5.1 A High Throughput Approach for Bioinformatic Design and Cloning of Protein Epitope Sequence Tags Suitable for Antibody Generation

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The Swedish Human Proteome Resource aims to explore the human proteome and produce a publicly available protein atlas of protein expression and localization in human normal and diseased tissues (www.proteinatlas.org). A high throughput strategy has been established for generation of protein epitope signature tags (PrESTs) for the non-redundant set of proteins. The strategy involves bioinformatical design, cloning and expression of PrESTs to be used for subsequent generation of corresponding monospecific antibodies and tissue micro-array stainings for the protein atlas.

The initial PrEST-design involves sequence similarity scanning towards the ENSEMBL database in order to select regions with lowest homology to other proteins. PrEST selection was focused on fragments of 25 to 150 amino acid residues excluding transmembrane regions and signal peptides of the native protein. After PrEST-design, the regions are amplified by RT-PCR with specific first strand priming for the non-redundant set of proteins. The strategy involves bioinformatical design, cloning and expression of PrESTs to be used for subsequent generation of corresponding monospecific antibodies and tissue micro-array stainings for the protein atlas.

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82% of the genes analyzed have been considered to be suitable for generation of PrESTs. For this set of theoretically predicted PrESTs RT-PCR with specific first strand priming has a success rate of approximately 80% and solid phase cloning yields nearly 100 success. In this presentation we show the methodology, statistics and success rates of PrEST-design and cloning.

5.2 Cell TMA; a Method Creating an Artificial Tissue of Cells for Use in a High Throughput TMA Setting

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Tissue Micro Array (TMA) is nowadays often used for immunohistochemical analysis of protein expression in various tissues (Kononen J. Nat Med 1998). The best morphology is achieved with fixed tissues, most often formalin, and embedded in paraffin. However, to analysis patient cell samples or cells from cell lines in a high throughput manner, in the same way as tissue, is more difficult. We developed a method to use cells as a tissue (Wester, K., J Pathol. 2000) and adapted it for a TMA application (manuscript in preparation). In this way the cells are spread in a three dimensional way in an agarose gel and then histoprocessed, resulting in a paraffin block with cells. From this it is possible to make cores to be used in a TMA. The cells in the gel will last longer than an embedded cell pellet would. The cell morphology in a cell-TMA is good and it is further investigated with the goal to use automated image analysis. For statistical analysis and image scanning issues, using cores with 0.6 mm, we found that a cell concentration of 100 million cells per ml was optimal. In conclusion, we developed a method suitable for using cells in a TMA-setting by preparing the cells in a gel that can be histoprocessed and subsequently used for cell-TMA construction.

5.3 Real-time RT-PCR of Protein Epitope Signature Tags

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The Swedish Human Proteome Resource (www.proteinatlas.org) is an initiative that aims to explore the human proteome with a large scale strategy that involves cloning and expression of protein epitope signature tags (PrESTs) and the subsequent generation of the corresponding monospecific antibodies. These antibodies are used to study expression profiling and localization in human normal and disease tissues and cells. The initial PrEST design involves sequence similarity scanning towards the Ensembl database in order to select the region with lowest similarity to all other proteins. The selected PrEST will have a length of about 100–150 amino acids. Before cloning, the PrEST templates are amplified from tissue derived mRNA by reverse transcriptase polymerase chain reaction (RT-PCR). Currently, reverse PrEST specific primers are used for first strand synthesis and different pools of mRNA are used to capture transcripts with a success rate of 83%. To optimise the RT-PCR, first strand priming with random hexamers, oligo-dT and reverse PrEST specific primers were compared. Also, real time RT-PCR has been used in quantitative and specificity studies with the above mentioned priming methods. The preliminary results show that the PrEST approach can be used for quantifying mRNA levels in tissues or cell lines. Furthermore reverse PrEST specific priming yielded higher specificity and will continue to be the first choice of priming for PrEST amplification, although random hexamers and oligo-dT can make good complements for transcripts with secondary structures that would not otherwise be amplified.
5.4

