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Development and application of an immuno-MS assay for analysis and quantification of RBM3

Marie Utterbäck

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Author

Marie Utterbäck

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Abstract

The protein RBM3 has been identified as a potential oncology biomarker since it has recently been demonstrated that high expression of RBM3 in cancer cells correlates with increased patient survival and sensitivity to platinum-based chemotherapy in several types of cancers. In this study, an immuno-MS assay has been developed in order to quantify RBM3 in cell lines. A QPrEST, which is a recombinant protein fragment labelled with heavy isotopes on arginine and lysine has been used as an internal standard to enable absolute protein quantification. Immuno-enrichment of peptides was performed in order to reduce the complexity of the samples. An antibody screening was performed in order to determine which antibodies that can bind tryptic peptides and thus can be used in the immuno-enrichment step of the assay. One polyclonal antibody showed affinity to one tryptic RBM3 peptide and was used in the enrichment step of the assay. The assay was tested on four colorectal cancer cell lines. The results showed that the number of RBM3 molecules differs between the cell lines, which also have been confirmed by previous experiments. The result of the MS analysis was compared with results from a WB analysis and the methods showed similar results.

Keywords

Antibody, Immuno-SILAC, Mass spectrometry, RBM3, QPrEST.

Supervisors

Dr. Tove Boström
Atlas Antibodies

Scientific reviewer

Prof. Sophia Hober
Royal Institute of Technolog

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Biology Education Centre Biomedical Center Husargatan 3, Uppsala
Box 592, S-751 24 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 471 4687

Development and application of an immuno-MS assay for analysis and quantification of RBM3

Marie Utterbäck

Populärvetenskaplig sammanfattning

Cancer är en utav de vanligaste dödsorsakerna i världen. 2012 upptäcktes 14 miljoner nya fall av sjukdomen och 8,2 miljoner människor dog av cancerrelaterade orsaker. Antalet fall av cancer är dessutom uppskattat att öka med ungefär 70 procent under kommande två decennier. Det är av stor vikt att hitta nya sätt att både diagnostisera och behandla cancer. Ett problem inom cancervården är att det ofta är svårt att veta vilken behandling som är bäst lämpad för patienten. Genom att analysera uttrycket av vissa proteiner, så kallade biomarkörer, går det att få information om en patient är sjuk i cancer men också prognosen för sjukdomen och vilken typ av behandling som är bäst lämpad för just den patienten.

Proteinet RBM3 har visat sig vara en potentiell biomarkör för flera olika typer av cancer och det är därmed av stort intresse att hitta metoder för att kunna detektera och kvantifiera proteinet i celler. Ett sätt att göra detta är att använda sig av masspektrometri, vilket är en metod som ger information om massa och laddning av molekyler, men kan också med hjälp av en intern standard användas för att ta fram relativa och absoluta koncentrationer.

Ett problem när cellprover ska analyseras med denna metod är att de innehåller väldigt många olika proteiner som kan störa analysen. Detta problem kan lösas genom att använda antikroppar som binder till det intressanta proteinet och som därmed kan sorteras ut från de andra proteinerna. När man analyserar proteiner med masspektrometri är det en stor fördel att först klyva proteinet till mindre peptider för att få en bättre analys. Dock kan detta leda till problem då de intressanta peptiderna ska sorteras ut innan MS analysen, det är nämligen inte säkert att antikroppar framtagna mot större proteinfragment kan binda de mindre peptiderna. En screening är i dessa fall nödvändig för att avgöra antikropparnas lämplighet i denna typ av assay.

I denna studie har en metod utvecklats för att kunna kvantifiera RBM3 i cellinjer. Ett antal antikroppar har testats för att hitta antikroppar som kan binda en mindre peptid. En antikropp av fyra visade sig fungera bra och fick därmed användas i metoden. Då metoden utvecklats har den också testats i fyra olika typer av cancercellinjer. Resultat av detta visade att mängden RBM3-protein överensstämmer med resultat från tidigare mätningar av proteinnivå i dessa cellinjer. Dessa resultat visar att metoden som utvecklats genom detta projekt kan användas för att kvantifiera RBM3 i cancerceller.

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1. Abbreviations

ABP	albumin binding protein§
Cam	chloramphenicol
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
FASP	filter-aided sample preparation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HER2	human epidermal growth factor receptor 2
His	hexahistidine
HPA	human protein atlas
IAA	iodacetamide
IHC	immunohistochemistry
IMAC	ion metal affinity chromatography
Kan	kanamycin
LC	liquid chromatography
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MS/MS	tandem mass spectrometry
OD	optical density
PrEST	protein epitope signature tag
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SILAC	stable isotope labeling by amino acids in cell culture
siRNA	small interfering ribonucleic acid
SISCAPA	stable isotope standards capture with anti-peptide antibodies
TMA	tissue microarray
TOF	time of flight
WB	western blot

2. Introduction

2.1. Cancer

Cancer is one of the most common causes of human death. In 2012 there were 14.1 million new cases worldwide and 8.2 million cancer related deaths. The cases of cancer are furthermore expected to increase by about 70 percent over the next two decades [1]. Cancer, which also is known as malignant tumor or malignant neoplasm are defined as a group of diseases where abnormal cells start to grow and divide without control. There are more than 100 types of disease that are defined as cancer and the cells can start to grow almost anywhere in the body. The most common cancer types are lung, breast, colorectal and prostate cancer [2].

The disease originates within a single cell, which by genetic changes has acquired the ability to ignore the normal regulatory signals that tell normal cells to stop grow and begin the process of apoptosis. Cancer cells that have started to proliferate can form tumors, which is masses of cells. The cancer cell can unlike normal cells invade nearby tissues and parts of the body. That type of cancer is called metastatic cancer [3].

Genetic changes that cause cancer can arise both by genetic inheritance from the parents or by damage of the DNA that has been caused by external factors like chemicals from cigarette smoke, UV-radiation and viruses [3].

Two types of genes that are associated with cancer are oncogenes and tumor suppressor genes. Oncogenes are mutated proto-oncogenes which are the genes encoding proteins important for cell growth. Tumor suppressor genes are the genes encoding proteins involved in reparation of damaged DNA and apoptosis. When these tumor suppressor genes are mutated it will lead to an inactivation of the genes and the transcription of this genes will thereby stop. When oncogenes are mutated it will instead lead to an activation of genes that allows the cells to ignore normal cell signals and the cells can thereby start to proliferate [4].

2.2. Cancer and biomarkers

The understanding of the cancer disease is important in order to select the optimal treatment for the patient. Cancer biomarkers are biological molecules that can indicate the state of the disease. A cancer biomarker can serve as a tool in order to diagnose cancer, estimate the risk of developing cancer, determine the prognosis of the disease or predict the response to therapy. One example of a biomarker that is used to predict the optimal type of therapy is the human epidermal growth factor receptor 2 (HER2). HER2 is a biomarker for breast cancer and is used to determine if the patient will respond to anti-HER2 antibody treatment. Biomarkers can be detected in the blood or other body fluids or tissues and it is produced either by the cancer cells itself or by the body as a response to the cancer [5].

Biomarker discovery is an emerging field since there is a big need of finding new ways to both treat and diagnose cancer. Although there is a lot of research going on the number of biomarkers available for clinical purpose is still limited. One big challenge with biomarker discovery is the need of finding affinity molecules with high specificity to the biomarker, since the detection of the biomarker in many cases relies on antibodies. Enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC) are two common methods to use in order to determine the amount of the biomarker and both these methods require affinity molecules targeting the protein of interest [6].

2.3. Platinum-based cancer treatment

Platinum-based chemotherapeutics is a group of cytotoxic drugs, which are derived from platinum. Platinum-based therapy is used to treat several types of cancer e.g. colon, breast and ovarian cancer. There are three different platinum-based drugs that are used for cancer treatment, cisplatin, carboplatin and oxaliplatin. All these drugs consist of platinum complexes with two amine ligands and two other ligands that can bind intracellular DNA [7]. Platinum-based drugs interact with the DNA to form crosslink adducts. The formation of these crosslink adducts results in activation of signal transduction pathways, which finally will lead to activation of apoptosis.

Platinum-based chemotherapy is an effective drug for treatment of cancer patients but one problem is that medication with cisplatin often will result in resistance. There are several mechanisms that can lead to resistance including increased DNA repair, aggravation of the binding of cisplatin and also changes that will lead to aggravated cellular cisplatin uptake [8].

Treatment with a platinum-based drug can result in many side effects that will cause the patient to suffer. It is therefore of great importance to be able to select only responsive patients for the treatment. Biomarkers that can indicate if a patient should be treated with platinum-based chemotherapy or not will lead to an improved patient care and are thus of great importance.

2.4. The Human Protein Atlas project

The human protein atlas (HPA) project is a research project founded in 2003 at the Royal Institute of Technology with the aim of exploring the whole human proteome by using antibody-based methods. The program is a collaboration project between the Royal Institute of Technology and Uppsala University, led by professor Mathias Uhlén and supported by Knut and Alice Wallenberg Foundation. The project is working in a gene-centric manner to map the whole human proteome and generate affinity-purified antibodies targeting all human proteins. The antibodies are then used to study protein expression in human cells and tissues assembled on tissue microarrays (TMAs). The atlas currently includes data based on 24028 antibodies targeting 16975 unique human proteins [9].

The HPA program workflow (see figure 1) starts by the generation of PrEST proteins. PrESTs are protein fragments that consist of a sequence of 50-150 amino acids identical to a part of the corresponding human protein. The sequence is unique and selected based on low homology to other proteins [10]. The gene complemented with a sequence encoding a His-tag for purification and an ABP-tag for solubility is

cloned into a plasmid. The plasmid in sequenced to verify that the correct gene is inserted. The PrEST proteins are then produced by expression in *Escherichia coli* and purified by immobilized metal ion affinity (IMAC) using the His-tag. The produced PrEST proteins are used for immunization of rabbits to generate polyclonal antibodies. The antibodies are purified by affinity purification using columns with immobilized PrEST antigens. The specificity of the antibodies to the PrESTs is verified by PrEST microarrays and western blot analysis using samples from human cell lines, human tissues and human plasma. Validated antibodies are then finally used to study protein expression by immunohistochemistry in human tissues, human cancer tissues and human cell lines. Immunofluorescence is used to study subcellular localization. All data and images are published on the HPA website (http://www.proteinatlas.org) and available for free [9].

