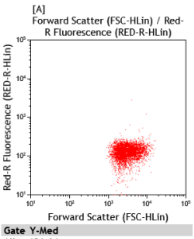
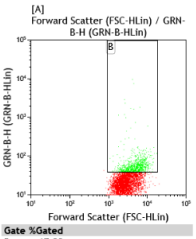
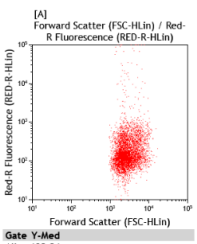
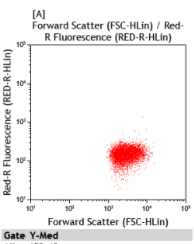
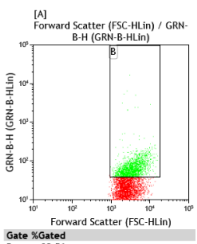
Clone	MsP	Flow. Cyt.	HC	LC	Primer	pTT5	Expression
Mal50B_4	yes	no					
Mal50B_5	yes	yes	yes	yes			
Mal50B_6*	yes	yes	yes	yes			
Mal50B_7*	yes	yes	yes	yes			
Mal50B_8	yes	yes	yes	yes			
Mal50B_9	yes	yes	yes	yes			
Mal50B_10*	yes	yes	yes	yes			
Mal50B_11	yes	yes	yes**	NR			
Mal50B_12	yes	yes	yes	yes			
Mal50B_13	yes	yes	yes	yes	yes	yes	yes
Mal50B_14	yes	yes	yes	yes	yes	yes	yes
Mal50B_15*	yes	yes	yes	yes			
Mal50B_16*	yes	yes	yes	yes			
Mal50B_17	yes	yes	yes	yes	yes	yes	yes
Mal50B_18	yes	yes	yes	yes			
Mal50B_19	yes	yes	yes	yes	yes	yes	yes
Mal50B_20	yes	yes	yes	yes			
Mal50B_21	yes	yes	yes**	yes			
Mal50B_22	yes	yes	yes**	yes			
Mal50B_23	yes	yes	yes**	yes			
Mal50B_24	yes	yes	yes	yes			

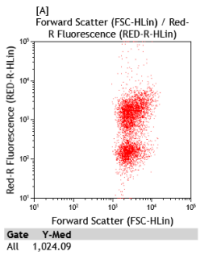
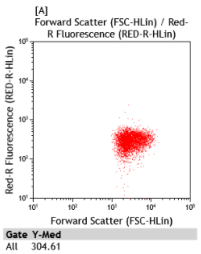
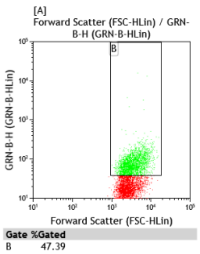
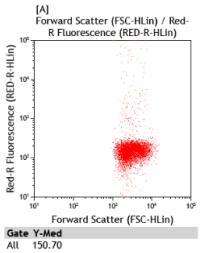
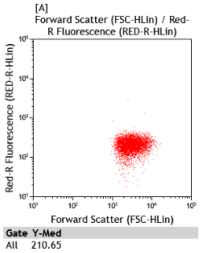
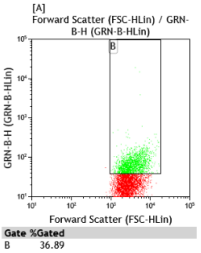
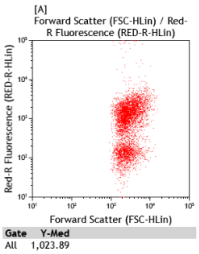
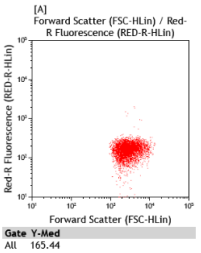
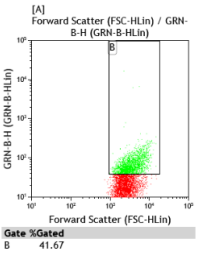
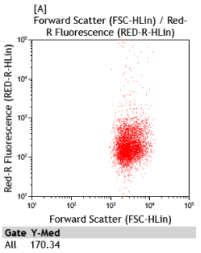
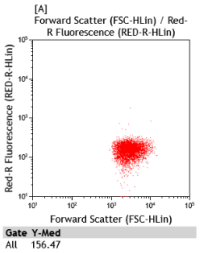
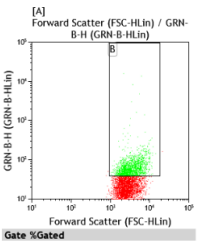
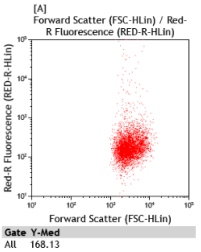
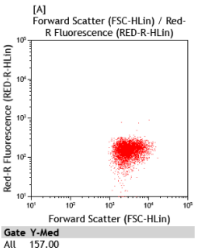
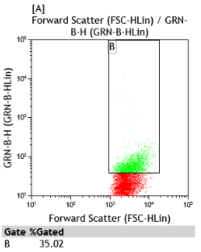
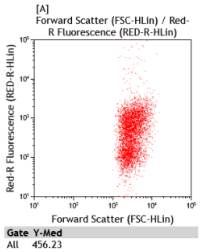
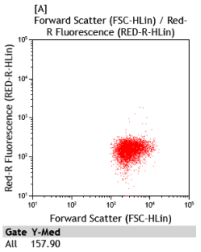
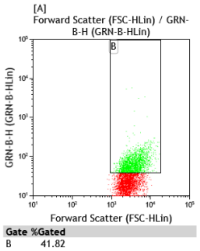
* These clones were analyzed by Gordana Wozniak-Knopp and Jonas Klammsteiner outside of this thesis. For characterization please refer to the bachelor thesis of Jonas Klammsteiner.
** All of these clones likely carry the same heavy chain, but none could be sequenced in one read. A consensus sequence was compiled out of Mal100_1, Mal100_8, Mal50_3.
*** Amino acid sequence of selected primer differed from the sequence contained within pYD at one position.

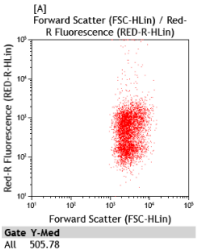
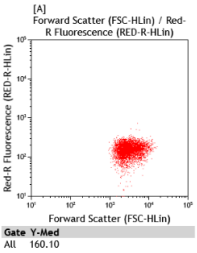
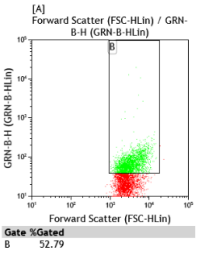
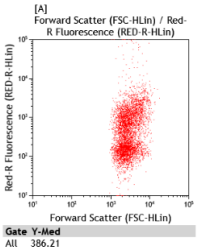
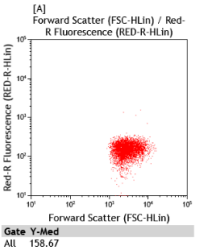
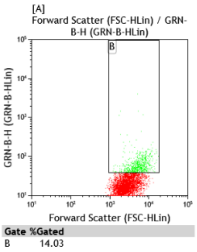
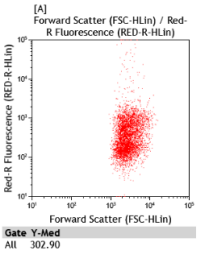
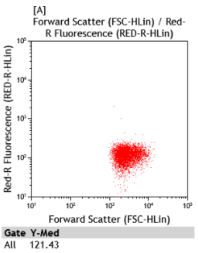
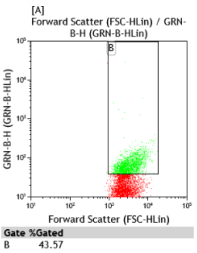
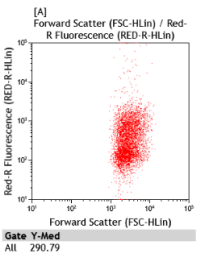
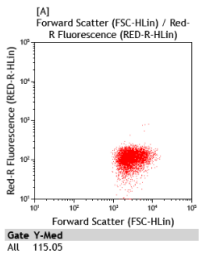
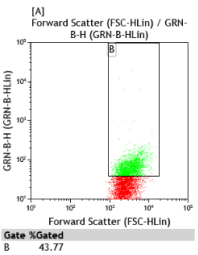
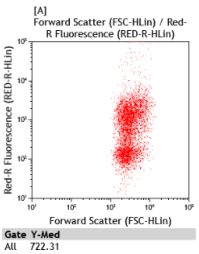
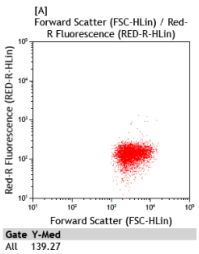
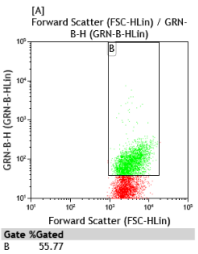
A.8 Flow Cytometric Screening of Individual Clones

