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Abstract

Over a decade after its start, Human Protein Atlas (HPA) has been developed as a universal database for human proteome. HPA has used both commercially designed and intra-designed antibodies for protein detection but both types should pass several validation tests like Western blot, immunohistochemistry, protein array and immunofluorescence. However, importance of data reliability requires more sensitive techniques to confirm specificity of antibodies to avoid any false data in HPA. Immunoprecipitation by help of magnetic beads (IP) coupled with Orbitrap Mass Spectrometry (MS) can be used as an antibody validation method with its incredible sensitivity and high capacity for different biological samples. In this study, it has been tried to test Orbitrap Mass Spectrometry and optimize a protocol for its future application as a validation method in HPA.

Four proteins ATP5B, DDX1, IL18 and KRT7 with high expression (according to RNA data and IHC staining) and high reliability score for their antibodies and four pancreatic markers, GPR44, StradB, TMEM100 and SerpinB10 were selected to be detected in lysates from the cell lines, SIHA, RH-30, BEWO and HACAT respectively (for each cell line one safe and one pancreatic marker). Protein lysates prepared by RIPA buffer were used for Immunoprecipitation and purified samples were run on SDS gel, stained with Coomassie blue and several bands were selected to be cut and prepared for MS run by in-gel digestion method. Peptides were run in Orbitrap Mass Spectrometry for detection.

In total three samples out of seven were detected as correct targets (one ATP5B, and two for KRT7), two samples with high molecular weight were detected as myosin which is a common contamination in proteomic lab and two bands belonging to GPR44 and TMEM100 failed in detection due to incorrect gel cutting.

In total, HPA antibodies were successful in detection of target proteins when gel bands were cut at correct molecular weight, but their mono-specificity should be further investigated since KRT7 came below contaminations. For a potential use of orbitrap MS as an antibody validation test, it is required to use clean facility, to preincubate dynabeads with antibody, to cut the gel at expected molecular weight for target protein and finally to filter out common contaminants in MS analysis to facilitate target protein detection.

Key words: Antibody Validation, Immunoprecipitation, Magnetic Beads, Orbitrap Mass Spectrometry

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1. Introduction
Protein expression profiling from human samples is a valuable asset for assessment of individual health status. In the post-sequencing era, one of the most challenging topics for scientists is to unravel proteomic content of human body, as a primary step to more precise protein expression profiling of individual cells. The whole Human genome sequencing project resulted in 20,364 genes, and this large number of genes can be translated into at least ~ 25000 protein products (Flicek et al., 2011). In reality, a much higher number of proteins is expected due to RNA splicing, post translational modification, protein degradation and last but not least the actual genetic variety among individuals ((Uhlén et al.a, 2005), (Uhlen et al.b, 2005)). Human Protein Atlas (HPA) is a comprehensive protein database in which a substantial number of human proteins have been mapped in normal tissues, cancer tissues and different human cell lines (Pontén et al., 2009). This project was first initiated by successfully mapping all protein products of the human chromosome 21 genes in 2003 (Agaton et al., 2003). HPA project has been extended gradually through these years to an inclusive database of nearly all known human proteins' expression patterns, subcellular localization and transcript expression in human tissues and cell lines ((Berglund et al., 2008), (Pontén et al., 2009)).

To map target proteins, the practical strategy is to generate polyclonal affinity-purified antibody against a very unique fragment of 100 to 150 amino acids long of target antigen. These fragments are called protein epitope signature tags (PrESTs), which are templates for synthetic peptides used for immunization in rabbits ((Uhlén et al.a, 2005), (Uhlen et al.b, 2005)).

1.1. Antibody Validation in HPA
Antibodies have to be validated through several steps to be able to use them for proteomic studies, and these methods are listed as following:

1.1.1. Western blot
Each antibody is used for Western blot against whole protein extract in two human cell lines RT-4, and U-251 MG, human plasma, liver and tonsil tissues. Usually a single band of correct molecular weight

is considered as the best result but multiple bands detection can be due to unknown splice variants or different post translational modifications rather than cross-reactivity ((Uhlen et al.b, 2005), (www.proteinatlas.org))

1.1.2. Immunohistochemistry

Each antibody is used to stain a tissue micro array (TMA) especially designed with duplicates or triplicates samples of 46 normal and 20 cancer human tissues and 66 normal human cell lines and later counter stained by hematoxylin-eosin to visualize cellular compartments. Staining pattern is compared to available literature to validate immunohistochemistry ((Kononen et al., 1998), (www.proteinatlas.org)).

