Application Forum

Prestige Antibodies™ - Monospecific Antibodies Designed for Immunohistochemical Analysis

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INTRODUCTION

Well-characterized antibodies are essential tools for protein studies, global proteomics analysis, as well as for clinical diagnostics. However, although production of antibodies is a well-established process and a large number of antibodies are commercially available through many vendors, specific antibodies still do not exist for the majority of human proteins. An underlying factor limiting the available antibody repertoire is that commercial production tends to focus on popular targets. The current needs of the proteomics community demand a far more global approach (Blow, Nature, Vol. 447, 2007, 741-742; Editorial, Nature Methods, Vol. 1, 2007, 1-2). A second significant issue is the lack of a universally defined standard for antibody quality. This makes it difficult to compare antibodies of various sources without committing resources to using the antibody in its final application. Initial standardized testing for specificity and sensitivity followed by a thorough characterization would be of great interest to the end user, but this is a costly endeavor and most efficiently accomplished on a larger scale.

At present, there are a handful of large-scale high-throughput antibody production efforts initiated around the world (Persson et al, Curr. Opin. Mol. Ther., Vol. 8, 2006, 185-190). One such initiative is the Swedish Human Protein Atlas (HPA) program (Uhlén et al, Mol. Cell Proteomics, Vol. 4, 2005, 1920-1932). The aim of this program is to explore the entire human proteome using an antibody-based proteomics approach (Kampf et al, Clin. Proteomics, Vol.1, 2004, 285-300; Uhlén and Pontén, Mol. Cell Proteomics, Vol. 4, 2005, 384-393). Specifically, the HPA program generates protein expression profiles of the non-redundant set of human proteins, presented as immunohistological images from the majority of human tissues. All images are annotated and made publicly available via an open access database, the Human Protein Atlas (www.proteinatlas.org). An ambitious and thorough quality-control process has been developed by which all antibodies have to pass a set of criteria prior to being tested by immunohistochemistry (IHC) and other methods (Hober and Uhlén, Curr. Opin. Biotech., Vol. 19, 2007, 1-6).

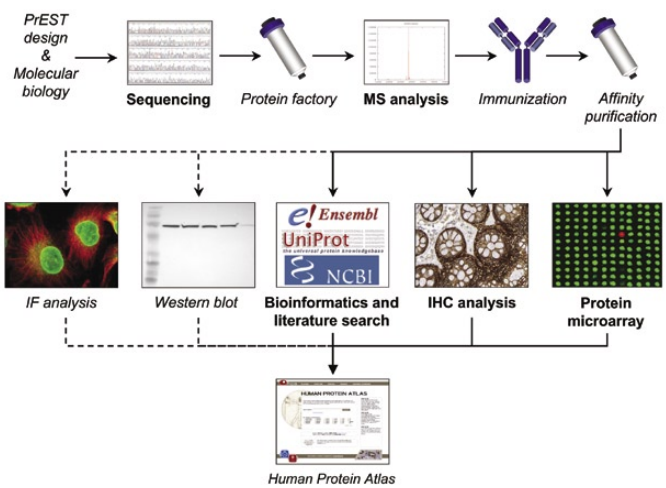

Figure 1. Schematic overview of the workflow and control steps in the HPA antibody development process (top row) and the various expression profiling analyses and validation steps (bottom row).

THE HUMAN PROTEIN ATLAS PROGRAM

The Swedish Human Protein Atlas (HPA) program is an academic initiative, headed by Professor Mathias Uhlén at the Royal Institute of Technology in Stockholm, Sweden and the Rudbeck Laboratory in Uppsala, Sweden. The vision of the HPA program is to systematically generate quality-assured antibodies to all non-redundant human proteins, and to use these reagents to functionally explore human proteins, protein variants and protein interactions. At present, 50 new antibodies are generated per week along with 50,000 new IHC images. In order to manage this large amount of material and data generated, methodologies have been developed to support high-throughput systems including data collection, image handling and storage.

