Multiplex Methods to Replace or Enhance Western Blot Analysis

Introduction

The Western blot has remained the most common method to analyze protein expression levels since its original development in late 1970s.[1-3] Western blots are most common in basic scientific research, but they are also used in areas like clinical diagnoses and antibody validation.[1-4] During the Western blot process, gel electrophoreses separates a biological sample by size, the sample is transferred to a solid membrane, blotted with an antibody that labels the target protein, and then the protein is visualized.[3] Running known standards alongside the sample can enable quantitative protein detection.[3]

Western blotting remains popular because its high specificity and straightforward procedure requires no special instrumentation.[3] However, the process also has distinct disadvantages. Western blotting is time-consuming and only capable of detecting proteins at high concentrations. Additionally, the method does not work as well for examining multiple proteins simultaneously, especially when targets have similar or unknown sizes.[1] Researchers can strip the blot and probe again for additional targets to combat this limitation. Although this process can be effective, it is also cost-prohibitive, time-intensive, and the stripping process can damage target proteins and potentially impact the results.[1, 5]

xMAP Technology offers an effective alternative to traditional Western blots. These methods enable the evaluation of up to 500 analytes simultaneously in a single well, known as multiplexing. xMAP Technology uses carboxylated polystyrene microspheres that have been fluorescently dyed into up to 500 spectrally distinct sets, or "regions," allowing them to be individually identified. In the multiplex assay environment, the microspheres act as both the surface for the solution phase assay and the spectral identifier that the instrument detects. The open architecture of xMAP Technology enables compatibility with both protein and nucleic substrates. The microspheres are individually read in a xMAP instrument. There are more than 1,400 commercially available xMAP-based kits in the xMAP Kit Finder.

Multiplexing with xMAP Technology offers distinct advantages to classical Western blots. Multiplexing is more affordable, sensitive, and reproducible than Western blotting. The method is also faster and has high throughput capability. Additionally, the ability to use small sample sizes is great for situations where sample material is limited.[3, 5, 6] Below are summaries of recent publications that explore how multiplexing methods can replace or enhance Western blot analysis. These publications highlight the diverse applications of xMAP Technology and how researchers have developed their own custom multiplex assays or used the wide variety of available kits to replace or supplement classical Western blots.

Creation of custom multiplex assays to replace or enhance Western blots

Examples of Customized Assays

Western blots are commonly used in the clinical diagnosis of different infectious diseases.[3] For example, a recent study utilized multiplexing to supplement Western blots during the diagnosis of human immunodeficiency virus (HIV).[7] Western blots are the gold standard for confirming an initial serodiagnosis with an HIV immunoassay, and they help reduce false positive results and improve overall specificity. However, Western blots also have relatively high rates of indeterminate results during HIV screening, possibly because of contamination of viral lysate-based blot strips with cellular antigens and other biological compounds.[7]

Kong et al. developed a sensitive and affordable bead-based assay using XMAP Technology and used the custom assay against a panel of specimens that tested positive during initial screening with the HIV immunoassay. For the assay, Luminex MicroPlex® Microsphere regions were coupled with a selection of HIV recombinant antigens, including viral coat proteins. After incubating microspheres with samples, biotin-labeled secondary antibody and SAPE enabled the detection of results that could be analyzed with Luminex 100 and Bio-Plex® manager software.[7] The custom xMAP assay exhibited superior sensitivity when compared to Western blot analysis, correctly verifying 72 of 87 HIV blood donor positive cases with 82.8% sensitivity while Western blot analysis only verified 65 cases with 74.7% sensitivity. Furthermore, the xMAP assay confirmed 19 of 33 specimens collected from men who have sex with men with 57.6% sensitivity while Western blot only confirmed 13 specimens with 39.4% sensitivity. The study concluded that using both methods simultaneously enhanced HIV screening and reduced the chance of any missed diagnoses.[7]

Similarly, Western blots are frequently used to confirm an initial serum enzyme immunoassay diagnosis of Lyme disease. However, the Western blot confirmation step has a low sensitivity for early disease detection, is prone to false positive results, and interpretation of results can sometimes be subjective.[8, 9] Porwancher et al. therefore set out to develop an alternative assay using Luminex-based multiplex microspheres. The assay used antibodies that target the peptides VlsE1 and pepC10, representing conserved portions of surface antigens expressed by *Borrelia burgdorferi* during early mammalian infection. The antigens were covalently bonded with the surfaces of separate bead sets and adapted to the AtheNA Multi-Lyte® Test System, a sandwich immunoassay that separates microparticles with xMAP Technology using flow cytometry.[8] The authors examined the effectiveness of their assay with a study population of 208 patients in different stages of the disease, 34 patients that had undergone antibiotic treatment, and 794 controls. The specificity of the customized assay was comparable to Western blotting but 12.5% more sensitive overall and 20.7% more sensitive for early-convalescent-phase disease.[8]

