World Scientific Research Journal (WSRJ) ISSN: 2472-3703
www.wsr-j.org

# Method Exploration for Assessing the Specificity of Antibody by Immuno-Mass Spectrometry

Wenyan Cai

Fudan University, Shanghai 200438, China

*Abstract: Objective: Using the Immuno-MS (Immuno-Mass Spectrometry) to establish a method for evaluating the specificity of antibody. Method:The target antibody coupled with protein G or protein A(GDPDH, Glyceraldehyde-3-phosphate dehydrogenase; VDR, Vitamin D receptor)is being used for enriching the antigen of the protein lysate of positive control cell, then the enriched antigen be treated for Immuno-MS analysis. The final data be analyzed with standard database for ensuring if the specific antigen is exist. Result:This method can assess the specificity of target antibody fully which make up for deficiencies of ELISA and Western Blot.*

*Keywords: Antibody specificity, Immuno-Mass Spectrometry, Western Blot*

## 1. INTRODUCTION

Post-genomic era, the function of protein research has become an important part of life science research, antibody as an important tool for protein research, its importance is more and more prominent; a good antibody is not only an important tool for basic research, but also clinical diagnosis and antibody Drug development of the core protagonist.

The parameters that determine the importance of antibody quality are antibody specificity and antibody titers, where the specificity is the first indicator of the antibody to be met.

Antibody preparation generally used as a polypeptide or whole protein as an immune antigen to immunize animals, and synthetic peptides as immune antigens, with convenient and efficient, easy to prepare the characteristics of many antibody preparation companies used; but this method will be due to antigen table Inappropriate selection of non-specific antibodies that produce cross-reactions with other antigens.

Our general antibody-specific assessment method uses ELISA [1] or Western Blot [2] method. The ELISA method will be used in the immunization process to assess the binding properties of immune sera and antigens, but the non-specific cross-reactivity cannot be assessed. WB method can do a systematic analysis of the specificity of the antibody, But only according to the size of the strip to make judgments, and the size of the band is a very rough basis, under normal circumstances, we often find the target protein and the expected size is

inconsistent, such as protein glycosylation, phosphorylation, etc. will have a certain impact on the size of the protein [3-4]; and WB man-made operation links, long cycle, more likely to produce errors. Therefore, these two assessment methods have some limitations, the objective need for more perfect way to replace.

Protein spectrum technology, the protein analysis technology into high specificity, high sensitivity era [5-8], can be a stable identification and differentiation of glycosylation and phosphorylation and other protein modification.

Immuno-mass spectrometry (Immuno mass spectrometry, Immuno-MS) [9-13] combines antigen-antibody immunoassay with bio-mass spectrometry to give full play to the high sensitivity of mass spectrometry techniques, coupled with the gradual refinement of the mass spectrometry database, so that this method can make accurate qualitative and quantitative analysis of the target protein. Immuno-MS is used for accurate quantitative analysis of antibody-specific targets for quantitative analysis of tumor markers, disease-specific target proteins [14-16], and has not been reported for antibody specificity. We used this method directly for antigen-specific analysis and found that it can make up for the lack of Western Blot and ELISA detection and evaluation methods, qualitative analysis of the target antigen, if there is no target antigen in the mass spectrometry list, then the antibody The specificity is questionable.

## 2. MATERIALS AND METHODS

### 2.1 Instruments and reagents

Table 1. Instrument Information for this Project

| Equipment name | Supplier |
|---|---|
| 4 degrees frozen centrifuge | Hunan Xiang Yi |
| Vertical electrophoresis tank | Shanghai Peiqing Technology Co., Ltd |
| Pure water meter | Millipore |
| Constant temperature mixing instrument | ABSON |
| Frozen Centrifugal Concentration Dryer | Taicang City Huamei Biochemical Instrument Factory |
| Desktop Fast Centrifugal Concentration Dryer | Taicang City Huamei Biochemical Instrument Factory |
| Automatic digital gel image analysis system | Shanghai Peiqing Technology Co., Ltd |
| Digital steady - state steady current electrophoresis | Shanghai Peiqing Technology Co., Ltd |
| Tandem mass spectrometer Thermo Scientific Q-Exactive | Thermofishier Scientific |

Table 2 Reagent information for this project

| Reagent name | Supplier |
|---|---|
| IAM iodoacetamide | Sigma |
| modified Trypsin protease, mass spectrometry | Promega |
| SDS-PAGE gel preparation kit | Beyotime |
| Coomassie brilliant blue dye solution (conventional method) | Beyotime |
| Protein molecular weight standard | ThermoFisher Scientific |
| Protein Ladder | ThermoFisher Scientific |
| Protease Inhibitor Cocktail | ThermoFisher Scientific |
| TECP | ThermoFisher Scientific |
| Acetonitrile LC/MS | ThermoFisher Scientific |
| Water-LC/MS | ThermoFisher Scientific |
| Methanol LC/MS | ThermoFisher Scientific |
| Formic acid LC/MS | ThermoFisher Scientific |
| Triethylammonium bicarbonate buffer | Sigma |