ANTIBODY DEVELOPMENT AND QUALITY CONTROL

The HPA proteomics approach is based on affinity-purified polyclonal antibodies (mono-specific antibodies, msAbs) raised toward bioinformatically-designed Protein Epitope Signature Tag (PrEST) antigens. The PrEST antigens, the monospecific antibodies and the resultant images for the Human Protein Atlas are generated in a high-throughput manner as outlined in Figure 1.

The initial step of the process is the antigen design. Tailor-made bioinformatics software based on the Basic Local Alignment Search Tool (BLAST) function is used to design the

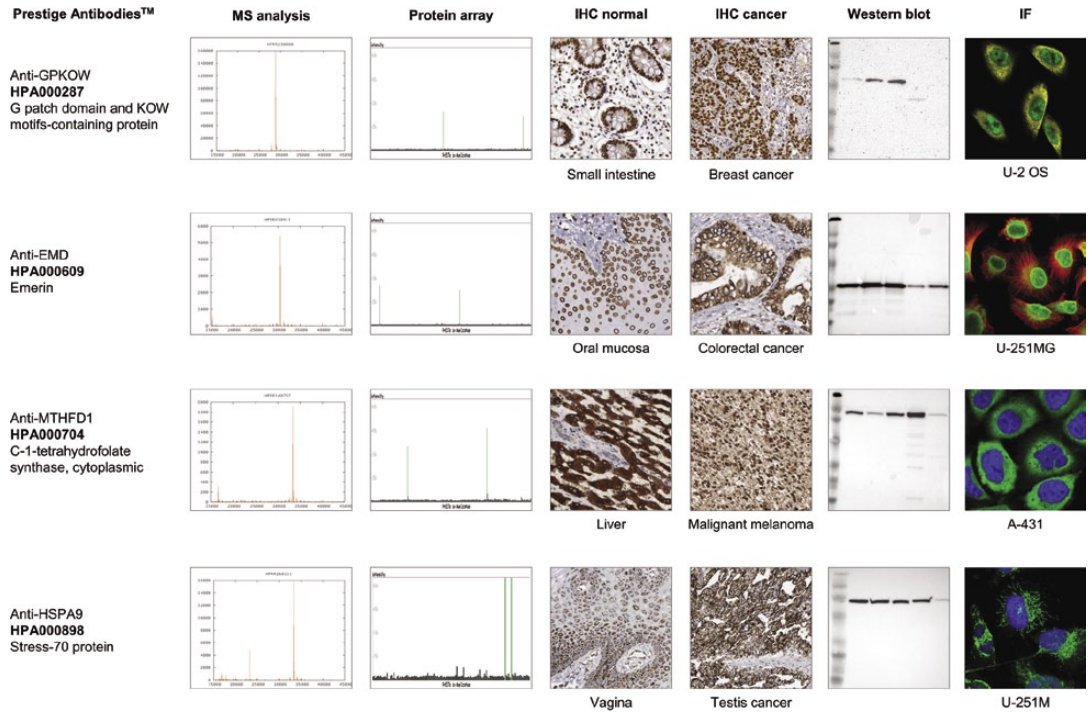

Figure 2. Four examples of Prestige Antibodies™ with corresponding quality control data and expression profiling results.

MS analysis. Mass spectrometry analysis of the PrEST antigens are presented in the first lane (MS analysis). A single peak of correct mass verifies production and purification of the correct PrEST antigen.

Protein array. Binding specificity for each purified Prestige Antibody is verified by protein microarray analysis (lane 2; Protein array). In the initial phase of the HPA program, each PrEST antigen was spotted in duplicate and two peaks verify antigen-specific binding as shown for each of the four Prestige Antibodies™ in Figure 2. However, presently 384 different PrEST antigens are spotted, each as a single spot, allowing analysis of background reactivity towards a larger set of PrEST antigens. Depending on PrEST array set up, the resulting images on the Human Protein Atlas will show two specific peaks (duplicate spots) or one specific peak (single spot).