1.1.3. Protein Array

384 different PrESTs are spotted on a glass slide divided into 14 subarrays enabling simultaneous analysis of 14 different antibodies. Primary antibody and fluorescently labeled antibody are added and signal intensity for each spot is measured as an indication of antibody mono-specificity ((Nilsson et al., 2005), (www.proteinatlas.org)).

1.1.4. Immunofluorescence

Each antibody has been tested for immunofluorescent staining of eleven types of human cells and localizing target protein within the cell. To facilitate protein visualization in cell, its nucleus, microtubules and endoplasmic reticulum are stained by different fluorophores than of target protein. Subcellular position of target protein is compared to available literature and eventually antibody is validated ((Barbe et al., 2007), (www.proteinatlas.org u.d.)).These validation methods resulted in dismissal of a substantial number of generated antibodies due to failure in validation tests and the rest of them are scored as highly to lowly reliable antibodies (Uhlén et al.a, 2005). Since HPA plays an important role in research and diagnostics further validation of antibodies by siRNA technique or Mass Spectrometry can be of great advantage in particular for novel proteins with limited literature about them or proteins with many paralogues or orthologues which are quite tricky for antibody development.

1.2. Mass Spectrometry

Mass spectrometry receives increasing popularity in proteomics as method of choice and it has been welcomed by researchers in this area for its incredible sensitivity for identification, characterization or quantitation of proteins in sophisticated biological samples. Many different mass spectrometry instruments have been launched and one of the latest versions is Orbitrap Mass Analyzer in which ions derived from the sample orbit by an electrostatic force around a central electrode while centrifugal force of another electrode (encapsulating the central electrode) influences them. Ions' oscillations frequencies are measured by a detector and m/z ratio of ions can be read with high accuracy. Method of choice for peptide identification is usually bottom-up procedure in which purified sample is digested in order to obtain smaller peptides, Orbitrap instrument is able to detect peptide of 1ppm concentration accurately. In the Figure 1, Orbitrap main parts have been pictured very simply. In principle, Orbitrap Mass Analyzer consists of two electrodes, one electrode looks like a barrel ensheathing a cylinder shape inner electrode. The outer electrode is split into two halves by an insulating layer of ceramic and provides an empty space for injected ions to orbit around the inner electrode, and by help of Fourier transform for each ion's frequency of oscillation, its respective mass to charge ratio can be measured (Scigelova et al., 2006).

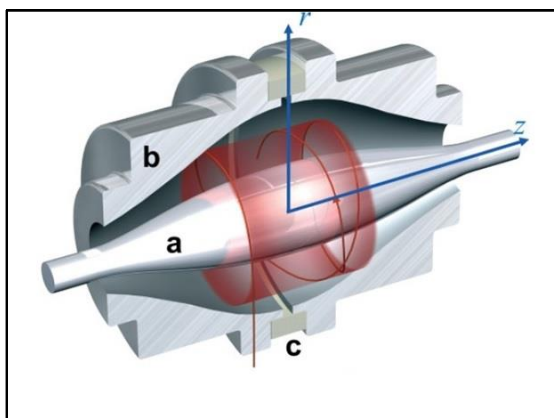

Figure 1:Schematic picture of orbitrap MS. Three main parts are shown; (a) inner electrode, (b) outer electrode , and (c) isulator layer of ceramic dividing outer electrode into two halves , injected ions move in speiral patterns around inner electrode ((Scigelova et al., 2006) with official permission from Thermofisher company)

1.3. Biomarkers to test Mass Spectrometry

To test the Orbitrap Mass Spectrometry method several proteins were chosen. They were selected from HPA database based on their relevantly good expression in corresponding cell lines (based on RNA concentration). These proteins can be categorized into two groups, one group containing safe markers with assured high expression in one specific cell line including ATP5B, DDX1, KRT7 and IL18. The other group consists of pancreatic proteins with apparently high and specific expression in the same cell line as safe marker; this group of markers includes GPR44, StradB, TMEM100 and SerpinB10. A brief description for each marker presented below.

