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ACCELERATING FOOD SAFETY AND QUALITY CONTROL:

The use of antibody engineering techniques in the detection of low-molecular weight food contaminants

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To Hanna and boys

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, referred in the text by roman numerals (**I-IV**)

I. Leivo, J., Chappuis, C., Lamminmäki, U., Lövgren, T., Vehniäinen, M. (2010) Engineering of a Broad-specificity Antibody: Detection of 8 Fluoroquinolone Antibiotics Simultaneously. Analytical Biochemistry 409:14-21

II. Leivo, J., Lamminmäki, U., Lövgen, T., Vehniäinen, M. (2013) Multiresidue Detection of Fluoroquinolones: Specificity Engineering of a Recombinant Antibody with Oligonucleotide-Directed Mutagenesis. Journal of agricultural and food chemistry 61:11981-11985

III. Leivo, J., Mäkelä, J., Rosenberg, J., Lamminmäki, U. (2016) Development of recombinant antibody-based enzyme-linked immunosorbent assay (ELISA) for the Detection of Skatole. Analytical Biochemistry 492:27-29

IV. Leivo, J., Vehniäinen, M., Lamminmäki, U. (2015) Detection of deoxynivalenol with small-molecule based inhibition: Generation of anti-idiotype antibodies from synthetic repertoire (Manuscript)

In addition, some unpublished results are presented

ABBREVIATIONS

ALP	Alkaline phosphatase
CDR	Complementarity determining region
CIP	Ciprofloxacin
DAN	Danofloxacin
DIF	Difloxacin
DON	Deoxynivalenol
EC	European Commission
ELISA	Enzyme linked immunosorbent assay
EMA	European medicines agency
ENR	Enrofloxacin
EP-PCR	Error-prone polymerase chain reaction
Fab	Fragment antigen-binding
FLU	Flumequine
GC	Gas chromatography
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
HTS	High throughput screening
IC50	Analyte concentration which produces 50% inhibition of the maximum signal
JEFCA	Joint Expert Committee on Food Additives and Contaminants
Kd	Dissociation constant

LOD	Limit of detection
MAR	Marbofloxacin
MRL	Maximum residue limit
MS	Mass spectrometry
NAL	Nalidixic acid
NGS	Next generation sequencing
NIV	Nivalenol
NOR	Norfloxacin
OS-ELISA	Open-sandwich ELISA
SAR	Sarafloxacin
scFv	Single-chain variable fragment
sdAb	Single domain antibody
SKA	Skatole
SOE-PCR	Strand overlap extension PCR
TRF	Time-resolved fluorescence
WHO	World health organization

ABSTRACT

The increased awareness and evolved consumer habits have set more demanding standards for the quality and safety control of food products. The production of foodstuffs which fulfill these standards can be hampered by different low-molecular weight contaminants. Such compounds can consist of, for example residues of antibiotics in animal use or mycotoxins. The extremely small size of the compounds has hindered the development of analytical methods suitable for routine use, and the methods currently in use require expensive instrumentation and qualified personnel to operate them. There is a need for new, cost-efficient and simple assay concepts which can be used for field testing and are capable of processing large sample quantities rapidly. Immunoassays have been considered as the golden standard for such rapid on-site screening methods. The introduction of directed antibody engineering and *in vitro* display technologies has facilitated the development of novel antibody based methods for the detection of low-molecular weight food contaminants.

The primary aim of this study was to generate and engineer antibodies against low-molecular weight compounds found in various foodstuffs. The three antigen groups selected as targets of antibody development cause food safety and quality defects in wide range of products: 1) fluoroquinolones: a family of synthetic broad-spectrum antibacterial drugs used to treat wide range of human and animal infections, 2) deoxynivalenol: type B trichothecene mycotoxin, a widely recognized problem for crops and animal feeds globally, and 3) skatole, or 3-methyindole is one of the two compounds responsible for boar taint, found in the meat of monogastric animals.

This study describes the generation and engineering of antibodies with versatile binding properties against low-molecular weight food contaminants, and the consecutive development of immunoassays for the detection of the respective compounds.

TIIVISTELMÄ

Elintarvikkeiden turvallisuuteen ja laadunvalvontaan kiinnitetään muuttuneiden kuluttajatottumusten vuoksi yhä enemmän huomiota sekä tuottajien että viranomaisten toimesta. Elintarvikkeiden turvallisuutta heikentää laaja kirjo erilaisia pienikokoisia haitta-aineita kuten antibiootteja ja homemyrkkyjä, jotka kulkeutuvat elintarvikkeiden mukana ihmisiin. Globaalien markkinoiden ja lisääntyvän tutkimustiedon myötä näiden pienikokoisten haitta-aineiden lukumäärä kasvaa jatkuvasti, mikä tekee elintarvikkeiden turvallisuuden- ja laadunvalvonnasta haastavaa.

Elintarvikkeissa esiintyvien haitallisten yhdisteiden pieni koko vaikeuttaa etenkin uusien elintarvikkeiden turvallisuutta ja laatua valvovien määritysten kehittämistä. Useat nykyisin käytössä olevat tekniikat vaativat kalliita laitteita ja niiden käyttöön koulutettua henkilökuntaa, joten nykyisten menetelmien rinnalle on tarpeellista kehittää uusia halvempia ja helppokäyttöisempiä määrityksiä, joilla voidaan käsitellä suuria näytemääriä kerralla ja jotka soveltuvat myös elintarvikkeiden tuottajien käyttöön. Vasta-ainemääritykset ovat laajasti käytössä oleva seulontamenetelmä, jolla voidaan saavuttaa edellä mainittu tavoite. Käyttökelpoisten vasta-ainemääritysten kehittämistä etenkin pienmolekyylien kohdalla on kuitenkin hidastanut kohteena olevien yhdisteiden erittäin pieni koko ja joissain tapauksessa myös niiden myrkyllisyys. Vasta-ainemuokkaustekniikoiden kehitys on mahdollistanut vasta-aineiden kehittämisen lähes mille tahansa kohteelle. Lisäksi tekniikoiden avulla on mahdollista muokata vasta-aineiden sitomisominaisuuksia kuten affiniteettia ja spesifisyyttä monipuolisesti.

Väitöstutkimuksessa tavoitteena oli muokata pienikokoisia haitta-aineita tunnistavien vasta-aineiden sitomisominaisuuksia faaginäyttö- ja proteiininmuokkastekniikoiden avulla elintarviketurvallisuuden ja laadunvalvonnan edistämiseksi. Tutkimuskohteena oli kolme yleistä elintarvikkeiden laatuun ja turvallisuuteen haitallisesti vaikuttavaa yhdistettä: 1) fluorokinolonit, laajakirjoinen antibioottiluokka, joita käytetään yleisesti sekä ihmisten että eläinten infektioiden hoidossa, 2) trikotekeenit, homemyrkkyluokka, joka aiheuttaa huomattavia elintarvikkeiden turvallisuuteen ja tuotantoon liittyviä haittoja, ja 3) karjunhajua lihatuotteissa aiheuttava skatoli.

Väitöstutkimuksessa kehitettiin sitomisominaisuuksiltaan monipuolisia vasta-aineita pienikokoisille elintarvikkeiden haitta-aineille, sekä sitojiin pohjautuvia määrityksiä kyseisten yhdisteiden seulontaan.

1 INTRODUCTION

Safety and consumer acceptance are one of the key elements of successful food production. The globalized supply chain for food products together with growing number of contaminants have created a demand for more stringent control. In order to guarantee the high level safety and quality of the produced food stuffs, several legislative regulations have been set by the European Commission (EC). The integrated approach in European Union (EU) is aimed to provide safe and high quality food products, while ensuring the welfare of animals and environment. Part of creating this well-established food regulatory system has been the development of different analytical methods used to enforce the regulations. The safety and quality monitoring is done at all stages of the food supply process as stated in the topic of the food safety strategy "from farm to fork" established by the EC. Particular concern is related to contaminants which are hard to detect due to the low-molecular weight of the compounds. The low-molecular weight of these food contaminants create demand for adequate detection methods and an additional challenge to the stakeholders and food safety managers.

The most commonly used methods for the analysis of low-molecular weight food contaminants can be roughly divided in to two groups: (i) confirmatory, or reference methods, which are most often based on the combination of liquid chromatography (LC) to various detection technologies such as fluorescence detection or mass spectrometry (MS). These methods are lab based and have exceptional sensitivity and reliability for the determination of the contaminating compound, and as such have become golden standard for food safety management. Due to the high performance, these methods are also used as a research tool for the precise identification of new unknown contaminants. (ii) Screening methods, or high throughput screening (HTS) methods on the other hand rely on the specific capture of the analytes with the use of various bioaffinity molecules such as antibodies. Screening methods emphasize simplicity and cost-efficiency and are the method of choice for large sample quantities and field testing. There is a need for rapid and cost-efficient analytical methods for the high-throughput screening of low-molecular weight food contaminants as a part of safety and quality assurance.

The use of immunochemical technologies for the analysis of food products have expanded significantly during the last three decades. A wide range of novel immunoassay concepts and label technologies have been developed which contribute to the extremely sensitive and specific detection of wide range of analytes. Immunoassays can be flexibly modified to meet the most relevant

parameters involved, such as large sample quantities and matrix interference, to establish an optimal platform for the detection of the respective contaminant. As the quality of the immunoassay is primarily dependent on the binding properties, such as affinity and specificity, of the antibody used to detect the contaminating compound, it is often feasible to modify these binding properties to enhance the desired parameter. The high performance and cost efficiency have made immunoassays one of the most fundamental technologies in food diagnostics.

2 REVIEW OF THE LITERATURE

2.1 Antibody and immunoassay development in food diagnostics

This literature review focuses on the advancements in antibody engineering, recombinant antibody technologies and immunoassay development for the detection and screening of low-molecular weight food contaminants. The spotlight is on three different types of compounds which have a major effect on the safety and quality of widely used food products.

2.1.1 The classification, function and structure of antibodies

Antibodies or immunoglobulins are a group of vertebrate immune-system related glycoproteins secreted by the plasma B-cells. The primary biological function of antibodies as a part of the immune system is to bind and inactivate foreign, harmful pathogens such as infectious agents and microbial toxins. This is accomplished by high sequential differentiation on amino acid level, which ensures the efficient recognition of various foreign pathogens. Antibodies also have regulatory role in the effector cell and complement activation. The immunoglobulins are classified in to five categories according to the biological functionality and the structure of the constant heavy regions. The five different classes are IgA (α), IgD (δ), IgE (ε), IgG (γ) and IgM (μ), which are further divided in a set of sub-classes. In addition, the constant light chains can be divided in two structural categories: lambda (λ) and kappa (κ).

The tertiary protein structure of antibodies consists of four polypeptide chains, two heavy and two light chains, connected by disulfide bridges (Figure 1). The four connected domains form a symmetrical and flexible Y-shaped tertiary structure, approximately 440 amino acids long and 150 kDa in weight, which is the basic unit of an antibody molecule. The two structural regions, constant region (C-region) and variable region (V-region), of the antibody have different functionalities in immune regulation (Figure 1). The C-region interacts with cell surface receptors and is involved in the complement and B-cell activation process during the immune response. The V-region on the other hand, which contains six polypeptide loops the complementarity determining region (CDR) is involved in the antigen binding. However, it has been proposed that the two regions have more complex interaction than the proposed basic model of two regions with independent functionalities (Woof *et al.,* 2004). These interactions have been reported to have effect both to the affinity and

specificity of the antibody. The six CDR loops are located in the peripheral surface of the heavy and light V-regions in the antibody structure, three in each loop (CDR-L1-3 and CDR-H1-3) intervened by conserved immunoglobulin framework β–sheets, which define the orientation of the CDR-loops. The high amino acid sequence and loop length diversity of the CDR regions forms the basis of the efficient recognition of wide selection of structurally variable antigens. This is especially the case for the third loop of both chains (CDR-L3 and CDR-H3) which have been found to be mainly responsible for the antibody antigen interaction. The six CDR loops are highly variable in length and amino acid composition and form the antigen binding site, or paratope of the antibody, which is structurally very adaptable to bind different targets (for a review see Janeway *et al.*, 2001).

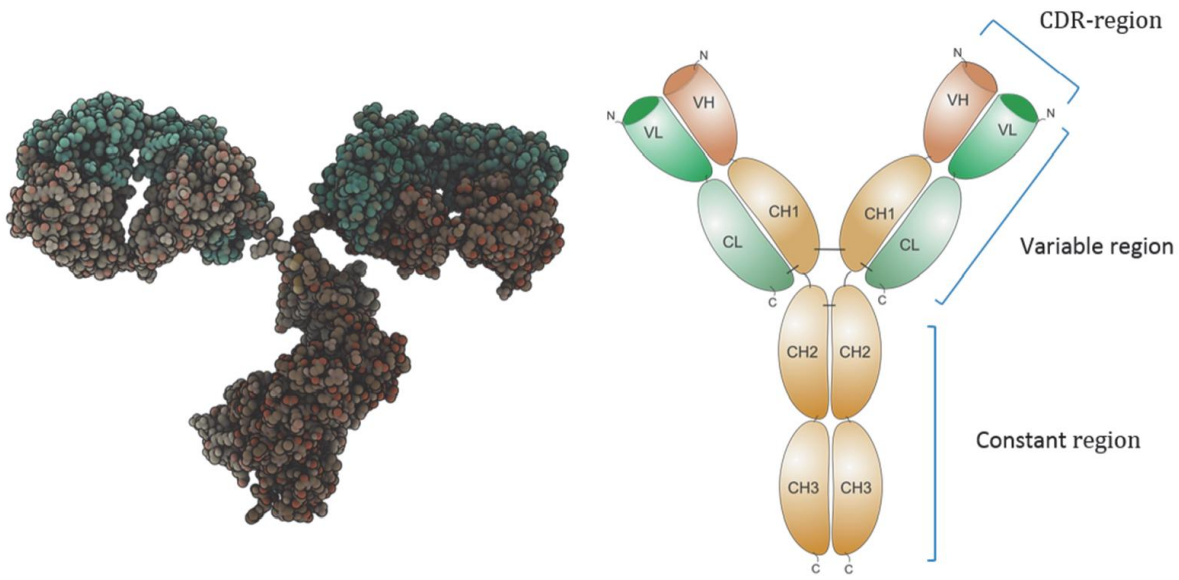


Figure 1. Structure of antibody. Immunoglobulin G (IgG) consists of four polypeptide chains connected by disulfide bridges and flexible hinge region. A) X-ray crystallography structure of an IgG molecule (PDB entry 1IGT modified with Qutemol software). B) Schematic representation of the individual domains and the functional regions of the antibody where the heavy chains are designated as VH, CH1-3 (Brown), and light chains VL, CL (Green).

2.1.2 Directed protein engineering techniques in antibody development

The introduction of recombinant antibody technologies and protein engineering techniques have enabled the flexible modification of antibodies in multidisciplinary research fields ranging from basic diagnostic tools to therapeutic use in humans. The inherent properties of antibodies, such as high affinity, stability and specificity make them an ideal tool for diagnostical applications. The functionality of the Fc region and bivalency of the intact IgG provides benefits for antibodies in therapeutic use, however in diagnostics it is usually desirable to use only the binding regions of the proteins for maximal functionality and binding capacity.