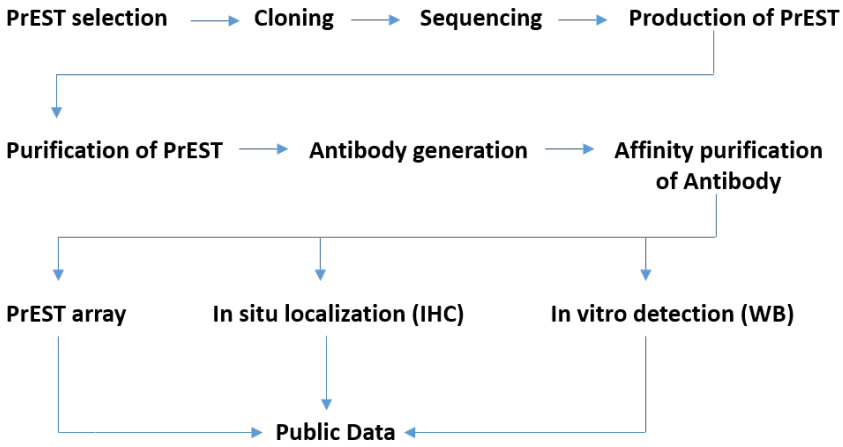

Figure 1. Workflow of the Human Protein Atlas program. Figure modified from [9].

2.5. Atlas Antibodies

Atlas Antibodies is a company founded in 2006 by researches from the HPA project with the mission of making the polyclonal HPA antibodies commercially available for researchers. Apart from the production and marketing of polyclonal antibodies Atlas antibodies also have their own production of monoclonal murine antibodies that also are available in the product catalog. Polyclonal and monoclonal antibodies are epitope mapped using synthetic overlapping peptides in a bead-based array [10].

In December 2014 the company launched two new products, PrEST antigens and QPrESTs. The PrEST proteins are the same antigens used within the HPA project for immunization to generate corresponding antibodies. The QPrEST proteins are a new type of standard for mass spectrometry (MS) based quantitative proteomics. QPrESTs have the same sequence as the corresponding PrEST but are labelled with heavy isotopes on arginine and lysine residues with 99 % isotope incorporation. After accurate determination of QPrEST concentration they can be used as standards for MS-based absolute quantification [11].

Additional to the production, marketing and sales of current products Atlas antibodies also conducts their own research. The exploration of protein expression in human cancer tissues performed within the HPA project has revealed some interesting

protein expression patterns and potential oncology biomarkers. Atlas antibodies is working with these potential biomarkers in collaboration with the HPA project, with the mission of finding oncology biomarkers that can be used to diagnose cancer, giving prognosis of the cancer progression and predict which treatment that is optimal for the patient [11].

2.6. The RNA binding motif protein 3 (RBM3)- A potential oncology biomarker

RBM3 is a glycine rich protein that has a RNA recognition motif (RRM), which enables binding to both RNA and DNA. It has been seen that the protein is expressed during cellular stress like hypothermia and hypoxia but its function is not fully understood [4]. It seems like RBM3 helps stressed cells to survive by facilitating synthesis of proteins important for survival [12].

The HPA project has identified RBM3 as a potential oncology biomarker. It has been shown in a study where two independent groups of patients diagnosed with colorectal cancer were analysed by microarray-based immunohistochemistry that high expression of RBM3 in tumours correlates with increased patient survival (see figure 2). In colorectal cancer there is a big need of finding ways to indicate the prognosis of the disease. Colorectal cancer is in many cases only treated with surgery but for high risk patients adjuvant treatment is often given after surgery. [13]

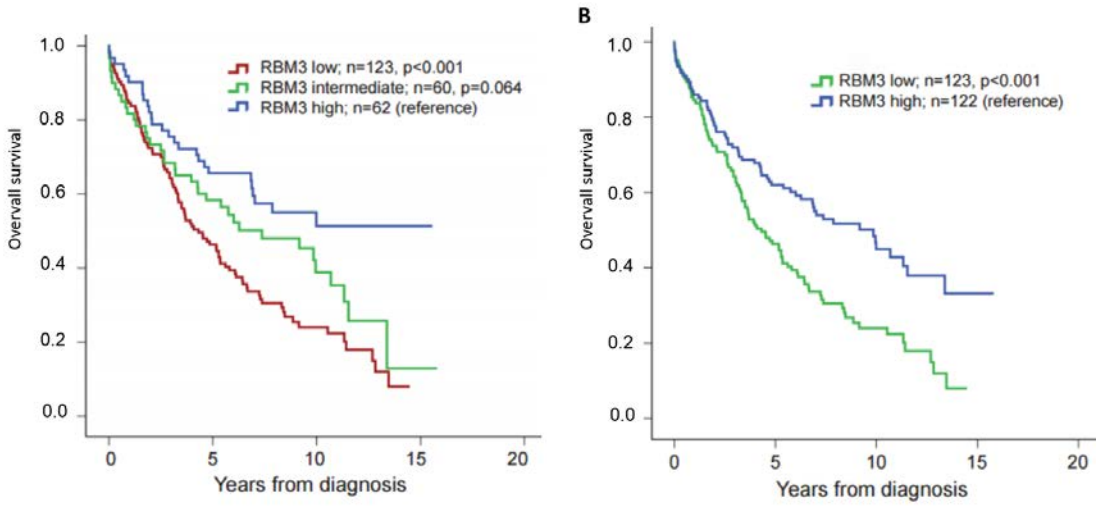

Figure 2. Overall survival of 305 patients diagnosed with colorectal cancer. A, Patients divided into three groups (high, intermediate or low) according to RBM3 expression. B, Patients divided into two groups (high or low) according to RBM3 expression.

Due to difficulty of categorizing patients that are in need of additional treatment, many patients are adjuvant treated although they do not need it. RBM3 is a potential biomarker that can identify high risk patients and thereby predict if adjuvant treatment should be given [13].

Similar studies have been performed on patients diagnosed with other types of cancers. These studies have demonstrated that high expression levels of RBM3 in

cancer cells correlates with increased patient survival in breast [14] ovarian [15], testicular, urothelial bladder [16], prostate [17] cancer and malignant melanoma [18].

In addition to the correlation between patient survival and high RBM3 expression, one study has also shown a correlation between high expression levels of RBM3 and platinum-based therapies. In this study cancer cells from patients diagnosed with ovarian cancer were analysed. The most common treatment for ovarian cancer is cisplatin so most of the patients included in the study were thus treated with cisplatin. The analysis shows a correlation between cisplatin sensitivity and high expression of RBM3 [19].

Further studies on this correlation have been performed by analysing the ovarian cancer cell line A2780 and the cisplatin resistant ovarian cancer cell line A2780-Cp70. The gene encoding RBM3 was also silenced by siRNA to further show the correlation between high RBM3 expression and cisplatin sensitivity. These studies confirmed the correlation since cells with high expression of RBM3 showed a lower viability than cells with low RBM3 expression when cisplatin was added at different concentrations (see figure 3) [19]. RBM3 is according to these facts a possible predictive biomarker that can be used to decide which type of treatment that is optimal for the patient.

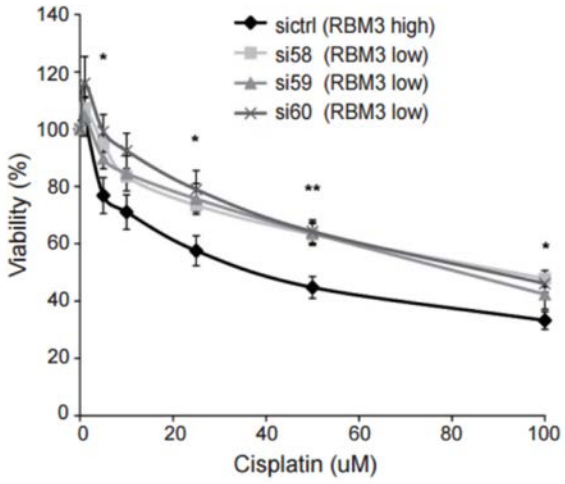

Figure 3. Viability of ovarian cancer cells (RBM3 was silenced by siRNA transfection) at different concentrations of cisplatin.

2.7 Mass-spectrometry based proteomics.

Mass-spectrometry (MS) is the most common used technique within the field of proteomics. When analyzing proteins using MS the mass-to-charge (m/z) ratio is measured of gas phase ions. The instrumentation set up consists of an ion source, a mass analyzer and a detector. At the ionization part analyte ions are produced [10]. The ionization can occur in different ways but the most widespread techniques for analysis of biomolecules are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). MALDI is often used when analyzing more simple peptide samples, while ESI is advantageous to use when analysing more complex samples, often connected with a liquid chromatography (LC) system for further separation [20]. In the mass analyzer analyte ions are separated based on their m/z ratio. The most common types of mass analyzer used when performing MS-based proteomics are orbitrap. Other common mass analyzers are Time of flight (TOF), quadrupole, ion trap, and fourier transform ion cyclotron resonance (ICR), these analyzers are often used in combination with the Orbitrap.

Tandem mass spectrometry or MS/MS includes two steps of analysis. At the first analyzing step (MS1) specific ions (precursor ions) are selected. The precursor ions are then fragmented and the fragments are analysed by a second analyser (MS2). The ions analysed by the second mass analyser are called the product ions. After the mass analysing step the detector will generate a mass spectrum where the intensity of the product ions is plotted against m/z [21]. Peptide ions can be detected directly without fragmentation (MS1) or a fragmentation step can be included to enable peptide sequencing (MS2) [22].

MS-based proteomics can be used for both identification and quantification of proteins. However it is not possible to identify or quantify all types of proteins by just performing a MS-analysis on full-length proteins. The most common strategy when performing MS-based proteomics is to digest the peptides before the MS-analysis. Peptides are easier to both ionize and fragment and are due to complicated protein modification patterns easier to map than full-length proteins. Digestion is most commonly performed by addition of trypsin, which is a proteolytic enzyme that cleaves the protein after arginine and lysine residues [22]. Peptides generated by tryptic digestion are detected by a second mass analyzer and can then be compared to a peptide sequence database. By comparing generated MS-spectra to databases is it possible to identify unknown proteins in complex samples. Proteins can also be identified by a method called peptide mass fingerprinting where tryptic peptides are mapped to full-length proteins only by peptide mass information [23].