## 2.2 Methods

### 2.2.1 Antibody and antigen preparation

Antibody was purchased from well-known brand antibody, we selected GAPDH and VDR target antibody; sample selection of cultured human cervical cancer cells Hela (manufacturers recommended positive control cells), with protein lysate (RIPA, Pik Wan days) treatment of cells, protein G / A Conjugated antibody to antigen. The process is as follows:

1) Wash the cells in the 10 cm Petri dish twice with pre-cooled PBS (about 107 cells).

2) Add pre-cooled IP cell lysate (P0013) and protease inhibitor (107 cells by adding 1 ml).

3) Scrape the cells from the culture medium with pre-cooled cells and transfer them to a clean 1.5 ml EP tube. And placed in low-speed shaker, 4°C slow shaking 15min.

4) 4°C, 12000rpm centrifugation 15min, transfer the supernatant to the new pre-cooled tube.

5) Use the BCA method or other methods to determine the total protein concentration.

6) Reduce the total protein to 1 μg/μl with PBS to reduce the concentration of detergent in the lysate.

7) Add a certain volume of the primary antibody (VDR concentration 0.2mg / ml, take 10ul) to the fourth step with the supernatant tube.

8) Slowly shake the antigen-antibody mixture with a shaker and overnight at 4°C.

9) Add 40 μl of fully resuspended protein A/G-agraose at 4°C for 3 hours.

10) 2500 rpm for 5 min, the precipitate was collected and washed three times with pre-cooled PBS (800 [mu] l each time).

11) Elution: add 40μL of low pH glycine buffer (100mM glycine/HCl, pH 2.5-3.0), mix thoroughly, centrifuge at 2500 rpm for 5 min, and add 4 μl of 1 MTris/HCl pH 8.5 to neutralize the eluent, an antigen sample for mass spectrometry analysis was obtained.

### 2.2.2 Antigen sample digestion

1) Take 30μl of the purified antigen sample and add the volume to 50μl with the lysate;
2) The final concentration of 10mM TCEP was added and reacted at 37°C for 60min.
3) The final concentration of 40mM iodoacetamide (Iodoacetamide) at room temperature under the light reaction for 40min;
4) Each tube was added with pre-cooled acetone (acetone: sample volume ratio = 6: 1), -20°C for 4 hours;
5) 10000g centrifugation for 20 minutes, take precipitation;
6) 90% acetone cleaning twice, 4°C, 10000g centrifugation 10min, takes precipitation;
7) Completely dissolve the sample with 100μl of 100 mM TEAB;
8) Add Trypsin at 37°C overnight at a mass ratio of 1:40 (enzyme: protein).

### 2.2.3 Desalination

1) After trypsin digestion, the peptide was pumped with a vacuum pump;
2) After digestion of the extracted peptide 2% ACN, 0.1% TFA complex peptides;
3) Activate the column with methanol (sep-pak), loading;
4) Eluted with 40% ACN, 0.1% TFA, collected;
5) Vacuum concentrator dry.

### 2.2.4 LC-MS / MS analysis

Data Acquisition Software: Thermo Xcalibur 4.0 (Thermo, USA)
Reverse column information: C18 column (75 μm x 25 cm, Thermo, USA)
Chromatography apparatus: EASY-nLC 1200
Mass Spectrometer: Q-Exactive (Thermo, USA)
Chromatographic separation time: 90 min
A: 2% ACN with 0.1% formic acid
B: 80% ACN with 0.1% formic acid
Flow rate: 300nL / min
Gradient:

Table 3 EASY-nLC liquid phase gradient

| Time (min) | B (%) |
|---|---|
| 0 | 0 |
| 1 | 5 |
| 41 | 23 |
| 51 | 29 |
| 59 | 100 |
| 65 | 100 |
| 90 | Stop |

MS scanning range (m/z) 350-1300, acquisition mode DDA;

Top 20 (select the strongest signal in the parent ion for secondary fragmentation)

First - order mass spectrometry resolution of 70000, fragmentation HCD;

Secondary resolution of 17500, the dynamic exclusion time 18s.