In natural immune response antibodies are produced by multiple B-cells simultaneously, forming a polyclonal mixture of antibodies with multiple specificities. The development of hybridoma technologies revolutionized the field of immunochemistry with the introduction of monoclonal, or monospecific antibodies, which enabled the efficient generation of antibodies specific to a single antigen in large quantities (Köhler *et al.,* 1975). Another significant advance in the field was the possibility to isolate the antibody gene, which enabled the cloning and expression of antibody fragments in bacteria (Ward *et al.,* 1989, Orlandi *et al.,* 1989, Plückthun *et al.,* 1989). This provided the means to dissect the intact IgG in to smaller fragments and fusion to other functional proteins, such as reporter enzymes or alternative binding motifs and purification tags (Figure 2). With the use of flexible polypeptide linker it possible to express only the V-domains of heavy and light chain to form a single chain fragments (scFv) (Bird *et al.,* 1988, Huston *et al.,* 1988). Similar approach has been used to increase the valency, and consecutively the affinity of the antibody constructs, by adding a connecting peptide or protein linker which enables the expression of multiple binding units of a single scaffold (Zhang *et al.,* 2004). The most basic binding unit in antibody, the V-region can be produced as a monomeric binding unit. This single-domain antibody (sdAb) or its commercial alternative, nanobody, were first found from the immune systems of camelids (Hamers-Casterman *et al.,* 1993). As sdAbs are less than 1/10 of the intact IgG (12-15 kDa), it has proven to be especially useful in applications which are size dependent or require high densities of the binding protein (Verheesen *et al.,* 2003). Most of the before mentioned antibody fragments can be expressed individually or in fusion with proteins or peptides. The use of fusion proteins and peptides may be used for example, to immobilize or purify the desired antibody, with the use of purification tags such as hexa-histidine peptide or glutathione s-transferase (GST) or intrinsically

biotinylating domains (Santala *et al.,* 2004) and peptides (Cloutier *et al.,* 2000). The same method can be used to detect antibody binding to specific target with the fusion to reporter molecules such as alkaline phosphatase (ALP), beta-lactamase (BLA) or green fluorescent proteins (GFP) and its analogs (Suzuki *et al.,* 1997, Goshorn *et al.*, 1993, Casey *et al.,* 2000).

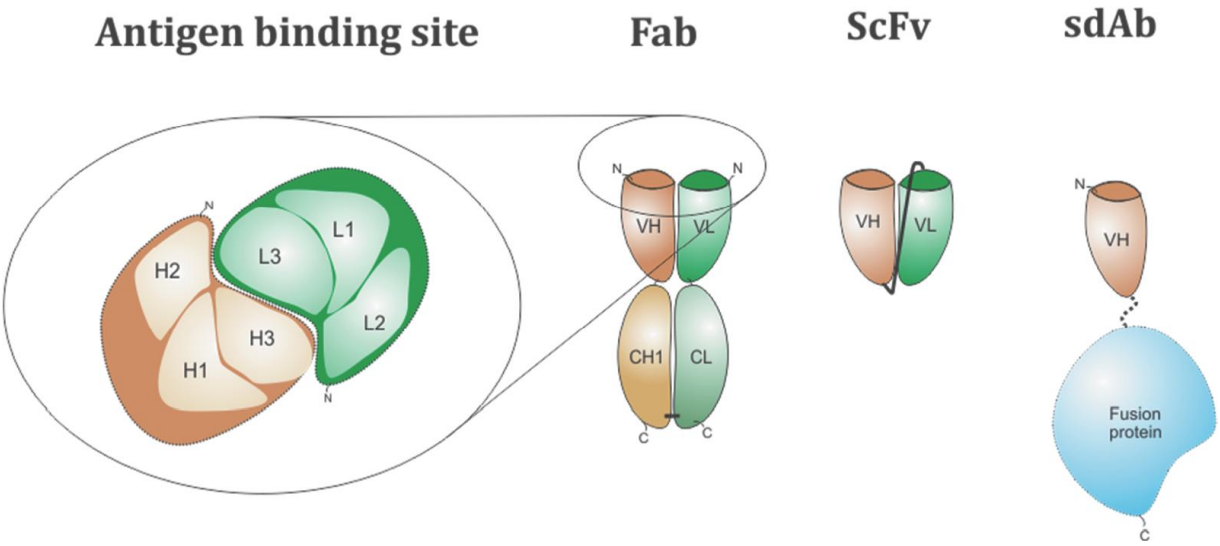


Figure 2. Representation of the most commonly used antibody fragments generated by antibody engineering techniques. The paratope, or antigen binding site, is formed by six hypervariable loops (CDRs) abbreviated according to light chain (L1-L3) heavy chain (H1-H3) location. Fragment antigen binding (Fab) consists of the V-region of intact IgG protein. Single-chain fragment (scFv) contains the variable domains from both heavy (VH) and light (VL) chains connected by flexible polypeptide linker. Single-domain antibody (sdAb) contain only single monomeric variable domain. The antibody fragments can be expressed in fusion with various proteins (blue).

Alongside with the development of hybridoma technology and monoclonal antibodies, the generation and tailoring of antibodies from antibody libraries has made the process rapid and more controllable. Antibody libraries can be constructed on the basis of natural sources, where the immunized antibody gene pools are isolated from the B-cells of a source animal and used to construct the antibody library (Huse *et al.,* 1989). The library construction can be also done completely *In Vitro,* in non-immune/single-pot manner (Griffiths *et al.,* 1994, Vaughan *et al.,* 1996). These non-immunized, semi-synthetic or synthetic antibody libraries contain higher sequential diversity and are designed to derive antibodies to a wide range of targets. Furthermore in the case of synthetic antibody libraries, the use of single framework and carefully targeted mutagenesis in predefined regions enables to circumvent the limitations of natural immune response related to biases and redundancies, and can be used to generate binders against virtually any antigen (Huovinen *et*

al., 2012). The concept has also been applied to the construct antibody libraries which have certain prerequisites towards the finding of binders against difficult targets such as haptens (Persson *et al.,* 2006), peptides (Cobaugh *et al.,* 2008) or carbohydrates (Schoonbroodt *et al.,* 2008). Although the use of large synthetic libraries for the antibody generation process has several benefits, the diversity of the library needs to be high in order to find binders with adequate affinity. The construction of such libraries is technically difficult and is limited by the efficiency of the transformation (Sidhu *et al.,* 2000, Benatuil *et al.,* 2010). As a result, for the generation of antibodies from synthetic libraries against difficult targets, it is often feasible that the technique is coupled with methods which can be used to enhance the affinity of the found antibodies further (Brockmann *et al.,* 2011).

As the antibody libraries consists of millions to billions different clones it needs to be coupled to a technology which can be used to efficiently enrich and isolate the antigen specific binders. *In Vitro* display technologies has provided the means for this, enabling even greater control over the selection conditions and various parameters used to modify the antibody properties. Antibody development with *In Vitro* display technologies has proven to possess several benefits over the traditional immunization based technologies. Most notably the selection of antibodies with picomolar (Hanes *et al.,* 2000) or even femtomolar (Boder *et al.,* 2000) affinities has expanded the limits in assay development. The antibody selection process itself is rapid and easily controllable, which provides the means to raise antibodies against challenging antigens which are either toxic or dependent on certain physical charasteristics such as pH (Kehoe *et al.,* 2006). Alternatively, the binding properties of the antibody itself can be designed to generate binding site where the functionality is dependent in certain physical attributes of the surrounding environment such as pH (Bonvin *et al.,* 2015). The generation of antibodies *In Vitro* is based on the efficient expression or display of the binding regions, which is usually achieved with the use of recombinant forms of the antibody fragments in microbial environment, such as bacteria (Francisco *et al.,* 1993), yeast (Van Antwerp *et al.,* 2000) or phages (Smith, 1985). In addition, techniques which rely on cell free display of the antibody fragments have been introduced (Hanes *et al.,* 1997). From these techniques, perhaps the most widely used technology for *In Vitro* antibody development is phage display, which was originally described by Smith (1985) and further enhanced in other studies by McCafferty *et al.,* (1990) and Clackson *et al.,* (1991). Phage display allows the enhancement and development of antibodies *In Vitro* with virtually unlimited specificity and high affinity regardless of the originating species. The technique introduces a physical link between the genotype and the phenotype

of the antibody fragment, which is co-expressed with one of the surface proteins of M13-bacteriophages. With consecutive rounds of phage propagation and selection against immobilized antigens it is possible to enrich target specific antibodies very efficiently.

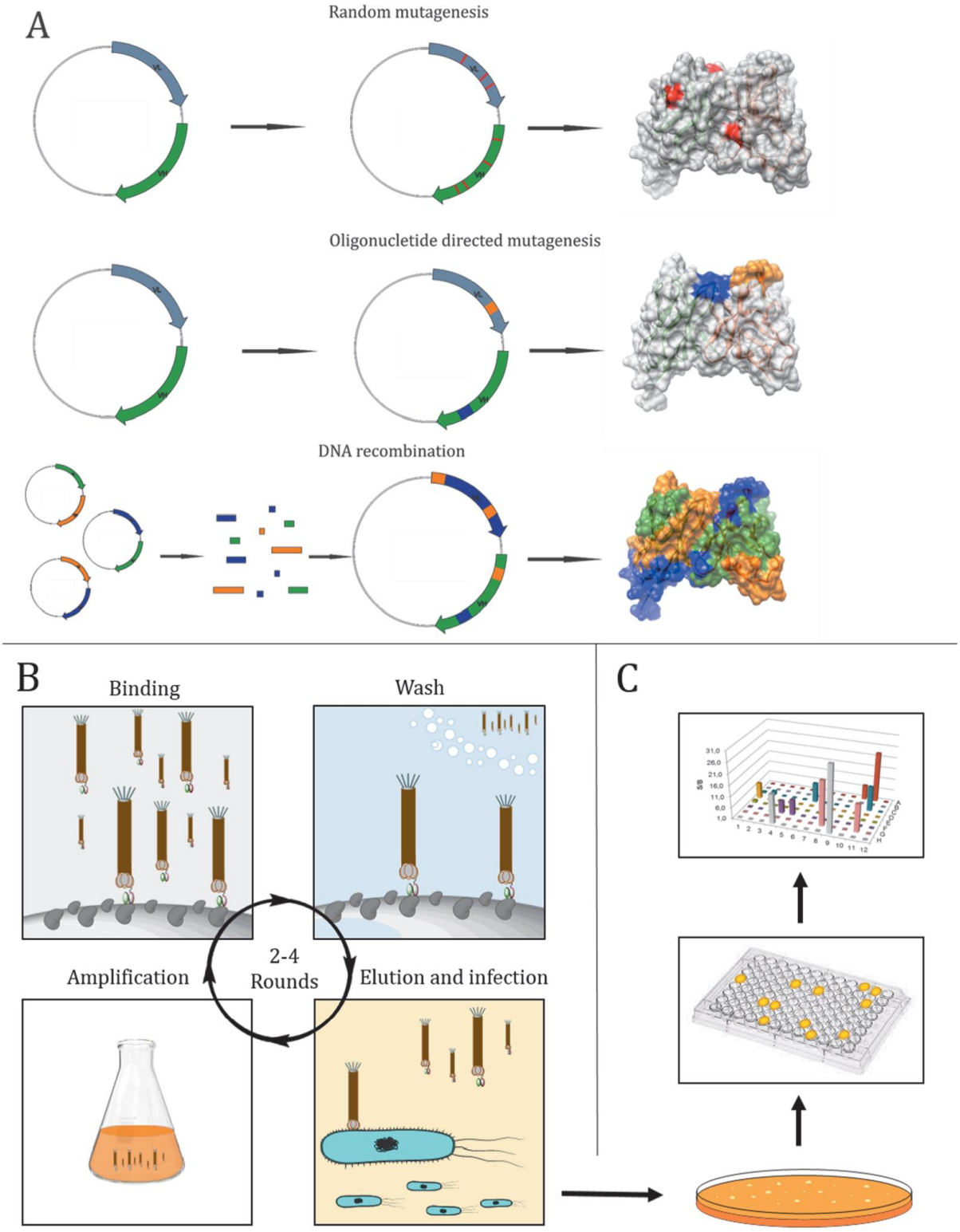


Figure 3. *In Vitro* antibody generation process. (A) Illustration of the most common *In Vitro* mutagenesis methods used for the antibody library construction. (B) Phage display selection process used for the panning of antigen specific antibodies consist of 2-4 rounds of consecutive enrichment steps. (C) Antigen specificity of individual antibodies can be determined from the enriched phage pool with single colony screening.

2.1.3 Engineering of the antibody-antigen binding interaction

The binding between antibody and antigen is energetically complex interaction which is affected by several parameters both in the surrounding environment and in the surfaces of the binding structures. The variation in the amino acid composition of the CDR-region makes the surface very adaptable to binding. The antibody surface can either be planar, cavity or a wedge-like depending on the antigen structure and the antibody function (MacCallum *et al.,* 1996). Based on the studies in structural biology, majority of the inter-molecular bonds formed between the antibody and the antigen have been found to be weak, non-covalent interactions, such as hydrophobic, electrostatic interactions or hydrogen and Van der Waals bonds (Davies *et al.,* 1990). The general parameters most often affiliated to the antibody binding process are specificity and affinity, both of which can be modified with the use of antibody engineering techniques (Valjakka *et al.,* 2002). The engineering of the target antibody gene requires the introduction of mutations to the certain areas or residues of the protein. In the case of antibodies, usually it is preferred that the variable region of the antibody is targeted for mutagenesis, with either randomization of the whole domain, or with designed amino acid substitutions in the antigen binding residues (Figure 3). The most widely used *In Vitro* mutagenesis methods can be roughly divided in three categories and are reviewed here.

2.1.3.1 Random mutagenesis

Controlling the amino acid substitution pattern in random mutagenesis of the target gene is crucial for the functionality and quality of the antibody library. Random mutagenesis provides assets for the fine tuning of antibodies properties or alternatively gives insight when the exact function or structure of the protein is unknown. Most widely used randomization methods are based on catalytic reactions of DNA modifying or replicating enzymes (Cadwell *et al.,* 1992, Hughes *et al.,* 2003). The main pitfall in random mutagenesis methods in general results from "mutational bias" on DNA and amino acid –level, which results from various factors involved in the mutagenesis process (Vanhercke *et al.,* 2005). Due to the limitations of the genetic code, single point mutations on DNA cause higher prevalence of certain amino acids codons. In optimal conditions mutations would be introduced as a balanced pattern of transitions and transversions (1:3, respectively) throughout the DNA region targeted for randomization (Wong *et al.,* 2006). The randomization method should also provide an evenly distributed selection of all amino acids without an excess of "hot-spots", frame shifts or stop-codons. The mutational bias problem has been circumvented to some extent with the use of engineered DNA modifying

enzymes as a source of mutations (Leung *et al.,* 1989). Alternative methods relying on *Escherichia coli* mutator strains have also been described (Irving *et al.,* 1996, Greener *et al.,* 1997). The introduction of mutations is fairly simple, however the mutation frequency is low in comparison to the enzyme based mutagenesis methods. Low-fidelity DNA polymerases can be used to create point mutations in error-prone polymerase chain reaction (EP-PCR). The frequency of mutations can be controlled with different ways: i) the cofactor of the DNA polymerase Mg^{2+} can be replaced by Mn^{2+} which induces higher rate of mutations during the amplification. ii) The use of nucleotide analogues or unbalanced concentration of dNTPs correlates with the incorporation of nucleotides to the amplified DNA strands (Lin-Goerke *et al.,* 1997). iii) Controlling the amount of target template in the amplification. iv) Increasing the amount of cycles and the use of consecutive PCR reactions may be used to achieve higher error rates.

Low fidelity polymerases have been modified to create versatile nucleic acid mutation patterns with controllable mutation frequency (Biles *et al.,* 2004, Rasila *et al.,* 2009). Most commonly used enzyme in EP-PCR methods is Taq –polymerase, although biased towards transition mutations (results in higher prevalence of stop-codons), the mutation frequency can be controlled to generate high diversity libraries in a single round of mutagenesis. Commercially available Mutazyme –polymerases are efficient for low frequency libraries due to favorable mutational bias (Cline *et al.,* 1996). Combination of previously mentioned polymerases has resulted in development of moderate frequency and balanced mutation pattern in terms of functionality and quality of library mutagenesis. Another enzyme derived randomization method is based on the rolling circle amplification (RCA) where circular DNA can be rapidly amplified (Fujii *et al.,* 2004, Huovinen *et al.,* 2011). The mutations are introduced with the use of manganese, which reduces the fidelity of ϕ29 DNA polymerase, in a similar way to EP-PCR.

2.1.3.2 Oligonucleotide directed mutagenesis

When the structure or the active site of the target protein is known it is often preferable to designate certain areas or amino acids for randomization. This can be achieved with oligonucleotide directed mutations where the amino acid changes can be controlled by the use of degenerate oligonucleotides. Selected areas in target gene are replaced with synthetic DNA with either complete randomization of the codons (Matteucci *et al.,* 1983) or with designed amino acid composition (Fellouse *et al.,* 2004). Several methods have described since the 1980s based on oligonucleotide derived enzymatic modifications of

proteins (Kunkel *et al.,* 1987, Horton *et al.,* 1990, Sarkar *et al.,* 1990). Although the mutational bias is not as influential as in random mutagenesis methods, genetic code intrinsically sets certain limitations for the library design.

