Kim Kataja

Development, production and characterisation of novel triiodothyronine (T_3) -specific recombinant antibodies

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This Bachelor's thesis was carried out at VTT Technical Research Centre of Finland. The purpose of the thesis was the development, production and characterisation of novel 3,3,5-triiodo-L-thyronine (T3) –specific recombinant antibodies.

A semi-automated selection method called biopanning was applied to the *in vitro* development of the novel antibodies from the previously constructed and validated phage displayed recombinant antibody (Fab) library. The Fab library, constructed from an immunised source, has been successfully panned against T3 before, with an outcome of multiple novel anti-T3 Fabs (Henri Arola 2011). Here two different selection strategies were studied in order to optimize the amount of novel antibodies for T3 thyroid hormone.

After selections, 768 individual recombinant antibody clones were produced in *E.coli* bacteria cells and their binding activities against T3 were assayed from the culture supernatants by ELISA with the robotic workstation. Number of the screened clones was reduced by affinity and specificity rankings with ELISAs. The heavy and light chains of the twenty most interesting clones were sequenced. Finally, three anti-T3 clones with different binding characteristics were selected for a small-scale production, purification and the further characterisations.

As a result, one T3-specific and one high-affinity multi-specific clone were obtained. These antibodies together with the previously developed anti-T3 antibodies provide valuable information regarding to the amino acid sequences responsible for the molecular recognition of the T3 thyroid hormone.

Keywords	Phage display, antibody, biopanning, hapten

Tekijä Otsikko Sivumäärä Aika	Kim Kataja Trijodityroniini (T3) –spesifisten rekombinanttivasta-aineiden eristys, tuotto ja sitomisominaisuuksien tutkiminen 50 sivua + 2 liitettä 25.5.2015
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Opinnäytetyö tehtiin valtion teknillisessa tutkimuskeskuksessa (VTT) bioanalytiikan tutkimusryhmässä. Työn tarkoituksena oli uusien kilpirauhashormoni (3,3,5-trijodityroniini, T3) –spesifisten rekombinanttivasta-aineiden kehittäminen, tuottaminen ja tutkiminen.

Vasta-aineita eristettiin *in vitro* aiemmin tehdystä ja validoidusta vasta-ainekirjastosta faaginäyttötekniikan avulla. Työssä käytettyä vasta-ainekirjastoa on aiemmin käytetty onnistuneesti T3-spesifisten vasta-aineiden eristykseen (Henri Arola 2011). Tässä työssä käytettiin kahta eri valintamenetelmää parantamaan eristettävien T3-spesifisten vasta-aineiden saantoa.

Eristyksen jälkeen 768 yksittäistä rekombinanttivasta-ainekloonia tuotettiin *E.coli* bakteerissa. Vasta-ainekloonien T3-aktiivisuutta arvioitiin ELISA-testeillä, jotka toteutettiin robottiaseman avulla. Tutkittavien kloonien määrää vähennettiin arvioimalla kloonien aktiivisuutta ja spesifisyyttä T3:a kohtaan erilaisilla ELISA-testeillä. Jäljelle jääneiden kahdenkymmenen kiinnostavimman kloonin kevyiden ja raskaiden ketjujen aminohappojärjestykset määritettiin. Lopulta kolme sitoutumisominaisuuksiltaan ja aminohappojärjestykseltään erilaista anti-T3-kloonia valittiin pienen mittakaavan tuottoon. Tuotetut vasta-aineet puhdistettiin, jonka jälkeen niiden sitomisominaisuuksia määritettiin tarkemmin ELISA-testien avulla.

Tuloksena saatiin yksi T3-spesifinen ja yksi korkea-affiniteettinen moni-spesifinen anti-T3 klooni. Yhdessä aiemmin eristettyjen kloonien kanssa työ tuotti arvokasta tietoa T3 kilpirauhashormonin tunnistamisesta vastaavista aminohapposekvensseistä.

Avainsanat	Faaginäyttötekniikka, vasta-aine, hapteeni

Contents

List of abbreviations

ABTS	A water-soluble peroxidase substrate that yields a green end product
AMP	Ampicillin
AP	Alkaline phosphatase, an enzyme that catalyses the hydrolysis of phosphate groups
BCA	Protein quantitation assay based on the reduction of Cu^{2+} to Cu^{1+} by protein
BSA	Bovine serum albumin
CA buffer	Cuts all buffer (appendix 1)
cDNA	Complimentary DNA, double-stranded DNA synthesized from a mRNA
CARB	Carbenicillin
DDIW	Double de-ionized water
DDT	1,4-dithiothreitol, a strong reducing agent, reduces disulfide bonds
DEA	Diethanolamine buffer
DMSO	Dimethyl sulfoxide,
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen-binding fragment
HEPES	Zwitterionic organic chemical buffering agent
HAS	Human serum albumin
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IgG	Immunoglobulin G
IMAC	Immobilized metal affinity chromatography
IPTG	Protein expression inducer, isopropyl-beta-D-thiogalactopyranoside
KANA	Kanamycin
LB	Luria broth (appendix 1)
LSB	Laemmli Sample Buffer (appendix 1)
MALDI	Matrix-assisted laser desorption/ionization
Milli-Q	Ultrapure water, Millipore Corporation
MWCO	Molecular weight cut off
mRNA	Messenger ribonucleic acid, subtype of RNA

M13	Filamentous bacteriophage, a common vector for *E. Coli*
PBS	Phosphate-buffered saline, an isotonic buffer solution
PBST	Phosphate-buffered saline supplemented with Tween® 20
pKKTac	Histidine tagged plasmid vector
pNPP	*Para*-Nitrophenylphosphate, a chromogenic substrate for phosphatases in ELISA
rATP	Ribose adenine triphosphate, a building component of RNA
RB	Reducing Buffer (appendix 1)
RT	Room temperature
RV308	Production strain of *E. coli*
SAMI	An automated robotic working station
SB	Super Broth (appendix 1)
SDS	Sodium dodecyl sulfate
scFv	Single-chain variable fragment
SOB	Super Optimal Broth (appendix 1)
SOC	Super Optimal broth with Catabolite repression, SOB with glucose (appendix 1)
TB	Terrific Broth (appendix 1)
TBST	Tris-Buffered Saline + Tween® 20 (appendix 1)
TEA	Tetraethylammonium hydroxide
TET	Tetracycline
T_2	Diiodothyronine
T_3	Triiodothyronine
T_4	Tetraiodothyronine, also known as thyroxine
VCSM13	Interference-resistant helper phage
XL1-Blue	Competent *E. Coli* strain

1 Introduction

Twenty-six years ago, the production of recombinant antibody fragments in bacteria was reported for the first time [**1**; **2**]. Rapid development of modern molecular biology methods for modifying and expressing recombinant DNA has revolutionized the development of the recombinant antibodies. The global market for research antibodies and pharmaceutical antibody drugs has reached the worth of 71 billion dollars [**3**; **4**] and the share of recombinant antibodies is showing increasing trend in the market.

Although the conventional immunoglobulin G (IgG) format still predominates in the therapeutics, smaller antibody fragments, such as antigen-binding fragment (Fab) and single-chain variable fragment (scFv) fusion-protein, are providing multiple advantages over the whole Ig molecules, especially, on application areas where small size, modifiability, and faster and more economical production are preferred over the IgG [**5**].

The introduction and development of efficient antibody library display methods (e.g. phage display) and selection methods (e.g. biopanning) have enhanced dramatically the development of recombinant antibodies [**6**]. Respectively, in this study, phage display and biopanning are applied to the development of novel triiodothyronine (T_3)-binding antibodies from the previously constructed and validated recombinant antibody (Fab) library (Henri Arola, 2011). The library was constructed from a multi-immunized mouse, and it has already been successfully employed to the development of T_3-specific Fabs (Henri Arola, 2011).

The target molecule of the study, T_3, is widely considered to be one of the most active forms of biologically highly important thyroid hormones [**7**] and thus also an important analyte. The absence and weak availability of T_3 binders with high affinity and specificity leaves a demand for better T_3-specific antibodies (Tarja Nevanen, personal communication, 2014).

Besides creating novel anti-T_3 clones, the development and characterisation process is expected to yield valuable information concerning the structures responsible for the antigen binding abilities. This type of information will be useful for the understanding and modification of the antigen-binding domains of T_3-specific antibodies.

The biopanning from a previously employed library will also provide information about the reproducibility and reliability of the methods and protocols that are applied to the antibody development process.

2 Theory

2.1 The molecule of interest: T3

T3 belongs to the family of thyroid hormones. These tyrosine-based signalling molecules are the principal secretory products of the thyroid gland in vertebrates [**8**; **9**]. Thyroid hormones are playing crucial role in the metabolism, growth, development, differentiation, and physiological function of virtually all organ systems and tissues [**10 – 12**]. They consist of two benzene rings and are generally named after the number and the conformation of the iodine atoms bound to them. The quantity of the iodine atoms can vary from one to four. Thyroid hormones are accordingly named as mono- (T_1), di- (T_2), tri- (T_3) and tetraiodothyronine (also known as thyroxine, T_4). The number of the bound iodine atoms strongly affects to the biological functionality of the hormone. According to the current knowledge, the biologically most active forms of the thyroid hormones are T_4's more potent metabolites T_3 and T_2 [**13**]. Majority of the thyroid gland secreted thyroid hormones are in the form of T_4, but it is estimated that more than 70 % of it will be deiodinated in peripheral tissues to T_3 and part of T_3 subsequently to T_2 [**7**; **13**]. T_3-specific antibodies were the main targets of this study, but to estimate their cross-reactivity, they were also tested against T_4. T_3 and T_4 are illustrated in Figure 1.

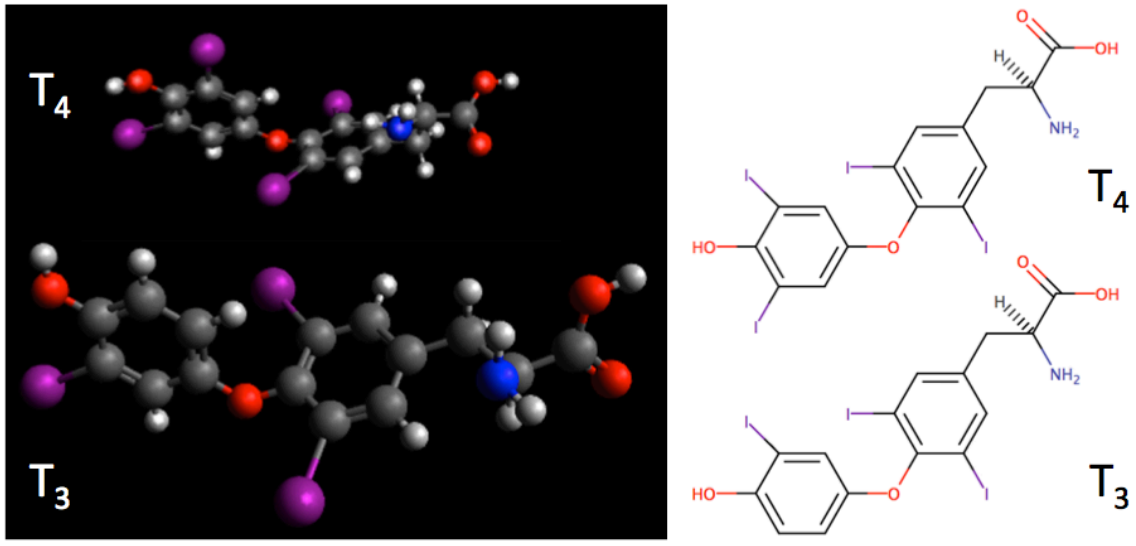


Figure 1. T_3 and T_4 molecules. In contrast to T_4, T_3 has only three iodine atoms.

Due to their broad range of biological functions, thyroid hormones have been excessively studied for several decades. Based on its active role and high importance, T_3 has a great demand for applications especially in fields such as diagnostics and therapeutics. In diagnostics, reliable, sensitive and efficient detection methods are needed to determine hormone levels from samples. T_3 tests are particularly important in diagnosing and monitoring of hyperthyroidism, a condition in which the thyroid gland secretes excessive amounts of free thyroid hormones (T_3 and T_4). The opposite of hyperthyroidism is called hypothyroidism, a condition caused by insufficient production and secretion of thyroid hormones. The detection of free T_3 is challenging due to its low levels in circulation. Free T_3 levels of adults are typically ranging from 3 to 8 pmol / L [**14**]. Antibody fragments with high affinity and specificity against the T_3, could be applied to create more sensitive and specific tools for the detection of the T_3 levels.

2.2 Antibodies

Antibodies, also called immunoglobulins, are immunologically active glycoproteins that are the most important part of humoral (antibody-mediated) immune system. They are produced and secreted by differentiated B-lymphocytes. The activation and differentiation is triggered by the recognition of the antigen by the cell surface antigen receptor of the B-lymphocyte and usually requires helper T cells.

Antibodies act in three main ways in the humoral immune system that protects the body from pathogens and other foreign particles. They can bind to the antigen and inhibit its toxic effects or pathogenicity (neutralization), invite phagocytic cells to destroy the recognized and antibody coated (opsonisation) pathogenic cells or particles, or activate the complement system that can strongly enhance the opsonisation or directly kill some pathogens.

2.2.1 The general structure of an antibody

Antibodies typically consist of two identical heavy and two identical light chains (Figure 2). The polypeptide chains are connected *via* disulfide bonds by their cysteine residues. Each chain, both heavy and light, is constructed from series of approximately 110 amino acids long sequences [**12**; **15**]. These repeats form the discrete and compactly folded domains of the antibody protein structure. The heavy and light chains are highly variable between antibodies, but the variability is focused mainly on the first pro-

tein domains, also called the functional domains, of the amino-terminal ends of the chains, while the remaining domains are much more constant [16; 12]. The amino-terminal variable domains of heavy (V_H) and light chain (V_L) form together the structures responsible for antigen binding abilities of the antibody, while the constant parts (C_H and C_L) correspond for the effector functions [16].

Each variable domain (V_H or V_L) can be divided to smaller subregions. Conserved framework regions are responsible for the tertiary structure of the variable domains. Three hypervariable loops, also called complementary determining regions (CDRs), in each chain are located between the framework regions, and together these six loops of the heavy and light variable domains form the antigen-binding site of the antibody [15].

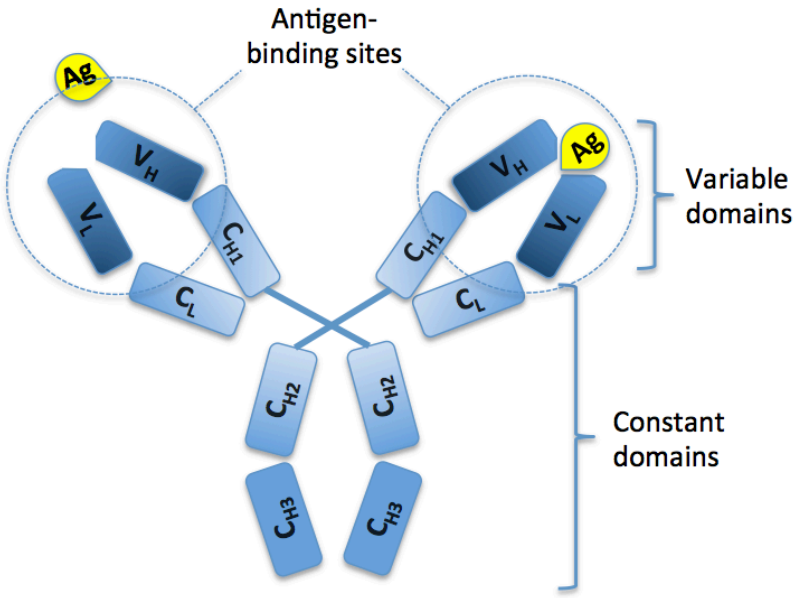


Figure 2. Schematic of IgG structure. Both the heavy and light chains consist of constant and variable domains (Heavy of C_{H1-3} and V_H, light of C_L and V_L). Variable domains are responsible for the antigen binding abilities of the antibody.

2.2.2 Recombinant antibodies

Recombinant antibodies are antibodies produced by genetic engineering. Structures and properties of antibodies can be genetically engineered by recombinant DNA techniques. Due to the domain structure of antibodies, it is particularly suitable to modify or, like in the most of the cases, to produce only the domains of the antibodies. Respectively, majority of the recombinant antibodies, such as Fab and scFv, consist only of the functional fragments of antibodies [17].

A vast number of structural variations have been developed, but the most widely used format of recombinant antibodies is the Fab. Among with complete Ig molecules, Fabs have already gained regulatory approvals and they have been successfully applied as a treatment of cancer, infectious disease and inflammatory disease [5]. Fabs and other recombinant antibodies have been applied to create multivalent high-avidity reagents and fused with wide range of molecules and platforms [5]. Besides the Fab and scFv, the diverse group of recombinant antibodies include many other variations as well. The most common ones, Fab and scFv are illustrated in Figure 3.

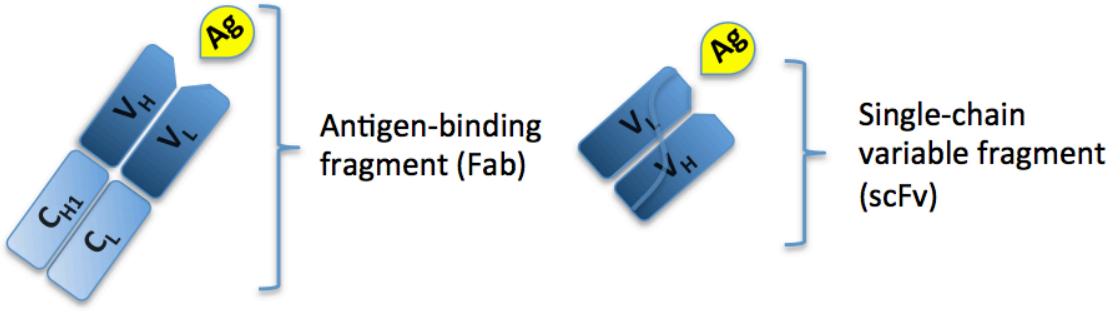


Figure 3. Basic structures of Fab and scFv molecules. Fab consists of a whole arm of an antibody, but scFv is a fusion protein including only the variable domains of a single antigen-binding site. Despite their small size, both structures retain the antigen binding abilities of a whole IgG.