A.8.1 Flow Cytometric Analysis of Isolated Mal1x Clones

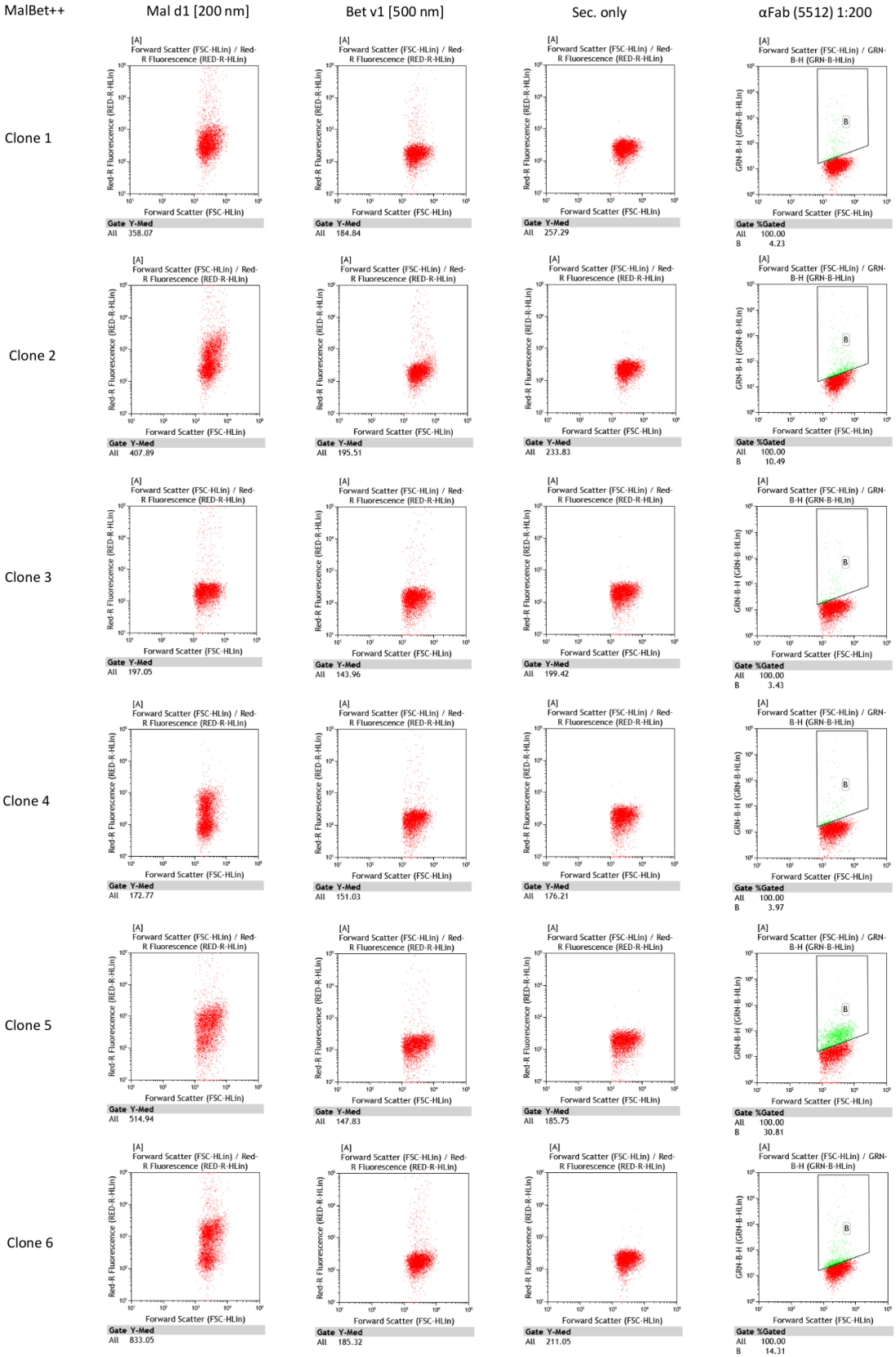


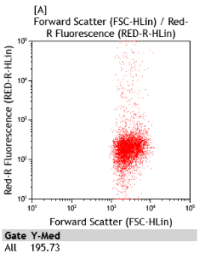
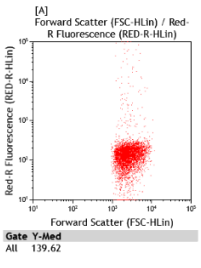
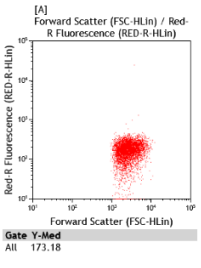
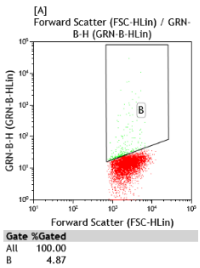
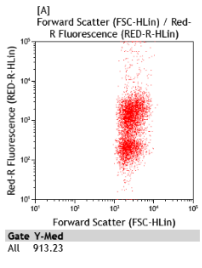
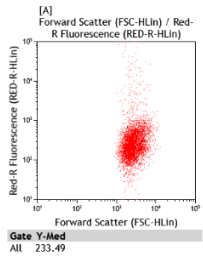
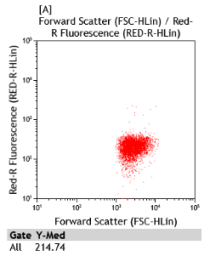
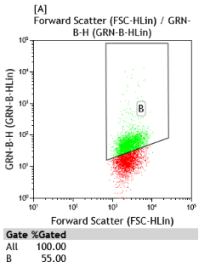
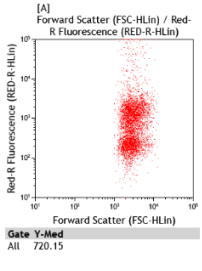
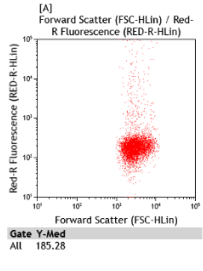
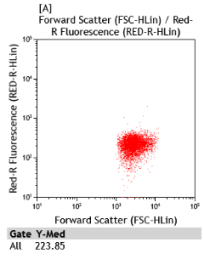
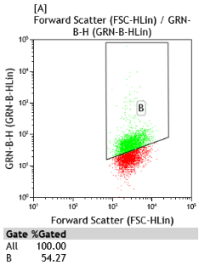
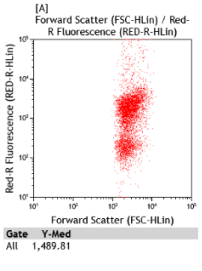
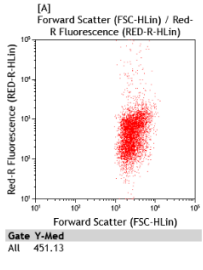
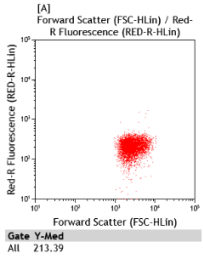
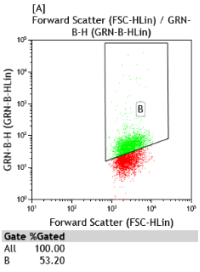
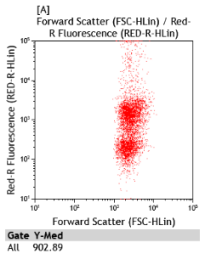
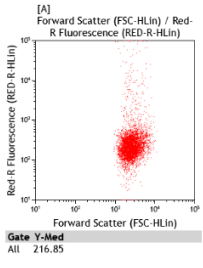
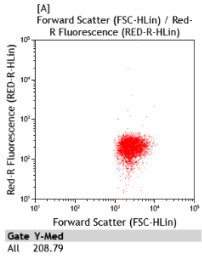
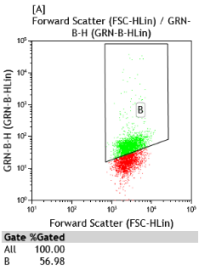
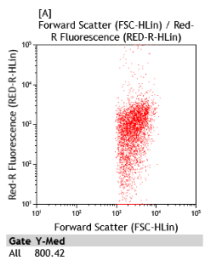
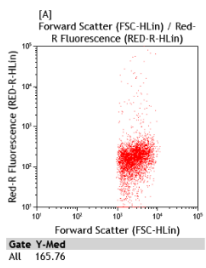
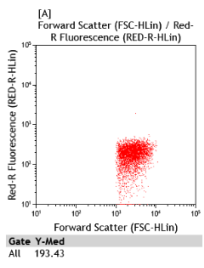
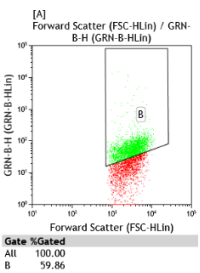
ELHE Mal 1x	Mal D1 [200 nm]	Sec. only	αFab (5512) [1:200]
Clone 7	 Gate Y-Med All 1,326.21	 Gate Y-Med All 145.78	 Gate %Gated B 53.77
Clone 8	 Gate Y-Med All 1,155.30	 Gate Y-Med All 129.18	 Gate %Gated B 63.33
Clone 9	 Gate Y-Med All 1,135.29	 Gate Y-Med All 133.29	 Gate %Gated B 57.82
Clone 10	 Gate Y-Med All 607.41	 Gate Y-Med All 132.39	 Gate %Gated B 52.43
Clone 11	 Gate Y-Med All 535.54	 Gate Y-Med All 131.64	 Gate %Gated B 17.90
Clone 12	 Gate Y-Med All 138.94	 Gate Y-Med All 152.48	 Gate %Gated B 39.56

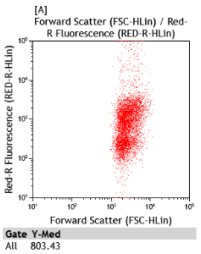
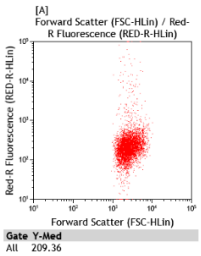
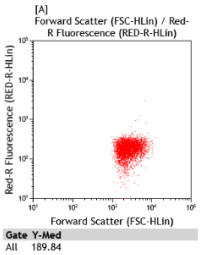
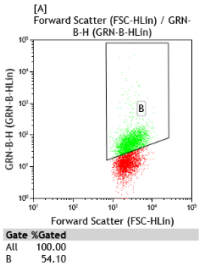
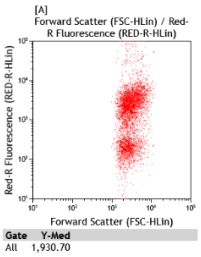
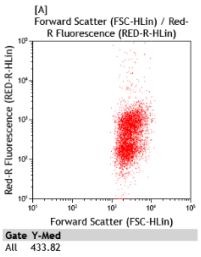
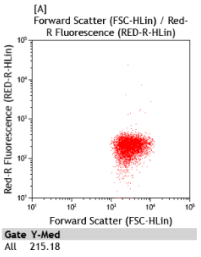
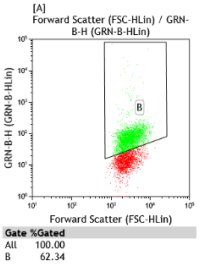
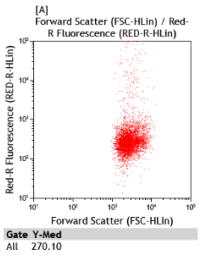
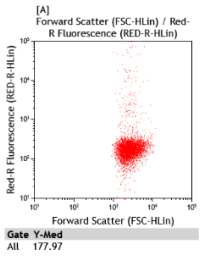
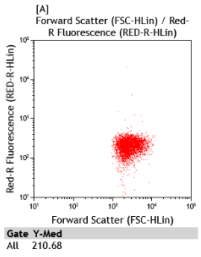
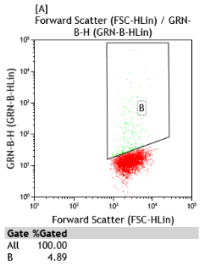
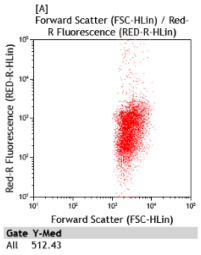
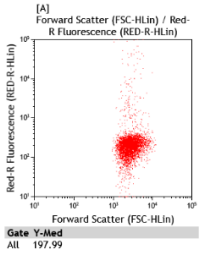
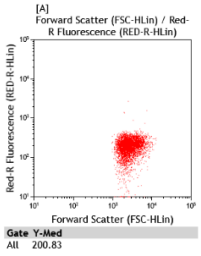
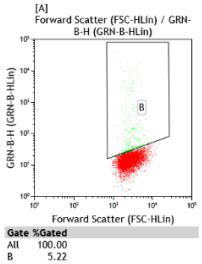
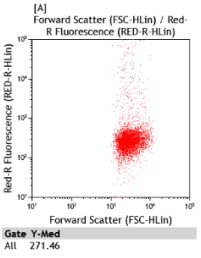
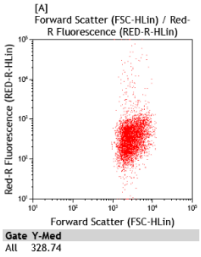
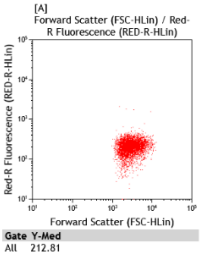
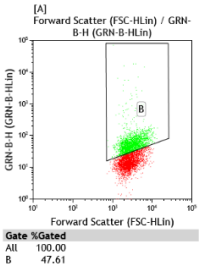
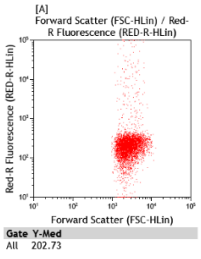
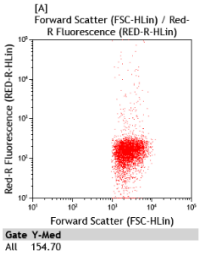
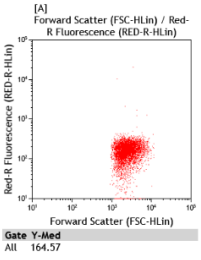
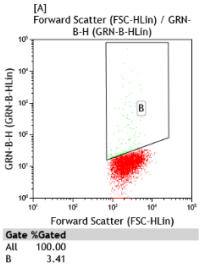
ELHE Mal 1x	Mal D1 [200 nm]	Sec. only	αFab (5512) [1:200]
Clone 13	 Gate Y-Med All 1,024.09	 Gate Y-Med All 304.61	 Gate %Gated B 47.39
Clone 14	 Gate Y-Med All 150.70	 Gate Y-Med All 210.65	 Gate %Gated B 36.89
Clone 15	 Gate Y-Med All 1,023.89	 Gate Y-Med All 165.44	 Gate %Gated B 41.67
Clone 16	 Gate Y-Med All 170.34	 Gate Y-Med All 156.47	 Gate %Gated B 34.49
Clone 17	 Gate Y-Med All 168.13	 Gate Y-Med All 157.00	 Gate %Gated B 35.02
Clone 18	 Gate Y-Med All 456.23	 Gate Y-Med All 157.90	 Gate %Gated B 41.82

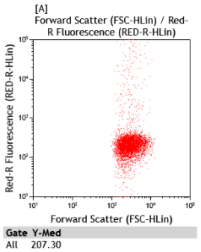
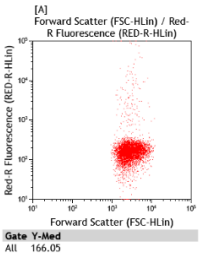
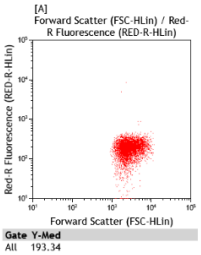
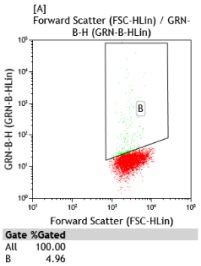
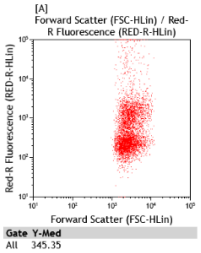
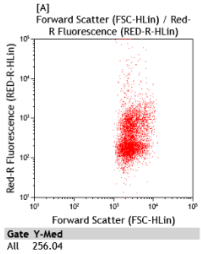
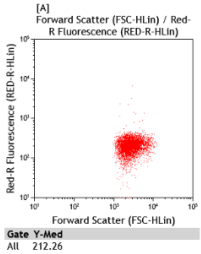
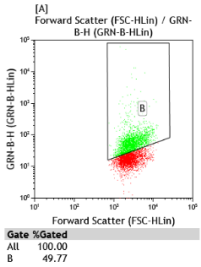
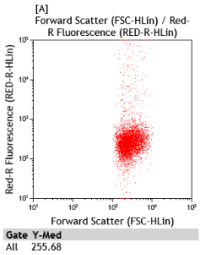
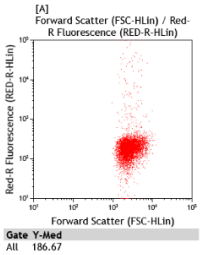
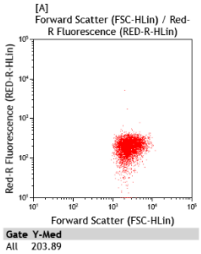
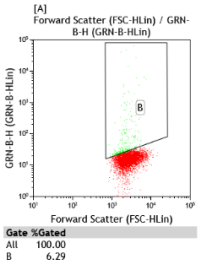
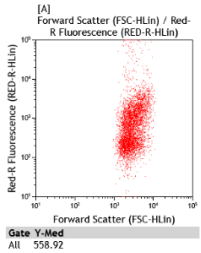
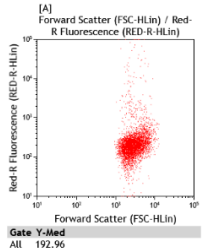
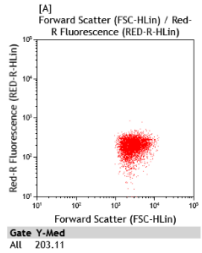
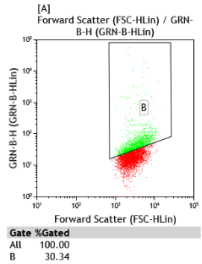
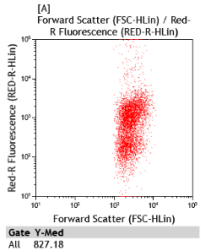
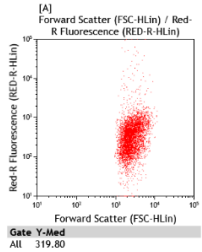
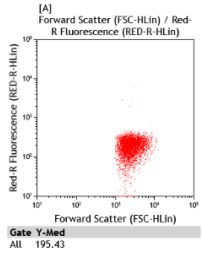
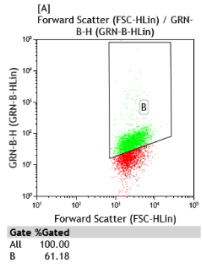
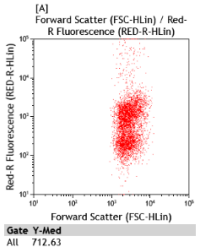
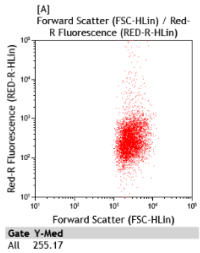
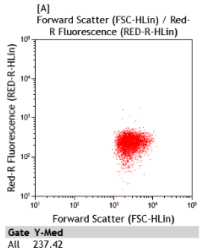
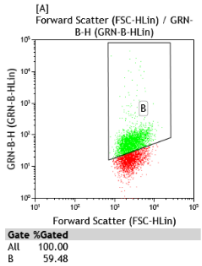
ELHE Mal 1x	Mal D1 200 nm	Sec. only	αFab (5512) 1:200
Clone 19			
Clone 20			
Clone 21			
Clone 22:	Did not grow		
Clone 23			
Clone 24			