When using MS in order to quantify proteins it is not possible to convert the intensity of certain proteins directly to a concentration since the ionization efficiency varies between peptides. A strategy to overcome this problem is by using an internal standard when performing the MS analysis. The internal standard should have the same properties as the targeted peptide or protein since it is important that the standard has the same ionization efficiency. One common strategy is to use a peptide with the same sequence as the targeted peptide but that is labeled with incorporated heavy isotopes. The labeled peptide will have the same ionization efficiency as the targeted peptide but with a small mass-shift that makes it possible to distinguish between the “heavy” and “light” peptide. The intensity ratio between heavy and light can then be used for relative quantification. If the concentration of the standard is known it is then possible to convert the intensity ratio to a concentration ratio and thereby generate an absolute concentration [24].

A commonly used method where metabolically labeled proteins are used is stable isotope labeling of amino acids in cell culture (SILAC). When performing this method for quantification of proteins, the heavy standard is produced by letting cells grow in medium containing metabolically labeled amino acids. Light proteins are produced in the same way but are cultured in medium containing only light amino acids. Heavy and light proteins are combined before digestion and an MS-analysis is performed. The generated intensity ratio between heavy and light peptides is used for relative quantification [25].

When analysing protein in complex samples by MS a common problem is that other proteins of high concentration will interfere with the analysis. This problem can be solved by enriching the peptides of interest by immuno-affinity using polyclonal and monoclonal antibodies targeting the peptide of interest. Immuno-SILAC (see figure 4) is one method where antibodies are used for enrichment before MS-analysis. The workflow described in figure 4 starts by production of heavy recombinant proteins. Heavy proteins will be spiked into cell samples consisting of the corresponding light protein. Proteins will be mixed and digested by a protease, heavy and light peptides are enriched by using the corresponding antibody immobilized on magnetic beads. Finally the sample is analysed by MS and the intensity ratio of the heavy and light peptide is used to determine the relative concentration of the protein of interest [25]

One similar method to immuno-SILAC is the Stable Isotope Standard Capture with Anti-Peptide Antibodies (SISCAPA) method. The difference between these methods is first that the internal standard is added after digestion when performing the SISCAPA instead of before digestion like when performing immuno-SILAC. It is advantageous to add the peptide standard early in the workflow since the losses during sample preparation and the cleavage efficiency for heavy and light peptides will be more equal. One more difference between these two methods is that when performing the SISCAPA method antibodies are generated by immunization of one selected tryptic peptide. The antibodies used when performing the immuno-SILAC method are instead generated by immunization of a PrEST including a minimum of two tryptic peptides. The disadvantage of both these methods is that there is a need of generating affinity molecules against all proteins that will be quantified, which is challenging. For immuno-SILAC it is an even bigger challenge to find antibodies that can bind the shorter digested peptides since it is possible that the epitope of the antibodies is located at a sequence including a cleavage site [25].

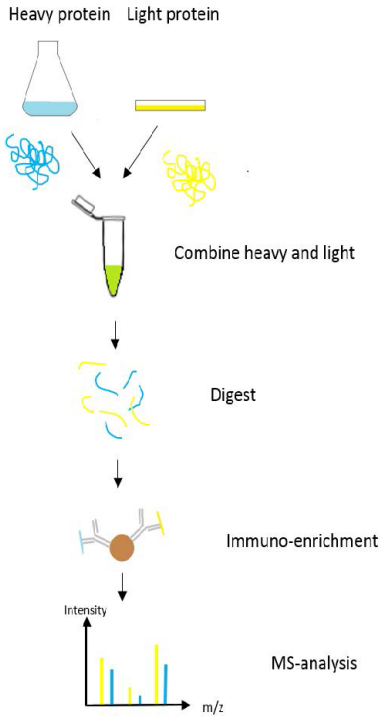

Figure 4. Description of the Immuno-SILAC. Figure modified from [25].

2.8. Aim of the project

The aim of this project is to develop an immuno-MS assay for quantification of RBM3 in cells. RBM3 has in earlier studies been analysed by immunohistochemistry [15, 16, 17] however mass spectrometry is advantageous in this application as it enables a more accurate determination of protein concentration. Since the expression level of RBM3 can indicate the state of a cancer disease is it important to find effective ways to determine the amount of the protein.

The immuno-MS assay will include the immuno-SILAC method and QPrEST proteins will be used as the internal standard. Atlas antibodies have antibodies targeting RBM3 in their product catalog that will be used in this study. Antibodies will be screened to investigate if some of the antibodies can bind digested peptides and then be used as affinity molecules in the assay. The assay will be tested in a real application by using the assay to analyse four colorectal cancer cell lines.

3. Materials and methods

3.1. Production of PrEST and QPrEST proteins

3.1.1. Transformation

E. coli cells containing the plasmid encoding the PrEST proteins were available as glycerol stocks and therefore the transformation of the plasmid encoding the protein was only performed for the QPrEST protein. QPrEST proteins are expressed in a strain auxotroph for arginine and lysine and a glycerol stock of that strain with the right plasmid was not available.

Cells from a glycerol stock containing the plasmids encoding the PrEST protein was re-streaked on agar plates containing kanamycin and chloramphenicol to a concentration of 50 µg/ml and 10 µg/ml. Plates were incubated at 37 °C over night.

40 µl freeze competent ΔArg ΔLys Rosetta *E coli* cells were thawed on ice. 4 µl DNA plasmid were gently mixed with the competent cells and incubated for 5 min on ice. Cells were heat chocked in a 42 °C water bath and then incubated on ice for 2 min. 80 µl Super Optimal Broth (SOC) media (Novagen) were added to cells and DNA. Cells were incubated at 37 °C at 250 rpm for 60 min. Cells were streaked on agar plates complemented with kanamycin to a final concentration of 50 µg/ml and chloramphenicol to a final concentration of 10 µg/ml. Plates were incubate at 37 °C over night.

3.1.2. Inoculation

One colony from each plate was inoculated in 5 ml TSB+Y (0.3 mg/ml Tryptic Soy Broth, 5 µg/ml Yeast Extract, distilled H_2O) containing kanamycin and chloramphenicol to a concentration of 50 µg/ml and 34 µg/mg. Cultures were incubated at 150 rpm at 37 °C over night.

3.1.3. Overexpression of PrEST proteins

1 ml over night culture was added to a baffle flask containing 100 ml TSB+Y complemented with kanamycin and chloramphenicol to a concentration of 50 µg/ml and 10 µg/ml. Cells were incubated at 37 °C at 150 rpm until the Optical density (OD) reached approximately 1. Protein expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Protein expression was performed at 150 rpm at 25 °C over night.

3.1.4. Overexpression of QPrEST proteins

10 µl over night culture were added to 10 ml QPrEST medium (500 mM Na_2HPO_4, 500 mM KH_2PO_4, 250 mM $(NH_4)_2SO_4$, 5 % Glycerol, 0.5 % Glucose, 2 % Lactose, 200 mM $MgSO_4$, 50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 2 mM $CoCl_2$, 2 mM $CuSO_4$, 2 mM $NiSO_4$, 2 mM $(NH_4)_6Mo_7O_{24}$, 2 mM Na_2SeO_3, 2 mM H_3BO_3, 200 µg/ml Heavy isotope labeled (^{13}C and ^{15}N) lysine and arginine (Cambridge Isotope Laboratories), 200 µg/ml of each remaining amino acid (Sigma-Aldrich)) complemented with kanamycin and chloramphenicol to a concentration of 50 µg/ml and 34 µg/mg. Cultures were incubated at 150 rpm at 37 °C for 22 hours. Protein expression was induced by auto induction by lactose when glucose was consumed.

3.1.5. Harvest and cell lysis

Cells were harvested by centrifugation at 2700 x g for 10 min at 4 °C. Pellets were resuspended in 5 ml lysis buffer (7 M Guanidiniumchloride, 47 mM Na_2HPO_4, 2.65 mM NaH_2PO_4, 10 mM Tris-HCl, 100 mM NaCl) complemented with 20 mM β-mercapthoethanol. Harvested cells were lysed by incubation at 37 °C at 150 rpm for 2 hours. Cell debris was centrifuged at 17100 x g for 40 min. Cell lysates were transferred to falcon tubes.

3.1.6. Protein purification

2 ml HisPur™ cobalt resin slurry (Thermo Scientific, Rockford, USA) were added to PD10-flowthrough columns. The matrix slurry was washed with 2 ml Immobilized Metal Ion Affinity Chromatography (IMAC) wash buffer (6 M guanidiniumchloride, 46.6 mM Na_2HPO_4, 3.4 mM NaH_2PO_4, 300 mM NaCl). The lysates mixed with 5 ml IMAC wash buffer were loaded onto the plugged columns and resuspended in the gel. The gels were set to sediment for 20 min. The plugs were removed to let the unbound protein flow through. The gels were washes with 100 ml wash buffer. The columns were plugged and 3 ml IMAC elution buffer was added and resuspended in the gels. Gels were set to sediment for 10 min and the elutes were then collected in falcon tubes. The elutes were diluted in 15 ml 1 x PBS to a final concentration of 1 M urea. PrEST and QPrEST proteins were concentrated with Pierce concentrators 9K MWCO (Thermo Fisher) down to a volume of approximately 2 ml. Absorbance were measured by NanoDrop spectrophotometer at 280 nm. Absorbances were converted to concentrations by using the absorbance coefficient for respectively PrEST. The absorbance coefficient was determined by using the Expasy ProtParam tool (2).

3.1.7. Purity estimation by SDS-PAGE

1.5 µg of the PrEST and QPrEST were mixed with 7.5 µl 4 x Lammeli Sample Buffer (Bio-Rad), 1.5 µl 1 M Ditiotreitol (DTT) and H_2O up to a volume of 30 µl. Samples were heated at 95 °C for 5 min and then centrifuged at 13 400 x g for 1 min. 20 µl of the samples were loaded on a Criterion™ TGX Precast Gel (Bio-Rad). The gel was assembled in a gel tank filled with cold running buffer (10 % 10 x TGS (Tris/Glycine/SDS (Bio-Rad)), 90 % H_2O). 10 µl PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) were loaded into the first and last lane. Samples were run at 200 V for 40 min. The gel was washed with water 3 x 5 min, stained with SimplyBlue SafeStain (Life technologies) and then washed 2 x 60 min. The imaging was performed by a ChemiDoc MP camera (BioRad) and the image was analyzed by the software ImageLab 5.1 (BioRad).