1.3.1. ATP5B

This marker attributes to subunit β from Adenosine triphosphate synthase (ATP synthase), a house keeping gene and the main intracellular energy producer located in mitochondria. ATP synthase is composed of two major entities; $F_{\circ}$ and F_1. $F_{\circ}$ location is inside the inner membrane of mitochondria and consists of several subunits and β subunit is a major subunit for $F_{\circ}$ entity characterized as a 56.6 kD peptide ((Jonckheere et al., 2012) (www.ensembl.org)).

1.3.2. DDX1

Dead (Asp, Glu, Ala, Asp) box helicase1 is a member of RNA helicase family acting by unwinding RNA-RNA or RNA-DNA helices (Kitajima et al., 1994). As a RNA helicase, it has much to do with virus replication within cells, there are a several reports showing its role in increasing cells susceptibility to different viruses like HIV or JCV (Sunden et al., 2007). It has been reported that in retinoblastoma and neuroblastoma, cells have very high expression of it and it has been proposed as a potential prognostic marker for early stages of primary breast cancer , it has been characterized as~ 82 kD protein ((George et al., 1997), (Germain et al., 2011)).

1.3.3. KRT7

Keratin 7 has been classified as a simple type II Keratin filament expressed quite extensively in different types of epithelial cells from simple one layered cells to more stratified epithelial cell e.g. in stomach. It has been characterized as a 55 kD protein with high similarity

to Keratin 8 ((Smith et al., 2002), (Porter et al., 2003)). Its function is still quite unknown but it appears to be quite replaceable according to knockout mouse models although its absence might slightly affect urothelium of bladder (Sandilands et al., 2013).

1.3.4. IL18

Interleukin 18, a 22 kD protein classified as a member of IL-1 super-family, is a cytokine with prominent role in regulation of both innate and acquired immune responses. Its expression is reported in a wide range of type of cells but its seems that its mRNA needs an especial machinery which could be found in immune system cells like B cells, T cells and Kupffer cells ((Biet et al., 2000), (Matsui et al., 2003)). Several important functions have been reported for it like maturation of NK and T cell or cytokine production (Biet et al., 2000).

1.3.5. GPR44

G protein-coupled 44 was discovered quite recently. It is a 43 kD surface marker expressed particularly on Th2cells in human but can be found on other immune cell types e.g. eosinophils. Studies have shown that it has a prominent role in activation and chemotaxis of Th2cells in humans and its abundance increases in response to allergens ((Vinall et al., 2007), (Pettipher, 2008)).

1.3.6. StradB

STE20-related kinase adaptor beta was first discovered in a genomic region highly associated to Amyotrophic lateral sclerosis (ALS) (Hadano et al., 2001). This 47 kD kinase seems to be an isoform of more common pseudokinase and can act as an activator for AMP-activated protein kinase (AMPK) ((Boudeau et al., 2003), (Hawley et al., 2003)). It has been shown that STRADB10 presence has protective effect for cells against apoptosis by interacting with an inhibitor of apoptosis called XIAP (Sanna et al., 2002).

1.3.7. TMEM100

Transmembrane100 protein has been recently discovered and this 14 kD protein is believed to be crucial for normal embryonic vascular development (Somekawa et al., 2012). Knockout mice embryos of TMEM100 die indicating its imperative role for healthy early stage

development. Embryos show deficiencies in arte-rial endothelium and vascular system morphogenesis (Somekawa et al., 2012). Study of its expression profile in human and mouse show its mRNA presence in only enteric neurons of gastrointestinal tract and also in mouse neuronal cell bodies and fibers in the brain and basal ganglia root (Eisenman et al., 2013).

