Patobiochemistry, practice 1: Immunochemical detection of p53 protein on nitrocellulose membrane (dot blot)

Aim

Immunochemical detection of oncogenic form of p53 protein

Introduction

p53 protein is encoded by *TP*53 gene and plays crucial role especially as a **tumor suppressor**. p53 regulates expression of many target genes responsible for cell growth and apoptosis thanks to its role of transcription factor. Protein also participates in DNA reparation process. More than 50% of tumors is associated with mutation in *TP*53 gene.

Dot blot is simplified version of western blot serving for detection and identification of proteins. In difference with western blot, there is no electrophoretic separation taking place, thus it´s impossible to determine size of target protein. Anyway, the presence of given protein (p53) can be confirmed. Drop of the sample is loaded dirrectly onto the nitrocellulose membrane where subsequently protein of interest can be immunochemically detected.

Principle of the method

Using of **antigen-antibody** reaction is principle of immunochemical detection. **Antigens** are compounds recognized by organism as a foreign ones. Their presence stimulates antibody synthesis. Every antigen consists of antigenic determinants (**epitops**) containing 5-8 aminoacids. The ability of antibodies to distinguish even a small difference in epitops is basic principle of immunodetection methods.

Antibodies fall into **immunoglobulins** and are produced as a part of immunity response. Several classes can be distinguished - IgG, IgM, IgA, IgE a IgD. Antibodies are defined by an **affinity** – strength of interaction between the antibody and the antigen binding determinant, an **avidity** – overall strength of the antigen-antibody complex (majority of antigens is multivalent i.e. there is several binding determinants for different antibodies) and a **specifity** – antibodies show only a low interference with compounds which the antibody isn´t intend for. Antibody can be divided to polyclonal and monoclonal ones.

Polyclonal antibodies are produced by immunisation of animals i.e. a particular antigen is applied to a mammal. During immunisation time (2-6 months) antibodies against different antigenic determinants of antigen are produced in blood serum. Generally, polyclonal antibody can interact with **several**

antigenic determinants. On the plus side of polyclonal antibodies is higher sensitivity and avidity. On the other hand there is an individual immune response thus irreproducibility.

Monoclonal antibodies aren´t produced by an organism but by cell culture. A mouse is immunised by antigen then lymphocytes producing antibodies are taken out of spleen. By its hybridisation with myeloma cells both cell types combine. By cloning only the cells producing antibodies against particular antigenic determinant of antigen can be picked. This way produced antibody contains only one type of binding side thus only **one antigenic determinant can be recognized**. Monoclonal antibodies are characterized by higher purity, affinity, specifity and reproducibility.

Nitrocellulose membrane surface binds proteins thus **blocking** of membrane is necessary after loading a sample. Membrane is immersed to some cheap protein solution e.g. BSA (bovine serum albumine) or low-fat powdered milk in PBS with detergent addition to reduce background noise. Proteins BSA or milk casein bind all the empty sites where proteins of our sample didn´t bind. After antibody addition nonspecific binding to membrane surface is excluded. Antibody needs to seek specifically its epitop placed at antigens of the sample.

Using specific primary antibody against particular protein and **secondary antibody conjugated with reporter enzyme** is crucial for target protein detection. In this case, DO1 is primary antibody recognizing specifically N-terminus of wild type protein p53 as well as its mutant form. It is about **mouse monoclonal antibody** of IgG class. Secondary antibody is produced by immunisation using particular primary antibody. Immunisation takes place in another type of host organism than it was in case of primary antibody. In this case, DO1 primary antibody was produced in mouse thus **secondary polyclonal antibody** (anti-mouse IgG) was produced in goat who was injected with primary antibody. Overall, secondary antibody binds primary antibody and is conjugated with reporter enzyme allowing detection, in this case with **alkaline phosphatase**.
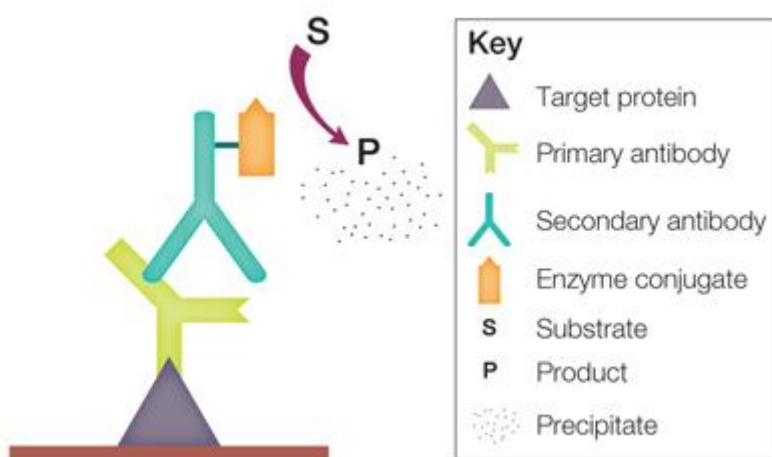
Pic. 1: Immunochemical detection of protein principle