2.3 Antibody gene libraries

2.3.1 Antibody gene library

Antibody gene library is a library constructed from a repertoire of antibody encoding genes. Complimentary DNA (cDNA) synthesized from murine or human B-lymphocyte's total RNA and the antibody genes are amplified by PCR (polymerase chain reaction) with special primers for heavy and light chains.

The DNA of heavy and light chains are cut with specific restriction enzymes and ligated into a vector, which in phage display method is a phagemid plasmid. After the phagemid contains both of the chains, it can be used to express the antibodies in a host organism, such as *E. coli*. When displayed on phages, antibodies can be selected by their affinity against the desired antigen (see section 2.4). Different types of libraries can be constructed, but Fab and scFv libraries are the most common ones. Fab library

contains only the structures responsible for the antigen-binding abilities of an antibody, the first constant and variable regions from both, the light and the heavy chain (V_H – C_{H1} and V_L – C_{L1}), whilst the scFv contains only the variable regions (V_H and V_L).

Recombinant antibody library, such as Fab or scFv library, is typically constructed from immunized or *naïve* sources, however, they have also been successfully constructed completely synthetically [**18**] and combinatorially [**19**]. Immunized source, most commonly a mouse, is immunized with a desired immunogenic antigen to activate the production of antigen-specific antibodies. Natural source, such as a mouse or a human that is not immunized, is called *Naïve.* Affinities of antibodies produced from immunized sources are corresponding to secondary immune response, whereas libraries of *naïve* sources produce antibodies against diverse group of antigens, but with weaker affinities usually corresponding to the affinities of primary immune response [**15**].

Unlike immunized and *naïve* sources, which are entirely based on naturally occurring sequence diversity, synthetic and combinatorial libraries are diversified by genetic engineering, either according to design, randomisation or their combination [**20**]. In combinatorial approaches, naturally occurring sequences are combined to designed ones, but the fully synthetic libraries are completely based on computational *in silico* design and the composition of their antibodies is precisely defined and controlled [**20**].

Although antibody repertoires with remarkably high diversity can be achieved with gene libraries, the diversity varies significantly between libraries. Typical library size for a phage-displayed library, constructed from an immunized source, is in the range of 10^8. Respectively, libraries provide a huge potential to the search of new recombinant antibodies. The number and type of antibody clones obtained, are highly dependent on the size and diversity of the antibody library and it has been acknowledged that the quality of the library is one of the most important factors in all of the *in vitro* approaches of developing antibodies [**20**].

2.3.2 Developing of hapten-specific antibodies from an immunized source

Haptens, such as thyroid hormones, are low molecular weight molecules, too small to induce a humoral immune response without assistance of a larger carrier. Due to their size, haptens are not recognizable to antigen receptors, unless presented as an epitope of larger structure. Therefore, haptens must be linked to macromolecules, such as immunogenic proteins or synthetic polymers [**21**], before immunizations [**15**]. Classi-

cally, haptens are covalently bound to these carrier molecules *via* linkers; therefore, both the hapten and the carrier must have a reactive group for the attachment [**22**]. Linking of the haptens to other molecules can be significantly challenging and requires sophisticated chemistry [**15**].

The linking itself may affect to the immunization and the properties of produced antibodies [**15**]. Linking may, for example, cause the anti-hapten antibodies to recognise hapten and part of the linker with higher affinity than the native form of the hapten ('bridge effect'), or distort the structure of the hapten and produce antibodies that recognise the conjugated form of the hapten with higher affinity and decrease the affinity for the hapten alone [**15**].

In comparison with immunogenic target molecules, developing of hapten-specific antibodies and their fragments can be significantly more challenging [**15**]. The methods and molecules applied to the conjugation of the hapten affect significantly to the antibodies developed [**23**; **15**]. It has been acknowledged that several factors, such as the carrier molecule, hapten-carrier ratio, linker molecule, the site of conjugation, and the number of carriers applied, affect the properties of the conjugate and therefore to the immunization and generated antibodies [**23**]. To raise the possibilities for success, it is highly advisable to carefully analyse and validate the hapten-protein conjugates; the degree of hapten conjugation should be estimated e.g. mass spectrometrically with matrix-assisted laser desorption/ionization (MALDI) (Tarja Nevanen, personal communication, 2014).

2.3.3 *In vitro* display methods of recombinant antibody gene libraries

Although various *in vitro* library display methods have been introduced and successfully applied for the development of recombinant antibodies, the fundamental idea behind them is the same. Practically, all of the display techniques link the phenotype to its genotype (DNA or mRNA). Antibodies have been displayed, i.e. expressed, not only *in vivo* on microorganisms such as yeast, viruses and bacteria, but also *in vitro* by ribosome display and mRNA display [**15**]. The most popular display technologies are phage (bacteriophage) display and yeast display [**6**]. Also, the antibody (Fab) gene library of this study was displayed on the surface of a filamentous phage (M13), as a fusion with its coat protein (pIII).

2.4 *In vitro* selection of binders from phage-displayed libraries

Antigen-specific antibodies (Fab, scFv etc.) can be selected from the phage-displayed antibody library *in vitro* according to their antigen binding properties with a method called “biopanning”. Biopanning involves library’s incubation with target antigen, removing of nonspecific phages (e.g. by washing), collection of specific binding phages, and enrichment of binders by propagation in host bacteria (e.g. *E. coli*) [**24**].

Usually 3 – 4 rounds of panning are sufficient to enrich the binders [**24**]. Panning can be conducted with different techniques that differ in terms of selection method, washing protocol and elution methods. The antigen library can be panned *in vitro* against antigens that are coated on a solid support (e.g. microplates, column matrices, sensor chips, polyester tubes or 96 well microtitre plates), in solution with biotinylated antigens, on intact cell surfaces, on Western blots and on tissue sections [**24**]. Antibody clones expressing specificity against the antigen are collected or retained and the unspecific clones removed. Method applied to remove the unbound clones can vary between different assay types, but usually unspecific clones are washed with suitable solution.

After removing the unspecific clones, selected clones are collected by elution. Elution method has proven to be one of the most important factors affecting to the antibody outcome of the panning [**15**]. Due to its highly important role, multiple elution techniques have been developed and applied to the panning of antibody libraries. Both high [**25**] and low pH [**26**] methods, different methods utilizing enzymatic cleavage such as trypsin [**27**] and DNAse [**28**], DTT method [**29**] and antigen competition [**25**] have been successfully applied as elution techniques. The efficiency of elution methods tends to vary case by case; thus the elution method should be carefully planned for each panning [**28**], and for the best possible results, multiple elution methods should be applied or at least tested [**30**].

In this study, the phage-displayed library was panned *in vitro* against T_3-alkaline phosphatase (AP) conjugates. The conjugates were immobilized on the surface of streptavidin magnetic beads (Life Technologies). For clarification of the reaction conditions, main reaction components (hapten-protein conjugate (T_3-AP), vector (filamentous bacteriophage M13), linker molecules and magnetic streptavidin beads are depictured below (Figure 4 and Figure 5).

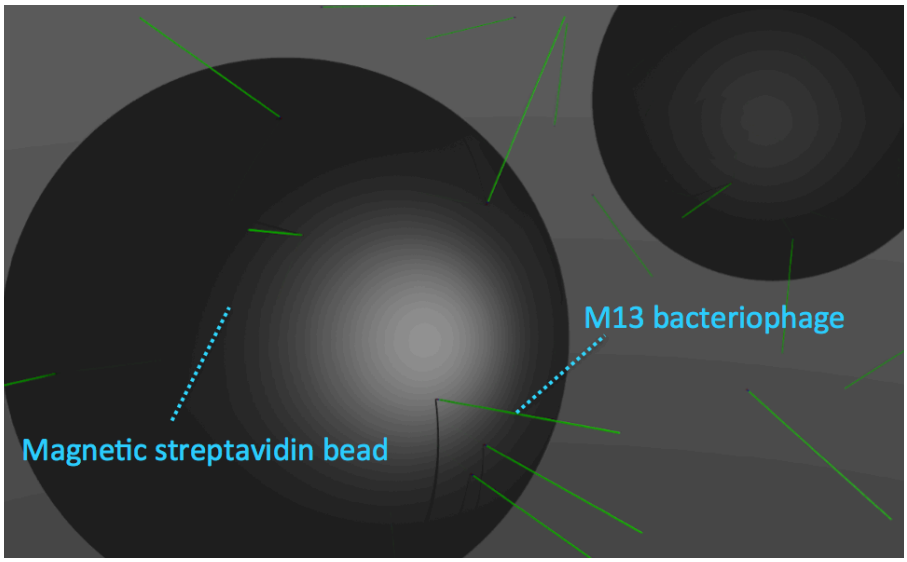


Figure 4. Simplified schematic presentation of magnetic streptavidin beads (Dynabeads® M-270 Streptavidin, diameter 2,8 µm) and M13 filamentous bacteriophages (880 nm in length and 6,6 nm in diameter [**31**]).

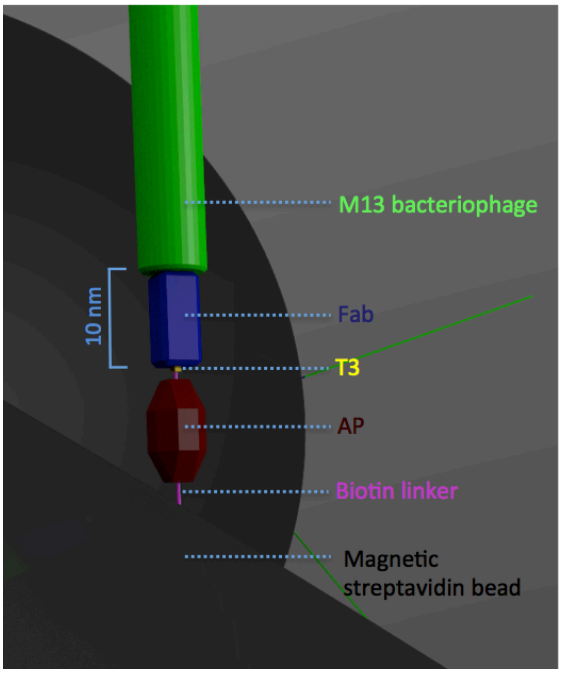


Figure 5. Schematic presentation of molecules involved to the biopanning, enlargement from Figure 4. This simplified picture presents the binding of a hapten-specific Fab clone to an immobilized hapten-protein conjugate. The purpose of the picture is to clarify particle sizes and the panning process. In reality, the surface of the magnetic streptavidin bead would be coated almost completely with the biotinylated hapten-protein conjugates (T_3-AP + biotin linker). Dimensions of the structures are in realistic scale to each other (M13 [**31**], Fab [**32**], T_3 calculated with MarvinSpace 15.3.30.0, AP [**33**], biotin linker and magnetic streptavidin beads (manufacturers specifications, EZ-Link Sulfo-NHS-SS-Biotin and Dynabeads® M-270 Streptavidin).

2.5 Applications of phage-displayed recombinant antibodies

Since its development in 1985, phage display has become the most successful *in vitro* antibody selection method [**34**]. It has proven to be a powerful tool in the development of recombinant antibodies, and has already proven its value in fields of research, diagnostics and therapeutics [**34**]. All *in vitro* methods have two major advantages: they can be applied to an enormous range of antigens and the broad flexibility in the selection conditions that can be adapted to adjust the selection pressure [**20**]. Some of phage display's advantages over the other *in vitro* methods are robustness, which gives it a great potential for automation, and the high stability of phage, which enables applying extreme conditions to the selection procedure [**20**; **34**].

Antibody phage display can be applied to produce a range of recombinant antibody formats limited only by *E. coli's* folding machinery and the periplasmic expression efficacy [**20**; **34**]. However, due to the phage displays ability to link the phenotype to the genotype, this limitation can be overcome by reconverting the recombinant antibody fragment to the antibody format of interest (e.g. Fab or scFv) and producing it in different production host capable to correct glycosylation and expression (e.g. modified yeast or human cell) [**34**; **6**].

The diversity of recombinant antibody applications is enormous. Different fields of applications and the desired application define the specific requirements for the particular antibody; for example, in therapeutics special attention must be given to the safety, manufacturability, and formulation of the antibody product [**20**]. Immunogenicity and safety are particularly problems with nonhuman antibodies and their fragments. In such cases, antibodies are usually modified either by chimerisation or humanisation [**34**]. In chimeric antibody the constant regions are human and the variable regions murine, whereas in humanisation human counterparts replace also the murine framework regions of the variable domains [**34**].

Although Ig molecules still predominate in the therapeutics, there are many recombinant antibodies in preclinical and clinical development [**5**]. Besides the growing interest in their development, for example Fabs have already gained clinical approvals from FDA, e.g. as an antidote for rattlesnake bite (CroFab®, BTG International Inc.), and as a treatment of digoxin overdose (DigiFab®, BTG International Inc.), cardiovascular dis-

ease (ReoPro®, Janssen Biologics B.V.), rheumatoid arthritis (Cimzia®, UCB, Inc.), and age related macular degeneration (Lucentis®, Genentech USA, Inc.).

Due to modifiable structure and size, faster and more economical production, and retained targeting specificity of whole Ig molecules, recombinant antibodies provide powerful tools also for research and diagnostic applications [**5**]. Especially promising application areas are biosensors [**5**; **35**], *in vivo* medical imaging [**5**], *in vitro* immunoassays [**5**; **35**], *in vivo* tumor and clot-imaging [**5**], and tuning and detecting the activity of cell proteins *in vivo* [**35**]. Antibodies are already standard tools for many methods of basic research such as immunoblot, ELISA, immunofluorescence microscopy, flow cytometry, purification of molecules or cells by affinity chromatography, and microarrays [**34**].

3 Materials and methods

The main steps of the thesis are presented in the flow diagram (Figure 6) below.

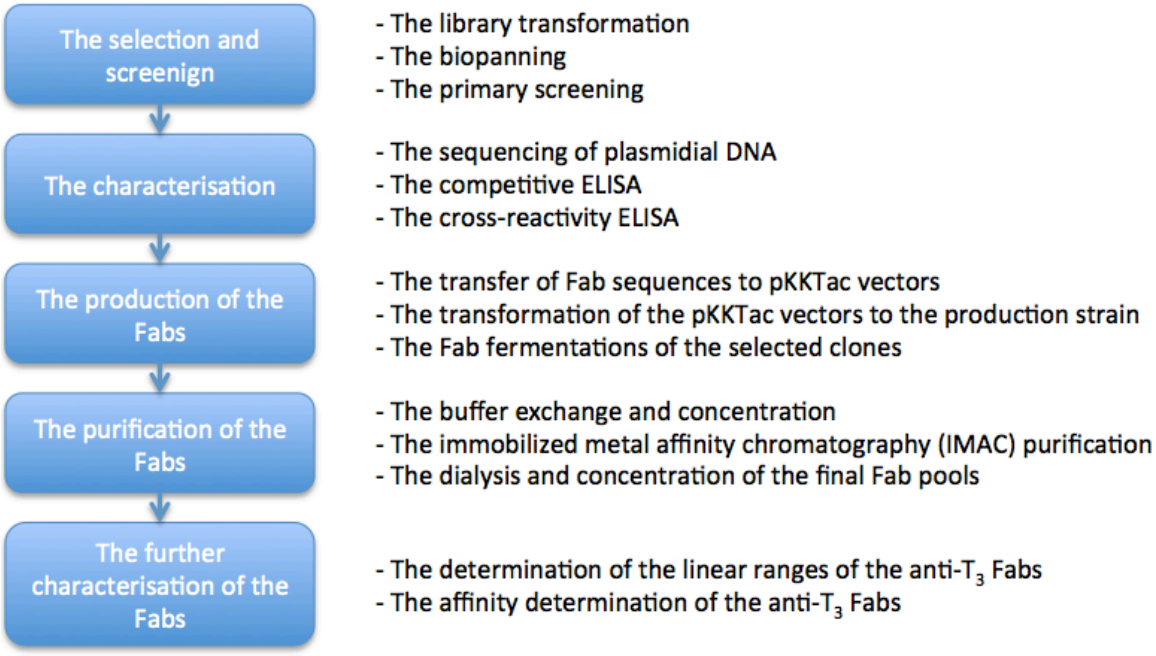


Figure 6. Flow diagram of the main steps of the thesis.

3.1 The selection and screening of the T_3-specific clones

3.1.1 The phage library transformation

An antibody library used for screening and selection was built by combining four differently cloned pools of the Addams library, a multi-immunized library previously constructed and validated by Henri Arola (2011). Transformation of the library was implemented *via* electrotransformation. Cells applied for the transformation were from a commercial electrocompetent *E. coli* strain, XL1-Blue (Agilent).

For the transformation, one µg of DNA was used per 100 µL of the XL1-Blue cells. The DNA-libraries were mixed with the cells and the tubes were kept on ice for a minute. The cells were transferred to precooled Ø 0,2 cm electrocuvettes (Cuvettes Plus™, Model No. 620, BTX), in which the electroporation was carried out with an initial voltage of 2,5 kV. After the electric shock, the cells were immediately gathered and washed

three times with 1 mL of Super Optimal broth with Catabolite repression (SOC) and transferred within the solution to 8 mL test tubes. The cells were incubated at 250 rpm shaking at a temperature of 37 °C for an hour.