1.3.8. SerpinB10

Serine protease inhibitor B10 or Bomapin belongs to the SerpinB10 superfamily, a large family of protease inhibitors found in nearly all organisms and its main function is to inhibit thrombin and trypsin within cells (Law et al., 2006). There are 35 Serpin genes in human genome and all of them have at least one structure in common, a mobile reactive center loop (RCL) supported by a β sheet structure. Bomapin (a 45kD protein) has quite restricted expression in human body and can be found in bone marrow, and in leukoctes of patients with myeloid leukemia (Przygodzka et al., 2010).

1.4. Aims of Study

In this study the primary goal was to optimize Orbitrap Mass Spectrometry potential to become part of routine antibody validation procedure in Human Protein Atlas. Immunoprecipitation was chosen as method of choice for protein purification for Mass Spectrometry run. The second aim was to check efficiency of detection for some pancreatic markers expression in specific cell lines.

2. Methods and Materials

2.1. Cell culture

Four different human cell lines were used. Their maintenance conditions and harvesting method are described below.

2.1.1. HACAT

HACAT cells (derived from human keratinocytes) were grown in sterile Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% Heat inactivated(h.i.) Fetal Bovine Serum (FBS), 1% Penicillin and 2mM L-Glutamine at 37℃ and 5% CO_2.

Cell medium was changed every 2- 3 days and, cells were split 1:7 every three days.

2.1.2. RH-30

RH-30 cells (derived from human rhabdomyosin sarcoma) were grown in sterile RPMI-1640 medium supplemented with 10% (h.i.) FBS, 1% Penicillin, and 2mM L-Glutamine at 37ºC and 5% CO_2. Cell culture medium was changed every two days and cells were split 1:6 every three days.

2.1.3. BEWO

BEWO cells (derived from human placenta, choriocarcinoma) were grown in sterile Nutrient Mixture F-12 Ham medium (Sigma) supplemented with 10% (h.i.) FBS, 1% Penicillin, 2mM L-Glutamine and 1.5 g/L $NaHCO_3$ at 37ºC and 5% CO_2. Cell culture medium was changed every other day and cells were split 1:2 every 3-4 days.

2.1.4. SIHA

SIHA cells (derived from cervix carcinoma, squamous cells) were grown in Minimum Essential Medium (Sigma) supplemented with 10% (h.i.) FBS, 1% Penicillin, 2mM L-Glutamine, 1 mM Sodium Pyruvate, 0.1 mM non-essential amino acids (Life technologies), and 1.5 g/L $NaHCO_3$ at 37ºC and 5% CO_2. Cell culture medium was changed every other day and cells were split 1:6 every three days.

2.2. Cell split and harvesting

To split each plates, cell culture medium was aspirated and cells were washed with 5 ml of sterile phosphate buffer (PBS). After PBS removal, 3 ml of Trypsin- EDTA was added and the flask was incubated at 37º C for 5 minutes. Trypsin- EDTA was aspirated; this step was repeated again for HACAT and SIHA cells. 4 ml of fresh cell culture medium was added to detached cells and the cells were transferred to new plates and fresh cell culture medium was added up to 10 ml. Cells were harvested when they were 70 % confluent by washing them in sterile PBS, addition of 3 ml of PBS and scraping the flask by a plastic cell scraper, this step was repeated again. Cells were centrifuged at 1500 x g for 10 minutes. Supernatant was removed and the cell pellet was kept in a -70º C freezer for future use.

2.3. Safe Marker selection

All candidate markers were selected for detection in specific cell line based on strong RNA expression evidence in the corresponding cell line, supportive immunohistochemistry and Western blot data in Human Protein Atlas (http://www.proteinatlas.org/).

2.4. Protein isolation by RIPA buffer

Cell culture medium was aspirated and cells were washed by PBS, after PBS removal fresh PBS was added to harvest the cells. Cells were mechanically harvested by a plastic cell scraper and cell suspension was centrifuged at 1500 x g for 10 min, PBS was aspirated and cells were frozen for protein lysate preparation. RIPA buffer was prepared by addition of 1ml of RIPA (Sigma, R0278), 10 µl of Protease Inhibitor Cocktail and 1.5 µl of Benzonase (CalBioCHEM) were added to a 2ml Eppendorf tube. One cell pellet (containing ~ 10- 15 x 10^6 cells) was thawed on ice and based on pellet size 200 to 400 µl of RIPA buffer was added to it. The cell suspension was pipetted up and down several times, transferred to an Eppendorf tube and put on a shaking table for at least 20 minutes. The tube was centrifuged at 20 x 10^3 g for 20 minutes. Supernatant was transferred to a new Eppendorf tube.