### 2.2.5 Database search parameters

2.2.5.1 Database selection

Currently used to the database can be divided into two categories, one is NCBI to maintain the other one is responsible for the maintenance by the EBI. The database is built with the following methods:

NCBInr full library;

NCBInr classification library, including the whole animal, the whole plant, microbial library, the whole library of bacteria;

SwissProt/UniProt classification library, including animal whole library, plant whole library, microbial library, bacteria library;

NCBInr corresponding species library, including human, wheat, yeast, E. coli;

Other species such as self-test genome, transcriptome database.

In the choice of the database, follow the following principles, if the sequencing of biological, direct selection of the species database, if non-sequencing organisms, then select the sample is most relevant to the large proteome database.

This experiment uses the database link: http: //www.uniprot.org/proteomes/UP000005640

2.2.5.2. Database search

The library version is ProteomeDiscovererTM Software 2.1. When the library is submitted to the ProteomeDiscoverer server, select the database has been established, and then searches the database. The relevant parameters are as follows:

Table 4 ProteomeDiscoverer search parameters

| Item | Value |
|---|---|
| ProteomeDiscverer version: | 2.1 |
| Protein Database | uniprot-proteome_UP000005640-Homo sapiens-20160619-71913s |
| Cys alkylation | Iodoacetamide |
| Dynamic Modification | Oxidation (M), Acetyl (Protein N-Terminus) |
| Static Modification | Carbamidomethyl(C) |
| Enzyme Name | Trypsin (Full) |
| Max. Missed Cleavage Sites | 2 |
| Precursor Mass Tolorance | 10 ppm |
| Fragment Mass Tolorance | 0.05Da |

Note: The result filter parameters for the Peptide FDR≤0.01.
VDR antibody is administered in conjunction with GAPDH antibody

## 3. RESULTS

### 3.1 SDS-PAGE of purified antigen (GAPDH, VDR)

Polyacrylamide gel is a network structure with molecular sieve effect. In SDS-polyacrylamide gel (SDS-PAGE), the electrophoretic mobility of the protein subunit is mainly dependent on the molecular weight of the subunit. Purification of the antigen sample by 1.2.1, take 1/5 samples for PAGE gel electrophoresis, that is, loading 8 μl, electrophoresis patterns as follows:

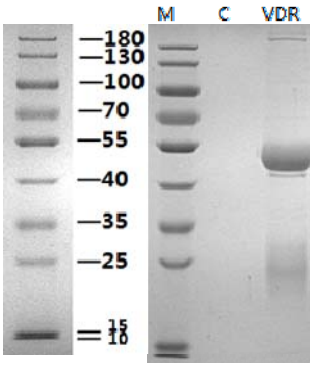


Figure 1 SDS-PAGE Figure

Note: the amount of loading are: 7μl (take the sample used 1/5); loading order from left to right: Marker, C, and VDR.

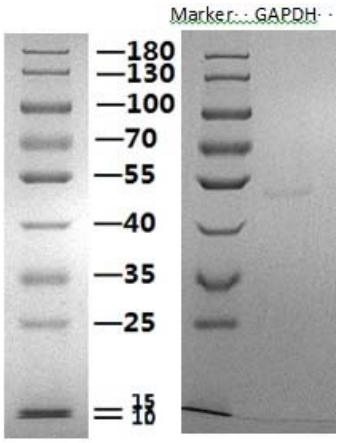


Figure 2 SDS-PAGE Figure

Note: the amount of loading are: 7μl (take the sample used 1/5); loading order from left to right: Marker, GAPDH.

### 3.2 Mass spectrometry database search results

The GAPDH mass spectrometry data search results are as follows:

Table 5 List of search results

| Total Spectrum | Identified Spectrum | Peptide number | Protein number | Protein group number |
|---|---|---|---|---|
| 13459 | 1028 | 525 | 742 | 161 |

VDR mass spectrometry data search results are as follows:

Table 6 List of search results

| Total Spectrum | Identified Spectrum | Peptide number | Protein number | Protein group number |
|---|---|---|---|---|
| 22647 | 1152 | 759 | 772 | 152 |

Search Unirpot database, the following part of the Uniprot search results as an example of the protein information part of the header list, as shown below (see the search list for details).