**Inhibition of Angiogenesis by a Novel Neutralizing Antibody**

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Vascular endothelial growth factor receptor 3 (VEGFR-3) is a receptor for the VEGF-C and D and plays a critical role in the development of the embryonic vascular system. In this report, we generated a novel panel of 17 monoclonal antibodies (mAbs) against the human VEGFR-3 and characterized their ability to inhibit the proliferation of human erythroleukemia (HEL) cells and angiogenesis of chick embryo chorioallantoic membrane (CAM). Among these mAbs, BDD073 was demonstrated to inhibit the interaction of soluble VEGFR-3 with VEGF-D and the proliferation of HEL cells. In CAM angiogenesis experiments, the angiogenesis induced by recombinant GST-VEGF-D was decreased in the presence of antibody BDD073. These data indicate that this novel neutralizing antibody against human VEGFR-3 not only might be a potential compounds in blocking the VEGF-D-induced angiogenesis and lymphangiogenesis, but also be a tool for the investigations of biology of VEGFR-3.

5.5

**Rapid and Efficient Production of Mouse Antibodies Specific for Human Membrane Receptors and “Difficult to Express” Proteins by Optimized Genetic Immunization Methods**

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The genetic immunization methods were optimized using cDNA clones encoding HIV-1 reverse transcriptase (RT) and human ErbB2. Immunization of mice with novel plasmid vectors containing RT and ErbB2 genes resulted in rapid and strong antibody responses to RT and ErbB2, respectively; antibodies in sera were detected at 3 weeks after the first immunization and reached maximum levels by 5 weeks with more than 10,000 antibody titers and the significant levels were sustained for more than 6 months. To explore applicability of the methods to proteome research, we immunized mice with the vectors containing cDNA encoding endotherin receptors A (ETAR) and B (ETBR) as a model of human G protein-coupled receptor proteins and a kinase SIK protein as a model of difficult to express proteins. With the help of additional molecular adjuvants, rapid and strong antibody responses were induced against all of these proteins. The polyclonal antibodies induced by ETAR and ETBR genes were highly specific for conformational epitopes on the each molecule expressing on the cell surface; ETAR antibodies were absorbed by ETAR-expressing 293T cells and not by non-transfected or ETBR-expressing 293T cells. In contrast, ETBR antibodies were absorbed only by ETBR-expressing 293T cells. Expression of ETAR on normal human aortic smooth muscle cells was demonstrated either by FACS analysis or immunohistochemistry using paraffin sections. The optimized genetic immunization protocols with adequate molecular adjuvants will be widely applicable to proteome research and functional proteomics to new drug targets.
5.6 Preparation and Characterization of Monoclonal Antibodies against LSECtin Molecule
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Liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin), a new C-type lectin-like gene encoding 293 amino acids, is a type II integral membrane protein of approximately 40 kDa in size with a single C-type lectin-like domain at the COOH terminus, closest in homology to DC-SIGNR, DC-SIGN, and CD23 (Liu, W., et al., J Biol Chem 2004; 279: 18748–58). LSECtin is expressed predominantly by sinusoidal endothelial cells of human liver and lymph node and co-expressed with DC-SIGNR. LSECtin binds to mannose, GlcNAc, and fucose in a Ca (2+) -dependent manner but not to galactose, and might function in vivo as a lectin receptor.

Aim: To prepare and characterize the monoclonal antibodies (mAbs) against LSECtin protein.

Methods: BALB/c mice were immunized with protocaryon expressed LSECtin protein. The splenocytes from immunized mice were fused with Sp2/0 cells. The hybridomas secreting mAbs against LSECtin were selected by indirect ELISA. The mAbs specifically reacting to LSECtin were further characterized by Western blot and immunohistochemical staining.

Results: Nine hybridoma cell lines secreting mAbs against LSECtin were established. One of these mAbs could recognize LSECtin by Western blotting, and seven out of them could react with the LSECtin in the sinusoidal endothelial cells of human liver.

Conclusion: A novel panel of mAbs recognizing LSECtin was generated, and these mAbs may provide a useful tool for studying the functions of LSECtin.

