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Use of Immunocapture Techniques to Enable Efficient Sample Preparation for LC-MS/MS Analysis of Oxytocin in Human Plasma

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Abstract

Oxytocin (OT) is a small neuropeptide, which is present in human plasma at extremely low endogenous levels of only a few pg/ml. It is mainly involved during labour and lactation but it has recently also been found that OT is involved in social behaviours like bonding and feeling empathy. During the last couple of years, the interest in OT has been raised and due to the matrix complexity, it is very challenging to develop a highly sensitive assay to be able to measure endogenous OT. An established quantitative tool for the detection of peptides in biological samples is liquid chromatography (LC) combined with tandem mass spectrometry (MS/MS). However, when working with complex matrices and concentrations in the low pg/ml range, an efficient sample preparation is required to remove endogenous matrice components which interfere with the detection of the analyte by LC-MS/MS analysis. In this project the potential of using immunocapture as sample preparation was investigated. The immunocapture techniques investigated where Dynabeads® and MSIA™ pipette tips. To evaluate the success of the developed immunocapture technique a SPE method for OT, used for comparison, was optimized. Using commercially available antibodies, the Dynabeads® showed a great potential whereas the results for MSIA™ were inconclusive. The Millipore antibody MAB5296 used together with Dynabeads® Protein G was found to generate the highest recovery. To increase the recovery during optimization of the immunocapture method, parameters like elution, incubation time and sample volume were investigated. It was found that these parameters did not have a great impact on recovery but an increase in sample volume, without changing the eluate volume, did generate approximately a twofold increase in signal, making it possible to obtain a LLOQ of 10 pg/ml. The final immunocapture method showed a good linearity in the concentration range of 10 to 100 pg/ml using seven calibration points. The precision and accuracy were qualified using four quality control (QC) samples (10, 30, 45 and 85 pg/ml). The precision ranged from 12 to 15% and the accuracy ranged from -8 to 2% except for the HIGH QC-sample where it ranged from +23 to 33%. This was probably due to the use of wrongly spiked QC-samples (100 pg/ml). When comparing the final method with the SPE it was found that a much cleaner extract was obtained with the immunocapture method where endogenous OT was detected whereas no endogenous OT could be seen when using SPE. The S/N for 5 and 10 pg OT/ml plasma was 4.01 and 0.97 for the SPE prepared samples compared to 25.3 and 41.4 for the immunocapture method. These observations show that immunocapture is an efficient technique for selective extraction of a target peptide enabling LC-MS/MS detection at very low concentrations in complex matrices such as plasma.

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Preface

The experimental work was performed at the Department of Bioanalysis at Ferring Pharmaceuticals A/S, Copenhagen, Denmark. Firstly I would like to thank my supervisors at Ferring, Anders Sonesson and Karl Petterson, for their great contributions and support provided throughout the entire project. Further, I would like to thank Sara Stensson for her guidance and assistance during the SPE development and Elica Nesevska for her helpfulness regarding purchase of materials at the required time. Last but not least I would like to thank the management team Alf Carlshaf, Magnus Knutsson and Birgitte Buur Rasmussen for giving me the opportunity to perform my Master´s Thesis project in the Bioanalysis Department.

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Abbreviations

Ar: Argon

BSA: Bovine serum albumin

EIA: enzyme immunoassay

ELISA: enzyme-linked immunosorbent assay

ESI: Electrospray ionization

Fc-region: Fragment crystallisable region

Ig: Immunoglobulins

IS: Internal Standard

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LLOQ: lower limit of quantification

m/z: mass-to-charge ratio

MRM: multiple reaction monitoring

MSIA™: Mass Spectrometric Immunoassay

N_2: nitrogen gas

OT: Oxytocin

PBS: Phosphate buffered saline

QC: quality control

RIA: radioimmunoassay

S/N: signal-to-noise ratio

SPE: Solid Phase Extraction

TFA: Trifluoroacetic acid

1. Introduction

Oxytocin (OT) is a biologically significant neuropeptide, which is mainly involved during labour and lactation. Recently, it has also been found that OT is involved in several social behaviours like bonding and increasing trust and empathy in human. In addition, a possible link between OT and neurological disorders is being investigated (Zhang et al., 2011).

Oxytocin is present in human plasma at extremely low endogenous levels of only a few pg/ml. Since 2004 there are several commercially available methods for measuring OT including ELISA, EIA and RIA. Unfortunately, evaluation of these methods has shown a lack of reliability and specificity (McCullough et al., 2013). Due to the matrix complexity, it is very challenging to develop a highly sensitive assay to be able to measure endogenous OT. Liquid chromatography (LC) with tandem mass spectrometry (MS/MS) detection is an established quantitative tool for peptides in biological samples with high selectivity and sensitivity in detection. However, for the determination of peptides at low pg/ml levels in plasma samples, several studies have stressed the need for (1) efficient sample preparation techniques to provide a clean sample extract for analysis and (2) selective LC analysis using coupled columns with orthogonal separation capability, in order to establish a sensitive and robust method (Szapacs & Kellie, 2014) (Bylda et al., 2014).

Immunocapture has proven to be an efficient sample clean-up technique for the analysis of proteins and peptides in biological samples using LC-MS/MS. The immunocapture can be performed both as off-line sample preparation and on-line in a coupled column LC system (Winther et al., 2009). The goal of this project is to investigate the potential of off-line immunocapture techniques for efficient sample purification of oxytocin from human plasma to prepare a clean extract for LC-MS/MS analysis.

1.1.Problem formulation

In the pharmaceutical industry the use of lower doses of potent peptide drugs is becoming more and more interesting. Thus, this places higher demands on sensitive quantitative assays to measure peptides at low pg/ml or even lower levels which in turn requires efficient sample preparation when working with complex matrices such as plasma. This project focuses on developing a sensitive assay for the quantification of OT with aims of implementing a successful method on the company's own peptide drugs.

1.2. Aim

The aim of this project is to:

- explore different possibilities for the purification of OT from plasma samples using the off-line immunocapture techniques; magnetic beads and pre-coated pipette tips.
- explore different coating techniques in order to optimize the immunocapture process.
- develop a quantitative assay for the quantification of OT in human plasma samples with high sensitivity using LC-MS/MS.

2. Theory

2.1. Bioanalysis in the pharmaceutical industry

Bioanalysis is the measurement of drug concentrations in biological matrices such as serum, plasma, blood, urine, saliva and tissue. In the pharmaceutical industry, bioanalysis is conducted to support the development of new drugs and drug formulations.

Plasma and serum are the two most common bio-specimens for bioanalysis. The measurement of low abundance drugs in plasma is a difficult and challenging task as plasma contains endogenous lipids, peptides and proteins of a large dynamic range. Therefore, efficient sample preparation procedures are required to extract the target molecule from the biological matrix of interest before the measurement with a sensitive instrument, like LC-MS/MS, can be performed for large sample series using a robust method (Hansen & Pedersen-Bjergaard, 2015).

For bioanalysis of therapeutic peptides present at low pg/mL concentrations in plasma, the complex matrice makes the identification and quantification highly challenging.. A successful bioanalysis procedure require selective isolation of the target peptide (Hansen & Pedersen-Bjergaard, 2015).

2.1.1. Matrix Effects

To describe the problems encountered during analysis of complex biological samples, such as plasma, with variation in ionization efficiency and thus on the signal provided by the MS detector, the term matrix effects is used. Matrix effects can be caused by both endogenous and exogenous compounds eluting in the LC separation with the same retention time as the target analyte. Endogenous compounds causing interference could be metabolites of the target analyte, lipids, peptides or proteins. The exogenous compounds are all the substances introduced during sample preparation and analysis. Studies have shown that the most important interferents are phospholipids. These polar lipids are very difficult to remove from the biological samples. Phospholipids are very abundant in serum and plasma since they are major constituents of cell membranes. Phospholipids consist of two functional groups, a hydrophilic head made up of choline and phosphate units, and a hydrophobic tail composed of fatty acyl chains. These two functional groups cause ion suppression effects during LC-MS/MS analysis especially when using electrospray ionisation (Bylda C. et al., 2014).

Several approaches can be taken to reduce or eliminate matrix effects. One strategy is to optimize the chromatographic separation so that the target analyte and interfering compounds are separated in time but unfortunately this can result in very long run times. Another strategy is to instead optimize the sample preparation to obtain a clean extract of the target analyte. A proper sample preparation can eliminate or strongly reduce the matrix effects.

2.1.1.1. Internal Standards

An internal standard (IS) is used in a quantitative LC-MS/MS method to compensate for any variation caused during sample preparation, sample injection and the final LC-MS/MS detection. A good IS usually has a structure or physical/chemical properties similar to the analyte to be measured. Therefore stable isotope IS such as ^{2}H, ^{13}C and ^{15}N-labeled analogues are the preferred IS. An internal standard is added in a constant concentration to the samples.

A good IS should compensate for endogenous and exogenous differences between samples and sample handling reproducibility during the analysis procedure. The ratio of IS to given analyte concentration remains constant because the same fraction of each is lost during sample preparation and sample analysis (Harris D. C., 2010). However, a large variability in the IS response through a sample series (e.g. 96 samples) can cause concerns regarding the assay's resilience to subtle changes in the matrix composition and may raise questions about the trueness of the results. A Standard Operating Procedure (SOP) describing the acceptance criteria of IS variation between prepared calibration and quality control samples and real-life samples is common in regulatory bioanalysis (White et al., 2014)(Harris D. C., 2010).

2.2. Oxytocin

Oxytocin (OT) (Greek, "quick birth") is a cyclic neuropeptide consisting of nine amino acids, which was isolated and synthesized biologically by Vincent du Vigneaud in 1953, a work awarded with the Nobel Prize in Chemistry year 1955 (peptidesciences.com). It is synthesized in the hypothalamus together with its carrier protein neurophysin I and transported axonally to the posterior pituitary gland, also called the neurohypophysis, where it is either secreted or stored. OT has both peripheral (hormonal) – and brain actions. It acts on smooth muscle cells causing for example uterine contractions and milk ejection in women (Bjålie et al., 1998) but it also acts within the brain where it is involved in e.g. social recognition and bonding in both men and female. The action of oxytocin is mediated by the oxytocin receptor which belongs to the class I G-protein coupled receptor family. It is a high-affinity receptor that requires both Mg^{2+} and cholesterol to function (Gimpl & Fahrenholz, 2001).

2.2.1. Structure and properties

As mentioned, OT is a peptide consisting of nine amino acids with the molecular formula $C_{43}H_{66}N_{12}O_{12}S_2$. See figure 1 for 2D & 3D-structure. OT has a molecular weight of 1007 Da and consists of the amino acids in the order of cysteine-tyrosine-isoleucine-glutamine-asparagine-cysteine-proline-leucine-glycine-amine. The cysteine residues form a disulfide bond. The OT molecule is very similar to the vasopressin molecule, which retains water in the body and constrict blood vessels, and they are the only known hormones to act at a distance. The only difference between the two hormones is the two amino acids at residue 3 and 8.

OT is rapidly metabolized in the liver and in the plasma by oxytocinases. It is excreted unchanged in the urine and has a half-life of 6 minutes but this is decreased during lactation and in late pregnancy. Other properties include a hydrophobicity of -2.7, meaning that the OT molecule is polar, and an isoelectric point of 5.51, meaning that the oxytocin molecule carries zero net electrical charge at pH 5.51 (DrugBank, 2014).

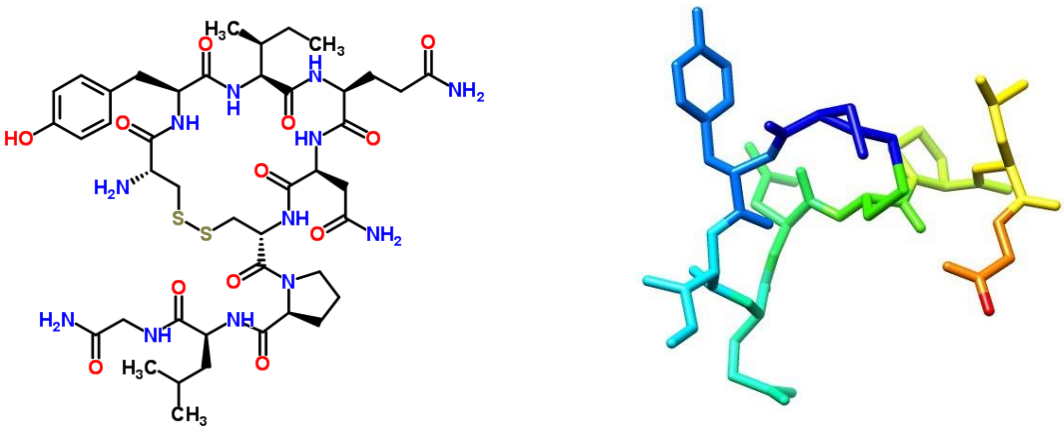


Figure 1. 2D (left) & 3D (right) - structure of OT (ChemSpider)(DrugBank, 2014).

2.3. Antibodies

Antibodies, also known as immunoglobins (Ig) are Y-shaped glycoproteins with a molecular weight of approximately 150 kDa and are produced in response to an antigen. The antibody can recognize and bind to the antigen that caused its production. There are several Ig isotypes: IgG, IgA, IgM and IgD, where IgG is the most common one in human serum. The isotypes differ in molecular size, structure, charge, amino acid composition and carbohydrate content but all Ig have a basic structure composed of four polypeptide chains connected to each other by disulfide bonds, creating a Y-shape. The basic structure includes a constant region and a variable region, where the antigen is recognized via the variable region. The antigen is recognised by the antibody through its epitope, which is the specific region on the antigen to which the antibody binds. There are both monoclonal and polyclonal antibodies. A monoclonal antibody recognizes one specific epitope of the antigen while a polyclonal antibody recognizes multiple epitopes on any one antigen. Monoclonal antibodies normally have a higher specificity toward its antigen while polyclonal antibodies may cause cross-reactivity. Specific antibodies can be produced by immunization or artificially through hybridoma formation (Prescott et al., 2002).

2.4. Proposed Approach

OT is an interesting peptide with low endogenous levels of a few pg/ml. Since it is of interest to determine OT in human plasma with high selectivity and with a limit of quantification below endogenous levels, a bioanalysis method will demand an efficient sample preparation procedure before analysis with LC-MS/MS. Plasma mostly contains water (90%) but also proteins (8%), mainly albumin (35-54 mg/ml), immunoglobins, and inorganic and organic ions (2%) dominated by sodium and chloride (Dugdale, 2013) (Hansen & Pedersen-Bjergaard, 2015). These compounds cause major matrix effects during analysis and therefore a proper sample preparation is required.

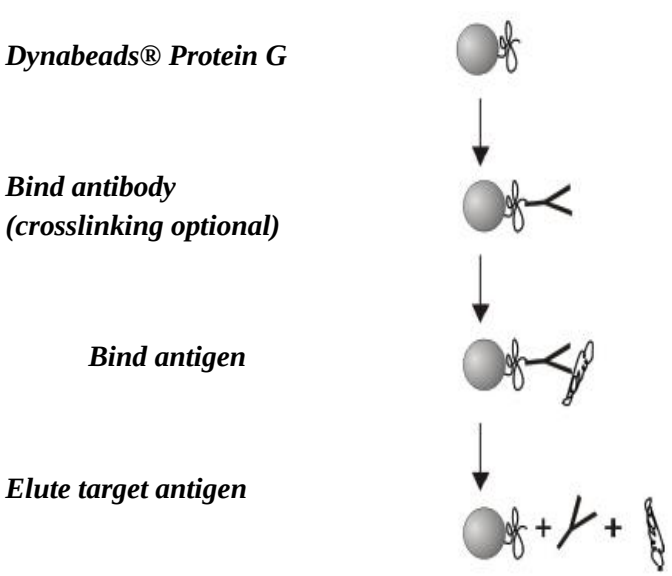
To purify OT from plasma, novel sample preparation methods like immunocapture techniques can be used. In this project, sample preparation with magnetic beads and pre-coated pipette tips will be studied. These techniques are both based on antibodies and antibody-antigen interactions. To evaluate the efficiency of the immunocapture sample preparation, the techniques will be compared not only to each other but also to solid phase extraction (SPE). SPE is the sample preparation technique used by the company - prior to this project. The purified sample, containing the target analyte (OT) and an IS, will then be analysed using LC-MS/MS.

2.4.1. Dynabeads® Magnetic Separation Technology

In year 1976 the Norwegian professor John Ugelstad achieved something that prior to this year only had been achieved by NASA in the weightless conditions of space. Professor Ugelstad succeeded in making spherical polystyrene beads of exactly the same size. These beads were later made magnetisable and in year 1982 the first commercially supermagnetic beads were developed. The Dynabeads® are today owned and produced by Invitrogen™ and Novex® – both a part of Thermo Fisher Scientific (Life Technologies). There are several types of Dynabeads® with varieties in size and surface functionalities, which gives these magnetic beads a wide application range. In this project four different types of Dynabeads® were evaluated. The Dynabeads® are used as a magnetic separation technology providing a consistent surface on which various bioreactive molecules e.g. antibodies, proteins, DNA/RNA probes can be adsorbed or coupled on. The beads are monosized, spherical and superparamagnetic, meaning that they only exhibit magnetic properties in a magnetic field leaving no residual magnetism once the field is removed. The magnetic material is encased in a thin polymer shell and is evenly dispersed in each bead providing a specific and defined surface for binding of e.g. an antibody. The full control of parameters such as bead size, surface area, iron content and magnetic mobility during production of the beads provides consistent physical and chemical properties which in turn secures reproducibility and quality of the results. Thanks to the strictly controlled production the same surface area for each experiment can be offered, leading to efficiency and a unique reproducibility of the results (Life Technologies, 2015).

2.4.1.1. Dynabeads® Protein G (novex®)

Dynabeads® Protein G are 2.8 µm in size and have recombinant Protein G, with a size of approximately 17 kDa, covalently coupled to the bead surface. The Dynabeads® Protein G suspension contains PBS, pH 7.4 with 0.01% Tween®-20 and 0.09% sodium azide as preservative and should be stored in 2 to 8 degrees Celsius. The principal of sample purification using Dynabeads® Protein G can be seen in figure 2 (left figure). The beads have a binding capacity of approximately 8 µg human IgG per mg beads where the antibody, during incubation, binds to the beads via its Fc-region. The binding strength of Protein G to different species of antibodies and their subclasses can be seen in figure 2 (right figure). When using a magnet the magnetic beads migrate towards the magnet and create a pellet allowing an easy removal of supernatant. The recombinant Protein G does not contain any albumin binding sites hence albumin is not co-purified during the process (Life Technologies Corporation, 2011).



Ig origin	Affinity for Protein G
Human IgG1,2,4	+++
Human IgD	-
Human IgA, E, M	-
Human IgG3	+++
Mouse IgG1	+++
Mouse IgG2, 2b, 3	+++
Mouse IgM	+
Rat IgG1	+
Rat IgG2a	+++
Rat IgG2b	+
Rat IgG2c	+
Bovine IgG1	+++
Bovine IgG2	+++
Chicken IgY	-
Dog IgG	+
Goat IgG1	+++
Goat IgG2	+++

Figure 2. Principle of sample purification using Dynabeads® Protein G (left). Different species of Ig´s and their subclasses and their affinity for Protein G (right).

2.4.1.2. Dynabeads® M-280 Sheep anti-Mouse IgG (novex®)

Dynabeads® M-280 Sheep anti-Mouse IgG have the same size as Dynabeads® Protein G, i.e. 2.8 µm, but instead of recombinant Protein G, affinity purified polyclonal sheep anti-mouse IgG is covalently coupled to the bead surface. The bead suspension contains 6-7 x 10^8 beads/ml in PBS (pH 7.4) with 0.1% BSA and 0.02% sodium azide. The sheep anti-Mouse IgG works as a secondary antibody and bind both light and heavy chain to mouse IgG_1, IgG_{2a} and IgG_{2b} with minimal cross reactivity to human IgG. These beads can be used with two different approaches, either a direct or an indirect approach. The indirect approach can be of benefit for example if the antibody concentrations is low or the antibody-antigen affinity is weak. If this approach is taken the primary antibody is first allowed to bind to the antigen prior to

addition of the Dynabeads®. In this project however, the direct approach has been used meaning that the sample containing the antigen is incubated with the Dynabeads® already containing the primary antibody. This approach is preferred when the affinity of the primary antibody is high. The primary antibody can be covalently cross-linked to the secondary antibody on the beads but this may affect the binding capacity of the antibody (Life Technologies Corporation, 2012).

2.4.1.3. Dynabeads® M-280 Streptavidin & MyOne™ Streptavidin T1 (Invitrogen™)

The Dynabeads® M-280 Streptavidin have a bead diameter of 2.8 µm compared to the MyOne™ Streptavidin beads that have a diameter of 1 µm. These two bead types have a monolayer of recombinant streptavidin coupled to its surface and have been further blocked with BSA. The advantage of this monolayer is that the majority of the biotin binding sites have been left sterically available for binding of e.g. biotinylated antibodies. The streptavidin-biotin interaction has a very high binding affinity allowing a direct and fast isolation of any biotinylated molecule. One mg of the Dynabeads® M-280 Streptavidin binds approximately 10 µg biotinylated IgG while one mg of the Dynabeads® MyOne™ Streptavidin T1 bind double the amount, approximately 20 µg. The Dynabeads® MyOne™ Streptavidin T1 are as mentioned smaller in size and therefore have a slower sedimentation rate during incubation (Life Technologies) (Life Technologies Corporation, 2011).