For antibody engineering oligonucleotide directed mutagenesis provides optimal tools to create libraries with high diversity since the hypervariable loops are often compact enough for the complete randomization of the entire loop with single degenerate oligonucleotide. Also applications of oligonucleotide directed mutations such as CDR-walking (Yang *et al.,* 1995) or alanine scanning (Cunningham *et al.,* 1989) have been developed for studying the binding properties of the antibody or pointing out certain key residues primarily in contact with the antigen. This can be achieved by using degenerate codons which only translate to small side chain amino acids such as alanine. For high diversity antibody libraries it would be preferable to mutate more than one CDR–region at the time. For most oligonucleotide directed mutations –methods, such as strand overlap extension PCR (SOE-PCR) (Horton *et al.,* 1990) and megaprimer extension (Sarkar *et al.,* 1990), the incorporation of the synthetic DNA in to the full length gene can be done at one site at a time. Randomization of multiple areas at once is more complex, however methods such as parsimonious and Kunkel mutagenesis (Balint *et al.,* 1993, Kunkel *et al.,* 1987) have overcome this problem to some extent, making the construction of high diversity libraries in single round of mutagenesis possible.

2.1.3.3 DNA recombination

Techniques which are based on DNA recombination can be narrowed down to methods using the natural evolution or recombination of the target gene. These techniques can be used either to recombine homologous gene segments in hope to emphasize the desired property, or to shuffle together heterologous genes to create new diversity. By far the most widely used methods is DNA shuffling (Kang *et al.,* 1991, Stemmer *et al.,* 1994), where homologous DNA segments are digested with DNAase and reassembled with ligation to produce novel combination of parental properties. Zhao *et al.* (1998) described a modified PCR method which is based on staggered extension with the use of heat denaturation-hybridization of target template and primers resulting in recombination of target genes. DNA recombination is a useful tool for example in the affinity maturation process of antibodies. The high resemblance of the mutant clones to the parental counterparts and high degree of functionality makes it a lucrative option to be used in combination with random or oligonucleotide directed mutagenesis (Ness *et al.,* 2002).

2.1.4 The use of directed antibody engineering techniques in food diagnostics

Immunoassays are one of the most fundamental platforms for the development routine screening tools used in food diagnostics. Monoclonal antibody based assays, which are designed to detect food contaminants in various food matrixes have been in routine use since early 1980s (Hitchcock *et al.,* 1981, Meulenberg, 1997). Although antibodies against low-molecular weight compounds have been around for the past three decades, the development of reliable and rapid immunoassays is still limited by the availability of antibodies with high enough affinity and desired specificity. Low-molecular weight food contaminants are haptens, and as such do not inflict immunoresponse when injected to animals. To overcome this problem, the antigens need to be conjugated to protein or polypeptide carriers for antibody generation. The small size of the haptenic antigens and the use of carrier proteins can result in bridge-effect, where the antibodies have specificity towards the surrounding carrier protein and linker regions, causing interference in the assay performance (Franek, 1984, Moghaddam *et al.,* 2001). Additional challenge can arise when the target compound is toxic to the animal which is used for the immunization. To overcome these challenges, the limitations of immunoassays developed for the detection and screening of low-molecular weight contaminants have recently been expanded with the use of recombinant antibodies and antibody engineering in the sense of both sensitivity and amount of detectable compounds.

Many of the commonly found food contaminants are composed from a group of structurally relevant molecules rather than a single compound. These kinds of compounds can consist of different kinds of classes of, for example antibiotics, pesticides or toxins. In order to detect majority or all of the contaminants in a single immunoassay the specificity of the antibody needs to more promiscuous or broader than usual (Kramer *et al.,* 1996). This can be achieved by generating antigens which contain only the most structurally similar regions of the desired class of compounds (Spinks *et al.,* 1999, Wang *et al.,* 2007a). The successful generation of these broadly specific antibodies is highly dependent on the structure of the target antigen and such antibodies have been applied for the detection of pesticides (Garrett *et al.,* 1997, Yau *et al.,* 1998), antibiotics (Franek *et al.,* 2006, Cao *et al.,* 2009) and mycotoxins (Jiang *et al.,* 2013a). Furthermore, molecular modelling assisted design of the target antigen has facilitated the generation of antibodies which have a broader specificity to the target compounds (Pinacho *et al.,* 2012, Wang *et al.,* 2007b).

The design and synthesis of antigen derivatives which are useful for the development of specific antibodies is laborious and the resulting antibodies do not always have the desired binding properties. An alternative approach to detect the binding of the analyte without the antigen derivatives is to use anti-idiotype antibodies. This method is especially useful when the detectable analyte is toxic or unsuitable for chemical conjugation. The method relies on the possibility to generate antibodies against the variable region or the antigen binding site of another antibody. The resulting secondary antibodies can be used to develop a competitive immunoassay without the need for labeled analyte (Kohen *et al.,* 2000). Such assays have been described for the detection of aflatoxin (Wang *et al.,* 2013) and fumosinin (Shu *et al.,* 2015). Another variation of the anti-idiotype detection method has been described by Self *et al.* (1994), where the binding of the secondary antibody is dependent on the presence of the analyte. These immunocomplexes can be used to develop simple homogenous detection methods for low-molecular weight analytes (Pulli *et al.,* 2005). The variable regions of an antibody can also be expressed separately as described by Ueda *et al.* (1996), which provides more versatility to the assay design. This method termed open-sandwich ELISA (OS-ELISA) has been used for the simple detection of low-molecular weight compounds, such as mycotoxin zearalenone (Suzuki *et al.,* 2000, Suzuki *et al.,* 2007).

Several studies have described the cloning of existing, previously validated monoclonal antibodies against food contaminants in to recombinant form and the characterization of the performance in the respective format (Kramer *et al.*, 1996, Garrett *et al.*, 1997, Chambers *et al.,* 1999). However the conversion of intact antibody in to a smaller binding unit is not always straightforward and may result in a significant reduction in binding kinetics or aggregation during antibody production (Choi *et al.,* 2004, Borras *et al.,* 2010). In addition the loss of the intrinsic bivalency of the native IgG molecule has a direct effect on the functional affinity of the antibody and may result in decreased assay performance.

Relatively few studies have focused in the use of directed evolution to enhance the binding properties of monoclonal or recombinant antibodies used in the detection of food contaminants. In one of the first studies, Wyatt *et al.* (1999) used oligonucleotide-directed mutagenesis targeted to CDR-H3 loop of an organophosphorus insecticide parathion specific scFv. Significant enhancement was observed in a study, where the functional affinity of an antibody specific to commonly used herbicide atrazine was improved over 25-fold with the use of chain shuffling (Kramer *et al.,* 2000). The engineering of a *p*-azophenyarsonate specific antibody with EP-PCR resulted in over 10-fold increase in affinity (Parhami-Seren *et al.,* 2002). Similar to the other general

properties of proteins, protein engineering can be used to either increase or decrease the binding specificity of the antibody. Saviranta *et al.* (1998) described the use of antibody engineering to enhance the steroid specificity of an anti-estradiol antibody. The opposing effect with the use of similar techniques could be observed when Korpimäki *et al.* (2002) engineered the binding properties of a broad specificity sulfonamide antibody. In a later study the antibody was optimized and ultimately validated to detect 13 different sulfonamide antibiotics from milk samples (Korpimäki *et al.,* 2004). These studies show the versatile benefits gained from the use of antibody engineering to tailor the binding properties of a moderate monoclonal antibody.

2.2 Fluoroquinolones

2.2.1 Background

Quinolones and fluoroquinolones are a class of synthetic antimicrobial compounds, originally derived from nalidixic acid, a side-product in the synthesis of antimalarial compound (Figure 4). The finding of the antibacterial effect of nalidixic acid led to the development of a new class of broad spectrum antibiotics (Lesher *et al.,* 1962). Since the discovery in 1962, more than 30 derivatives of quinolones in four generations have been synthetized. The addition of fluorine atom to the position C-6 consecutively led to the development of fluoroquinolones which increased the overall therapeutic efficacy substantially (Andriole *et al.,* 2000). Fluoroquinolones are effective against wide range of both gram-positive and gram-negative bacteria, which has led to wide spread use in treatment in both human and animal diseases. The therapeutic uses include common bacterial infections in respiratory and urinary tracts.

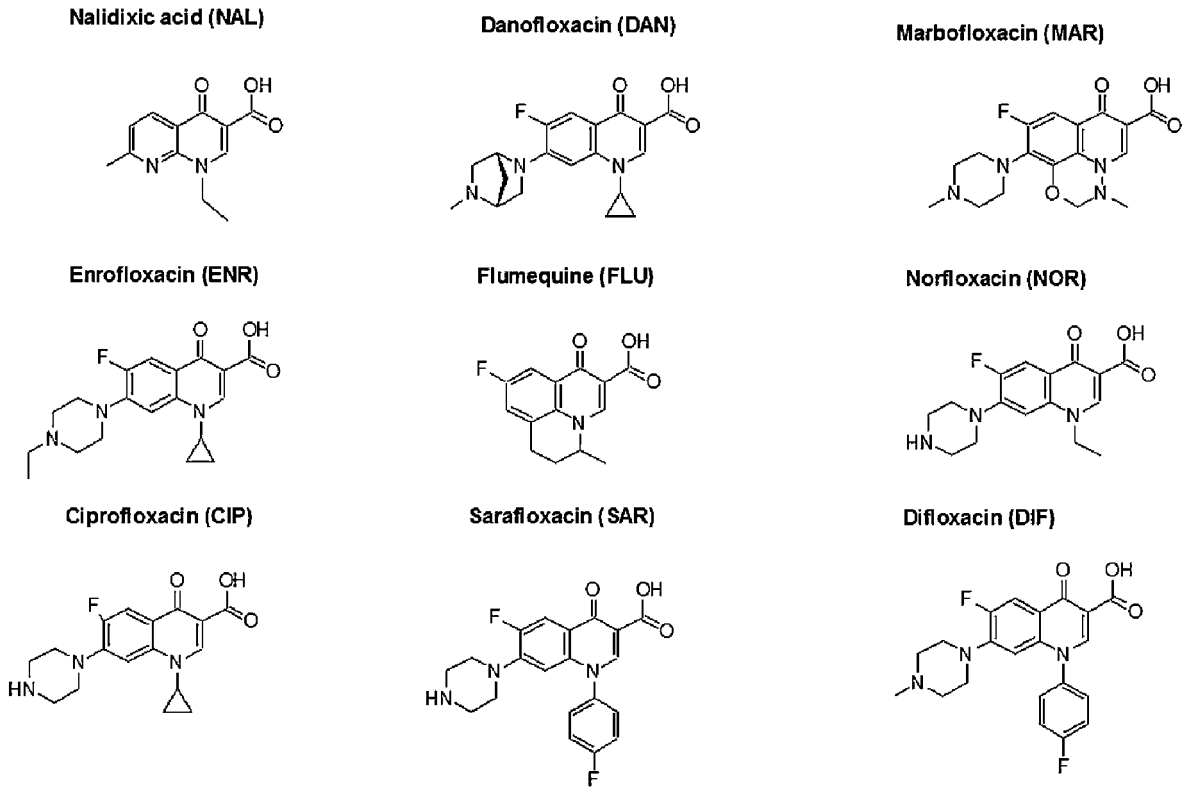


Figure 4. The structures of nalidixic acid and the eight EMA controlled fluoroquinolone antibiotics. Abbreviations in captions.

The molecular mechanism of action is result of the non-covalent binding of the quinolone structure with DNA gyrase, or topoisomerase II enzyme, which is crucial for the replication of the bacteria. The binding of quinolone to topoisomerase II stabilizes the cleaved form of the enzyme and consecutively leads to the accumulation of single stranded DNA precursors inside the cells which ultimately prevents the replication of the cell (Crumplin *et al.,* 1976). With the development of generation three and four fluoroquinolones the drugs have been more selective to topoisomerase IV.

The molecular weight of fluoroquinolones range between 260-360 g mol^{-1}. All fluoroquinolones share a common 4-quinolone ring structure accompanied by side groups commonly attached to positions C-7, C-8 or N-1, and a fluorine group in position C-6. The addition of side-groups have shown to change the pharmacokinetic efficacy and lower the adverse side-effects of the first generation quinolones (Brighty *et al.,* 2000). Most notably the addition of piperazine ring at position C-7 has been shown to increase the activity against aerobic bacteria in *staphylococci* and *pseudomonas* species (Domagala *et al.,* 1994). More recently the studies have been oriented to overcome the emergence of fluoroquinolone resistant strains and have led to the development of fourth generation of quinolones (Zhao *et al.,* 1997). Fluoroquinolones also endow pH dependent physical attributes which affect their solubility, ionization state and chelating properties, all of which have an impact to the functionality of fluoroquinolones (Pinacho *et al.,* 2012).

2.2.2 The use and control of fluoroquinolones

The overall consumption of fluoroquinolones for medical use in humans and animals has not been studied extensively and the data currently available is contradictory. However the emergence of fluoroquinolone resistant bacterial strains has increased the need for controlling the fluoroquinolone usage in both clinic and agriculture (Radostits *et al.,* 2004). The use of fluoroquinolones as antibiotic growth promoters in animals is another possible source of antibiotic resistance. The stable structure of fluoroquinolones remains mainly unchanged after usage (Sörgel *et al.,* 1993) which provides a source of resistance for bacteria. Since fluoroquinolones are an important class of antibiotics for the treatment of many severe and invasive infections, this proposed health risk has consequently led to the precautive control of fluoroquinolones in food-producing animals (FAO/WHO, 2003). Furthermore European commission directives (Council Regulation No. 2377/90) has set maximum residue limits (MRLs) ranging from 10-400 µg kg^{-1} for the occurrence of fluoroquinolones in different food matrices.

2.2.3 Fluoroquinolone detection methods

The increased legislative control over the use of fluoroquinolones in food producing animals and the emergence of antibiotic resistant bacterial strains has resulted in the development of variety of analytical technologies for the identification and quantification of fluoroquinolones in food products (for a review see Andreu *et al.,* 2007). The development of analytical methods, both for confirmation and screening of fluoroquinolones are mainly dependent on the matrix which the antibiotic is to be screened. The most common and perhaps widely accepted confirmatory method is based on the physical separation of the analyte with the use of chromatography, namely GC and HPLC. HPLC methods for the detection of fluoroquinolones have been described for environmental samples (Golet *et al.,* 2001, Turiel *et al.,* 2006), food products (Jehl *et al.,* 1985, Horie *et al.,* 1994, Hassouan *et al.,* 2007) and biological fluids (Morton *et al.,* 1986, Samanidou *et al.,* 2003). More recently, the these methods have focused in the coupling of HPLC to mass spectrometric (MS) or tandem mass spectrometric (LC–MS/MS) analysis (Schneider *et al.,* 2002, Ye *et al.,* 2007, Bogialli *et al.,* 2009). The main benefit of using HPLC-MS method is the possibility to identify the compound in addition to the quantitation. Even though these methods are reliable and enable the specific identification of the contaminant, they require both expensive instrumentation and solvent based sample extraction followed by different clean-up steps before analysis, and as such are not suitable for high throughput screening.

The screening of fluoroquinolones with methods which are both cost-efficient and simple are mainly ligand binding antibody based assays. Multiple studies have been conducted which describe the detection of single fluoroquinolone residues (Duan *et al.,* 2001, Haasnoot *et al.,* 2007). However, due to the large amount of different fluoroquinolones used in clinic and agriculture, the trend has been to develop methods for multiresidue detection of fluoroquinolones. The possibility to raise antibodies which have more generic specificity enable the screening of multiple fluoroquinolone residues with the use of monoclonal antibodies (Holtzapple *et al.,* 1997). More recently, as the interest for multiresidue detection of antibiotic residues has increased, studies which describe more detailed antigen synthesis or conjugation based on structural data analysis has led to the generation of broad specificity antibodies against up to 20 fluoroquinolones (Wang *et al.,* 2007b, Cao *et al.,* 2009, Tao *et al.,* 2013). The studies of the specific chemical and structural properties of fluoroquinolones with the use of molecular modeling have also contributed to the optimization of antigen synthesis and assay development (Wang *et al.,* 2007b).