5.7 Improved Phage Display Systems for the German NGFN Proteome Antibody Initiative
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In the framework of the German national genome research network (NGFN), antibody phage display will be adapted to the generation of antibodies optimised for proteome research. In particular, the opportunities arising from the in vitro selection process will be evaluated. Despite the fact that a multitude of antibody phage display libraries has been built, systematic comparisons of critical design parameters are rare, in particular for systems based on the presentation of Fab fragments. Various parameters, including vector design, growth parameters and phII::antibody fusion protein design, were systematically compared and optimised. The significant differences found for phage yield, display of Fabs on the phage and expression of soluble Fabs suggest to use a bicistronic vector with an fd-fragment::phII fusion for the construction of future Fab phage display libraries. The design of the antibody fragment itself was adapted as well, with a novel antibody design proving to be superior to the commonly used Fab format. We further compared the dependence of various quality-related parameters when using different helperphage. Best presentation of Fab-Fragments and scFv fragments was achieved by using the “Hyperphage” system. The system was validated by the generation of antibody fragments to viruses which have been selected because it was very difficult to obtain antibodies against their relevant antigens in animal based antibody generation systems.

5.8 Generation and Characterization of Antibodies against CXCR3-B NH2 Terminus
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Platelet factor 4 (PF4) is an antiangiogenic ELR-negative chemokine with unusually high affinity for heparan sulfates. It has been shown that PF4 inhibits proliferation and migration of endothelial cells, and angiogenesis in vitro and in vivo. Endothelial cells in some vascular beds, including those in heart and skeletal muscle, express the PF4 receptor which is an alternatively spliced product from the CXCR3 gene termed CXCR3B. CXCR3B is 51 amino acid residues longer at the NH2-terminus compared with CXCR3A. CXCR3A binds to CXCL9, CXCL10, and CXCL11 exclusively, but not to PF4. Furthermore, unlike activation of CXCR3A, which is pertussis toxin-sensitive and which activates cellular proliferation, activation of CXCR3B is pertussis toxin-resistant and leads to inhibit cellular proliferation. Thus, 51 amino acid residues of CXCR3B NH2-terminus are very important in CXCR3B binding to PF4. In the present study, three peptides of CXCR3B NH2-terminus (a.a. 1–19, a.a. 17–35, and a.a. 33–51) were conjugated with KLH and a GST-tagged fusion protein of CXCR3B (a.a 1–51) were used as immunogens for generation of polyclonal and monoclonal antibodies against CXCR3B (a.a. 1–51). Two mAbs reacted specifically with the CXCR3B a.a. 1–19, and one with the CXCR3B a.a. 33–51. Western blot results showed that the antibodies could recognize the protein with molecular weight about 50 KD in the total lysates of human fetal heart. CXCR3B recognized by the antibodies were localized in the vascular endothelial cells of human fetal heart tissues. These antibodies could be useful tools for the functional studies of human CXCR3-B.

5.9 IGG Purity Assay Using a New High Resolution SDS Gel
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Capillary SDS-Gel electrophoresis of proteins has been an important analytical method used to characterize, monitor process control and provide quality control for the production of immunoglobulins. In this poster we present the development of a new polymer formulation and standardized methodology to assess the purity and heterogeneity of IgG and its isoforms. This methodology provides increased resolution of the IgG isoforms from typical developmental impurities like non-glycosylated heavy chain, and low molecular weight impurities associated with the IgG light chain. The assay includes an artificially engineered IgG control with a fixed percentage of non-glycosylated heavy chain to provide assay suitability determination prior to the analysis of unknowns. All aspects of the methodology from sample preparation to automating data analysis will be discussed.
Monoclonal antibodies (mAbs) will be the most powerful tools in the proteomic research for protein expression, quantification, localization, and modification, as well as protein-protein interactions. We have reported a strategy to establish hybridoma cell lines using mixed and fractionated native liver proteins as immunogens and several approaches to identify the mAb’s specificity by ELISA, immunoprecipitation plus MS, and screening of cDNA expression library (Uni-ZAP XR). In the present studies, we established and optimized a new method to identify the mAb targets by biopanning of T7Select human liver cDNAphage display library (Novagen). In the first round of biopanning, phages displaying antigens were captured by using mAb-Protein G sepharose. The phages rescued from first round were incubated with the same mAb to be identified in ELISA plate well for 2 hours in the second round. Bound phages were eluted and plated on agar plate containing BLT5615 cells and molten agarose. The plate was overlaid with nitrocellulose membrane, and subjected to Dot blot using the mAb to be identified. The positive PFUs were further confirmed by Western blot, and the cDNA inserts from these clones were amplified using PCR and subjected to sequencing. Most importantly, not only a panel of liver protein antigens including albumin, fibronectin, and ADH1A has been identified, but also the mAb epitopes have been mapped by using this approach. We believe that this approach is time, cost, and labor saving and may be a promising alternative to screening of λ phage cDNA expression library in identification of mAbs’ specificities for the establishment of mAb bank for liver proteins.