After the incubation, 7 mL of Super broth (SB) containing 20 µg / mL of carbenicillin (CARB, Sigma-Aldrich Co.) and 10 µg / mL tetracycline (TET, Sigma-Aldrich Co.) was added to the tubes. The antibiotics were added to prevent the growth of untransformated cells. After the addition, 100 µL of the cells were cultivated on Luria broth (LB) plates with 100 µg of TET per 1 mL of LB. The plate count was applied to determine the transformation efficiency and the library size.

The tube cultivation of the cells was continued under the previously described conditions for an hour before the addition of CARB to the final concentration of 50 µg / mL. The antibiotic addition was followed by a one-hour incubation in the conditions analogous to the previous one. The incubated cells were infected with 1 mL of VCSM13 helper phages (Stratagene, ~1012 phage particles / mL) and the tubes were held for 30 minutes at a temperature of 37 °C without shaking to enhance the infection.

After the infection, the cultures were transferred to 90 mL of preheated (37 °C) SB containing the same concentrations of antibiotics as the cultivation media. The cultures were incubated in the volume of 100 mL for two hours under the incubation conditions mentioned above. After the incubation, the cultures were treated with 70 µg / mL kanamycin (KANA, Sigma-Aldrich Co.) and cultivated overnight at a temperature of 37 °C and 250 rpm shaking. KANA was added to the cultivation media by means of allowing the surviving and proliferating only for the VCSM13 infected cells.

The cultures were centrifuged for 15 minutes with 4 000 rpm to separate the cells from the cultivation media. The supernatant containing the cell-secreted phages was separated from the cultivated cells by pouring and mixing it to 25 mL of precooled precipitation solution, which was containing 2,5 M NaCl and 20% of polyethylene glycol (PEG 6000, Sigma-Aldrich Co.). In order to efficiently precipitate the phages, the mixture was kept on ice for 30 minutes. The precipitated phages were centrifuged to the bottom of the tube with 9 000 rpm at 4 °C for 20 min. The supernatant was disposed from the centrifugation tube and the pellet was resuspended to 2 mL of phosphate-buffered saline (PBS). The 2 mL of phages was then divided to two 1,5 mL Eppendorf tubes. The tubes were centrifuged with 14 000 rpm for 5 minutes and reprecipitated as before with

250 µL of the precipitation solution. The phages were centrifuged for 8 min with 11 000 rpm and the supernatant was carefully removed. The compounded pellet was resuspended to 1 mL of PBS and stored at a temperature of 4 °C.

3.1.2 The biotinylation of the hapten-protein conjugates

Hapten-protein conjugates were constructed by combining protein carriers, AP molecules (Sigma-Aldrich Co.), to haptens, T_3 molecules (Sigma-Aldrich Co.). The mass spectrometrically (matrix-assisted laser desorption/ionization, MALDI) validated hapten-protein conjugates were provided by Antti Tullila. The conjugates and free AP molecules were biotinylated for the selection phase.

0,2 mg of the T_3-AP-conjugates and AP molecules were diluted to 200 µL of double deionized water. A Twenty-fold molar excess of Sulfo-NHS-SS-biotin (EZ-Link™, Thermo Fisher Scientific Inc.) was added and mixed to the dilutions. The additions and mixings were conducted in three steps. After the last additions, the tubes were held for two hours in shaking at a room temperature (RT).

The solutions were transferred from Eppendorf tubes to Millipore's 10 000 MWCO centrifugal filter tubes (Amicon® Ultra - 0,5 mL 10 K Centrifugal Filters) and washed 5 times with 500 µL of PBS before resuspending to 160 µL of PBS.

The quantity of the proteins obtained was determined by Thermo Scientific's Pierce™ BCA Protein Assay kit with human serum albumin (HSA) standards (4, 2, 1, 0,5 and 0,25 mg / mL) provided by Hannakaisa Jaatinen. The assay was conducted according to the manufacturer's instructions. The detection was exploited after 30 minutes at a wavelength of 560 nm with SPECTROstar Omega spectrometer (BMG LABTECH GMBH).

The degree of the biotinylation was measured with an ELISA test. A streptavidin coated 96-well plate (KaiSA96-C96 Clear, Kaivogen Oy) and an AP control were applied for the assay. 0,5 µg of the analyte and the AP were diluted to 100 µL of PBS and incubated on the plate for 20 min with shaking at a RT. 100 µL of *Para*-Nitrophenylphosphate (pNPP, Sigma-Aldrich Co.) diethanolamine (DEA, Reagena) solution (2 mg / mL) was implemented for the detection. Absorbance values were measured at a wavelength of 405 with SPECTROstar Omega spectrometer after 20 and 30 minutes of incubation.

3.1.3 The hapten-protein conjugate coating of the magnetic beads

Magnetic streptavidin beads (Dynabeads® M-270 Streptavidin, Invitrogen™, Life Technologies), which were to be used in the selection phase, were coated with the biotinylated hapten-protein conjugates (section 3.1.2) to enable the capturing of hapten-specific Fab clones. The control beads were coated with biotinylated AP molecules (section 3.1.2). The goal was to saturate the surfaces of the beads with the biotinylated molecules. After the coating, all of the possibly uncoated surfaces were blocked with the biotin. KingFisher™ Duo was applied to efficiently combine and perform the protocols for both the coating and blocking.

KingFisher™ Duo's program created for the selection phase included 8 steps and took approximately 1 h and 20 min. The whole process was designed to be implemented on a deep 96-well plate (MASTERBLOCK®, 2 ml V-Shaped, Greiner Bio-one), compatible with KingFisher™ Duo. The plate was prefilled with desired reagents, and the premeditated order of the reagents enabled the right reactions when magnetic beads were shifted from a well to another. The main working steps of KingFisher™ Duo's program are described below in table Table 1.

Table 1. Summarization of the KingFisher™'s program designed for the coating and blocking of magnetic streptavidin beads.

KingFisher™: Coating and Blocking of Streptavidin (SA) Beads					
Step #	**Function**	**Mixing included (Yes / No)**	**Reagents**	**Volume**	**Duration**
1	Collection of beads	Yes	100 µl / ml of SA-Beads	250 µl	30 sec
2	Coating of SA-beads	Yes	50 µg / ml biotinylated protein molecules	250 µl	1 h
3	Washing	Yes	PBST (0,05 % of Tween20)	500 µl	20 sec
4	Washing	Yes	PBST (0,05 % of Tween20)	500 µl	20 sec
5	Blocking	Yes	Biotin + PBST (0,05 % of Tween20)	500 µl	15 min
6	Washing	Yes	PBST (0,05 % of Tween20)	500 µl	20 sec
7	Washing	Yes	PBST (0,05 % of Tween20)	500 µl	20 sec
8	Ejecting of processed beads	Yes	PBST (0,05 % of Tween20)	100 µl	1 min

After completing the protocol, successful coating of the beads was ensured by applying an ELISA-test. Uncoated SA-beads were utilized as controls. pNPP DEA solution (2 mg / mL) was applied to detect the amount of AP in the sample and control solutions. Absorbance values were measured at a wavelength of 405 with SPECTROstar Omega spectrometer.

3.1.4 The selection of specific anti-T_3 clones through biopanning

The selection, also called as biopanning, was applied to enrich the bacteriophage M13 clones that expressed the T_3-specific antibodies. As a starting material for the first selection round, was the bacteriophage pool from the phage library transformation (section 3.1.1). The clones obtained as a result of each selection round were applied as a starting material for the following round. Thereby, the selection enriched only the selected clones of each round. The needed amount of the clones for each of the selection rounds was obtained by utilizing *E.coli* (XL1-Blue) cells to produce the selected phages. The selection was conducted to the clones by utilizing two different elution methods in parallel. The elution methods applied were the alkaline and acidic elution.

The KingFisher™ Duo was applied for the selection step. The biopanning was conducted with the previously created hapten-protein coated magnetic streptavidin beads (Section 3.1.3) and it was consisted of five selection rounds. In order to increase the selection pressure between the rounds, reaction conditions of the selection were gradually hardened for each of the rounds.

Unlike the reaction conditions, the basic structure of the protocol was remained mainly the same during all of the rounds. Each selection round was began with depletion and binding, either simultaneous or as separated steps. The amounts of the phages and hapten-protein coated magnetic streptavidin beads used to the binding were utilized to affect the selection pressure. After the binding phase, three washing cycles followed. The washings were gradually elongated towards the fifth round. The final step of KingFisher™'s protocol was the 30 min elution of the selected phages from the hapten-protein coated beads. After the elution, the detached phages in 100 µL of the elution mixture, either glycine-HCl (0,1 M pH 2,2) or tetraethylammonium hydroxide (TEA, ~ 2,7 M, pH 11,7, Sigma-Aldrich Co.), were manually neutralized either with 8,5 µL of 1 M Tris pH 9,5 or with 135 µL of 1 M Tris pH 7,4 depending on the elution method used. The specific conditions and the differences between each round are described in detail below in Table 2.

Table 2. Detailed protocols for each of the selection rounds of the biopanning. The enriched clones were produced in XL1-Blue cells between each round.

Selection: Rounds I - V, T3								
Round	**Depletion**		**Binding**		**Washes**		**Elutions**	
I	**type**	AP-SS-Biot-SA-beads	**beads (T3-AP-SA)**	5 µl	**cycles**	3	**reagents**	Acidic: Glycine-HCl (pH 2.2) Alkaline: TEA (pH ~11,75)
	reagents	10 µl AP-SS-Biot-SA-beads 300 µl PBST (0,05 % Tween20) 200 µl phage pool	**depleted phage pools (µl)**	200 µl	**duration per cycle**	20 sec		
	duration	2 h	**duration**	o/n			**duration**	30 min
II	**type**	AP-SS-Biot-SA-beads	**beads (T3-AP-SA)**	5 µl	**cycles**	3	**reagents**	Acidic: Glycine-HCl (pH 2.2) Alkaline: TEA (pH ~11,75)
	reagents	10 µl AP-SS-Biot-SA-beads 300 µl PBST (0,05 % Tween20) 200 µl phage pool	**depleted phage pools**	200 µl	**duration per cycle**	20 sec		
	duration	2 h	**duration**	o/n			**duration**	30 min
Round	**Combined depletion and binding**				**Washes**		**Elutions**	
III	**type**	soluble combined with binding			**cycles**	3	**reagents**	Acidic: Glycine-HCl (pH 2.2) Alkaline: TEA (pH ~11,75)
	reagents	20 µg soluble AP 4µl T3-AP-SA-beads 200 µl bacteriophages 300 µl PBST (0,05 % Tween20)			**duration per cycle**	1 min		
	duration	2 h					**duration**	30 min
IV	**type**	soluble combined with binding			**cycles**	3	**reagents**	Acidic: Glycine-HCl (pH 2.2) Alkaline: TEA (pH ~11,75)
	reagents	20 µg soluble AP 3 µl T3-AP-SA-beads 200 µl bacteriophages 300 µl PBST (0,05 % Tween20)			**duration per cycle**	1 min		
	duration	2 h					**duration**	30 min
V	**type**	soluble combined with binding			**cycles**	3	**reagents**	Acidic: Glycine-HCl (pH 2.2) Alkaline: TEA (pH ~11,75)
	reagents	20 µg soluble AP 2 µl T3-AP-SA-beads 2 µl bacteriophages 498 µl PBST (0,05 % Tween20)			**duration per cycle**	5 min		
	duration	1 h					**duration**	30 min

For the production of the selected phages, XL1-Blue cells were cultivated at 30 mL of SB containing 10 µg / mL TET. The cells were cultivated 2,5 hours until the cell culture's absorbance, measured at wavelength of 600 nm (OD600), had reached the value of 1. 3 mL of the XL1-Blue cells were infected with the neutralized bacteriophages for 30 min at 37 °C. Also, 1 mL of the cells were infected with the same protocol, but instead of the elution mixture with 1:10 000 dilution of the original input phage pool of the current round.

After the infection 7 mL of SB with 10 µg / mL TET and 20 µg / mL of CARB was mixed to 3 mL of the cultivation. 100, 10 and 1 µL of the cells from the 10 mL cell culture and 10, 1, 0,1 and 0,01 µL from the 1 mL culture were cultivated on LB plates with 100 µg of TET per mL of LB. With the plate cultivation it was possible to estimate the effect of the selection on the ratio between input and output of each round.

After increasing the CARB's concentration to 50 µg / mL, the 10 mL cultivations were incubated one hour at 37 °C with 225 rpm shaking. The incubation was followed by an infection with helper phage VCSM13. The infection and cultivation after it were performed similarly to the protocol of the phage library transformation, which is described in section 3.1.1.

After the overnight production of the selected bacteriophages, they were precipitated with the same protocol as during the phage library transformation (section 3.1.1). The precipitated phage pools were applied as a starting material for the next round of the selection. After five selection rounds the pools were tested with a phage ELISA (section 3.1.5).

3.1.5 The phage ELISA

Two MaxiSorp® NUNC-IMMUNO C96 (Thermo Scientific Ltd.) 96-well plates were coated overnight with 3 µg / mL T_3-HSA and HSA Na-bicarbonate (0,1 M pH 9,6) solutions, 100 µl per well.

The precipitated phage pools, both the alkaline and acidic eluted (section 3.1.4), were diluted to three dilutions (1:10, 1:100, 1:1000) and the 96-well plates were washed three times with 0,05 % PBST and blocked for one hour in shaking at RT with 280 µl of SuperBlock® (Thermo Scientific Ltd.) 0,05 % Tween® 20 (Sigma-Aldrich Co.) solution per well. Plates were washed as before and 100 µl of phage dilutions were incubated on the plates for an hour in conditions described above. After incubation, plates were washed as described before and 100 µl of HRP-conjugated anti- M13 detection antibody (Sigma-Aldrich Co.) was added per well. Plates were incubated and washed analogously to earlier steps, and 100 µl of ABTS substrate (1 mg ABTS and 7 µl of double de-ionized water (DDIW) in 4,4 mL of 50 mM citrate buffer) was added per well. Intensities of the occurred colour reactions were measured at a wavelength of 405 nm with SPECTROstar Omega spectrometer.

3.1.6 The primary screening (ELISA)

Primary screening was conducted only to the phage pools indicating specificity against T_3 in the phage ELISA (section 3.1.5). Respectively, the rounds from two to five from both, the acidic and the alkaline elutions, were represented and the first rounds were excluded. SAMI, an automated robotic working station (specifications classified), was employed for the ELISA screenings, which were exploited to estimate the T_3-specificity and affinity of the clones.

As a starting material were the output plate cultivations from the T_3-specific pools (rounds II – V). 96 XL1-Blue clones from each of the plate cultivations were collected and transferred to 96-well plates (Pharmacia Ab) filled with 100 µL of SB containing 100 µg / mL ampicillin (AMP), 10 µg / mL TET and 1 % of glucose. The cells were incubated overnight at 37 °C, with 620 rpm shaking and at a relative humidity level of 80 %. For the overnight cultivations, the plates were sealed with gas exchange allowing membranes (4titude®, Gas Permeable Seal) and incubated in plastic bags to prevent unwanted evaporation of the media.

On the second day, 10 µL of the cultivated cells were transferred to deep incubation plates (MASTERBLOCK®, V-shape 2 mL, Greiner Bio-one) containing 500 µL of SB supplemented with 10 µg / mL TET and 100 µg / mL AMP. 20 µL of 85 % glycerol was added to the remaining 90 µL of the cultivations. The cultures were stored at -20 °C. The Induction plates were incubated 2,5 hours at 37 °C with 620 rpm shaking at 80 % of relative humidity. After the incubation 10 µL of induction media, 10 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma-Aldrich Inc.) SB containing 100 µg / mL AMP, was added to the cultivations by the robot. The induction plates were sealed with the gas exchange allowing membranes (4titude®, Gas Permeable Seal) and incubated overnight in plastic bags at 37 °C with 620 rpm shaking at 80 % of relative humidity.

On the same day, a total of 24 96-well plates (NUNC-IMMUNO PLATE C96) were coated, eight plates with each of the conjugates (1 µg / mL of T_3-HSA, T_4-HSA or HSA in 0,1 M sodium bicarbonate buffer, pH 9,6). The conjugates were provided by Antti Tullila and the coating was implemented overnight at 4 °C.

On the third day, before proceeding to SAMI, the cells were separated from the secreted Fabs (supernatant) by centrifuging the plates with 4 000 rpm for 10 min. SAMI's ELISA protocol for 24 96-well plates was previously programmed by Henri Arola. Otherwise the protocol was identical to the phage ELISA's (section 3.1.5), but 0,5 % bovine serum albumin (BSA, Sigma-Aldrich Co.) in PBS was applied for the 30 min blocking step and Anti-kappa AP 1:1000 (SouthernBiotech) in 0,5 % BSA PBS was employed as the detection antibody and incubated on the plates for 30 min. Detection solution, 2 mg pNPP per 1 mL of DEA, revealed the amount of the attached detection antibody in each well. Intensity of the occurred colour reaction was measured after 30 min at a wavelength of 405 nm. Execution of the whole 24 plate ELISA protocol took approximately 7,5 hours. Henri Arola's Anti-T_3 Fab (Anti-T_3 DTT T_3.K4.C5) was employed as a control.

According to results of the primary ELISA, twenty of the most promising or interesting clones were selected for sequencing and further characterization. The decisions were made by comparing the Fabs' specificities against the T_3-HSA, T_4-HSA and HSA.

3.2 The characterization of the screened anti-T_3 clones

3.2.1 The sequencing of plasmidial DNA

The selected anti-T_3 XL1-Blue clones were picked from the -80 °C storage and cultured in 4 mL of SB with 100 µg/ mL AMP, 10 µ / mL TET and 1 % glucose overnight at 37 °C. Plasmidial DNA of phagemid (9.2, VTT) vectors was isolated from the cultivated cells by applying Plasmid & DNA Purification kit (NucleoSpin® Plasmid, MACHEREY – NAGEL). The isolated DNA was sequenced with specific primers (GATC-1867 and GATC-6044, GATC Biotech AG). Data obtained from the sequencing was analysed with Geneious 6.1.8.

