

Immunohistochemical staining of Neurofilaments (green) in an organotypic chicken spinal cord culture, DAPI in blue indicates cell nuclei, Propidium iodide in red also stains nuclei.

Antibodies

A Tool for Molecular Biology

Antibodies are immunoglobulins, which build an important component of an organism's immune system. They are produced by B lymphocytes, a specialized type of white blood cells.

When foreign macromolecules enter the body, antibodies are produced to identify and to tag invading macromolecules for elimination by other parts of the immune system. Each antibody recognizes very specifically a particular epitope (a 3 dimensional surface structure build by a certain amino-acid sequence) on its antigen (the foreign macromolecule). This makes antibodies interesting tools in molecular biology because by their aid proteins of interest can be identified in a crude mixture of many proteins. Proteins of interest can be visualized and quantified using antibodies.

Structure

Antibodies are large Y-shaped monomeric glycoproteins with a molecular weight of approximately 150 kDa. Typically two identical heavy chains build the centre of the „Y“, each accom-

panied by a light chain on the outer side (fig.1) All chains of the immunoglobulin are linked to each other by disulfide bridges.

Both tips of the „Y“ bear a paratope, a highly specific binding region for the epitope, to which this antibody binds. You can imagine this paratope-epitope binding as uniquely as a key fitting in a particular lock. The tips including the paratopes are also called Fab (fragment antigen binding) regions. As this region is shaped by amino acids from both heavy and light chain, it becomes clear that interaction between both chains is an essential requirement for spatial recognition of the epitope.

Production

In order to generate an antibody an antigen of interest first must be introduced into a host



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Useful hints



- Avoid temperature extremes.
- Freeze antibodies in aliquots to avoid repeated freeze-thaw-cycles.
- It may be useful to add some non-disturbing protein to the buffer to prevent antibody precipitation at very low antibody concentrations.
- Closely related species can exhibit cross reactions, e.g. secondary antibody (anti mouse) may also detect rat immunoglobulins (strong background staining / low signal to noise ratio). Even traditional blocking agents (bovine serum albumin or non-fat dry milk) might show cross reactions (bovine immunoglobulins contaminations) with anti-bovine, anti-goat, anti-horse or anti-sheep secondary antibodies. Pre-absorption with serum from the species you work with can minimize high background staining but this can also result in very low signals.
- High antibody concentrations can result in high background. Prepare dilution series for new antibodies. For ELISAs and CLIA a checkerboard pipetting scheme with different concentrations of primary and secondary antibody is recommended.
- Reliable controls are essential; As positive control recombinant protein or transiently transfected cells and their supernatants can be used. As negative control tissues from knockout (protein of interest) animals are useful. Detection of CD47 can serve as an example. CD47 is upregulated on the surface of cells to avoid phagozytosis by macrophages. Some cancer cells also show enhanced expression of CD47 to escape the immune system. Therefore CD47 expression in mice can be endogenous, pathological or nonspecific at all. Using a CD47 deficient mouse can solve this problem. The gold standard is the comparison of a knockout and wildtype littermates. Knockout should be confirmed on DNA, RNA and protein level to prove the knockout. Tissue-specific or conditional knockouts triggered for example by temperature changes are also often available, if complete deletions are lethal.

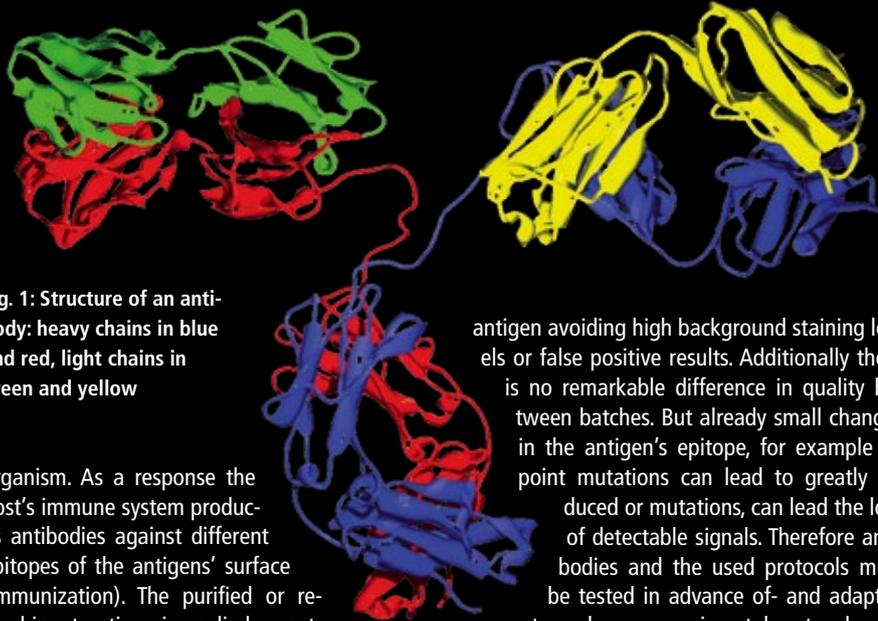


Fig. 1: Structure of an antibody: heavy chains in blue and red, light chains in green and yellow

organism. As a response the host's immune system produces antibodies against different epitopes of the antigens' surface (immunization). The purified or recombinant antigen is applied repeatedly to a suitable animal by injection until a strong immune response with high antibody titers in serum is reached. Rabbits, goats and sheep show strong immune responses and large serum volumes. When the titer is high enough, blood is extracted from the animal and the serum is purified by chromatography. The result is a mixture of antibodies from different B lymphocytes (polyclonal) directed against different epitopes of the same antigen.