A.8.2 Flow Cytometric Analysis of Isolated MalBet++ Clones

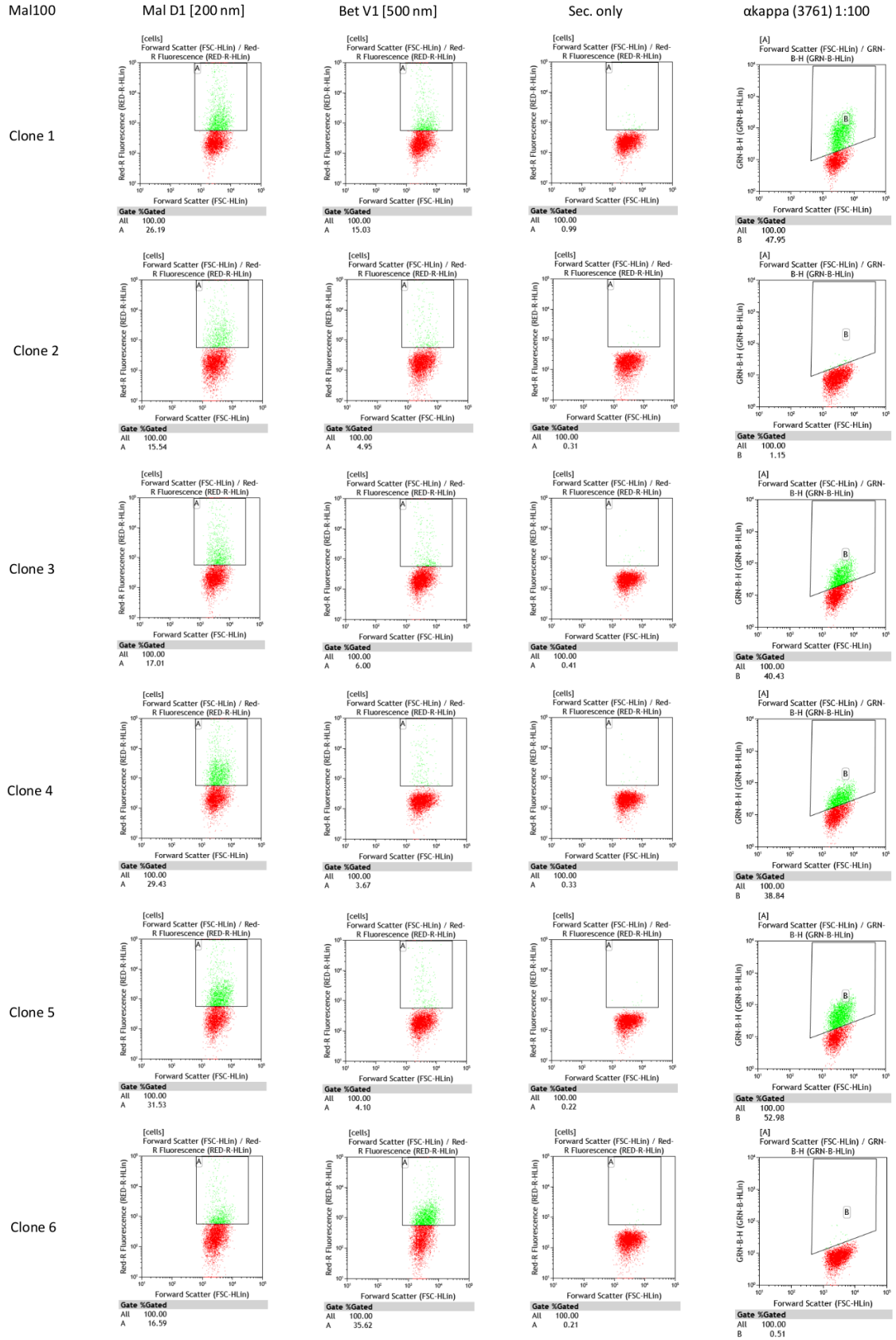


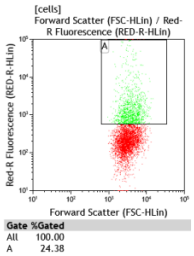
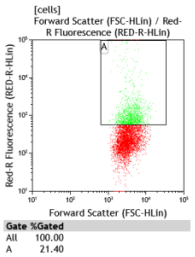
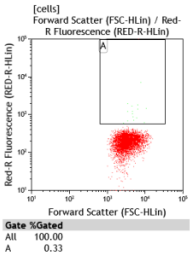
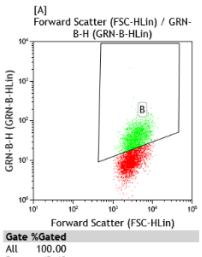
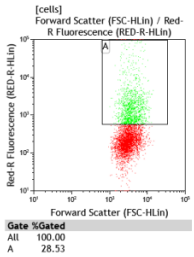
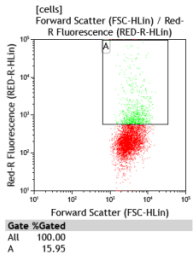
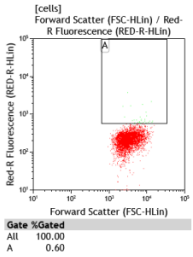
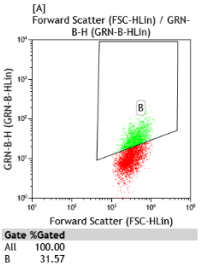
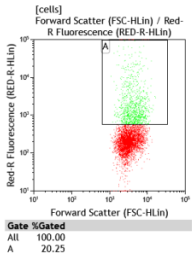
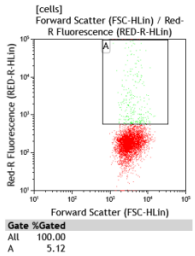
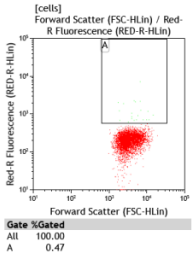
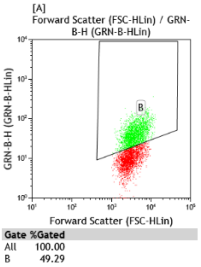
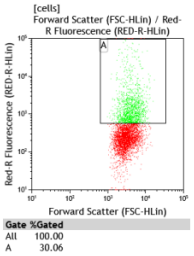
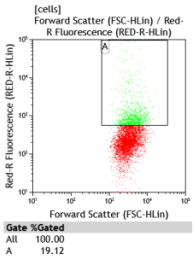
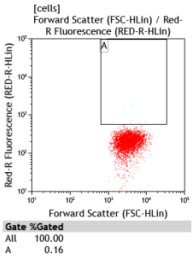
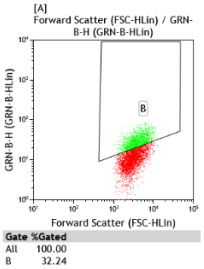
MalBet++	Mal d1 [200 nm]	Bet v1 [500 nm]	Sec. only	αFab (5512) 1:200
Clone 7				
Clone 8				
Clone 9				
Clone 10				
Clone 11				
Clone 12				

MalBet++	Mal d1 [200 nm]	Bet v1 [500 nm]	Sec. only	αFab (5512) 1:200
Clone 13				
Clone 14				
Clone 15				
Clone 16				
Clone 17				
Clone 18				

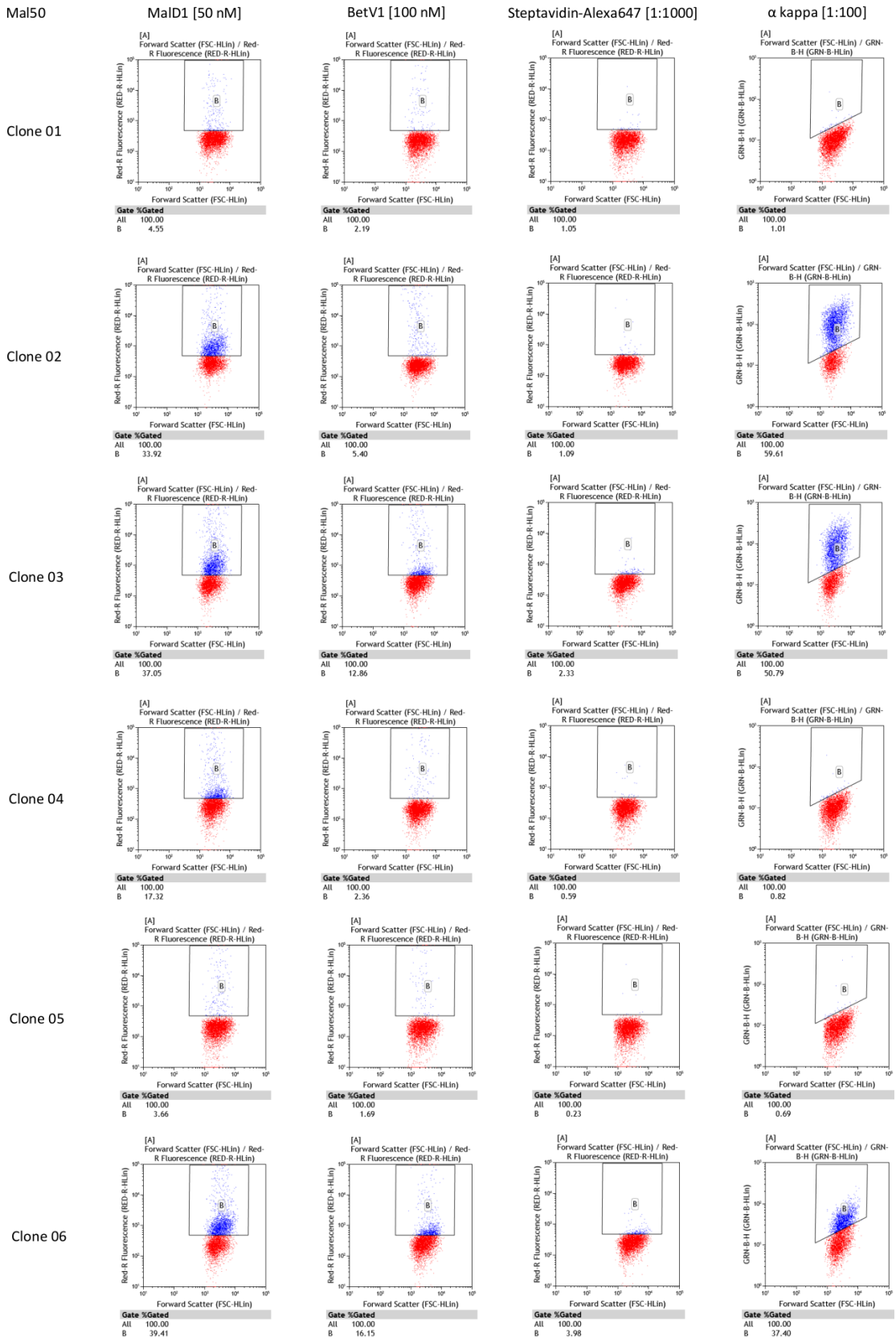
MalBet++	Mal d1 [200 nm]	Bet v1 [500 nm]	Sec. only	αFab (5512) 1:200
Clone 19				
Clone 20				
Clone 21				
Clone 22				
Clone 23				
Clone 24				

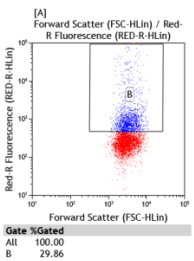
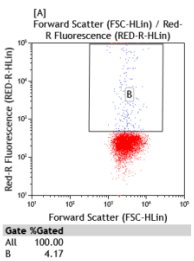
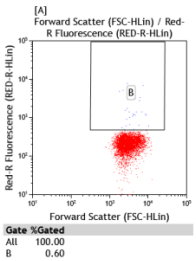
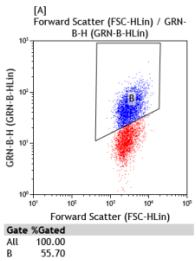
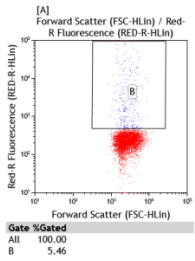
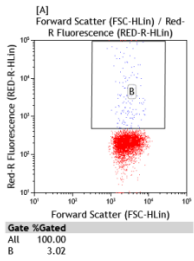
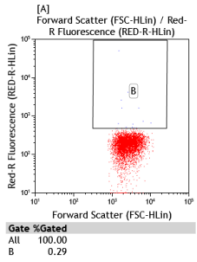
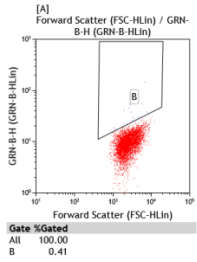
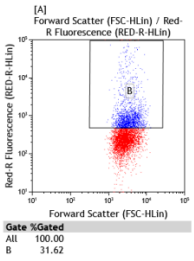
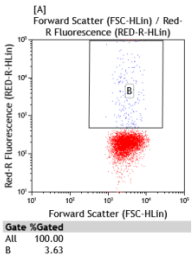
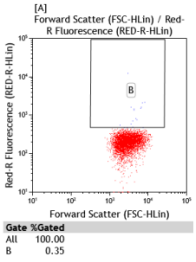
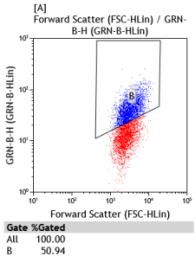
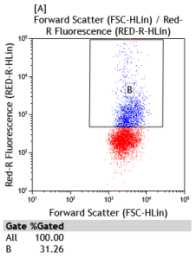
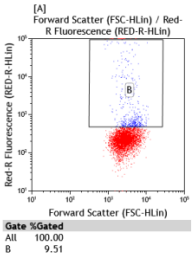
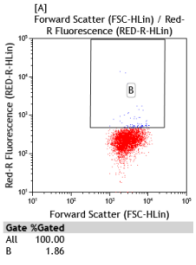
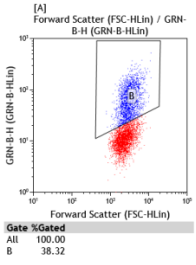
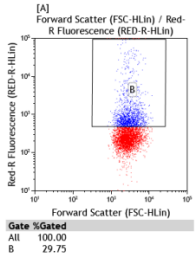
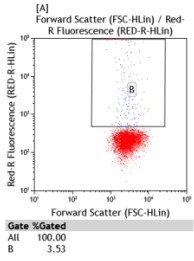
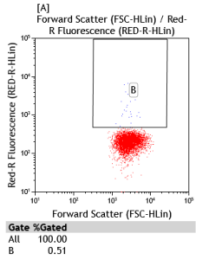
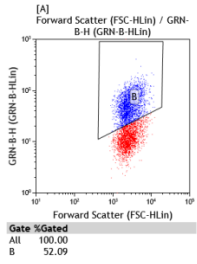
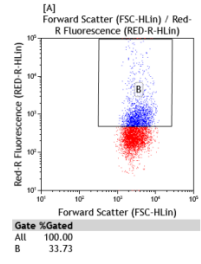
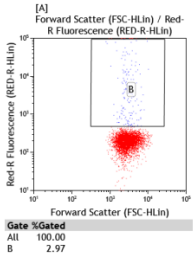
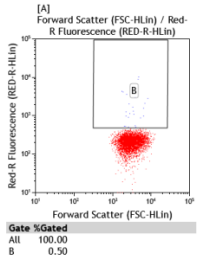
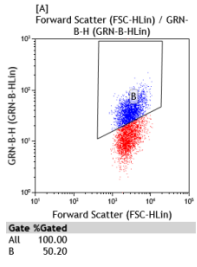
A.8.3 Flow Cytometric Analysis of Isolated Mal100 Clones