3.2.2 The competitive ELISA

In order to produce the Fabs, the twenty selected anti-T_3 clones were cultivated in 4 mL of SB with 100 µg / mL AMP and 10 µg / mL TET for 2,5 h at 37 °C with 220 rpm shaking. Induction of the cultivated cells was implemented by adding protein expression

inducer (IPTG) to the final molar concentration of 1 mM. The cell cultures were incubated overnight at 30 °C with 220 rpm shaking.

During the anti-T_3 cell cultivation MaxiSorp® NUNC-IMMUNO C96 plates were coated with T_3-HSA (100 ng T_3-HSA (Antti Tullila) / 100 µl sodium bicarbonate, pH 9,6) and incubated overnight at + 4 °C.

After the overnight incubation, the cultivations were centrifuged for 7 minutes with 6 000 g and supernatant was collected to 2 mL tubes. Right amounts of the T_3 and T_4 molecules, which were provided by Antti Tullila, were diluted to PBS containing 1 % of dimethyl sulfoxide (DMSO, Sigma-Aldrich Co.) to obtain the final molar concentration of 20 µM of each hapten. 125 µL of each supernatant, containing the produced fab, was mixed and preincubated with 125 µL of each hapten dilution for 1 h.

During the preincubation, the coated 96-plates were first washed three times with 300 µL of PBS and then blocked with 285 µL of PBS with 0,5 % BSA. The blocking was applied for 30 min at RT with 220 rpm shaking and followed by washings identical to the previous ones.

After the preincubation, the competed supernatants were transferred to the coated and blocked ELISA plates, 100 µL of each to two wells. The ELISA plates were incubated for 1 h at RT with 220 rpm.

After the incubation the plates were washed as described before. Secondary antibody, 1:2000 dilution of Anti-kappa AP (SouthernBiotech) in 0,5 % BSA PBS, was added to the plate (100 µl per well). The plates were incubated for 1 h at the same conditions as before and washed as described above. The amount of AP was detected by adding 100 µL of the detection dilution, 2 mg pNPP per 1 mL of DEA solution, to each well. Intensities of occurred colour reactions were measured at a wavelength of 405 nm with SPECTROstar Omega spectrometer.

According to the results of the competitive ELISA and the sequencing data, six of the most promising clones were selected for T_3 and T_4 cross reactivity testing with a secondary ELISA.

3.2.3 The cross reactivity testing of the T_3-specific clones

The protocol of this ELISA is described in the previous section. The only change to the previous protocol was that the blocking solution (0,5 % BSA in PBS) was replaced with SuperBlock® + 0,05 % Tween® 20. Competiveness of the 6 previously selected clones was measured with T_4 and T_3 in three different concentrations, 20, 2 and 0,2 µM.

As a result, the ELISA gave valuable information about the specificity of Fabs' against the T_3 and T_4. On the basis of the obtained data, three of the six tested Fab clones were chosen for a small-scale production (2 L) and further characterization.

Plasmidial DNA, the phagemid (9.2, VTT) vectors, of the selected clones was isolated by applying MACHEREY – NAGEL's Plasmid & DNA Purification kit (NucleoSpin® Plasmid).

3.3 The production of the T_3-specific Fabs

3.3.1 The transfer of the Fab coding sequence from phagemid to pKKTac

In order to enable the later purification of the produced Fabs, Fab coding sequences were transferred from the phagemids (9.2, VTT) to histidine tagged pKKTac vectors.

The isolated plasmidial DNA of the clones selected to the larger scale Fab production, was digested for 4,5 hours at 37 °C and stored at 4 °C. Digestion mixture contained 2 µL of both endonucleases *Nhe*I (New England Biolabs) and *Not*I (New England Biolabs), 5 µL CA (cuts all) buffer (pH 7,5) with β-mercaptoethanol (Fluka BioChemika), 0,5 µL BSA (20 mg / mL) and 15,5 µL DDIW.

The Fab coding regions of the phagemid plasmids were separated by agarose gel electrophoresis. The electrophoresis was carried out with MSCHOICE (Cleaves Scientific Ltd.) and GeneRuler™ 1 kb DNA Ladder (Thermo Scientific). 7 µL of DNA Safe colour was added per 100 mL of the 1 % agarose gel.

The samples, digested plasmidial DNA, and the control, 4 µL of undigested plasmidial DNA, were prepared by adding 1/6 volume of 6x DNA Loading Dye (Thermo Scientific) to them. The samples were kept in a +65 °C heat block for 10 minutes and cooled back

to RT before loading them into pre-cast wells in the agarose gel. Applying of an electric field separated the DNA fragments. The electrophoresis was run with the voltage of 80 V.

UV-light table of Molecular Imager Gel Doc™ XR+ (Bio-Rad Laboratories Inc.) was exploited for visualization and cutting off of the separated DNA bands from the agarose gel. The severed inserts were purified with MACHEREY – NAGEL's NucleoSpin® Gel and PCR Clean-up kit.

In order to determine the approximate DNA concentration of the purified inserts, NanoDrop 1000 spectrophotometer (Thermo Scientific) was applied. Absorbance was measured from a sample volume of 2 µL by using NanoDrop's preset configurations for nucleic acid measurements.

The mixtures with total volume of 10 µL contained 50 ng of the insert DNA, 100 ng of twice NheI and NotI digested pKKTac vector, 1 µL of 10x buffer, 1 µL T_4 ligase, 1 µL rATP (ribose adenine triphosphate, Thermo Fisher Scientific Inc.) and for the rest of the volume DDIW. The ligation mixtures were incubated 2 h at RT. The efficiencies of the ligations were estimated by selective plate cultivations after the transformation of the ligated pKKTac plasmids to the XL1-Blue competent cells (section 3.3.2).

3.3.2 The heat shock transformation of the pKKTac plasmid vectors

The ligated pKKTac plasmid vectors were transformed to XL1-Blue competent cells (VTT) *via* heat shock transformation. 200 µL of the heat shock competent XL1-Blue cells were added to 5 µL of each of the ligation mixtures and incubated on ice for 30 min. The tubes were then transferred for two minutes to a 37 °C heat block and then stabilized on the ice for two minutes. 400 µL of SOC was added to each of the tubes and the cells were cultivated for one hour at 37 °C with 220 rpm shaking. The cells were diluted in SB to 1:10 and 1:100. 100 µL of the dilutions were cultivated on LB plates supplemented with 100 µg of AMP per mL of LB and incubated at 37 °C over-night. The colonies were counted to enhance the ratio of the transformation.

3.3.3 The cultivation and selection of the pKKTac containing clones

Four colonies from each of the plate cultivated pKKTac containing XL1-Blue clones (section 3.3.2) were isolated and cultivated separately in 4 mL of SB with 10 µg / mL TET, 100 µg / mL AMP and 1 % glucose. The cells were incubated overnight at 37 °C with 220 rpm shaking. The plasmidial DNA of 2,5 mL of the cells was purified by applying MACHEREY – NAGEL's Plasmid & DNA Purification kit (NucleoSpin® Plasmid).

100 µL of the cells were transferred to 3900 µL of SB supplemented with 10 µg / mL TET, 100 µg / mL AMP and 0,01 % glucose. The cells were incubated at 37 °C with 220 rpm shaking until the cultivation media reached the absorbance of one. The absorbance was determined with the OD_{600} method. In order to produce the Fabs, 4 µL of 1M IPTG and AMP (final concentration of 100 µg / mL) was added to the media. The clones were incubated overnight at 37 °C with 220 rpm shaking.

On the next day, the cells were centrifuged from the supernatant (1 min with 13 000 rpm). The supernatants were retained and a primary ELISA with protocol identical to section 3.1.5, differing only by the coating solution, 1 µg / mL T_3-HSA and HSA Na-bicarbonate (0,1 M pH 9,6) in this assay, and the detection antibody, Anti-kappa AP 1:2000 (SouthernBiotech) in SuperBlock® supplemented with 0,05 % Tween® 20 in this assay, and by the detection substrate, 2 mg pNPP per 1 mL of DEA solution in this assay, was applied to the twelve undiluted supernatants.

Every set of four clones, collected from the same plates, consisted of theoretically identical clones, but the ELISA-test was performed to ensure it. An immunoblot (protocol described in section 3.4.2) was also conducted to enhance the uniform and sufficient production rates of the Fabs. The most specific and sufficiently producing individual was selected from each of the three clones.

3.3.4 The transformation of the pKKTac plasmids to production strain

The selected and purified pKKTac plasmids (section 3.3.3) were transformed to a production strain of *E. Coli* (RV308) by heat shock transformation (protocol described in the section 3.3.2). After the transformation the RV308 clones were plate cultivated overnight on LB AMP (100 µg/ mL) plates at 37 °C. Colonies were counted to enhance the ratio of the transformation.

3.3.5 The small-scale fermentations for the three selected clones

Small-scale (2 L) Fab fermentations were conducted with the three selected clones, HD9, EH8, and EA4 (section 3.2.3). The fermentations were set up on different days.

One colony was selected from the RV308 plate and cultivated in 50 mL of LB (100 µg/ mL CARB, 1 % glucose) overnight at 37 °C with 220 rpm shaking.

The cells were transferred to 1,8 L of Terrific Broth (TB) (100 µg/ mL CARB, 0,01 % glucose). The 1,8 L cultivation was divided to six 2 L erlemayers, 300 mL of media to each of the bottles. The cells were incubated at 37 °C with 220 rpm shaking until their growth reached an exponential phase. Growth rate of the bacteria was determined by measuring absorbance values with the OD_{600} method.

IPTG (300 µL, 1 M) was added to each of the 300 mL cultivations. The fermentation was performed overnight at 30 °C with approximately 220 rpm shaking. The fermentation media was centrifuged 20 min at 4 °C with 8 000 rpm. After the centrifugation cell pellets were discarded, and combined supernatants were treated with 1 mg of DNAse I (Roche Oy) and 0,5 mL of 1 M $MgCl_2$ per 1 L of the supernatant. After the enzyme addition, the supernatant was incubated 1 h at 37 °C with 140 rpm shaking. The supernatant was filtered with vacuum flask equipped with two filter papers (Whatman™, GLASS MICROFIBRE FILTERS, GF/C Circles Ø110 mm) and stored at 4 °C.

3.4 The purification of the produced T3-specific Fabs

3.4.1 Concentration and buffer exchange with Pellicon PLCGC cassette

The supernatant, containing the secreted proteins, from the 2 L fermentation (section 3.3.5) was refiltered with a vacuum flask as previously described (section 3.3.5).

Filter cassette's (Pellicon PLCGC 10K 0,5 m^2, regenerated cellulose, Merck Millipore Ltd.) storage buffer (0,02 % NaN_3) was rinsed with 3 L of ultrapure water (Milli-Q, Millipore Corporation). Another 1 L of Milli-Q was recirculated through the cassette for half an hour. After the washing the cassette was equilibrated to a new buffer, 10 mM Hepes 0,5 M $NaCl_2$, by rinsing it with 500 mL and recirculating with 500 mL of the buffer.

The supernatant was concentrated by filtration with the Pellicon cassette. Retentate was discarded and permeate returned to the concentrated supernatant until the total volume of the supernatant was quartered. After the filtration step, the volume of the supernatant was increased back to the original by an addition of the buffer. In order to exchange the buffer to an adequate extent, the filtration and buffer addition was repeated two more times. After the filtration of the last round, the total volume was adjusted to 1 L. The concentrate was stored at a temperature of 4 °C.

3.4.2 The immobilized metal affinity chromatography purification

Immobilized metal affinity chromatography (IMAC) purification was applied to the three Fab-containing concentrates obtained from the cassette filtrations (section 3.4.1). The IMAC purification was executed on different days for each of the concentrates.

The IMAC purification was preceded by charging of Chelating Sepharose® Fast Flow (GE Healthcare) with nickel ions. 15 mL of the Sepharose was washed two times with 15 mL of Milli-Q. 7,5 mL 0,2 M $NiCl_2$ solution was added to the Sepharose. To remove excess of the Ni-ions, the Sepharose was washed three times with 30 mL of 0,05 M sodium acetate 0,1 M $NaCl_2$ solution (pH 4, pH adjusted with 50 % acetic acid). The next washing, 3 x 15 mL, was implemented with charged Sepharose's storage buffer (10 mM Hepes pH 7,4, 1 M $NaCl_2$, 10 % (v/v) glycerol and 1 mM imidazole pH 7,4, Sigma-Aldrich Co.). The Sepharose was equilibrated to the same buffer and stored at 4 °C.

As much as 4 mL of the Ni^{2+} coupled Sepharose was added to 1 L of the concentrate (section 3.4.1) and the mixture was incubated overnight at 4 °C with vertically rotational mixing (< 10 rpm). After the incubation, the mixture was left to sediment for 1 h at RT with no mixing. The mixture was slowly poured to 50 mL NUNC column (Bio-Rad). While the Sepharose was caught in the filter of the column, liquids flowed through and were discarded. The column was washed with four washing solutions containing gradually increasing concentrations (1, 5, 10 and 20 mM) of imidazole, 10 mM HEPES pH 7,4 and 1 M $NaCl_2$. After the washings, twelve elution buffers, with imidazole concentrations varying from 50 mM to 500 mM, were applied to release the Fabs, which had bound to the charged Sepharose. As well as the washing fractions, all of the elution fractions were collected during the purification. The formulas and the volumes of the elution and washing fractions are described more detailed in Appendix 2.

Protein concentrations of the collected fractions were estimated with Pierce™ BCA Protein Assay kit. The assay was conducted with a protocol analogous to the section 3.1.2. The fractions expressing the highest protein concentrations were selected for SDS-PAGE. Fab-specific western blot was applied to estimate the efficiency of the purification and elution steps from the beginning of the IMAC purification.

Samples were prepared simultaneously for SDS-PAGE and western blot, and Henri Arola's anti-T3 Fab was utilized as a control. Each IMAC fraction was diluted in DDIW to obtain the right sample concentrations, 1 µg per well for SDS-PAGE and 200 ng per well for Western blot. Final sample volume for each fraction was 20 µl, including 15 µl of the sample dilution and 5 µl of 4x Laemmli Sample Buffer (LSB, without β- mercaptoethanol, VTT). Sample dilutions were mixed to 4 x LSB and heated at 88,5 °C for five minutes. Samples and ladders (7 µl of Precision Plus Protein™ Dual Color Standards, Bio-Rad) were loaded into the pre-cast wells in 15 % SDS gel provided by Armi Boman. Empty wells were loaded with 15 µl of DDIW supplemented with 5 µl of 4 x LSB. Electrophoresis was conducted with Mini-PROTEAN Tetra System (Bio-Rad) and PowerPac Basic (Bio-Rad) in 1 x RB with 0,001 % SDS. For the first half an hour the current was set to 10 mA per gel, and then increased to 50 mA per gel.

After the electrophoresis the protocols for SDS-PAGE and Western blot separated. In Western blot the proteins were transferred to a nitrocellulose paper (Trans-Blot® Turbo™ Mini Nitrocellulose Transfer Packs, Bio-Rad) by plotting (7 min, 2,5 A and 25 V, Trans-Blot® Turbo Transfer System, Bio-Rad). Blotting was followed by blocking with 1,5 % milk powder (Fat-free milk powder 500 g, Valio Oy) in DDIW for 15 min. The nitrocellulose paper was washed three times for 5 minutes in TBST (0,05 % Tween 20) and incubated for an hour with detection antibody (GoatAnti-Mouse Kappa-AP diluted to 1:2000 in 0,05 % TBST, SouthernBiotech). Free detection antibodies were washed with the protocol described above. A detection solution, 33 µl of BCIP mixed to 66 µl of NBT in 10 mL of AP buffer (100 mM Tris pH 9,5, 100 mM NaCl and 5 mM $MgCl_2$), was applied on the paper and after approximately five minutes of incubation, the developed colour reaction was terminated by washing the nitrocellulose paper with DDIW.

In SDS-PAGE, three ten-minute washings with DDIW followed the electrophoresis. Washed gel was fixated in 20 % isopropanol 10 % acetic acid solution (10 mL, 5 min). After fixation the gel was washed with DDIW and coloured in PageBlue solution (Ther-

mo Scientific Inc.) for one hour at + 50 °C. The excess of the PageBlue was rinsed with DDIW and the gel was stored in 10 % glycerol at least overnight before its drying.

The richest Fab fractions with the least impurities were selected according to the SDS-PAGE and western blot results. By combining the selected fractions, one pool was created from each of the Fabs.

3.4.3 The dialysis, concentration and SDS-PAGE for the Fab pools

The buffers of the Fab pools, which were created from the IMAC fractions (section 3.4.2), were exchanged by a dialysis with Visking Size 3 dialysis tube (Medical International Ltd., molecular weight cut off (MWCO) of 12 – 14 kD) in 2 x 5 L of PBS with magnetic stirring.

The protein concentrations of the pools were estimated with Pierce™ BCA Protein Assay kit with Fab samples (4, 2, 1, 0,5, and 0,25 mg / mL, Helena Henno) as protein standards. The pools were concentrated to ~ 2 mg / mL with 10 000 NMWL Amicon® Ultra – 15 Centrifugal Filters (Merck Millipore Ltd.) according to the manufacturer's instructions. The Efficiency of the concentration was estimated with Pierce™ BCA Protein Assay kit (protocol identical to section 3.4.3.).

Overall purity and the protein concentrations of the pools were estimated with SDS-PAGE (protocol analogous to the section 3.4.2) and anti-T_3 control Fab.