2.5. SDS-PAGE

Protein lysates prepared by RIPA were measured by Qubit 2,0 fluorometer (Invitrogen, life technologies) and based on the lysate concentration an appropriate amount of lysates was used for sample preparation to have 20 µg of protein lysate per well. Lysate was mixed with 87% glycerol (to the final concentration of 20% of glycerol) and 4 µl of 5x Red. Samples were heated on a heating block at 95 ºC for 5 minutes and subsequently cooled down by leaving it on room temperature for few minutes. Samples were loaded on an 18-well comb 10-20 % Criterion™ SDS-PAGE gradient gel (Biorad) with running buffer of Tris /Glycine/ SDS (Biorad). For each set of samples at least one well was loaded with 5 µl of Page Ruler Marker, and 20 µl of prepared sample was loaded in the well. Gel tank (Biorad, Mini Trans Blot Electrophoresis Transfer Cell) was put in a 4 °C cold room and it was run at 130 V for 90 minutes.

2.6. Western blot

After gel run, gel was released from its frame and was equilibrated in Transfer buffer (Tris /Glycine (Biorad)) for 15 minutes. All the Fiber pads, paper pads and the cassette for assembly were equilibrated in transfer buffer as well. Proteins were transferred to the membrane (Biorad Mini Trans Blot Electrophoresis Transfer Cell) at 75 V for 45 minutes. Membrane was blocked with 5% skim milk in Tris based saline with 0.5% Tween 20 (0.5% TBST) on a rock ´n´ roll shaker for 1 hour, then block buffer was removed and 2-3 ml of diluted antibody was added to the membrane and the mixture was incubated for 1 hour on the rock ´n´ roll shaker. Membrane was washed with 0.05% TBST four times for 5 minutes and incubated with 5 ml of HRP-conjugated secondary antibody on the rock ´n´ roll shaker for 1 hour. Membrane was washed with 0.05% TBST four times for 5 minutes and incubated in the development solution (Super Signal West Dura-kit) for 5 minutes and possible bands were detected by Biorad molecular imager Chemidoc XRS device and Quantity One software. To check protein concentration of selected markers in cell lines each marker concentration was checked in all four cell lines lysates, the list of used antibodies is shown in Table 1.

2.7. Immunoprecipitation

1-2 µg of antibody was added to each protein lysate prepared by RIPA buffer and incubated on a rotating table in the cold room for 3-4 hours. Lysate- antibody mix was added to 50 µl of Dynabeads protein A coupled (Invitrogen), incubated on rotating table in the cold room for 1 hour, and washed as instructed in the Dynabeads protocol (Cat. no.100.06D, Invitrogen). Samples were collected from beads by eluting in 20 µl of elution buffer and stored at -70ºC freezer before running in comassie gel.

List of antibodies used for each proteins are shown in Table 1.

Table 1: list of antibodies for safe markers and pancreatic proteins

Safe Marker	Antibody	Pancreatic Protein	Antibody
ATP5B	HPA001520	**GPR44**	HPA014259

DDX1	HPA034502	StradB	HPA 026549
KRT7	HPA007272	**TMEM100**	Origine-2D3 (mouse-monoclonal)
IL18	Epitomics – EP523Y (rabbit-monoclonal)	**SerpinB10**	HPA015480

*All HPA antibodies are from rabbit.

2.8. Coomassie blue staining

Purified samples (20 µl) were prepared for SDS-PAGE as mentioned above and gel was run at 130 V for 90 minutes. Gel was released from the frame, washed with 100 ml of miliQ water 3 times for 5 minutes. 20 ml of Simply Blue Safe Stain was added to the gel and put on the gently shaking table in room temperature for 1 hour. Gel was washed with water four times for 1 hour (Figure 3). Some bands were cut from the gel and stored in the fridge.