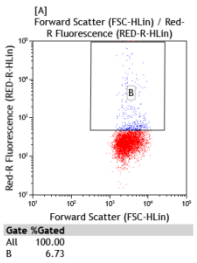
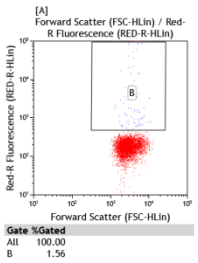
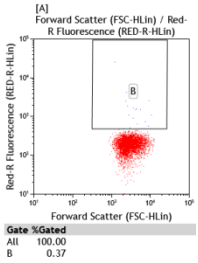
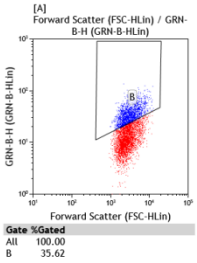
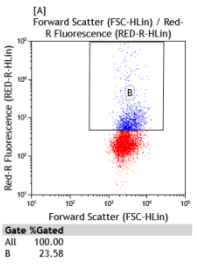
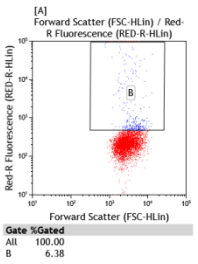
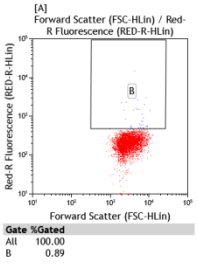
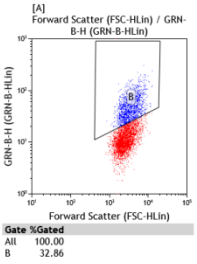
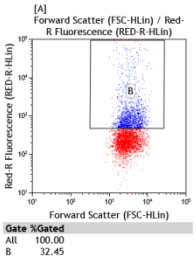
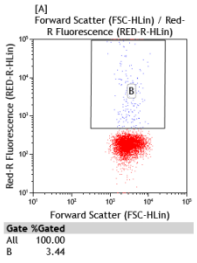
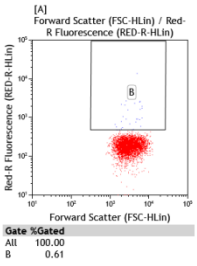
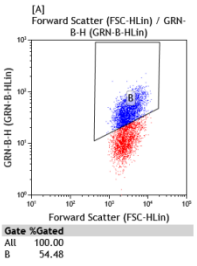
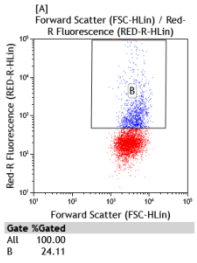
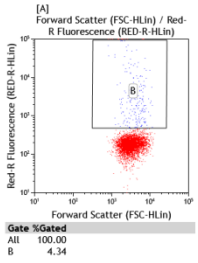
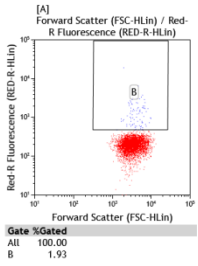
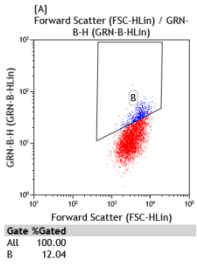
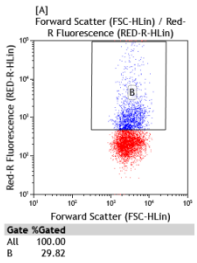
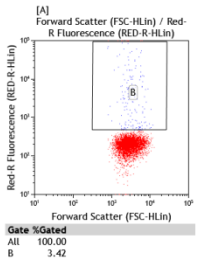
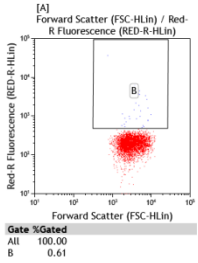
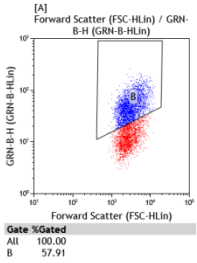
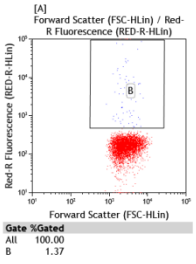
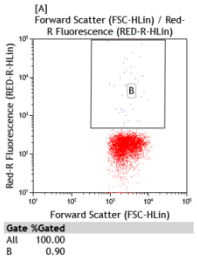
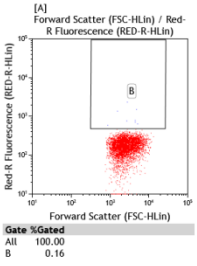
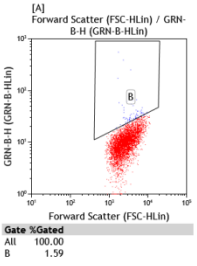


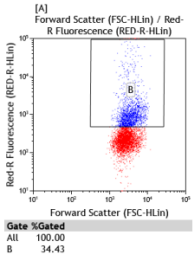
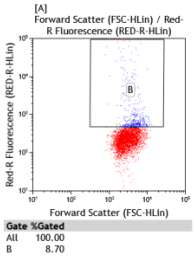
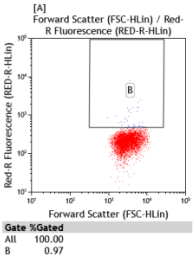
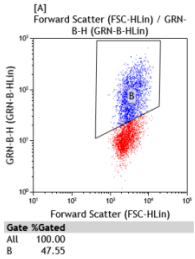
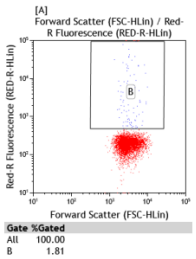
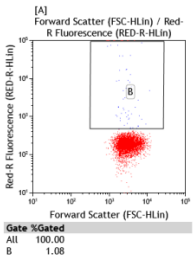
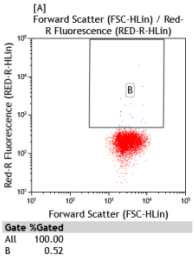
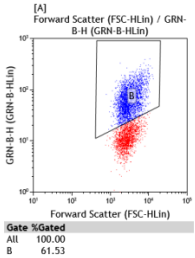
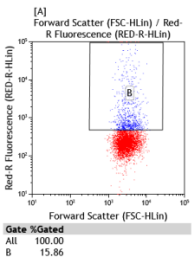
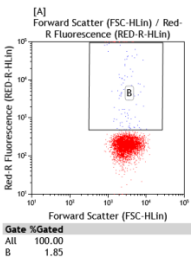
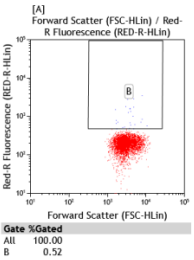
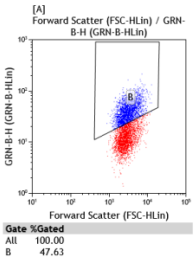
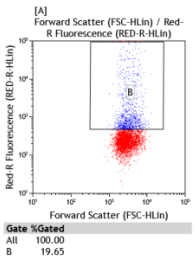
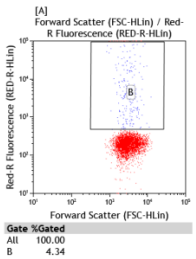
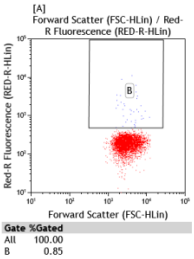
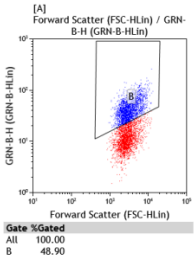
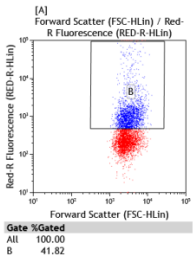
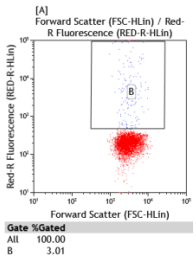
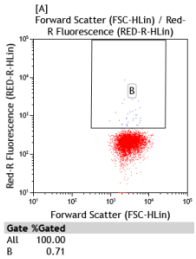
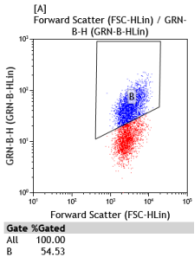
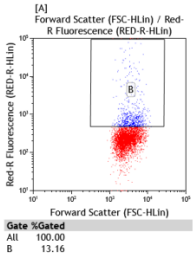
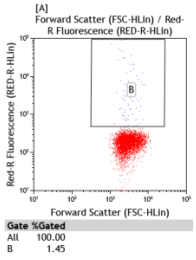
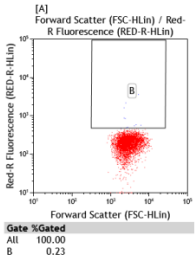
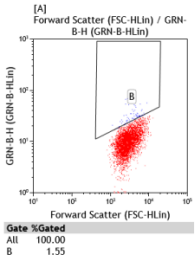
Mal100	Mal D1 [200 nm]	Bet V1 [500 nm]	Sec. only	αkappa (3761) 1:100
Clone 7				
Clone 8				
Clone 9				
Clone 10				

A.8.4 Flow Cytometric Analysis of Isolated Mal50 Clones

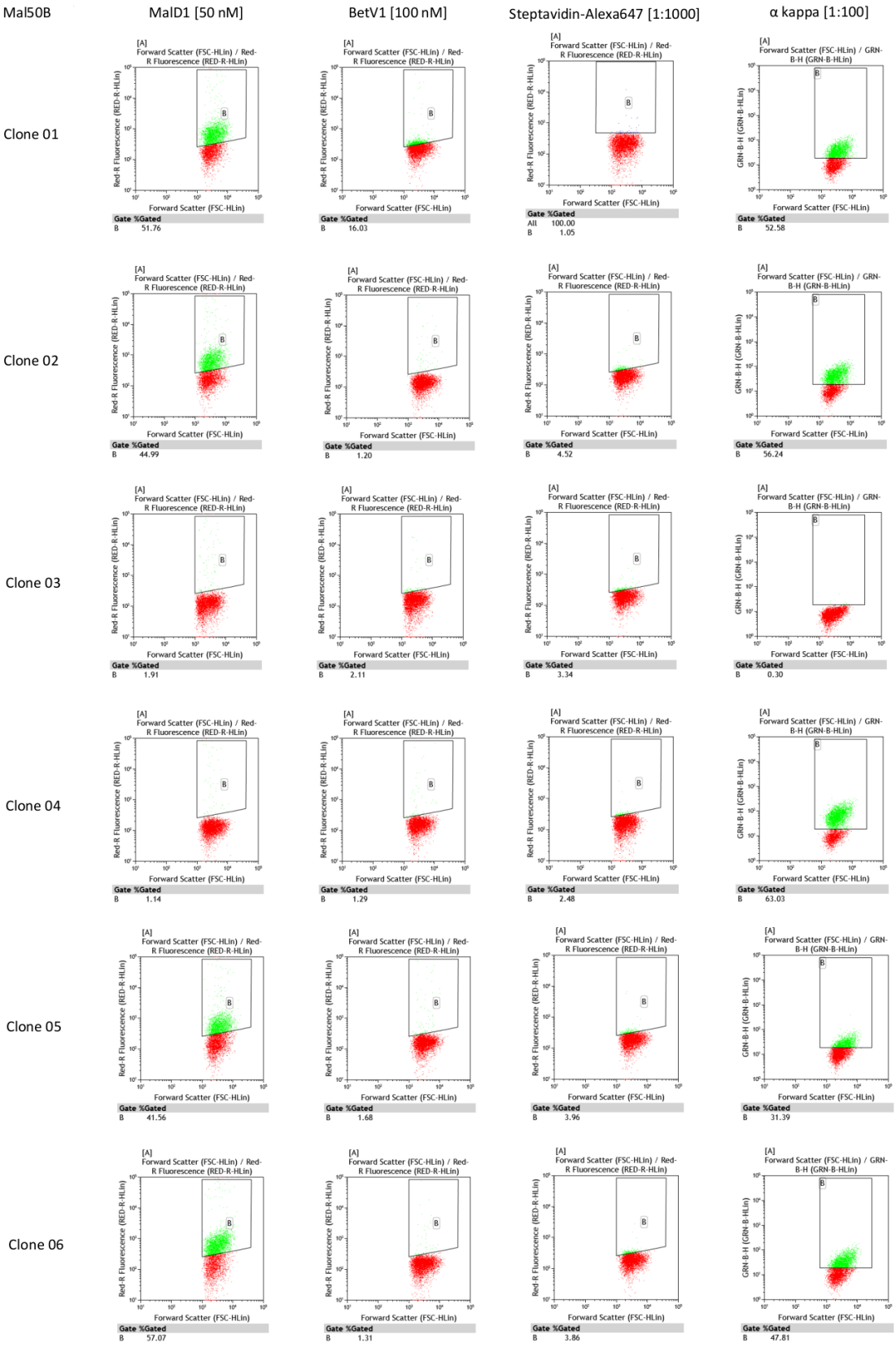


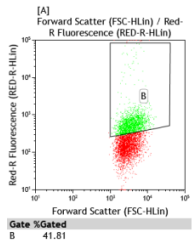
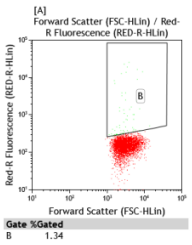
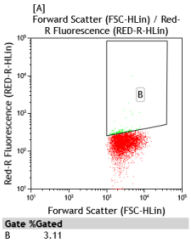
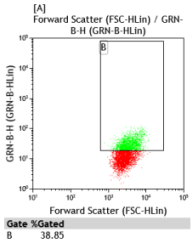
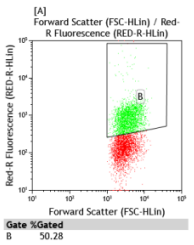
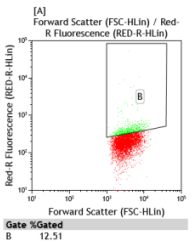
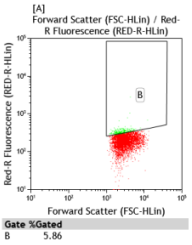
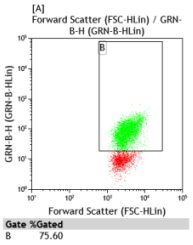
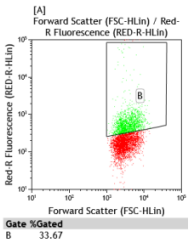
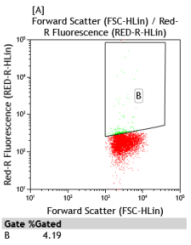
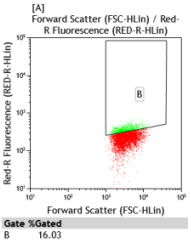
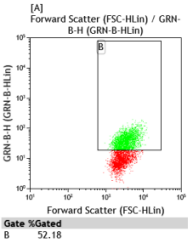
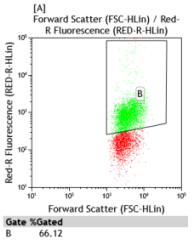
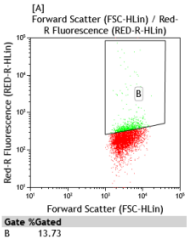
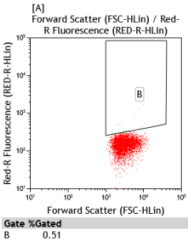
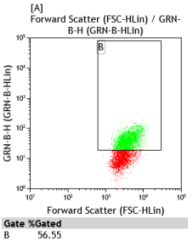
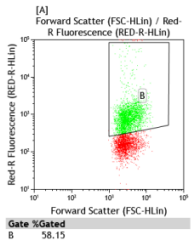
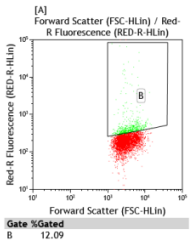
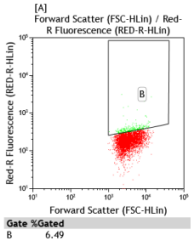
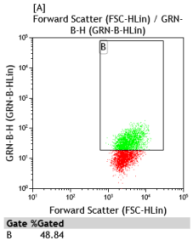
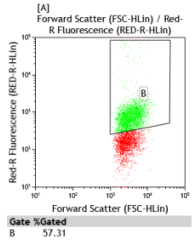
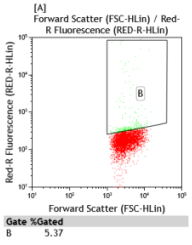
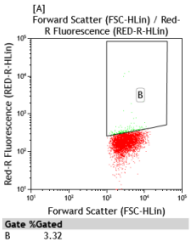
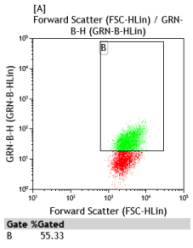
Mal50	MalD1 [50 nM]	BetV1 [100 nM]	Steptavidin-Alexa647 [1:1000]	α kappa [1:100]
Clone 07				
Clone 08				
Clone 09				
Clone 10				
Clone 11				
Clone 12				

