Development of novel Fluorescence-activated cell sorting (FACS) and Immunoprecipitation (IP)/Western-Blot assays to characterize antibody therapies

by Ralf Geiben Lynn, Ph.D., MBA, Cambridge Biomedical Inc.

Background:

Biotech industry is in a boom, and we at Cambridge Biomedical are proud that we can contribute to it. The arrival of new antibody therapies feels like a fresh ocean breeze at Biarritz, France, which we can still feel here in Boston. In our role as a bioanalytical assay CRO, we are involved in the development of novel therapeutic antibodies that show examples of how the industry creates sustainable growth, which drive this boom.

There are multiple new antibodies that boost the immune system and that are part of novel therapeutic interventions. Some of these antibodies enhance T-cell immune responses: Pembrolizumab (Keytruda, MK-3475, Merck) is approved to treat metastatic melanoma. New applications may include lung cancer and mesothelioma. Nivolumab (Opdivo, Bristol Myer Squibb) reduced non-small cell lung cancer, melanoma, renal-cell cancer in a clinical trial. Pidilizumab (CT-OII, Cure Tech) MPDL328OA (Roche) and BMS936559 (Bristol Myers Squibb) are also tested for other novel applications.

Method I:

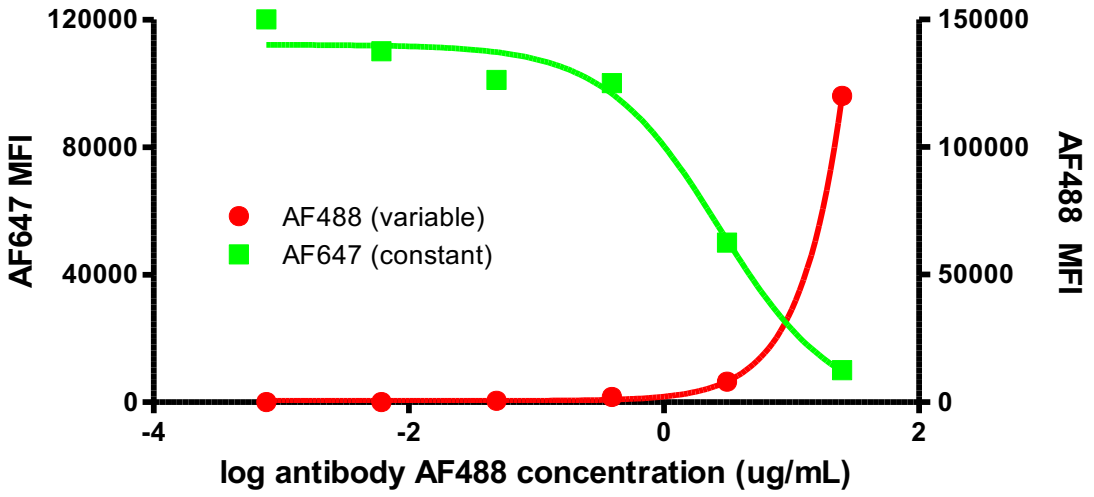
Development of a FACS assay to compare binding affinities of antibody therapies

Fluorescence-activated cell sorting (FACS) uses flurochrome labeled antibodies to determine the phenotype of cells by measuring cell membrane receptor densities. In this method a mixture of different dye-labeled antibodies recognizing specific membrane proteins is incubated with cells. In our model system (Fig. 1) we utilize two dyes to distinguish the binding of two antibodies. One antibody carries the Alexa Fluor (AF) dye with an optimal excitation wavelength of 488 nm and is called AF488. The other antibody is linked to an AF with an optimal excitation wavelength of 647 and therefore called AF647. The amount of dye bound to the cells allows us to characterize the cells through specific light scattering and fluorescent characteristics after passing a laser beam. Another usage of this technology is to measure intracellular expression of a target protein with an antibody after cell membranes are permeablized to allow passage of antibody into the cell.

We have extended on these applications and have developed a method to compare therapeutic antibodies binding by competitive antibody binding assays using FACS: (I think a better connecting sentence is needed here) known binding affinities of a

reference antibody are used to calibrate the system. This allows us to determine, but not to distinguish, if antibodies have similar binding domains or if there is steric hindrance. Both the reference antibody and the therapeutic antibody are mixedunder experimental conditions where one antibody is kept at a constant concentration andthe other antibody is serially diluted. For the antibody which is kept at one concentration most often the 95 % saturation concentration of the median fluorochrome intensity (MFI) is used for our experiments. The loss of MFI of the reference antibody is then used to measure competitive binding.