2.9. Mass Spectrometry sample preparation

Gel bands were cut into smaller pieces, incubated with 150 µl of 25 mM of NH_4HCO_3 for 5 minutes. After removal of supernatant the gel pieces were incubated with 150 µl of Acetonitrile (ACN) for 5 minutes. Destaining step was repeated twice. Gel pieces were dried in SpeedVac® for 10-15 minutes. Dried gel was covered with 10 mM dithiothreitol (DTT), incubated at 50° C for 1 hour, and supernatant was discarded. 55mM Iodoacetamide was added to gel pieces to cover them and they were incubated in darkness for 1 hour. Then the gel pieces were washed with 150 µl of 25 mM of NH_4HCO_3 for 5 minutes, supernatant removed and gel bands were incubated with 150 µl of Acetonitrile (ACN) for 5 minutes. Gel pieces were dried in SpeedVac® for 10-15 minutes. Proteins were partially digested by a solution of trypsin and Ammonium Hydrogencarbonate (12 ng/µl of trypsin in 25 mM of NH_4HCO_3) and incubated at room temperature

for 1 hour. 25 mM of NH_4HCO_3 was added to gel bands to cover them and the gel pieces were incubated at 37ºC overnight. Supernatants were collected the day after, to gel bands a solution of 60% ACN and 5 % formic acid was added. The mixture was incubated for 5 minutes and supernatant was pooled with previous fractions. Samples were dried in SpeedVac®. The rest of preparation for running samples in MS was done by MS facility personnel.

3. Results

All markers were tested by Western blot in the four cell lines to check their expression level. Ideally, highest expression should be in the cell line that marker was going to be purified later with only single band of correct size. In figure 2 KRT7 Westren blot image is shown as an example.

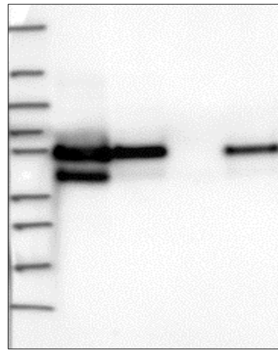

Figure 2: Western blot image of KRT7 with HPA007272 antibody. On the left side of the image molecular marker is shown (250, 130, 100, 70, 55, 35, 25, 15, 10 kD), KRT7 in SIHA, BEWO, RH-30 and HACAT cells (left to right) was tested and it showed two bands for SIHA , one strong band for BEWO, no band in RH-30 and one band for HACAT

Purified samples were stained by Coomassie blue and several bands were cut. Due to technical limitation seven samples belonging to 5 markers out of 8 were selected to be analyzed in MS run. For some of the markers more than one band were present in the gel which were cut and sent for Mass Spectrometry analysis. In Figure 3 a Coomassie gel image is shown.

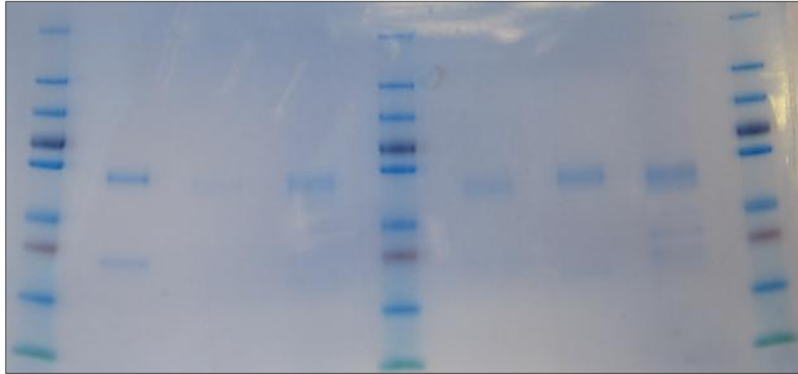

Figure 3: Coomassie gel image of purified lysates after Immunoprecipitation. Three lanes of marker were loaded (first, last and the fifth lanes (250, 130, 100, 70, 55, 35, 25, 15, 10 kD)). After staining, spots with high concentration of proteins turned blue. Mass Spectrometry samples were cut based on their bands position in Western blot.