3.1.8. Molecular weight determination by MS full-length analysis

PrEST and QPrEST proteins were diluted in 1 M urea, 0.1 M NH_4HCO_3 to a final volume of 50 µl and a final concentration of 30 µg/ml. Samples were reduced by addition of 1.5 µl 400 mM DTT followed by incubation for 1 h. Proteins were alkylated by addition of 1.25 µl 400 mM iodoacetic acid (IAA) followed of incubation for 30 min in dark. Samples were neutralized by addition of 2.5 µl DTT. Protein samples were diluted ten times in MS buffer (5 % Acetonitrile, 0.1 % Formic acid, 95 % H_2O) to a final volume of 100 µl. Samples were analysed by ESI-QTOF and then Deconvolution was performed by the software MassHunter.

3.1.9. Quality control by MS quantification of QPrEST protein

5 µl QPrEST proteins (approximately 16 µM) and 50 pmol light HisABPOneStrep were diluted in 0.1 M NH_4HCO_3 to a final volume of 23 µl. The sample was reduced

by addition of 1 µl 250 mM DTT followed by incubation for 1 hour. Proteins were alkylated by addition of 0.5 µl 1.2 mM iodoacetic acid (IAA) followed of incubation for 30 min in dark. 2.5 µl 10 % acetonitrile were added before addition of 100 ng trypsin. Samples were incubated at 37 °C over night and then analysed by ESI-QTOF. Data analysis was performed by using the software APP [28] and the search engine X!Tandem was used with the human UniProt database complemented with the HisABP sequence. The minimum peptide length was five amino acids and maximum of two miss cleavages was allowed. Heavy to light ratio was generated using the XPRESS software.

3.2. Antibody screening

3.2.1. Digestion of PrEST proteins

10-kDa FASP (filter-aided sample preparation) filter (Millipore) were washed with 200 µl UA (0.1 M Tris-HCl, 8 M Urea) and centrifuged at 11 200 x g for 15 min. 10 µg of 8 PrEST proteins were pooled and mixed with 200 µl UA complemented with DTT to a final concentration of 10 mM. The Sample was added onto the column and centrifuged at 11 200 x g for 15 min. 100 µl UA complemented with DTT was added to a final concentration of 10 mM. The column was centrifuged at 11 200 x g for 1 min. The filter was washed with 200 µl UA and centrifuged at 11 200 x g for 15 min. 100 µl UA complemented with IAA to a final concentration of 50 mM was added onto the filter and incubated for 20 min in dark. The sample was centrifuged at 11 200 x g for 10 min. The filter was washed with 100 µl ABC (0.1 M NH_4HCO_3) and centrifuged for 10 min. The wash step was performed three times. 20 µl ABC and 20 µl 100 ng/µl trypsin was added to the filter. The sample was incubated at 37 °C in a wet chamber over night. Digested peptides were collected by centrifugation at 11 200 x g for 10 min. 60 µl H_2O was added onto the filter and the sample was centrifuged at 11 200 x g for 10 min. Trypsin was heat inactivated at 95 °C

3.2.2. Antibody immobilization

22 monoclonal murine antibodies were pooled into six pools. Seven rabbit polyclonal antibodies were pooled into one pool (three monoclonal RBM3 antibodies and one polyclonal RBM3 antibody). Each pool containing a total amount of 6 µg antibody were diluted in washing buffer (1xPBS, 0.03% Chaps) to a final volume of 200 µl. Each monoclonal pool was added to 1.17 mg Dynabeads® Protein G (Life Technologies) washed with 3 x 500 µl washing buffer using a magnet. The polyclonal pools were added to 1.17 mg Dynabeads® Protein A (Life Technologies). Beads and antibodies were incubated for 2 hours on a rotor mixer.

3.2.3. Peptide enrichment

Beads corresponding to 300 ng/antibody were washed with 3 x 45 µl washing buffer. 1.5 µl of the digested peptides sample were added to each sample. Antibodies immobilized on beads and digested peptides were incubated over night on a rotor mixer. Beads were washed with 2 x 45 µl washing buffer and 2 x 45 µl 50 mM NH_4HCO_3. Peptides were eluted by addition of 40 µl 0.1 % formic acid and incubation for 180 s on a rotor mixer. Antibodies were heat inactivated at 95 °C for 5 min. Formic acid was removed from the sample by vacuum centrifugation for 1 hour. Peptides were resuspended in 10 µl MS buffer (5 % Acetonitrile, 0.1 % Formic acid, 95 % H_2O).

3.2.4. MS-Analysis

Three screenings were performed, the samples from the first screening were analysed by an Orbitrap Velos (Thermo Scientfic) MS instrument, samples from the second and third screening were analysed by a QExactive HF (Thermo Scientfic) instrument. The injection volume was 2 µl for all samples. Data analysis was performed by the software MaxQuant, which includes the search engine Andromeda. A human Uniprot database was used for the search. The minimum peptide length was six amino acids and maximum two miss cleavages were allowed.

3.3. Cultivation of cells and preparation of cell lysates

3.3.1. Cultivation of cells

WiDr and Widroxt24 cells (kindly donated by Lars Ekbladh, Lunds University) were cultivated in tissue culture flask (TC-flask) (Sarstedt) for adherent cells containing 20 ml Dulbecco's modified Eagle's medium (EMEM) (Sigma-Aldrich) complemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich), 1 % glutamine (Sigma-Aldrich) and 1 % no essential amino acids (NEAA) (Sigma-Aldrich). SW480 and SW620 cells were cultivated in cell cultivation TC-flasks for adherent cells containing 20 ml RPMI-1640 medium (Sigma-Aldrich) complemented with 10 % FBS and 1 % glutamine.

3.3.2. Preparation of cell lysates

Cells were removed from cultivation flasks by addition of 5 ml trypsin-EDTA (Sigma-Aldrich) followed by incubation at 37 °C for approximately 10 min. Cells were resuspended in 5 ml medium (EMEM for WiDr and Widroxt24 and RPMI for SW480 and SW620). Amount of cells were determined by using a Scepter™ 2.0 Cell Counter (Millipore) and cells were then centrifuged at 3000 x g, at 20 °C for 10 min in aliquots of 1 million cells. The supernatants were removed and cells were washed with 100 µl 1 x PBS two times. Cell pellets were stored at -20 °C. Stored cells were thawed on ice for 30 min. Thawed cells were resuspended in 1 ml lysis buffer (0.1 M Tris-HCl pH 7.6, 4% SDS) and reduced by addition of 10 µl DTT followed by incubation at 95 °C for 3 min. Cells were sonicated for approximately 1 min, 1 s pulse and 1 s rest, amplitude 30 %. Cells were stored at -20 °C in aliquots of 100 000 cells.

3.4 RBM3 quantification by using the immuno-MS assay

100 fmol RBM3 QPrEST protein were reduced by addition of DTT to a final concentration of 10 mM followed by incubation for 30 min. Reduced protein was added to thawed cell lysates. Samples were diluted to 300 µl by addition of UA and centrifuged at 11 200 x g for 15 min through spin filters. Samples were then digested using the FASP filter method described above.

2 µg RBM3 polyclonal antibody were immobilized on 720 µg magnetic beads (24 µl bead solution) using the same antibody immobilization protocol described above. 15 µg digested protein samples were added to beads corresponding to 250 ng antibody. Incubation, elution and MS-analysis were performed as described in section 3.2.

3.5. Western blot analysis

3.5.1. Protein separation by SDS-PAGE

20 µg trypsin digested sample were mixed with 7.5 µl 4 x Lammeli Sample Buffer (Bio-Rad), 1.5 µl 1 M DTT and H_2O to a volume of 30 µl. Samples were heated at 95 °C for 5 min and then centrifuged at 16 100 x g for 1 min. Samples were loaded on a Criterion™ TGX Precast Gel (Bio-Rad). The gel was assembled in a gel tank filled with cold running buffer. 10 µl PageRuler™ Plus Prestained Protein Ladder were loaded into the first and last lane. The gel was run at 200 V for 40 min.

3.5.2 Western Blot transfer and detection

The gel was placed in the middle of a Trans-Blot® Turbo Midi PVDF transfer pack (BioRad) and the membrane was placed on top of the gel. The transfer was run at 100 V for 8 min. The membrane was rinsed in deionized water and then placed on a kimwipe to dry. The membrane was activated in methanol for a few minutes and then placed in block buffer (1 x TBST (H_2O, mM Tris Base, 15 mM NaCl, 0.1 % tween) + 5 % low fat dried milk powder) on a rocking shaker for 30 min. The membrane was washed with 1 x TBST before incubation with a monoclonal RBM3 antibody (primary antibody) diluted 1:3000 with 3.5 ml blocking buffer. Antibody and membrane were incubated for 1 hour on a roller mixer. The membrane was washed with 1 x TBST for 5 min on the rolling mixer and then incubated with the secondary antibody, diluted 1:3000 in 3.5 ml blocking buffer on a rolling mixer for 30 min. The membrane was then washes 3 x 5 min with 1 x TBST and placed in a tray consisting of 10 ml Immobilon Western Chemiluminescent HRP substrate (Millipore). After one minute the membrane was moved to a plastic cover and placed in the ChemiDoc MP camera (BioRad). The membrane was analysed by the Image Lab software (BioRad). Images of the gel were saved and the membrane was incubated with the GAPDH primary antibody, which will serve as a control. Incubation and washes of the primary and secondary antibody was performed in the same way as described earlier. The membrane was analysed again and the RBM3 data were normalized using the GAPDH data.

4. Results

4.1. Production of PrEST proteins

PrESTs are protein fragments that consist of a sequence of 50-150 amino acids identical to a part of a corresponding human protein. PrEST proteins (see supplementary material, table 1) are used in this study for screening of antibodies against digested peptides. The proteins are commonly used as antigens for antibody development and the antibodies produced by immunization of the PrEST proteins will also be used in this study for enrichment of target peptides.