3.5 The further characterization of the T_3-specific Fabs

3.5.1 The determination of the Fab concentrations of the pools

In order to estimate the affinities of the T_3-specific Fabs, the Fab concentrations had to be measured as accurate as possible. In the erstwhile conditions, the available time was the determinative factor in the consideration of the best quantifying methods.

The Fab concentrations were determined by measuring the protein concentrations from three dilutions, 1:5, 1:10 and 1:20, of each pool with two spectrophotometric devices, NanoDrop 1000 Spectrophotometer and BioPhotometer (Eppendorf AG). The optical

densities of the samples were measured at a wavelength of 280 nm and an average was calculated from the results of both devices.

Fab concentration was calculated by proportioning the averaged protein concentration by an extinction coefficient. The extinction coefficients, dependent on the amino acid sequences of the Fabs', were obtained from ExPASY Bioinformatics Resource Portal, by exploiting their ProtParam tool.

3.5.2 The determination of the linear ranges of the anti-T_3 Fabs

The linear range of an antibody refers to the range of antibody concentrations that can be used for accurate measurements in a particular assay. It is a reflection of the quality of the standard curve, which should have a defined linear portion indicating the area where the antibody is giving predictable responses, dependent upon the concentration of the antigen. A successful and reliable characterisation of the affinity of an antibody by ELISA requires that the antibody is applied to the assay within its linear range.

For the determination of the linear ranges of Fabs *via* ELISA, MaxiSorp® NUNC-IMMUNO C96 96-well plate was coated with 300 ng of T_3-HSA per well. T_3-HSA was diluted to final concentration of 3 µg / mL with 0,1 M sodium bicarbonate buffer (pH 9,6). The coating was implemented overnight at 4 °C. After the coating, the plate was washed three times with 280 µl of PBST (PBS + 0,05 % Tween® 20).

100 µl of each Fab dilution (4, 3, 2, 1, 0,5, and 0 µg of Fab per 1 mL of PBS + 1 % DMSO) was pipetted per well. Henri Arola's anti-T_3 and anti-T_4 Fabs were applied as controls. After a one-hour incubation, unbound Fabs were washed off with PBST (0,05 % Tween® 20) in the manners described before. 100 µl of detection antibody (1:1000 dilution of Fab-specific Anti-mouse IgG AP, Sigma-Aldrich Co.) was pipetted to each well and the plate was incubated for an hour. Washing, identical to the washes before, followed the incubation. 100 µl of pNPP DEA solution (2 mg / mL) was added to each well and the A405 values were measured with SPECTROstar Omega spectrometer.

3.5.3 The affinity determination

96-well ELISA plate, MaxiSorp® NUNC-IMMUNO C96, was prepared for the assay as described in section 3.5.2.

According to the linear ELISA and previous studies (Henri Arola, 2011), the Fabs were diluted to concentrations within the linear ranges, T_3-specific Fabs, K4HD9 and anti-T_3 control Fab, to 1,5 and 3 µg / mL and multispecific T_3-binders, K4EH8 and anti-T_4 control Fab, to 0,25 and 0,5 µg / mL PBS.

The Fabs (350 µL) were preincubated for an hour with 350 µl of each of the T_3 dilutions (final 0,0001, 0,001, 0,01, 0,1, 1 and 10 µM in 2 % PBS DMSO). Preincubation was implemented at RT with 220 rpm shaking. Three parallel samples, *á* 100 µl, from each mixture were transferred to the coated and washed ELISA plate. The mixtures were incubated on the plate for one hour at the same conditions as the preincubation.

The detection step was conducted with a protocol identical to the section 3.5.2

4 Results

4.1 The transformation efficiency

Transformation efficiency was calculated according to the quantity of the exogenous DNA (1 µg), XL1-Blue cells (100 µL) and the number of colonies formed during the plate cultivation. Transformation was conducted with four differently ligated Addams libraries (LH1, LH2, HL1 and HL2, Henri Arola). After the transformation, the cell cultures were combined to create an antibody library with a higher diversity. The transformation of the HL1 library is selected as an example. The calculation of the library size and transformation efficiency is described below (Table 3.). All of the transformations (LH1, LH2, HL1 and HL2) are summarized in Table 4.

V_{tot} = 100 µL XL1-Blue cells + 3 mL SOC + 7 mL SB = 10,1 mL
m_{DNA} = 1 µg DNA
m_{DNA} / V_{tot} = 0,0990099 µg / mL = 99,0099 ng / mL = 99,0099 pg / µL

Table 3. Library size and transformation efficiency of Addams HL1:

Dilution	V_cells (µL)	m_DNA (µg)	Colonies	cfu / mL	cfu / µg DNA
$2 * 10^{-6}$	0,01	9,90E-04	323	3,23E+07	3,26E+05
$2 * 10^{-7}$	0,001	9,90E-05	43	4,30E+07	4,34E+05
$2 * 10^{-8}$	0,0001	9,90E-06	5	5,00E+07	5,05E+05
			Average:	**4,18E+07**	**4,22E+05**

Table 4. Summary of the transformations. The library sizes and transformation efficiencies for Addams LH1, LH2, HL1 and HL2 transformated libraries.

	LH1	LH2	HL1	HL2
Library size (cfu / mL)	3,09E+07	1,81E+07	4,18E+07	2,28E+07
Transformation efficiency (cfu / µg DNA)	3,12E+05	1,83E+05	4,22E+05	2,30E+05
Sum of the library sizes (cfu / mL)	1,14E+08			

According to the results, the transformation was successful and efficient. Total library size was at the same range with the original Addams library (10^8 phage particles per mL).

4.2 The results of the phage ELISA

The phage ELISA was applied to all of phage pools obtained from the biopanning. The assay was conducted to detect the phage pools' specificities against the T_3-HSA and the HSA, which was applied as a background for the ELISA. The A405 values were measured for three phage pool dilutions (1:10, 1:100 and 1:1000) after 10, 20 and 35 minutes. The results of the last measurements of both the acidic and alkaline eluted pools are illustrated in Figure 7. Ratios between the A405 averages of T_3-HSA and HSA (background) are presented in Figure 8.

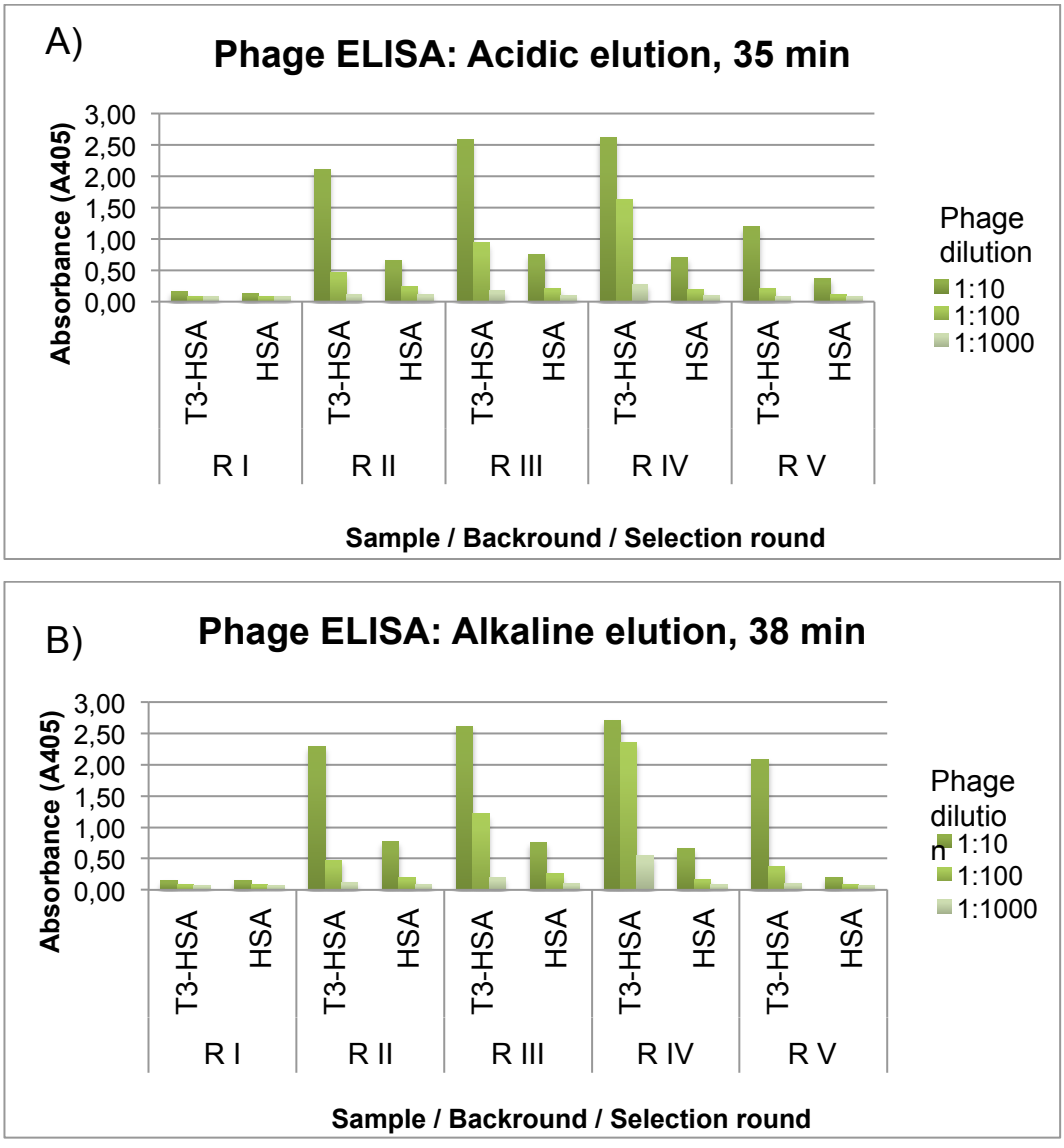


Figure 7. The results of the primary ELISA. The pools obtained with acidic elution (A) and the pools obtained with alkaline elution (B).

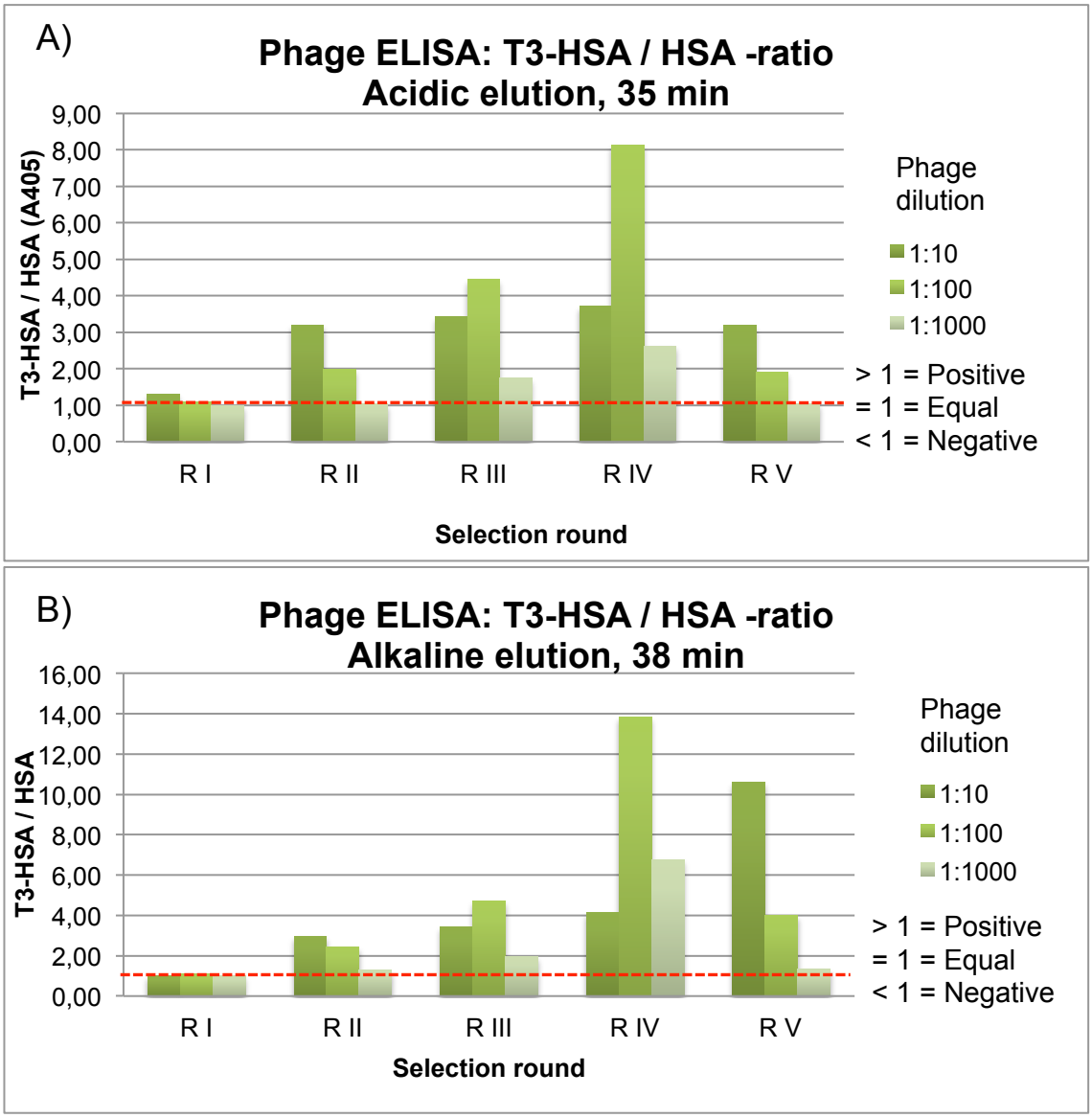


Figure 8. The results of the primary ELISA applied to the phage pools eluted with the acidic elution mixture presented as ratio between the averages (A405) of T_3-HSA and HSA (background). Red line represents the zero point, where the absorbance values are not differing between T_3-HSA and HSA. Upper in the Figure are the pools obtained with acidic elution (A) and lower are the pools obtained with alkaline elution (B).

The ELISA clearly shows the enrichment of T_3-specific Fabs, between the rounds II – V. According to the results, rounds from II to V from both elutions expressed significant specificity against T_3-HSA, when compared to the specificity against HSA. The first rounds of both elutions gave 'negative', although expected, results and thus were left out from the further screenings.

4.3 The results of the primary screenings against T_3-HSA, T_4-HSA and HSA

96 XL1-Blue clones from each of the output plate cultivations of the T_3-specific pools (rounds II – V) were collected and screened against T_3-HSA, T_4-HSA and HSA with the primary ELISA. As an example of the results, A405 absorbance values of 16 clones of the fourth alkaline eluted round are presented in Figure 9. A405 values of the best 20 clones selected according to this primary screening assay are gathered to Figure 9. The results suggest, that the clone K4HD9 (HD9, blue-framed star in Figure 9) seems to be the most specific against T_3-HSA and that the clone K4EH8 (EH8, red-framed star in Figure 9) binds T_4-HSA with the highest affinity.

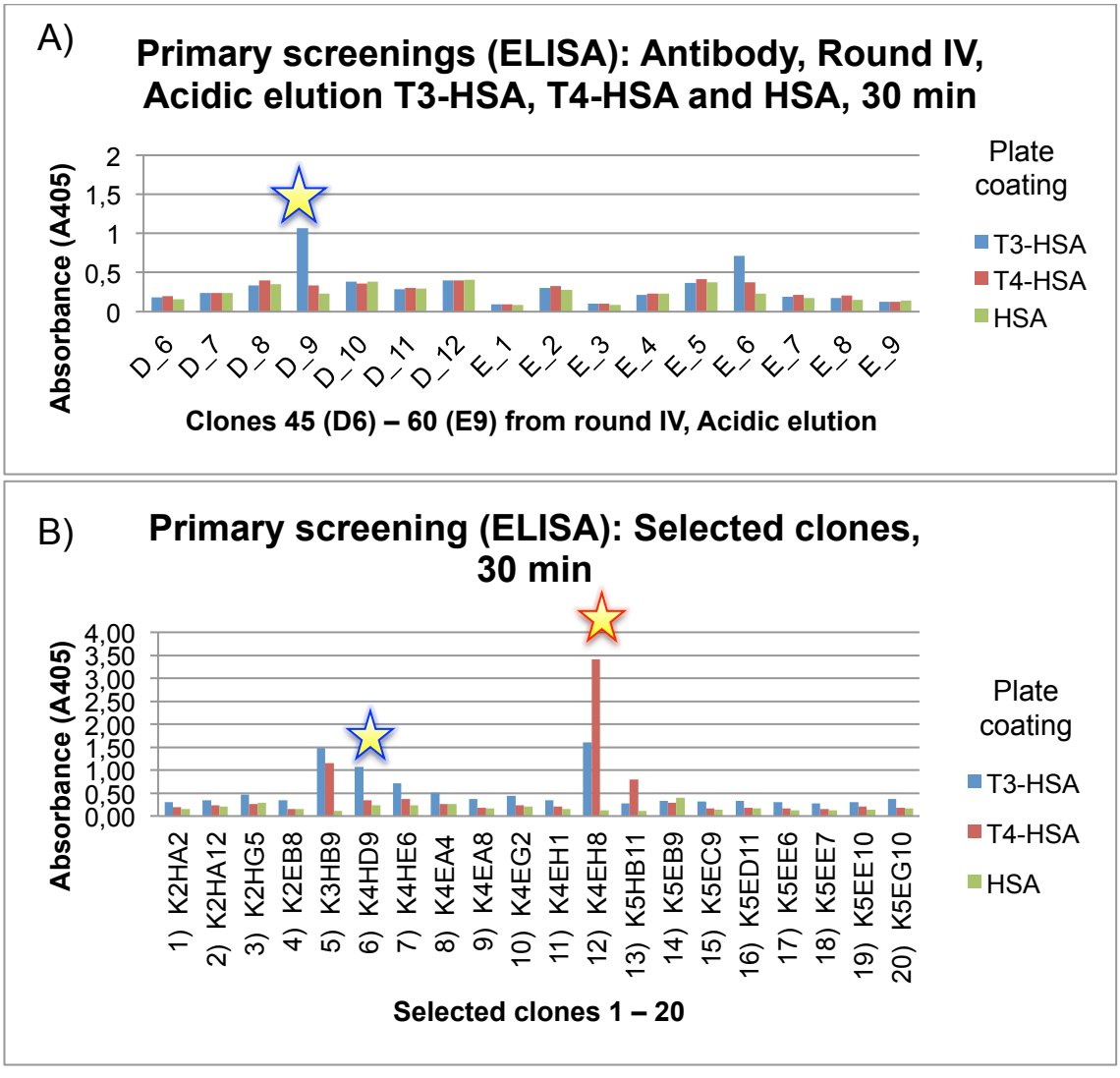


Figure 9. Primary screening results: A) is an example of fourth alkaline eluted round (only clones 45 – 60). The clone with highest affinity against T_3-HSA (HD9) is marked with blue-framed star. The 20 clones, selected according to the screenings, are presented in B). Multispecific high-affinity clone (EH8) is marked with the red-framed star.