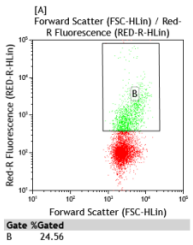
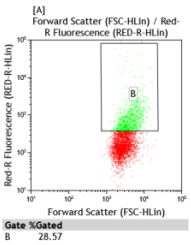
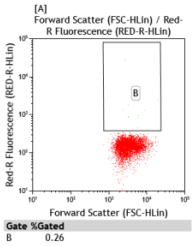
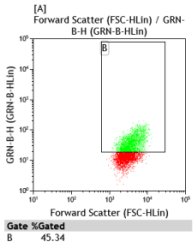
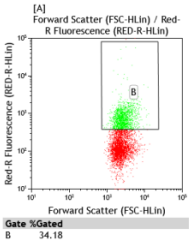
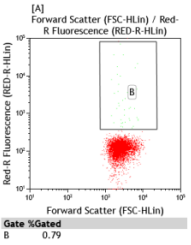
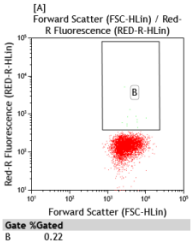
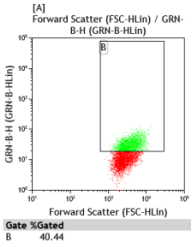
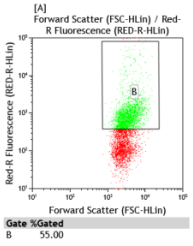
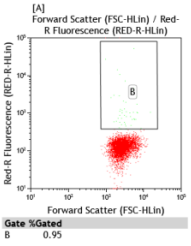
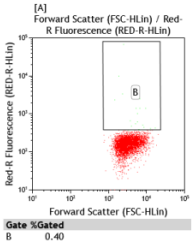
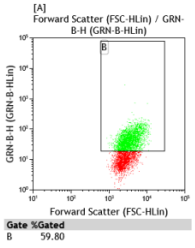
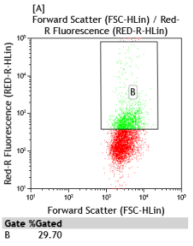
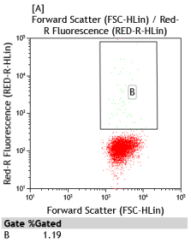
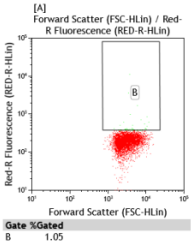
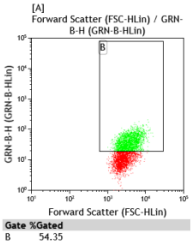
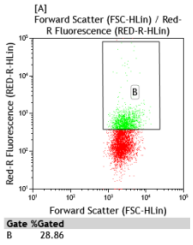
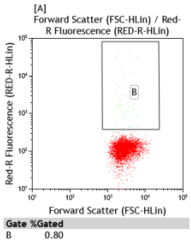
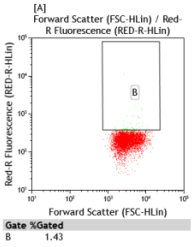
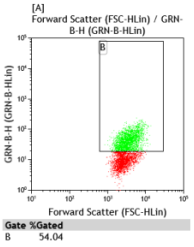
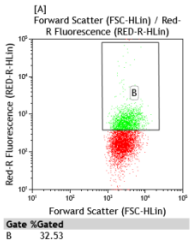
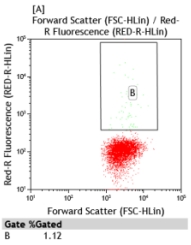
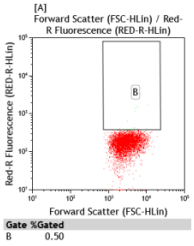
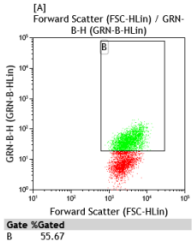
Mal50	MalD1 [50 nM]	BetV1 [100 nM]	Steptavidin-Alexa647 [1:1000]	α kappa [1:100]
Clone 13				
Clone 14				
Clone 15				
Clone 16				
Clone 17				
Clone 18				

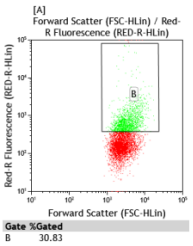
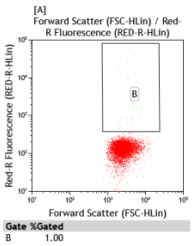
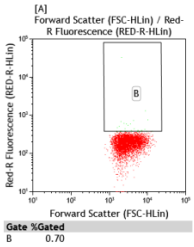
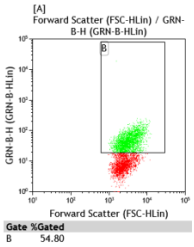
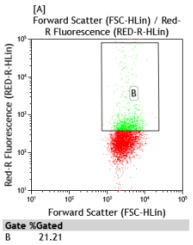
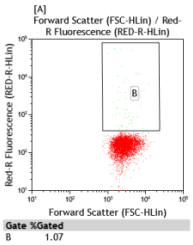
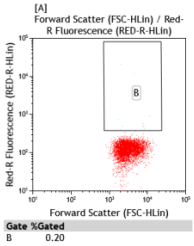
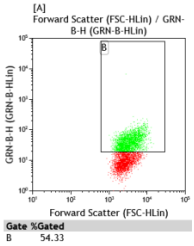
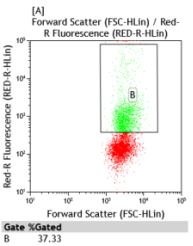
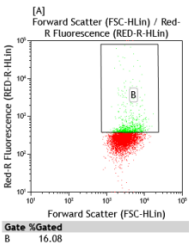
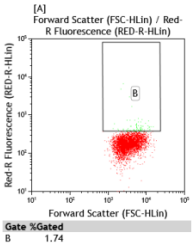
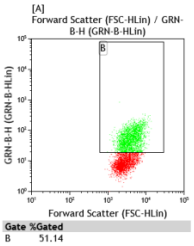
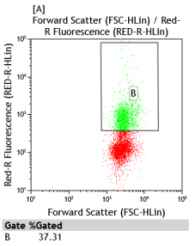
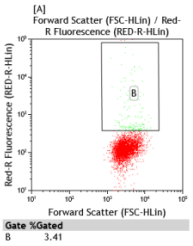
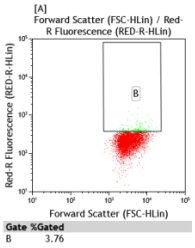
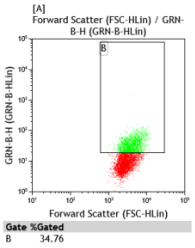
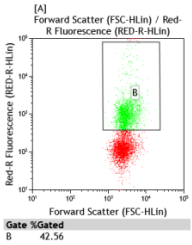
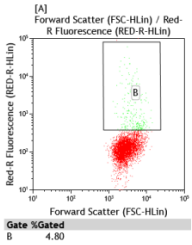
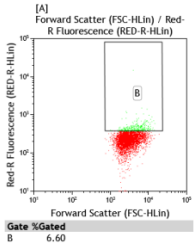
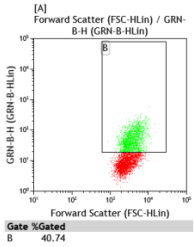
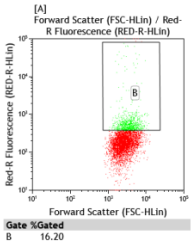
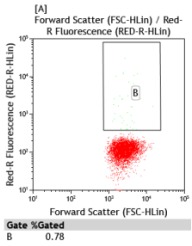
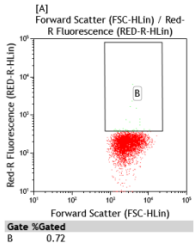
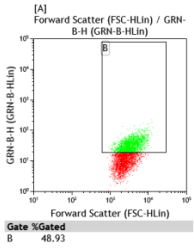
Mal50	MalD1 [50 nM]	BetV1 [100 nM]	Steptavidin-Alexa647 [1:1000]	α kappa [1:100]
Clone 19				
Clone 20				
Clone 21				
Clone 22				
Clone 23				
Clone 24				

A.8.5 Flow Cytometric Analysis of Isolated Mal50B Clones



Mal50B	MalD1 [50 nM]	BetV1 [100 nM]	Steptavidin-Alexa647 [1:1000]	α kappa [1:100]
Clone 07				
Clone 08				
Clone 09				
Clone 10				
Clone 11				
Clone 12				

Mal50B	MalD1 [50 nM]	BetV1 [100 nM]	Steptavidin-Alexa647 [1:1000]	α kappa [1:100]
Clone 13				
Clone 14				
Clone 15				
Clone 16				
Clone 17				
Clone 18				

Mal50B	MalD1 [50 nM]	BetV1 [100 nM]	Steptavidin-Alexa647 [1:1000]	α kappa [1:100]
Clone 19				
Clone 20				
Clone 21				
Clone 22				
Clone 23				
Clone 24				

A.9 Amino Acid Sequences of Screened Antibodies

Table A.9.1: Amino acid sequences of the V-domains of all clones that were considered for recloning.

Clone		Amino Acid Sequence
Mal1x_2	HC:	QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYYMHWVRQAPGQKLEWMGIINPSGGTTS YAQKFQGRVTMTRNTSTSTVYMELSSLRSEDTAVYYCARSLTVAGWWATWGQGTLVTVS S
	LC:	DIVLTQTPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDIATYYCQQYDNLLPTFGPGTKVEVKRT
Mal1x_3	HC:	QVQLVQSGGGLVQPGGSLRLSCAASRFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	TTLTQSPGTLSVSPGEGATLSCRASQSISNKVAWYQHRPGQAPRLLIYGASTRAAGIPA TFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPVTFGPGTKVDIKRT
Mal1x_5	HC:	EVQLVESAGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGRGLEWVSGINWKGDRTY YADSVEGRFTISRDNSKNTLFLQMNSLRAEDTAVYYCAKVIGTYTIYYWGQGTTVAVSS
	LC:	DIQMTQSPSSLSASVGDRVTITCRASQTISTYLNWYQQKPGKAPKVLIYDAYSLQSGVP SRFSGSGSGTDFALTISSLQPEDVATYFCQQGHSTPPLTFGGGTRVEIKRT
Mal1x_6	HC:	EVQLVESGGGVVQPGRSLRLSCAASGFIFSNYAMSWVRQAPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGRGVVRYFDWFVLGQGTLVT VSS
	LC:	DIRVTQSPSSVSASVGDRVTITCRASQHVSNWLAWYQQKPGKAPTFLIFAASTLQSGVP SRFSGSGSGTEFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRT
Mal1x_7	HC:	QVQLVQSGAEVKKPGSSVRVSCKASGGTFSRYPVGWVRQAPGQGLEWMGGIIPFSGAAF STQKFRDRVTITADGSTGTVYMELSSLRSEDTAVYYCARSLTVAGWWATWGQGTLVTVS S
	LC:	DIVMTQSPSTLSASVGDRVAITCRASQSIRDSLAWYQQKPGKAPNLLIYRASILENGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQYYGSVWTFGQGTKVEIKRT
Mal1x_8	HC:	QVQLVQSGGDLIQPGGSLRLSCAASGFTVSRHYMSWVRQAPGKGLEWVSGISGSGGATY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	EIVMTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIP ARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPPSYTFGQGTKLEIKRT
Mal1x_9	HC:	EVQLVQSGAEVKKPGSSVKVSCKVSGGTFTSYYMHWVRQAPGQGLEWMGIINPSGGSTS YAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARSLTVAGWWATWGQGTLVTVS S
	LC:	DIQMTQSPSTLSASIGDRVTITCRASQDIISWLAWYQQKPGKAPKRLIYAASSLQGGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQSHSTPRLTFGGGTKVEIKRT
Mal1x_10	HC:	EVQLVESGGGLVQPGMSLRLSCEASGFTFRSYGMHWVRQTPGKGLEWVATISYDENYTY YADSVKGRFTISRDNSKNTLYLQMNSLSAEDTAVYYCAKTKPYGTTWYGGIDVWGQGTT VTVSS
	LC:	TTLTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKLLIYVASELNTGVPS RFTGSGSGTDFTLTITSLQSEDFATYYCLQDYNYPWTFGQGTKVEINRT
Mal1x_11	HC:	QVQLVQSGRGVVQPGRSLRLSCAASGFTFSSYAMHWVRQVPGKGLEWVALISYDGTNKY YADSVKGRFTISRDNSKNTLYLQMNSLRGEDTALYYCARDSSGYGGYISDYWGQGTLVT VSS
	LC:	DIVMTQSPSTLSASVGDRVTITCRASQSISSWLAWYQQKPGKAPKLLIYKASSLESGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQQYNSYPWTFGQGTKVEIKRT
Mal1x_12	HC:	QVQLVESGGGLVQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSVIYSGGSTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCSRAEKGATYYWGQGTLVTVSS
	LC:	TTLTQSPVTLSVSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTRATGIPA RFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPLTFGGGTKVEIERT
Mal1x_15	HC:	QVQLVESGAEVKKPGSSVKVSCKASGGTFSRYPIGWVRQAPGQGLEWMGVIDPRGGATS YAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARSLTVAGWWATWGQGTLVTVS S
	LC:	DIRVTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTLALTFGGGTKVEIKRT

Clone		Amino Acid Sequence
Mal1x_16	HC:	QVQLVQSGGGLVQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSVIYSGGSTYY ADSVKGRFTISRDNGKNSLYLQMNSLRAEDTAVYYCARGSQKLGPYYYGMDVWGQGTTV TVSS
	LC:	DIVMTQSPSTLSASVGDRVTITCRASQSISSWLAWYQQKPGKAPKRLIYAASSLQSGVP SRFSGSGSGTEFTLTISSLQPEDFATYYCQQSYSTPRYTFGQGTKLEIKRT
Mal1x_17	HC:	QVQLVQSGGGLVQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSVIYSGGSTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCSRAEKGATYYWGQGTLVTVSS
	LC:	DVVMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYVASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYVTPITFGQGTRLEIKRT
Mal1x_18	HC:	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYYIHWLRQAPGQGLEWMGIINPSGGSTS YAQKFQGRVTITADASTTTAYMELSSLRSEDTAVYYCARSLTVAGWWATWGQGTLVTVS S
	LC:	DIVLTQSPSFVSASVGDRVTITCRASQSINNWLAWYQQKPGKAPKSLIYAASSLQSGVP SRFSGSRSGTDFTLTISSLQPEDFATYYCQQANSFPHTFGQGTKLEIKRT
Mal1x_19	HC:	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYTISWVRQAPGQGLEWMGGITPIFGTPT YAQKFQGRVTITADESTSTAYMEVSSLRPEDTAVYYCVRGFWSGHYALGWWGQGTLLTV SS
	LC:	DIQLTQSPSFLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFTGSGSGTEFTLTITSLQPDDFATYYCQQLNSYPITFGQGTRLEIKRT
Mal1x_20	HC:	QVQLMQSGGGLVQPGGSLRLSCEASGFIFNNYALSWVRQAPGKGLEWVSVIYSGGSTYY ADSVKGRFTISRDNSKNTLHLQMNSLRVDDTAVYYCARVPYYCTSSSCYFEYWGQGTLV TVSS
	LC:	DIQMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIYDASNLETGVP SRFSGSGSGTDFTFTISSLQPEDIATYYCQQYDNLPHTFGQGTKLEIKRT
Mal1x_21	HC:	QVQLVESGGGVVQPGRSLRLSCAASGFPFSSYGLHWVRQAPGKGLEWVAVISYDGSYKY YADSVKGRFTISRDNSKTTLYLQMNSLRAEDTAVYYCAKELGRGYSYGSGVDYSGQGTL VTVSS
	LC:	DIVMTQSPSSVSASVGDRVTITCRASQHVSNWLAWYQQKPGKAPTFLIFAASTLQSGVP SRFSGSGSGTDFTLTISSLQPEDSATYYCQQANSFPITFGQGTRLELKRT
Mal1x_23	HC:	QVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSYISSGGSSIY YADSVKGRFTISRDNAKNSLYLQMNSLRGEDTAVYYCARGSQKLGPYYYGMDVWGQGTT VTVSS
	LC:	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSSLAWYQQKPGQAPRLLISGASSRASAI PDRFSGSGSGTDFTLTISRLEPEDLAVYYCQQYGRSPWTFGQGTKVEIKRT
MalBet++_14	HC:	QVQLVQSGGGVVQPGGSLRLSCAASGFTFRSYWMSWVRQAPGKGLEWVANIKRDGTETY YADSLRGRVTISRDNAKNTLYLQMNSLRAEDTAIYFCVAGRGWQPDYWGQGTLVTVSS
	LC:	DIVMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCLQDYNYPYTFGQGTKLEIKRT
MalBet++_17	HC:	QVPLVQSGGGVVQPGRSLRLSCAASGFTFSRYAMHWVRQAPGKGLEWVATISYDENYKY YTDSVKGRFTVSRDNSKNTLFLQMNSLKAEDTAVYYCSNDMYCGGDCLAGWGQGAQVTV SS
	LC:	TTLTQSPGTLALSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLLYGASGRATGVPD RFIGSGSGTEFTLTISSLESEDCAIYYCQQYGSSPLFTFGPGTKVEIKRT
MalBet++_20	HC:	QVQLVQSWGGLVKPGGSLRLSCAASGFTFSNAWMSWVRQAPGKGLEWVAVISYDGSYKY YADSVKGRFTISRDNSKNTLYLQMNDLRAEDTAVYYCARGSQKLGPYYYGMDVWGQGTT VTVSS
	LC:	DVVMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPETFGQGTKVEIKRT
MalBet++_24	HC:	QVQLVQSGAEVKKPGSSVRVSCKASGGTFSRYPVGWVRQAPGQGLEWMGGIIPFSGAAF STQKFRDRVTITADGSTGTVYMELSSLRSEDTAVYYCARSLTVAGWWATWGQGTLVTVS S
	LC:	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIP ARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPLGYTFGQGTKLEIERT
Mal100_1	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIQMTQSPSSLSASVGDRVTITCRASQDISHYLAWFQQKPGKAPKSLIYDASSLQSGVP SKFSGRGSGTTFTLTISSLQPEDFATYYCQQYNSYPLTFGGGTKVEIKRT

Clone		Amino Acid Sequence
Mal100_4	HC:	QVQLVQSGAEVKKPGASLKVSCKASRYTFTNYYMHWVRQAPGQGLEWLGVINPGGGSTS YPQKFQDRVTMTSDTSTSTVHMKLSSLRSEDTAVYYCARSLTVAGWWATWGQGTLVTVS S
	LC:	DIQMTQSPSTLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQVGVP SRFTGSGSGTDFTLTIRGLQPEDSATYYCQQSYSASYTFGQGTKVETTRT
Mal100_5	HC:	QVQLVQSGAEVTKPGASVKLSCKASGYTFSNYHIHWVRQAPGQRLEWMGIINPSGGSTS YAQKFQGRVTMTRDTSTSTVYMELRSLTSDDTALYYCARRSKWGGLFRSMAYFFDSWGQ GTLVTVSS
	LC:	DIQMTQSPSSVSASVGDRVTITCRASQHVSNWLAWYQQKPGKAPTFLIFAASTLQSGVP SRFSGSGSGTDFTLTISSLQPEDSATYYCQQANSFPITFGQGTRLELKRT
Mal100_7	HC:	QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWLRQAPGQGLEWMGWINPNSGGTN YAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARSLTVAGWWATWGQGTLVTVS S
	LC:	DIQMTQSPSSLSASVGDRVTITCRASQSISRYSNWYQQKPGKAPKLLIYTASSLQSGVP SRFSGSGSGTDFTLTITTLQPEDFATYYCQQSDSFPYTFGQGTNLEIKRT
Mal100_8	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	EIVMTQSPATLSLSPGERATLSCGASQSVSSSYLAWYQQKPGLAPRLLIYDASSRATGI PDRFSGSGSGTDFTLTISRMQPEDFAVYYCQQYGSSLPYTFGQGTKLEIKRT
Mal100_9	HC:	EVQLVESGGGLVQPGGSLRLSCAASGFSISPFWMTWVRQAPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIQMTQSPSTLSASVGDRVTITCRASQSISRWLAWYQQKPGKAPKLLIYKASTLEYGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQQYNSYRTFGQGTKVEIKRT
Mal100_10	HC:	EVQLVESAGGVVQPGRSLRLSCAASGFTFSDYSMNWIRQVPGKGLEWVAGIGGSGSKTY YADSVKGRFTISRDNSRDTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIVMTQSPSSVSASVGDRVTITCRASQGINSWLTWYQQEPGKAPKLLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSLPTFGQGTRLEMKRT
Mal30/30_2	HC:	QVQLVQSGGGAVQPGTSLRLSCTTSGINFRTSGMNWVRQVPGKGLEWLAIISSDGSKKF YADSVKGRFTISRDNSKNTLYLQMNSLRVEDTAVYYCARDRGIGSSWYELDYWGQGTLL TVSS
	LC1:	TALTQSPATLSVSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTRATGIPA RFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPLTFGGGTKVEIKRT
	LC2:	EIVMTQSPGTLSLSPGERATLSCRASQSVGNYLGWYQQKPGQAPRLLISDASNRATGVP TRFSGSGSGTDFTLTISSLEPEDFAVYYCQQYGYSPPITFGQGTRLDIKRT
	LC3:	DIQLTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKLLIYDASNLETGVP SRFSGSGSGTDFTFTISSLQPEDIATYYCQQYDNLLALTFGGGTKVEIERT
Mal50_2	HC:	QVQLVQSGRGVVQPGRSLRLSCAASGFTFSSYAMHWVRQVPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIVLTQSPSSLSASVGDRVTIACRASQTIGSYLNWYQQKPGKAPKLLIYATSTLQSGVP SRFSGSGSGTDFTLTITSLQPEDFATYYCQQYYSPPPITFGQGTRLEIKRT
Mal50_3	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIVMTQSPGSLSLSPGERATLSCRASQSVSSSYLAWYQQKPGKAPKRLIFTATTLQSGV PSRFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIKRT
Mal50_6	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIVMTQSPSSLSASVGDRVTITCRASQGISNYLAWYQQKPGKVPKLLIYAASTLQSGVP SRFSGSGSGTDFTLTISSLQREDSATYFCQQSYSTPPTFGGGTKVEIRRT

Clone		Amino Acid Sequence
Mal50_7	HC:	QVQLLESGGGLVQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSVIYSGGSTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQGT TVTVSS
	LC:	TTLTQSPDTLSLSPGERATLSCRASQSISSSYLAWYQQKSGQAPRLLIYGASSRARNIP DRFSGSGSGTDFTLTISGLEPEDCAIYYCQQYGSSPLTFGGGTKVEFKRT
Mal50_9	HC:	EVQPVESGGGLVQPGKSLRLTCAASGFTFSNHAMTWVRQAPGKGLEWVAAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	EIMLTQSPATLSLSPGERATLSCRASQSVANFLAWYQQIPGQAPRLLISGASNRATGIP ARFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPMYTFGQGTKLEIKRT
Mal50_10	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	ETTLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGI PDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSLLYTFGQGTKLEIERT
Mal50_11	HC:	QVQLVQSGGGLVQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSVIYSGGSTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQGT TVTVSS
	LC:	EIVLTQSPSTLSASVGDRVTITCRASQSISRWLAWYQQKPGKAPKRLIYAASSLQSGVP SRFSGSGSGTDFTLTITTLQPEDFATYYCQQSDSFPYTFGQGTNLEIKRT
Mal50_12	HC:	QVQLVQSGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTALYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	EIVLTQSPATLSLSPGERATLSCRASQSVVRFLAWYQQKPGQAPRLLIYDTSNRASGIP ARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRKDWPPLTFGGGTKVEIKRT
Mal50_14	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIVLTQTPGTLSVSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTRATGIP ARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPPAFGQGTRLEIKRT
Mal50_15	HC:	EVQLLESGGGLVQPGGSLRLSCAVSGFTFNNFDLNWFRRAPGKGLEWVSAISRSGGGTY YADSVKGRFTISRDNSKNTLYLQMNSLRAGDTAVYYCATRRKNQYSGYAYGMDVWGQGT TVTVSS
	LC:	EIVLTQSPATLSVSPGERATLSCRASQSVSSNLAWCQQKPGQAPRLLIYAASTRATGIP ARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPSTFGQGTKLEIKRT
Mal50_16	HC:	EVQLVESGGGLVQPGMSLRLSCEASGFTFRSYGMHWVRQTPGKGLEWVATISYDENYTY YADSVKGRFTISRDNSKNTLYLQMNSLSAEDTAVYYCAKTKPYGTTWYGGIDVWGQGTT VTVSS
	LC:	DIVMTQSPSSLSASIGDRVTITCRASQYINTFLNWFQQKPGEAPKLLIYAASHLQNGVP PRFSGGGSGTEFTLTISSLQPDDFATYYCQQYNSYPWTFGQGTSLEIKRT
Mal50_17	HC:	QVQLVDSGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	EIVMTQSPSFLSAPVGDRVTITCRASQGISSYLAWYQQKPGKAPKLLIYAASILQSGVS SRFSGSGSGTDFTLTISSLQPEDFATYFCQQTYSTPSTFGQGTKLDIERT
Mal50_19	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIQMTQSPSTLSASVGDRVIITCRASQSITSHLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGSGSGTQFTLTINSLQPDDFATYHCQQYNSYSPTFGQGTKLEMKRT
Mal50_21	HC:	QVQLVQSGGGVVQPGRSLRLSCEASGFTFRSYGMHWVRQTPGKGLEWVATISYDENYTY YADSVKDRFTISRDNSKNTLYLQMNSLRAEDTAVYYCSRAEKGATYYWGQGTLVTVSS
	LC:	DIVLTQTPSSLSASVGDRVTITCRASQSISNYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGNGSGTDFTLTISSLQPEDFATYYCQQSYSSPPHFGGGTKVEIKRT