2.4.2. Mass Spectrometric Immunoassay (MSIA™) Pipette Tips

Sample preparation using immunocapture can also be made possible by using the MSIA™ Disposable Automation Research Tips (D.A.R.T'S™). A MSIA™ pipette tip has a volume of 300 µl and is embedded with a highly porous immunoaffinity column on which an antibody of choice is immobilized by following a straight forward protocol. The MSIA system is said to be highly effective due to the micro-fluidic immunoaffinity column which enhances antibody/antigen kinetics and increases signal-to-noise ratio by improving the specificity and reducing the background. The protein recovery and reproducibility is also said to be superior compared to bead-based methods. The procedure includes 3 main steps: loading of the affinity ligand, purification and elution of the target analyte. To reduce sample complexity, antibodies are immobilized on a proprietary microcolumn surface to purify target analytes. Biological samples, such as plasma, are directed through microfluidic channels to expose the target analyte to the antibody which captures the analyte with high selectivity and specificity. The purified target is then eluted from the microcolumn and ready for MS analysis. (Thermo Fisher Scientific Inc. , 2014).

The MSIA D.A.R.T's™ can be used manually together with the Thermo Scientific Finnpipette Novus i Multichannel Electronic Pipettes (8 or 12 well sample processing) (figure 3) or automated at using the Thermo Scientific Versette Automated Liquid Handling Platform. The pipettes employ a repetitive cyclical pipetting motion with up to 999 aspirations in one cycle. This motion allows for coupling of antibody, sample purification and enrichment of the target analyte, elution as well as washing steps where the tips are rinsed. There are different types of MSIA tips available depending on what kind of antibody coupling is preferred. In this project streptavidin MSIA-tips and protein G and A MSIA-tips have been evaluated (Thermo Fisher Scientific Inc., 2013).

Figure 3. Thermo Scientific Finnpipette Novus i Multichannel Electronic Pipettes (Thermo Fisher Scientific Inc., 2013)

2.4.2.1. Protein G and A/G MSIA™-tips

The protein G and A MSIA™-tips contain covalently immobilized recombinant Protein G and A/G respectively. The recombinant Protein G has a molecular weight of approximately 21,600 Da and contains two Fc-binding domains that can interact immunoglobins. In order to reduce nonspecific binding the albumin and cell binding sites have been eliminated. The recombinant Protein A/G has a molecular weight of approximately 50,000 Da and contains four Fc-binding domain, two from Protein A and two from Protein G (Thermo Fisher Scientific Inc., 2012).

2.4.2.2. Streptavidin MSIA™-tips

As mentioned before the streptavidin-biotin interaction has a very strong binding affinity and this complex is one of the strongest non-covalent interactions in biology. This interactions is taken advantage of when using the MSIA Streptavidin D.A.R.T'S together with a biotinylated antibody. The streptavidin immobilized onto the tips is a Thermo-Pierce affinity-purified, recombinant streptavidin, isolated from *Streptomyces avidinii*. Streptavidin has four potential biotin binding sites and less non-specific binding than avidin. The MSIA Streptavidin D.A.R.T'S can be used in either a forward or reverse approach. Figure 4 shows an illustration of the forward approach where the biotinylated antibody first is coupled to the micro-column and then used to capture the antigen from a biological sample. This allows for simultaneous purification and enrichment of the analyte and this is the approach chosen in this project. In

the reverse approach the biotinylated antibody is first incubated together with the antigen prior to coupling on to the micro-column on the MSIA Streptavidin D.A.R.T'S (Thermo Fisher Scientific Inc., 2013).

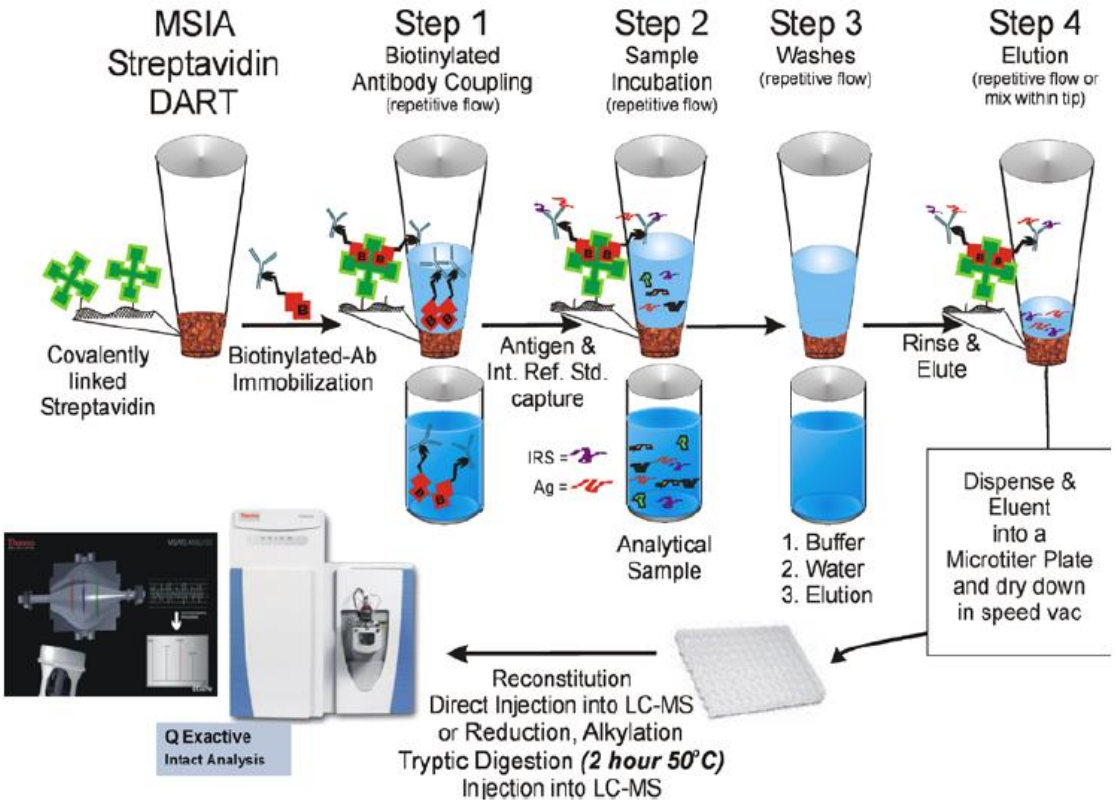


Figure 4. Overview of the forward MSIA Streptavidin approach (Thermo Fisher Scientific Inc., 2013).

2.4.3. Solid Phase Extraction (SPE)

Solid Phase Extraction (SPE) is a chromatographic technique that is used for sample preparation. Typically a cartridge like device is used. The cartridge contains chromatographic packing material which is used to chemically separate the compounds. The SPE purification reduces or eliminates matrix effects which is crucial when working with biological samples. The SPE enables a sensitive, selective and robust LC-MS/MS analysis and at the same time removes interference (Harris D. C., 2010). In this project Waters Oasis SPE products have been used to optimize a method for oxytocin, which in turn will be compared to the sample preparations using immunocapture.

Phospholipids and lysophospholipids are key contributors to ion suppression during LC-MS/MS and by using Oasis SPE they can successfully be removed. Waters have patented five SPE chemistries and introduced the first water-wettable – yet hydrophobic – polymeric sorbent. Two of these chemistries were optimized in the project; *HLB* – Hydrophilic-Lipophilic-Balanced reversed phase and *WCX* – Mixed-Mode Weak Anion-eXchange reversed

phase. Reversed phase SPE separates the analyte based on its polarity (Waters Corporation, 2011). The water-wettable Oasis sorbents holds great retention of polar compounds in addition to having a relative hydrophobic retention capacity that is three times higher than for traditional silica-based SPE-sorbents like $C_{18.}$ A high retention capacity means that more analytes are retained with less breakthrough which in turn improves the recovery and overall reproducibility. One advantage with the Water-wettable Oasis sorbents is that even if the sorbent bed runs dry during conditioning or sample loading the retention capacity is not effected, giving a more robust method. Further, the water-wettable sorbents have a great stability both at pH extremes and in a wide range of solvents. When working with traditional silica-based sorbents the long-term, batch to batch reproducibility may be compromised by e.g. hydrolytic instability at pH extremes. The Oasis sorbents have demonstrated a consistent long term, batch-to-batch reproducibility for over 15 years (Waters Corporation, 2011).

Oasis HLB is made from a specific ratio of two monomers, the *hydrophilic* N-vinylpyrrolidone and the *lipophilic* divinylbenzene. The Oasis HLB enhances the retention of polar analytes and provides superior reversed-phase capacity thanks to a neutral polar ”hook”. All Oasis mixed-mode sorbents are built upon this unique HLB copolymer, see figure 5 (left figure). The Oasis WCX holds all the advantages of Oasis HLB (figure 5). It was designed to provide sample preparation for strong bases and quaternary amines and employs both ion exchange and reversed phase (Waters Corporation, 2011).

There are several Oasis formats that can be used; 96-well extraction plates, syringe barrel – or glass cartridges, on-line columns and µElution plates that enables elution volumes of clean extracts to be as low as 25uL.

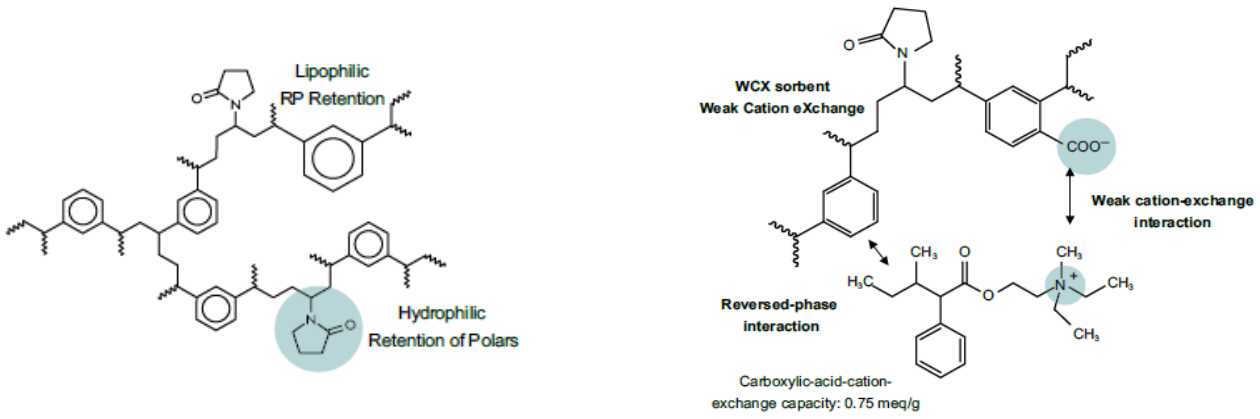


Figure 5. Structure of HLB Copolymer (left) and WCX (right) (Waters Corporation, 2011).

2.4.4. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS is a common analytical technique with a very high sensitivity and selectivity and it is used in many application areas especially for quantitative analysis, one of them being bioanalysis of peptides. LC-MS/MS combines the capabilities of two different techniques, the separation capability of LC and the detection capability of MS/MS. MS is commonly used as a detector when working with chromatography and can provide both quantitative and qualitative information (Harris, 2010).

2.4.4.1. Liquid Chromatography

To be able to analyze a certain analyte, in a sample containing hundreds of other compounds in varying concentrations in 10-fold scale, effective separation prior to detection is required. Liquid chromatography is a chromatographic separation technique which involves two phases, a mobile phase and a stationary phase that move relative to each other. The sample to be analyzed is forced by a liquid at high pressure (mobile phase) through a closed column containing fine particles (stationary phase) which in turn enables a high resolution separation. The separation of the components of the sample is due to their different degrees of interaction with the stationary phase. The interactions between the sample components and the stationary phase is highly influenced by the composition and temperature of the mobile phase. The mobile phase typically consists of water/organic solvents (like acetonitrile or methanol) mixtures. Acetonitrile is typically the first choice since it has a low viscosity, which allows a low operating pressure and permits ultraviolet detection down to 190 nm. At this low level many analytes have some absorbance. It is also common that the mobile phase contains acids like TFA to assist in the separation (Harris, 2010) and avoid peak distortion due to interaction between basic groups in the analyte and the Si surface of the stationary phase of the column.

The LC-system enables purification of the sample on-line and consists of an autosampler, a solvent delivery system (pumps), a sample injection valve and a high pressure chromatography column. The LC-system is then coupled to a MS/MS instrument as a detector, giving the powerful analytical tool LC-MS/MS. There are several types of columns that can be used. However, when working with MS/MS is it most common to use a column with a diameter of 2.1 mm since the typical interface of the MS/MS instrument i.e. electrospray ionization (ESI) requires low mobile phase flow < 1 ml. Other advantages with a narrow column is that lower use of mobile phase solvents is needed. The column contains the stationary phase which typically consist of microporous spherical, silica particles with diameters of 1.5-5 μm. The column temperature effects the efficiency regarding to mass transfer between the two phases and an elevated temperature enables a high-speed chromatography (Harris, 2010).

The stationary phase can either be polar (e.g. silica surface) or nonpolar (coated with a bonded phase of e.g. carbon chains such as C_4, C_8 or C_{18}). The solvent molecules compete with solute molecules for sites on the stationary phase and when solvent displaces solute from the

phase, elution occurs. A chromatogram is a graph that shows the detector response as a function of elution time. Depending on the identity of each compound in the sample, they will have different elution times and correspond to different peaks in a chromatogram. The time that elapses between injection of the mixture onto the column and the arrival of that compound to the detector is called retention time, t_r. Using the same chromatographic conditions for each sample injection the t_r should be identical for the analyte of interest and provides a mean for identification. The chromatographic separation should be optimized so that the peak for the target analyte is selective separated from other compounds in the sample.

For very complex samples and in bioanalysis of low concentrations analytes, coupled column chromatography can be used to increase the selectivity of the chromatographic separation. Typically, the sample is injected on column no 1 and a fraction containing the analyte of interest and IS eluting from column no 1 is transferred to a trap column (column no 2) by valve switching of the LC-flow (heart-cutting). By using additional LC solvent pumps, the analyte and its IS is eluted off the trap column into a third analytical column for the final separation. By using columns with different stationary phases, orthogonal chromatography, highly selective on-line separation can be obtained for complex samples.

2.4.4.2. Tandem Mass Spectrometry (MS/MS)

Mass spectrometry (MS) is a technique used for studying the masses of atoms or molecules or fragments of molecules. The principle of the technique is that gaseous species are ionized in a ion source and the formed ions are then accelerated by an electric field and separated in a mass analyzer according to their mass-to-charge ratio, *m/z*. The separated ions are detected by a ion counting device (e.g. electron multiplier). In a mass spectrum graph the detection response versus the *m/z* value is shown. The area of each peak is proportional to the abundance of each isotope. The MS used as a detector can be highly selective for the analyte in focus and this increases the signal-to-noise ratio (Harris D. C., 2010).

The ion separation of gaseous ions in a MS instrument requires high vacuum. When solvent vaporizes, in the interface between the column in the LC-system and the mass spectrometer, a huge volume of gas is created. The majority of this gas must be removed before the ion separation in the mass spectrometer can take place. One way of introducing liquid from the LC into the MS is by using a method called electrospray ionization (ESI; nobel prize John Fenn, 2002). An electrospray is used to produce ions by applying high voltage to a liquid to create an aerosol. Liquid from the LC-column enters a steel nebulizer capillary along with a coaxial flow of an inert gas such as nitrogen. When working with positive ion mass spectrometry the spray chamber is held at approximately – 3500 V. Together with the coaxial flow of nitrogen gas, a strong electric field at the nebulizer outlet creates a fine aerosol of charged particles. The ions from the aerosol are attracted toward a small orifice such as a capillary leading into the mass spectrometer by a strong potential (Harris D. C., 2010).

A tandem mass spectrometry consists of two mass analyzers and a collision cell between the mass analyzers. In a triple quadrupole MS/MS instrument the first mass analyzer is named Q1, the second, functioning as a collision cell, is named Q2 and the third mass analyzer is named Q3. Detection by multiple reaction monitoring (MRM) using a triple quadrupole mass spectrometer is a powerful method for quantifying target peptides. In the first step a mixture of ions from the electrospray ionization, enters quadrupole Q1. Only one selected precursor ion (the ion of interest) is then passed to the collision cell – quadrupole Q2. Q2 is a pressurized collision cell and this is where the precursor ion collides with a neutral gas, like N_2 or Ar, and breaks into fragments called product ions. In quadrupole Q3 only a small number of specific product ions are selected to reach the detector. MRM is highly selective for the analyte of interest enhancing the lower detection limit for peptides by improving the signal-to-noise in detection compared to full scan MS analysis. See figure 6 for an overview.

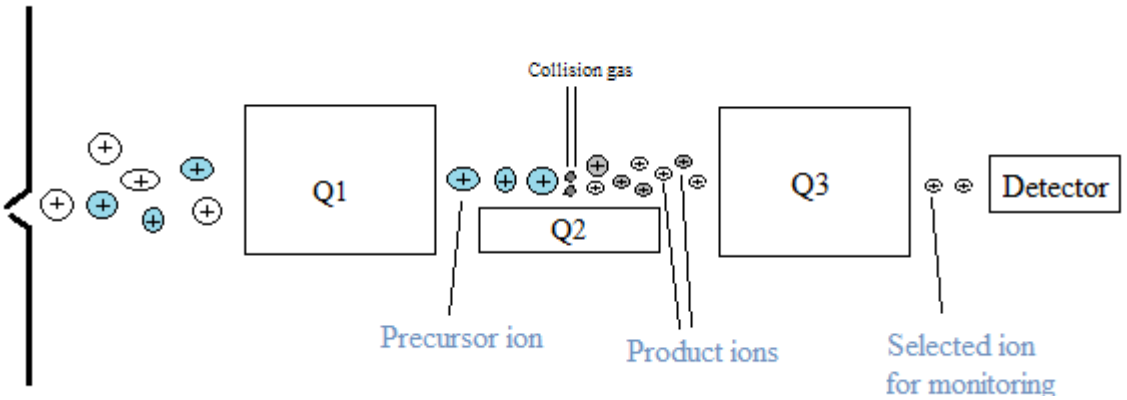


Figure 6. Principle of multiple reaction monitoring (MRM) using a triple quadrupole mass spectrometer.

3. Materials and Methods

3.1. Chemicals and reagents

The chemicals used for preparation of buffers required for the peptide purification and LC-MS/MS were ammonium formate (NH_4COOH) (Sigma Aldrich), ammonia solution (25%; NH_4OH)(Merck) acetonitrile *hypergrade for LC-MS/MS* (C_2H_3N) (Merck), formic acid (98-100%; CH_2O_2) (Merck), methanol *hypergrade for LC-MS/MS* (CH_4O) (Merck), acetic acid (100%; ($C_2H_4O_2$) (Merck), trifluoroacetic acid ($C_2HF_3O_2$) (Merck), potassium chloride (KCl), sodium chloride (NaCl) (Merck), glycine ($C_2H_5NO_2$) (Sigma Aldric), citric acid ($C_6H_8O_7$) (Sigma Aldrich), phosphoric acid (H_3PO_4), isopropyl alcohol (C_3H_8O)(iPrOH) and ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich).

3.2. Equipment

HulaMixer® Sample Mixer (Life Technologies), Allegra X-15R Centrifuge (Beckman Coulter), Sorvall RC-4 (Thermo Scientific), VMS-C4 Advanced (VWR), PHM220 LAB pH Meter (MeterLab®), API 5000 (Applied biosystems), Xevo TQ-S (Waters) LC-20AD UFLC (Shimadzu), DHU-20A_3 Degassr (Shimadzu), CTC Analytics HTS (Pal Systems), PB403-S (Mettler Toledo), MX5 Microbalance (Mettler Toledo), TurboVap®96 (Zymark), Vortex Maxi Mix II Type 37600 Mixer (Thermolyne) and Positive Pressure-96 Processor (Waters).

3.3. Preparation of standards and plasma samples

Stock solutions of oxytocin and internal standard were prepared in 10mM ammonium formate pH 4.4. The internal standard used was oxytocin-d5. The stock solutions were prepared in concentrations of 1 mg/ml and stored in - 20°C. Care was taken to avoid excessive freeze-thaw cycles. The same stock solutions for OT and IS were used during the entire project. The stock solutions of OT were used to spike plasma samples for the immunocapture experiments. Plasma samples were spiked in various concentrations along with the experiments. The dilutions were done in plasma using serial dilution. The stock solutions were also used to spike controls in different concentrations. The controls were diluted in Acetonitrile: 0.01% BSA in 1% formic acid (20:80, v/v), using serial dilution, except for the last dilution were the elution buffer used for the specific experiment was used as dilution buffer. This was done to prevent unspecific binding of the OT or IS to the tube walls during the dilution steps. The plasma samples as well as the controls were stored in - 20°C in volumes of 200 µl to avoid excessive freeze-thaw cycles.