2.3 Skatole

2.3.1 Background

The metabolism of L-tryptophan in the rumen of animals can lead to the production of several indolic compounds, such as indole (IND), 3-methylindole (SKA), indole-3-acetic acid (IAA) and indole-3-propionic acid (IPR) (Yokoyama *et al.,* 1979). These natural side products of microbial L-tryptophan degradation route is found mainly in monogastric animals (Carlson *et al.,* 1975). The other indolic compounds are metabolized by many types of ruminal bacteria, however only a few strains of two genera of bacteria, *Lactobacillus* and *Clostridium*, have been reported to produce skatole (Fellers *et al.,* 1925 and Kowalewska *et al.,* 1985). The structure of skatole has a methyl group attached to C-3 position of indole ring with the molecular weight of 131 g mol^{-1} (Figure 5).

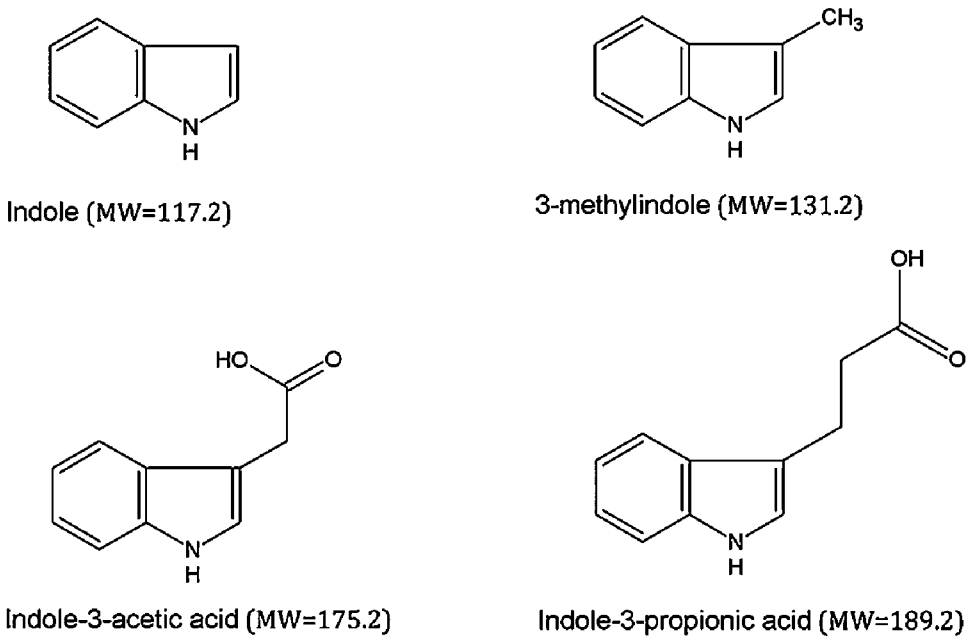


Figure 5. The structures and molecular weight of the most common indolic compounds produced by bacterial metabolism. Indole (IND), 3-methylindole (SKA), indole-3-acetic acid (IAA) and indole-3-propionic acid (IPR)

2.3.2 Skatole and boar taint

Odor and taste are the two most important attributes to cause negative consumer experiences in meat products. In non-castrated male pigs the accumulation of skatole and testicular steroid androstenone to the adipose tissue can cause unpleasant odor in processed meat products when heated (Lundström *et al.,* 1996). Meat containing this fecal odor, more commonly known as boar taint, prevents the breeding of entire male pigs and reduces the quality of the meat which has only limited use in the food industry (Lundström *et al.,* 2009). While the individual perception of boar taint varies, the negative effect is more commonly caused by skatole than androstenone (Dijksterhuis *et al.,* 2000 and Matthews *et al.,* 2000).

The prevention of boar taint has traditionally relied mainly on the surgical castration of male piglets. Other common methods to control the emergence of boar taint compounds include reduced slaughter age, feed and breeding control (Aldal *et al.,* 2005). In addition medical companies have more recently introduced immunological castration based method, where the boars are vaccinated against gonadotropin-releasing hormone (GnRH). The vaccination prevents the release of gonadotropin related steroids and consecutively lowers the overall production of androstenone and skatole in the animal (Dunshea *et al.,* 2001). These methods used for the prevention of boar taint have several productional, economical and animal welfare related hindrances compared to the breeding of non-castrated pigs. These include lower feed to meat conversion and faster growth rates (Andersson *et al.,* 1997), lower total meat yield and also negative impact for the consumer acceptance.

Recently European Commission assembled a consortium consisting of members of farmers, meat producers, retailers, researchers, veterinarians and animal welfare organizations to discuss the possibility to desist surgical castration and to develop possible alternatives to overcome the boar taint problem. The declaration concluded to recommend the discontinuation of surgical castration from the beginning of 2018 (European Declaration on alternatives to surgical castration, 2011). To ensure the successful transfer to other techniques, new standardized methods need to be established for the control and detection of boar taint related compounds. European Commission has not set official MRLs for skatole or androstenone, and the generally approved limits of 0.25 μg L^{-1} and 0.1 μg L^{-1} respectively, are not directly applicable for all samples due to differences in slaughter weight and pre-analytical methodology (Walstra *et al.,* 1999).

2.3.3 Skatole detection methods

The approaching legislative regulation concerning the ban of surgical castration creates an urgent need for rapid, simple and cost-efficient methods for the detection of boar taint related compounds. As more than 250 million pigs are being processed annually in the EU countries alone (2013, Source: Eurostat), the established method should be suitable for automated screening of very large sample amounts. In addition the more demanding sample pretreatment for adipose tissue as sample matrix has brought additional limitations to the detection of skatole with the most commonly used techniques. Optimally the method could be used to sort out boar taint containing carcasses directly from the slaughter line. Although several approaches have been developed, some of which have also been automated, there is still a need for a rapid method which could be used as a basis for standardization for the screening of skatole and androstenone. Comparison of different analytical methods for the detection of boar taint related compounds was recently reviewed by Haugen *et al.* (2012).

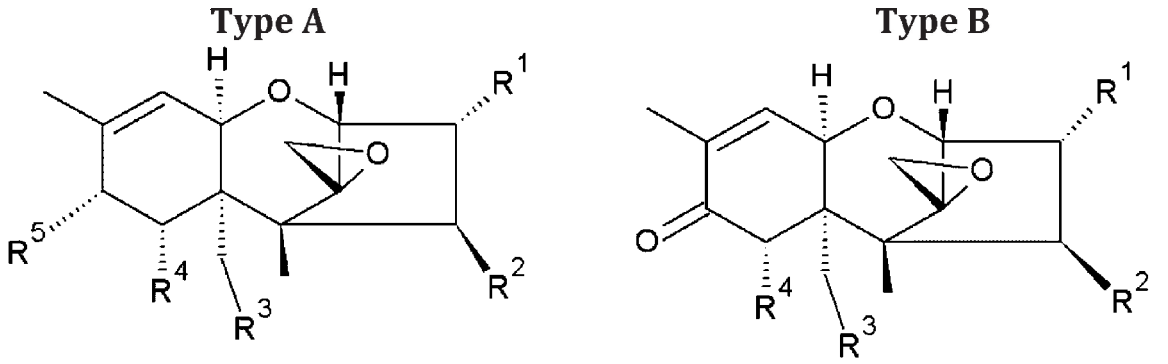
A number of studies which describe the use of chromatographic methods for the detection of skatole from plasma or serum samples have been described. These methods include both GC (Zabolotsky *et al.,* 1995), HPLC (Claus *et al.,* 1993, Tuomola *et al.,* 1996) and the use of each separation method in combination with MS (Peleran *et al.,* 1985, Verheyden *et al.,* 2007). Chromatographic methods can efficiently be used to detect and identify all boar taint related compounds simultaneously, however as stated previously, these methods are rarely suitable for routine screening and with current technology they are too expensive and slow for high throughput analysis.

Contrary to the two other classes of low-molecular weight food contaminant introduced in this review, only a few studies have been published which describe the use of immunological methods for the detection of skatole (Singh *et al.,* 1988, Tuomola *et al.,* 2000 and Aguilar-Caballos *et al.,* 2002). These methods however are either not sensitive enough or suffer from unwanted cross-reactivity from other indolic compounds, and as such have not been applied for routine screening. The lack of immunological detection methods for skatole has been mainly hindered by the difficulties in antibody generation process resulting from the low-molecular weight of the compound.

2.4 Trichothecenes

2.4.1 Background

Fusarium species molds are a common fungi found globally which are known to produce a class of mycotoxins. Trichothecenes are one group of these mycotoxins and a common contaminant of dry food and animal feeds. Trichothecenes contain more than 200 different derivatives which are divided in four types (Pestka, 2010). All trichothecene toxins share similar tetracyclic 12,13-epoxytrichothec-9-ene structure with varying side groups and hydroxylations around the core and molecular weight in the range of 200-500 g mol^{-1}. The intake of toxin containing feed cause feed refusal, impaired growth and various diseases resulting from the immunosuppressive effect of trichothecenes in food producing animals (Dersjant-Li *et al.,* 2003). The more specific biological mode of action has been shown to be the cause of protein synthesis inhibition in eukaryotic cells via binding to the peptidyl transferase of 60S ribosome subunit (Feinberg *et al.,* 1989). The most common trichothecenes responsible for the majority of health hazard cases to humans and animals are deoxynivalenol (DON), nivalenol (NIV), T-2 toxin and HT-2 toxin (Figure 6), DON being the most common cause of trichothecene spoiled foodstuffs in Europe (Streit *et al.,* 2012).



Name	R1	R2	R3	R4	R5
T-2	OH	OCH_3	OCH_3	H	OC_5H_9
HT-2	OH	OH	OCH_3	H	OC_5H_9
Nivalenol	OH	OH	OH	OH	O
Deoxynivalenol	OH	H	OH	OH	O
3-Acetyldeoxynivalenol	OCH_3	H	OH	OH	O
15-Acetyldeoxynivalenol	OH	H	OCH_3	OH	O

Figure 6. The structures of type A and type B trichothecenes and the common metabolic isoforms.

2.4.2 Trichothecene contaminations in food products

Trichothecene contaminations continue to be a worldwide problem for human food and animal feed, as it has been estimated that nearly 25% of all crops are exposed to fungal growth (Mannon *et al.,* 1985). These contaminations have been mainly found from cereals such as wheat, barley, oats, rye, maize and rice. In addition trichothecenes can enter the food chain through animal products (milk, meat) from livestock that are fed with trichothecene containing feeds (Meneely *et al.,* 2011). The synthesis of trichothecenes in the mold is a complex, species dependent process. In addition to the environmental factors such as temperature, moisture and surrounding microbiota contribute to the process. Any single species is capable of producing more than one type of toxin. As moisture and temperature are the two main factors affecting the fungal growth, prevention of growth during the storage of the foodstuffs has proved out to be the most effective mean in the control of toxin production (Christensen *et al.,* 1982). The high stability of trichothecenes has limited the use of detoxification approaches which include physical, biological and chemical methods (He *et al.,* 2010). The trichothecene contaminations also have a significant economical and societal impact. The resulting decrease in crop yields and animal productivity in combination with tightened legislative regulations, all have increased the workload for food producers. Ultimately all these factors have an impact to the consumer prices.

Due to the potential health risks for humans, international organizations have published several risk assessments for the prevalence and analysis of trichothecenes in human food, such as WHO/FAO/OIA and Joint Expert Committee on Food Additives and Contaminants (JECFA, 2001) and Scientific Committee for Food (SCF, 2002) in European Union. According to the assessment publication, regulatory limits have been set for deoxynivalenol residues in different food products (Commission Regulation, 1881/2006). According to the regulation tolerable daily intake values for trichothecenes were set between 200-1750 $\mu g\ kg^{-1}$, depending on the sample matrix. Detection of trichothecenes is especially challenging in baby foods due to the low regulatory limits. Significant effort has been made during the past 10 years in order to enforce these regulatory limits with the development wide range of different confirmatory and screening methods for the control of trichothecene contaminations in food products.

2.4.3 Trichothecene detection methods

The very stable structure of trichothecenes makes them difficult to remove from contaminated food stuffs, and although the prevention of mycotoxin formation has been considered to be the best method to control these contaminations, complete prevention is often not possible (Bullerman *et al.,* 1984). This creates a need for sensitive and robust analytical methods which would be suitable for routine use in the field. Chromatographic methods are considered as the reference method for trichothecene detection on account on the high sensitivity and reliability of the techniques. Furthermore, the use of MS or tandem MS in combination with GC and LC enables the detection and quantification of multiple trichothecenes in a single run (Nielsen *et al.,* 2001, Berthiller *et al.,* 2005, Ren *et al.,* 2007). A more detailed comparison of the performance of different analytical methods have been recently reviewed by Meneely *et al.* (2011) and Ran *et al.* (2013).

Immunochemical methods for trichothecenes are often preferred for the routine analysis, but these have mainly been limited to the detection of single or structurally similar trichothecenes. Majority of the methods found in the literature have been established for the screening of DON, being the most prevalent contaminant of food related products. Ideally the assay would be capable of detecting all of the most relevant trichothecenes in a single assay, but to achieve this, the capture antibody would need to have broad specificity against the structurally similar regions of the type A and B trichothecenes (Figure 6). The toxicity of the compounds has hindered the development of high quality antibodies. Nivalenol especially has proven out to be extremely challenging target for antibody development with relatively few successful studies. One of the rare exceptions is a study where Maragos *et al.* (2006) describe the generation of nivalenol specific monoclonal antibody. The antibody was later used as a starting point for the development of a SPR assay immunoassay for the detection of DON and NIV (Kadota *et al.,* 2010). Recently Maragos (2014) described the use of anti-idiotype antibodies for the detection of deoxynivalenol in three different immunoassay platforms. Methods using SPR (Meneely *et al.,* 2010), lateral-flow (Kolosova *et al.,* 2008, Liu *et al.,* 2012), ELISA (Sinha *et al.,* 1995), electrochemical sensor (Romanazzo *et al.,* 2010) and time-resolved fluorescence (Wang *et al.,* 2009) based detection have also been described. In addition several studies have focused in the use of recombinant antibodies for the detection of DON (Choi *et al.,* 2004, Wang *et al.,* 2007c, Romanazzo *et al.,* 2010).

2.5 Future perspectives in the use of directed antibody engineering techniques for the detection of low-molecular weight targets

Majority of the commercially available immunoassays for the detection of low-molecular weight food contaminants are still relying in the use of monoclonal or polyclonal antibodies. Although directed antibody engineering technologies have proven to be a powerful platform for the development of antibodies with novel binding properties and enhancement of the existing binders, the wide spread use of these technologies is still hindered by a challenging patent situation (Bradbury *et al.,* 2011). However, the most relevant patents have, or will be soon expiring, which will most likely increase the commercial availability of recombinant antibodies.

The evolvement of antibody engineering techniques have provided new ways to raise antibodies against challenging targets as well as created versatile tools to tailor the properties of existing monoclonal binders. The size reduction itself of IgG into a Fab or scFv can enhance the binding capacity of the assay and consecutively lead to increased sensitivity (Härmä *et al.,* 2000, Brockmann *et al.,* 2010). The expression of recombinant antibody fragments can provide site-specific modifications to the C-, or N-terminus of the antibody enabling targeted labeling of certain amino acid residues (Karyakin *et al.,* 2000). The possibility to easily clone the antibody to different expression vectors has also enabled versatile co-expression of different fusion proteins and tags. With this method, the production of binder-reporter proteins can be done in a reproducible manner, keeping the molar ratio of the tracer invariable. The most straight forward example of this system is the expression of scFv fragments in fusion with alkaline phosphatase or beta-lactamase, both well characterized enzymes used in various simple immunoassay platforms such as ELISA and lateral flow. The use of antibody-reporter enzyme fusions in immunoassays can increase the assay sensitivity considerably, enabling the detection of low-molecular weight analytes at attomolar level (Kobayashi *et al.,* 2006). Another innovative example of the use of reporter enzymes was recently described by Jiang *et al.* (2013b) in a study of dual-colorimetric ELISA which can be used to detect 13 fluoroquinolone and 22 sulfonamide residues in a single assay. The assay uses monoclonal and polyclonal antibodies labeled with ALP and HRP enzymes which gives a colorimetric result rapidly without the need of specialized instrumentation. More advanced reporter systems have also been described with the use of GFP fusions (Casey *et al.,* 2000) of recombinant antibodies and with the use of reporter peptide tags (Los *et al.,* 2008, Gautier *et al.,* 2008).