5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT) are commonly used for colorimetric detection of alkaline phosphatase tagged molecules. If these compounds are incubated with alkaline phosphatase insoluble diformazan NBT producing purple colouring is formed.
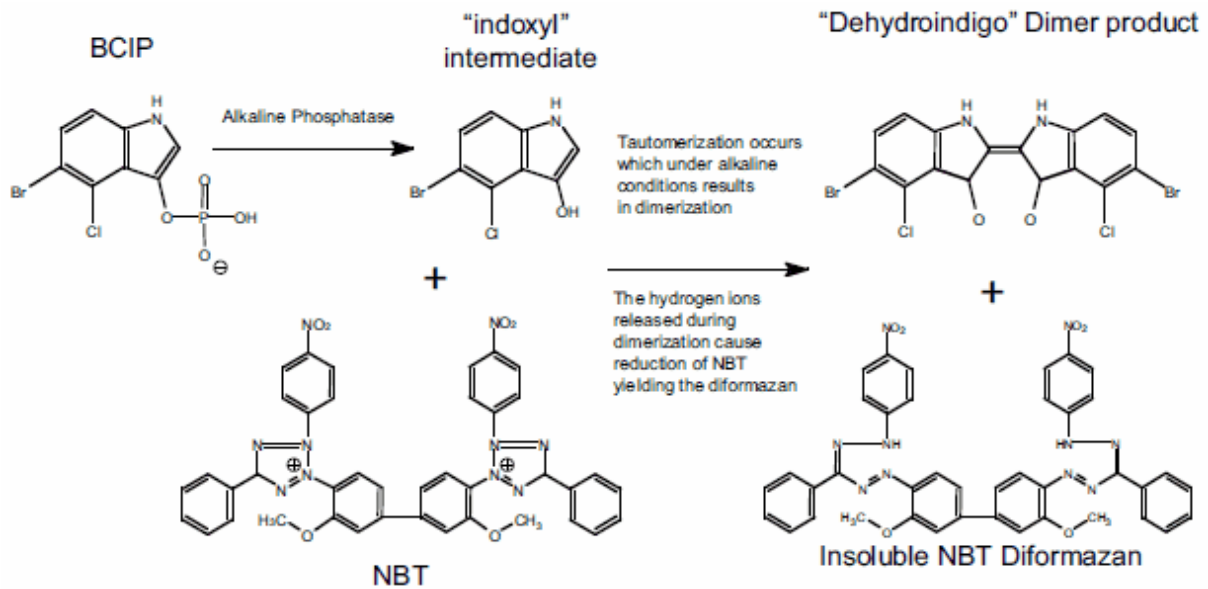
Pic. 2: BCIP/NBT reaction

Working procedure

1. Every pair has A and B sample. One of them contains oncogenic form of p53 protein while the second one serves as a check lacking any protein. Dilute both samples 10x (23 µl of sample + 207 µl of 1x PBS).

2. Take a piece of nitrocellulose membrane, describe it with a pencil, mark sample A and B at concentrations 1x (undiluted protein) and 10x. Vortex diluted samples. According to caption onto the membrane load 2µl of sample A and B at dilution mentioned above (see pic. 3). Overall, there will be 4 drops onto the membrane. Touch the membrane as little as possible. Load sample at dilution 10x first then use the same tip for loading sample diluted 1x.

1x   **10x**

Pic. 3: Drops of p53 at different concentration detected onto a membrane after colouring by BCIP/NBT solution.

3. Use tweezers to place the membrane into a 15 ml falcon flask marked „milk" and pour approximately 5 ml of 5% PBS milk. Incubate for 15 min at shaking machine.

4. Add 1 µl of primary antibody DO1 to a milk and incubate at least for 30 min at shaking machine.

5. Pour the milk containing antibody into a prepared 15 ml falcon flask marked „milk + DO1" and pour approximately 5 ml of washing buffer 1x PBST onto the membrane, leave wash for 5 min at shaking machine. Pour out the buffer and repeat washing step twice more.

6. Pour approximately 5 ml of milk onto the membrane and add 1 µl of secondary antibody anti-mouse IgG. Incubate for 30 min at shaking machine.

7. Milk containing secondary antibody pour to 15 ml falcon flask marked „milk + anti-mouse" and repeat washing step, wash membrane 2x after 5 min with 1x PBST and 1x with substrate buffer.

8. Detection will be performed for three pairs together. Place 3 membranes into a Petri dish/falcon flask. Prepare developing solution by adding 33 µl of BCIP and 330 µl of NBT to 10 ml of substrate buffer. Incubate membrane in this the solution at shaking machine as long as purple colouring observed (about 3 min).

9. Stop the reaction by washing with distilled water.

Used chemicals

1x PBST – 137 mM NaCl, 12 mM phosphate, 2,7 mM KCl, 0,05% Tween, pH 7.4
5% PBS milk – 5% powdered milk in 1% PBS
BCIP – 50 mg/ml water solution
NBT – 10mg/ml water solution
Substrate buffer – 0,1 M Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9,5

Evaluation

Determine which sample contains oncogenic form of p53 protein.

Report

Name:

Date:

Method principle:

Conclusion:

Questions:

1. **What do you know about p53 protein?**

2. **What is dot blot?**

3. **Explain immunochemical detection of protein principle.**

4. **What is milk/BSA used for during immunochemical detection of protein?**

5. **Which enzyme catalyze purple coloured diformazan NBT formation?**