Objective: The mapping of the genetic code including approximately 22000 genes offers new opportunities to explore function and communication within the proteome. To be able to functionally explore the human proteome, there is a great necessity to generate protein-specific antibodies. The Swedish human protein resource project (HPR) systematically generates mono-specific antibodies (mAbs) using antibody proteomics. One aim of the HPR project is to create a protein atlas displaying expression and localization patterns of proteins in normal human tissues, the 20 most prevalent cancer forms and a selection of 50 cell lines.

Methods: A set of standardized TMAs was produced to systematically explore clinical samples to allow for rapid screening of a multitude of different tissues using immunohistochemistry. By using an automated scanning technology, large numbers of digital images corresponding to immunohistochemically stained normal and cancer tissues were generated.

Results: These images were displayed and annotated with an in-house web-based annotation tool. Protein expression patterns were evaluated with respect to staining intensity, conformity, reliability, quantity, and localisation. Since annotation speed was of importance, a brief general set of tissue structures were included in the annotation tool.

Conclusion: By using a Web-based annotation system, we conclude that virtual pathology could be a useful tool in mapping the human proteome. Combined with the image annotation analysis systems, TMAs are powerful profiling tools.

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Construction of a monoclonal antibody (mAb) bank containing a vast variety of antibodies against human tissue proteins is important for proteomic research. A novel strategy of subtractive immunization using fractionated native proteins was developed for high throughput generation of monoclonal antibodies against human plasma proteins. By this approach, the major bottleneck of antigen preparation can be overcome by combining repeated immunization of animals with subtracted fractions of plasma/tissue proteins and identification of target antigens by immunoprecipitation/Mass Spectrum. Until now, 350 strains of hybridomas secreting mAb against fractionated plasma proteins were obtained after two-round subtractive immunizations and cell fusions. In the first round, mice were immunized with the fractionated plasma proteins HPS-I, HPS-II and HPS-III respectively and 110 strains of hybridomas were established. For the second round immunization, the immunogen HPS-I-P, HPS-II-P and HPS-III-P were obtained by absorb the target proteins in fractions HPS-I, HPS-II and HPS-III with the mAbs against high abundant plasma proteins obtained from the first round. After identification of the target antigens of the mAbs got from this round cell fusions with ELISA and immunoprecipitation combined with mass spectrum, 80% antibodies obtained from this round were identified as new antibodies when compared with mAbs obtained from the first round immunization with HPS-I, HPS-II and HPS-III. The results suggest that subtractive immunizations with fractionated plasma proteins may be an effective approach for rapid preparation of mAbs against plasma and tissue proteins.
Antibody-based Proteomics for Human Tissue Profiling; the Swedish Human Proteome Resource Project (HPR)