3.4. LC-MS/MS

Two different LC-MS/MS systems were used during the project.

3.4.1. Triple quadrupole – API 5000

The LC-MS/MS system consisted of a PAL HTS autoinjector, two Shimadzu LC-20AD pumps, a Shimadzu DGU-20A_3 degasser and Applied Biosystems API 5000 triple quadrupole mass spectrometer. Data acquisition and processing were carried out using SCIEX Analyst® software version 1.4.2. The ESI source was operated in positive ionization mode and the MS/MS was set to monitor MRM transition *m/z* 1007.6 → *m/z* 723.60 for OT. The MS/MS operating conditions were as follow: Ion spray voltage 5500 V, collision energy 20.00 V, temperature 600°C, ion source gas 1 20.00 and ion source gas 2 40.00.

3.4.2. Triple quadrupole – Xevo TQ-S

The LC-MS/MSsystem consisted of ACQUITY UPLC I-class system with XEVO TQ-S triple quadrupole mass spectrometer (all Waters). System control and data acquisition were performed with MassLynx® (Waters) version 4.1. The ESI source was operated in positive ionization mode and the MS/MS was set to monitor the two MRM transitions *m/z* 1007.6 → *m/z* 285.24 and *m/z* 1007.6 → *m/z* 723.30 for OT. The optimized collision energy was 45 eV for the *m/z* 285.24 fragment and 28 eV for the *m/z* 723.30 fragment. The MS/MS operating conditions were as follows: nebuliser gas 7.0 bar, collision gas (Ar) 0.15 ml/min, cone gas (N_2) 150 l/h, desolvation gas (N_2) 800 l/h, source temperature 150°C and desolvation temperature 500°C.

3.5. Chromatographic conditions

Two different LC configurations were used: A single LC column system was used with the API 5000 system and a coupled column LC system was used with the Xevo TQ-S system.

3.5.1. API 5000 system

LC separation was performed with a Phenomenex Aeris Widepore, 3.6µm, XB-C18 column with column dimensions 50 mm x 2.1 mm. The mobile phases used were A: MQ-water/acetonitrile 95/5 (v/v) with 0.2% formic acid and B: acetronitrile with 0.2% formic acid. The flow rate was set to 0.400 ml/min and the injection sample volume was 50 µl. The autosampler (Pal Systems) wash consisted of a weak wash with MQ-water/acetonitrile 90/10 (v/v) and a strong wash with MQ-water/acetonitrile/acetone/iPrOH/formic acid 240/250/250/250/10 (v/v). The gradient table can be seen in table 1.

Table 1. Gradient table. Solvent A: MQ-water/acetonitrile 95/5 (v/v) with 0.2% formic acid, solvent B: acetronitrile with 0.2% formic acid. Column: Phenomenex Aeris.

	Time (min)	%A	%B
1.	0.20	96.5	3.5
2.	0.60	89.5	10.5
3.	0.90	84.6	15.4
4.	2.70	58	42
5.	2.72	58	42
6.	2.75	0	100
7.	4.00	0	100
8.	4.10	96.5	3.5
9.	6.00	Stop	Stop

3.5.2. Xevo TQ-S system

Chromatographic separation was carried out using a three-column coupled system. The columns used were BetaBasic™ (Thermo Scientific Fisher) CN, 5µm, with column dimensions 50mm x 2.1mm, XBridge™ (Waters) C18, 10 m Direct Connect HP column with column dimensions 2.1 mm x 30mm (trap column) and ACQUITY (Waters) UPLC HSS T3 Column, 1.8 µm with column dimension 2.1 mm X 50 mm. Since three columns were used, three binary pumps were used as well. The injection sample volume was set to 50 µL. The gradient tables for each pump can be seen in table 2-4. The column switching from the CN column to the trap column, i.e. a heartcut, was performed by valve switching between 1.10 and 1.60 min, directing the flow from the CN column to the trap column. Back flush of the CN-column was performed between 4.00 and 6.00 min while the gradient was running on the analytical column.

Table 2. Gradient table – pump A. Solvent A: 0.05% TFA in MQ-water, solvent B: acetonitrile. Column: BetaBasic™

	Time (min)	Flow Rate	%A	%B	Curve
1.	Initial	0.300	82.5	17.5	Initial
2.	2.20	0.300	82.5	17.5	6
3.	2.50	0.300	20.0	80.0	11
4.	3.90	0.300	20.0	80.0	11
5.	4.00	0.300	82.5	17.5	11
6.	7.00	0.300	82.5	17.5	11

Table 3. Gradient table – pump B. Solvent A: 1mM ammonium formate pH 4.6, solvent B: acetonitrile. Column: XBridge™.

	Time (min)	Flow Rate	%A	% B	Curve
1.	Initial	0.300	90.0	10.0	Initial
2.	1.60	0.300	90.0	10.0	6
3.	5.00	0.300	70.0	30.0	6
4.	5.10	0.300	10.0	90.0	11
5.	7.00	0.300	90.0	10.0	11
6.	8.00	0.300	90.0	10.0	11

Table 4. Gradient table - pump C. Solvent A: 1 mM ammonium formate pH 6, solvent B: acetonitrile/MQ-water/formic acid 70/28/2. Column: ACQUITY.

	Time (min)	Flow Rate	%A	%B	Curve
1.	Initial	0.600	50.0	50.0	Initial

Waters ACQUITY FTN autosampler was used for injection. Both a pre and post-inject wash of 10 seconds respectively was applied using acetonitrile(methanol/isopropanol/MQ-water/formic acid 25/25/24/24/1 (v/v/v/v).

3.6. SPE

SPE was carried out by using Oasis HLB and WCX 10 mg 96-well extraction plates. All resins were conditioned with 500 µl methanol and 500 µl Milli-Q water prior to use. Each step in the SPE procedure was carried out by loading the solution in the wells of the Oasis plate used. In order to press the liquids through the wells, the plate was placed on Waters Positive Pressure-96 processor. Pressure was applied by manually increasing the pressure of the instrument. The flow-through fractions were collected using a deep-well plate and the flow-through fractions to be analysed were collected and saved in separate, clean deep-well plates.

3.6.1. Determination of optimal SPE conditions

Optimization of the SPE procedure was performed with both Oasis HLB (10 µg) 96-well plate and WCX (10 µg) 96-well plate to determine the optimal SPE plate for OT. The optimization is done to determine the optimal percentage of methanol and acetonitrile to be used in the SPE and also to determine whether the HLB or WCX plate should be used. The determination is done by analysing the extracts with LC-MS/MS (the Xevo TQ-system).

15 ml pre-spiked human plasma with an OT concentration of 100 pg/ml was thawed and was together with 150 µL IS (100 ng/ml) diluted 1:1 with 4% H_3PO_4.. This gave a final concentration of 1000pg/ml IS in all samples. Further, 2 ml plasma, including 20 µl IS (100 ng/ml) was diluted 1:1 with 4% H_3PO_4 as well, this was used as zero plasma (plasma with 1000pg/ml IS but no OT added). Both plates were first conditioned with 500 µl methanol followed by a condition with 500 µl MQ-water. Then 200 µl of the diluted plasma samples (100pg/ml OT) were added to both plates at positions 1,2,4,5 A:H and 3,6 A:D, see table 5. In positions 3,6 E:H 200 µl of the zero plasma sample.

This was followed by a first wash with 500 µl 0.5% ammonia solution/acetic acid pH 6.3 in each well for both plates. The flow-through after each step was collected using a deep-well plate. With the HLB plate the second wash consisted of 10-100% methanol and 10-100% acetonitrile as can be seen in table 5. This last flow-through was collected in a separate clean deep-well plate and the solutions were evaporated to dryness using TurboVap®96 (Zymark) under a stream of N_2 gas at 50°C.

For the WCX plate the second wash also consisted of 10-100% MeOH as in table 5 but instead of adding the acetonitrile, columns 4-6 were washed with 10% MeOH. The flow-through from columns 1-3 was saved. After this columns 4-6 were washed with 10-100% acetonitrile with 1% TFA and the flow-through was saved. The saved flow-through fractions were evaporated to dryness.

To reconstitute the samples, 100 µl 5mM ammonium formate pH 4.6:acetonitrile (90/10, v/v) was used. For the recovery samples (REC), a concentration of 100 pg/ml OT in the final samples was added as well.

Table 5. Overview over the samples used for SPE optimization.

	1	2	3	4	5	6
A	10%	50%	90%	10%	50%	90%
B	10%	50%	90%	10%	50%	90%
C	20%	60%	100%	20%	60%	100%
D	20%	60%	100%	20%	60%	100%
E	30%	70%	50% Blank	30%	70%	50% Blank
F	30%	70%	50% Blank	30%	70%	50% Blank
G	40%	80%	50% REC	40%	80%	50% REC
H	40%	80%	50% REC	40%	80%	50% REC
	% Methanol in wash 2			**% Acetonitrile in wash 2**		

3.6.2. Final SPE procedure

The final SPE procedure was done using the Oasis HLB (10 µg) 96-well plate. Plasma and spiked plasma samples with the desired OT and IS concentrations were thawed and diluted 1:1 with 4% H_3PO_4 in separate tubes. The plate was then conditioned as in the SPE optimization before the plasma samples were added. The first wash was also done according to the SPE optimization, followed by a second wash with 30% methanol for all samples. To elute the OT and IS, 500 µl 30% acetonitrile was used and the flow-through was saved and evaporated to dryness and reconstituted in 100 µl 2.5% formic acid, 10% acetonitrile, pH 2.5. The wells containing the zero plasma samples were reconstituted with the same buffer including OT with the same concentration as in the spiked plasma samples and used as controls during analysis.

3.7. Immunocapture assay development and optimization

After sample preparation using either Dynabeads® or MSIA™ the samples were centrifuged for 2 min at 1500 rpm and stored at 4°C before analysis with either the API 5000 or the T-QS system.

3.7.1. Antibodies

In table 6 the tested antibodies and their respective suppliers can be seen. Further, the clonality, specificity, source, isotype and presentation for each antibody is shown. The antibodies were stored at -20°C and thawed the same day as the sample preparation was performed.

Tabel 6. Antibodies and their suppliers, clonality, specificity, source and isotype.

Antibody (supplier)	Clonality	Specificity	Source	Isotype	Presentation
MAB5296 (Millipore)	Monoclonal	Anti-OT (clone 4G11)	Mouse	IgG	Ascites fluid, 0.1% sodium azide
Ab67457 (Abcam)	Polyclonal	Anti-OT	Rabbit	IgG	Immunogen affinity purified. 0.02% sodium azide, 1% BSA, PBS pH 7.4
Ab124771 (Abcam)	Monoclonal	Anti-OT	Rabbit	IgG1	Tissue culture supernatant. 0.01% sodium azide, 50% glycerol, 0.05% BSA
RB-13-0010B-50 (RayBiotech)	Polyclonal	Anti-OT (+Biotin)	Rabbit	IgG	Affinity purified.
RB-13-0010 (RayBiotech)	Polyclonal	Anti-OT	Rabbit	IgG	Affinity purified.
SA1-9507 (Thermo Scientific)	Polyclonal	Anti-Mouse (+Biotin)	Bovine	IgG	Affinity purified. 0.02% sodium azide, PBS

3.7.2 Buffers

The buffers used during immunocapture assay development and optimization can be seen in table 7.

Tabel 7. Buffer names and their contents.

Buffer Name	Content
Washing Buffer 1	PBS 0.05% Tween, pH 7.4
Washing Buffer 2	PBS 0.1% BSA and 2mM EDTA, pH 7.4
Buffer 1	PBS 0.01% BSA, pH 7.4
Buffer 2	PBS 0.05% Tween , 1 % BSA, pH 7.4
Elution Buffer 1	2.5% formic acid, 10% acetonitrile, pH 2.5
Elution Buffer 2a	0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, 0.2 M acetic acid, 0.2 M glycine, pH 2.5
Elution Buffer 2b	0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, 0.2 M acetic acid, 0.2 M glycine, 10% acetonitrile, pH 2.5
Elution Buffer 3	0.1mM Citric acid pH 3.0

3.7.3. Dynabeads®

For a visual flowchart of the Dynabead® principle, see figure 2.

The development of a sample preparation procedure using Dynabeads® was initially based on *Life Technologies* existing protocol for each Dynabeads® type. The *Life Technologies* protocols can be seen in Appendix I. The tubes used during sample preparation were 1.5, 2 and 5 ml Eppendorf tubes and for plates Thermo Scientifics 96-Well Non-Skirted PCR plate (0.3ml) was used. The magnets used were DynaMag™ -2 Magnet for the tubes and DynaMag™-96 Side Skirted magnet for the 96-well plates. The Dynabeads® were always placed at least 2 min on the magnet to ensure that all beads were collected on the tube or well walls. Great caution was taken to ensure that no beads were lost during pipetting. During antibody-bead incubation the antibody was always added in excess to ensure optimal binding to the beads. An example of how the amount of beads in relation to the amount of antibody was calculated can be found in Appendix II. The amount of beads and antibody used for a specific experiment is shown in *Results*. The Dynabeads® and the immuno-extracted samples were stored at +4°C until usage. When analysing the samples, controls for each concentration were used.

For the analysis of the samples during assay development the API 5000 system was used. The final method was then optimized and evaluated using the Xevo TQ-S system.

3.7.3.1. Dynabeads® M-280 Streptavidin & MyOne™ Streptavidin T1

The Dynabeads® were resuspended in its original vial by vortexing for 60 sec. The desired volume was transferred to a 1.5 ml tube and 1 ml *Washing Buffer 1* was added and vortexed together with the Dynabeads® for 10 sec. The tube was then placed on a magnet and the supernatant was discarded while keeping the tube on the magnet. The beads were then resuspended in same volume of *Buffer* as the initial volume of Dynabeads® taken from the vial. The washing step was repeated 2 times for MyOne for a total of 3 washes.

The beads were placed on the magnet and the supernatant was discarded. The beads were then incubated with RB-13-0010B-50 at room temperature for 1-3 hours using the HulaMixer® or manual mixing by inversion every 10 min. After incubation the tube was placed on the magnet and the supernatant was removed. The antibody-coated beads were then washed 4-5 times using 1 ml *Washing Buffer 1* for each washing step. The beads were then resuspended in 1 ml *Buffer* and stored.

The desired amount of antibody-coupled beads were transferred to 13 tubes: triplicates of LOW, MID and HIGH and duplicates of buffer and plasma blank. 200 µl of sample was added in each tube and incubated together with the beads for 1-3 hours in room temperature with either manual mixing every 10-20 min or by using the HulaMixer®. After incubation the beads were washed 3 times with 200 µl *Washing Buffer* 1 and the supernatant from wash 1 and 3 was saved to analyse a potential loss of OT during washing. After the last washing step the supernatant was removed and 200 µl *Elution Buffer 2* or *3* was added and incubated together

with the beads for 5-10 min. The supernatant was then transferred to an analysis plate and stored until analysis (maximum one day after sample prep.).

3.7.3.2. Dynabeads® Protein G

The Dynabeads® were resuspended in its original vial by vortexing for 60 sec. The desired amount of beads were transferred to a tube, placed on the magnet and the supernatant was removed. The antibody of choice (diluted in *Washing Buffer 1*) was added to the beads and incubated with rotation using the HulaMixer® for 30-60 min. After incubation the antibody-coated beads were placed on the magnet and the supernatant was removed and the beads were washed once with 1 ml *Washing Buffer 1*. After the washing step the supernatant was removed and the antibody-beads were resuspended in 1 ml *Washing Buffer* 1 and stored. The antibodies tested with the Dynabeads® Protein G were: AB911, MAB5296, Ab67457, Ab124771 and RB-13-0010 (see table 6).

As described earlier the desired amount of antibody-coated beads were transferred to either 1.5 ml tubes or a 96-well plate depending on the amount of samples to be analysed and the supernatant was removed. 100-500 µl plasma sample was added in at least one LOW and one HIGH concentration of OT. All concentrations were analysed in at least duplicate samples. The OT was allowed to incubate with the antibody-coated beads for 1-24 hours in room temperature using the HulaMixer® (tubes) or by mixing using a pipette (plates) every 10-20 min depending on the incubation time. The 24 hour incubation was only tested with tubes and the HulaMixer®. After incubation the OT-antibody-bead-complex was washed 3 times according to previous described washes using *Washing Buffer 1*. When working with the 96-well plate 100 µl washing buffer was used. After washing, 100-200 µl *Elution Buffer 1, 2* or *3* was added and incubated together with the OT-Ab-bead-complex for 5-10 min in room temperature. After incubation the beads were placed on a magnet, the supernatant was removed and transferred to an analysis plate. The analysis plate was centrifuged for 2 min at 1500 rpm to spin the entire sample down into the wells, leaving no residues on the well walls. A silicone lid was used when centrifuging.

3.7.3.3. Dynabeads® M-280 Sheep anti-Mouse IgG

To prepare the beads they were handled and washed as described for the Streptavidin-beads but instead of using *Washing Buffer 1*, *Washing Buffer 2* was used. Antibody MAB5296 was used to test the potential of the Dynabeads® M-280 Sheep anti-Mouse IgG by comparison with the Protein G-beads. The desired amount of beads were incubated with the antibody for 24 hours at +4°C using the HulaMixer®. After incubation the antibody-bead complex was washed three times using 1 ml of *Washing Buffer 2*. After the last washing step the complex was resuspended in 1 ml of the same buffer.

The antibody-bead complex was incubation for 2-4 hours together with 100 µl plasma sample and washed three times after incubation as described for the Protein G-beads, however *Washing Buffer 2* was used instead. To elute the OT, 100 µl of *Elution Buffer 3* was used and incubated together with the OT-Ab-bead complex for 10 min. After incubation the beads were

placed on a magnet, the supernatant was removed and transferred to an analysis plate. The analysis plate was centrifuged for 2 min at 1500 rpm to spin the entire sample down into the wells, leaving no residues on the well walls. A silicone lid was used when centrifuging.

3.7.4. MSIA™

The MSIA™ was performed using the Finnpipette Novus i Multichannel Electronic Pipettes (8 well sample processing) and the MSIA™-tips mentioned in section *2.4.2.1. & 2.4.2.2.* Before starting with the MSIA™ a few preparative actions were taken. The plasma samples were centrifuged for 3 min at 2600 rpm to remove unwanted particles. All reagents used, including the MSIA™-tips, were brought to room temperature. The pipetting heights were controlled so that no air passed through the microcolumn during pipetting. To ensure that the tips were inserted properly onto the pipette , manual checking was performed. Throughout the MSIA™ procedures pipette speed 1 was used. In the washing steps *Washing Buffer 1* was used. For the elution *Elution Buffers 1* and *2b* were tested.

Firstly, the MSIA™-tips were prewashed using 10 iterations, a microplate volme of 250 µl and a pipetting volume of 150 µl. This was followed by binding of the antibody using 700-999 iterations, a microplate volume of 120 µl and a pipetting volume of 100 µl. The antibodies were diluted in *Washing Buffer 1* and 1.2-12 µg antibody per Protein G(or A) tip was tested. The Abs tested were MAB5296 and Ab124771. Due to the lack of ready-made biotinylated antibodies that showed good performance, or antibodies that were pure enough to enable biotinylation a different approach was taken with the Steptavidin-tips. SA1-9507 was pre-incubated with MAB5296 in *Buffer 2* for 2 hours in room temperatur using the Sample Hula Mixer. Excess of the anti-mouse antibody was used to ensure that enough MAB5296 was bound to capture the total amount of OT. One Ab124771 antibody had the capacity to bind two MAB5296 antibodies.

Following the antibody capture a wash step, identical to the prewash was performed to prepare for the OT binding, where the plasma samples was diluted 1:1 with PBS and 400 µl was added in each well. The pipetting volume was 250 µl and 999 iterations was used. This was followed by two additional washing steps and completed with an elution using 100 µl elution buffer, a pipetting volume of 75 µl and 100-200 iterations.

3.7.5. Final immunocapture procedure

The final immunocapture procedure was performed using Dynabeads® Protein G and antibody MAB5296. The principle of the procedure was as described in section *3.6.3.2. Dynabeads® Protein G* with the following specifics: 500 µl beads were used and incubated together with 100 µl antibody stock solution (1-3 mg/ml) pre-diluted in 2900 µl *Washing Buffer 1*. The antibody-bead incubation time was 30 min using a 5 ml tube and the HulaMixer®. After incubation the antibody-bead-complex was resuspended in 5 ml *Washing Buffer 1*. For the sample processing a 96-well plate was used and 60 µl of the antibody-bead suspension was added in each well and the supernatant was removed before 200 µl of the

plasma samples were added. The OT-antibody-bead incubation time was 1 hour using manual mixing by pipetting every 10 min. The elution was done using 100 µl *Elution Buffer 1* with an incubation time of 10 min. The analysis was performed using the Xevo TQ-S system.