Summary of the 33 positive and 4 T_3-specific clones discovered with the screening is presented in Figure 10. The *clones whose A405 response against T_3-HSA was at least twofold compared to the response against HSA were categorized as positive. All **positive clones whose T_3-HSA / HSA –ratio was at least twofold to its T_4-HSA / HSA –ratio, were considered as T_3-specific.

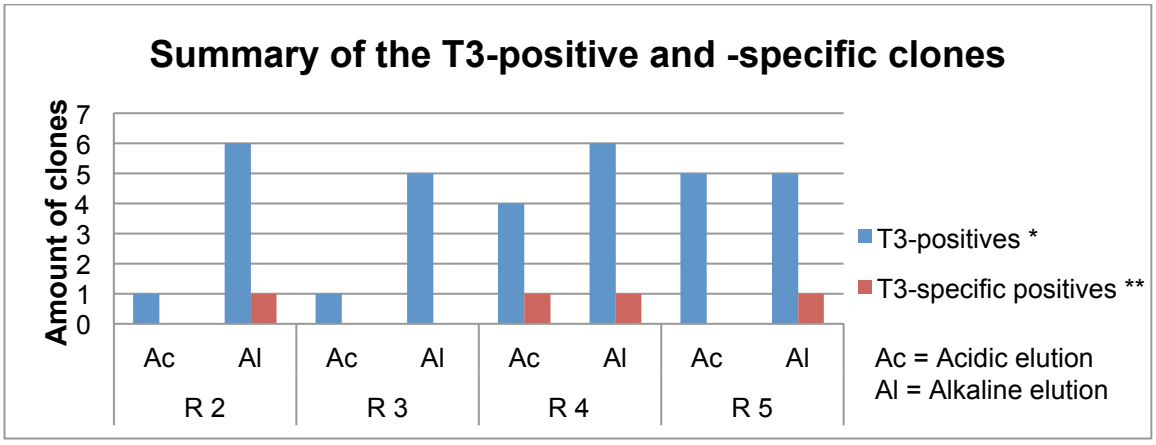


Figure 10. Summary of the 33 T_3-positive and 4 T_3-specific clones of each selection round from both of the elution methods, the acidic (Ac) and alkaline (Al).

4.4 The T_4 and HSA cross-reactivities of the selected clones

In order to reduce the number of the selected clones, the phagemid DNA of the 20 clones was sequenced and their cross-reactivities were tested against T_3, T_4, and HSA. Fabs' specificities against T_3 and T_4 were tested by competitive screening with only one hapten dilution (final 20 µM) for each hapten. The ELISA plates were coated with 100 ng of T_3-HSA per well. The results of the competitive cross-reactivity ELISAs are presented as A405 values below in Figure 11. In the figures the height of the bar is indicating the clone's affinity against the corresponding hapten applied to the pre incubation (T_3 and T_4). The lower the bar, the bigger the affinity is against the hapten. In Figure 11, the most specific clone, K4HD9, is marked with blue-framed star, multispecific K4EH8, expressing relatively high affinity against all of the haptens, with red-framed star and K4EA4, an interesting clone expressing specificity against T_3 with green-framed star.

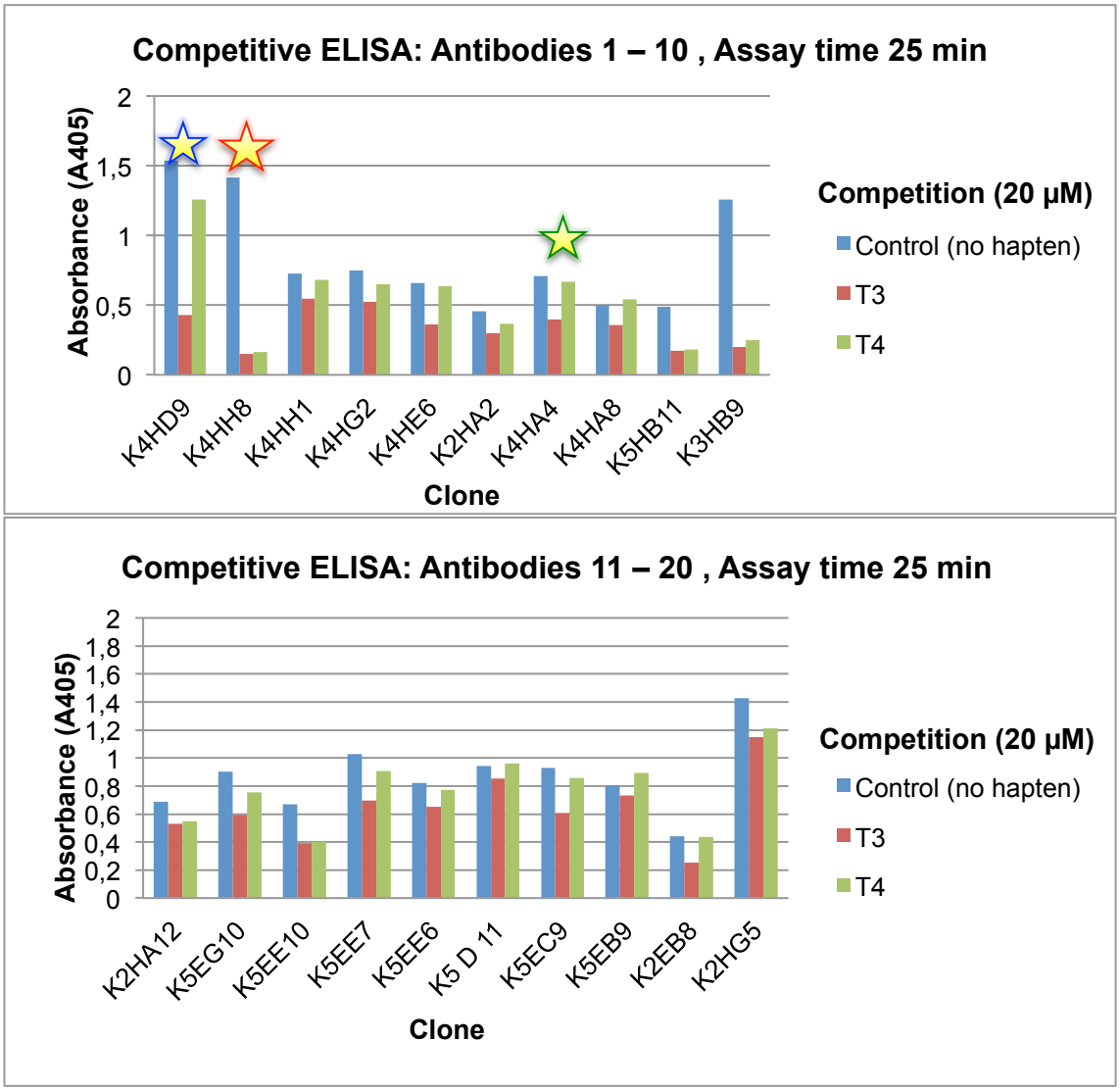


Figure 11. A405 Results of the cross-reactivity ELISA: A) the clones 1 – 10, B) the clones 11 – 20.

K4EA4 seems to behave similarly to the K4HD9, but with overall lower responses. When compared to the results of the clones 1 – 10, the clones 11 – 20 do not appear to have significant specificities against any of the haptens. K5EC9 and K2EB8 are presenting minor specificity against T_3. Despite that K2EB8's relative specificity against T_3 is around 2-fold to the specificity against T_4, the antibody suffers from the low total response (A405 < 0,5) and therefore was not chosen for further studies.

4.5 The DNA analysis

Phylogenetic trees were created based on the analysis conducted to the sequencing data of the heavy and light chains of the Fabs. As an example, the phylogenetic tree presented in Figure 12, is created from the data obtained from the light chain sequencing. The sequencing of the heavy chains encountered difficulties and failed, at least partly, for twelve of the twenty clones, including K4HD9.

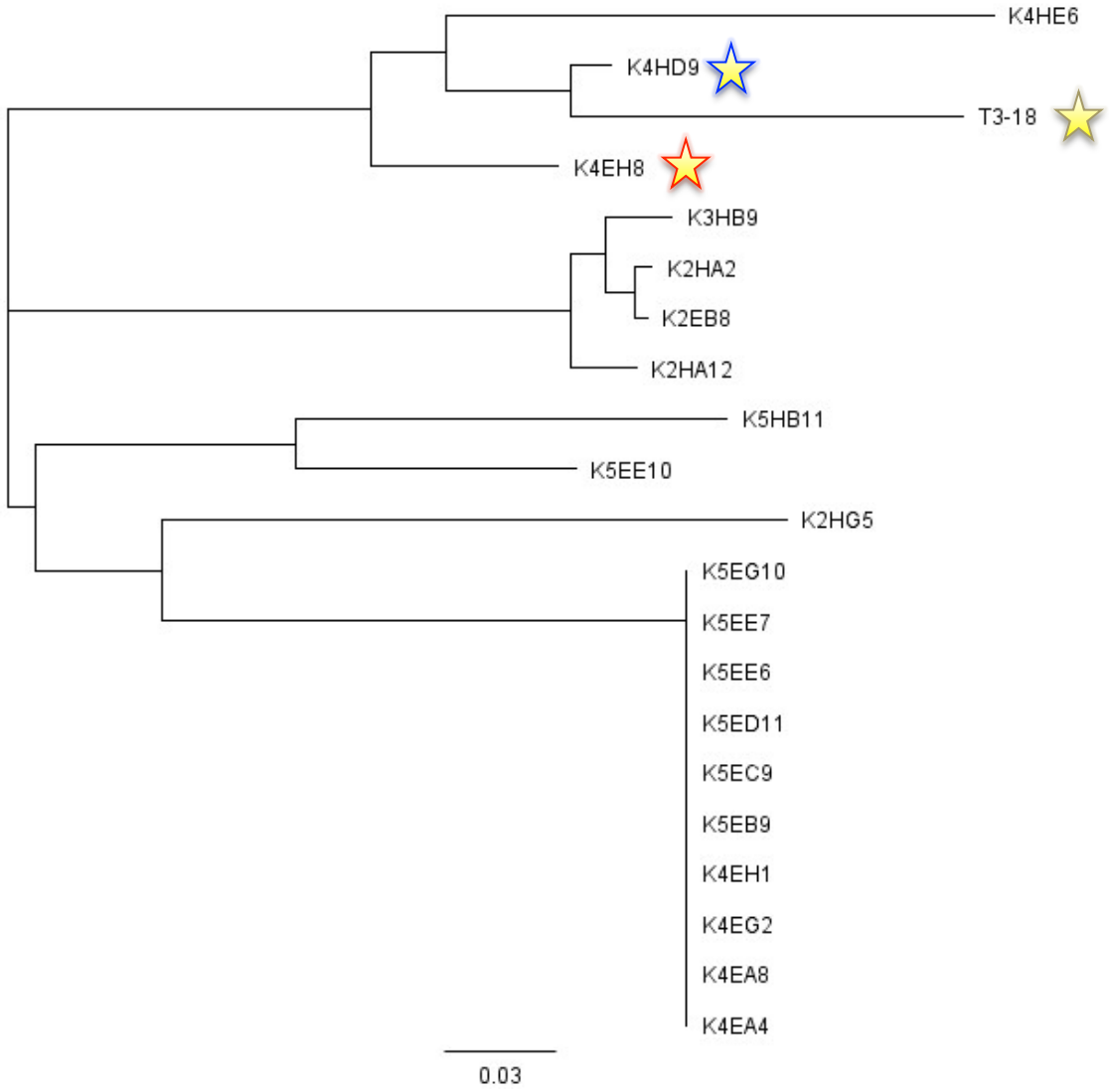


Figure 12. Phylogenetic tree created based on the light chain sequencing data. Picture illustrates relations between the light chain sequences of the clones.

In comparison, T_3-specific Fab clone, previously obtained from the same library by Arola, was included to the analysis of the light chains. The sequencing data revealed that ten of the twenty clones were identical by their light chain sequences. The phylogenetic

tree illustrates similarities in the genotypes, and suggest that the most promising and interesting clones, the T_3-specific K4HD9 (blue-framed star in picture) and the highest affinity clone K4EH8 (red-framed star in picture) were closely related to the anti-T_3 control Fab (light brown-framed star in picture). According to the sequencing data and the ELISAs, the six most interesting clones were selected to further analysing.

4.6 The competitiveness of the selected anti-T_3 clones

A more detailed competitive ELISA was implemented for the clones selected with the cross-reactivity ELISA. Competiveness was measured against free T_3 and T_4 with three concentrations (10 µM, 2 µM and 0,2 µM). A405 results of the assay are presented in Figure 13.

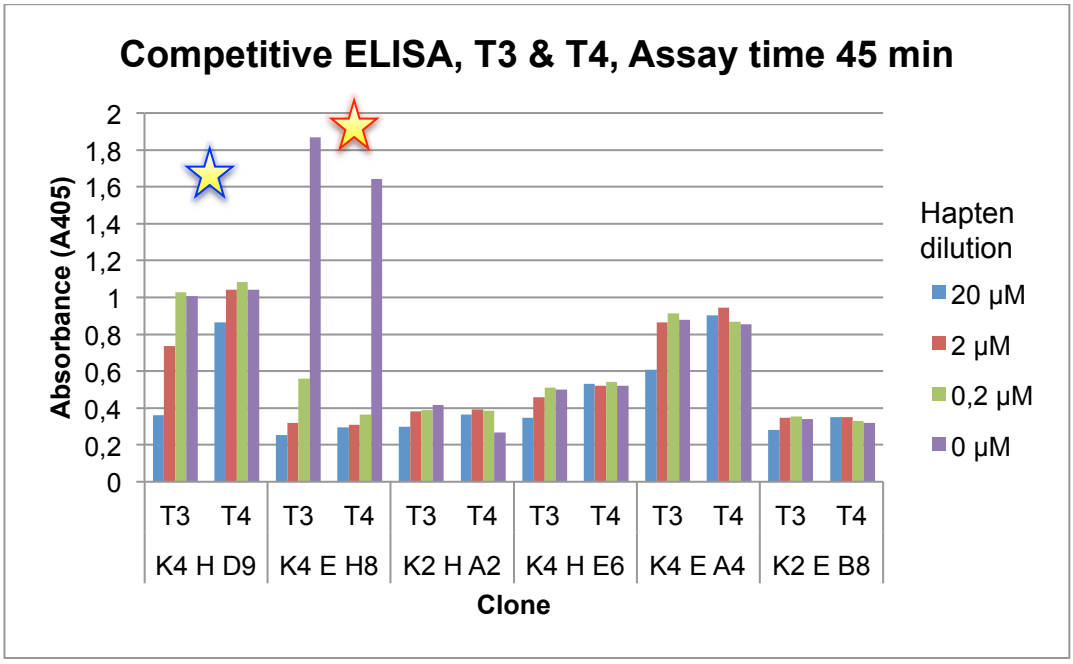
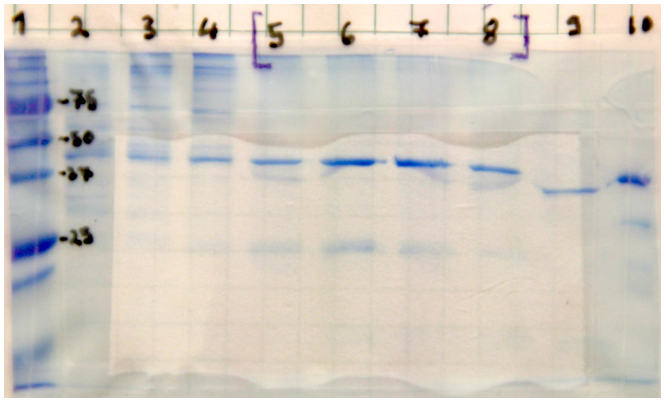


Figure 13. The results of the competitive ELISA. Competiveness was determined against T_3 and T_4 with three different concentrations (20, 2 and 0,2 µM). T_3-specific K4HD9 is marked with the blue-framed star and multispecific K4EH8 with the red-framed star.

According to the ELISA, K4HD9 and K4EA4 are the most T_3-specific clones. The results also confirm the previous conclusions about the multispecificity of the K4EH8. Based on the results, K4HD9, K4EH8 and K4EA4 were selected for the small-scale production in *E.coli* and characterization phase. These clones are later referred as HD9, EH8 and EA4.

4.7 The IMAC purification

After the small-scale production in *E.coli*, the Fabs were purified from the buffer solution by IMAC. The protein concentrations of the IMAC fractions were first estimated with Pierce™ BCA Protein Assay kit and according to the results of the BCA, the most promising fractions with highest protein concentrations were selected for the SDS-PAGE. An example of the SDS-PAGE results is illustrated in Picture 1.



