

## LECTURE-26

### Microarrays for Autoantibody Profiling

#### Handout

#### PREAMBLE

Autoimmune diseases and cancers lead to expression of mutated and aberrant proteins, and these proteins act as self-antigens evoking host immune response. These self-antigens are known as autoantigens and the corresponding antibodies to these autoantigens are known as autoantibodies. The presence of autoantibodies have been reported in several diseases such as rheumatoid arthritis, systemic lupus erythematosus, thyroiditis, sclerosis and several cancers; and have the potential to develop as serological diagnostic tool. These autoantibodies may appear in the blood stream before the onset of disease in many cases. So these autoantibodies can be used as biomarkers for many autoimmune diseases. Autoantibodies detection at early stage of cancer may have diagnostic and therapeutic potentials. There are several reasons for the immunogenicity of the self-antigens such as mutations, alternative splicing, deregulation of apoptotic process, etc. Although, several potential biomarkers have been reported from various studies, the sensitivity and specificity of these reported proteins are low. Such issues can be avoided by using a panel of proteins instead of single protein marker. Protein arrays provide a high-throughput platform, which offers rapid and efficient detection of autoantibody.

## **OUTLINE OF LECTURE**

- I. INTRODUCTION
- II. Autoantibodies in diseases
- III. Methods for autoantibody detection
- IV. Applications
- V. Advantages and challenges
- VI. Conclusions

## **I. INTRODUCTION**

The recent development of protein microarrays offers the ideal tool for screening for immune responses to tumor antigens. These arrays have the advantage that hundreds to thousands of different proteins can be printed on a standard microscope slide and only require a few microliters of serum for screening assays. There are two approaches that employ microarrays to detect cancer autoantibodies. The first approach is to spot microarrays with lysates from tumor specimens or cancer cell lines using reverse phase protein blot, and then use serum to determine any reactivity. Even after fractionation, each RPP spot contains multiple protein species, and identity of the detected antigens is established using mass spectrometry. A complementary protein microarray approach is to assemble protein microarrays with known proteins, and screen these target protein microarrays from patient serum. This approach has the advantage that the identity of the antigen at each spot is known in advance, such that no further characterization is needed to identify the detected antigen.

## **II. AUTOANTIBODIES**

Autoantibodies are defined as aberration of an important physiological and protective process representing immunoglobulins reacting against self-molecules. These are generated when immune system is unable to distinguish between 'self' and 'non-self'. Antibodies to tumor antigens have advantages over other serum proteins as potential biomarkers for cancer and autoimmune diseases as they are stable, highly specific, easily purified from serum, and are readily detected with well-validated secondary reagents. The role of antibodies directed at self-antigens, are referred to as autoantibodies.

### **AUTOANTIBODIES IN DISEASES**

The pathophysiology that underlies the development of autoantibodies is poorly understood, but may relate to antigen mutation, over-expression, altered antigen folding and degradation as well as genetic factors. Autoimmune diseases are also characterized by presence of multiple autoantibodies. Examples of different autoimmune disease are rheumatoid arthritis, systemic lupus erythematosus, thyroiditis and scleroids. Autoantibodies are one of the diagnostic tools and an indicator of activity for rheumatic and non-rheumatic autoimmune disorders. Rheumatoid factors are used as biomarkers for Rheumatoid arthritis (Steiner *et al.*, 2002). Autoantibodies may act as an important prognostic tool for patients suffering from diabetes as Islet-cell autoantibodies are strongly responsible for the development of type1 diabetes (Pihoker *et al.* 2005). The detection of autoantibodies is an important method in the diagnosis, monitoring and prognosis of the disease in patients with autoimmune disorders. This

has stimulated search for novel antibodies as diagnostic indicators and hence are also called as predictor of diseases. Proteomic approaches aids in identification and characterization of individual proteins and their potential biomarkers. Various groups around the globe have identified autoantibodies from the serum samples of autoimmune diseases and cancers and some of these studies are summarized in table 1.