3.8. Qualification of the Final Method

To evaluate the potential of immunocapture as sample preparation prior to LC-MS/MS analysis, precision and accuracy were tested at 10 (lower limit of quantitation; LLOQ), 30(LOW), 45(MID) and 85(HIGH) pg/ml using six replicates. Concentration of the low QC was within three times the LLOQ concentration and the high QC was within 80% of the upper limit of quantitation (ULOQ). Accuracy was evaluated by calculating the percent deviation from the nominal concentration, and is reported as relative error (RE). The mean value should be within 15% of the nominal value, except at LLOQ, where it should be within 20% of the nominal value. Precision was determined by calculating the coefficient of variation (CV). The precision at each concentration level should not exceed 15% CV, except for the LLOQ, where it should not exceed 20% CV.

In addition, the linearity of the response was evaluated at seven concentrations (n=2): 10, 25, 40, 55, 70, 85 and 100 ng/ml using duplicates. Calibration curve performance was assessed by evaluating deviation of standards from the nominal concentration and evaluating the slope, intercept, and coefficient of determination (r^2) of the weighted $1/x^2$ linear regression lines. where ± 20% from the nominal value was acceptable at the LLOQ and ±15% from the nominal value accepted at all other concentrations. If a calibrator did not meet these criteria, it was dropped from the calibration curve and the curve was recalculated. The method evaluation was performed using the TQ-S system.

To compare the final immunocapture method with the final SPE method two OT concentrations were compared at 5 and 10 pg/ml using six replicates. The comparison was done using the TQ-S system.

4. Results

4.1. SPE Optimization

To determine the optimal conditions i.e. the percentage of methanol and acetonitrile to be used in the SPE, an optimization of both Oasis HLB and Oasis WCX was performed. Figure 7 and 8 shows the OT/IS ratio versus the methanol used in percentage for both HLB and WCX. As can be seen in both figures, elution of OT occurs after washing with 40% methanol and more.

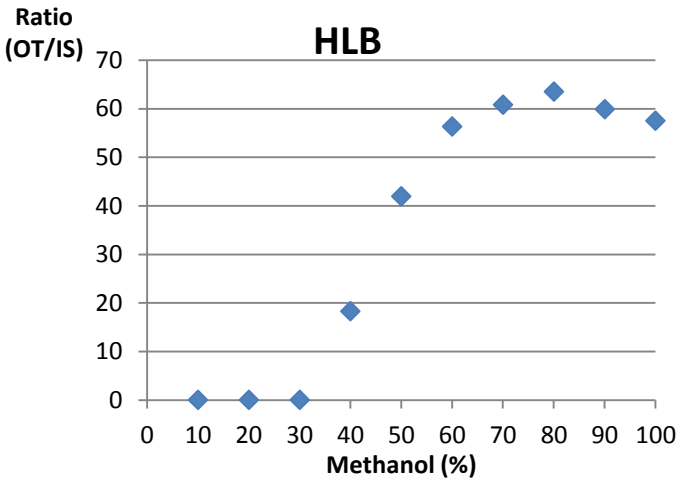


Figure 7. Ratio of OT/IS versus methanol (%) for Oasis HLB.

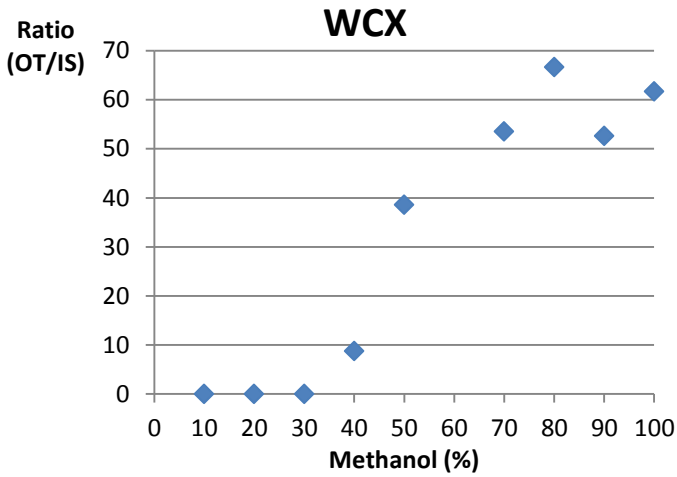


Figure 8. Ratio of OT/IS versus methanol (%) for Oasis WCX.

Figure 9 and 10 shows the OT/IS ratio versus the acetonitrile used in percentage for both HLB and WCX. Further, it can be seen that after 20-30% acetonitrile is used the elution of OT remains relative constant for both methods.

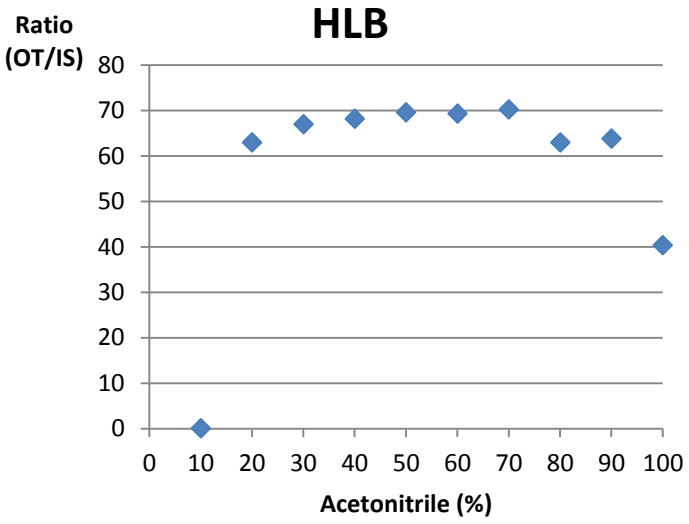


Figure 9. Ratio of OT/IS versus acetonitrile (%) for Oasis HLB.

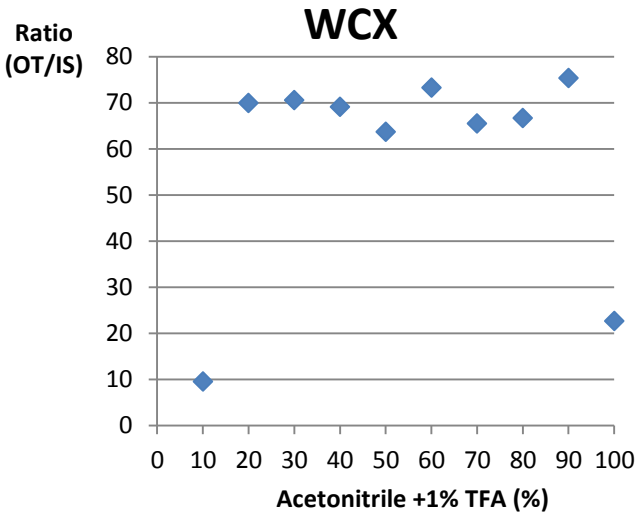


Figure 10. Ratio of OT/IS versus acetonitrile (%) for Oasis WCX.

4.2. Immunocapture Assay Development

During the immunocapture assay development several experiment were conducted. In this section a selection of these experiments are presented. This selection is based on the importance of each experiment in regard to developing and choosing the final method.

4.2.1. Dynabeads® M-280 Streptavidin & MyOne™ Streptavidin T1

The experiments carried out using the Streptavidin-beads and the biotin labelled anti-OT Ab RB-13-0010B-50 demonstrated inconclusive results. A selection of these results, shown in chromatograms generated by API 5000, can be seen in Appendix III.

4.2.2.Choice of Antibody

During the assay development using the Protein G-beads, four different antibodies were tested to determine the antibody with greatest potential for further development and optimization. In table 8, the recoveries for each antibody is shown. The recovery is the amount of pure product recovered versus the amount of crude material used. The recovery is a mean value calculated from duplicate samples (1 ng/ml OT). During the Ab-bead-complex incubation (1 hour) 1.2 mg beads were incubated with 13.75 µg Ab124771, 20 µg Ab67457 and 20 µg RB-13-0010B-50 respectively. Since MAB5296 had an unknown concentration of 1-3 mg/ml stock solution, due to the fact that it was presented in ascites fluid, three different concentration ranges were tested: 10-30, 12.5-37.5 and 20-60 µg. The recovery for MAB5296 in table 8 is a mean value calculated from the average value of duplicate samples for each concentration range.

During the incubation with OT, 100 µl plasma + 100 µl PBS was used to enable sufficient mixing since 1.5 ml tubes were used. The incubation time was 30 min and *Elution Buffer 2a* was used. The analysis was performed using the API 5000 system and the recovery was calculated using the chromatograms generated. The immune-prepared sample was compared to a control sample (1 ng/ml OT). As can be seen in table 8, the Millipore antibody MAB5296 showed the greatest recovery (11%).

Table 8. Antibody, the amount of antibody-bead-complex per well in µg and their respective recovery.

Antibody	Ab-bead-complex per well (ug)	Recovery
MAB5296 (Millipore)	36	11%
Ab67457 (abcam)	36	3.5%
Ab124771 (abcam)	36	2.6%
RB-13-0010B-50 (RayBiotech)	36	7.0%

4.2.3. Comparison of Coupling Mechanisms

4.2.3.1. Dynabeads® Protein G and Sheep Anti-Mouse

Since the Streptavidin-beads showed no potential the protein G-beads were compared to the Sheep Anti-Mouse beads, using the antibody exhibiting the greatest potential (MAB5296). During the preparation of antibody-bead-complex 50 µl of bead stock solution was incubated with 10-30 µg Ab each for 30 min. For the incubation with the plasma samples a 96-well plate was used and 100 µl plasma (1 ng/ml OT) was added in each well. The samples were incubated for four hours in duplicates. For the Protein G-beads 100 µl *Elution Buffer 2a* was used and for the Sheep Anti-Mouse-beads 100 µl of *Elution Buffer 3* was used. Two different amounts of Ab-bead-complex were tested and analysed using API 5000. Table 9 shows the recovery (%) for each bead type and Ab-bead-complex amount. As can be seen, the Dynabeads® Protein G beads show a greater recovery compared to the Sheep Anti-Mouse-beads with a recovery ranging from 38 to 40% compared to 21 to 22%.

Table 9. Antibody, the amount of antibody-bead-complex per well in µg and their respective recovery.

Dynabeads® type	Antibody-bead-complex per well (µg)	Recovery
Sheep Anti-Mouse	25	22 %
Sheep Anti-Mouse	35	21 %
Protein G	25	40 %
Protein G	35	38 %

4.2.3.2. MSIA Protein G, AG & Streptavidin

To compare the Protein G-beads with MSIA a couple of experiments using MSIA were conducted. Unfortunately, the experiments carried out using the MSIA-tips generated inconclusive results. The Protein A and AG tips were tested with 2 different antibodies, one of them being the MAB5296 antibody. The streptavidin-tips were tested using a pre-incubated solution of the biotinylated SA1-9507 and the MAB5296. Different concentrations of the antibodies and a few different elution buffers were tested. A selection of these results, shown in chromatograms generated by the API 5000 system, can be seen in Appendix III.

4.3. Immunocapture Optimization

Since the Dynabeads® Protein G together with MAB5296 was the method showing greatest potential during assay development, this was the immunocapture method optimized. During optimization the focus was on increasing the yield and the signal. All optimization experiments were analysed using Xevo TQ-S system. In this section a selection of these experiments are presented. This selection is based on the importance of each experiment in regard to developing and choosing the final method.

4.3.1. Elution

The first step in the optimization was to determine which elution buffer to move forward with. Therefore three experiments were conducted using *Elution Buffer 1, 2b* and *3*. The plasma samples were analyzed in duplicates using 30 µl antibody-bead-complex in each well.

During the preparation of antibody-bead-complex 1.5mg beads/ml and 10-30µg antibody/ml was used. The plasma samples contained 100 pg IS/ml plasma as well. The standard samples were OT spiked solutions of each elution buffer, including 100 pg IS/ml.

It was quickly noticed that *Elution Buffer 2a* was not suited for the TQ-S system due to the high salt content and therefore no reliable results were obtained. The results for the other elution buffers can be seen in table 10. Here it should be noted that unacceptable variations occurred at three concentrations for *Elution Buffer 1* and at four concentrations for *Elution Buffer 3* (red values). For the concentrations were the variation in QC area was acceptable, the ratio of the QC area/Standard area was calculated. For *Elution Buffer 1* the ratio ranged from 19-28% and for *Elution Buffer 3* it ranged from 21-25%. No larger difference was found between the two elution buffers and since *Elution Buffer 1* has shown to be successful (Osgood, et al.), it was chosen for the sample preparation.

In order to be able to calculate the yield correctly the IS (100 pg/ml) was chosen to be added together with the elution instead.

Table 10. The QC – and Standard areas for seven OT concentrations for Elution Buffer 2a and 3 and the QC Area/Standard Area ratio (%) for the areas with acceptable variation.

Elution Buffer 1				
OT conc. (pg/ml)	**QC-area 1**	**QC-area 2**	**Standard-area**	**QC-area/Standard-area (%)**
10	28	11	180	
25	60	80	380	21
40	170	95	640	
55	190	180	860	25
70	220	210	1030	24
85	280	320	1170	25
100	270	520	1380	

Elution Buffer 3				
OT conc. (pg/ml)	**QC-area 1**	**QC-area 2**	**Standard-area**	**QC-area/Standard-area (%)**
10	47	18	190	
25	84	30	340	
40	160	80	480	
55	200	120	740	
70	220	180	890	19
85	300	260	1230	24
100	390	370	1390	28

When using IS (100 pg/ml) with the elution (100 µl) instead a much smaller variation was obtained (see Appendix IV) and the yield could be calculated correctly. The QC area/Standard area varied from 29-37%, an improvement from previous experiment using IS during the sample preparation instead. Still, a low percentage of OT seemed to be extracted during the sample preparation and therefore two more experiments were conducted: 150 µl elution buffer was used instead of the normal volume of 100 µl and 50 µl 0.03% BSA was added in each well of the analysis plate prior to addition of the sample to be analyzed. These experiment were conducted to (1) investigate if a greater volume of elution buffer improved the recovery and (2) to analyze if BSA reduced a potential unspecific binding. When using 150 µl elution the ratio varied from 26-41% and when adding 50 µl 0.03% BSA the ratio varied from 25-35%. These experiments did not improve the amount of OT being extracted and therefore continuation with 100 µl of *Elution Buffer 1* proceeded. The results can be seen in table 11.

Table 11. OT QC Area/OT standard Area (%) for 100µl, 150µl and 100µl + 50µl 0.03% BSA *Elution Buffer 2a*.

OT conc. (pg/ml)	OT QC Area/OT standard Area (%) **100ul *Elution Buffer 1***	OT QC Area/OT standard Area (%) **150ul *Elution Buffer 1***	OT QC Area/OT standard Area (%) **100ul *Elution Buffer 1* + 50uL 0,03% BSA**
10	37	26	34
25	36	29	35
40	34	41	28
55	32	32	32
70	31	36	31
85	30	36	28
100	29	33	25

4.3.2. Antibody-bead-complex Amount

To investigate how the amount of antibody-bead-complex per well influenced the signal, three different amounts were evaluated. During the preparation of antibody-bead-complex 50 µl of bead stock solution was incubated with 10-30 µg antibody for 30 min. For the incubation with the plasma samples a 96-well plate was used and 100 µl spiked plasma, in seven different concentrations, was added in each well. The samples were incubated for 1 hour in duplicates. Elution was done using 100 µl *Elution Buffer 1* including 100 pg/ml IS.

Figure 11 shows the signal to noise ratio (S/N) for each concentration and antibody-bead-complex amount: 60 µg, 90 µg and 120 µg. As can be seen, there is no larger difference in S/N. However, 90 µg display the highest signal for all OT concentrations except 55 pg/ml where 60 µg gives the highest signal. Based on this, 90 µg antibody-bead-complex was used for the remaining optimization experiments.

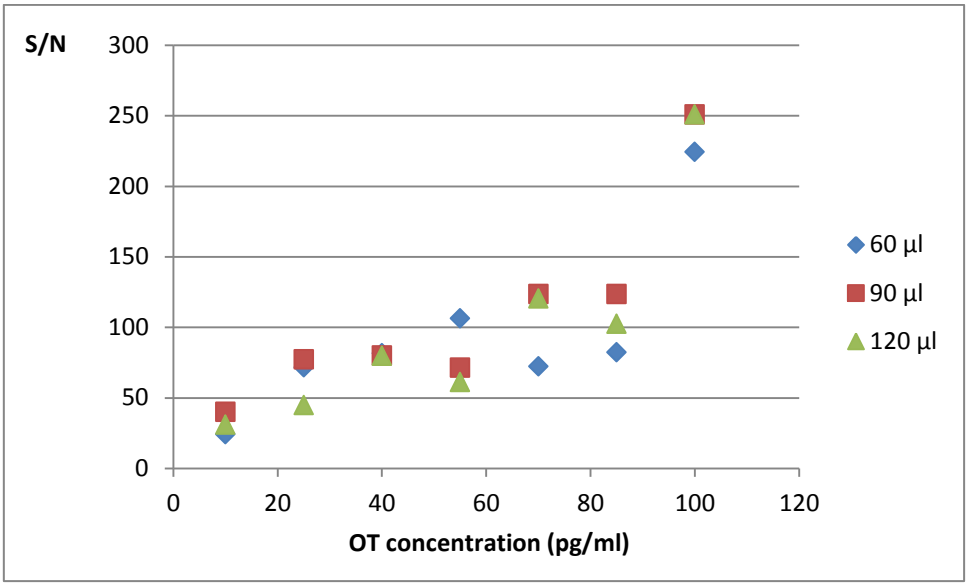


Figure 11. S/N for seven OT concentrations (10, 25, 40, 55, 70, 85 and 100 pg/ml) for three different antibody-bead-complex amounts (60, 90 and 120 µg)

4.3.3. Incubation Time

The recovery when using 1 hour incubation in room temperature can be seen in table 12. When incubating the samples a 96-well plate was used and manual mixing by pipetting every 10-15 min was performed. Table 11 shows that there is a small decrease in recovery with increasing OT concentration. The recovery is calculated as an average from duplicate samples compared to a control sample for each concentration. The recovery ranges from 25.6-47.5%.

Table 12. The recovery (%) after sample preparation (1 hour incubation) for different OT concentrations.

OT Concentration (pg/ml)	Recovery (%)
10	47.5
25	37.5
40	31.8
55	31.9
70	31.1
85	30.6
100	25.6

Two more experiments were conducted where manual mixing by pipetting was done with less frequency (two vs. three times during the 1 hour incubation). Unfortunately, this did not show an increase in recovery. To investigate the influence of incubation time and mixing on recovery an experiment using continuous rotation with the HulaMixer® for 24 hours was done. To decide the potential of this experiment, two concentrations were analysed: 10 and 100 pg/ml. The results showed that the recovery was 28.2 and 19.1% for 10 and 100 pg/ml respectively (table 13).

Tabel 13. The recovery (%) after sample preparation (24 hour incubation) for different OT concentrations.

OT Concentration (pg/ml)	Recovery (%)
10	28.2
100	19.1

4.3.4. Sample Volume

As a last step in the optimization, 200 µl plasma sample was used instead of 100 µl while still remaining an elution buffer volume of 100 µl i.e. concentrating the sample to be analysed with a factor 2. This was performed in order to reach a detection signal that would enable quantification with a LLOQ of 10 pg/ml. Duplicate samples were tested in seven concentrations and the response of the analyte (OT) was compared to the response obtained when using 100 µl sample volume. The response was calculated as a mean value using duplicate samples for each OT concentration. The results showed more than a two-fold increase for all concentrations when using 200 µl sample volume, except for 10 pg/ml where the increase was 42%, still a clear improvement compared to previous experiments (table 14). The increase in response was the ratio calculated as Response 200 µl/Response 100 µl. Based on this improvement in OT response this was chosen to be the final method to qualify i.e. using 200 µl sample volume while keeping an elution volume of 100 µl.

Table 14. The OT response when using 100 µl and 200 µl sample volume respectively and the increase in response calculated as the ratio (Response 200 µl/Response 100 µl).

OT nominal conc. (pg/ml)	Response (100 µl)	Response (200 µl)	Increase in response (Response 200 µl/Response 100 µl ratio)
10	0.06	0.08	1.42
25	0.11	0.29	2.73
40	0.16	0.37	2.30
55	0.21	0.47	2.21
70	0.28	0.65	2.29
85	0.28	0.63	2.21
100	0.31	0.83	2.67

4.3.5. Bead Capacity

As a final step the antibody-bead-complex capacity was tested since an increase in plasma volume indicated an unexploited bead capacity. The experiment was done by increasing the plasma sample volume and investigating the OT response. Sample preparation using 300, 400 and 500 µl plasma was tested. The result for the samples containing 10 pg OT/ml (mean value of duplicates) can be seen in figure 12 and the figure shows an increase in response with increasing plasma volume.