In Vitro display technologies have evolved rapidly during the past decade and several novel approaches have been described since. One interesting branch of this field is the development of cell free display systems, where the binding protein is physically linked to its coding gene without the need for cellular translation. The most extensively studied technique being ribosome display (Hanes *et al.,* 1997). In addition other methods using similar methodology based on protein-DNA complexes have been introduced (Odegrip *et al.,* 2004, Reiersen *et al.,* 2005). These techniques rely to the use of *In Vitro* translation combined with artificial compartmentalization of the individual translated units (mRNA or cDNA). The use of *In Vitro* translation system makes the selection process both extremely rapid and easily automated. In addition the diversity of the libraries is much higher in comparison to other display techniques, as the construction is not limited by cellular transformation. The amplification between each selection round is usually done with PCR, which enables the use of directed evolution to introduce additional mutations to the gene pool, and consecutively the possibility to direct the selection pressure more efficiently. The down side of these techniques is the lack of robustness in comparison to phage display: the stability of the translated proteins is very low and requires fixed environmental conditions for successful selection process.

The recent advances in molecular biology have created new, information based methods for the engineering of antibodies (Weinstein *et al.,* 2009). Next generation sequencing (NGS), or deep sequencing, has been used to study the sequence-structure-function relationship based on the enrichment of antibodies with desired properties (Mathonet *et al.,* 2013). NGS techniques have also shed light to the common problem related to phage display where the so-called "parasitic" sequences create unwanted biases in the selection process during the amplification steps (Matochko *et al.,* 2014). In addition, the NGS has brought alternative methods to control the enrichment and selection of antigen specific antibodies (Ravn *et al.,* 2010). The most notable benefit in analyzing the sequence diversity of all the clones in consecutive selection rounds, is the possibility to bypass of laborious single clone screening. As the technical advancement continue to reduce the prices of commercially available services for the construction of complex gene constructs has become more trivial. Construction of focused antibody libraries based on sequences of existing monoclonal antibodies are readily available from multiple biotechnology companies, providing tools for the rapid enhancement of immunoassay performance. These synthetic and bioinformatic technologies will further expedite the design and construction of the next generation antibody libraries, where the sequence repertoire can be designed to contain

even more focused functionality, and when coupled with efficient display and affinity maturation platforms, the technologies create a genuinely competitive alternative to the monoclonal antibodies.

3 AIMS OF THE STUDY

The main objective of this thesis was to study the use of directed antibody engineering techniques for the generation and tailoring of antibodies against low-molecular weight food contaminants.

The more specific aims of the study were:

I. Engineering of the binding properties of a broad specificity monoclonal antibody for the detection of multiple fluoroquinolone antibiotics in a single assay.

II. Development of a TRF immunoassay for the screening of eight structurally similar fluoroquinolones controlled by European medicines agency (EMA).

III. Generation of antibodies specific to low-molecular weight food contaminants with the use of synthetic antibody libraries and phage display.

IV. Development of TRF and ELISA based immunoassays for the detection of skatole and deoxynivalenol.

4 SUMMARY OF MATERIALS AND METHODS

This section gives an overview of the general materials and methods used in this study. The more detailed description of all the materials and methods can be found in the respective original publications (I-IV).

4.1 Synthesis and labeling of antigen derivatives (I, III)

The following unconjugated low-molecular weight target antigens used in the publications were obtained from commercial vendors: Sigma-Aldrich: difloxacin (DIF), sarafloxacin (SAR), ciprofloxacin (CIP), danofloxacin (DAN), enrofloxacin (ENR), norfloxacin (NOR), flumequine (FLU) and marbofloxacin (MAR), skatole (SKA), indole (IND), indole-3-acetic acid (IAA), indole-3-propionic acid (IPR), 5-hydroxyindole (5HI) and 6-methoxyindole (6MI). Biopure: nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), T-2 toxin and HT-2 toxin.

For the immobilization and detection of the antibodies and determination of the assay performance, derivatives of certain target antigens were made (Figure 7). The biotinylated analogs, bio-NOR, bio-DIF, bio-cFQ, bio-SKA_1 and bio-SKA_2, used in the enrichment and immobilization steps of the antibody generation were labeled with the use of sulfo-NHS-LC-LC-, or amine-PEG11-biotin conjugated to the amine orcarboxylic acid groups of the respective antigens (Table 1). In addition europium labeled tracer analogs the fluoroquinolone antigens of NOR and cFQ were made with the use of 9-dentate europium chelate {2,2`,2``,2```-{[2-(4-isothiocyanatophenyl) ethylimino]-bis(methylene)bis{4-{[4-(α-galactopyranoxy)phenyl]ethynyl}- pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetate)}europium(III) (Von Lode *et al.,* 2003). Purification of the derivatives was done with the use of Dionex Ultimate LC3000 (Thermo Scientific) HPLC with the use of C18 10x15 or Hypurity C18, 150 x 4.6 column (Thermo Scientific). The derivatives were analyzed with JEOL JNM-GX-400 for the ^{1}H and for ^{13}C NMR spectra and with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems).

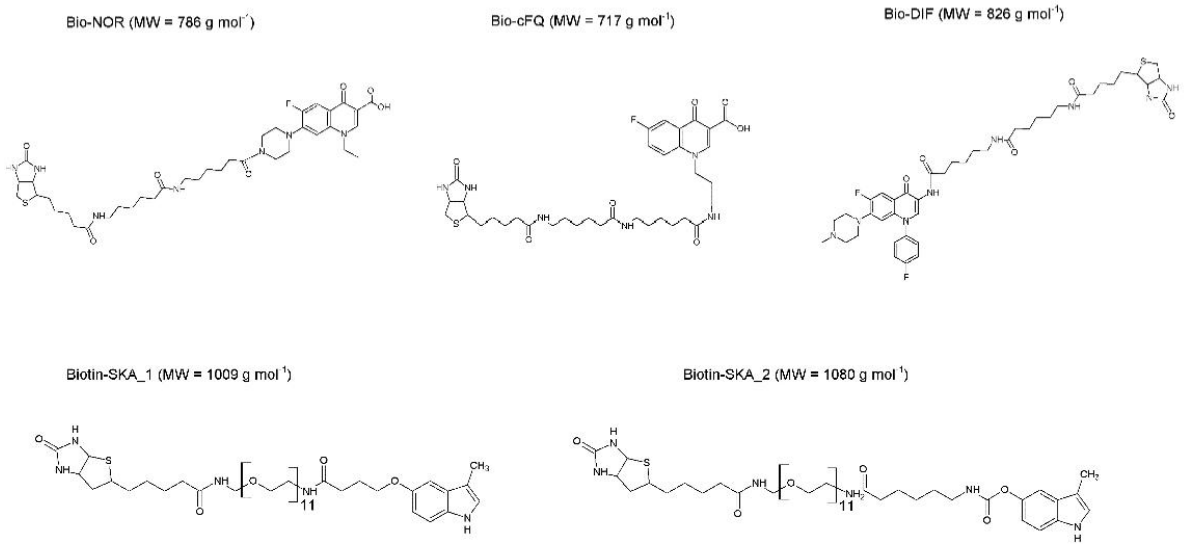


Figure 7. Structures of the biotinylated fluoroquinolone and skatole derivatives used for the immobilization and detection of the antibodies and phages during the antibody development process described in publications I-III.

Table 1. The biotinylated antigen derivatives used in the phage display selections and immunoassays.

Antigen	*Description*	*MW (calculated)*	*MALDI-TOF (found)*	*Study*
Bio-NOR	Sulfo-NHS-LC-LC-biotin conjugated to the N-2 of the piperazine ring of norfloxacin	786	773	I, II
Bio-DIF	Sulfo-NHS-LC-LC-biotin conjugated to the C-3 carboxylic group of difloxacin	826	825	I, II
Bio-cFQ	Sulfo-NHS-LC-LC-biotin conjugated to the N-1 amino group of the core fluoroquinolone	717	717	I, II
Bio-SKA_1	Amine-PEG11-biotin linked 3-methylindole with ether bond containing spacer	1009	1008	III
Bio-SKA_2	Amine-PEG11-biotin linked 3-methylindole with carbamate bond containing spacer	1080	1080	III

4.2 Antibody library construction and expression (I, II, III)

Three different EP-PCR and one oligonucleotide directed antibody libraries were constructed on the basis of previously generated antibodies. Two of the libraries were used to broaden the specificity of monoclonal fluoroquinolone antibody with low and high mutation frequencies (I), and one was constructed for the affinity maturation of a skatole specific antibody (III). The fluoroquinolone antibody was later enhanced further with the use of oligonucleotide directed mutagenesis to emphasize the broad specific binding of fluoroquinolones (II). In addition, two previously established synthetic single framework antibody libraries (Brockmann *et al.,* 2011 and Huovinen *et al.,* 2013) were used in this study for the development of antigen specific antibodies described in articles III and IV. The antibodies were displayed in Fab (fluoroquinolone) and scFv (SKA, DON) format fused with either pIII or pIX coat protein of the M13 bacteriophage. The DNA vectors used for the phage display selections and expression of the antibodies are summarized in table 2.

Table 2. DNA vectors used in the phage display selections and expression of the soluble antibodies described in the studies.

Vector	*Functionality*	*Study*	*Reference*
pAK100	Monovalent display with VCSM13 phage coat protein pIII	I	Krebber *et al.,* 1997
pAK200	Monovalent display with VCSM13 phage coat protein pIX	II	Krebber *et al.,* 1997
pEB32x	Monovalent display with VCSM13 phage coat protein pIII	III, IV	Brockmann *et al.,* 2011
pAK400	Periplasmic expression with His(6)-tag	I, II, III	Krebber *et al.,* 1997
pLK06H	Periplasmic expression in fusion with bacterial alkaline phosphatase with His(6)-tag	IV	Huovinen *et al.,* 2013

The predefined regions of fluoroquinolone and skatole antibody genes were targeted for random mutagenesis. The libraries were constructed with the use of commercial EP-PCR kit, Mutazyme II, supplied by Stratagene where the mutation frequency can be controlled with initial template concentration and nucleotide composition. The construction of the libraries followed the same protocol with following conditions: (I) for the fluoroquinolone antibody the region for randomization was the VH-region of the 6H7 Fab fragment. The oligonucleotides used for the amplification were WO1132-primer 5´-CGGCCTGCTGCTGCTG and WO415-primer 5´-TGGGCAGCAGATCCAGGG (TAGC, Denmark). Two libraries were constructed parallel with low (Lib 1) and high (Lib 2) mutation frequencies. (III) The skatole scFv antibody SKA.C9 EP-PCR library was randomized for both VL-, and VH-regions with the use of JLe01_ss 5´-CGGCAGCCGCTGGATTGTTATTAC and JLe01_as 5´-ACCAGAACCGCCACGAC CTTC (Oligomer, Finland) oligonucleotides. The genes were targeted for low-medium randomization, with 2-5 nucleic acid substitutions per 1000 bases according to the manufacturer's instructions. The randomized gene constructs were cloned to phagemid vectors pAK100 (fluoroquinolone) and pEB32x (skatole) with *SfiI* digestion for co-expression with truncated phage surface protein pIII.

To further enhance the binding properties the CDR-H3 region of a fluoroquinolone antibody (m81516) was diversified with the use of Kunkel mutagenesis (Figure 8. Kunkel *et al.,* 1987). Prior to the randomization, stop mutation and a *SacII* restriction site was introduced to the CDR-H3 loop in order to ensure that only the mutated clones would be functionally displayed on the phages. The degenerate oligonucleotide was designed to introduce 1-3 nucleotide substitutions to the central areas of the CDR-H3 loop with the following sequence, randomized area is shown underlined: 5´-TTGGCCCCAGTAGTCAAA<u>AAAAATCTCCCCGTAGTAACCATCATAGAT</u>GGGTCGA ACACAGTAATATG. The pAK200 phagemid vector with pIX surface expression was used for the phage display selections.

XL1-Blue and BL21 *Escherichia coli* –strains were used to produce the antibodies of interest after the selections. The expression vectors used for the soluble production of the antibodies was done in either monomeric form (pAK400) or as a dimer in fusion with bacterial alkaline phosphatase (pLK06H). Both expression systems contained a hexa-histidine peptide tag for the purification of the proteins. Commercial kits containing Ni-NTA matrix were used to purify the antibodies from the periplasmic extract of the cell cultures.

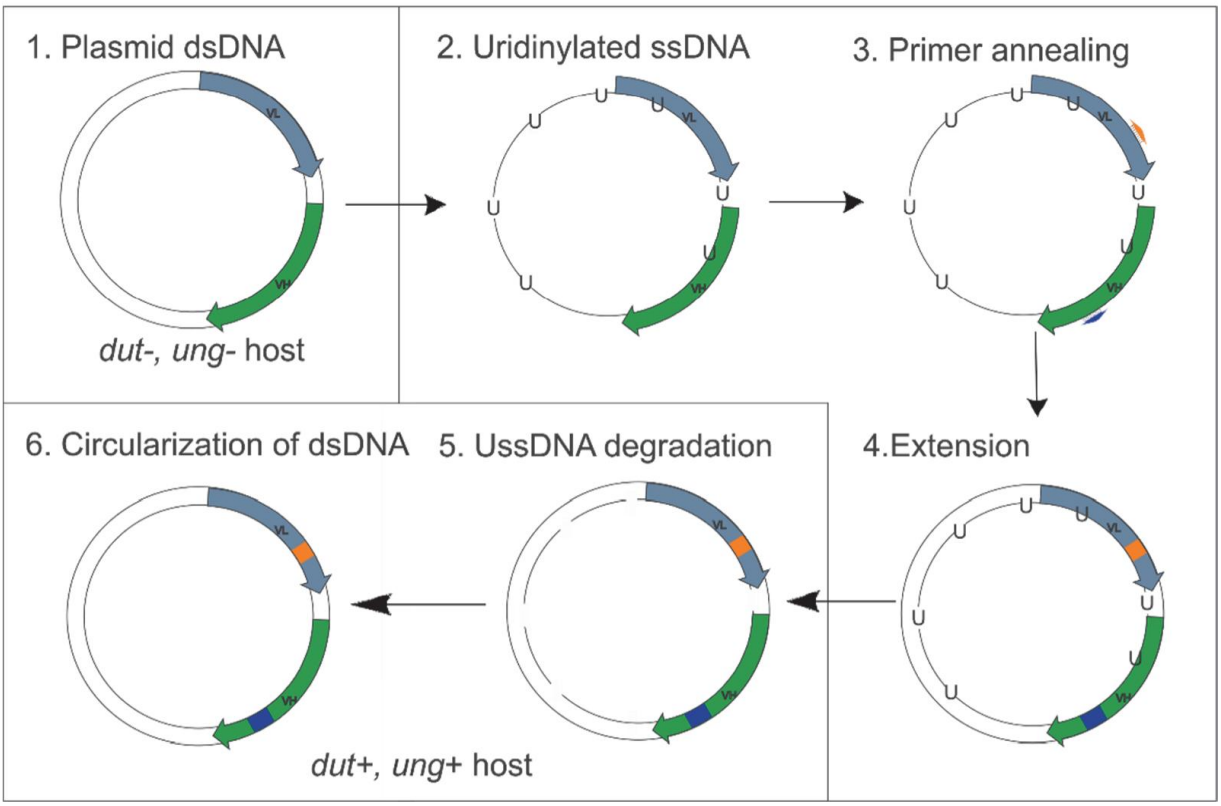


Figure 8. Kunkel mutagenesis method. 1. The target DNA is repropagated in a bacterial host containing *dut- ung-* genotype. 2. Uracil containing ssDNA can be isolated from e.g. M13 phages. 3. The mutagenic oligonucleotides are annealed to the ssDNA. 4. Mesophilic DNA polymerases is used for the extension of the new strand. 5. After the transformation of the dsDNA to a new host, the parental uracil containing DNA is preferentially degraded. 6. The recircularized dsDNA contains the mutations introduced with the mutagenic oligonucleotides.