**Table 1:** Autoantibodies identified in various autoimmune diseases and cancers

<b>Diseases</b>	<b>Autoantibodies</b>	<b>References</b>
Rheumatoid arthritis	AAb against citruline  Rheumatoid factor (RF) against the Fc portion of IgG and anti-nuclear antibodies (ANAs)	Schellekens GA et al. 1998,  Shmerling RH et al. 1991,  Tan EM et al. 1997
Graves disease	Thyroid stimulating hormone (TSH)	Stassi et al. 2002
Type-1 diabetes	AAb against Glutamic acid decarboxylase (GAD), the protein tyrosine phosphatase-like molecule, IA-2, and insulin	Leslie, R.D.G et al. 1999
Celiac disease	AAb against tissue transglutaminase 2 (TG2)	A Alaedini et al. 2008
Breast, ovarian and prostrate cancers	AAb against aberrant O-glycoforms of extracellular mucin (MUC1)	Wandall et al. 2011
Hashimoto thyroiditis	AAb thyroid peroxidase and thyroglobulin	Dayan CM et al. 1996
Pernicious anemia (PA)	AAb against gastric H <sup>+</sup> /K <sup>+</sup> -ATPase and intrinsic factor	Toh BH et al. 1997
Breast cancer	P53, C-myc, NY-EOS-1, BRCA1, BRCA2, HER2, MUC1	Chapman C et al. 2007
Small-cell lung cancer	P/Q type voltage-gated	Bazhin AV et al. 2001,

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(SCLC)	calcium channel (VGCC), $\alpha$ -Enolase, Recoverin (Antiretinal)	Adamus G et al. 1996, Monstad SE et al. 2004
Breast cancer	ASB-9, SERAC 1 and RELT	Zhong Li et al. 2008
Devic's disease or Neuromyelitis optica (NMO)	NMO-IgG which binds to Aquaporin-4	Lennon VA et al. 2005

## **III. METHODS FOR AUTOANTIBODY DETECTION**

There are several methods, which are employed for the identification of autoantibodies from the serum of the patients. Some of these methods are mentioned below:

1. Indirect Immunofluorescence (IIF)
2. ELISA
3. Western Blotting
4. Serologic expression cloning (SEREX) and Serological Proteomic Analysis (SERPA) Latex Agglutination
5. Protein microarray

Although there are many methods which can be used for autoantibody screening, here, we'll discuss protein microarray approach in detail. Microarrays provide high-throughput identification of autoantibodies in a single experiment. The amount of reagents and the sample volumes required for the assay is very low. Recent advancements in the field of cell-free expression systems have boosted the usage of microarrays in the field of biomarker discovery.

## **AUTOANTIBODY DETECTION PROCEDURE USING PROTEIN MICROARRAYS**

A protein microarray or proteome chip has several thousands of features correspond to thousands of proteins along with positive and negative controls. Serum screening is performed for autoantibody screening and comparison is made between patients and healthy controls using following procedure.

**Blocking:** Blocking is performed by immersing slides in blocking buffer (5% milk in PBST) on rocking shaker at RT for 1 hr.

**Incubation with serum:** 5 ml of serum (diluted 500-fold in 5% milk in PBST) is applied and slides are kept for incubation for 1 hr at RT on rocking shaker.

**Washing:** After incubation with serum, slides are washed three times, each time for 10 min washes with PBST at 40°C with a change of buffer in between at 5 min. Washing can be performed at room temperature as well while washing steps at 40°C may help in background reduction. Third washing is followed by secondary antibody incubation.

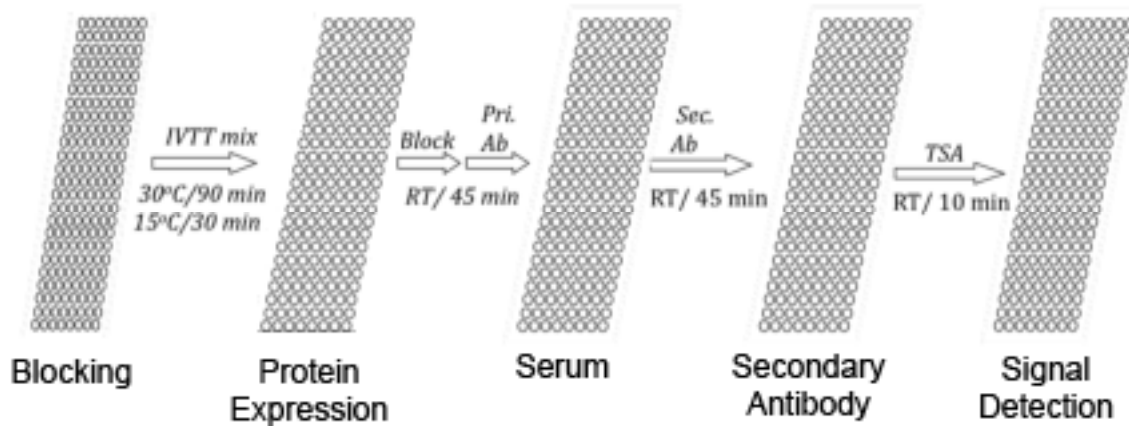
**Incubation with antibody:** Anti-human (if serum screening is performed for human diseases) IgG antibody labeled with Cy-3/Cy5/Alexa flour is diluted in superbloc and added on protein chip and keep for incubation at RT for 1 hr in dark. Detection can also be performed using Tyramide Signal Amplification (TSA) system (Fig 1).