Names and positions of analysed bands are summerized in Table 2 below. Gel bands for markers were cut according to Western blot result.

Table 2: List of selected gel bands for MS analysis. Selected gel bands for MS run are listed below, their marker type shown in first column, approximate size of each band is shown in second column and results of MS run are shown in last column. For markers more than one band was present in gel and all bands were cut and they are shown in table with a number after marker's name.

Sample	Molecular weight of marker	Approximate size of gel band (kD)	Mass Spectrometry result
ATP5B	56	50-55	ATP5B
GPR44	43.3	50-55	ND
KRT7(1)	55	~47-50	KRT7
KRT7(2)	55	~50-53	KRT7
KRT7(3)	55	≤250	ND
STRADB10	47	≤250	ND
TMEM	~14.4	45	ND

Based on the MS results three of the samples were sucessfully detected, bands cut for ATP5B and KRT7 were correctly detected as their corresponding proteins, yet one heavy band from KRT7 located around 250 kD was not detected as KRT7. This heavy band was present in other marker's lanes and it was cut for StradB10 as well. The heavy bands were detected as Myosin by MS instead of their corresponding marker. It should be mentioned for first Kerain7 band located around 47 kD the first few hits in MS run were common contaminants such as actins, tubulins and Keratin8 while KRT7 scored lower than contaminations.
GPR44 and TMEM bands were cut according to Western blot results and observation of a faint band yet common contaminents were detected by MS.

4. Discussion

In this study, we aimed to test Orbitrap Mass spectrometry as a potential antibody validation method in Human Protein Atlas. Four proteins were selected as safe markers based on their high expression in a specific cell line and robust Western blot results according to HPA, these markers are positive controls and should be detected in a Mass Spectrometry run. Four pancreatic markers with no certain information on their antibodies' specificity were selected to be checked. All samples were tested by Western blot in all cell lines to check their expression and purified by Immunoprecipitation before running in orbitrap Mass Spectrometry.
One sample from ATP5B and two from KRT 7 were detected in Mass Spectrometry and for both safe markers HPA antibodies were used for pull-down. HPA001520 (ATP5B antibody) was able to pull it down with accuracy and ATP5B came as the first detected protein in Mass Spectrometry results. This can prove that HPA001520 antibody as a mono-specific and reliable antibody which can pull down its target in complex biological combination (whole SIHA cells lysate) with great accuracy.
KRT7 samples contained impurities that made its identification results less solid than ATP5B but it should be noted that Keratins are quite difficult proteins to be pulled down due to their unique characteristics; this family is one of the most common proteins in human

samples (Hannan et al., 2009). Moreover, their presence as contamination in purified samples is a recurrent problem in proteomic labs ((Hodge et al., 2013) (Mellacheruvu et al., 2013)). Keratins have various types, yet they show amazing similarity in structure (Porter et al., 2003) which complicates antibody development for this protein family. Not surprisingly, there is evidence that keratin 18 antibody (clone CY-90 Sigma Aldrich) shows strong affinity for Keratin 13 as well (Fulzele et al., 2012). In this study, KRT7 was chosen for two reasons, first, to check Fulzele's study results which are critical for antibody development and validation, for Keratins or similar proteins and to test HPA antibody reliability. Second, Keratins are biomarkers of epithelial cells and KRT7 is a unique biomarker for BEWO cell line (Hannan et al., 2009) which makes it an interesting protein to study. HPA007272 antibody was successful in pulling down KRT7 in BEWO cell lysate for both gel bands yet KRT7 was not the top protein in the Mass Spectrometry results. Actin and other types of Keratins (like KRT8) came before KRT7 indicating that there might be unspecific binding of these proteins. Since KRT7 should be present in BEWO cells in high concentration ((Hannan et al., 2009), (www.proteinatlas.org)), its low abundance should not be the problem for detection.