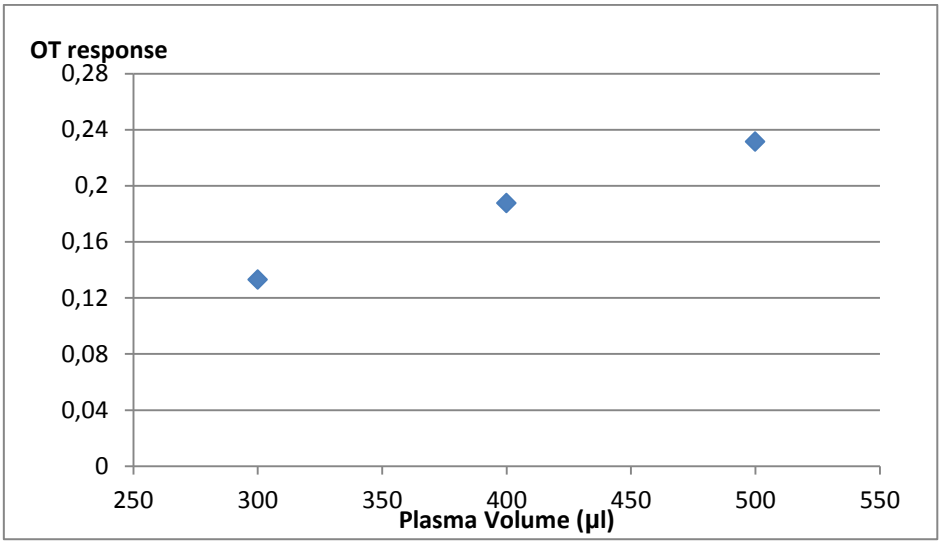


Figure 12. OT response for sample preparations using 300, 400 and 500 µl plasma containing 10 pg OT/ml plasma.

4.4. Qualification of the Final Method

The qualification of the final method was performed as described in section *3.7. Qualification of Final Method* using the experimental method described in section *3.6.3. Final immunocapture procedure.*

A $1/x^2$ weighting was used to fit a linear least squares regression calibration curve to the response versus concentration data. Two versus three calibration points were deleted in run 1 and 2 respectively. After removal of these points good linearity in the range of 10 pg/mL to 100 pg/mL was achieved, with r^2 values of 0.9857 and 0.9866 for the two qualification runs. These two qualification runs yielded lines with slopes of 0.006399 and 0.006927 and y-intercepts of 0.02808 and 0.02608 respectively. Table 15 shows the calibration points for the two runs, where the deleted calibration points are shown in red color. The CV and RE are calculated without these deleted points and ranges from 1 to 14% and -4 to 6% respectively.

Table 15. Standard curve results. SD, standard deviation; CV, coefficient of variation; RE, relative error. Calibrations points deleted are shown in red.

		CAL 1	CAL 2	CAL 3	CAL 4	CAL 5	CAL 6	CAL 7
Nominal conc. (pg OT/mL plasma)		10	25	40	55	70	85	100
Observed conc. (pg OT/mL plasma)	**RUN 1**	4,10	21,9	33,6	42,0	75,2	82,6	96
		10,3	26,5	43,3	51,9	76,1	92,8	98,6
	RUN 2	8,7	23,5	30,0	57,8	68,6	72,8	98,7
		11,3	18,3	24,3	59,0	77,6	85,9	98,9
Average:		8,6	22,6	32,8	52,7	74,4	83,5	98,1
SD:		3,2	3,4	8,0	7,8	4,0	8,3	1,4
CV (%):		**13**	**10**	**14**	**7**	**5**	**10**	**1**
RE (%):		**1**	**-4**	**-4**	**2**	**6**	**-2**	**-2**

n	4	4	4	4	4	4	4

For the QC samples the precision (reported as CV%) did not exceed 15% , while the accuracy (expressed as relative error, RE) did not vary more than -8% from the expected value, except for the highest QC were the accuracy varied +33% and +23% from the expected value. Analysis of the four QC samples yielded a precision between 12% and 15% and an accuracy ranging from 92 to 102% (excluding HIGH QC) (table 16).

Table 16. Precision and Accuracy. LLOQ, lower limit of quantification; QC, quality control; SD, standard deviation; CV, coefficient of variation (measure of precision); RE, relative error (measure of accuracy).

	LLOQ	**LOW QC**	**MID QC**	**HIGH QC**
Nominal conc. (pg OT/mL plasma)	10,0	30,0	45,0	80,0
RUN 1				
Observed conc. (pg OT/mL plasma)	9,40	25,7	41,7	95,6
	10,6	23,7	39,8	84,7
	6,90	27,1	37,3	120
	9,20	29,3	39,8	96,1
	9,90	34,6	43,2	96,9
	9,30	33,2	53,2	97,4
Mean:	9,20	28,9	42,5	98,4
SD:	1,25	4,28	5,61	11,5
CV (%):	**14**	**15**	**13**	**12**
RE (%):	**-8**	**-4**	**-6**	**23**
n	6	6	6	6
RUN 2				
Observed conc. (pg OT/mL plasma)	9,90	27,5	44,8	103
	11,1	25,4	42,7	91,4
	7,20	29	40,1	129
	9,60	31,5	42,8	104
	10,5	37,2	46,5	105
	9,70	35,6	57,3	105
Mean:	9,70	31,0	45,7	106
SD:	1,33	4,64	6,08	12,4
CV (%):	**14**	**15**	**13**	**12**
RE (%):	**-3**	**3**	**2**	**33**
n	6	6	6	6

4.5. Comparison of Final Immunocapture Method and SPE Method

When comparing the immunocapture and SPE method, samples with plasma blank and OT concentration of 5 and 10 pg/ml were tested. OT had a t_r of approximately 4.6. Figure 13 shows the results for the plasma blanks. In the upper chromatogram corresponding to the SPE method no detectable OT peak can be seen. However, in the lower chromatogram corresponding to the immunocapture method an OT peak is detected with a S/N of 2.89. Figure 14 shows the result for the samples with 5 pg OT/ml plasma. For the SPE (upper chromatogram) the results show a S/N of 4.01 and for the immunocapture (lower chromatogram) the S/N is detected to be 25.45. For the samples containing 10 pg OT/ml the S/N is 0.97 for the SPE and 41.44 for the immunocapture method (figure 15).

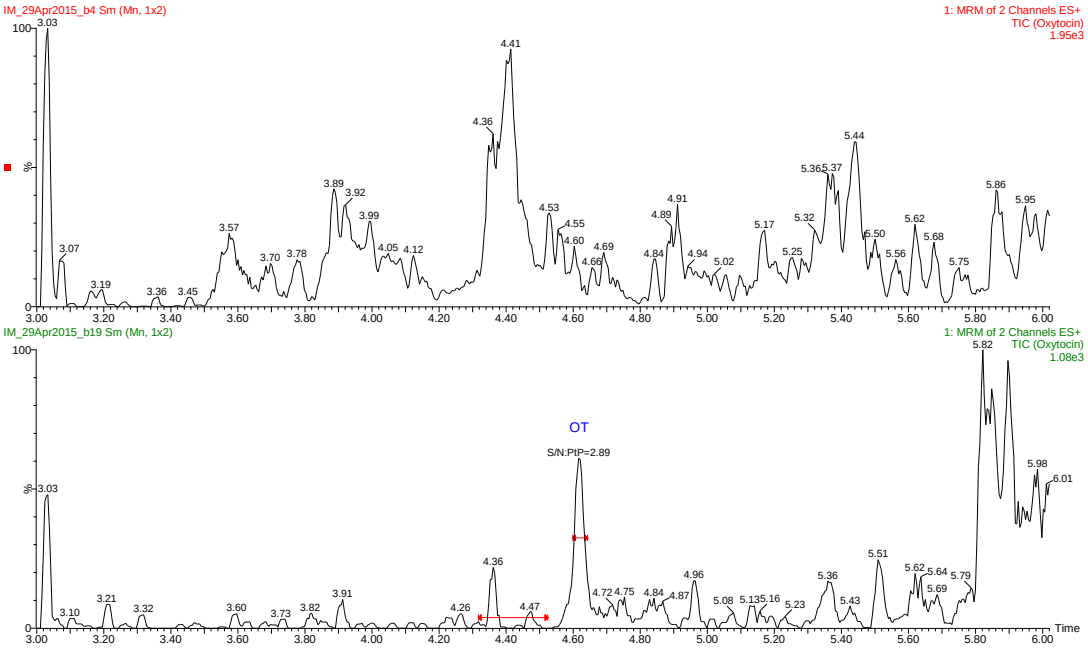


Figure 13. Chromatogram for the plasma blanks of the SPE(upper) - and immunocapture(lower) method respectively.

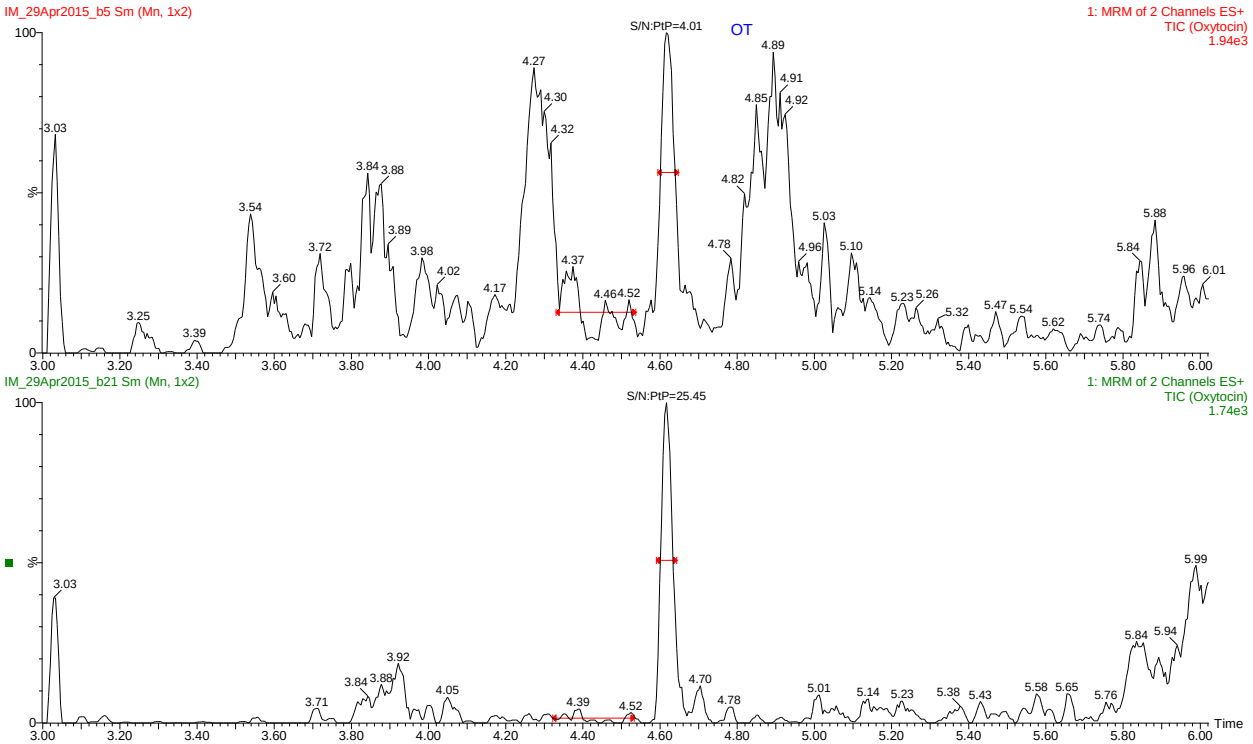


Figure 14. Chromatogram for plasma samples containing 5pg OT/ml of the SPE(upper) - and immunocapture(lower) method respectively.

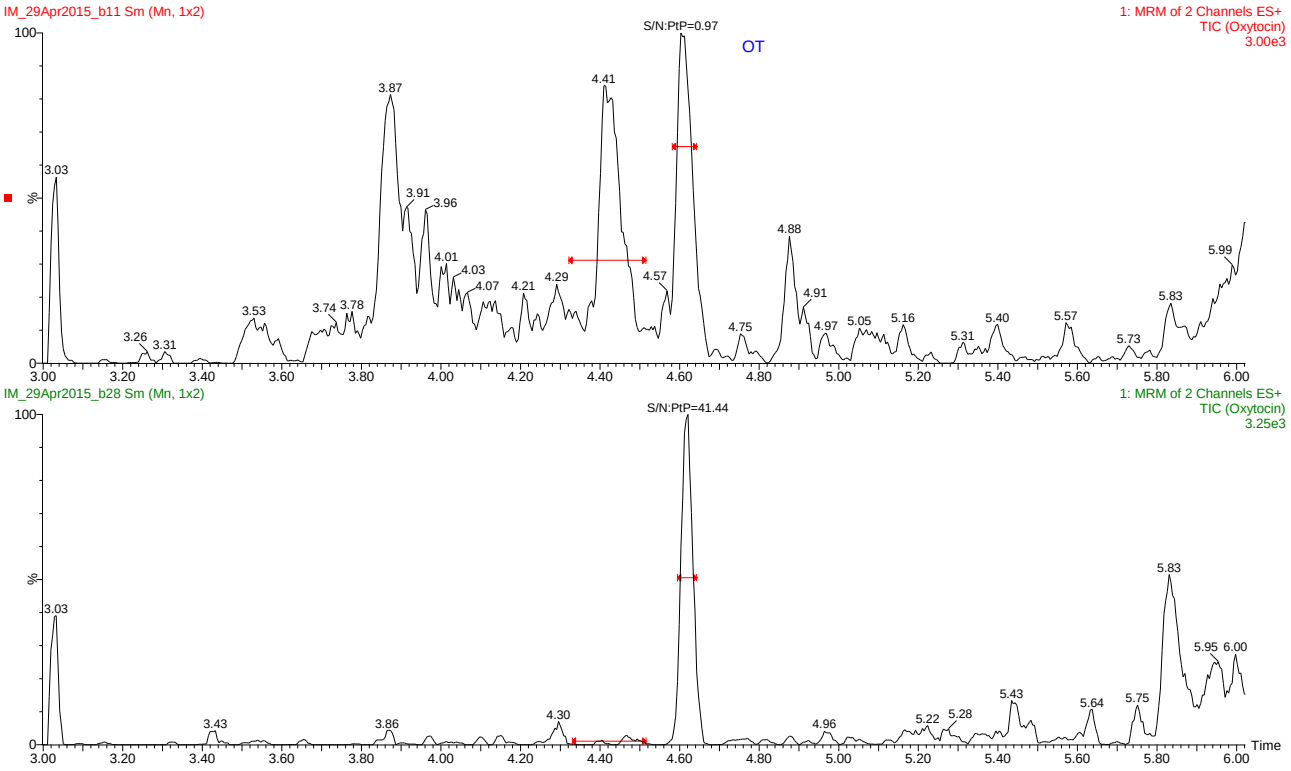


Figure 15. Chromatogram for plasma samples containing 10pg OT/ml of the SPE(upper) - and immunocapture (lower) method respectively

5. Discussion

During the immunocapture assay development and optimization a great amount of experiments were conducted, some more successful than others. To investigate the potential of immunocapture techniques as sample preparation both SPE optimization and immunocapture assay development and optimization was performed. The SPE method, the previously established sample preparation method prior to LC-MS/MS analysis was used to evaluate the successfulness of the developed immunocapture assay.

5.1. SPE Optimization

As can be seen in figures 7-10 the results are more or less identical for both HLB and WCX. This confirms that OT does not bind stronger to the WCX and thus does not take advantage of the ion binding mechanism of WCX. The core of the WCX material is in fact HLB and this explains the similar results. In consultation with Research Scientist Sara Stensson the HLB plate was chosen as the final SPE plate, since the WCX plate did not improve the results. Elution occurred after washing with 40% methanol (figure 7) and therefore 30% methanol was chosen for the second wash. It is crucial not to elute OT during the washing steps. For the elution 30% acetonitrile was chosen. A higher acetonitrile percentage did not increase the elution of OT and is therefore not necessary (figure 9).

5.2. Immunocapture Assay Development

During the immunocapture assay development the potential of different coupling mechanisms for both the Dynabeads® and the MSIA were investigated. Further, the potential of different antibodies were tested. Due to the time limit of the project, choosing of the coupling mechanism and the antibody having the greatest potential, had to be made based on a limited amount of experiments.

Unfortunately, the Dynabeads® experiments carried out utilizing the streptavidin-biotin interaction gave inconclusive results. There was no success in capturing OT during the sample preparation. To make sure that the OT was not washed off during the washing steps, the supernatant from wash 1 and 3 was analysed. No OT was detected in the supernatant obtained from the washing steps either. Also, two different elution buffers were tested giving no difference in the results. Since two different beads were tested with different antibody concentrations and elution buffers, the conclusion is that the problem lies in the capacity of the biotinylated anti-OT antibody. The supply of biotinylated anti-OT antibodies in the commercial market was found to be very poor and no replaceable antibody could be found. Based on the results and the lack of biotinylated anti-OT antibodies on the market no more experiment were conducted using these types of Dynabeads®. The strong streptavidin-biotin interaction would have made these beads a greater candidate than the protein G-beads since the interaction between protein G and antibody is not near the strength of streptavidin-biotin. If a proper biotinylated OT-antibody was available on the market this would probably be the

best choice since the antibody would not have been washed off during elution and the beads could have been reused saving both time and money.

Due to the fact that the biotinylated antibody was found to be unsuccessful, the choice of antibody was done by testing different antibodies with the Protein G-beads. The Protein G-beads were used to be able to compare the results using the same coupling technique with MSIA™. The choice of antibody was based on recovery calculated in regard to the signal for the immunocapture-prepared sample compared to the control signal (1 ng OT/ml). As can be seen in table 8 the antibody showing the greatest potential is the Millipore antibody MAB5296, with a recovery of 11%, this can be explained by the fact that the antibody is monoclonal and therefore likely to be very specific towards OT. This antibody was chosen for further development and optimization. Due to the time limit it was assumed that MAB5296 was the antibody best suitable for the Sheep Anti-Mouse-beads as well. This was done to determine whether to proceed optimization with the Protein G-beads or the Sheep Anti-Mouse-beads.

To determine which Dynabeads® type to continue the optimization with, the Protein G-beads and the Sheep Anti-Mouse-beads were compared using the chosen antibody MAB5296. Two different amount of antibody-bead-complex per sample was tested and the results showed that the Protein G-beads gave a higher recovery of 38 and 40% compared to 21 and 22% (table 9) i.e. the Protein G-beads showed a recovery twice as high. Further, it can be seen that the recovery when using the smaller amount of antibody-bead-complex per well is 1 and 2 % higher for the Sheep Anti-Mouse and Protein G-beads respectively. Since it only differs 1 and 2% it is not possible to state that the difference in recovery is due to the antibody-bead-complex amount used.

To investigate the potential of MSIA™ a couple of experiments were conducted using both Protein A, Protein AG and Streptavidin tips. Unfortunately, the results were disappointing and could not compete with the potential of the Protein G-beads. During the development of MSIA™ a Field Application Specialist (MSIA Consumables & Platform) from Thermo Fisher was of assistance. Even though the successful antibody MAB5296 was used for the MSIA experiments, there was no detectable OT in the chromatograms. As the Field Application Specialist stressed, the capacity and purity of the antibody is of great importance during MSIA™ development. Why MAB5296 did not work with MSIA™ may be explained by the presentation of the antibody. Since the antibody is presented in ascites fluid, it is not affinity purified and may contain other IgG that are not specific to OT as well. Therefore there is a risk of other competing mouse IgG binding to the Protein G. It was discussed whether or not to affinity purify the antibody but with risk of losing the antibody e.g. if the antibody-antigen interaction is too strong, causing an unsuccessful elution of the antibody, this approach was not taken.

Due to the lack of ready-made biotinylated antibodies that showed good performance or antibodies that were pure enough to enable biotinylation, a different approach was tested

with the streptavidin-tips where pre-incubation of MAB5296 and biotinylated anti-mouse antibody was performed. Still, inconclusive results were obtained. When pre-incubating, the binding of the two antibodies may have caused steric hindrance for the OT binding. Instead of pre-incubating, the antibodies can be used directly with MSIA™ instead. Prior to capturing the anti-OT antibody the anti-mouse antibody is captured instead. Even though further development of MSIA™ could have yielded in detecting OT, the limitation of antibodies and time limit lead to the decision to focus on optimizing the Protein G beads instead. However, if having a successful antibody, MSIA™ would have been simple to automatize and handle.

5.3. Immunocapture Optimization

Even though the monoclonal antibody chosen for optimization is presented in ascites fluid and may therefore contain other antibodies as well, the affinity of the antibody seems high. During the optimization parameters such as elution, antibody-bead amount, incubation time, sample volume and bead capacity were tested and the influence of each parameter was analyzed. The biggest problem during the optimization was to increase the OT recovery. The yield, when testing different elution experiments, was calculated as the ratio between the OT QC-area and OT standard-area and ranged from 19 to 28% when using IS during the sample preparation and 25 to 41% when using IS in the elution. To be able to correctly calculate the yield, it was necessary to add IS to the elution buffers instead. The elution as parameter did not seem to influence the yield. No clear pattern in recovery could be seen when changing the elution buffer or the elution buffer amount. Therefore the low recovery does not seem to be caused by the elution process.

The second parameter to be tested was the antibody-bead-complex amount and to examine whether the capacity was too low to capture the total amount of OT, even if working with in a very low concentration level. Three different amounts were tested but the results showed no sign of an increased signal with an increased amount. The difference in signal between the different amounts was very small but for all concentrations except one (55 pg/ml) 60 µg showed a slight increase in signal. Due to the minor increase in signal it is not possible to state that the increase depends on the amount of antibody-bead-complex used, it can simply be a coincidence. Even if no explanation can be made, 90 µg showed a minor increase and was the amount chosen. If the problem of the low yield was the antibody-bead-complex amount the highest amount of 120 µg would have generated the highest signal.