SDS-PAGE (1 µg / well)

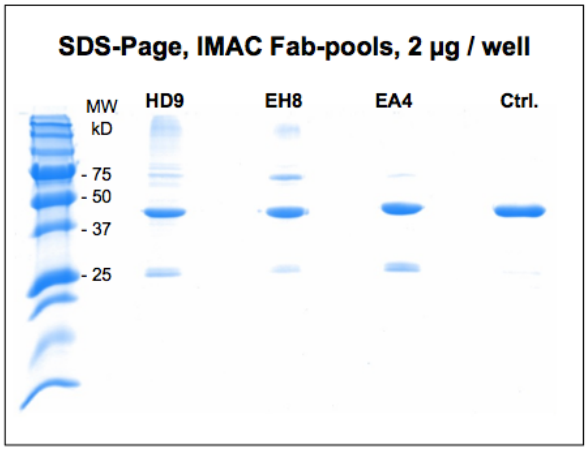
1: Ladder, Precision Plus Protein™
2: Elution 1
3: Elution 2
4: Elution 3
5: Elution 4
6: Elution 5
7: Elution 6
8: Elution 7
9: Elution 8
10: Ctrl

Picture 1. Example of SDS-PAGE applied to IMAC fractions: The IMAC fractions of HD9. Fabs, separated by their molecular size, are located between the 37 and 50 kD markers (the first column). Legends of the wells are displayed on the right side. The gel was bent between columns 8 and 10; therefore, Elution 8 does not contain lower molecular weight antibodies than the others.

Since the total protein concentration is approximately the same in each well, it can be observed from the picture, that the elution fractions from 5 to 8 have the highest Fab concentrations with the least impurities. Western blots were applied to ensure the success of the purifications (data not shown). Fab pools were created by combining the most pure IMAC fractions of each clone, as an example, fractions from 5 to 8 for HD9 (Picture 1).

4.8 Purity of the Fab pools

After the dialysis and concentration of the Fab pools, an SDS-PAGE with a protein load of 1 µg per well was implemented to visualize and compare the purity and protein concentration of the created Fab pools. The SDS-PAGE gel is presented in Picture 2.



Picture 2. SDS-PAGE of the Fab pools. SDS-PAGE gel was photographed with the light table of Molecular Imager Gel Doc™ XR+ (Bio-Rad Laboratories Inc.). SDS-PAGE verified the successful purification of the produced Fabs.

The blue smear on the third lane (Picture 2) of the gel indicates that the pool of HD9 probably contained some impurities with relatively large molecular size. The small bands, below the actual Fabs, are separate heavy and light chains. Overall, the band of the interest, the Fab-fragment, is clearly the major band, and the purity level was sufficient for the further characterisation steps (ELISAs).

4.9 The linear ranges of the anti-T_3 Fabs

Competitive ELISA with six Fab dilutions (4, 3, 2, 1, 0,5, and 0 µg of Fab per 1 mL of PBS + 1 % DMSO) was conducted to determine the linear ranges of the Fabs. The ELISA plates were coated with 300 ng of T_3-HSA per well. Henri Arola's clones with similar functional profiles, T_3-specific (T3-18 in Figure 14) and multispecific (not shown) Fabs were applied as controls for HD9, EA4 and EH8. As an example, a point-to-point fitting of the A405 results of HD9's ELISA is presented in Figure 14.

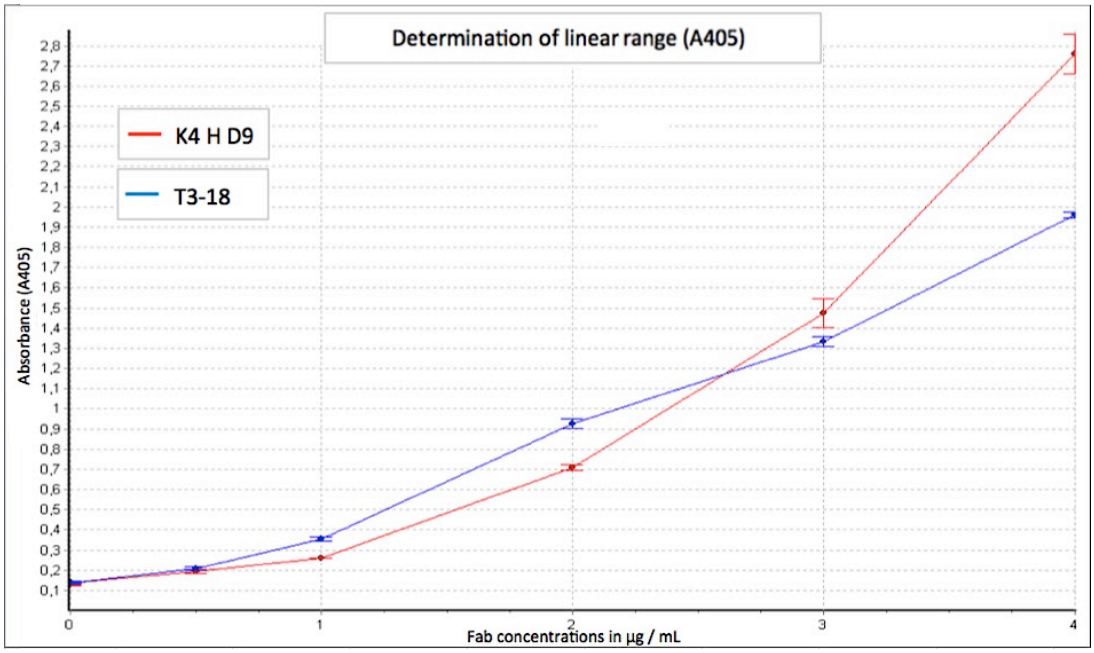


Figure 14. Determination of the linear range: HD9. Henri Arola's anti-T_3 (T3-18 in picture) as a control.

The determination did not give an unambiguous answer about the linear range of HD9 and could have been more accurate if the number of the Fab dilutions would have been increased. Also, the impurities in the HD9's Fab pool might have affected to the results.

According to the results of the ELISAs, the linear ranges, the Fabs' concentration ranges where the absorbance is linearly increasing with respect to the quantity of the substrate, were suggested to be between 1 – 2,5 µg / mL for HD9 and 0,2 – 1,5 µg / mL for EH8. EA4's responses in ELISA were too weak for the determination, and therefore this clone was not further studied. However, all of the ELISA results are depending on the assay conditions, such as buffer composition and pH; therefore, the same assay conditions were applied for the competitive ELISA.

4.10 The determined affinities of HD9 and EH8 against the T_3

Linear ranges of the Fabs were estimated with the primary ELISA. Fab concentrations for the competitive ELISA were selected from within the Fabs' linear ranges. Linear range determination failed for the EA4 and thus it was rejected from the assay. Competiveness of HD9 and EH8 against T_3 is illustrated in the bar diagrams (Figure 15).

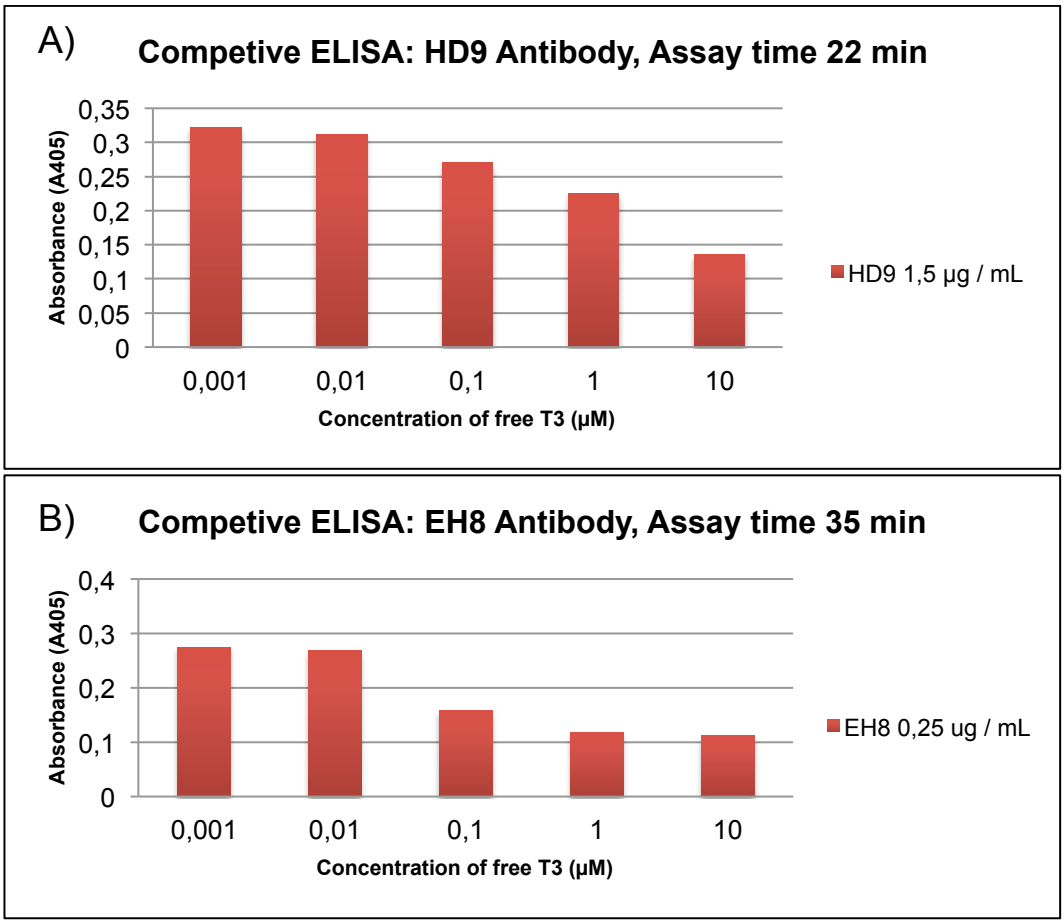


Figure 15. Competitive ELISA: A) antibody HD9 1,5 µg / mL, T_3 0,001, 0,01, 0,1, 1 and 10 µM, B) antibody EH8 0,25 µg / mL, T_3 0,001, 0,01, 0,1, 1 and 10 µM.

The results suggest that with both of the clones the free T_3 starts to compete at submicromolar concentrations. However, the amount of applied EH8 was only one-sixth of the HD9's; and hence the competition can be stated to be remarkably stronger with EH8 than with HD9. Another aspect seconding the estimate is, that with HD9 a clear inhibition can be observed at 1 µM and with EH8 at 0,1 µM T_3 concentration. The assay was not optimized to the required level to make more accurate determinations on Fabs' affinities against the T_3. Although, it clearly indicates that the Fabs (HD9 and EH8) are T_3-binders.

5 Discussions

5.1 The library transformation

Recombinant DNA can be introduced into competent *E.coli* cells either by chemical transformation or electroporation. The chemical method, also known as heat shock method, is cost-effective and reliable, but electroporation can be over ten times more efficient. As described in section 2.3.1, the size and the quality of the library are one of the main factors affecting to the outcome when developing antibodies with *in vitro* techniques [**20**; **15**]1. As such, the electroporation was selected to maximize the library size.

Transformation was conducted with four differently ligated Addams libraries (LH1, LH2, HL1 and HL2, Henri Arola). After the transformation, the cell cultures were combined to create an antibody library with higher diversity. The total library size (10^8 cfu / mL) was at the same range with the previous transformation of the same library (Henri Arola, 2011). As the transformation succeeded, it provided a good basis for the work.

5.2 The biopanning

A semi-automated selection method was applied to enrich the T_3-specific antibody clones. The outcome of the positive clones depends mostly on the library size, antigen, selection conditions and the number of selection rounds [**15**]. The library size was calculated to be sufficient and the antigen, AP conjugated with T_3, was provided and validated by Antti Tullila. The validation and estimation of the conjugation degree was estimated mass spectrometrically with MALDI. T_3-AP conjugates were biotinylated and attached to magnetic streptavidin beads, which were used to enrich the positive clones. To increase the efficiency of the panning, it was conducted on semi-automated platform with two elution methods in parallel (alkaline and acidic). Five panning rounds with gradually hardened panning conditions were applied to enrich the positive clones.

Since the elution method affects significantly to the antibody outcome of the panning and the efficiency of elution methods tend to vary case by case [**15**], the elutions were designed carefully. Multiple efficient elution techniques have been introduced and successfully applied to the development of recombinant antibodies (section 2.4), but each employed elution method multiplies the amount of work. Thus, it was decided here to

do the biopanning with two different elution methods in parallel. The acidic and the alkaline elutions were selected mainly based on the positive results gained from the previous panning of the same library (Henri Arola, 2011).

As assumed, the elution methods affected significantly to the enrichment of clones. The antibody clones obtained by acidic elution were not obtained by alkaline elution and vice versa. Both methods yielded several positive clones and the outcome of the whole panning was 33 positive clones. The alkaline elution yielded 22 out of the 33 positives and, according to the characterisations (ELISAs), the clone with the highest affinity against the T_3 (EH8), but the acidic elution provided the most T_3-specific clone. Respectively, discarding either of the elution methods would have had remarkable influence to the outcome of the work.

The selection pressure was controlled during the biopanning by adjusting the panning conditions (section 3.1.4). The panning conditions were hardened for each of the five rounds, to gradually increase the selection pressure against the clones with the highest affinity. Besides finding the highest affinity clones, it is desirable to maintain the diversity of clones high throughout the panning; therefore, it is not ideal to forcefully harden the reaction conditions immediately, but rather to use multiple panning rounds. Here, the enriching itself did not take into account all properties of the antibodies (e.g. cross-reactivity or specificity against the free hapten) and thus favoured clones with even undesired features. According to the primary screenings of the study, the most enriched clones from the last panning round were neither the most specific against T_3 nor the ones with the highest affinities. All the most interesting clones were from the fourth panning round.

With diverse range of clones it is possible to make further analyses to the interesting individuals to compare their differences. For example, by mirroring the differences in functional properties to the differences in amino acid sequences, it is possible to obtain valuable information about the amino acid sequences affecting to the antibody's properties, such as affinity and specificity.

5.3 The primary screening with SAMI

ELISA assays were applied for the primary screening of the selected panning rounds (rounds II – V). The enriched Fabs were screened in ELISAs based on their specificity against T_3. The specificity (cross-reactivity) was estimated by testing the reactivity against the T_3 and by comparing the results to the reactivity against T_3's carrier molecule HSA and T_3's metabolic precursor T_4. Reactivity against T_4 was tested based on the previous studies wherein several antibodies reacting with T_3 also interacted with T_4 (Tarja Nevanen, personal communication, 2014). T_4's structure differs from T_3's only by one iodine atom (section 2.1.1), which may increase the possibility of cross-reactivity. Reactivity against the carrier molecule was tested to ensure that the antibodies were recognizing only the hapten and not the carrier.

The screening was conducted with the robotic workstation (SAMI), which enabled a comprehensive approach. A total of 768 clones, were screened on 24 96-well plates. Although employing a robot to do the laborious part of the work saves time, it also increases the amounts of reagents used. Particularly, in the development of novel antibodies the antigen can often be expensive or difficult to arrange in big quantities and thus may delimit or make it more unattractive to use automated systems, such as SAMI (Tarja Nevanen, personal communication, 2014).

The primary screening with the robot provided a rough estimation about the cross-reactivity of the clones against T_4-HSA and of their affinity against HSA in comparison with T_3-HSA. Twenty of the most promising clones were selected for the further characterization according to the results. The effect of varying production levels was minimized during the evaluation process by emphasizing the intensity ratios between antigens (T_4-HSA / T_3-HSA and T_3-HSA / HSA). However, if the production level or stability of an antibody is low in the assay conditions, the antibody may not be able to give clear positive results even if it has high affinity against the antigen.

According to this assay, the T_3-positive (T_3-HSA had at least twofold increase in intensity when compared to HSA) clones distributed quite unevenly between the two elution methods. Alkaline elution yielded twice as much of positives (22 clones) as the acidic did (11 clones). The acidic elution has been more widely applied to elutions (Tarja Nevanen, personal communication, 2014) than the alkaline, but in this case the alkaline yielded bigger number of positive clones. Also, other elution methods such as using an

excess of free antigen in elution [**36**] could have been worth of trying. For example, the use of free antigen in excess would not require additional modification for the conjugate and could provide a different outcome in contrast to the methods such as acidic and alkaline elutions that apply such harsh reaction conditions before the neutralisation (in this work pH 2,2 and pH 11,75).

5.4 The characterisation of the anti-T3 clones

Properties of the selected clones were estimated with ELISA assays. Applied assays included both linear and competitive ELISAs. Although ELISAs are convenient for ranking the clones by their affinities and cross-reactivity, they do not provide sufficient accuracy and reliability for kinetic and affinity analyses (Tarja Nevanen, personal communication, 2014). For such purposes, more precise and reliable immunoassays, such as surface plasmon resonance (SPR) methods (e.g. Biacore™) or so-called kinetic exclusion assay (KinExA), are usually required [**37 – 39**].

Since the results of ELISAs' are depending on the assay conditions, they might not be valid in other conditions; therefore, they may not be generalized. In addition, our assay was not optimized to the required level to make highly accurate determinations on Fabs' affinities against the T_3. However, this does not mean that the applied assays would not have provided good estimations about the properties of the selected clones. Due to the reference samples, which were previously selected and characterized Fab clones of the same library, the obtained data concerning the Fabs is especially useful and comparable to the results of the previous studies carried out by Henri Arola in 2011.

According to the results of the competitive ELISA, the selected Fab clones (HD9 and EH8) are clearly T_3-binders. With both of the competitive ELISA tested clones the free T_3 started to compete at submicromolar concentrations. According to the ELISAs, the behaviour, competitiveness of T_3 and the cross-reactivities against T_4, of the Fabs was remarkably similar to the reference clones provided by Arola (data not shown).