However, considering the persistent presence of different Keratins in most biological samples as contamination and the fact that Keratins are amazingly similar in structure, HPA007272 antibody is quite successful in detection of KRT7 in BEWO cell lysate which is quite sophisticated sample with high concentration of different keratins in it (www.proteinatlas.org). It is worth mentioning that the first KRT7 band (located at~47 kD) was not exactly at the right molecular weight and this could contribute to detection of lower concentration of KRT7 and identification of contaminants instead of target protein. But extra band presence after Coomassie staining, and results of Western blot in SIHA protein lysate made the other band an interesting sample to study.

The other analyzed bands belonged to GPR44 (Mw 43.3) and TMEM100 (Mw 14.4) (www.ensembl.org). Location of their gel bands and real molecular weight were not in line with each other and consequently none of them were detected in Mass Spectrometry run.

The reason for cutting bands in position other than their real molecular weight was the results of Western blot. Western blot is one of the validation methods in HPA and these two proteins showed bands in positions totally different than expected molecular weight. Bands were cut according to Western blot leading to detection of common contaminants in low concentrations in Mass Spectrometry. The two remaining samples were heavy molecular weight bands which were showing up in almost all lanes and after Mass Spectrometry analysis; they were detected as myosin contamination. Myosin contamination similar to Keratins, is reported as one of the most recurrent and common contaminants in different proteomics labs (Mellacheruvu et al., 2013).

Immunoprecipitation by magnetic beads was selected as protein purification method and magnetic beads have showed superiority to conventional sepharose beads by taking up less non-specific nuclear proteins, however they are not perfectly specific for antibody-protein complex and can take up proteins associated with cell structure or motility such as Actin, Myosin and Tubulin ((Trinkle-Mulcahy et al., 2008) (Mellacheruvu et al., 2013)).

Human protein Atlas (HPA) has extremely important responsibility of authentic information provision for scientists, researchers and clinicians and to accomplish this role, it has to set up a thorough antibody validation process to ensure accuracy of the data. In this study, we tried to address this need by means of orbitrap Mass Spectrometry and checking HPA antibodies efficiency. HPA have been mostly relied on immunoassays like Immunohistochemistry or Western blot for antibody validation which without any doubts have certain merits ((Matos et al., 2010), (Bordeaux et al., 2010)), yet they have their own flaws and one important one is low sensitivity for distinguishing between markers with similar molecular weight or staining pattern ((Hoofnagle et al., 2009) (Becker et al., 2012)). Mass Spectrometry receives more attention as method of choice in high-throughput proteomic labs due to its sensitivity and high capacity for simultaneous detection of several targets in complex samples. Orbitrap Mass Spectrometry can be a useful asset in any lab with its incredible sensitivity (capable of protein detection in ppm concentration). In this study, Orbitrap MS was able to identify right targets when gel bands were

cut at correct molecular weight and revealed samples ‘contaminants with incredible precision. However, it cannot be denied that its application in HPA lab needs more trials to prepare an efficient protocol for it. To improve future Immunoprecipitation and Orbitrap Mass Spectrometry some suggestion could be applied:

It is necessary to use laminar flow hood to reduce risk of entering contaminations from lab environment. Lab technician responsible for Immunoprecipitation should use long sleeved clean gloves.

The gel should always be cut where the expected protein should be present regardless of Western blot results or Coomassie blue staining results.

To avoid unspecific binding of proteins to dynabeads, incubate antibody with magnetic beads to cover beads surface with antibody prior to addition of protein lysate, to hide all potential interaction sites for non-target proteins on the beads.

To skip detection of contaminants in Mass Spectrometry common contaminants can be filtered out ((Trinkle-Mulcahy et al., 2008), (Mellacheruvu et al., 2013))

In conclusion, the total success rate of orbitrap Mass Spectrometry run was about 43% and three samples out seven were successfully detected. HPA antibodies were successful in detection of correctly cut bands of safe markers yet their mono-specificity and affinity for just one target protein could be further tested with Mass Spectrometry since it clearly detect co-purified proteins and contaminations. Pancreatic markers failed in Mass Spectrometry due to incorrect gel cutting and consequently no data is available on their antibody’s efficiency yet modification of Immunoprecipitation protocol and Mass Spectrometry sample analysis could be of great help.

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