IHC normal tissue. In lane 3 (IHC normal), one out of 144 images from IHC stained normal tissue samples are shown. The tissue localization of a protein is shown by specific binding of an antibody to its corresponding antigen in IHC, indicated by brown staining. The tissue section is counter-stained with hematoxylin to enable visualization of microscopical features. Hematoxylin stains both cells and extracellular material in blue.

IHC cancer tissue. IHC expression analysis of cancer tissue samples is performed the same way as IHC staining of normal tissue, with the binding of the antibody to its antigen resulting in a brown staining pattern. One out of 432 images from IHC stained cancer tissue samples is shown in lane 4.

Western blot. The Prestige Antibodies are analyzed by Western blot against total protein lysates from two human cell lines (RT-4 and U-251MG), two human tissues (liver and tonsil) and human plasma in a high-throughput standardized manner (lane 5, Western blot).

Immunofluorescence (IF). A large number of Prestige Antibodies have been used for subcellular localization by immunofluorescence (lane 6, IF). The protein targeted by the Prestige Antibody is shown in green, the endoplasmic reticulum in yellow, cytoskeleton in red and nuclei in blue.

PrEST antigens. The scanning procedure permits selection of fragments of a specified size, between 50-150 amino acids, with a minimal sequence similarity to other human proteins. Further, this program allows for the avoidance of certain restriction enzyme sites, transmembrane regions, and signal peptides.

The computer selected PrEST regions are RT-PCR amplified from pools of human total RNA and cloned into an expression vector as a fusion to a histidine tag and an albumin binding protein (ABP) (Agaton et al, Mol. Cell Proteomics, Vol. 2, 2003, 405-414). All recombinant PrEST clones are fully sequenced to verify the correct insert and that no polymerase-introduced mutations are present. The PrEST sequence analysis is the first of several quality control steps that must be passed before further processing (Figure 1).

The sequence-verified PrEST clones are expressed in *E. coli* and the produced PrEST antigens are affinity purified by immobilized metal ion chromatography under denaturing conditions. The purified PrEST antigens are quality controlled by mass spectrometry (MS) for sequence accuracy, by SDS-PAGE analysis for protein purity analysis, and by bicinchoninic acid assay (BCA) for protein concentration determination.

The purified PrEST proteins serve various purposes in the downstream antibody development and control process, first as antigens for immunizations, secondly as PrEST-ligands coupled to affinity columns for antibody purification and thirdly as ligands on protein arrays used to help ensure specific antibody binding to the complementary antigen.

Mono-specific polyclonal antibodies are purified from raw antiserum in a three-step process, starting with depletion of unwanted specificities, i.e. His_6-ABP affinity tag-specific antibodies. The flow-through is then passed through a PrEST antigen column, allowing capture of anti-PrEST specific antibodies. Finally, after washing, captured antibodies are eluted and loaded onto a desalting column for buffer exchange. The purified mono-specific polyclonal antibodies are then analyzed for specificity on a protein array consisting of 384 different PrEST antigens spotted on glass slides, including the matching PrEST antigen to the msAb to be tested (Nilsson et al., Proteomics, Vol. 5, 2005, 4327-4337). An example of a typical PrEST array analysis is shown in Figure 2. It illustrates specific reactivity between the msAb and the corresponding PrEST protein, which is contrasted against low background reactivity to other PrEST proteins.