4.3 Library sorting and screening

The *Escherichia coli* –strains and bacteriophages which were used for the phage display selections and antibody expression are summarized in table 3. All phage display selection steps were done with the use of magnetic nanoparticles manufactured by Dynal (Invitrogen) either by immobilizing the biotinylated small molecule derivatives to streptavidin coated beads (I, II, III) or by directly conjugating the beads with antigen recognizing antibody (IV). Consecutive rounds of phage display selections were carried out to enrich antigen specific phage populations where enrichment was monitored by calculating the amount of colonies on the plated infections (output) and with the use of TRF-based immunoassay. The selection stringency was controlled by increasing the amount of washes and decreasing the antigen concentration 2-10-folds between the selection rounds. In addition, depletion steps were

included for the synthetic antibody library selections, where the phages were incubated with streptavidin surface prior to the addition of antigen to prevent the enrichment of the unspecific binders.

Table 3. Following *Escherichia coli* –strains and bacteriophages were used in the construction, sorting and expression of the antibody libraries:

	Strain/phage	*Genotype*	*Study*	*Vendor/reference*
Escherichia coli	XL1-Blue	(recA1, endA1, gyrA96, thi-1, hsdR17, relA1, lac [F`, TetR]), SS320 (galE15, galK16, rpsL, hsdR2, mcrA, mcrB1, lac [F`, TetR]).	I-IV	Agilent technologies, USA
	SS320	(galE15, galK16, rpsL, hsdR2, mcrA, mcrB1, lac [F`, TetR]).	I, III	Sidhu *et al.*, 2000
	BL21	(F-, dcm, ompT, hsdS[rB-mB-], gal [malB+],K-12[λS]).	III, IV	New England Biolabs, USA
	KJ236	(ung-, relA, dut-, thi-, spoT, mcrA, [FΔ(HindIII), Tra+, Pil+, CamR]).	II	New England Biolabs, USA
Helper phages	VCS-M13	KanR	I-IV	Agilent technologies, USA
	Hyperphage VSC-M13	KanR, K07ΔpIII	III	Progen Biotechnik, Germany

Individual antibody clones were screened from the enriched binder pool with two step with immunoassay. i) In primary screening the antigen specificity of the antibodies, either displayed on phages or expressed in soluble form, were determined. ii) The secondary screening was done with the use of free antigens in a competitive immunoassay where specific binding properties, such as the cross-reactivity and IC50 were preliminarily assessed. Antibodies with most promising binding properties were produced in larger scale and selected for further characterization with sequential and structural analysis, and validation of assay performance.

4.4 Immunoassays

Immunoassays were used in all studies to follow the enrichment of antigen specific binders between the selection rounds, to screen antibodies with desired binding properties and to characterize the performance of the found antibodies (Figure 9). The raw milk which was used to determine the matrix effect and analysis of assay performance of the fluoroquinolone antibody (II) was obtained from a local farmer which was freshly milked (<16 h) and assumed free of any antibiotic residues. All immunoassays were done in 96-well microtiter wells coated with streptavidin or rabbit anti-mouse IgG (RAM). DELFIA series instrumentation and reagents were used for the assays. The TRF and absorbance measurements were done with 1420 Victor Multilabel Counter (Perkin-Elmer, Finland).

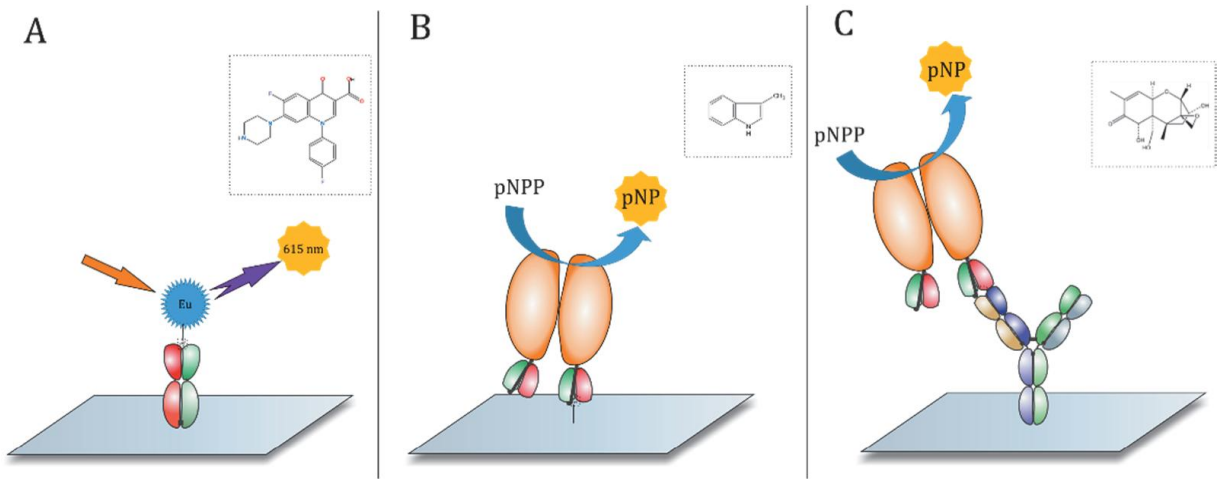


Figure 9. The principle of the competitive immunoassays developed in the publications. (A) Broadly specific Fab (mH3D7) was used as a capture in a TRF based assay using europium labeled fluoroquinolone analogs (Publications I and II). (B) Competitive ELISA developed for the detection of skatole (Publication III). ScFv-BALP fusion protein (ESKA.H6) were used as reporter for the the detection of biotinylated skatole derivatives. (C) Concept of the assay developed for the detection of DON and int two metabolic isoforms (Publication IV). Monoclonal 10B5 IgG was used as a capture for the detection of scFv-BALP (cDON_1). The binding interaction can be inhibited with the use of free forms of DON.

5 SUMMARY OF RESULTS AND DISCUSSION

5.1 Synthesis of antigen derivatives (I, III)

One of the key elements in the successful development of antibodies with generic binding specificity is the design of an antigen which contains all the relevant regions of the target compounds while omitting all the potentially immunogenic side-chains (Pinacho *et al.,* 2012). In order to broaden the specificity of the monoclonal 6H7 antibody towards the recognition of all eight target fluoroquinolone compounds, a synthetic small molecule derivative was designed. The aim was to generate a structure which would contain the 4-quinolone ring with exposed carboxylic acid group attached at position C-3 and fluorine group at position C-6, which are shared by 27 different fluoroquinolones (Cao *et al.,* 2009). This synthesized derivative was named core-fluoroquinolone (cFQ) which was used as a precursor for the biotin and europium labeled derivatives (bio-cFQ and eu-cFQ). The synthetic route and the structure of the cFQ is described in more detail in figure 10 and in publication I.

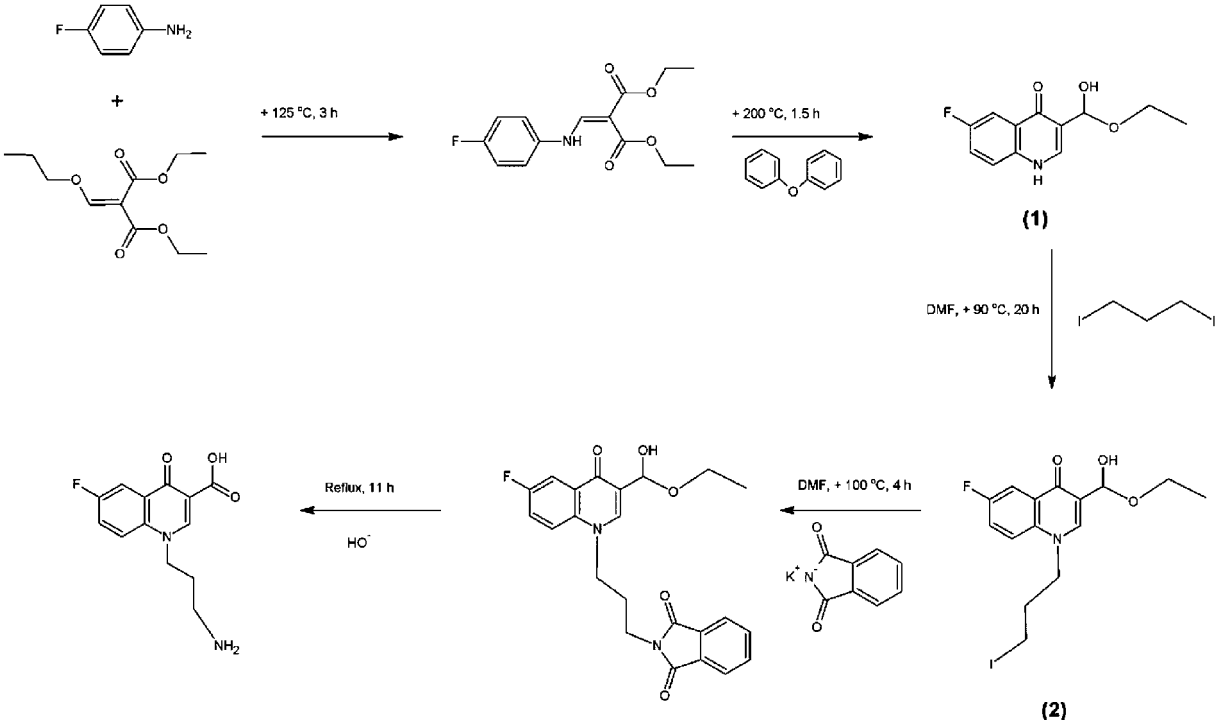


Figure 10. Synthetic route for cFQ precursor development. The precursor (1-[3-aminopropyl]-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid) was used to create the biotin and europium labeled derivatives

The antibodies generated against haptenic structures may have only limited specificity towards the target compound as the paratope of the antibody can be considerably larger than the antigen. This can cause unwanted recognition of the regions surrounding the antigen or the linker used to immobilize the antigen (Franek, 1987). To overcome this problem, novel skatole derivatives were designed to be used as selection antigens in the generation of novel binders against the 3-methyindole structure derived from synthetic antibody repertoire. Two structures were designed with different spacer regions to direct the selection pressure towards the regions available in the free form of skatole. The synthesized structures contain different bonds (ether and carbamate) in the C-5 position of the indole ring (Figure 11). The synthetic structures were labeled with amine-PEG11-biotin and used as selection antigens in the phage display selections.

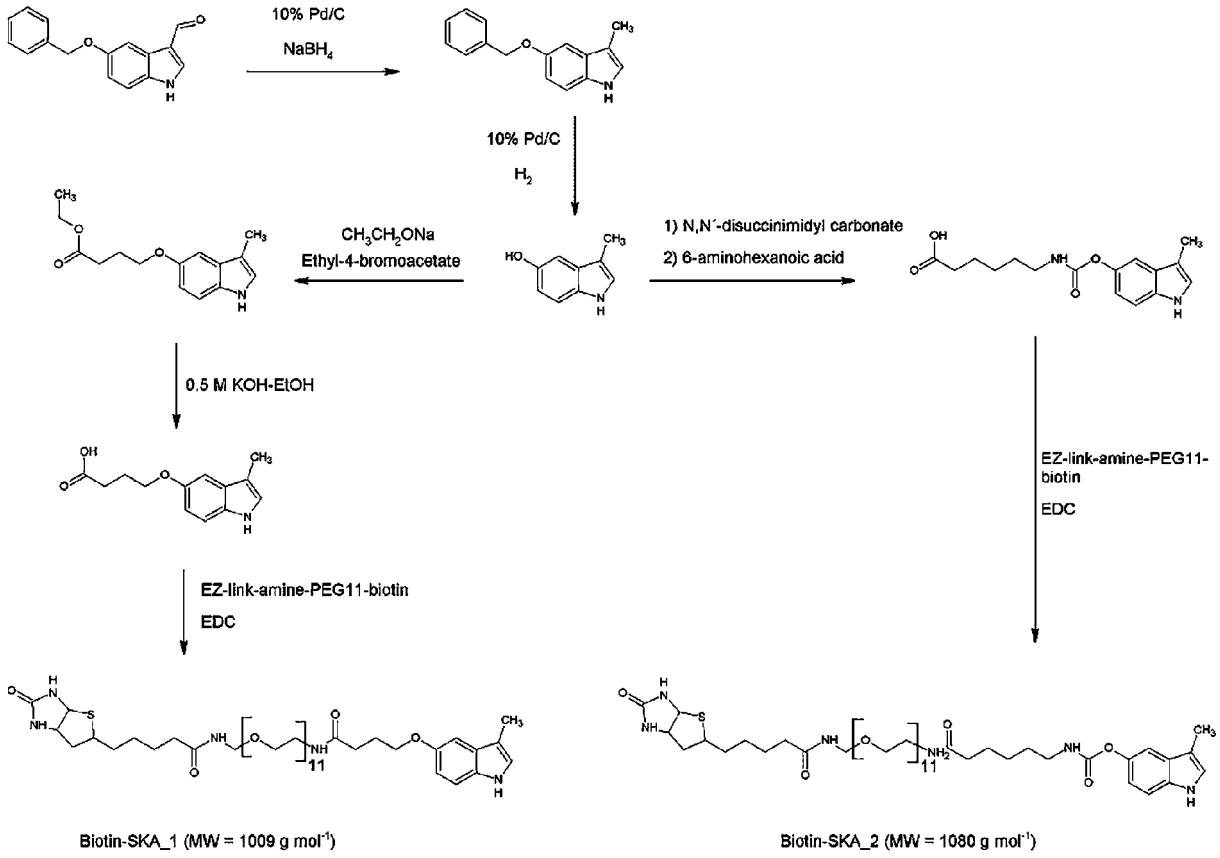


Figure 11. Synthetic route of the biotinylated skatole derivatives. The two biotinylated compounds biotin-skatole_1 (Bio_SKA_1) and biotin-skatole_2 (Bio_SKA_2) were synthesized from the same precursor. The two structures differ in the linker region connected to the C-5 position of the indole ring.

5.2 Engineering of fluoroquinolone specific antibodies (I and II)

The starting point for the study was a monoclonal antibody 6H7, raised with the use of cHSA conjugated sarafloxacin as immunogen. The aim of the study was to broaden the specificity of the wild-type 6H7 with random mutagenesis to enable the simultaneous recognition of all eight target fluoroquinolones. The VH and VL regions of the IgG were cloned in to Fab format for bacterial expression and the construction of antibody libraries. The functionality of the 6H7 Fab was analyzed with the use of biotinylated and europium labeled fluoroquinolone derivatives. In the preliminary characterization of the antibody, the affinity of the 6H7 against one of the target antigens, eu-NOR was found to be 1.13 x 10^9 nM (Figure 12). In addition, the binding specificity against other biotinylated fluoroquinolone derivatives was assessed confirming that the antibody was capable of detecting all of the biotinylated derivatives except for bio-DIF, which was later omitted from the phage display selections. The binding of bio-NOR and lack of specificity for bio-DIF, gave the first indication that the binding regions of the antibody, or epitope, was located in the vicinity of the carboxylic acid group attached at position C-3 of the 4-quinolone ring.

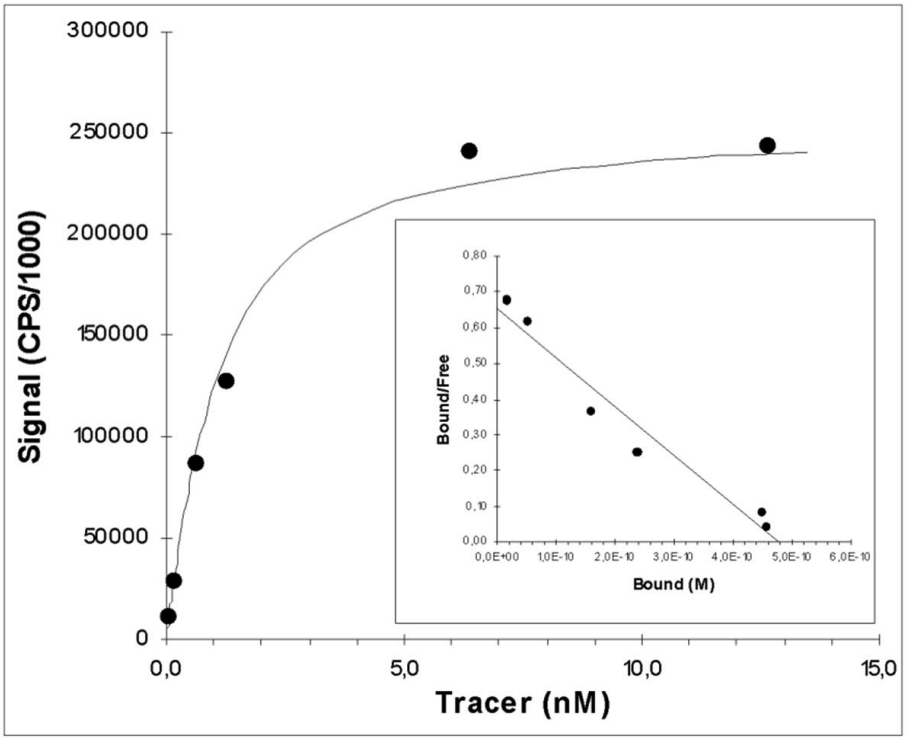


Figure 12. Schatchard plot of the 6H7 Fab fragment. Europium labeled NOR was used as a tracer to determine the dissociation constant for the antibody calculated from the slope.