HD9 seemed to be much more specific against the T_3 when compared to the multi-specific EH8 that appeared to recognize even more efficiently the T_4 molecule (section 4.6). Although being more specific, HD9 expressed lower affinity against the T_3 than EH8 (sections 4.6 and 4.10). The selected Fabs were purified before the assays by

IMAC-purification and dialysis, but the minor impurities in the Fab pools (visible in SDS-PAGE, section 4.8) may have had a slight effect to the obtained results.

By recognizing and characterizing different behaviour profiles, it is possible to efficiently compare and study the amino acid sequences between clones. This increases the knowledge concerning particular structures and their effects to the functionality and binding abilities of the antibodies. This type of knowledge could be utilized, for example, to the designing of recombinant antibodies expressing higher specificity and affinity against the T_3.

5.5 The comparison of the DNA sequences

By comparing the amino acid sequences and the functional properties (e.g. affinity and specificity) of the clones with the Fabs sequenced and characterized during the previous panning of the same library (Henri Arola, 2011), it is possible to connect the differences in the amino acid sequences to the properties of the antibodies.

Analysis of the sequencing data included formation of phylogenetic threes. Heavy and light chain sequences of Arola's T_3-specific Fab were included to the analyses. The sequence analysis revealed the interesting fact that both, the clone with the highest affinity against the T_3 (EH8) and the most T_3-specific clone (HD9) were genetically closely related to Arola's anti-T3 Fab. The difference between HD9's and the anti-T3 Fab's light chain amino acid sequences was approximately 6,1 % and between K4EH8's and the anti-T3 Fab's 7,5 %. Unfortunately, some difficulties occurred in the sequencing of HD9's heavy chain and thus a reliable comparison between it and the anti-T3 Fab is not yet possible. Another comparison between the similarly acting multispecific clones showed, that the total difference between Arola's anti-T_4 Fab and EH8, was only 3,4 %.

The total difference in the amino acid sequences of EH8 and the anti-T_3 Fab was relatively small (11,2 %) when compared to the differences of their functional properties. While EH8 is a multispecific T_3-binder with the highest affinity from the clones obtained, the anti-T_3 is the most specific of Arola's clones. When examined closer, it can be clearly seen that the differences in the sequences are focused on particular areas of the sequences. For example, in the heavy chains, more than half (65,2 %) of the changes are located in two of the CDRs (CDR2 and CDR3), areas that together consist

of 32 (17 and 15) amino acids (Kabat definition) and affect strongly to the binding abilities of the antibody [**15**]. The difference between the equivalent areas of EH8's and the anti-T_4 Fab's sequences was 0 %; the heavy chains of the multispecific clones were identical regarding to the sequences of the CDRs. Light chains differed by 6,6 %, which is not much smaller than the difference between EH8 and the nati-T_3. Only one third of these changes were on CDRs, which might indicate that the heavy chain might be more dominant in the directing of the T_3-specificy.

This type of information can be collected from multiple clones, combined, and then utilized to determine the amino acids and antibody structures that are directing the specificity exclusively towards the T_3. By determining the roles of certain amino acids, it can be possible to modify antibodies towards the desired direction, for example, to increase the specificity of a multispecific high affinity antibody, such as EH8.

6 Conclusions

The study succeeded in the development and characterisation of novel T_3-binding recombinant antibodies. One T_3-specific (HD9) and one multispecific fab clone (EH8), recognizing both the T_3 and T_4, were selected, produced and characterized from the previously constructed and validated recombinant antibody (Fab) library provided by Henri Arola.

The clones were panned from the phage-displayed library by semi-automated biopanning. A robotic workstation was employed to the primary screenings. The final selection of the clones and the further characterisation was based on DNA sequencing and competitive and linear ELISAs. According to the results of the competitive ELISAs, the selected Fab clones (HD9 and EH8) are clearly T_3-binders. With both of the clones, the free T_3 started to compete at submicromolar concentrations. The behaviour, competitiveness of free T_3 and cross-reactivity of the Fabs were remarkably similar to the clones obtained from the previous study conducted with the same library (Henri Arola, 2011).

The amino acid sequence comparisons between EH8 and Arola's multispecific anti-T_4 Fab and T_3-specific Fab, revealed interesting differences in the sequences focusing especially on the two CDRs (CDR2 and CDR3) of the heavy chains. It is very likely that in the future, this type of information could be applied, for example, to the designing and modification of recombinant antibodies towards higher specificity and affinity against the T_3.

To increase the value of the information obtained from the study, kinetics and affinity of the selected clones (HD9 and EH8) could be analysed more deeply, for example, with Biacore™. For a more complete analysis of the obtained T_3-specific clone, HD9, successful sequencing of the heavy chain would be mandatory. Amino acid sequence comparison between high-affinity multispecific clones, such as EH8 and the anti-T_4 Fab, and HD9 could probably provide valuable information about the amino acid sequences responsible for the antigen binding abilities.

References

[1] J S Huston et al., "Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli.," vol. 85, no. 16, pp. 5878-5883, August 1988.

[2] RE Bird et al., "Single-chain antigen-binding proteins.," *Science*, vol. 242, no. 4877, pp. 423-426, October 1988.

[3] BCC Research LLC, "Global Markets for Research Antibodies," Wellesley, MA, Market research 2015.

[4] BCC Research LLC, "Antibody Drugs: Technologies and Global Markets," Wellesley MA, Market research 2015.

[5] Phillipp Holliger and Peter J Hudson, "Engineered antibody fragments and the rise of single domains," *Nature Biotechnology*, vol. 23, no. 9, pp. 1126-1136, September 2005.

[6] Andrew R.M. Bradbury, Sachdev Sidhu, Stefan Dübel, and John McCafferty, "Beyond natural antibodies: the power of *in vitro* display technologies," *Nature Biotechnology*, vol. 29, no. 3, pp. 245-254, March 2011.

[7] G. S. Kelly, "Peripheral Metabolism of Thyroid Hormones: A Review," *Alternative Medicine Review*, vol. 5, no. 4, pp. 306-333, August 2000.

[8] J. H. Oppenheimer, "The nuclear receptor-triiodothyronine complex: relationship to thyroid hormone distribution, metabolism, and biological action.," in *Molecular Basis of Thyroid Hormone Action*, J. H. Samuels and H. H. Oppenheimer, Eds. New York, United States: Academic Press, Inc., 1983, pp. 1-35.

[9] R. Pitt-Rivers and J. R. Tata, "Physiological Actions of Thyroid Hormones," in *Thyroid Hormones*, R. Pitt-Rivers and J. R. Tata, Eds. St Albans, Great Britain: Pergamon Press, Inc, 1959, pp. 59-92.

[10] H. H. Samuels, "Identification and characterization of thyroid hormone receptors and action using cell culture techniques.," in *Molecular Basis of Thyroid Hormone Action*, J. H. Oppenheimer and H. H. Samuels, Eds. New York, United States, 1983, pp. 35-64.

[11] H. H. Samuels, B. M. Forman, Z. D. Horowitz, and Z. Ye, "Regulation of Gene Expression by Thyroid Hormone," *Perspectives*, vol. 81, no. 11, pp. 957-967, April 1988.

[12] P. M. Yen, "Physiological and Molecular Basis of Thyroid Hormone Action,"

Physiological Reviews, vol. 3, pp. 1097-1126, July 2001.

[13] F. Goglia, "Review: Biological Effects of 3,5-Diiodothyronine (T2)," *Biochemistry (Moscow)*, vol. 70, no. 2, pp. 164-172, September 2005.

[14] Klaus Kapelari et al., "Pediatric reference intervals for thyroid hormone levels from birth to adulthood: a retrospective study," *BMC Endocrine Disorders*, vol. 8, no. 15, November 2008.

[15] Tarja K. Nevanen, *Enantioselective antibody fragments*, Tarja K. Nevanen, Ed. Espoo, Finland: VTT Technical Research Centre of Finland, 2004.

[16] Susumu Tonegawa, "Somatic generation of antibody diversity," *Nature*, vol. 302, no. 14, pp. 575-581, April 1983.

[17] M Sergey and Kipriyanov and Melvyn Little, "Generation of Recombinant Antibodies," *Molecular Biotechnology*, vol. 12, no. 2, pp. 173-201, June 1999.

[18] Achim Knappik et al., "Fully Synthetic Human Combinatorial Antibody Libraries (HuCAL) Based on Modular Consensus Frameworks and CDRs Randomized with Trinucleotides," *Journal of Molecular Biology*, vol. 296, no. 1, pp. 57-86, February 2000.

[19] Frederic A. Fellouse et al., "High-throughput Generation of Synthetic Antibodies from Highly Functional Minimalist Phage-displayed Libraries," *Journal of Molecular Biology*, vol. 373, no. 4, pp. 924-940, November 2007.

[20] Dirk Ponsel, Julia Neugebauer, Kathrin Ladetzki-Baehs, and Kathrin Tissot, "High Affinity, Developability and Functional Size: The Holy Grail of Combinatorial Antibody Library Generation," *Molecules*, vol. 16, no. 5, pp. 3675-3700, May 2011.

[21] Shan S. Wong and David M. Jameson, "Applications of Chemical Conjugation in the Preparation of Immunoconjugates and Immunogens," in *Chemistry of Protein and Nucleic Acid Cross-Linking and Conjugation, Second Edition*. Florida, United States: CRC Press, 2011, pp. 366-368.

[22] Hans Ulrich Weltzien, "Hapten and Carrier," in *Encyclopedia of Immunotoxicology*, Hans-Werner Vohr, Ed. Berlin, Germany: Springer Berlin Heidelberg, 2014, pp. pp 1-4.

[23] Jean W. Lee and Wayne A. Colburn, "Immunoassay Techniques," in *Handbook of Pharmaceutical Analysis*, Jean W. Lee and Wayne A. Colburn, Eds. New York, United States: Marcel Decker, Inc., 2001, pp. 234-235.

[24] Don L. Siegel, "Diagnostic and Therapeutic Applications of Phage Display

Technology," in *Emerging technologies in transfusion medicine*. Maryland, United States: American Association of Blood Banks (AABB Press), 2003, pp. 55-93.

[25] Claudia Sheedy, Roger MacKenzie, and J. Christopher Hall, "Isolation and affinity maturation of hapten-specific antibodies," *Biotechnology Advances*, vol. 25, pp. 333-352, February 2007.

[26] Laura Turunen, Kristiina Takkinen, Hans Söderlund, and Timo Pulli, "Automated Panning and Screening on Microplates for Antibody Generation from Phage Display Libraries," *Journal of Biomolecular Screening*, vol. 14, no. 3, pp. 282-293, February 2009.

[27] Engberg J et al., "Phage-display libraries of murine and human antibody Fab fragments," *Molecular Biotechnology*, vol. 6, no. 3, pp. 287-310, December 1996.

[28] Ville Santala and Petri Saviranta, "Affinity-independent elution of antibody-displaying phages using cleavable DNA linker containing streptavidin beads," *Journal of Immunological Methods*, vol. 284, no. 1, pp. 159-163, 2004.

[29] Ruan B., Hoskins J., Wang L., and Bryan P. N., "Stabilizing the subtilisin BPN' pro-domain by phage display selection: how restrictive is the amino acid code for maximum protein stability?," *Protein Science*, vol. 7, no. 11, pp. 2345-2353, November 1998.

[30] Jianlong Lou et al., "Antibodies in haystacks: how selection strategy influences the outcome of selection from molecular diversity libraries," *Journal of Imunological Methods*, vol. 253, pp. 233-242, March 2001.

[31] Byung Yang Lee et al., "Virus-based piezoelectric energy generation," *Nature Nanotechnology*, vol. 7, no. 6, pp. 351-356, June 2012.

[32] Mark K Boehm, Jenny M Woof, Michael A Kerr, and Stephen J Perkins, "The fab and fc fragments of IgA1 exhibit a different arrangement from that in IgG: a study by X-ray and neutron solution scattering and homology modelling," *Journal of Molecular Biology*, vol. 286, no. 5, pp. 1421-1447, March 1999.

[33] The Israel Structural Proteomics Center (ISPC), Weizmann Institute of Science in Israel. (2015, January) Proteopedia. [Online]. http://www.proteopedia.org/wiki/index.php/Alkaline_phosphatase

[34] Thomas Schirrmann, Torsten Meyer, Mark Schütte, André Frenzel, and Michael Hust, "Phage Display for the Generation of Antibodies for Proteome Research, Diagnostics and Therapy," *Molecules*, vol. 16, no. 1, pp. 412-426, January 2011.

[35] Ario de Marco, "Biotechnological applications of recombinant single-domain

antibody fragments," *Microbial Cell factories*, vol. 10, no. 44, pp. 1-14, June 2011.

[36] Tim Clackson, Hennie R. Hoogenboom, Andrew D. Griffiths, and Greg Winter, "Making antibody fragments using phage display libraries," *Nature*, vol. 352, pp. 624-628, August 1991.

[37] Béatrice Luginbühl et al., "Directed Evolution of an Anti-prion Protein scFv Fragment to an Affinity of 1 pM and its Structural Interpretation," *Journal of Molecular Biology*, vol. 363, no. 1, pp. 75-97, July 2006.

[38] Phinikoula S. et al. Katsamba, "Kinetic analysis of a high-affinity antibody/antigen interaction performed by multiple Biacore users," *Analytical Biochemistry*, vol. 352, no. 2, pp. 208-221, May 2006.

[39] Andrew W Drake, David G Myszka, and Scott L Klakamp, "Characterizing high-affinity antigen/antibody complexes by kinetic- and equilibrium-based methods," *Analytical Biochemistry*, vol. 328, no. 1, pp. 35-43, May 2004.

[40] Charles A Janeway, Jr Paul Travers, Mark Walport, and Mark J Shlomchik, *Immunobiology: The Immune System in Health and Disease*. New York, USA: Garland Science, 2001.

Appendices

Appendix 1: Reagent list

Cuts All (CA) buffer:

200 mM Tris-HCl, pH 7,5
70 mM $MgCl_2$
1000 mM KCl
20 mM 2-mercaptoethanol
Store at +4 °C

4 x LSB - β-mercaptoethanol

23,4 mL DDIW
5 mL 1M Tris, pH 6,8
11,6 mL 87 % Glycerol
2 g SDS (or 10 mL 20 % SDS)
10 mg Bromphenol blue
Store at - 20 °C

Luria Broth (LB):

5 g / 1 000 mL Yeast Extract
10 g / 1 000 mL Tryptone
10 g / 1 000 mL NaCl
15 g / 1 000 mL Agar (granulated)
For Luria Broth ampicillin plates: 100 mg / mL AMP

10 x PBS:

0,12 M $Na_2HPO_4 \bullet 2\ H_2O$, 42,7 g
0,03 M $NaH_2PO_4 \bullet H_2O$, 8,3 g
1,5 M NaCl, 175,3 g
DDIW 2 000 mL
(Diluted to 1 x PBS, pH 7,30)

10 x Reducing Buffer (RB):

250 mM Tris, 151,3 g
1,92 M Glycine, 721 g
DDIW, 5 000 mL
Do not require pH adjustment, Store at RT or + 4 °C

Super Optimal Broth (SOB):

Tryptone, 20 g
Yeast extract, 5 g
NaCl, 0,58 g
KCl, 0,18 g
DDIW 1 000 mL
Add 1M $MgCl_2$ and 1 M $MgSO_4$, 1 mL / 100 mL before use

Super Optimal broth with Catabolite repression (SOC):

SOB + 20 % Glucose (49:1 = 0,4 % final)

Super Broth (SB):

30 g / 1 000 mL Tryptone
20 g / 1 000 mL Yeast Extract
10 g / 1 000 mL 3-(N-Morpholino)propanesulfonicacid
(MOPS, Sigma-Aldrich Co.)
pH adjusted to 7,0 with 5 M NaOH

10 x TBS (and 1 x TBST):

500 mL 1M Tris-HCl, pH 8,0
438,3 g NaCl
DDIW to 5 000 mL final volume
Diluted to 1 x TBS when used
For 1 x TBST: add 0,5 mL Tween® 20 / 1 000 mL 1 x TBS before use

Appendix 2: Solutions and fractions of the IMAC purification

Table 1. Formulas of the washing and the elution solutions. The stock solutions were 2 M imidazole, 1 M HEPES (pH 7,4) and 1 M $NaCl_2$.

Name of the buffer	C_imidazole		V_tot (mL)	V_Imidazole (mL)	V_HEPES (mL)	V_NaCl2 (mL)	V_MilliQ (mL)
Washing_buffer 1	1	mM	300	0,15	3	150	146,85
Washing_buffer 2	5	mM	200	0,5	2	100	97,5
Washing_buffer 3	10	mM	200	1	2	100	97
Washing_buffer 4	20	mM	200	2	2	100	96
Elution_buffer 1	50	mM	200	5	2	100	93
Elution_buffer 2	75	mM	150	5,625	1,5	75	67,875
Elution_buffer 3	100	mM	150	7,5	1,5	75	66
Elution_buffer 4	200	mM	200	20	2	100	78
Elution_buffer 5	500	mM	200	50	2	100	48

Table 2. Volumes and imidazole concentrations of the washing and the elution fractions of the IMAC purification.

Washing #	C_Imidazole	V_tot (mL)
1.	1 mM	60
2.	5 mM	25
3.	10 mM	25
4.	20 mM	25
Elution #	**C_Imidazole**	**V_tot (mL)**
1.	50 mM	10
2.	50 mM	10
3.	50 mM	10
4.	75 mM	10
5.	75 mM	10
6.	75 mM	10
7.	100 mM	10
8.	100 mM	10
9.	200 mM	10
10.	200 mM	10
11.	500 mM	10
12.	500 mM	10