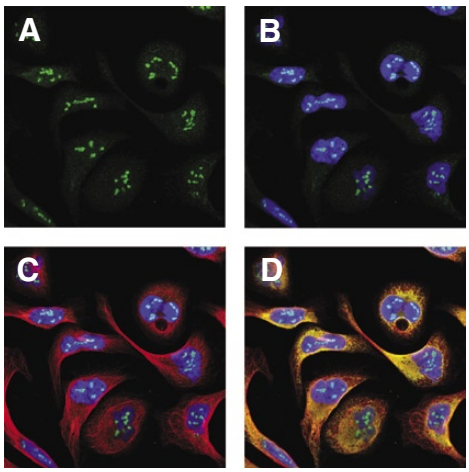

Figure 3. Confocal images of immunofluorescent staining of the human cell line U-2OS using the nucleoli specific Prestige Antibody anti-HMGB2 (Cat no HPA0003506) in combination with organelle probes specific for the endoplasmic reticulum, cytoskeleton and nuclei. The four-color image is acquired in four separate channels that can be viewed separately or in different combinations.

Image A. View of one channel showing the Prestige Antibody staining of nucleoli in green.

Image B. A second channel is turned on for an overlay view of the control staining of nuclei (blue) and the anti-HMBG2 Prestige Antibody (green).

Image C. A third channel is turned on for a three-color overlay view of cytoskeleton (red), nuclei (blue) and the anti-HMBG2 Prestige Antibody (green).

Image D. The fourth channel is turned on for a four-color overlay view of endoplasmic reticulum (yellow), cytoskeleton (red), nuclei (blue) and the anti-HMBG2 Prestige Antibody (green).

HUMAN PROTEIN EXPRESSION PROFILING

All mono-specific antibodies that pass the protein array control step are tested on a standard set of human Tissue Micro Arrays (TMAs), carefully selected to be tissue representative. The final set of TMAs stained for full protein expression profiling includes formalin-fixed paraffin-embedded samples from 48 non-neoplastic, morphologically normal human tissues in triplicate and tissues from 20 common cancer types derived from 4-12 individuals performed in duplicate. In addition, each antibody is used to stain Cell Micro Arrays (CMAs) from 47 cell lines and 12 samples of primary blood cells, each performed in duplicate (Andersson et al., J. Histochem. Cytochem., Vol. 54, 2006, 1413-1423).

In total, more than 700 high-resolution IHC images, each representing a tissue or cell section, are generated per antibody, and uploaded onto the Human Protein Atlas. With the aid of a web-based annotation program, certified pathologists or specially trained personnel annotate all newly tested antibodies. One or several representative cell types are annotated for each tissue and given a staining score. To ensure high quality, all annotated tissue images are curated by a second person. Annotation of cell sample images is done automatically by image analysis software (Strömberg et al., Proteomics, Vol. 7, 2007, 2142-2150).

Each antibody in the Human Protein Atlas is given a validation score. A validation score is assigned based on (i) the IHC staining pattern, (ii) available bioinformatic data, and (iii) whether supporting data exists in the literature. The validation score indicates how well the quality assurance data supports the specificity of the antibody against the expected human target protein.

In order to obtain a ***high*** validation score, two independent antibodies targeting the same protein and showing similar staining patterns are required. In addition, the staining pattern must be consistent with experimental and/or bioinformatic data.

A ***medium*** score is assigned when the staining pattern is consistent with experimental and/or bioinformatic data.

When the staining pattern is only partially consistent with experimental and/or bioinformatic data, a ***low*** score is assigned.

For profiling of human proteins where experimental and/or bioinformatic data is not available, or the staining pattern is not consistent with such data the antibody is assigned a ***very low*** score.

All results generated, including the PrEST array result, IHC images, annotation results, literature summary and validation score, are published by the Human Protein Atlas (www.proteinatlas.com).

ADDITIONAL APPLICATIONS

All new antibodies are analyzed by Western blot in a high-throughput standardized manner. Total protein lysates from two human cell lines (RT-4 and U-251MG), two human tissues (liver and tonsil) and human plasma are used to evaluate the antibody target binding in a Western blot setting. The Western blot analysis for each antibody is performed using identical set-ups with no optimization, i.e. the same five protein lysates, as well as the same conditions are used for all antibodies. As the protein samples and running conditions are not specifically selected and adapted for each antibody and its corresponding protein, not every antibody has been successfully validated for Western blot application. However, the Western blot data does have an impact on the validation score.