**Washing:** After antibody incubation slides are washed three times, each time for 10 min washes with PBST at room temperature.

**Rinsing with distilled water:** After washing with PBST, slides are rinsed with double-deionized water followed by drying.

**Scan:** Slides are scanned at suitable wavelength. For the visualization of antibodies labelled with Cy3 or Cy5 one needs to use the wavelengths 532 nm or 635 nm, respectively.





**Fig 1.** Autoantibody screening using NAPPAs. The assay involved various steps like blocking, expression of the proteins, serum incubation, secondary antibody (anti-human IgG) labeled with Cy3/ Cy5 or detection system using Tyramide Signal Amplification (TSA).

### **IV. APPLICATIONS**

Antibodies to tumor antigens have been detected in early stages of multiple types of cancer and may correlate with disease progression. Few representative examples are described below.

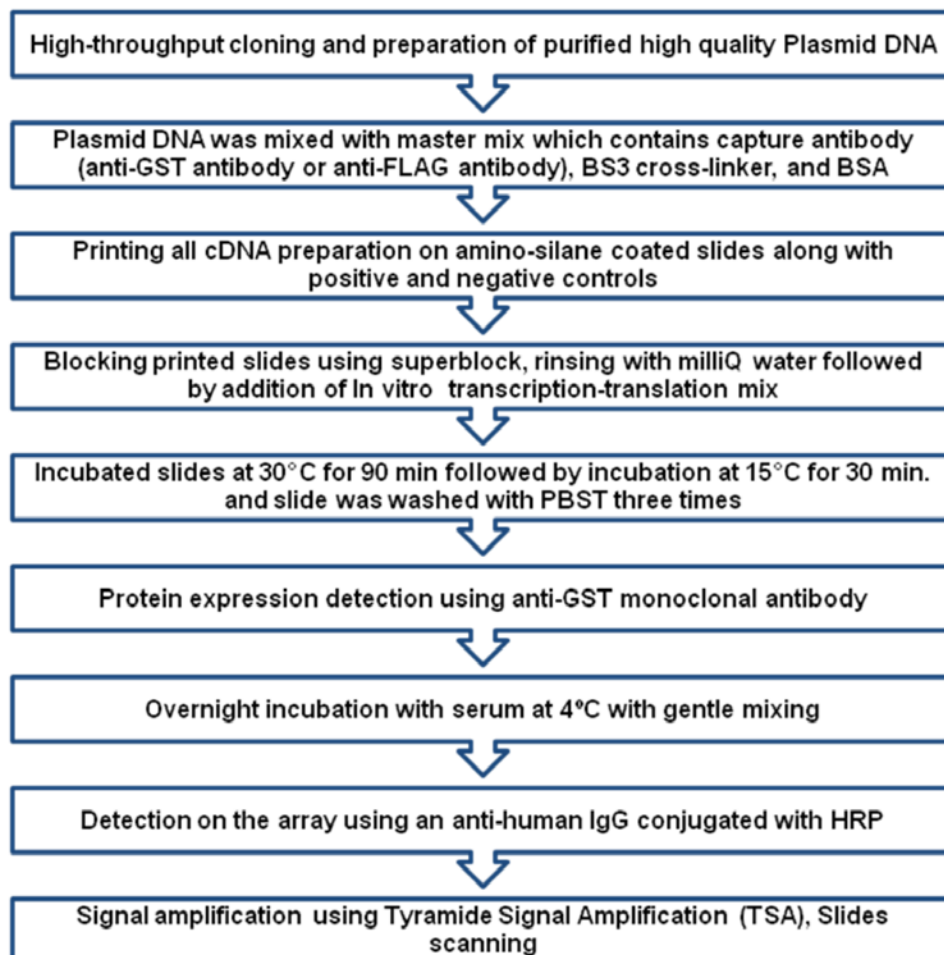
#### **Serum screening for autoantibody profiling for early detection in ovarian cancer with microarray**