When working with a large amount of samples, a 96-well plate was used during sample preparation. Unfortunately no appropriate mixer could be found and sedimentation of the beads occurred between every manual mixing occasion. According to Life Technologies, a continuous mixing/rotation of the beads is very important during incubation to enable a sufficient capture of OT. To determine if an increase in incubation time and continuous mixing had an impact on the recovery a 1 hour incubation, using a 96-well pate and mixing by pipetting every 10 min, was compared to a 24 hour incubation using the HulaMixer

(continuous rotation of the tubes). Both incubations were performed in room temperature. The results show that an increase in incubation time did not have a positive effect on the recovery that ranged from 19.1 to 28.2% compare to the 1 hour incubation were the recovery was 25.6-47.5%. For the 24 hour incubation only two concentrations were tested at: 10 and 100 pg OT/ml plasma, to explore the potential of this method change. The negative effect on recovery may be due to a too long incubation in room temperature causing a dissociation of the antibody and the Protein G. If this was the case the antibody, together with the OT, was washed off during the washing steps and an incubation at +4°C could possibly have prevented it.

To ensure that the low recovery was not due to a capacity issue with the antibody, the sample volume was increased and the antibody-bead-complex capacity was investigated. When increasing the sample volume from 100 to 200 µl approximately a twofold increase in OT response was detected, meaning that the capacity of the antibody-bead-complex was not the problem. This increase in response and hence the detection signal, made it possible to use a LLOQ of 10 pg/ml.

The qualification of the method was therefore done using 200 µl plasma but to further investigate the antibody-bead-complex capacity an additional experiment was done where 300, 400 and 500 µl plasma was used. The increase in response when using 300 and 400 µl plasma was approximately 40% while the difference between using 400 and 500 µl plasma was approximately 25%. An increase in response indicates that the used amount of antibody-bead-complex has a capacity to endure more plasma than the original amount used. To determine the capacity an experiment using larger volumes of plasma should be conducted. The increase in response shows that the low recovery is not due to capacity issues of the antibody-bead-complex. Why then, is the recovery only between 30-40% when working with 100 µl plasma?

The most probable explanation is the unsufficient mixing during incubation generating an unsatisfactory binding of the OT. Due to the time limit no more experiments to investigate this were conducted, however additional experiments testing different incubation times and using the HulaMixer® for continuous mixing can be done to optimize the incubation. Another explanation can be unspecific binding, which often is a problem when working with small peptides like OT. One experiment was conducted where BSA was added in the analysis plate prior to addition of the purified sample to reduce the unspecific binding but the results showed no improvement in recovery. Additional experiment to investigate the unspecific binding can be done. A third explanation can be that the antibody and antigen (OT) reach equilibrium when 30-40% of the OT in the sample has bound to the antibody, nevertheless to determine this, the mixing and unspecific binding should be investigated first.

5.4. Qualification of the Final Method

The qualification of the final method was done by testing the linearity at seven concentrations and precision and accuracy at four QC-levels. The back calculated concentration should be within ±15% of the nominal concentration except for LLOQ where it should be within ±20%. At least 75% of the calibration samples, with a minimum of six concentration levels, must fulfil this criterion. After removing 4 calibration points, good linearity in the range of 10 to 100 pg/ml was achieved. The CV was no more than 14 % including the LLOQ and the RE varied no more than -4 to 6%. For the QC-samples the precision and accuracy was qualified. The precision was measured in CV with the highest value at 15% (30 pg/ml) which fulfilled the criterion for precision. The accuracy was measured in RE and varied no more than -8% from the expected value except for the HIGH QC-sample where the accuracy was found to be +23% an +33% from the expected value even though the precision was according to the criterion. When looking at the measured concentration at QC 80pg/ml, it seems to be varying very close to 100 pg/ml and since linearity was achieved at these concentration (both at 85 and 100 pg/ml) it seems highly likely that the QC samples used as 80 pg/ml was in fact samples spiked with 100pg/ml. This would explain the good precision but the great margin of error in accuracy. Excluding the QC-samples at 80pg/ml the final immunocapture method exhibited a good linearity and a precision and accuracy according to the criterions. A further improvement to improve precision would be to include the IS during the immunocapture to compensate for variations during this process.

5.5. Comparison of Final Immunocapture Method and SPE Method

To determine if the developed immunocapture method was an improvement from the optimized SPE method they were compared at two concentrations: 5 and 10pg/ml as well as endogenous levels in blank plasma. When analysing the blank plasma samples no endogenous levels of OT could be detected with the SPE method, however when using the developed immunocapture method OT was detected with a S/N of 2.89 (figure 13). For the samples containing 5 pg/ml the S/N was 4.01 for the SPE and 25.45 for the immunocapture method. By looking at the background noise in figure 14 it is very clear the sample prepared with immunocapture shows a very clear OT peak distinctly distinguish from the background while the sample prepared with SPE has disturbing peaks almost as high as for the OT. The same goes for the sample were 10 pg OT/ml plasma was used. Here the S/N for the SPE was 0.97 compared to 41.44 for the immunocapture method (figure 15). A high S/N indicates a high signal for the analyte compared to the background signal. These impressive results for the immunocapture method shows that a highly pure sample containing OT was generated and that even low endogenous levels of OT in blank plasma could be detected. The background noise generated by the complex matrix plasma, was drastically reduced compared to the SPE and the immunocapture method seems to have a great potential for a method able to quantify levels down to the low pg/ml.

6. Conclusion

The proposed approach to use immunocapture techniques for sample preparation prior to LC-MS/MS analysis was shown to have great potential for detecting OT at low concentration levels. With the available commercial antibodies in mind, the Dynabeads® were successful whereas the MSIA™ had higher demands on the antibody and the supply of antibodies on the market was unsatisfactory. The immunocapture method showing the most promising results were the Dynabeads® Protein G together with the monoclonal antibody MAB5296. Problems with low recovery was come across but to be able to quantify the method using a LLOQ of 10 pg/ml, the sample volume was increased to 200 µl generating a two-fold increase in OT response. The qualification of the final method showed a good linearity and precision and accuracy when excluding the HIGH QC-sample that most likely was wrongly spiked. When comparing the developed method with the SPE method is was very clear that the immunocapture technique enabled a very pure extract where endogenous levels of OT was detected in blank plasma whereas OT levels of 10 pg/ml could not be distinguished from the background noise when using SPE. These observations show that immunocapture is a successful technique for extracting an analyte at very low concentrations in complex matrices such as plasma. The specificity towards the analyte enables a very pure extract to be analysed.

For future research, a full validation of the final method should be conducted. Also, further optimization of the Dynabeads® would be interesting. A development of a pure presented monoclonal antibody with high specificity would be very satisfying, enabling a biotinylation and also the possibility to do a re-evaluation on the merits of the MSIA™ system. With MSIA™ an automation would be simple to implement whereas it would be more of a challenge, but still interesting to try to automate the Dynabeads® method for future use. Further, it would also be interesting to develop an on-line immunocapture method coupled to a LC-MS/MS system.

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Appendix I: Life Technologies protocols for the different Dynabeads® types

The different protocols from Life Technologies as referred to in section *3.6.3. Dynabeads®* can be seen below in the order.

invitrogen
by life technologies

Dynabeads® M-280 Streptavidin

Catalog nos. 11205D, 11206D, 60210 **Store at 2 to 8°C**

Rev. Date: October 2011 (Rev. 015)

Product Contents

Cat. no.	Volume
11205D	2 mL
11206D	10 mL
60210	100 mL

Dynabeads® M-280 Streptavidin contains 10 mg (~6–7 × 10^8) Dynabeads®/mL in phosphate buffered saline (PBS) pH 7.4, with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide as preservatives.

Product Description

Dynabeads® M-280 Streptavidin are ideal for numerous applications, including purification of proteins and nucleic acids, protein interaction studies, immunoprecipitation, immunoassays, phage display, biopanning, drug screening and cell isolation.

Add Dynabeads® to a sample containing biotinylated molecules, e.g. peptides, proteins, antibodies, sugars, lectins, oligonucleotides, DNA/RNA. During a short incubation, the biotinylated molecule will bind to the beads. Separate the molecule-bead capture, washing, and detection can be optimized for manual or automated use. With indirect capture, mix the biotinylated molecule with the sample to capture the molecule-target complex before adding Dynabeads®. Indirect target capture is an advantage when molecule-target kinetics are slow, affinity is weak, molecule concentration is low, or molecule-target binding requires optimal molecule orientation and true liquid-phase kinetics.

Required Materials

- Magnet (DynaMag™): See www.lifetechnologies.com/magnets for magnet recommendations.
- Mixing device with tilting and rotation (e.g. HulaMixer® Sample Mixer).
- Buffers and Solutions (see Table 1).
- Biotinylated compounds. For advice on biotinylation, see www.lifetechnologies.com/Dynabeads.
- For biotinylation details, download the Molecular Probes® Handbook from www.lifetechnologies.com/handbook.

Table 1: Recommended buffers and solutions

For coupling of nucleic acids	For Dynabeads® treatment before RNA manipulations	For coupling of proteins and other molecules
Binding and washing (B&W) Buffer (2X): 10 mM Tris-HCl (pH 7.5) 1 mM EDTA 2 M NaCl	**Solution A:** DEPC-treated 0.1 M NaOH DEPC-treated 0.05 M NaCl **Solution B:** DEPC-treated 0.1 M NaCl	PBS buffer pH 7.4 These buffers can also be used for your application if needed: PBS/BSA (PBS, pH 7.4 containing 0.01% (w/v) BSA) PBST (PBS pH 7.4 containing 0.01% (v/v) Tween®-20)

The salt concentration and pH (typically 5–9) of the chosen binding/washing buffers can be varied depending on the type of molecule to be immobilized. Beads with immobilized molecules are stable in common buffers.

General Guidelines

- Keep the tube on the magnet for 2 min to ensure that all the beads are collected on the tube wall.
- For diluted samples, increase the incubation time or isolate in smaller batches using the same beads in each batch.
- Avoid air bubbles during pipetting.
- Free biotin in the sample will reduce the binding capacity of the beads. A disposable separation column or a spin column will remove unincorporated biotin.
- For some applications it can be an advantage to add a detergent such as 0.01–0.1% Tween® 20 to the washing/binding buffers to reduce non-specific binding.
- Run the PCR with limiting concentrations of biotinylated primer, or remove free biotinylated primer by ultrafiltration, microdialysis or other clean-up protocols. PCR Clean Up products are available from www.lifetechnologies.com.

Both the size of the molecule to be immobilized and the biotinylation procedure will affect the binding capacity. The capacity for biotinylated molecules depends on steric availability and charge interaction between bead and molecule and between molecules. There are two or three biotin binding sites available for each streptavidin molecule on the surface of the bead after immobilization.

- Optimize the quantity of beads used for each individual application by titration.
- Use up to two-fold excess of the binding capacity of the biotinylated molecule to saturate streptavidin.
- Binding efficiency can be determined by comparing molecule concentration before and after coupling.

Protocol

Recommended Washing Buffers

- Nucleic acid applications: 1X B&W Buffer (see Table 1 for recipe). Dilute to 1X B&W Buffer with distilled water.
- Antibody/protein applications: PBS, pH 7.4.

Wash Dynabeads®

Calculate the amount of beads required based on their binding capacity (see Table 2), and transfer the beads to a new tube.

1. Resuspend the Dynabeads® in the vial (i.e. vortex for >30 sec, or tilt and rotate for 5 min).
2. Transfer the desired volume of Dynabeads® to a tube.
3. Add an equal volume of Washing buffer, or at least 1 mL, and mix (vortex for 5 sec, or keep on a roller for at least 5 min).
4. Place the tube on a magnet for 1 min and discard the supernatant.
5. Remove the tube from the magnet and resuspend the washed Dynabeads® in the same volume of washing buffer as the initial volume of Dynabeads® taken from the vial (step 2).

Table 2: Typical binding capacities for one mg of Dynabeads®.

Biotinylated target	Binding/mL
Free Biotin (pmol)	650–900
Biotinylated peptides (pmol)	~200
Biotinylated antibody (µg)	~10
ds DNA (µg) *	~10
Ss oligonucleotides (pmol) *	~200

* **Oligonucleotides and DNA fragments**
For oligonucleotides, capacity is inversely related to molecule size (number of bases). Reduced binding capacity for large DNA fragments may be due to steric hindrance.

Product Use: For research use only. Not for human or animal therapeutic or diagnostic use.

Dynabeads® for RNA Manipulation

As Dynabeads® Streptavidin are *not* supplied in RNase-free solutions, perform the following steps after washing for RNA applications:

1. Wash the beads twice in Solution A for 2 min. Use the same volume of Solution A as the initial volume of Dynabeads® taken from the vial or larger.
2. Wash the beads once in Solution B. Use the same volume of beads as in step 5.
3. Resuspend the beads in Solution B.

The beads are now ready to be coated with the biotinylated molecule of your choice.

Immobilization Protocol

Wash the Dynabeads® according to "Wash Dynabeads®" section before use.

1. Add the biotinylated molecule to the washed Dynabeads®.
2. Incubate for 15–30 min at room temperature with gentle rotation of the tube.
3. Place the tube in a magnet for 2–3 min and discard the supernatant.
4. Wash the coated beads 3–4 times in washing buffer.
5. Resuspend to desired concentration in a suitable buffer for your downstream use.

Here are some examples of immobilization protocols for specific applications.

Immobilize Nucleic Acids

1. Resuspend beads in 2X B&W Buffer to a final concentration of 5 µg/µL (twice original volume).
2. To immobilize, add an equal volume of the biotinylated DNA/RNA in distilled water to dilute the NaCl concentration in the 2 B&W Buffer from 2 M to 1 M for optimal binding.
3. Incubate for 15 min at room temperature using gentle rotation. Incubation time depends on the nucleic acid length: short oligonucleotides (<30 bases) require max. 10 min. DNA fragments up to 1 kb require 15 min.
4. Separate the biotinylated DNA/RNA coated beads with a magnet for 2–3 min.
5. Wash 2–3 times with a 1X B&W Buffer.
6. Resuspend to the desired concentration. Binding is now complete. Resuspend the beads with the immobilized DNA/RNA fragment in a buffer with low salt concentration, suitable for downstream applications.

Immobilize Antibodies/Proteins

1. Incubate the beads and biotinylated antibodies in PBS for 30 min at room temperature using gentle rotation.
2. Separate the antibody-coated beads with a magnet for 2–3 min.
3. Wash the coated beads 4–5 times in PBS containing 0.1% BSA.
4. Resuspend to the desired concentration for your application.

Release Immobilized Biotinylated Molecules

The biotin-streptavidin bond is broken by harsh conditions. 5 min incubation at 65°C or 2 min at 90°C in 10 mM EDTA pH 8.2 with 95% formamide will typically dissociate >96% of immobilized biotinylated DNA. Alternatively, boil the sample for 5 min in 0.1% SDS for protein dissociation. Please note that proteins will be denatured by such treatment and Dynabeads® Streptavidin can not be re-used. It has also been reported that the biotin-streptavidin interaction can be broken by a short incubation in non-ionic water at a temperature above 70°C.

Automation

Magnetic separation and handling using Dynabeads® can easily be automated on a wide variety of liquid handling platforms. Dynabeads® MyOne™ Streptavidin T1 share similar properties to Dynabeads® M-280 Streptavidin but are smaller, making them ideal for automation applications due to their small size, low sedimentation rate and high magnetic mobility. Selected protocols are available at www.lifetechnologies.com/automation.

Description of Materials

Dynabeads® Streptavidin are uniform, superparamagnetic beads of 2.8 µm in diameter with a streptavidin monolayer covalently coupled to the surface. This layer ensures negligible streptavidin leakage while the lack of excess adsorbed streptavidin ensures batch consistency and reproducibility of results.

Related Products

Product	Cat. no.
Dynabeads® M-270 Streptavidin	65305
Dynabeads® MyOne™ Streptavidin C1	65001
Dynabeads® MyOne™ Streptavidin T1	65601
Dynabeads® Kit kilobaseBINDER™	60101
DynaMag™-2	12321D
HulaMixer® Sample Mixer	15920D

REF on labels is the symbol for catalog number.

Limited Use Label License

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Manufactured by Life Technologies AS, Norway. Life Technologies AS complies with the Quality System Standards ISO 9001:2008 and ISO 13485:2003.

SPEC-05738

For support visit www.lifetechnologies.com/support or email techsupport@lifetech.com

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Dynabeads® MyOne™ Streptavidin T1

Catalog nos. 65601, 65602 **Store at 2 to 8°C**

Rev. Date: October 2011 (Rev. 005)

Product Contents

Cat. no.	Volume	Concentration
65601	2 mL	10 mg/mL*
65602	10 mL	10 mg/mL*

* Corresponds to roughly 7–10 x 10^8 Dynabeads® per mL.

Dynabeads® MyOne® Streptavidin T1 contains 10 mg (~7–10 x 10^8) Dynabeads®/mL in phosphate buffered saline (PBS) pH 7.4, with 0.01% Tween®-20 and 0.09% sodium azide as a preservative.

Product Description

Dynabeads® MyOne™ Streptavidin T1 are ideal for numerous applications, including purification of proteins and nucleic acids, protein interaction studies, immunoprecipitation, immunoassays, phage display, biopanning, drug screening and cell isolation. MyOne® Dynabeads® offer increased binding capacity and slower sedimentation rate, making them ideal for automated applications and for when larger amounts of biotinylated ligand, or their specific target, need to be isolated.

Add Dynabeads® to a sample containing biotinylated molecules, e.g. peptides or oligonucleotides. During a short incubation, the biotinylated molecule will bind to the beads. Separate the molecule-bead complex with a magnet. Capture, washing, and detection can be optimized for manual or automated use.

With indirect capture, mix the biotinylated molecule with the sample to capture the molecule target complex before adding Dynabeads®. Indirect target capture is an advantage when molecule-target kinetics are slow, affinity is weak, molecule concentration is low or molecule-target binding requires optimal molecule orientation and true liquid-phase kinetics.

Required Materials

- Magnet (DynaMag™) for manual or automated protocols. See www.lifetechnologies.com/magnets for recommendations.
- Mixing device with tilting and rotation, e.g. HulaMixer® Sample Mixer.
- Buffers and Solutions, see Table 1. For many applications it can be an advantage to add a detergent, such as 0.01–0.1% Tween®-20 to the washing/binding buffers to reduce non-specific binding.
- For biotinylation details, download the Molecular Probes® Handbook from www.lifetechnologies.com/handbook.

General Guidelines

- Keep the tube on the magnet for up to 2 min to ensure that all the beads are collected on the tube wall.
- If you do not need to remove preservatives or change buffers, you can omit washing of Dynabeads ®.
- For diluted samples, increase the incubation time or aliquote the sample into several smaller volumes.
- Use a mixer to tilt/rotate the tubes so Dynabeads® do not settle at the tube bottom.
- Avoid air bubbles during pipetting.
- Free biotin in the sample will reduce the binding capacity of the beads.

Table 1: Recommended buffers and solutions

For coupling of nucleic acids	For Dynabeads® treatment before RNA manipulations	For coupling of proteins and other molecules
Binding and washing (B&W) Buffer (2X): 10 mM Tris-HCl (pH 7.5) 1 mM EDTA 2 M NaCl	Solution A: DEPC-treated 0.1 M NaOH DEPC-treated 0.05 M NaCl Solution B: DEPC-treated 0.1 M NaCl	PBS buffer pH 7.4 These buffers can also be used for your application if needed: PBS/BSA (PBS, pH 7.4 containing 0.01% (w/v) BSA) PBST (PBS pH 7.4 containing 0.01% (v/v) Tween®-20)

The salt concentration and pH (typically 5–9) of the chosen binding/washing buffers can be varied depending on the type of molecule to be immobilized. Beads with immobilized molecules are stable in common buffers.

A disposable separation column or a spin column will remove unincorporated biotin. Run the PCR with limiting concentrations of biotinylated primer, or remove free biotinylated primer by ultrafiltration, microdialysis or other clean-up protocols. PCR Clean Up products are available from www.lifetechnologies.com.

Both the size of the molecule to be immobilized and the biotinylation procedure will affect the binding capacity. The capacity for biotinylated molecules depends on steric availability and charge interaction between bead and molecule and between molecules. There are two or three biotin binding sites available for each streptavidin molecule on the surface of the bead after immobilization.

- Optimize the quantity of beads used for each individual application by titration.
- Use up to two-fold excess of the binding capacity of the biotinylated molecule to saturate streptavidin.
- Binding efficiency can be determined by comparing molecule concentration before and after coupling.

Protocol

Recommended Washing Buffers

- Nucleic acid applications: 1X B&W Buffer (see Table 1 for recipe). Dilute to 1X B&W Buffer with distilled water.
- Antibody/protein applications: PBS, pH 7.4.

Wash Dynabeads®

Calculate the amount of beads required based on their binding capacity (see Table 2), and transfer the beads to a new tube.