Clone		Amino Acid Sequence
Mal50_22	HC:	QVQLVQSGGGLVQPGGSLRLSCVASGFTVSGRYVIWVRQAPGKGLEWLSFTYADGTTYQ ADSMKGRLTVSRDTAKNTFFLQVNNLRPEDTAVYYCARGSQKLGPYYYGMDVWGQGTTV TVSS
	LC:	DIVMTQSPSTLSASVGDRVTITCRASQSISSWLAWYQQKPGKAPKLLIYKASSLESGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQQYNRYPYTFGQGTKLEIKRT
Mal50_23	HC:	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYWMYWVRQAPGKGLVWVSRINSHGSTTT YADSVKGRFTISRDNSKNTVYLQMNTLRTEDTAVYYCAKDALQKYFETSGRAFRPDVWG QGTTVTVAS
	LC:	DIVLTQSPSSLSASVGDRVTITCRAGQGIDRWLAWYQQKPGKAPKLLIYQASTLESGVP SRFSGSGSGTEFTLTISSLQPDDLATYYCQQYNSYLWTFGQGTKVEIKRT
Mal50B_1	HC:	EVQLVESGGGLVQPGGSLRLSCAASRFTFYRYAMTWVRQAPGEGLEWVSGISGSGDRTY YADSVEGRFTISRDNSRNTVYLQMNSLRDEDTAVYYCARSVFGFGRKVPKNILDLWGQG TPVTVSA
	LC:	DIRLTQSPSSVSASVGDRVTITCRASQGISNSLAWYQRKPGKGPKLLIYAASTLQSGVP SRFSGSGSGTDFTLLISSLQPEDVATYYCQQYNNYTGTFGQGTKVEFNRT
Mal50B_2	HC:	QVQLLESGGGLIQPGGSLRLSCAASGFTFRNFGMTWVRQAPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIVMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDSATYYCQQTYRTPLYTFGQGTKLEIKRT
Mal50B_5	HC:	QVQLVQSGGGLIQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSVIYSGGSTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQGT TVTVSS
	LC:	TTLTQSPATLSVSLGERATLSCRASQSVNRNLAWYQQKPGQAPRLLLYGASTRAAAIPV RFSGSGSGTEFTLTISSLQSEDFAVYYCQQYINWPRTFGQGTKLEIKRT
Mal50B_6	HC:	QVQLVQSGGGVVQPGRSLRLSCAASGFTFSRYAMHWVRQAPGKGLEWVAVISYDGSNKY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIVMTQSPSSLSASVGDRVTLTCRASQTIYTWLAWYQQKPGKAPKLLIYKASSLESGVP SRFSGSGSGTEFTLTISSLQPDDVATYYCQQYDTSSRTFGQGTRVEIKRT
Mal50B_7	HC:	EVQLVESGGGVVQPGRSLRLSCTASGFTFSSYAMHWVRQAPGRGLEWVAMIWFDGSHEY YKDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DVVMTQSPLSLPVTLGQPASISCRSSQSLVYSDGNTYLNWFQQRPGQSPRRLIYKVSNR DSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQGTHWPPTFGGGTKVEIKRT
Mal50B_8	HC:	QVQLVQSGGGLVQPGGSLRLSCAASKFTFNNYALSWVRQAPGKGLEWVSTISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIVLTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQKPGQSPQLLIYVGSTR ASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQIPLTFGPGTKVDIKRT
Mal50B_9	HC:	QMQLVQSGGGVVQPGRSLRLSCEASGFTFRSYGMHWVRQTPGKGLEWVATISYDENYTY YADSVEGRFTVSRDNSKNTLFLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIRMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQQKPGKVPKLLIYAASSLQNGVP SRFSGSRSGTDFTLTISSLRPEDFATYYCQQSYVTPITFGQGTRLEIKRT
Mal50B_10	HC:	QVQLLESGGGLVQPGGSLRLSCAASKFTFNNYALSWVRQAPGKGLEWVSTISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	TTLTQSPATLSVSPGERATLSCRASQRTSSSYLAWYQQKPGQAPRLLIYAASSRATGIP DRFSGSGSGTDFTLTISRLEPEDFAVYYCQHYGTSPYTFGQGTKLEIKRT
Mal50B_11	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	PCR amplification yielded insufficient material for sequencing reaction and was not repeated.

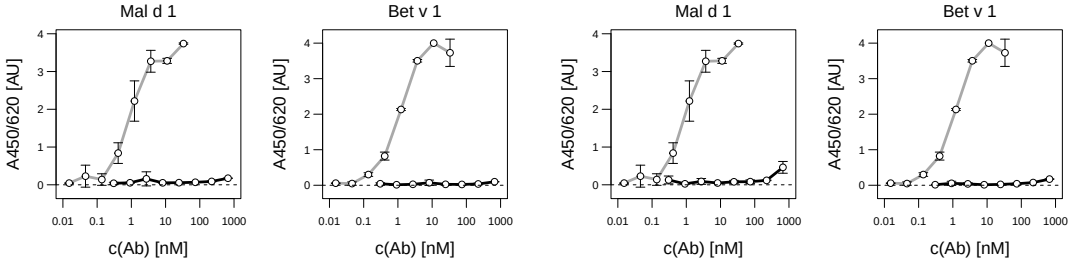
Clone		Amino Acid Sequence
Mal50B_12	HC:	QVQLVESGGGLVQPGGSLRLSCAASKFTFNNYALSWVRQAPGKGLEWVSTISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVAS
	LC:	DIVMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIYDASNLETGVP SRFSGSGSGTDFTFTISSLQPEDIATYYCQQYDNLPLTFGGGTKVEIKRT
Mal50B_13	HC:	EVQLVESGAEVKKPGSSVRVSCKASGYTFTKYGVHWARQVPGQRLEWMGWINPGNGNTK YSPQFQDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARDYDILTAYYRGYYGMDVWGQ GTTVTVSS
	LC:	TTLTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPS RFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPAFGQGTKVEIKRT
Mal50B_14	HC:	QVQLMQSGAEVKKPGASVKVSCKASGDTFTSYYIHWVRQAPGQRPEWMGIINPRGGSTT YAQKFQGRVTMTTDTSTSTVYMELSSLRYEDTAVYYCARGWGGRWGPLVGATRAKTYFF DYWGQGTPVTVSS
	LC:	DIQMTQSPSSVSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPRTFGQGTKLEIKRT
Mal50B_15	HC:	QVQLVESGGGLVQPGGSLRLSCAASGFRFSSYVMTWVRQAPGKGLEWVSAISGSGDRIY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	EIVLTQSPGTLSLSPGERATLSCRASQSLSSNYLAWYQQKPGQAPRLLIYGASSRATGI PDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSREFGQGTKVEIKRT
Mal50B_16	HC:	EVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	EIVMTQSPGTLSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYDASNRATGI PARFTGSGSGTDFTLTISRLEPEDFAVYYCQQYGNSPLTFGGGTKVEIKRT
Mal50B_17	HC:	EVQLVESGGDLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSGISGSGGGTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKVIGTYTIYYWGQGTMVTVSS
	LC:	DIVMTQSPSSVSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPPWTFGQGTKVEIKRT
Mal50B_18	HC:	QVQLVQSGGGVVQPGRSLRLSCAASGFTFSRYAMHWVRQAPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIRVTQSPSSLSASVGDRVTITCRASQGITIYLNWYQQKPGKAPNLLIYAASSLQSGVP SRFSGSGSGTDFALTISSLQPEDFATYYCQQTYKTPITFGQGTRLEIKRT
Mal50B_19	HC:	QVQLVQSGRGVVQPGRSLRLSCAASGFTFSSYAMHWVRQVPGKGLEWVAGIGGSGSKTY YADSVKGRFTVSRDNSKSTLYLQMNSLRAADTAVYYCVGGRGWLPDYWGQGTLVTVSS
	LC:	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPITFGQGTRLEIKRT
Mal50B_20	HC:	QVQLVQSGGGLVQPGGSLRLSCVASGFTVSGRYVIWVRQAPGKGLEWLSFTYADGTTYQ ADSMKGRLTVSRDTAKNTFFLQVNNLRPEDTAVYYCARGSQKLGPYYYGMDVWGQGTTV TVSS
	LC:	TTLTQSPATLSLSPGERATLSCRASQSVTKFAWYQQIPGQPPRLLISGASNRATGIPAR FSGSGSGTDFTLTISSLEPEDFAVYYCQQYGSSPYTFGQGTKLEIKRT
Mal50B_21	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPYTFGQGTKLEIKRT
Mal50B_22	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	TTLTQSPAFMSATPGDKVNISCKASQDIDDDMNWYQQKPGEAAIFIIQEATTLVPGIPP RFSGSGYGTDFTLTINNIESEDAAYYFCLQHDNFPLKFGQGTKVEIKRT
Mal50B_23	HC:	QVQLVESGGGLVQPGGSLRLSCTGSGFTFSSYAMTWVRQAPGKGLEWVSVISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	EIVMTQSPSSLSASVGDRATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTRATGIP DRFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPPITFGQGTRLEIKRT

Clone		Amino Acid Sequence
Mal50B_24	HC:	EVQLVESRGGLVQPGTSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRPAVAGGRYYYYGMDVWGQG TTVTVSS
	LC:	TTLTQSPAFMSATPGDKVNSSCKASQDIDDDMNWYQQKAGEAAIFIIQEATTLVPGIPP RFSGSGYGTDFTLTINNIESEDAAYYFCLQHDNFPTFGPGTKVDIKRT

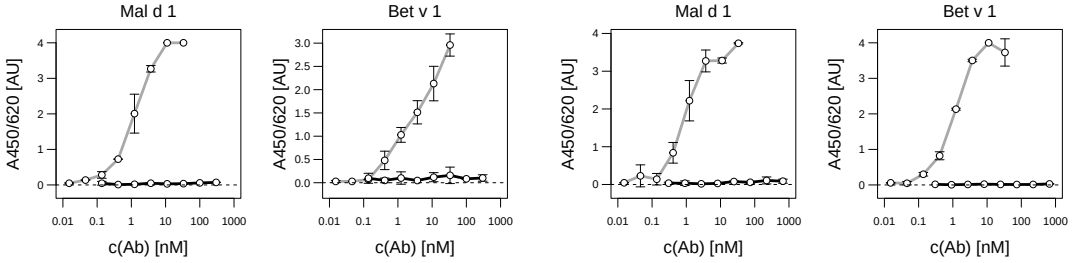
A.10 ELISA Data

A.10.1 Biotinylated Antigens

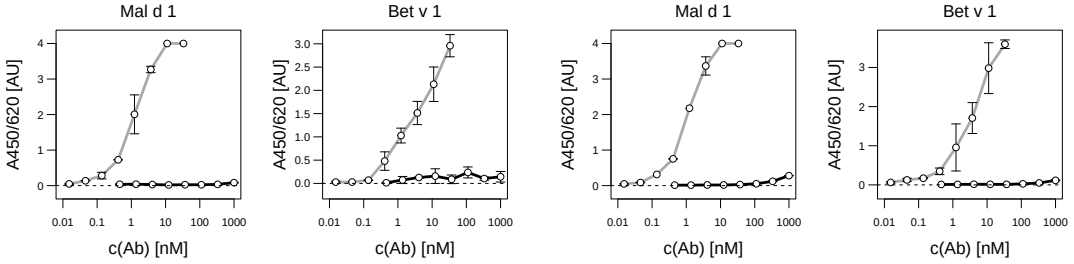
Immobilized antigen ELISA of all antibodies that could be successfully expressed as IgG (black lines). Biotinylated allergen was immobilized on streptavidin coated 96-well microplates. Then an eight-step 1:3-dilution series of antibody was added. Starting concentration was 1 µM, if sufficient antibody was available, otherwise antibody was added at the highest possible concentration. The cross-reactive mouse hybridoma antibody BIP-1 (gray, [80]) was used as a positive control. Data represent two replicates, errors are given as standard deviation.