QPrESTs are proteins used as standards for MS quantification (see supplementary material, table 2). The QPrESTs consist of the same sequence as the corresponding PrEST but are labelled with heavy isotopes on arginine and lysine residues with 99 % isotope incorporation. The QPrEST can be spiked into cell lysate in order to enable absolute protein quantification.

PrEST and QPrEST were both expressed in *E. coli* but the QPrEST proteins were expressed in an *E. coli* strain auxotrophic for arginine and lysine [26]. The expression medium was also different, the expression of QPrEST protein was performed in a defined medium containing heavy isotopes of arginine and lysine. The medium also contained glucose and lactose, which enabled auto induction of the protein expression. Glucose prevented the induction by lactose and acted as carbon source until it was consumed [24]. PrEST protein was expressed in TSB+Y. Both PrEST and QPrEST protein was purified by IMAC. The PrEST and QPrEST proteins are complemented with a His-tag for purification and an ABP-tag for solubility. The quality control was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine protein purity and MS-analysis for verification of protein molecular weight. Both the PrEST and the QPrEST showed distinct bands (minimum 80 % purity) at correct molecular weight of about 32 KDa (see figure 5). The result of the MS analysis showed peaks corresponding to 32168 Da for the PrEST (see figure 6) and 32511 Da for the QPrEST (see figure 7). Correct theoretical molecular weights are 32169 Da and 32514 Da. Five more PrEST and QPrEST proteins were also produced in this study, the SDS-PAGE analysis results for these proteins can be seen in supplementary material figure 18 and 19.

Accurate concentration of the QPrEST protein was measured by spiking in light HisABP to the QPrEST protein. The sample was analysed by MS and the intensity ratio between light ABP peptides and heavy ABP peptides were used to determine the concentration of the QPrEST. The quantification was performed on three replicates and resulted in a concentration of 11.7 µM and a variation coefficient below 10 % between the replicates.

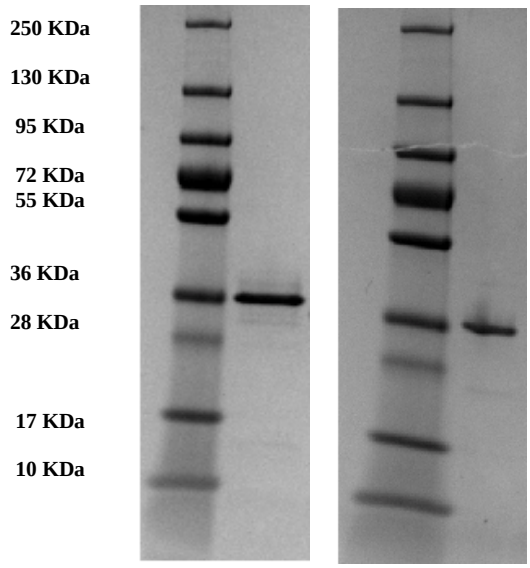

Figure 5. SDS-PAGE analysis of RBM3 PrEST to the left and QPrEST protein to the right. Both the PrEST and QPrEST protein showed a distinct band at the expected weight of 32 KDa.

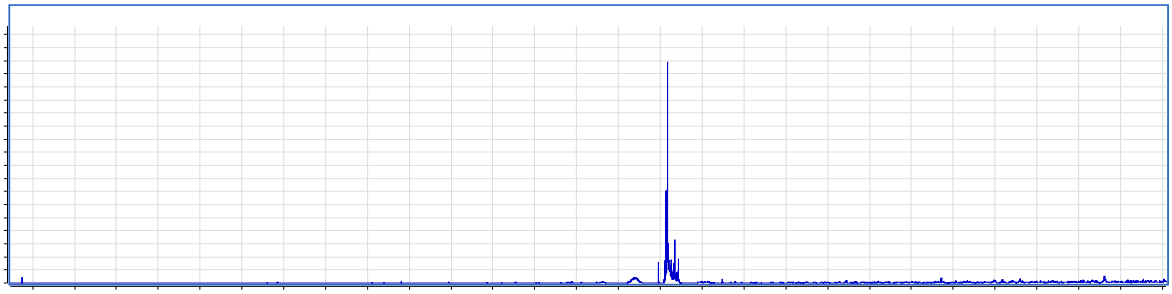

Figure 6. MS-spectra generated by ESI-QTOF analysis of the RBM3 PrEST protein. Deconvolution was performed by the software MassHunter.

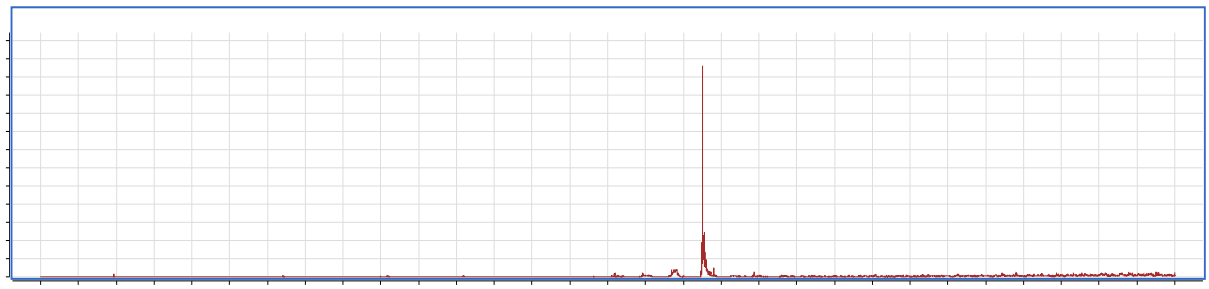

Figure 7. MS-spectra generated by ESI-QTOF analysis of the RBM3 QPrEST protein. Deconvolution was performed by the software MassHunter.

4.2. Antibody screening

An antibody screening was performed in order to investigate which antibodies against target proteins that had the ability to bind peptides generated by tryptic digestion of the PrEST protein. The antibodies were produced by immunization with the RBM3 PrEST protein but it has not previously been confirmed that the antibodies can bind the shorter tryptic peptides.

Before the screenings a feasibility study was performed where epitopes and tryptic peptides were compared in order to predict which antibodies that should have affinity to a tryptic peptide. The study showed that many of the tryptic peptides had a large part of an epitope sequence included (see figure 8 and 9). No tryptic peptides had the completely mapped epitope sequence of any of the corresponding antibodies included between cleavage sites. The first and the last peptide cannot be used for quantification since they will not be identical with the corresponding light peptides. It was also selected that only peptides consisting of more than six amino acids should be detected.

It is likely that the polyclonal antibody has affinity to some of the tryptic peptides since the antibody has more than one epitope, some of the corresponding peptides can therefore probably be used for quantification. The monoclonal antibody AMAb90656 has most of the epitope sequence included in one tryptic peptide sequence and is thus also a potential candidate to be used in this assay.

DEQALEDHFSSFGPISEVVVVK DR ETQSR GFGFITFTNPEHASVAMR AMNGESLDGR QIR VDHAGK SAR GTR GGGFGAHGR GR SYSR GGGDQGYGSGR YYDSR PGGYGYGYGR SR DYNGR NQGGYDR YSGGNY

Figure 8. Mapped epitopes of the polyclonal RBM3 antibody (HPA003624).

DEQALEDHFSSFGPISEVVVVK DR ETQSR GFGFITFTNPEHASVAMR AMNGESLDGR QIR VDHAGK SAR GTR
SGR YYD
GGGFGAHGR GR SYSR GGGDQGYGSGR YYDSR PGGYGYGYGR SR DYNGR NQGGYDR YSGGNY

Figure 9. Mapped epitopes of the three monoclonal antibodies against RBM3. AMAb90655 labeled in blue, AMAb90656 labeled in green and AMAb90657 labeled in orange

The RBM3 antibodies were pooled into pools also consisting of antibodies corresponding to 4 other proteins (see supplementary material table 3 and 4 for screening results of these 4 proteins) Six polyclonal and 22 monoclonal antibodies generated by immunization of the six PrEST proteins included were pooled into seven pools (see supplementary material, table 5) The RBM3 antibodies were included in four of the pools. One pool consisted of all the polyclonal antibodies and the remaining six pools contained the monoclonal antibodies. One polyclonal antibody targeting ANXA1 and one monoclonal antibody targeting EMD were included as positive controls. Both have in earlier experiments shown affinity for corresponding tryptic peptides. Every pool contained only one antibody recognizing each mapped epitope to make sure that each binding event could be accurately traced back to the correct antibody. The affinity of the antibodies to tryptic peptides were determined by performing the immuno-SILAC workflow by using a PrEST mix cleaved with trypsin and the pooled antibodies. Samples were analysed by MS and the data generated were analysed by the software MaxQuant [13], which includes the search engine Andromeda.

After data analysis the result showed that a lot of peptides were identified but some of them in the wrong pool. These results were difficult to analyse due to the high number of peptides in some of the pools and therefore an optimization of the protocol was necessary. One more screening was performed and this time protein G instead of protein A beads were used to immobilize the monoclonal antibodies. The change was performed for the monoclonal antibodies since it is expected that mouse antibodies will bind protein G with higher affinity than protein A. Polyclonal rabbit antibodies were still immobilized on protein A beads. In addition, the RBM3 polyclonal antibody was changed to a new lot. The new lot of the RBM3 antibody was generated by immunization of the same antigen as the first lot but purified from another animal, which can result in changes in the epitope localization [29].

The optimization of the protocol lead to better results from the screening. Two RBM3 peptides were detected, one with the corresponding polyclonal antibody epitope included and one peptide that did not have the corresponding epitope included. These two peptides and their corresponding antibodies were analysed one more time by a third screening. The peptide corresponding to the antibody used as positive control was also detected in the second screening but no peptides corresponding to the polyclonal antibody used as positive control were detected.

The last screening was performed in replicates of two to further verify the results. Two pools were also included as negative controls, one pool that consisted of an antibody targeting SIX1 and one pool with only magnetic beads added. The peptide corresponding to the RBM3 polyclonal antibody was identified in the right pool and in both the replicates of the pool. One more peptide targeting one of the RBM3 monoclonal antibodies with the corresponding epitope included was identified in the right pool but only in one of the replicates and with low signal intensity (YYDSRPGGYGYGYGR). Since this peptide has only been identified one time it will therefore not be used for quantification. Moreover, this peptide contained a missed cleavage site. The fully cleaved version of the peptide is only five amino acids in length (YYDSR) and does therefore not fulfil the search criteria of a minimum of seven amino acids. Since the digestion efficiency can vary between experiments, it is not surprising that the identified peptide was identified only in one experiment.