Table 7 Protein header information table

| Header | Description |
|---|---|
| # Protein Groups | Protein name |
| # AAs | The protein contains the number of amino acids |
| MW [kDa] | Theoretical molecular weight |
| calc. pI | Isoelectric point |
| Score Sequest HT | The scoring of the protein |

Table 8 Peptide groups table information table

| Header | Description |
|---|---|
| Annotated Sequence | Peptide sequence |
| Modifications | Modification information |
| # Protein Groups | The number of protein groups to which the peptide belongs |
| # Proteins | The number of proteins in the protein group |
| # PSMs | The number of spectra matched to the peptide |
| Master Protein Accessions | The name of the major protein to which the peptide belongs |
| # Missed Cleavages | Missing cut site |
| Theo. MH+ [Da] | Theoretical molecular weight |
| XCorrSequest HT | The peptide Xcorr score |
| Percolator q-Value Sequest HT | The peptide q value, the smaller the better |

Table 9 GAPDH protein

| Accession | Description | Sum PEP Score |
|---|---|---|
| P35908 | Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 | 85.10291344 |
| P04264 | Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 | 84.99855271 |
| P35527 | Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 | 65.19265276 |
| P05787 | Keratin, type II cytoskeletal 8 OS=Homo sapiens GN=KRT8 PE=1 SV=7 | 54.56666856 |
| P13647 | Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 | 50.25469508 |
| P13645 | Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 | 49.74532845 |
| P35579 | Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4 | 49.28390969 |

| | | |
|---|---|---|
| P60709 | Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1 | 39.00358016 |
| P08670 | Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4 | 38.46259642 |
| Q86YZ3 | Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 | 29.68429719 |
| Q15149 | Plectin OS=Homo sapiens GN=PLEC PE=1 SV=3 | 28.90949593 |
| P04259 | Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5 | 25.76867512 |
| P04406 | Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3 | 23.47524573 |
| P02533 | Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 | 22.31050817 |

Table 10 VDR protein search library list

| Accession | Description | Score |
|---|---|---|
| P60709 | Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1 - [ACTB_HUMAN] | 50.38 |
| P05787 | Keratin, type II cytoskeletal 8 OS=Homo sapiens GN=KRT8 PE=1 SV=7 - [K2C8_HUMAN] | 46.88 |
| P68032 | Actin, alpha cardiac muscle 1 OS=Homo sapiens GN=ACTC1 PE=1 SV=1 - [ACTC_HUMAN] | 36.28 |
| P04264 | Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN] | 24.56 |
| P13645 | Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN] | 24.26 |
| P05783 | Keratin, type I cytoskeletal 18 OS=Homo sapiens GN=KRT18 PE=1 SV=2 - [K1C18_HUMAN] | 20.22 |
| P35908 | Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN] | 12.64 |
| P35527 | Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN] | 12.25 |
| P08670 | Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4 - [VIME_HUMAN] | 11.10 |
| P62805 | Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2 - [H4_HUMAN] | 10.39 |
| P38646 | Stress-70 protein, mitochondrial OS=Homo sapiens GN=HSPA9 PE=1 SV=2 - [GRP75_HUMAN] | 10.00 |
| P68104-2 | Isoform 2 of Elongation factor 1-alpha 1 OS=Homo sapiens GN=EEF1A1 - [EF1A1_HUMAN] | 8.43 |
| P13647 | Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN] | 7.00 |
| P06899 | Histone H2B type 1-J OS=Homo sapiens GN=HIST1H2BJ PE=1 SV=3 - [H2B1J_HUMAN] | 6.15 |

## 4. DISCUSSION

Mass spectrometry found that some of the protein above some of the cell structure proteins, such as Keratin, Actin, Myosin, Vimentin, Spectrin, indicating that these structural proteins and antibodies have a certain degree of binding, this combination is non-specific. We are in the formal analysis, to remove the interference of these proteins.

In addition, mass spectrometry is a very sensitive technique, although our antigen-purified PAGE gel shows a single band, but in the results of mass spectrometry will still show a lot of protein, these proteins will appear depending on the frequency Score, the target protein will appear in the list of relatively advanced protein list. We found GAPDH in the results of GAPDH, found in the 14th position, but the exclusion of structural protein interference, ranking in the first place. The VDR antigen list, the top 15 are structural proteins, no target antigen, extended to the entire protein list, and no VDR appears, then the antibody specificity of the antigen will be questionable, the result is that we In practice, the positive control of the selection of manufacturers recommended cell lines, indicating that this situation is there, even the well-known manufacturers of antibodies may also produce errors, the antibody is actually against non-target antigen, and the molecular weight close to the size, and Using western blot, we generally tolerate a range of errors in the size of the molecular weight, so it is not complete to use western blot to assess the specificity of the antibody, while the mass spectrometry can give a qualitative analysis of the protein identity. There is a lot of interference number, is indeed a good complement to western blot technology.

With the maturation of mass spectrometry technology and the accumulation of large amounts of data, the database of protein spectrum search is becoming more and more mature, and the judgment of mass spectrometry is more and more accurate. Then we establish this method of antigen-specific analysis. A good qualitative method, can make a qualitative assessment of the specificity of the antibody to make up for the lack of ELISA and western blot methods, that is, antibody binding antigen components, by mass spectrometry must have an identity of the target antigen appears, otherwise The specificity of the antibody is questionable, which may be a necessary condition for antibody-specific assessment.

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