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A multi-disciplinary research program to create a “Human Proteome Resource” was started in July 2003. The aim of the program was to allow for systematic exploration of the human proteome using antibody-based tissue proteomics, combining high-throughput generation of mono-specific antibodies with protein profiling in human tissues and cells using tissue microarrays. Mono-specific antibodies (msAb) can be used for analysis of corresponding proteins in a wide range of assay platforms, including (i) immunohistochemistry for detailed tissue profiling, (ii) specific affinity reagents for various functional protein assays, and (iii) capture (“pull-down”) reagents for purification of specific proteins and their associated complexes for structural and biochemical analyses. The use of msAb, generated using recombinant protein fragments selected from unique regions called Protein Epitope Signatures Tags (PrESTs) as immunogens, with focus on the possibility to create a descriptive and comprehensive protein atlas for tissue distribution and sub-cellular localization of human proteins in both normal and cancer tissues will be discussed. An advantage of PrEST antibodies as compared to monoclonal and recombinant antibodies is that no screening is needed which facilitates attempts to scale-up to whole proteome applications. The possibility to detect and localize defined proteins at tissue, cellular and sub-cellular levels present a deeper insight into normal cellular functions and pathogenic mechanisms leading to different types of disease. Tissue microarray (TMA) technology provides an automated array-based high throughput technique for expression profiling of tissues and cells. Collection of tissues including production of TMAs and immunohistochemistry is fairly labor intensive, whereas slide scanning with subsequent image processing can be automated to a large degree. One important objective for clinical proteomics is to generate a protein atlas displaying expression and localization patterns of proteins in all or most human tissues and organs. A human protein expression atlas would function as a knowledge-base with regard to the structural and temporal expression of proteins in various cells and tissues. Strategies and examples illustrating the effort to create such a protein atlas will be discussed.

Expression of Three Unrelated Proteins in Mycoplasma-infected Cell Lines

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Most research laboratories involved in cell culturing are faced by complications such as interfering microorganisms, which impair cell growth and influence experimental results. Mycoplasmas are among the few prokaryotes that can grow in close interaction with mammalian cells without producing acute cytotoxic effects. Most of them are also resistant to the antibiotics commonly used in conventional cell culturing. The mycoplasmas are a large group of microorganisms, which are dependent on a host cell for metabolism and replication. Up to date, few studies have been performed investigating the influence of mycoplasma infection on gene and protein expression. In order to study the possible consequences of mycoplasma infection on protein expression of in vitro cultured cell lines, six leukemia cell lines were cultured each represented as a pair of one non-infected and one mycoplasma infected cell line, rendering a cell tissue microarray (TMA) containing 12 samples. Protein expression was analyzed by immunohistochemistry using in-house produced mono-specific antibodies included in the Swedish Human Proteome Resource (HPR). Screening of a large set of antibodies on this cell TMA revealed distinct protein expression with three different antibodies only in the mycoplasma-infected cell lines as compared to non-infected. The antibodies showed a membranous, granular staining pattern in all infected cell lines. These results will be further examined on both RNA and protein level. In conclusion, a clear visible difference in staining pattern between mycoplasma infected and corresponding non-infected cell line can be observed for antibodies to three proteins using immunohistochemistry.
Preparation, Characterization, and Application of Monoclonal Antibodies against Human Liver Mitochondrial Enzymes

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Mitochondria play crucial and diverse roles in many cellular processes and diseases, such as energy metabolism, apoptosis, free radical production, thermogenesis, and calcium signaling. Consequently, impairments of mitochondrial function have been associated with the emergence of severe human disorders. Despite a broad characterization of the genetic defects leading to mitochondrial disorders, few of their pathogenetic mechanisms are fully understood. The identification and analysis of the mitochondrial proteome would be invaluable for understanding the functions of mitochondrion and the mechanisms for mitochondrion-related diseases, and monoclonal antibodies (mAbs), with their highly specificities and sensitivities, are one of the most powerful tools in this field. The purpose of the present studies was to prepare mAbs against human liver mitochondria enzymes using unknown and native proteins as the immunogens. Normal human liver tissues were homogenized, and mitochondria were isolated by differential centrifugation. The total mitochondrial proteins were used to immunize mice. Hybridoma cell lines were established after cell fusion, screening, and cloning. All of the monoclonal antibodies were characterized by ELISA, immunohistochemistry, Western blot, and immunoprecipitation. The antibody-recognized mitochondrial proteins were identified by mass spectrometry following immunoprecipitation, and by screening of human liver cDNA expression library. The subcellular localization of these antigen proteins in mitochondria was further confirmed by double and mitochondrial specific staining on the acetone-fixed frozen sections of human liver tissues. Most of the protein antigens identified are mitochondrial enzymes, and a panel of antibodies were able to capture and deplete the targeting proteins or protein complexes from the total mitochondrial proteins by immunoprecipitation. For example, a complex with 20 proteins could be co-precipitated by a mAb against human carbamyl phosphate synthetase I, as analyzed on 2-D SDS-PAGE. These proteins further identified by MS included human carbamyl phosphate synthetase I, 78kDa glucose-regulated protein precursor, nonspecific lipid-transfer protein mitochondrial precursor, protein disulfide isomerase A3 precursor, argininosuccinate synthase, stress-70 protein mitochondrial precursor, hypothetical protein DKFZp686J18235. These enzymes are related with urinate cycle of glucose metabolism, TCA cycle, NH₃ metabolism, and lipid metabolism. We believe these well-characterized antibodies would be very useful in various applications for Human Liver Proteome Project (HLPP), especially in protein-protein interaction.