Based on the cross-reactivity profile of 6H7, the antibody was capable of recognizing six out of eight fluoroquinolones below the MRLs. Consecutively, the main focus for the mutagenesis and selections was to improve the binding of FLU and MAR. The engineering of the native 6H7 for more generic specificity was done in two phases described in more detail in publications I and II: Phase one consisted of the construction of two randomized libraries with EP-PCR, where the whole VH region was targeted for mutagenesis with mild and heavy randomization. In phase two the antibody which was shown to have most generic binding towards the eight target fluoroquinolones was selected to be the starting point for oligonucleotide directed mutagenesis.

The recombinant antibody format was selected to be Fab over scFv due to the ease of handling during screening and characterization steps. Also the potential loss of affinity and specificity might have hindered the assay performance (Choi *et al.,* 2004). However the display of the Fab fragment on the phages proved out to be low especially in the second phase mutagenesis. This may be the result of the pIX display, which was selected for display method in hope to emphasize the monovalent display, which in turn with successful selections should result in more high affinity binders. The display of the Fab fragment in fusion with pIX had a negative effect for both the enrichment of the antigen specific Fab-phages during the selections and to the overall number of clones found from the libraries.

The characterization of the found mutant antibodies was done on the basis of amino acid changes (table 4), IC50-values and cross-reactivity profile (table 5). From the clones analyzed after the first phase of mutagenesis, the mutations with most beneficial effect to the specificity of the antibody was shown to be in the framework region of the antibody. In phase two the amino acid residues most critical to the specificity of MAR was observed in the central region of the CDR-H3 loop. Mutations affecting residues Y105-F110 were found out to have a negative effect on the recognition of MAR. After phase two, the main focus for the antibody characterization was on two clones: mH3D7 and mH3G1, from which mH3D7 was ultimately selected as the most improved and used in the assay optimization steps.

Table 4. Observed amino acid changes in the variable heavy region of the fluoroquinolone antibodies. The most improved clones found after the selection phases are shown in bold.

	Observed mutations in variable heavy domain*						
Clone	**Framework 1**	**CDRH1**	**Framework 2**	**CDRH2**	**Framework 3**	**CDRH3**	**Framework 4**
6H7	-	-	-	-	-	-	-
m8042	-	-	-	-	I71V, V85I, A90P	-	T119I
m8158	T3S, T21S	-	-	-	L69F	-	-
m81516	G10E, T21A	-	G46D	-	-	-	-
m5A7	V2I	-	-	-	I71F	P100L	T118I
m5C4	S25T	-	-	-	T92I	P100I	-
m5H5	T3I	-	A51T	-	-	-	-
m5H6	-	-	A51T	-	-	-	K83N
mH3A1	-	-	-	-	-	Y106N, G107R	-
mH3A11	-	-	-	-	-	G104S, Y105E, Y106S	-
mH3B1	-	-	-	-	-	D103S, Y105N, G107R	-
mH3D7	-	-	-	-	-	G104Y, E108Q	-
mH3D9	-	-	-	-	-	D103Y, Y105F, Y106D, E108G, F110Y	-
mH3G1	-	-	-	-	-	D103Y, G104S	-

* Region definitions and amino acid changes according to Kabat nomenclature

Table 5. IC50 values and cross-reactivity profile of the clones found from the first phase of engineering.

	Antigen															
	Sarafloxacin		Difloxacin		Danofloxacin		Ciprofloxacin		Enrofloxacin		Norfloxacin		Flumequine		Marbofloxacin	
Clone	IC50 (µg L-1)	CR (%)	IC50 (µg L-1)	CR (%)	IC50 (µg L-1)	CR (%)	IC50 (µg L-1)	CR (%)	IC50 (µg L-1)	CR (%)	IC50 (µg L-1)	CR (%)	IC50 (µg L-1)	CR (%)	IC50 (µg L-1)	CR (%)
6H7	4,36	100,00	2,49	175,10	12,39	35,22	21,09	20,70	19,89	21,94	22,17	19,68	349,39	1,25	856,64	0,51
m8042	3,51	124,23	0,68	641,63	18,51	23,58	137,99	3,16	110,06	3,97	93,45	4,67	406,00	1,07	808,52	0,54
m8142	2,66	164,13	0,58	746,83	12,74	34,25	84,31	5,18	89,88	4,86	61,20	7,13	312,73	1,40	842,54	0,52
m81516	0,20	2206,15	1,24	352,58	7,51	58,10	19,57	22,30	12,87	33,92	10,44	41,81	337,95	1,29	549,58	0,79
m5A7	60,43	7,22	24,98	17,47	29,65	14,72	142,70	3,06	31,04	14,06	58,34	7,48	639,57	0,68	620,31	0,70
m5C4	16,37	26,66	7,86	55,55	12,06	36,18	84,66	5,16	13,01	33,53	15,01	29,08	187,40	2,33	1444,54	0,30
m5H5	0,34	1294,11	0,84	519,10	8,31	52,50	16,57	26,34	14,27	30,58	24,72	17,66	578,04	0,76	792,76	0,55
m5H6	8,10	53,87	1,71	255,97	17,60	24,80	17,29	25,24	25,62	17,03	40,56	10,76	399,37	1,09	744,20	0,59
MRL**	10/30		300		100/200		100**		100**		30		600/400		100	

* Set by *EU2377/90*

** Allowed sum for enrofloxacin and ciprofloxacin is 100 µg kg^{-1} in both fish and chicken. Other values are for fish/chicken muscle, respectively.

5.3 Optimization of broad-specificity immunoassay for fluoroquinolone detection (II)

The most enhanced mutant antibody, mH3D7, which was found from the antibody libraries showing broad specificity to the target fluoroquinolones was used in a TRF-immunoassay which was optimized and the matrix effect was determined with the use of spiked raw milk samples. As fluoroquinolones have been shown to possess pH dependency which has a strong effect on the antibody-antigen binding interaction (Pinacho *et al.,* 2012) the assay conditions were optimized. Parameters such as the amount of washes, salt concentration, pH and incubation time were tested for optimal assay performance. The pH dependency, was found to be crucial for the performance of the immunoassay. As shown in figure 13, the pH has very strong effect to the IC50 value of MAR when the assay buffer pH increases >7.0. On the basis of the assay optimization the pH of the buffer was set to 6.5 for the assay performance determination. In later studies, it has been noticed that the addition of chelating agents such as EDTA, could further improve the assay performance (Mi *et al.,* 2013).

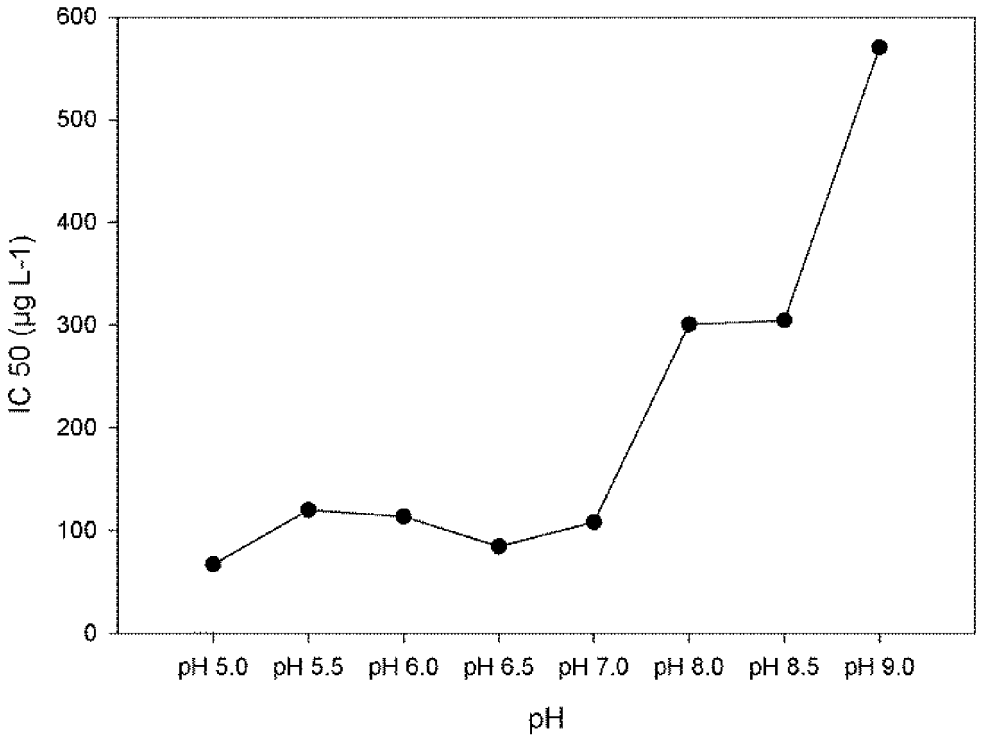


Figure 13. Correlation of the assay buffer pH in the determination of IC50-value for marbofloxacin.

The overall improvement resulting from the engineering of the antibody and optimization of the assay performance varied depending on the antigen. For SAR and DIF significant improvement in the respective IC50-values were achieved in comparison to the wild-type 6H7 and the most improved mutant mH3D7 (table 6). In addition, the initial problems regarding the poor recognition of MAR and FLU were resolved, as considerable improvement in the recognition of these two compounds was observed after the second phase mutagenesis. Additional improvement was gained from the optimization of the assay. With the use of mH3D7 and optimized assay setup it is possible to detect all eight EMA controlled fluoroquinolones simultaneously below the MRLs.

Table 6. The IC50 and cross-reactivity values of the optimized TRF-assay. The improvement resulting from the assay optimization for the clone mH3D7 is shown in parentheses.

FQ	mH3D7		m81516		6H7		MRL*
	IC50 (µg L^{-1})	CR (%)	IC50 (µg L^{-1})	CR (µg L^{-1})	IC50 (µg L^{-1})	CR (%)	(µg L-1)
DIF	0,22 (-0,09)	100,0	1,24	16,0	2,49	100,0	400
SAR	0,23 (-0,03)	92,5	0,20	100,0	4,36	57,1	100
CIP	5,03(-5,26)	4,4	7,51	2,6	21,09	11,8	100/100
DAN	4,69 (-4,00)	4,7	19,57	1,0	12,39	20,1	30/200
ENR	4,78 (-1,47)	4,6	12,87	1,5	19,89	12,5	100/100
NOR	5,14 (-0,13)	4,3	10,44	1,9	22,17	11,2	N/A
FLU	12,68 (-4,23)	1,7	337,95	0,1	349,39	0,7	50/100
MAR	68,05 (-120,76)	0,3	549,58	0,1	856,64	0,3	75/150

* Maximum residue limits for bovine milk/meat

Spiked raw milk samples were used to determine the assay performance and assess the effect of using raw milk as a sample matrix (table 7). Although some interference could be observed in a direct comparison of assay buffer and milk, satisfactory recoveries (>70%) of fluoroquinolones were obtained (table 8). The lower recoveries for DIF and SAR maybe the result of too high fortification level. Apart from DIF and NOR the CV% were also good for untreated samples. The pretreatment (Guanidine-HCl) of the raw milk samples prior to the use in assay was also tested with very low impact on the recoveries or sensitivity of the assay (data not shown).

Table 7. TRF-assay for the analysis of matrix effect to the limit of detection (LOD) and linear range in assay buffer and milk.

FQ	Assay buffer		Milk	
	LOD ($\mu g\ L^{-1}$)	Linear range ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	Linear range ($\mu g\ L^{-1}$)
DIF	<0.01	0.1-4.7	0.09	0.1-0.9
SAR	<0.01	0.1-3.4	0.11	0.1-0.8
CIP	0.32	0.7-52.5	0.34	0.5-14.6
DAN	0.33	0.6-48.9	1.30	2.1-86.9
ENR	0.39	0.8-39.0	1.34	1.9-39.6
NOR	0.32	0.7-67.4	2.27	3.2-51.8
FLU	0.60	1.2-77.1	3.80	4.8-37.5
MAR	2.30	8.5-505.0	33.90	44.0-275.0

Table 8. Detection of fluoroquinolones from spiked raw milk samples.

FQ	Spiked ($\mu g\ L^{-1}$)	Found ($\mu g\ L^{-1}$)	Recovery (%)	CV (%)
DIF	0.5	0.45	89.7	23.18
	2	1.31	65.4	4.29
SAR	0.5	0.39	77.5	8.21
	2	1.38	69.2	6.01
CIP	5	4.58	91.6	5.82
	10	9.79	97.9	5.11
DAN	5	3.62	72.3	4.11
	10	9.78	97.8	0.07
ENR	5	5.50	110.0	0.77
	10	9.16	91.6	1.06
NOR	5	4.52	90.4	16.02
	10	10.09	100.9	0.57
FLU	10	9.43	94.3	1.14
	20	14.65	73.3	3.09
MAR	50	37.07	74.1	2.23
	100	78.82	78.8	0.01

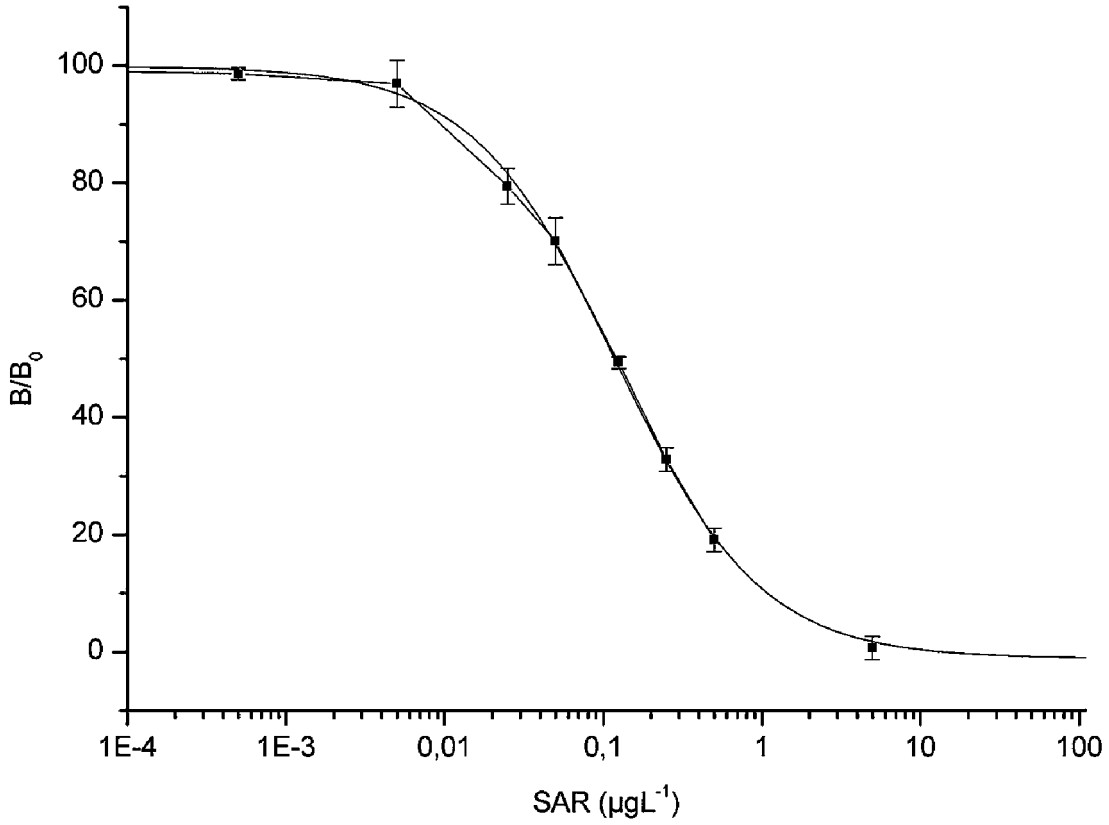


Figure 14. Inhibition curve of mH3D7 mutant antibody using SAR as competitior. The error bars indicate the standard deviation of three replicate wells.