In addition to the standard immunohistochemical expression profiling performed with each antibody, subcellular localization studies by confocal microscopy and immunofluorescence (IF) staining is performed for a large number of the Prestige Antibodies™ (Barbe et al., Mol. Cell Proteomics, Nov 19, 2007, Epub ahead of print), as shown in Figure 3. In the Human Protein Atlas, the cell lines used for IF staining can be visualized in several ways. The protein targeted by the Prestige Antibody is shown in green, nuclei in blue, cytoskeleton in red and endoplasmic reticulum in yellow. In order to optimize the view, one or several channels can be removed and/or combined.

HOW TO USE THE HUMAN PROTEIN ATLAS

The Human Protein Atlas currently consists of a collection of over 2.9 million IHC images covering the majority of normal and cancer tissues as well as a large selection of cell lines and primary cells. In the current version (3.1) more than 3,000 antibodies have been screened for protein profiling of more than 2,600 human proteins. More than half of these antibodies are Prestige Antibodies targeting approximately 1,600 human proteins. New data and new features are released on an annual basis.

There are three ways of searching the database; (i) by viewing the proteins by chromosome, (ii) by using the simple search box, or (iii) by advanced search. The simple search box

allows searches by gene name, gene description, antibody ID (product number) or Ensembl ID.

The advanced search tool is based on protein expression levels in all the included normal and cancer tissues, and a combination of searching criteria can be utilized, such as "and" or "and not" queries. In addition, it is possible to search for proteins differentially expressed within a tumor type, i.e. among different patients having the same cancer type. For example, the number of patients of a given tumor type that should show a specified staining intensity, higher or lower intensity as compared to the rest of the patients of the same tumor type can be selected. For a detailed description of the advanced search option and *in silico* biomarker discovery, refer to Björling and co-workers (Björling et al., Mol. Cell Proteomics, Oct 3 2007, Epub ahead of print).

A search query results in a list of proteins/antibodies fitting the search criteria. The expression profile overview page is viewed by clicking on the antibody ID. The normal and cancer tissues can be sorted in alphabetic or histogenic order. All tissues and cells are given a protein expression level score, visualized by a colored pie chart, ranging from strong staining (red) to no staining (white). The pie chart system gives a quick overview of the protein expression profile. An annotation summary provides a brief description of the major findings from the IHC analysis. The high-resolution IHC images are viewed by clicking on the tissue type name. To see specific detail, all images can be viewed at higher magnification.

CONCLUSIONS

The lack of high-quality protein-affinity reagents for extensive and efficient proteomics studies is a widely accepted problem (Blow, Nature, Vol. 447, 2007, 741-742; Editorial, Nature Methods, Vol. 1, 2007, 1-2). Here we have described one effort to increase the availability of anti-human protein antibodies, as well as the use of antibody-based proteomics to explore the human proteome.

With a proven high-throughput strategy for development of well-characterized antibodies against human protein targets, and the subsequent use of these antibodies in an extensive protein expression profiling study, the Human Protein Atlas (HPA) program will have a major impact on proteomics research for many years to come.

The current HPA antibody development pipeline generates about 2,000 new well-characterized anti-human antibodies every year. These Prestige Antibodies™ are valuable tools in protein research and proteomics studies, not only for the protein expression studies performed by the HPA program, but also for other scientific researchers around the world.

The main application for which all Prestige Antibodies™ are validated are immunohistochemical staining of paraffin-embedded, formalin-fixed tissue and cell samples. In addition, a large number of the Prestige Antibodies™ have been successfully used in Western blot analyses and immunofluorescence staining.

With the overall objective of achieving the first draft of a Human Protein Atlas covering the majority of the non-redundant human proteins by year 2014, the HPA initiative is well positioned to substantially increase the number of available anti-human protein-affinity reagents of exceptional quality and thus revolutionize how proteomic research is conducted.

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Sigma Life Sciences (www.sigma.com).

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