1. Resuspend the Dynabeads® in the vial (i.e. vortex for >30 sec, or tilt and rotate for 5 min).
2. Transfer the desired volume of Dynabeads® to a tube.
3. Add an equal volume of Buffer, or at least 1 mL and resuspend.
4. Place the tube on a magnet for 1 min and discard the supernatant.
5. Remove the tube from the magnet and resuspend the washed Dynabeads® in the same volume of Buffer as the initial volume of Dynabeads® taken from the vial (step 2).
6. Repeat steps 4–5 twice, for a total of 3 washes.

Table 2: Typical binding capacities for one mg of Dynabeads®.

Biotinylated target	Binding/mL
Free Biotin (pmol)	1100–1700
Biotinylated peptides (pmol)	~400
Biotinylated antibody (µg)	~20
ds DNA (µg) *	~20
ss oligonucleotides (pmol) *	~400

*** Oligonucleotides and DNA fragments**
For oligonucleotides, capacity is inversely related to molecule size (number of bases). Reduced binding capacity for large DNA fragments may be due to steric hindrance.

Dynabeads® for RNA Manipulation

As Dynabeads® Streptavidin are *not* supplied in RNase-free solutions, perform the following steps after washing for RNA applications:

1. Wash the beads twice in Solution A for 2 min. Use the same volume of Solution A as the initial volume of Dynabeads® taken from the vial or larger.
2. Wash the beads once in Solution B. Use the same volume as with Solution A.
3. Resuspend the beads in Solution B.

The beads are now ready to be coated with the biotinylated molecule of your choice.

Immobilization Protocol

Wash the Dynabeads® according to "Wash Dynabeads®" section before use.

1. Add the biotinylated molecule to the washed Dynabeads®.
2. Incubate for 15–30 min at room temperature with gentle rotation of the tube.
3. Place the tube in a magnet for 2–3 min and discard the supernatant.
4. Wash the coated beads 3–4 times in washing buffer.
5. Resuspend to desired concentration in a suitable buffer for your downstream use.

Here are some examples of immobilization protocols for specific applications.

Immobilize Nucleic Acids

1. Resuspend beads in 2X B&W Buffer to a final concentration of 5 µg/µL (twice original volume).
2. To immobilize, add an equal volume of the biotinylated DNA/RNA in distilled water to dilute the NaCl concentration in the 2X B&W Buffer from 2 M to 1 M for optimal binding.
3. Incubate for 15 min at room temperature (RT) using gentle rotation. Incubation time depends on the nucleic acid length: short oligonucleotides (<30 bases) require max. 10 min. DNA fragments up to 1 kb require 15 min.
4. Separate the biotinylated DNA/RNA coated beads with a magnet for 2–3 min.
5. Wash 2–3 times with a 1X B&W Buffer.
6. Resuspend to the desired concentration. Binding is now complete. Resuspend the beads with the immobilized DNA/RNA fragment in a buffer with low salt concentration, suitable for downstream applications.

Immobilize Antibodies/Proteins

1. Incubate the beads and biotinylated antibodies in PBS for 30 min at room temperature using gentle rotation.
2. Separate the antibody-coated beads with a magnet for 2–3 min.
3. Wash the coated beads 4–5 times in PBS containing 0.1% BSA.
4. Resuspend to the desired concentration for your application.

Release Immobilized Biotinylated Molecules

The biotin-streptavidin bond is broken by harsh conditions. 5 min incubation at 65°C or 2 min at 90°C in 10 mM EDTA pH 8.2 with 95% formamide will typically dissociate >96% of immobilized biotinylated DNA. Alternatively, boil the sample for 5 min in 0.1% SDS for protein dissociation. Please note that proteins will be denatured by such treatment and Dynabeads® Streptavidin can not be re-used. It has also been reported that the biotin-streptavidin interaction can be broken by a short incubation in non-ionic water at a temperature above 70°C.

Immunoassay Strategies

Due to their high surface area per weight, uniformity, excellent batch reproducibility and easy adaptation to automated processes, Dynabeads® have become the solid phase of choice for immunoassays (www.lifetechnologies.com) *in vitro* diagnostics.

Automation

Magnetic separation and handling using Dynabeads® can easily be automated on a wide variety of liquid handling platforms. Dynabeads® MyOne™ Streptavidin T1 share similar properties to Dynabeads® M-280 Streptavidin but are smaller, making them ideal for automation applications due to their small size, low sedimentation rate and high magnetic mobility. Selected protocols are available at www.lifetechnologies.com/automation.

Description of Materials

Dynabeads® MyOne™ Streptavidin T1 are uniform, superparamagnetic beads of 1.0 µm in diameter with a streptavidin monolayer covalently coupled to the hydrophilic bead surface. This layer ensures negligible streptavidin leakage while the lack of excess adsorbed streptavidin ensures batch consistency and reproducibility of results.

Related Products

Product	Cat. no.
Dynabeads® M-280 Streptavidin	11205D
Dynabeads® M-270 Streptavidin	65305
Dynabeads® MyOne™ Streptavidin C1	65001
Dynabeads® Kit kilobaseBINDER™*	60101
DynaMag™-2	12321D
HulaMixer® Sample Mixer	15920D

* For biotinylated DNA fragments >2 kb.

REF on labels is the symbol for catalog number.

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SPEC-05765

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Dynabeads® Protein G

Catalog nos. 10003D, 10004D, 10009D **Store at 2°C to 8°C**

Rev. Date: October 2011 (Rev. 007)

Product Contents

Cat. no.	Volume
10003D	1 mL
10004D	5 mL
10009D	50 mL

Dynabeads® Protein G contains 30 mg Dynabeads®/mL in phosphate buffered saline (PBS), pH 7.4, with 0.01% Tween®-20 and 0.09% sodium azide as a preservative.

Product Description

Dynabeads® Protein G are designed for immunoprecipitation of proteins, protein complexes, protein-nucleic acid complexes, and other antigens.

Antibody (Ab) is added to the Dynabeads® Protein G. During a short incubation, the Ab binds to the Dynabeads® via their Fc-region. The tube is then placed on a magnet, where the beads migrate to the side of the tube facing the magnet and allow for easy removal of the supernatant. The bead-bound Ab may now be used for immunoprecipitation. Bound material is easily collected utilizing the unique magnetic properties of the Dynabeads®.

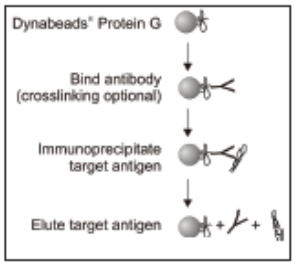

Figure 1: Principle of immunoprecipitation of antigen using Dynabeads® Protein G.

Required Materials

- Magnet (DynaMag™). See www.lifetechnologies.com/magnets for recommendations.
- Mixer allowing tilting and rotation of tubes (e.g. HulaMixer® Sample Mixer).

The following are general recommendations. Alternative buffers may also be used. See "General Guidelines" for details.

- Cell lysis buffer, e.g. Cell Extraction Buffer or NP-40 Cell Lysis Buffer.
- PBS pH 7.4 with and without 0.02% Tween®-20.
- 50 mM Glycine pH 2.8 (elution buffer).
- NuPAGE® LDS Sample Buffer and NuPAGE® Sample Reducing Agent (elution buffer).

General Guidelines

- Dynabeads® Protein G have a binding capacity of approximately 8 µg human IgG per mg beads. The amount of Ab captured depends on the concentration of Ab and Dynabeads® Protein G in the starting sample (see Table 1).
- For standard immunoprecipitation use PBS for antibody binding and washing steps. However, these may be substituted by other buffers of choice, such as alternative phosphate buffers, lysis buffer (e.g. RIPA, NP40), HEPES, Tris. The recommended elution buffer may also be substituted by alternative low pH-, high pH- or high salt buffers, depending on the application.
- Low-affinity antibodies require increased incubation time, thus it can be preferable to pre-incubate sample and antibody prior to bead capture. This improves binding kinetics for the antibody and minimizes non-specific binding. This approach is also recommended when working with protein/nucleic acid complexes, e.g. ChIP.
- Increasing incubation times during immunoprecipitation can improve yield when working with low affinity antibodies. An incubation time of only 10 minutes is sufficient for most applications.
  Increasing the incubation time to 20–120 minutes can increase yield, although non-specific binding may increase with increasing incubation times.
- For sensitive proteins and phosphorylation studies, the isolation protocol including elution may be performed at 4°C, to avoid protein complex dissociation and minimize enzymatic activity.

Protocol

This protocol offers a general guideline for immunoprecipitation. Optimization may be required for each antibody and target antigen. The protocol uses 50 µL of Dynabeads® Protein G, but this may be scaled up or down as required.

Cell Lysis

Cells may be lysed using any standard cell lysis protocol compatible with your starting material. We recommend the use of Cell Extraction Buffer or NP40 Cell Lysis Buffer. For protocols and additional information about cell lysis, see www.lifetechnologies.com/immunoprecipitation.

Prepare Dynabeads®

1. Resuspend Dynabeads® in the vial (vortex >30 sec or tilt and rotate 5 min).
2. Transfer 50 µL (1.5 mg) Dynabeads® to a tube.
3. Place the tube on the magnet to separate the beads from the solution, and remove the supernatant.
4. Remove the tube from the magnet.
5. Proceed directly to "Binding of Antibody".

Bind Antibody

1. Add your antibody (Ab) (typically 1–10 µg) diluted in 200 µL PBS with Tween®-20, to the Dynabeads® from step 4 in "Prepare Dynabeads®". The optimal amount of Ab needed depends upon the individual Ab used.
2. Incubate with rotation for 10 min at room temperature.
3. Place the tube on the magnet and remove the supernatant.
4. Remove the tube from the magnet and resuspend the beads-Ab complex in 200 µL PBS with Tween®-20. Wash by gentle pipetting.
5. Proceed to "Immunoprecipitate Target Antigen".

For storage of Ab-conjugated Dynabeads®, use PBS (pH 7.4) with 0.01–0.1% Tween®-20 to prevent aggregation.

Crosslinking

To avoid co-elution of your antibody, crosslink your antibody to the Dynabeads® before continuing with immunoprecipitation. Use the crosslinking reagent BS_3. For further information and procedure, visit www.lifetechnologies.com/crosslinking.

Immunoprecipitate Target Antigen

1. Place the tube (from step 5 in "Binding of Antibody") on the magnet and remove the supernatant.
2. Add your sample containing the antigen (Ag) (typically 100–1000 µL) and gently pipette to resuspend the Dynabeads®-Ab complex.
3. Incubate with rotation for 10 min at room temperature to allow Ag to bind to the Dynabeads®-Ab complex.
   Note: Depending on the affinity of the antibody, it may be necessary to increase incubation times for optimal binding.

4. Place the tube on the magnet. Transfer the supernatant to a clean tube for further analysis, if desired.
5. Wash the Dynabeads®-Ab-Ag complex 3 times using 200 µL Washing Buffer for each wash. Separate on the magnet between each wash, remove supernatant and resuspend by gentle pipetting.
6. Resuspend the Dynabeads®-Ab-Ag complex in 100 µL Washing Buffer and transfer the bead suspension to a clean tube. This is recommended to avoid co-elution of proteins bound to the tube wall.
7. Proceed to "Elute Target Antigen".

For storage of the immunoprecipitated protein, freeze the Dynabeads®-Ab-Ag complex after adding the elution buffer and sample buffer. For analysis of the sample, thaw and continue with the elution protocol.

EluteTarget Antigen

A. Denaturing elution

1. Place the tube (from step 7 in "Immunoprecipitation of Target Antigen") on the magnet and remove the supernatant.
2. Add 20 µL Elution Buffer, and 10 µL premixed NuPAGE® LDS Sample Buffer and NuPAGE® Sample Reducing Agent (mixed as per manufacturer's instructions).
3. Gently pipette to resuspend the Dynabeads®-Ab-Ag complex.
4. Heat for 10 min at 70°C.
5. Place the tube on the magnet and load the supernatant/sample onto a gel.

Note: As an alternative, the Dynabeads®-Ab-Ag complex can be resuspended in a sample buffer of your choice (e.g. SDS sample buffer). Follow the recommended temperatures and heating times for these buffers prior to gel loadi ng.

B. Non-denaturing elution

1. Place the tube (from step 7 in "Immunoprecipitation of Target Antigen") on the magnet and remove the supernatant.
2. Add 20 µL Elution Buffer and gently pipette to resuspend the Dynabeads®-Ab-Ag complex. Avoid foaming.
3. Incubate with rotation for 2 min at room temperature to dissociate the complex.
4. Place the tube on the magnet and transfer the supernatant containing eluted Ab and Ag to a clean tube. If the eluted protein is to be used for functional assays or stored, the pH of the eluate can be adjusted by adding 1 M Tris, pH 7.5.

Description of Materials

This product contains Dynabeads® Protein G for immunoprecipitation. Dynabeads® Protein G are uniform, 2.8 µm, superparamagnetic beads with recombinant Protein G (approximately 45 kDa) covalently coupled to the surface.

Related Products

Product	Cat. no.
Immunoprecipitation Kit – Dynabeads® Protein A	10006D
Immunoprecipitation Kit – Dynabeads® Protein G	10007D
Dynabeads® Protein A	10001D
DynaMag™-2	12321D
HulaMixer® Sample Mixer	15920D
Cell Extraction Buffer	FNN0011
NP40 Cell Lysis Buffer	FNN0021

REF on labels is the symbol for catalog number.

Table 1: Binding strength of Protein G to different species of Ig's and their subclasses.

Ig origin	Affinity for Protein G
Human IgG1,2,4	+++
Human IgD	-
Human IgA, E, M	-
Human IgG3	+++
Mouse IgG1	+++
Mouse IgG2, 2b, 3	+++
Mouse IgM	+
Rat IgG1	+
Rat IgG2a	+++
Rat IgG2b	+
Rat IgG2c	+
Bovine IgG1	+++
Bovine IgG2	+++
Chicken IgY	-
Dog IgG	+
Goat IgG1	+++
Goat IgG2	+++
Guinea Pig IgG	+
Hamster	NA
Horse IgG	+++
Monkey IgG	+++
Porcine IgG	+++
Rabbit IgG	+++
Sheep IgG1	+++
Sheep IgG2	+++

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SPEC-06726

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Dynabeads® M-280 Sheep anti-Mouse IgG

Catalog nos. 11201D, 11202D

Dynabeads® M-280 Sheep anti-Rabbit IgG

Catalog nos. 11203D, 11204D

Store at 2°C to 8°C

Rev. Date: May 2012 (Rev. 000)

Product Contents

Product contents	Cat. no.	Volume
Dynabeads® M-280 Sheep anti-Mouse IgG	11201D	2 mL
	11202D	10 mL
Dynabeads® M-280 Sheep anti-Rabbit IgG	11203D	2 mL
	11204D	10 mL

Dynabeads® M-280 Sheep anti-Mouse IgG and Dynabeads® M-280 Sheep anti-Rabbit IgG both contains 6–7 × 10^8 beads/mL (~10 mg/mL) in phosphate buffered saline (PBS) pH 7.4 with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide.

Caution: Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

Product Description

Note that this manual describes the protocols for two separate products: Dynabeads® M-280 Sheep anti-Mouse IgG and Dynabeads® M-280 Sheep anti-Rabbit IgG. The protocols and handling steps are the same; the only difference being different primary antibody targets:

- Dynabeads® Sheep anti-mouse IgG bind defined antigens via a mouse primary antibody.
- Dynabeads® Sheep anti-Rabbit IgG bind defined antigens via a rabbit primary antibody.

Dynabeads® M-280 Sheep anti-Mouse IgG and Dynabeads® M-280 Sheep anti-Rabbit IgG are designed as a solid support for simple and efficient binding of immunoglobulins (Ig) or other target molecules (see fig. 1).

The size of the beads (2.8 µm) makes them particularly suitable for isolation of antibodies (Ab) and their target proteins. The beads can also be used for cell isolation, but visit www.lifetechnologies.com/dynabeads to view our full range of cell isolation products.

The beads with primary antibody may be added directly to the sample containing your target antibody/antigen. The beads bind to the target during a short incubation, then the bead-bound target is separated by a magnet (direct technique). Alternatively, the primary antibody is allowed to bind to the target in suspension prior to adding the beads (see fig. 1).

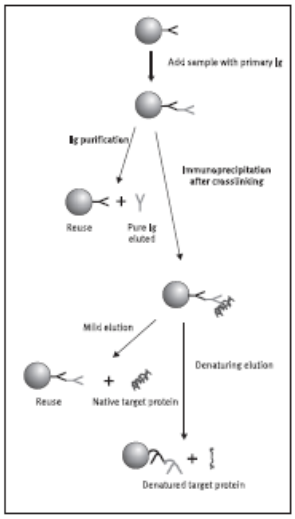

Figure 1: Overview of method

Required Materials

- Magnet (DynaMag™ portfolio). See www.lifetechnologies.com/magnets for recommendations.
- Mixer allowing tilting and rotation of tubes (e.g. HulaMixer® Sample Mixer).
- Primary mouse antibody for Dynabeads® M-280 Sheep anti-Mouse IgG.
- Primary rabbit antibody for Dynabeads® M-280 Sheep anti-Rabbit IgG.

Recommended Buffers

- Washing Buffer: Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.4.
  Note: BSA can be replaced by human serum albumin (HSA) or fetal calf serum (FCS). EDTA can be replaced by sodium citrate.
- Elution Buffer: 0.1 M citrate, pH 2-3.

General Guidelines

- Visit www.lifetechnologies.com/samplepreparation for recommended sample preparation procedures.
- Use a mixer that provides tilting and rotation of the tubes to ensure that the beads do not settle in the tube.
- These products should not be used with the MPC™-1 magnet (Cat. no. 12001D).
- Avoid air bubbles (foaming) during pipetting.
- Never use less than recommended volume of beads.
- Carefully follow the recommended volumes and incubation times.

Direct vs. Indirect Technique

- *Use the indirect technique when:* The concentration of antibody is low, the antibody-antigen affinity is weak, the binding kinetics is slow or the direct technique gives unsatisfactory purity.
- *Use the direct technique when:* The affinity of the primary antibody is high, there are high numbers of target antigens, or to make a larger stock preparation of primary coated beads (will generally have the same shelf life as stated on the beads vial).

Cross-linking prior to Immunoprecipitation

Immunoprecipitation (IP) is done by either adding the primary coated beads directly to a new sample containing the target protein, or by first covalently cross-linking the primary Ig to the antibody on the beads. Bound Ig will be co-eluted along with the target using different elution methods (e.g. for SDS-PAGE followed by Western blotting or autoradiography). For other applications (e.g. protein purification or amino acid sequencing) where co-elution of the Ig is not desired, the primary Ig should be cross-linked to the antibody on the beads prior to IP. If the Ig-coated beads are to be re-used, cross-linking is necessary.

Target Protein Elution

One of the major advantages of using Dynabeads® products in protein/Ig isolation is the possibility to elute in small volumes. Low pH (2.8–3.5), change in ionic strength, affinity elution, electrophoresis, polarity reducing agents, deforming eluents can be applied, or even boiling the bead-target complex in SDS-PAGE application buffer for direct characterization of protein on SDS-PAGE. The method of choice depends on the Ig's affinity for the specific target protein, stability of target protein and downstream applications and detection methods. Most proteins will be eluted at pH 3.1. Some protein functionality might be lost under these conditions. If maintaining functionality of the target protein is important, try milder elution conditions, such as high salt (e.g. 2 M NaCl) or step-wise elution reducing pH from 6 down to 3. This is also recommended if the bead-bound ligand must remain functional to allow re-use of the beads.

Protocol

Wash the Beads

See "Couple Beads with Target Ig" to determine the bead volume.

1. Resuspend the beads in the vial (i.e. vortex for >30 sec, or tilt and rotate for 5 min).
2. Transfer the desired volume of beads to a tube.
3. Add the same volume of Washing Buffer, or at least 1 mL, and resuspend.
4. Place the tube in a magnet for 1 min and discard the supernatant.
5. Remove the tube from the magnet and resuspend the washed beads in the same volume of Washing Buffer as the initial volume of beads (step 2).

Couple Beads with Target Ig

- This protocol is based on 50 µL Dynabeads® Sheep anti-Mouse IgG or Dynabeads® Sheep anti-Rabbit IgG, but is directly scalable. It is not recommended to work with lower volumes. When working with larger volumes, scale up all volumes accordingly.
- Use 0.4–4 µg Ig/50 µL beads. Optimize for your application.

6. Add the sample containing ~0.4–4 µg target-Ig (optimize) to 50 µL of pre-washed and resuspended beads.
7. Incubate with gentle tilting and rotation for 30 min or up to 24 hours at 2°C to 8°C.
8. Place the test tube on the magnet for 2 min and pipet off the supernatant.
9. Remove the test tube from the magnet; add 1 mL Washing Buffer and resuspend.
10. Repeat steps 4 and 5 twice.
11. Place the tube on the magnet and remove the supernatant.

Elute Isolated Ig

- Elute the isolated Ig off the beads using 0.1 M citrate (pH 23) to lower the pH. Most Ig will be eluted off at pH 3.1, but the degree of acidity required will depend on the specific Ig.
- This protocol is based on the 50 µL beads from the "Couple the Beads with Target Ig" section. If using higher volumes, scale up accordingly.