The peptide enriched by RBM3 polyclonal antibody (see figure 10) has been identified in both the second and the third screening and will be selected for quantification in the immuno-MS assay.

DEQALEDHFSSFGPISEVVVVK DR ETQSR **GFGFITFTNPEHASVAMR** AMNGESLDGR QIR VDHAGK SAR GTR GGGFGAHGR GR SYSR GGGDQGYGSGR YYDSR PGGYGYGYGR SR DYNGR NQGGYDR YSGGNY

Figure 10. The RBM3 PrEST sequence. Red labelled sequence is the sequence of the mapped epitope of the antibody. Underlined sequence is the tryptic peptide that will be used for quantification of the endogenous RBM3 protein.

4.3. Analysis and quantification of RBM3 in cell lysates

The assay that was developed by performing the antibody screening was tested by quantification of RBM3 in four different colorectal cancer cell lines. The cell lines that were analysed were WiDr, WiDroxt24 (see figure 11), SW480 and SW620 (see figure 12). WiDr is a human colorectal carcinoma cell line and WiDroxt24 is an oxaliplatin resistant cell line derived from WiDr. It has been seen that platinum resistant cells have lower expression of RBM3, wherefore it is interesting to compare the expression levels of RBM3 in these cell lines. SW480 is a primary growth colorectal cancer cell line and SW620 is a second growth cell line derived from the same patient. Second growth cancer has lower patient survival than primary growth cancer and investigating whether this can be explained by differences in RBM3 expression levels between these two cell lines is also of great interest. Cell lysates were prepared and heavy RBM3 QPrEST was spiked in. The protein mix was digested with trypsin and incubated with the antibody immobilized on magnetic beads. This time only the polyclonal RBM3 antibody that showed affinity to one tryptic peptide was used. Results from the MS-analysis showed that the peptide selected for quantification was identified in all cell lysate samples. The ratios of the intensities between heavy and light peptides were used to generate the number of RBM3 protein molecules per cell. The experiment was performed on replicates of two. Copy numbers of each replicate is shown in figure 13. Generated data shows that the number of RBM3 peptides is different in the cell lines. WiDr showed an expression of RBM3 that was about 30 % higher than the expression of RBM3 in the oxaliplatin resistant cell line WiDroxt24. The second growth cancer cell line SW620 showed an expression of RBM3 that was about 300 % higher than the expression of RBM3 in the primary growth cancer cell line SW480.

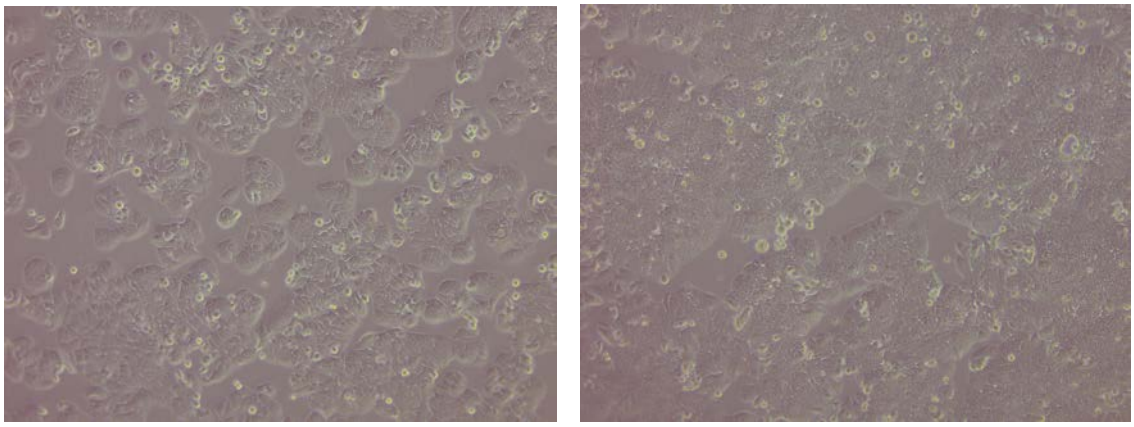

Figure 11. WiDr cells to the left and WiDroxt24 cells to the right. The photo was taken right before the cells were released from the TC-flask.

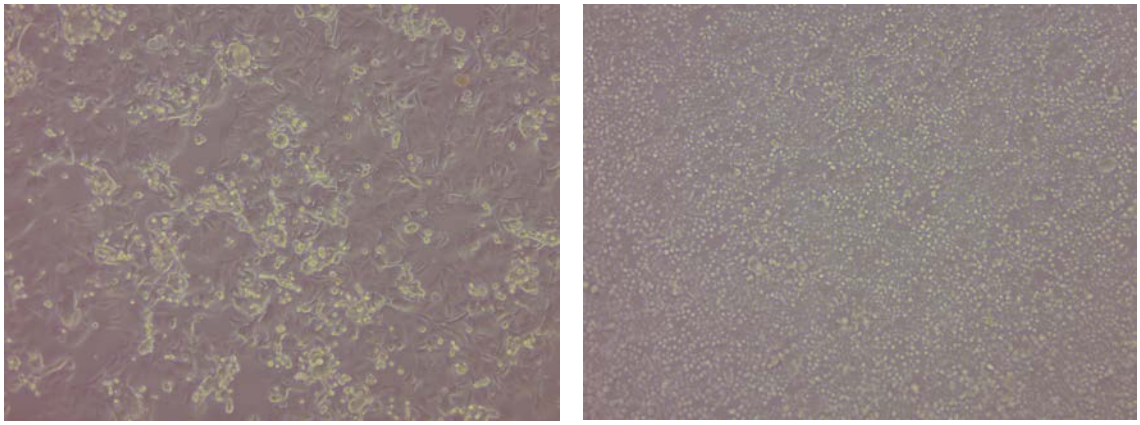

Figure 12. SW480 cells to the left and SW620 cells to the right. The Photo was taken right before the cells were released from the TC-flask.

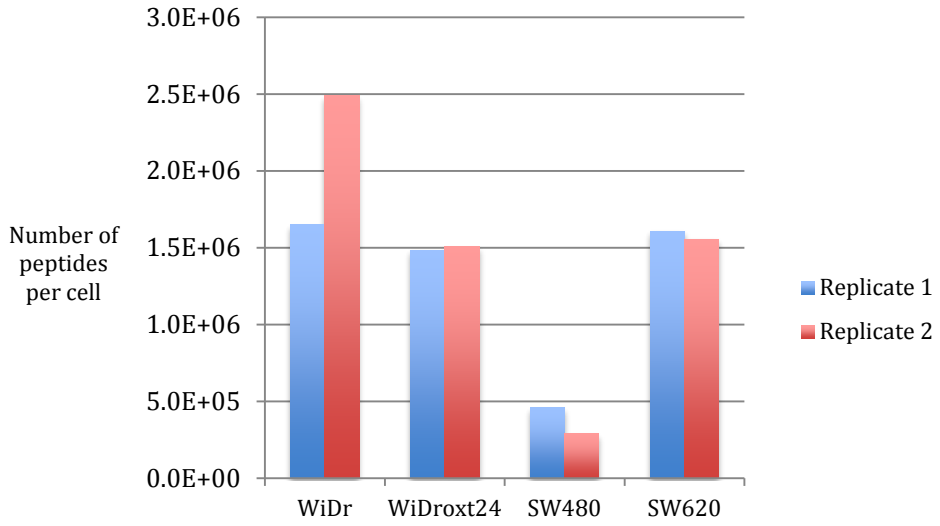

Figure 13. Number of RBM3 peptides per cell in two replicates of four different cell lines.

4.4. Western blot

A quantitative western blot analysis of the same lysates as analysed by MS were performed in order to compare the results. The western blot analysis will not give an absolute concentration of the protein but it is possible to compare the intensity of the band to get a relative concentration between the lysates. The concentration of the protein loaded on the gel when performing the SDS-PAGE analysis is measured by NanoDrop, so it is not an accurate measurement. To compensate for differences in the amounts of protein loaded on the SDS-PAGE gel, a control antibody was included in the analysis. The protein GAPDH has the same expression levels in almost all cell lines and can thereby be used to normalize the amount of RBM3. HRP-conjugated secondary antibodies were used for detection. The results of the analysis are shown in figure 14.

Bands corresponding to the molecular weight of RBM3 (17.7 KDa) were detected for all cell lines. The intensity of the bands is not consistent with the amount of protein since the loading amount of protein is not the same when looking at the loading control. Normalizing factors were generated by the BioRad software. The normalized data for WiDr and WiDroxt24 are shown in figure 15, normalized data for SW480 and SW620 are shown in figure 16.

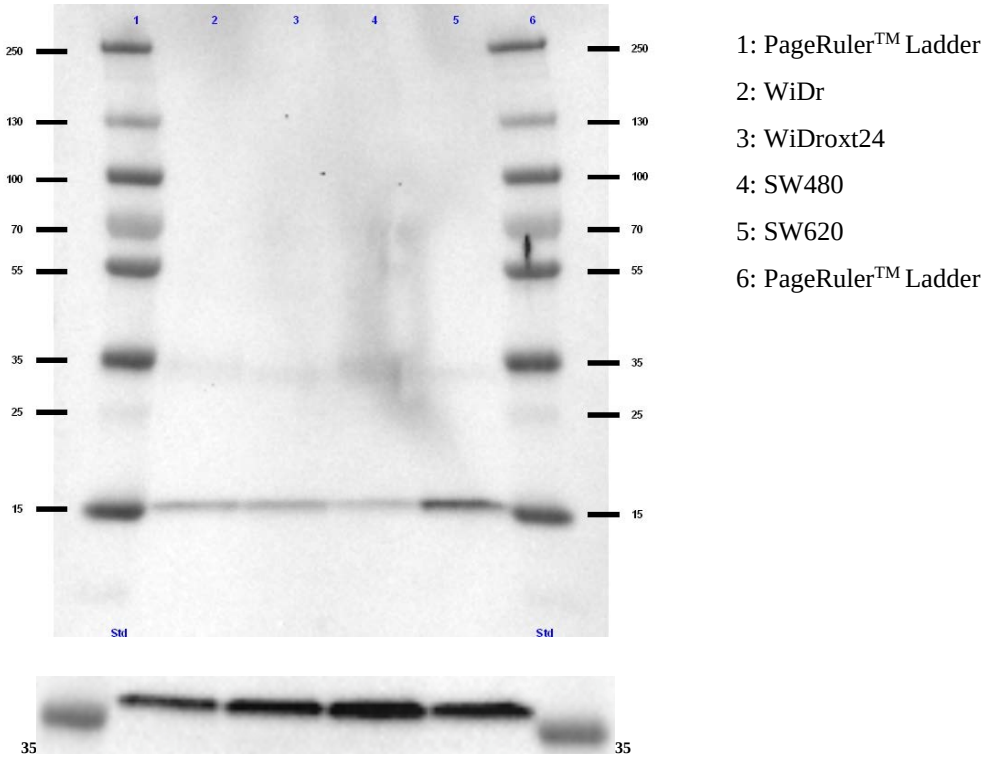

Figure 14. Western Blot analysis. Bands corresponding to the molecular weight of RBM3 (17. 7 KDa) are detected for all cell lines. The bands below correspond to the weight of the loading control GAPDH (37 KDa**)** and are used to normalize the amount of RBM3.