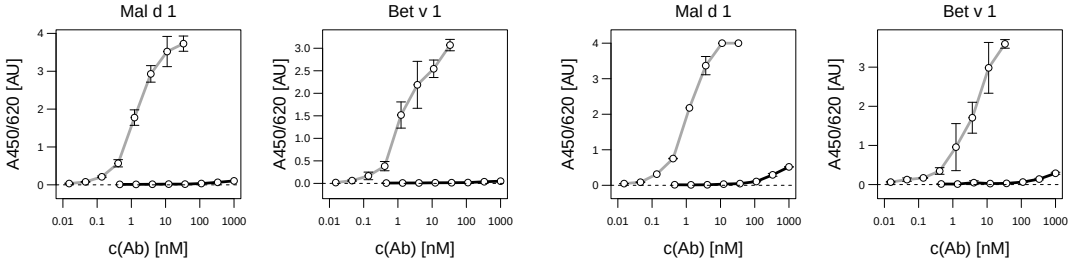
Mal1x_7 Mal1x_8



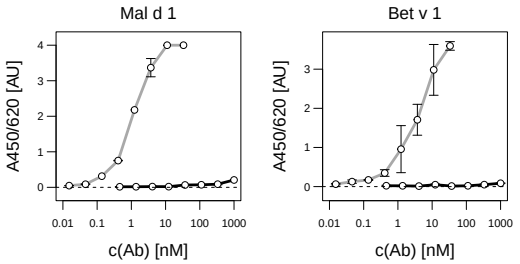
Mal1x_10 Mal1x_11



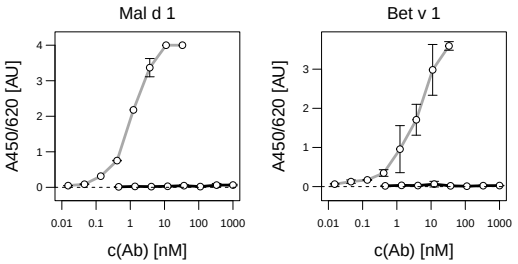
Mal1x_12 Mal1x_16



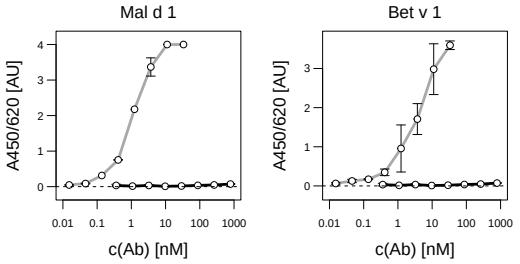
Mal1x_17 Mal1x_18



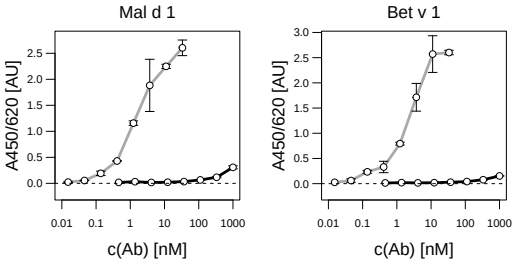
Mal1x_19



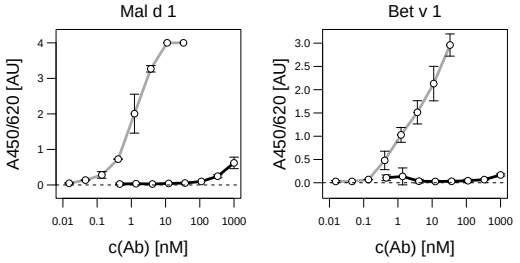
Mal1x_20



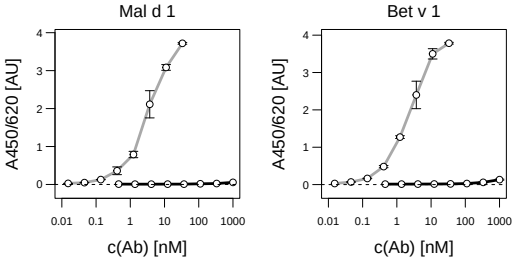
Mal1x_21



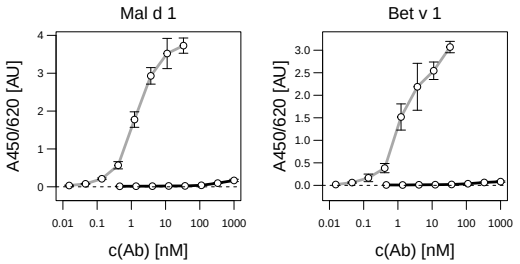
MalBet++_14



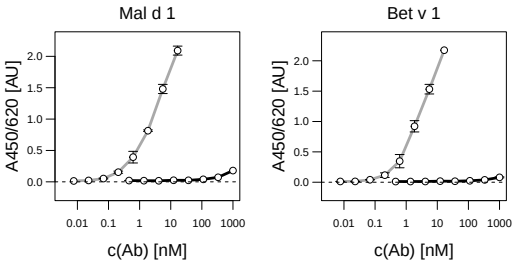
MalBet++_17



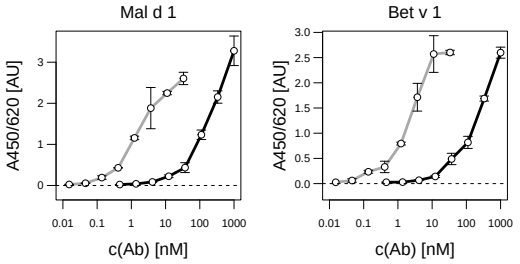
MalBet++_20



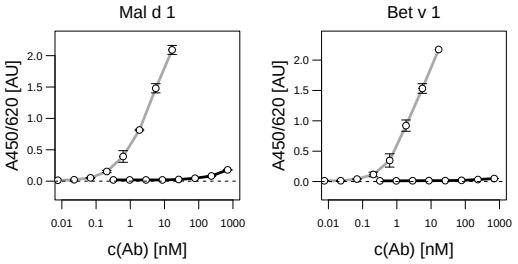
MalBet++_24



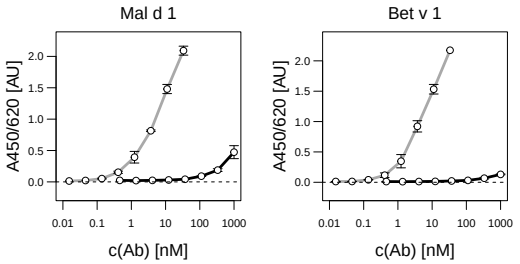
Mal100_1



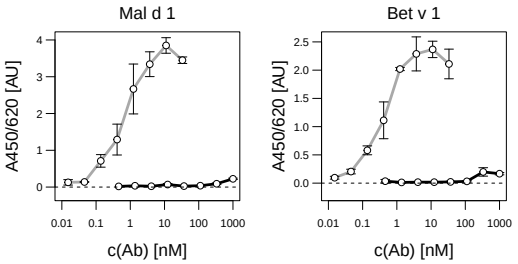
Mal100_5



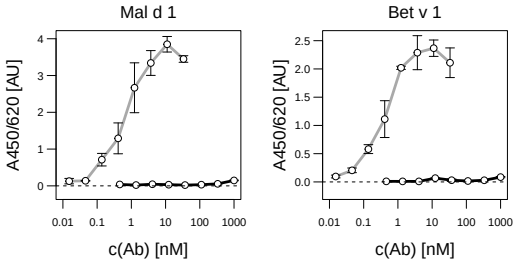
Mal100_8



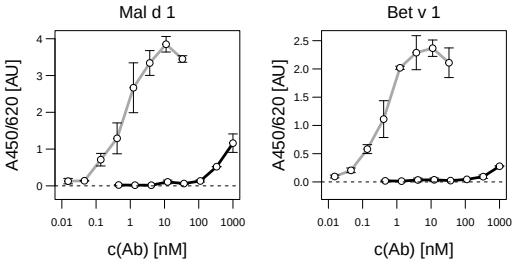
Mal50_6



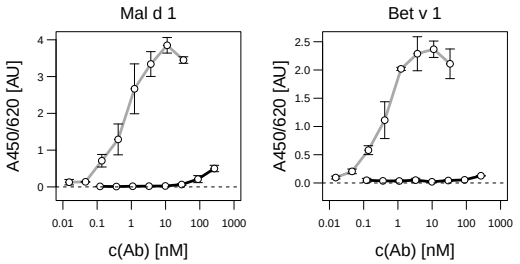
Mal50B_1



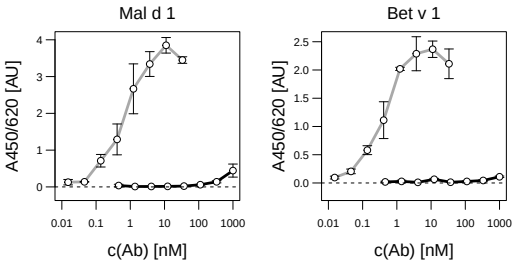
Mal50B_13



Mal50B_14



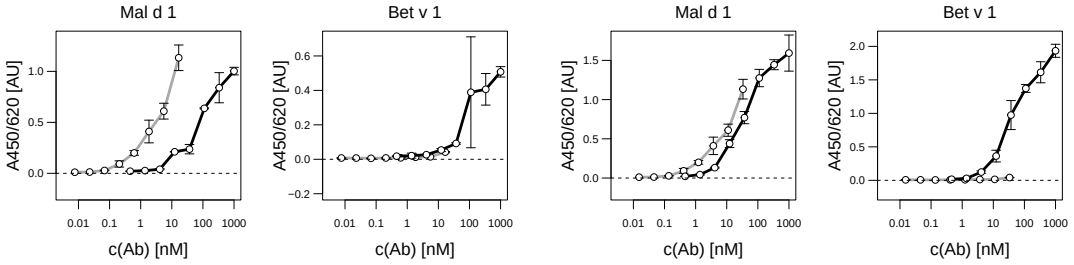
Mal50B_17



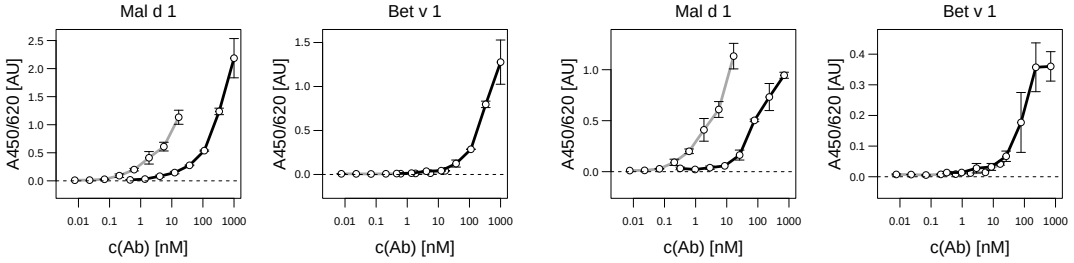
Mal50B_19

A.10.2 Native Antigen

Immobilized antigen ELISA of selected antibodies (black lines). Native allergen was immobilized on hydrophilic polystyrene 96-well microtiter plates. Then an eight-step dilution series (1:3) of antibody was added. Starting concentration was 1 μM, if sufficient antibody was available. The cross-reactive antibody BIP-1 (gray, [80]) was used as control. BIP-1 does not work with Bet v 1 in this setting, because Bet v 1 likely adheres to the plate in a way that prevents access of BIP-1 to its cognate epitope, but was included for completeness.



Mal100_1 Mal100_5



Mal50_6 Mal100_8

A.11 Modeling of EC_{50} Values

Sigmoid curves were modeled using the following equation :

$$y = \frac{\phi_1}{1 + exp(\phi_2 * (x - \phi_3))} \tag{1}$$

Binding curves were modeled with the R software's (version 4.1.2 - "Bird Hippie") non-linear least square (*nls*) function.

Table A.11.1: Quality metrics of models used to determined EC_{50} values

Model	Parameter	Estimate	Std. Error	P-value
Mal100_1: Mal d 1	ϕ_1	1.11708	0.09968	4.72e-08 ***
	ϕ_2	-2.16871	-6.154	3.47e-05 ***
	ϕ_3	0.12165	16.362	4.70e-10 ***
Bet v 1	ϕ_1	0.46853	0.05636	1.47e-06 ***
	ϕ_2	-5.29003	2.74944	0.0765 .
	ϕ_3	1.79429	0.13838	8.21e-09 ***
Mal100_5: Mal d 1	ϕ_1	1.63398	0.07153	7.06e-12 ***
	ϕ_2	-2.36669	0.28385	1.42e-06 ***
	ϕ_3	1.57180	0.06704	5.07e-12 ***
Bet v 1	ϕ_1	1.92637	0.09797	4.71e-11 ***
	ϕ_2	-2.37115	0.31689	4.61e-06 ***
	ϕ_3	1.63980	0.07583	1.42e-11 ***
Mal100_8: Mal d 1	ϕ_1	1.02214	0.07213	2.78e-09 ***
	ϕ_2	-2.65321	0.37787	9.05e-06 ***
	ϕ_3	2.11492	0.08216	1.54e-12 ***
Bet v 1	ϕ_1	0.3888	0.0405	2.88e-07 ***
	ϕ_2	-3.5645	1.1138	0.00697 **
	ϕ_3	2.0417	0.1105	1.03e-10 ***

A.12 SEC-HPLC Data

A.12.1 Chromatograms

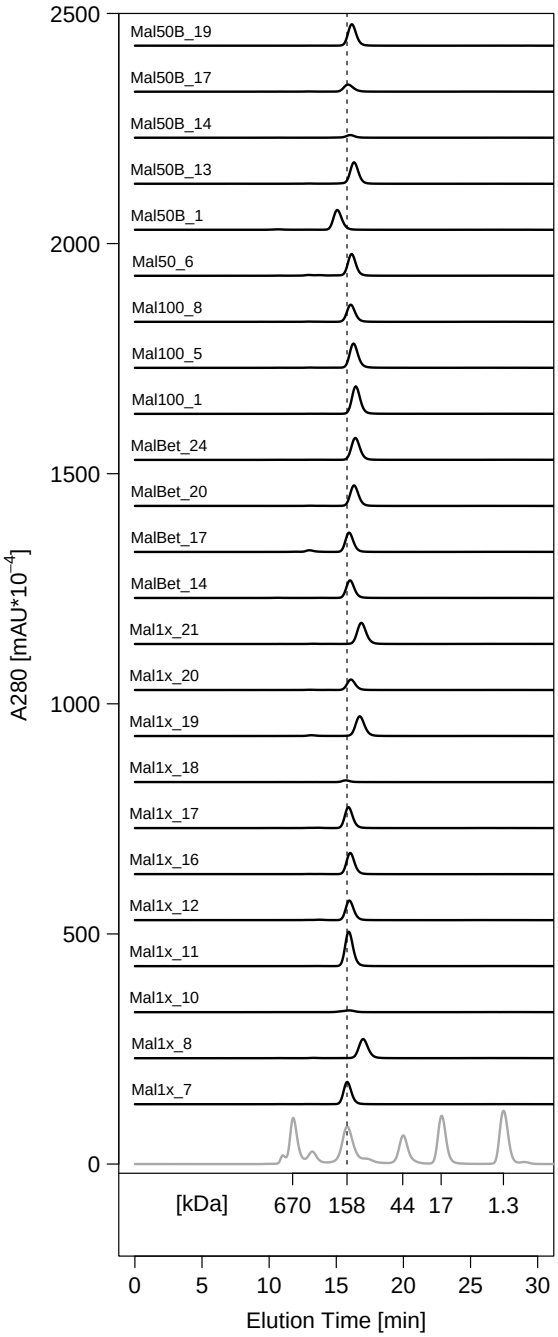


Figure A.12.1: Comparison of SEC-HPLC profiles of all antibodies that could be produced as full-length IgG. All antibodies eluted at times characteristic for IgG. Variation in the elution times might be due to differences in glycosylation or the hydrophobicity of the respective V-domains, but were not investigated further. Clones Mal1x_10, Mal1x_18, Mal100_8 and Mal50B_17 could not be expressed at concentrations high enough to load the standard amount 20 µg protein and were injected neat (see Tab. A.12.1). Additionally the reduced signal intensity of Mal1x_18 is likely caused by the formation of aggregates which were too large or to insoluble to even enter the column. For the other antibodies little dimerization and no fragmentation were observed, however, variation in absolute AUC might be due to aggregation similar to but to a lesser extent than Mal1x_18.

A.12.2 Evaluation

Table A.12.1: Analysis of SEC-HPLC chromatograms.

Name	$t_{elution}$ [min]	$m_{injected}$ [µg]	AUC_{IgG}/AUC_{tot} [%]
Mal1x_7	15.80	20.0	100
Mal1x_8	17.20	20.0	99.16
Mal1x_10	16.00	8.1	100
Mal1x_11	15.95	20.0	100
Mal1x_12	15.90	20.0	98.78
Mal1x_16	16.00	20.0	100
Mal1x_17	15.90	20.0	99.50
Mal1x_18	15.70	18.5	100
Mal1x_19	16.75	20.0	99.10
Mal1x_20	16.10	20.0	98.78
Mal1x_21	16.90	20.0	99.46
MalBet++_14	16.05	20.0	99.49
MalBet++_17	15.95	20.0	92.93
MalBet++_20	16.30	20.0	99.44
MalBet++_24	16.40	20.0	100
Mal100_1	16.50	20.0	100
Mal100_5	16.30	20.0	100
Mal100_8	16.01	17.5	99.49
Mal50_6	16.15	20.0	96.20
Mal50B_1	15.10	20.0	98.31
Mal50B_13	16.35	-*	-
Mal50B_14	16.05	-	-
Mal50B_17	15.90	7.2	100
Mal50B_19	16.15	20.0	100

*Information could not be recovered.

B Kurzbeschreibung (German Abstract)

8-16% der Bevölkerung in Nord- und Mitteleuropa sind durch saisonale Birkenpollenallergien betroffen. Zusätzlich können Birkenpollen komplexe Kreuzallergiemuster gegen ansonsten nicht allergene Nahrungsmittelallergene induzieren, am häufigsten gegen Äpfel. Es wird vermutet, dass die strukturelle Ähnlichkeit des Hauptallergens von Birkenpollen, Bet v 1, zu dem Hauptapfelallergen, Mal d 1, diese immunologische Kreuzreaktivität erlaubt. Kürzlich wurde gezeigt, dass im Voraus ausgesuchte, allergenspezifische monoklonale IgG Antikörper (blockierende Antikörper) zur Behandlung allergischer Erkrankungen verwendet werden können. Folglich sollten ausreichend affine kreuzreaktive Antikörper zur gleichzeitigen Behandlung mehrerer birkenpollen-assoziierter Allergien verwendet werden können. Da eine erfolgreiche allergen-spezifische Immuntherapie (AIT) mit der Induktion von allergen-spezifischen Antikörpern verbunden ist, verwendeten wir Hefe Display, um das IgG Repertoire eines Patienten, der AIT mit rekombinantem Mal d 1 durchlaufen hatte, abzubilden und durchzusehen. Bibliotheken der leichten und schweren Ketten wurden in Hefestämmen unterschiedlichen Paarungstyps hergestellt und mittels Hefepaarung zu einer kombinatorischen Fab Bibliothek vereinigt. Mittels einer Kombination von magnetischer und fluoreszenzaktivierter Zellsortierung gelang es uns zwei kreuzreaktive Antikörper zu identifizieren, die die gleiche schwere Kette, aber verschiedene leichte Ketten enthielten und Mal d 1 mit einem EC_{50} -Wert von jeweils 97 nM und 130 nM und Bet v 1 mit 62 nM und 110 nM im immobilisierten-Antigen-ELISA banden. Während die Affinitäten der entdeckten Antikörper weit über den Affinitäten liegen, die für den Einsatz als klinische blockierende Antikörper nötig wären, könnten sie nützliche Werkzeuge zur Kartographierung kreuzreaktiver Epitope sein.