1. Add 50 µL 0.1 M citrate to the Ig-coupled beads.
2. Mix well by tilting and rotation for 2 min.
3. Place the test tube on a magnjet for 2 min and transfer the supernatant containing the purified Ig to a new tube.
4. Add another 50 µL 0.1 M citrate to the bead fraction to elute any remaining Ig.
5. Mix well by tilting and rotation for 2 min.
6. Place the test tube on a magnet for 2 min, pipet off the eluate and pool the two supernatants containing pure Ig.

The Ig-eluted beads may be re-used at least five times. For re-use after elution, the beads should immediately be brought to neutral pH using a Na-phosphate buffer. For storage, the beads should be resuspended in Washing Buffer.

Cross-linking Ig to the Beads

If you want to avoid co-elution of the antibody, cross-link your antibody to the beads before continuing with e.g. IP. We recommend using the cross-linking reagent BS3.For further information and procedure, visit: www.lifetechnologies.com/crosslinking.

Antigen-Binding to Ig-Coated Beads

Use approximately 25 µg target antigen/mL beads to assure an excess of antigen. Dilute with PBS or 0.1 M phosphate buffer (pH 7–8), if necessary.

1. Add 25 µg target antigen/mL Ig-coupled beads.
2. Mix well by tilting and rotation for 1 hour. (Incubation time can be reduced to as low as 10 min, if the protein concentration is high).
3. Place the tube on the magnet for 2 min and pipet off the supernatant.
4. Remove the test tube from the magnet; add 1 mL Washing Buffer and resuspend.
5. Repeat steps 3–4 twice.

See "General Guidelines" for more information on elution of the target protein.

Description of Materials

Dynabeads® M-280 Sheep anti-Mouse IgG are uniform, superparamagnetic, polystyrene beads with affinity purified sheep anti-mouse IgG covalently bound to the bead surface. The antibody binds both heavy and light chains of mouse IgG1, IgG2a and IgG2b. They may have low reactivity towards mouse IgG3 and IgM. Human cross reactivity is minimal.

Dynabeads® M-280 Sheep anti-Rabbit IgG are uniform, superparamagnetic, polystyrene beads with affinity purified sheep anti-rabbit IgG covalently bound to the bead surface. The antibody binds all rabbit IgG subclasses.

Related Products

Product	Cat. no.
DynaMag™-2	12321D
DynaMag™-5	12303D
DynaMag™-15	12301D
Dynabeads® Protein A	10001D
Dynabeads® Protein G	10003D
HulaMixer® Sample Mixer	15920D

REF on labels is the symbol for catalog number.

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SPEC-07657

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Appendix II: Example of calculation for sample preparation using Dynabeads®

Dynabead® concentration:	30	mg/mL		
Capacity antibody:	8	ug Ab/mg beads		
Mw: IgG:	150 000	Da		
Mw: oxytocin:	1007	Da		
Example				
Desired HIGH-sample:	1	ng/mL		
Sample volume:	100	uL		
Bead volume:	30	uL		
Wanted oxytocin capacity (mass):	0,2	ng	Calculation:	Desired HIGH sample x Sample volume (ml)
Wanted oxytocin capacity (moles):	0,2	pmoles	Calculation:	(Wanted OT capacity (mass)x(10^-9))/Mw: OT
Required amount of antibody in each well:	0,01	ug	Calculation:	Mw: IgG/2*wanted OT capacity (moles)*1000000 2 mole OT/mole ab

Antibody-bead coupling

Used a higher amount antibody and beads than required to ensure sufficient binding to beads and also to ensure enough antibody-bead-complex to capture OT.

Total amount antibody required:	0,3 (used 10 instead)	ug	Calculation:	Required amount of antibody in each well/(bead volume/1000)
Total amount of beads required:	1,25 (10 ug Ab)	mg	Calculation:	Total amount antibody required/capacity of antibody
Bead volume required during coupling:	42(used 50 instead)	uL	Calculation:	Total amount of beads required/concentration of beads in original vial x 1000

Appendix III: Chromatograms

Figure 1-3 show the results from an experiment conducted with Dynabeads® Streptavidin M-280 using 1ng OT/ml plasma. Figure one, the control containing elution buffer spiked with 1 ng OT/ml, shows an intensity of 4945 cps and a t_r of 1.53 min for OT. Figure 2 represents the sample-prepared plasma sample (1ng OT/ml plasma) and the OT cannot be distinguished from the background noise even if the background noise only shows an intensity of 60 cps. Figure 3 shows the result of the supernatant collected from the first wash of the plasma sample in figure 2. No OT can be seen here either i.e. the OT has not been eluted during the washing steps.

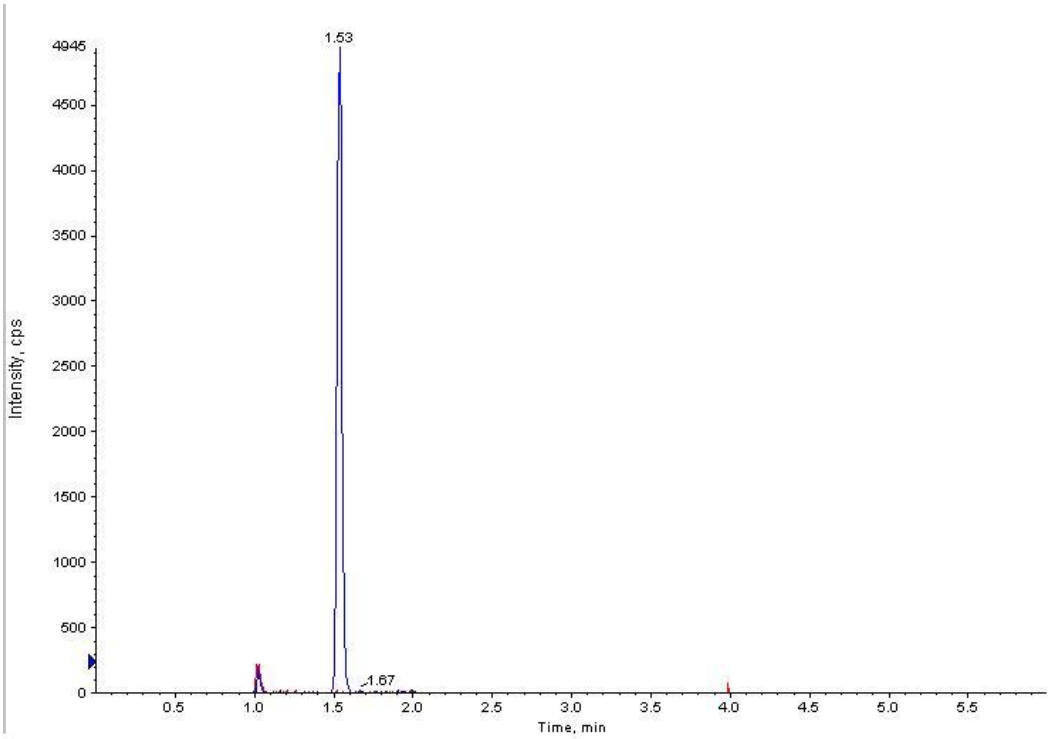

Figure 2. Streptavidin M-280 control (1 ng OT/ml). OT t_r 1.53 min.

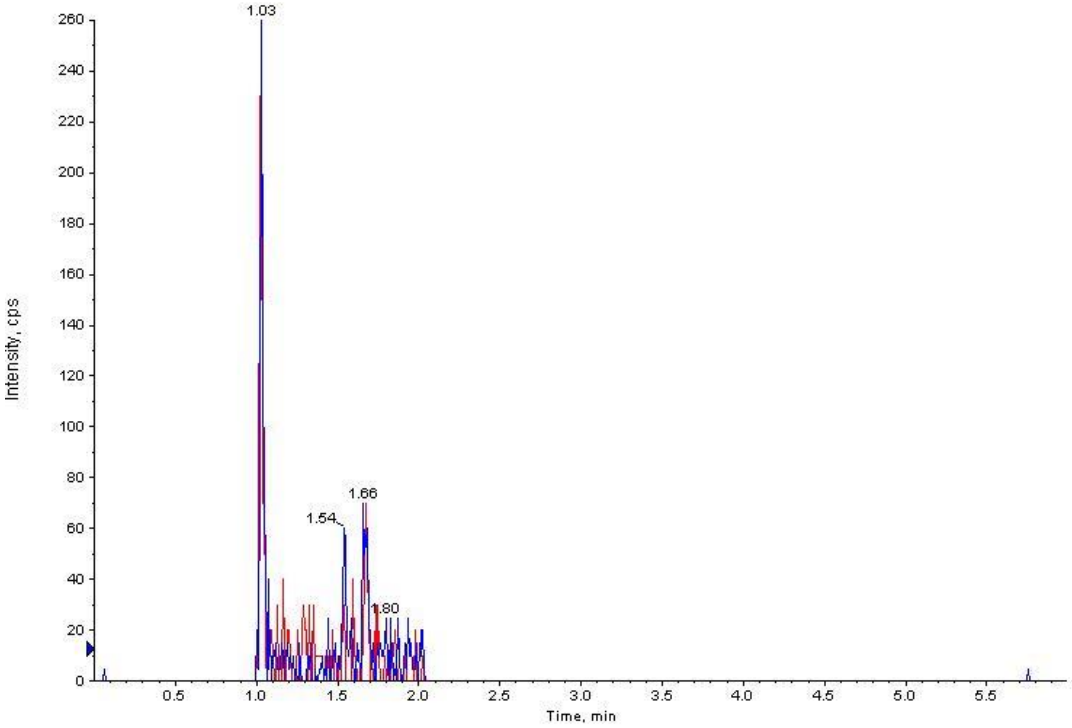

Figure 3. Streptavidin M-280 plasma sample (1ng/ml).

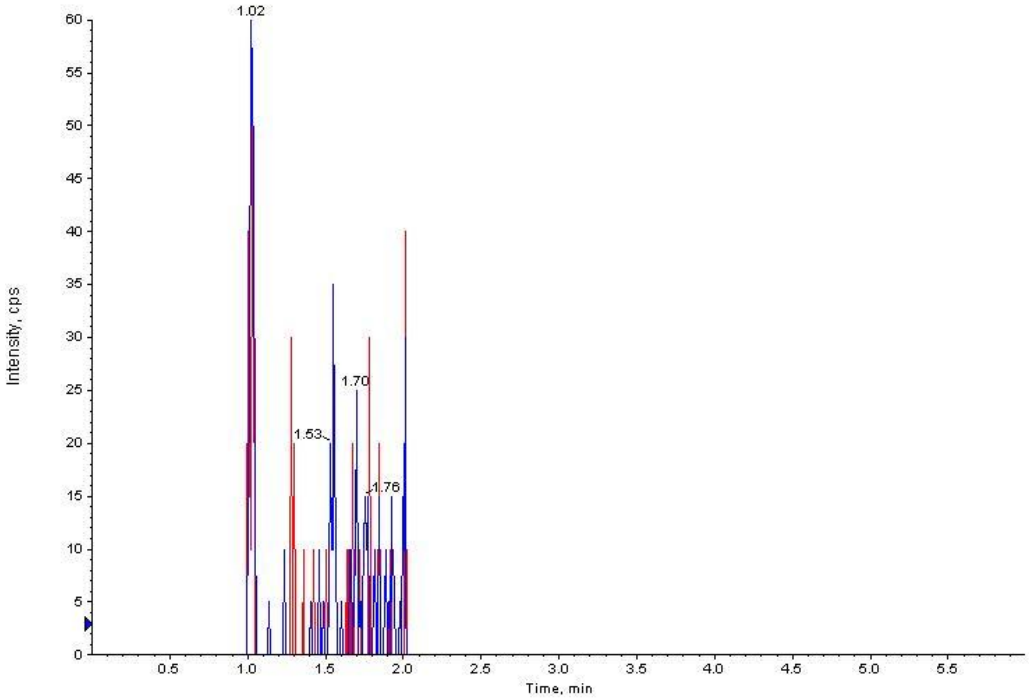

Figure 4. Streptavidin M-280 plasma sample (1ng/ml) wash 1.

Figure 4-6 show the results from a MSIA experiment conducted using Protein G and AG tips together with MAB5296. The control sample (1ng OT/ml) shows an intensity of 1300 cps at t_r 1.47 min for the OT peak (figure 4). Figure 5 and 6 show the result for the sample-prepared plasma sample (1ng/ml) using the MSIA™ Protein AG and Protein G pipette tips respectively. No OT was detected in either of them.

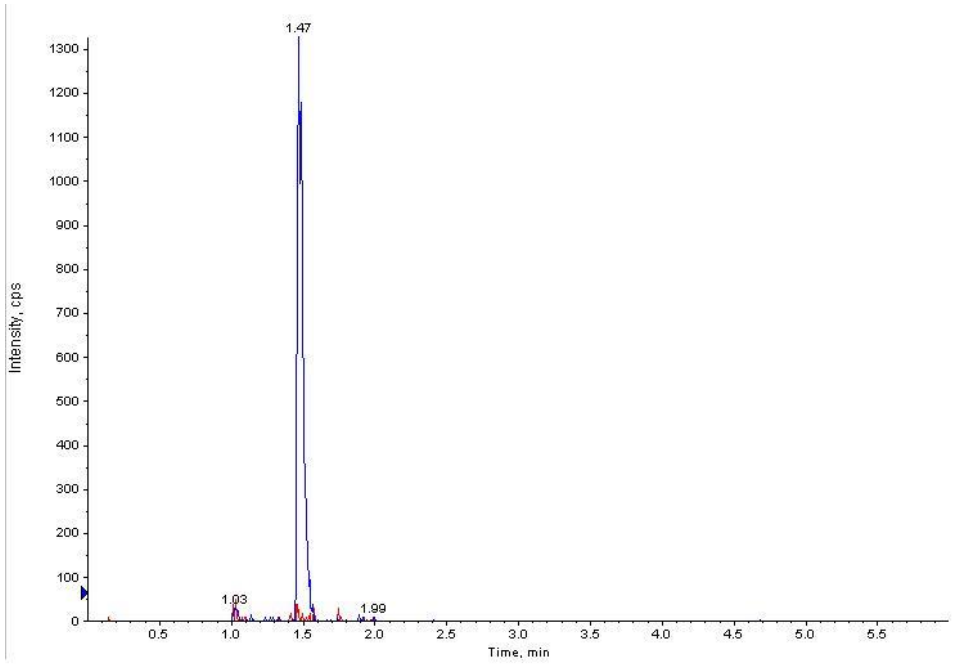

Figure 4. MSIA™ control 1 ng/ml. OT t_r 1.47 min.

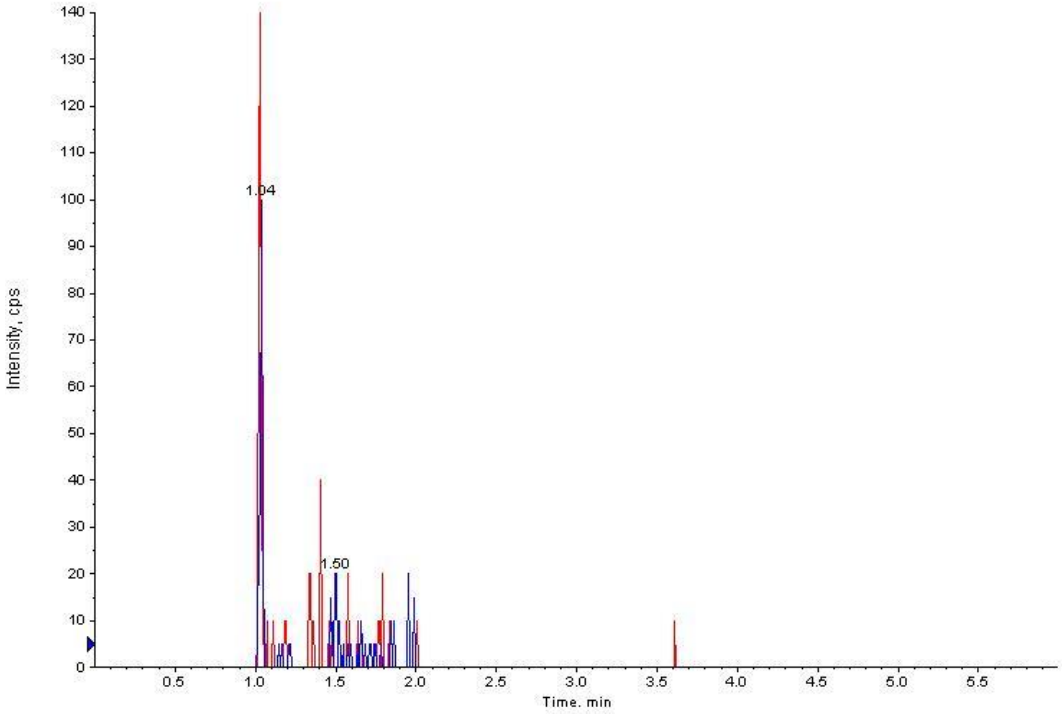

Figure 5. MSIA™ Protein AG-pipette tip – plasma sample 1 ng/ml.

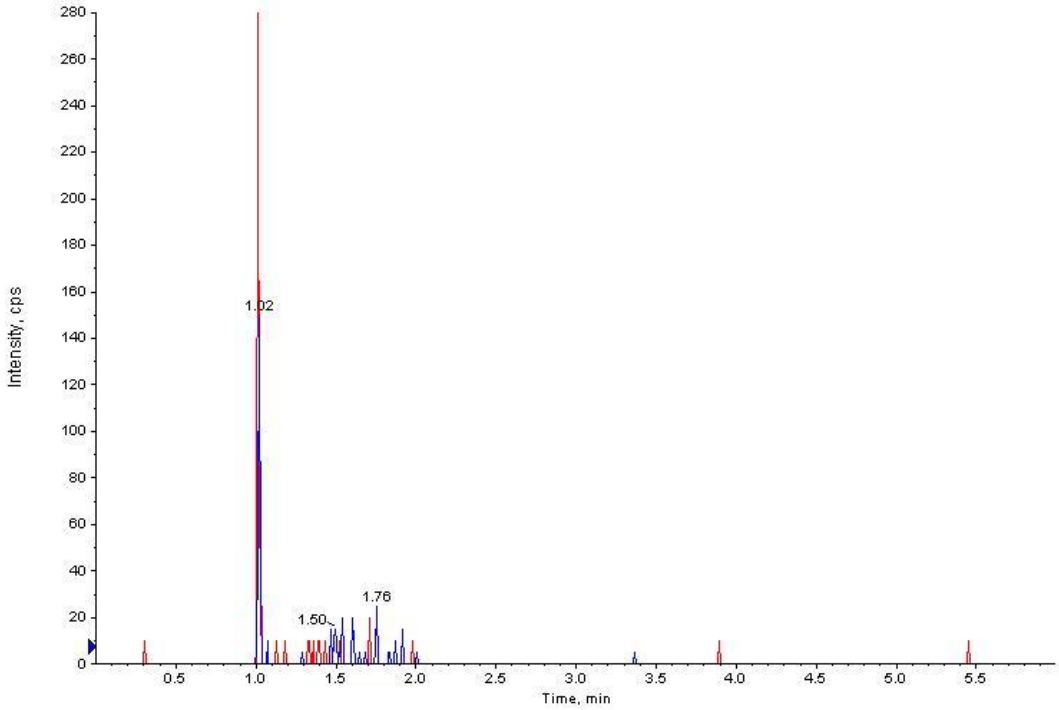

Figure 6. MSIA™ Protein G-pipette tip – plasma sample 1 ng/ml.

Appendix IV: Variation in QC-areas

Table 1 shows the variation between QC-areas for duplicate samples when using IS (100 pg/ml) in the elution. As can be seen there is very small variation between the duplicate samples (QC-area 1 & 2)

Table 1. The variation in QC-areas when using IS (100pg/ml) in the elution.

100ul Elution Buffer 2a				
OT conc. (pg/ml)	**QC Area 1**	**QC Area 2**	**Standard Area**	**OT QC Area/OT standard Area (%)**
10	64,8	41,2	141,5	37
25	129,2	124,9	349,6	36
40	183,5	177,5	533,5	34
55	264,7	234,6	771,3	32
70	302,1	295,9	957,5	31
85	333,9	386,8	1206,4	30
100	396,5	397,3	1363,7	29
150ul Elution Buffer 2a				
OT conc. (pg/ml)	***QC Area 1***	**QC Area 2**	**Standard Area**	**OT QC Area/ OT standard Area (%)**
10	40,9	35,6	146,0	26
25	92,5	79,6	299,5	29
40	171,9	174,0	423,2	41
55	226,5	216,3	683,4	32
70	265,0	251,6	718,3	36
85	355,9	344,0	963,1	36
100				
100ul Elution Buffer 2a + 50uL 0,03% BSA				
OT conc. (pg/ml)	**QC Area 1**	**QC Area 2**	**Standard Area**	**OT QC Area/ OT standard Area (%)**
10	51,0	43,2	140,4	34
25	116,6	115,5	334,5	35
40	133,3	120,5	457,8	28
55	208,1	211,6	653,0	32
70	247,50	287,7	874,6	31
85	316,4	235,0	978,9	28
100	358,6	286,3	1265,6	25