For the production of monoclonal antibodies often mice are immunized. Spleen cells are isolated and fused *in vitro* with tumor cells to give hybridoma cells cultures. The most effective clone is isolated for further use. Subsequently the antibody can be produced by immortal cells deriving from a single B lymphocyte clone. Both polyclonal and monoclonal antibodies have advantages and disadvantages. Polyclonal antibodies often have a higher sensitivity because different epitopes of the same antigen are recognized. This allows robust and enhanced antigen detection. On the other hand polyclonal antibodies often have a lower specificity in comparison to monoclonal antibodies and quality between different batches may change. Monoclonal antibodies are very specific for their

antigen avoiding high background staining levels or false positive results. Additionally there is no remarkable difference in quality between batches. But already small changes in the antigen's epitope, for example by point mutations can lead to greatly reduced or mutations, can lead the loss of detectable signals. Therefore antibodies and the used protocols must be tested in advance of- and adapted to each new experimental protocol.

Applications

Whenever the detection of an individual protein is needed, antibodies are the tool of choice for molecular biologists. There are different ways to detect antibodies bound to their target. Usually antibodies are not directly visualized. A secondary antibody, directed against a constant region in the backbone of the primary antibody is used. This gives the opportunity to use the primary antibody in combination of various detection methods. If the target protein is highly abundant in the sample, secondary antibodies coupled to fluorochromes or radioisotopes can be used. If the target epitope is very low abundant signal amplification is needed for proper detection. A strategy for such amplification is to couple the secondary antibody to an enzyme catalyzing a color reaction. Frequently used enzymes are Horseradish peroxidase and alkaline phosphatase (AP).

Antibodies can be used for the detection of secreted, extracellular and intracellular proteins, peptides and other molecules. They are used in immunohistochemistry, immunofluorescence, Fluorescence-activated cell sorting, Western



Fig. 2: In situ hybridization of the Cannabinoid receptor Type 1 (Cnr1) coding mRNA. An anti-sense RNA with a Digoxigenin (DIG) tag is visualized using an AP coupled anti-DIG antibody.

blotting, ELISAs, CLIA, RIAs, IRMAs, immunodiffusion assays and immunoelectron microscopy in aqueous solutions or in cells and tissues. Antibodies are used also for detection of specific DNA or RNA sequences. In these cases antibody targets are fused to antisense nucleotides, which are hybridized to the target sequence (*in-situ* hybridization). Then the antibody is used for detection. Moreover antibodies can be used for interventions such as inhibition and stimulation of protein function, e.g. in signalling pathways. Binding to a receptor for example can block the binding of the signaling molecule. This approach is increasingly used in medical applications.

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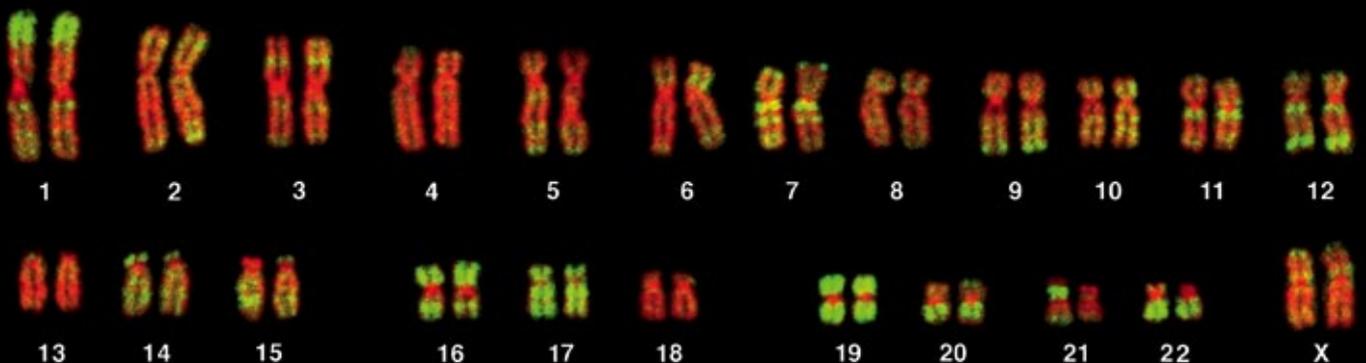


Fig. 3: Fluorescent in situ hybridization (FISH) of ALU sequences (green signals) in (female) human lymphocytes. ALU sequences are highly repetitive sequences in gene-rich chromosomal regions. DNA counterstaining in red. Bolzer et al., PLoS Biol 3(5) (2005)