In the optimized TRF assay setting the mH3D7 mutant has almost 20-fold improvement in IC50-value of SAR in comparison to the monoclonal 6H7 antibody, which was used as the starting point for this study. SAR, which was initially selected as the immunogen of 6H7 due to the lowest regulatory MRLs in aquaproducts (10 µg kg^{-1}), can be detected with good sensitivity (LOD<0.01 µg L^{-1}) with the use of mH3D7 (Figure 14).

5.4 Development and engineering of a skatole specific antibody (III)

One of the most common challenges in antibody generation with the use of synthetic antibody libraries and phage display selections is the unwanted enrichment of binders specific to the surrounding environment of the immobilized target antigen. The use of depletive selection steps prior the introduction of the antigen has shown to efficiently reduce the binders specific to the surfaces used for immobilization such as streptavidin. With low-molecular weight targets, as with skatole, the selection antigen itself should also be designed to provide means for the selection of binders in carefully predetermined regions of the target compound. In this study (III), two biotinylated skatole derivatives with structurally different spacer regions were designed to direct the selection pressure towards the region available in the free form of the antigen. In addition, the surface used to immobilize the biotinylated antigen was changed to neutravidin, a deglycosylated form of avidin, after the first selection round to reduce the amount of unspecific binders. After three rounds of phage display selections, clear enrichment of antigen specific binders could be observed from the TRF-assay. However, as shown in figure 15, even though the two selection antigens of skatole differ only in the spacer group attached to the C-5 position of the indole ring, the biotinylated skatole structure used in the first selection round (Bio-SKA_1) was recognized considerably stronger.

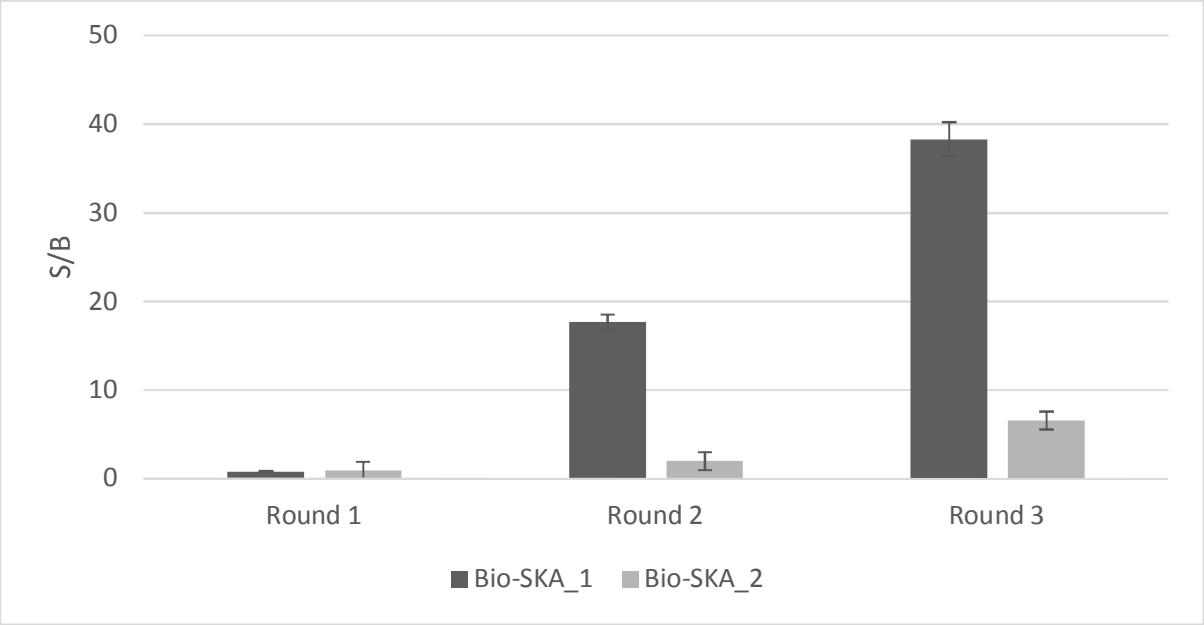


Figure 15. Signal to background (S/B) ratios of the phage stocks prepared after each phage display selection round.

On the basis of preliminary screening the antibody with the most promising IC50-value SKA.C9 was used as a starting point for further engineering. Due to the very limited amount of interacting amino-acids in the antigen binding site, only soft randomization by EP-PCR was introduced to the variable regions of the SKA.C9 scFv. On the basis of the sequence data of the ESKA.H6 contained only one amino acid change in the gene, which improved the IC50 value from 574 µg L^{-1} (SKA.C9) to 245 µg L^{-1} (ESKA.H6). The observed mutation, (D66E) was located in the end of CDR-H2 loop.

Table 9. IC50-values and cross-reactivity for different indolic compounds of the clone ESKA.H6.

Compound	*IC50* (*µg L^{-1}*)	*CR* (*%*)
3-methylindole	245	100
indole	3013	8.1
indole-3-acetic acid	>5000	<0.1
indole-3-propionic acid	>5000	<0.1
5-hydroxyindole	>5000	<0.1
6-methoxyindole	3707	6.6

The binding properties were further analyzed with cross-reactivity for other relevant indolic compounds. As the data from the table 9 indicates, the binding of the antibody ESKA.H6 is most likely interacting in the proximity of the methyl group attached to the indole ring. Only moderate cross-reactivity can be observed for indole (8.1%) and 6-methoxyindole (6.6%). The two most relevant structurally similar compounds which might cause cross-reactivity related interference in the assay, indole-3-acetic acid and indole-3-propionic acid, did not cross-react with in the assay. The performance of the antibody creates an excellent starting point for the further development of assay concept suitable for high-throughput screening of boar taint.

5.5 Detection of DON with small-molecule based inhibition (IV)

Synthetic antibody libraries have been shown to be useful in the generation of antibodies against targets which are difficult to develop with traditional immunization based methods. Anti-idiotype antibodies (anti-Id-Abs) are capable of detecting regions of another antibody are one example of such difficult antigens (Bona *et al.,* 1984). Traditionally, with immunization based methods, the generation of anti-Id-Abs relies on the chemical conjugation of the target antigen to the antigen binding site. The use of phage display enables the use of more targeted selection strategies as the conditions of the selection process can be easily controlled. This enables the use of more diverse antigenic structures, such as an existing antibody-antigen complexes. Previously constructed synthetic antibody library and phage display were used to generate anti-Id-Abs against DON specific monoclonal antibody (10B5). The selections were carried out in the presence of free DON. Additionally, the selection pressure was targeted towards the recognition of structures in the vicinity of the antigen-antigen binding site by depletion step, where the phages were incubated with 10B5 Mab. After three rounds of selections the binding of the parental mAb could be inhibited with the use of free DON (Figure 16).

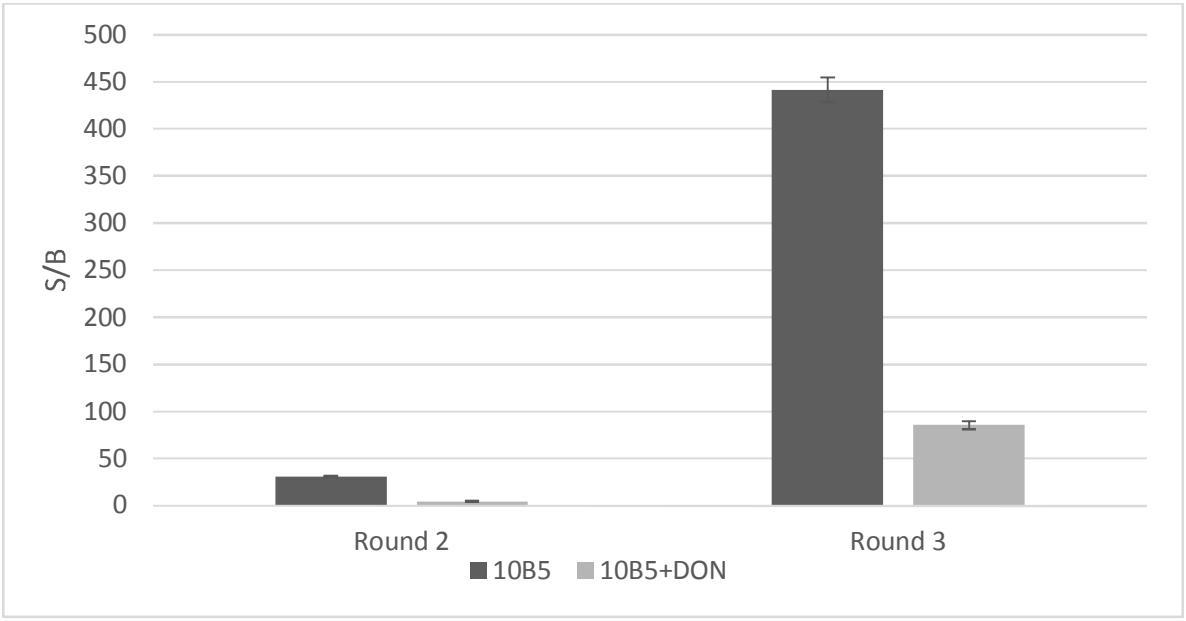


Figure 16. Enrichment of anti-idiotype antibodies subjected to inhibition of free DON. Signal to background (S/B) ratios obtained from the TRF-measurement of the phage stocks of rounds 2 and 3.

Four randomly selected antibodies were characterized further by cloning the antibody gene in fusion with BALP for soluble expression. The binding properties of the antibodies were analyzed in a competitive ELISA. On basis of the competitive immunoassay with free DON the clone showing the most efficient inhibition and was characterized further. The characterization was done with the use of two immunoassay setups: i) competitive ELISA using 10B5 as capture and BALP-scFv as reporter and ii) competitive TRF with biotinylated cDON_1 as capture and 10B5 Mab (detected with europium labeled rabbit anti-mouse IgG) as tracer. Based on the assay data the anti-idiotypic binding of the cDON_1 to 10B5 can be inhibited with all three DON isomers (Figure 17). However the binding is unaffected by other Type A and B trichothecenes (table 10). There is only minor difference in IC50-values between the two assay setups, although some decrease in the IC50-values was observed when cDON_1 is used as a capture.

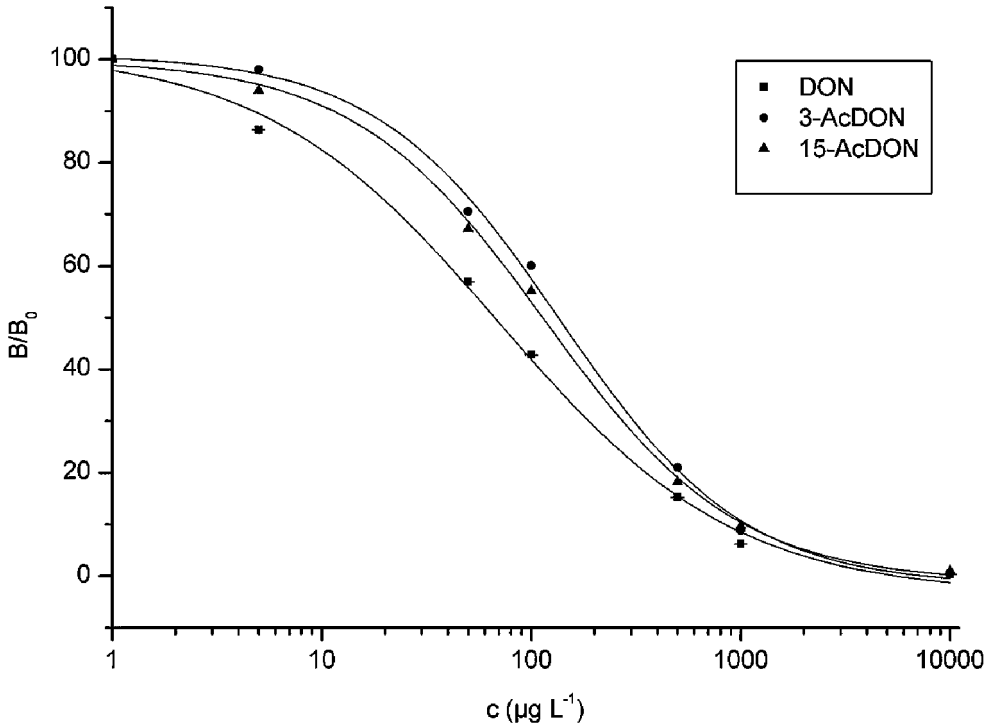


Figure 17. Inhibition curves from the competitive immunoassay (ELISA) against DON, 3-AcDON and 15-AcDON.

Table 10. The reactivity and IC50-values of different assay setups used for the detection of DON, 3-AcDON and 15-AcDON.

	cDON_1				10B5*	
	ELISA		TRF			
Target	Reactivity	IC50 (µg L^{-1})	Reactivity	IC50 (µg L^{-1})	Reactivity	IC50 (µg L^{-1})
DON	x	77,7	x	128	x	107
3-AcDON	x	111,5	x	168	x	123,8
15-AcDON	x	114,9	x	135	-	-
NIV	-	-	-	-	-	-
T-2	-	-	-	-	-	-
HT-2	-	-	-	-	-	-

*Values obtained by electrochemical immunosensor with monoclonal DON specific antibody (Romanazzo *et al.*, 2010)

6 CONCLUSIONS

Majority of screening methods used in food diagnostics rely in the use of antibodies for the specific detection of various contaminants. With the increasing amount of harmful compounds and global availability of consumer products, the detection of these contaminants needs to become more rapid and affordable, which in turn requires antibodies with exceptional binding properties. This study shows that directed antibody engineering techniques provides an efficient and versatile method both for the tailoring of the existing binders against difficult targets, and for the generation of antibodies with novel specificity.

Publications I and II show that directed antibody engineering techniques can be used to efficiently tailor the binding properties of existing binders in order to emphasize desired attribute, which in this case was generic binding of fluoroquinolones. The monoclonal broad specificity antibody was engineered with EP-PCR and oligonucleotide directed mutagenesis to recognize eight fluoroquinolone antibiotics controlled by EMA. The enhanced mutant antibody was used in a TRF based immunoassay which was optimized for the detection of fluoroquinolone residues directly from raw milk samples below the set MRLs. The performance of the assay would be especially useful for the screening of samples containing SAR, which is commonly used in aquacultures, due to the low MRLs set for marine products (10 µg/kg) and the exceptional sensitivity of the assay in the detection of SAR.

Synthetic antibody libraries can be efficiently used to create versatile binders with novel binding specificities as described in publications III and IV. Two low-molecular weight compounds were used as targets for the generation of antibodies specific to skatole and DON. In the case of skatole, the approaching legislative control of surgical castration creates an urgent need for novel high-throughput screening methods. As the availability of immunochemical methods for the detection of skatole is very limited, the antibody described in article III could, with further assay development, create a new method to avoid castration of piglets and a possible solution to the boar taint problem. The DON specific anti-Id-Abs generated in publication IV on the other hand demonstrate an interesting method for the development binders useful for the detection of low-molecular targets.

Although the assay concepts developed in publications III and IV need to be validated with the use of different sample matrixes and compared to reference methods, the studies show the high potential for the generation of antibodies

with novel binding properties directly from synthetic antibody libraries. As the selection process is rapid, the technique can be easily combined with other directed protein engineering methods. This enables the antibody generation process to become more trivial, resulting in the routine generation of high quality antibodies useful in wide range of applications.

In conclusion, this study emphasizes the great potential of directed antibody engineering techniques for the generation and tailoring of the binding properties of antibodies specific to low-molecular weight targets. These methods can be applied for the routine generation and modification of binders to create binding reagents for the development of novel assay concepts.

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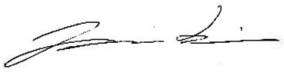
Turku, December, 2015



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