Novel Flow Cytometry-based Method for Analysis of Protein Production in Escherichia coli


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A method for measurement of soluble and precipitated protein levels in vivo by using a flow cytometer has been developed. The method has been used to analyse cell samples harbouring protein fragments fused to the C-terminus of enhanced GFP. Light scattering data, which gives information about inclusion body formation, was obtained as well as information about whole cell fluorescence. The latter gives a visualization of the intracellular concentrations of soluble fusion proteins. For evaluation of the method the cells were disrupted, separated into soluble and non-soluble fractions and analysed by gel electrophoresis. The correlation between fluorescence and soluble target protein was clear. The newly developed method was finally used for analysis of two different purification tags, His₆ and Zfusac, and four different promoters, T7, Trc, LacUV5 and Spa, and their impact on the expression pattern.
5.20
Multiplex Immunization Strategies for Generation of Monospecific Antibodies in Proteomics Research

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Monospecific antibodies generated through antigen specific purification of polyclonal antisera are valuable tools in proteome analysis. However, proteome wide generation of monospecific antibodies would require extensive immunization programs. Therefore, it is desirable to develop novel immunization strategies to reduce the number of animals needed for such antibody-based initiatives. Here we describe a novel multiplex immunization strategy for generation of monospecific antibodies towards recombinantly produced human gene fragments, denoted PrESTs. Antiserum from rabbits immunized with a mixture of two, three, five or ten unique PrESTs have been purified by a two-step immunoaffinity-based protocol to generate monospecific antibodies. Almost 80% of the animals immunized with antigens composed of two or three different PrESTs yielded antisera recognizing all the included PrESTs. For antigens containing five PrESTs, 25% of the immunizations resulted in antibodies towards all five PrESTs and 50% recognized four out of five PrESTs. The amount of purified antibodies from a single rabbit decreased with increased antigen complexity. This was expected since the total amount of immunized protein was kept constant regardless of antigen complexity. The specificity of purified antibodies was investigated using a two-color protein array concept and further characterized by western blot analysis using whole cell tissue lysates. Furthermore, a subset of the purified antibodies were used in cellular localization studies on tissue micro-arrays and compared with the previously obtained results from traditional immunization strategies. The results indicate that multiplex immunization strategies could become useful for antibody-based proteome initiatives, thus significantly reducing the number of animals needed in addition to providing a more cost-efficient method for production of monospecific antibodies.

5.21
Rabbit Monoclonal Antibodies for Entire Pathways in Human Proteome

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Rabbit monoclonal antibodies have been shown to play important roles as research reagents, diagnostic tools and in therapeutic development. The rabbit system has the advantages that each rabbit can offer 40 times as many lymphocytes in comparison to that of a mouse and recognize novel epitopes. It is also possible to carry out hybridoma fusion for multiplex antigens at a high throughput. One of our goals is to establish a monoclonal antibody bank against large number of human proteins by innovative technologies. So far Epitomics has successfully developed a large number of high quality rabbit monoclonal antibodies against difficult targets such as protein phosphorylation sites. Affinity of these rabbit MAbs are usually 10–100 times higher than mouse MAbs.

In this paper, we present examples of rabbit monoclonal antibodies and their applications in immunohistochemistry (IHC) and analyzing the entire signaling pathways. Availability of a large collection of high quality rabbit monoclonal antibodies should greatly facilitate research and development of novel therapeutics as well as new target discovery.