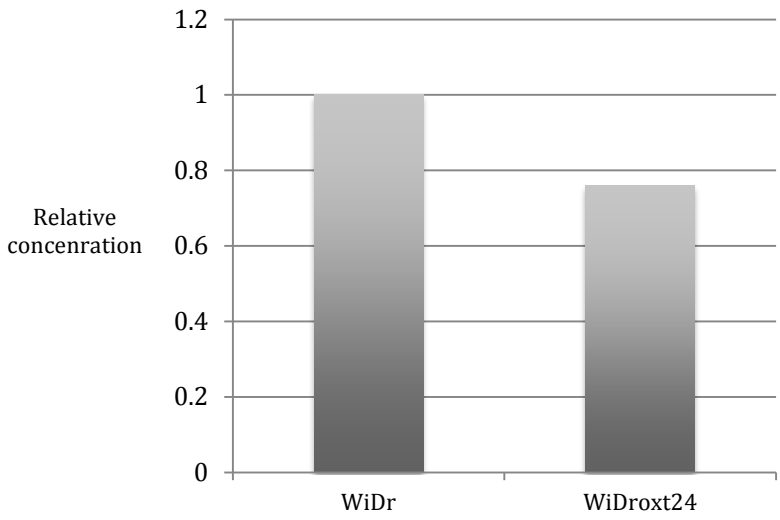

Figure 15. Relative concentrations of RBM3 in WiDr and WiDroxt24 cell lysates generated by Western Blot. Data was normalized using GAPDH as loading control

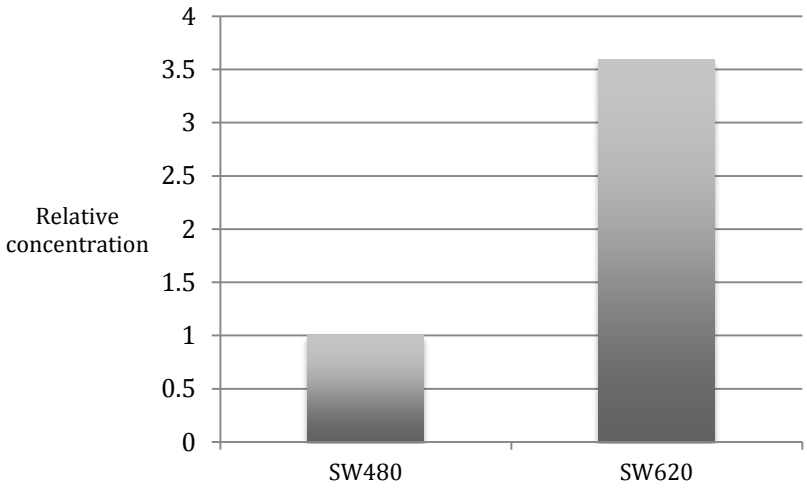

Figure 16. Relative concentrations of RBM3 in SW480 and SW620 cell lysates generated by Western Blot. Data was normalized using GAPDH as loading control.

4.5. Comparison of MS and WB generated data

The relative concentrations between the cell lines WiDr and WiDroxt24 and between the cell lines SW480 and SW620, determined by both WB and MS can be seen in figure 17. The relative concentrations generated by MS and by WB were compared and were found to be very similar. The difference between the relative concentrations generated by MS and WB were 0.4 for both the groups of cell lines, while the relative concentrations determined by western blot were a little higher for both groups of cell lines (WiDr, WiDroxt24 and SW480, SW620).

Figure 17. Comparison of the relative concentrations of RBM3 in the cell lines generated by MS and WB.

5. Discussion

In this study, an immuno-MS assay for detection and quantification of RBM3 has been developed. The possibility to be able to determine an absolute quantity of a target protein in a complex sample is of great importance in both proteomics and the clinical field. By using MS with an internal standard included accurate concentrations of proteins can be generated.

One common problem when analysing proteins in complex samples like cell lysates is that other proteins will interfere with the analysis. In this study this problem has been solved by enrichment of target peptides within the sample by using antibodies available in the product catalog of Atlas Antibodies. An antibody screening was performed where the affinity of the antibodies to tryptic peptides was tested. A prerequisite for this quantitative assay is that at least one antibody with the possibility to enrich a minimum of one tryptic peptide is needed. One peptide is minimum for quantification but it is advantageous if multiple peptides can be quantified since it will potentially result in a more accurate determination of protein concentration if more than one measurement value is generated.

Seven polyclonal antibodies and 22 monoclonal antibodies were included in the screening but only four were antibodies that target RBM3. The screening was performed three times, two times with the same pool composition of antibodies, and one time with only antibodies that seem to have affinity to a tryptic peptide according to the earlier screening results and the feasibility study of the mapped epitopes.

In the end one antibody targeting RBM3 with the possibility to enrich one tryptic peptide was identified and included in the assay. The antibody that was detected is a polyclonal antibody targeting RBM3. The targeted peptide was detected in the second and the third run and also in both the replicates analysed by the third screening. The reason why the peptide was not detected in the first screening is probably because the antibody was generated by immunization of another rabbit than the antibody used in the second and third screening. The antibodies were generated by immunization with the same antigen, but this can still result in antibodies targeting different epitopes since the antibodies were purified from serum taken from different rabbits [29]. Another explanation could be that the antibody has been degraded since it has been stored for a long time.

It was expected in the beginning of the study, that it should be easier to find polyclonal antibodies that can bind tryptic peptides, since the antibodies have the ability to bind more than one epitope. It has been shown by one study, where 150 polyclonal antibodies were analysed, that 50 % of the antibodies could bind minimum one peptide generated by tryptic digestion of HeLa cell lysates [25].

Results from all three screening experiments included detected peptides in pools where the corresponding antibody was not included. This could be explained by interaction between the peptides and the magnetic beads, the plastic of the tube wall or the constant parts of the antibody. During the third screening two negative control pools were included, one pool with only beads and one pool consisting of an antibody targeting the protein SIX1. The results showed that there were no peptides detected in the pool with only beads and some peptides in the pool with the SIX1 antibody. This indicates that the peptides to a larger extent interact with the unspecific part of the antibody compared to the beads or the plastic of the tube wall.

The problem with unspecific interactions is difficult to overcome, but can probably be reduced by optimization of the wash steps.

When the assay for quantification had been developed it was tested on four colorectal cancer cell lines, which in earlier experiments have shown RBM3 protein expression at different levels. The analysed MS data showed that the protein was detected in both replicates for all four cell lines, which demonstrates a robustness of the assay. The ratio between heavy and light peptides was used to generate an absolute concentration of the RBM3 protein. The concentration of QPrEST protein was used to calculate the copy number of peptides per cell, which was determined to be relatively equal between replicates, as can be seen in figure 14. The calculated concentrations of WiDr differs more between the two replicates than for the three other cell lines, this difference is probably a results of pipetting errors when the heavy standard was spiked in to the lysate. The analysis was performed on replicates of two and to further determine the performance of the assay more than two replicates should be analysed and compared.

A western blot assay was performed in order to compare the quantitative MS results with the results generated by another method for protein quantification. A loading control was included in order to compensate for different amounts of samples loaded in the wells. The relative concentrations between the cell lines WiDr and WiDroxt24 and between the cell lines SW480 and SW620 were determined and the results were compared with generated data from the MS analysis. The comparison viewable in figure 18 showed that the generated relative concentrations were very similar for both the methods. The Western blot analysis showed a little higher ratio between the cell lines than MS but the difference was the same for both the two groups of cell lines, which indicates a robustness of both the methods.

The generated relative concentration between WiDr and WiDroxt24 were similar to results generated in an earlier study were the relative concentration was determined. The study referred to was performed by Atlas Antibodies but has not been published. WiDr expressed 30 % more RBM3 protein than WiDroxt24, which was expected since the correlation between platinum resistance and low RBM3 expression is known. WiDroxt24 is an oxaliplatin resistant cell line and should thus show a lower expression of RBM3 than the WiDr cell line. The relative concentration between the SW480 and SW620 cell lines were also in the same range as shown in the earlier study. SW620 expressed 300 % more RBM3 than SW480, which is a big difference in protein expression. SW480 is a primary growth cancer cell line and SW620 is a second growth cancer cell line and has thus started to metastasize. According to the earlier study where it was shown that high expression of RBM3 correlates with high patient survival, the metastatic cancer cell line should express a lower level of RBM3 compared to the primary cancer cell line. In that study only about 10 % of the patients included in the study were diagnosed with second growth cancer [14] and it is probably the reason why the result from this project is not consistent with the published study. One theory is that the correlation between RBM3 expression and patient survival cannot be demonstrated by comparing cell lines if one of the cancers is metastatic and one is not. If the protein expression would be compared between two metastatic cell lines it would possibly result in a correlation between high expression of RBM3 and high patient survival.