Fig. 1: Model system to investigate competitive binding profile of antibodies labeled with two different dyes AF488 and AF647



During the development of our assays we include an optimization phase where the method is individually refined to adapt to the new therapeutic antibody we characterize. In a second phase the binding of a specific antibody is characterized in more detail by a validation process.

Method II:

Development of an immune-precipitation (IP)/Western Blot protocol to compare binding affinities of antibody therapies

Immuno-precipitations (IP) are normally used as a method that enables the purification of a protein. An antibody for the protein of interest is incubated with a protein solution bearing a mixture of proteins. Sepharose A or G beads, which bind antibodies, are then used to capture the antibody-target protein complex. To eliminate unwanted protein the bead-antibody-target protein complex is stringently

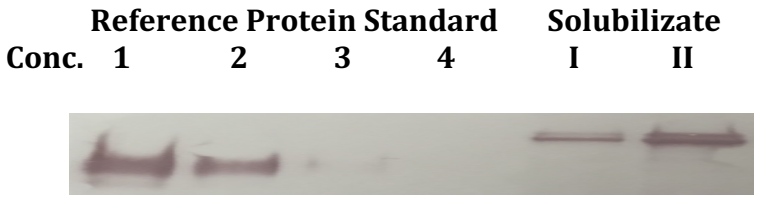
washed. To measure target protein recovery, bound protein is then separated by SDS-PAGE and transferred onto a cellulose membrane. The target protein now fixed in place is then identified and quantified by a combination of two antibodies. The first antibody is target protein specific and binds to the protein, a second antibody binds to first antibody. The last protein carries an enzyme function which allows after incubation with a substrate a color reaction. These two antibodies also enable an amplification of the signal.

We have expanded on the usage of the traditional IP/Western Blot. In a first step we crosslink the therapeutic antibodies to Sepharose beads. In parallel cells are solubilized to enrich intra-cellular (to my knowledge, therapeutics Abs in the clinic only target surface proteins so there is no need to permeabilize the cells) or membrane proteins dependent on the target of the therapeutic antibody. The solubilizate (is this a word?) is first incubated with different concentrations of a reference antibody then with constant amount of therapeutic antibody-beads complex. This allows a comparison of the binding affinities of the cross-linked therapeutic antibody with the reference antibody. Beads are precipitated by centrifugation and washed. Precipitate is separated by SDS-PAGE and blotted to a membrane and stained by the above-described two antibody detection system. Signal intensity is then semi-quantitatively measured by blackness quantification software. Purified target protein standard also blotted to the membrane is used to quantify amounts of precipitated target protein.

The binding of the therapeutic antibody to solubilizate I and II was determined by semi-quantitatively measuring image density then comparing with a protein standard (Fig. 3A/B). We used 100 (100 what?) for total image density on our intensity scale (Fig. 3B).

Fig. 3. Model system for software supported measurement of target protein recovery from IP eluates of Western Blot

A. Western Blot



B. Semi-quantitative stain intensity measurements

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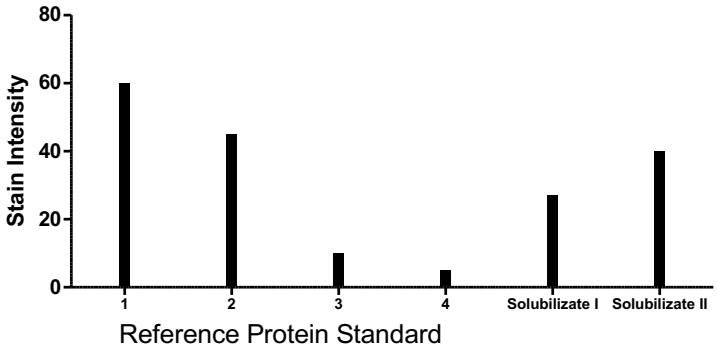

Summary:

We have expanded on the traditional use of FACS and the IP/Western Blot. We have developed usage to further compare binding characteristics of therapeutic antibody therapies.

For more information please contact us: info4@cambridgebiomedica.com

About the Author:

Ralf Geiben Lynn received his PhD. in Biology for the Justus/Liebig University in Giessen/Germany. He received a MBA from the Boston University. Ralf Geiben Lynn worked more than 10 years at the Harvard Medical School. He first worked as a post-doctoral fellow in the laboratory of Dr. Bruce Walker at the Massachusetts General Hospital, where he investigated human resistance factors against HIV. Afterwards, he worked with Dr. Norman Letvin as a Group Leader at the Beth Israel Deaconess Hospital and developed a program to study vaccine vectors and the biologic events that result in robust and durable immunogenicity. In these studies he developed an *in vivo* mouse imaging technology to follow the distribution and persistence of vaccine expressed antigen. Ralf Geiben Lynn was also involved in the formation of several biotechnology start-up companies in the US and Europe.