Autoantibodies detection has been performed in ovarian cancer using protein array approach by Hudson *et al* (Hudson *et al.* 2007). Serum samples collected from patients with various stages of cancer and normal sera were used for autoantibody profiling using proteome chip comprised of 5,005 human proteins. More than 90 candidates showed differential reactivity between patient and normal sera. Further, four candidates LaminA/C, Ral binding protein 1 (RALBP1), ZNF265 and structure specific recognition protein 1 (SSRP1) were confirmed using immunoblot analysis, immune-histochemistry staining and tissue microarrays. Lamin A/C and SSRP1 expression was examined in 30 cancer patients and 30 control sera and compared with CA-125 (ovarian cancer serum biomarker) using Alexa Fluor 594 goat anti-human IgG (H/L) antibody. Lamin A/C and SSRP1 showed a large difference in expression compared to CA-125 between cancer and control patients and present their candidature as potential marker for ovarian cancer. In another study by Draghici *et al.*, authors developed an assay for early detection of ovarian cancer using a panel of tumor markers such as CA125, carcinoembryonic antigen (CEA), M-CSF, LASA, etc which have been studied previously and were able to detect ovarian cancer at stage I by detecting autoantibodies for antigens with serum screening. These studies clearly show the potential of

microarray approach for developing diagnostic bioassay using a panel of analytes for early detection of diseases, and suggest that targeting a panel of serum markers is more reliable for diagnostic rationale instead of screening of a single biomarker (Draghici *et al.* 2005).

### **Serum screening for autoantibody detection in Breast cancer using NAPPA microarray**

Anderson *et al.* have used high-density NAPPA microarray comprised of 4,988 tumor antigens and performed screening with sera from 53 normal woman and 53 patients covered stage I-III of breast cancer, and 761 antigens were selected for further screening (Fig 2). Further screening of sera from 51 early stage breast cancers (IBC) and 39 benign breast diseases (BBD) revealed 119 potential candidates. Confirmatory screening was performed and 28 autoantibodies were identified, which showed high level of specificity and several of these candidates are associated with breast cancer tumor biology (Anderson *et al.* 2011).



**Fig 2.** Workflow of NAPPA assay: For the construction of NAPPA array, one needs to prepare plasmid DNA which is mixed with a capture antibody, BS3 cross-linker and BSA. This mixture is immobilized on amino-silane coated slide, which are then blocked. The blocking of slide would inactive the active surface of the slide and helps reducing the non-specific binding. To the blocked slide *the in vitro* transcription-translation (IVTT) mix was added. Later the protein expression was detected using anti-tag antibody. This is followed by serum incubation and detection is performed by using secondary antibody labeled with HRP or Cy3/ Cy5.

### ***Illustration: NAPPA Application – biomarker discovery***

*The authors generated protein microarrays based on NAPPA expression, which they probed with diluted sera of breast cancer patients having p53 autoantibodies. Detection was carried out by means of HRP-linked anti-human IgG. This study detected p53 autoantibodies by means of NAPPA microarrays, which was confirmed by ELISA. The p53 levels were found to be directly related to tumour burden with serum antibody concentration decreasing after neoadjuvant chemotherapy.*

### ***Illustration: Cell-free applications – immunological studies***

*The authors carried out cell-free expression of the PCR amplified vectors using an E. coli IVTT. They expressed 250 putative proteins that were printed directly onto microscopic array slides without any purification.*

*These arrays were probed with serum samples from patients who had been naturally exposed to Pf and who were experimentally exposed by means of radiation attenuated Pf. Authors successfully identified 72 highly immunoreactive protein antigens as well as 56 previously uncharacterized antigens that were serodominant, which can serve as potential vaccine targets.*

### **V. ADVANTAGES AND CHALLENGES**

Increasing number of evidences now suggest that protein microarray technology provides a powerful and versatile high-throughput platform that requires less sample consumption, cost-effective and highly efficient for autoantibody profiling. However, several challenges are associated with this technique to be used for autoantibody detection. Traditionally, antigen arrays were made by individually cloning and purifying proteins that is tedious, time consuming and costly method. Additionally, the recombinant proteins or synthetic peptides, which are arrayed on protein chip, lack the post-translational modifications that are present in native proteins and hence may not detect autoantibody (Qin S et al 2006). Therefore, for autoantibody profiling native protein microarrays should be used. Technical issues such as array-to-array less reproducibility, dynamic range of protein to be detected, less abundance of several proteins and delicate nature of proteins are some of the challenges that affect the results in microarray experiment. Nucleic Acid Programmable Protein Array (NAPPA) and other cell-free expression microarrays have overcome some of the mentioned challenges and has shown its application in the field of biomarker detection by performing serum screening.

## **VI. CONCLUSIONS**

Autoantibody profiling leads to the identification of tumor antigens that may have direct relevance to cancer and other autoimmune disease as these antigens are involved in cell-mediated immune reactions or tumor immunity; thus possess therapeutic applications. Protein microarrays have the potential to accelerate autoantibody serum biomarker screening in high-throughput manner. A panel of tumor antigens may be useful in cancer diagnosis, to develop clinical assay and immunotherapy.

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