In summary, an immuno-MS assay for quantification of RBM3 has been developed and tested in relevant research. The assay showed reliable results and can with some optimization probably be both more robust and more accurate. One project will be performed where the protocol used in this study will be optimized in order to be used for screening of many monoclonal antibodies. The goal is to develop a protocol that is robust and that generates result with high accuracy and precision.

The assay generated by this project can be a good method to use for quantification of RBM3 in order to investigate the state of a cancer disease or predict the optimal treatment for patients diagnosed with cancer.

6. Acknowledgements

Jag vill först och främst tacka min fantastiska handledare Tove som guidat mig genom mitt examensarbete på absolut bästa sätt samt alltid varit både inspirerande och motiverande. Jag vill tacka hela FoU-gruppen som alla varit otroligt hjälpsamma och både svarat på många frågor och hjälpt mig i det laborativa arbetet. Jag vill också tacka alla anställda på Atlas Antibodies som alla tagit emot mig på ett jättebra sätt och gjort att jag alltid känt mig välkommen och som en del av företaget. Slutligen vill jag tacka min ämnesgranskare Sophia Hober som gett mig mycket värdefull feedback samt mina examinatorer Lars-Göran Och Lena som alltid snabbt svarat på frågor och kommit med kloka råd.

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8. Supplementary material

Table 1. Produced PrEST proteins.

PrEST	Gene	Concentration (mg/ml)	Molecular Weight (theoretical)
HPRR232631	RBM3	4.91	32168
HPRR252371	SATB2	4.44	30638
HRRR252372	SATB2	9.09	31342
HPRR320022	HER2	7.94	31979
HPRR370117	PODXL	0.91	32556
HPR13700006	ANLN	4.51	32820

Table 2. Produced QPrEST proteins.

QPrEST	Gene	Concentration (µM)	Molecular Weight (theoretical)
HPRR232631	RBM3	11.7	32514
HPRR252371	SATB2	11.0	30913
HRRR252372	SATB2	10.7	31652
HPRR320022	HER2	13.3	32232
HPRR370117	PODXL		32816
HPRR1370006	ANLN	15.6	33191

Table 3. Peptides identified in the first and second screening. Pool 1-7 were analysed.

Tryptic peptide	Protein	Screening 1 Identified in pool:	Screening 2 Identified in pool:	Corresponding antibody included in pool:
GFGFITFTNPEHASVAMR	RBM3	-	1	1, 2, 4 and 5
AMNGESLDGR	RBM3	-	5	1, 2, 4 and 5
TASQSLLVNLR	SATB2	2	5	4, 5 and 7
ENLSDYCVLGQR	SATB21	2	-	1, 2, 3, 4, 5, 6 and 7
SMNPNVSMVSSASSSPSSSR	SATB2	2	-	4, 5 and 7
TSTPTTDLPIK	SATB2	5	-	4, 5 and 7
ATFNPAQDK	PODXL	-	5	1, 2, 3, 4 and 5
CEDLETQTQSEK	PODXL	5	5	1, 2, 3, 4 and 5
EITIHTK	PODXL	2	-	1, 2, 3, 4 and 5
LASVPGSQTVVVK	PODXL	2, 5, and 6	2 and 5	1, 2, 3, 4 and 5
LISLICR	PODXL	2, 3 and 6	6 and 7	1, 2, 3, 4 and 5

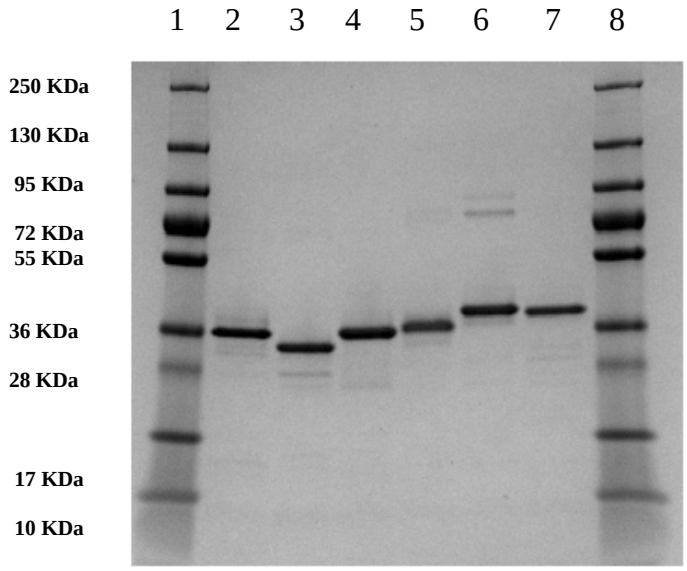
LPAKDVYER	PODXL	5 and 6	-	1, 2, 3, 4 and 5
TPSPTVAHESNWAK	PODXL	2	-	1, 2, 3, 4 and 5
AVTSANIQEFAGCK	HER2	2, 5, 6 and 7	5	1, 3 and 6
VCYGLGMEHLR	HER2	-	3	1, 3 and 6
GDADMYDLPK	EMD (PC)	5	6	6
KEDALLYQSK	EMD (PC)	5 and 6	5 and 6	6
TYGEPESAGPSR	EMD (PC)	-	2,5,6 and 7	6
TPIITPNTK	ANLN	2, 5 and 6	4, 5 and 6	1, 2, 3, 6 and 7
EICLQSQSK	ANLN	-	5	1, 3 and 6
ALYEAGER	ANXA1(PC)	-	5	1
SYPQLR	ANXA1(PC)	-	5 and 7	1

Table 4. Peptides identified in the third screening. Pool 1, 5, 8 and 9 were analysed. All pools were analysed in replicates of two. Pool 10, which consisted of the antibody SIX1 and pool 11, which consisted of only beads were used as negative controls.

Tryptic peptide	Protein	Identified in pool:	Corresponding antibody included in pool:
GFGFITFTNPEHASVAMR	RBM3	1.1, 1.2, 5.2	1
YYDSRPGGYGYGYGR	RBM3	5.2	5, 9
GDRSEDFGVNEDLADSDAR	ANAX1	8.1	1
GYNDDYYEESYFTTR	EMD		5, 9
SMNPNVSMVSSASSSPSSSR	SATB2	1.1, 1.2, 5.1, 5.2, 9.1, 10.1, 10.2	5, 8
TQGLLSEILR	SATB2	9.1	5, 8
TSTPTTDLPIKVDGANINITAAIYDEIQQEMKR	SATB2	5.1, 9.1	5, 8
QLVLNLTGNTLCAGGASDEK	PODXL	9.2, 10.1	5, 8

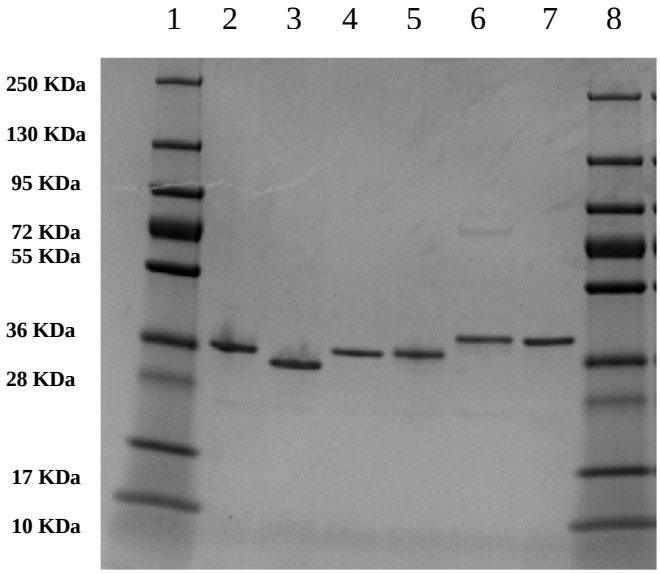
Table 5. Composition of antibody pools. Pool 1 consisted of all the polyclonal antibodies and pool 2-9 consisted of monoclonal antibodies. Pool 1-7 were analysed in the first and second screening. Pool 1,5 and 8,9 were analysed in the third screening

Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Pool 7	Pool 8	Pool 9
HPA003624 (RBM3**)**	AMAb90678 **(SATB2$_1$)**	AMAb90679 **(SATB2$_1$)**	AMAb90680 **(SATB2$_1$)**	AMAb90681 **(SATB2$_1$)**	AMAb90560 **(EMD PC)**	AMAb90683 **(SATB2$_1$)**	AMAb90614 **(SATB2$_2$)**	AMAb90627 **(HER2)**
HPA005680 (ANLN**)**	AMAb90659 **(ANLN)**	AMAb90660 **(ANLN**	AMAb90656 **(RBM3)**	AMAb90657 **(RBM3)**	AMAb90682 **(SATB2$_1$)**	AMAb90661 **(ANLN)**	AMAb90662 **(ANLN)**	AMAb90560 **(EMD PC)**
HPA029543 (SATB2$_1$**)**	AMAb90655 **(RBM3)**	AMAb90627 **(HER2)**	AMAb90643 **(PODXL)**	AMAb90614 **(SATB2$_2$)**	AMAb90662 **(ANLN)**	AMAb90635 **(SATB2$_2$**	AMAb90627 **(HER2)**	AMAb90657 **(RBM3)**
HPA001042 (SATB2$_2$**)**	AMAb90667 **(PODXL)**	AMAb90668 **(PODXL**	-	AMAb90644 **(PODXL)**	AMAb90628 **(HER2)**	-	-	-
HPA003091 (HER2**)**	-	-	-	-	-	-	-	-
HPA002110 (PODXL**)**	-	-	-	-	-	-	-	-
HPA011271 (ANXA1 PC)	-	-	-	-	-	-	-	-



1: PageRuler™ Ladder
2: HPRR232631 (RBM43)
3: HPRR252371 (SATB2)
4: HPRR252372 (SATB2)
5: HPRR320022 (HER2)
6: HPRR370117 (PODXL)
7: HPRR1370006 (ANLN)
9: PageRuler™ Ladder

Figure 18. SDS-PAGE analysis of the PrEST proteins produced



1: PageRuler™ Ladder
2: HPRR232631 (RBM43)
3: HPRR252371 (SATB2)
4: HPRR252372 (SATB2)
5: HPRR320022 (HER2)
6: HPRR370117 (PODXL)
7: HPRR1370006 (ANLN)
9: PageRuler™ Ladder

Figure 19. SDS-PAGE analysis of the QPrEST proteins produced.