In another example of the power of multiplexing to replace or enhance Western blots in the clinic, Anderson et al. created a custom bead-based assay for the diagnosis of roundworm infections.[10] *Toxocara canis* and *Toxocara cati* roundworms cause a range of disease in humans and can threaten children when their eggs contaminate playgrounds or sandboxes. Western blots for *T. canis*

excretory-secretory antigens (TES) are one effective method for diagnosis, but a limited availability of antigen made from parasite larvae prevents the widespread use of this and other detection methods. The custom multiplex assay eliminated reliance on native parasite materials and used recombinant immunoreactive proteins Tc-CTL-1 and Tc-TES-26 found in the TES. The assay had improved sensitivity to other testing methods with visceral larva migrans, but it was less sensitive than enzyme immunoassays and Western blots in detecting ocular larva migrans. However, the assay also had distinct advantages over other testing methods. It was more cost effective and enabled screening samples on a large scale. Most importantly, the assay reduced reliance on limited native parasite materials and provided the future possibility of tailoring screening to detect other human larval migrans syndroms caused by helminthes like *B. procyonis*.[10]

Kohlgraf et al. also illustrated the ability of multiplexing to replace or enhance Western blot analysis in the design of a novel xMAP particle-based capture and detection immunoassay of short palate lung and nasal epithelial clone 1 (SPLUNC1) in saliva.[11] The protein is elevated in a number of ailments, including chronic obstructive pulmonary disease and in various carcinomas. The assay could enable the study of the protein and help researchers understand its role in pathogenesis. Previous studies have utilized Western blots to detect the protein in saliva, but this method is only semi-quantitative and not practical for large-scale screening. A commercial goat anti-rhSPLUNC1 was linked to fluorescent polystyrene microspheres as a capture antibody while a biotinulated IgG2b anti-rhSPLUNC1 monocolongal antibody was used for detection. The xMAP assay and a comparative Western blot were used to detect SPLUNC1 in the saliva of 20 subjects. The protein was detected in the saliva at a variety of concentrations, demonstrating the effectiveness of the assay and its high throughput potential to give insight into the role of SPLUNC1 in various diseases.[11]

In addition to the aforementioned examples, a recent innovative study directly adapted the Western blot to a bead-based microarray platform known as DigiWest®. Treindl et al. combined the classical Western blot and xMAP Technology to enable hundreds of duplications of the initial blot to allow detailed analysis when limited sample material is available. In fact, they found that the new method had comparable sensitivity to traditional Western blots in validation experiments but required only 1/100th of the sample. The study also validated the effectiveness of the approach by analyzing the expression of nearly 200 proteins in tumor cells from human mammary carcinomas collected through laser capture microdissection.[12, 13]

This method has since been used to study the mode of action of preclinical cancer drugs,[14] understand the mechanism of Evi-deficiency mediated cell death,[15] develop a panel of platelet biomarkers for ovarian cancer diagnosis,[16] analyze the protein compositions of microvesicles and exosomes,[17] and uncover the mechanism of the constitutive androstane receptor.[18, 19]

Examples of Customized Assays with Luminex Partner Companies

Some research projects may have unique needs not met by the over 1000 available multiplexing kits. Assay development companies that partner with Luminex can help develop custom assays when researchers lack the time, desire, skills, or equipment to create a solution on their own.

In one intriguing example, the Food and Drug Administration joined with Luminex partner company Radix® BioSolutions to create a commercial multiplex test kit for the detection of allergens in foodstuffs.[20] Millions of people in the United States have food allergies and must follow strict diets to avoid allergic reactions. Food labels have to declare ingredients derived from major allergens to protect allergic consumers. The Food Allergen Detection Assay (FADA) commercial multiplex test kit was developed to enable simultaneous detection of up to 14 different allergens plus gluten.[20] The assay uses MagPlex® magnetic bead sets and established antibodies with two different extraction protocols and results that can be analyzed with the Bio-Plex® 200. The effectiveness of the assay was verified by screening cumin for the presence of undeclared antigens.[20]

The assay was later used alongside ELISA, Western blot analysis, mass spectrometry, microscopy, and DNA-based methods in a large-scale 2014 effort to identify materials with cumin-containing taco spice suspected of undeclared peanut contaminants. The screening effort ultimately led to the recall of over 675 products, making it one of the largest food safety recalls in history. The assay's ability to generate antigenic profiles was advantageous over other methods and enhanced the screening process. Meanwhile, Western blotting with a polyclonal antibody probe instead of relying on the presence of two antibody-binding sites to detect antigens enhanced the visualization of differences between samples.[21]

The FADA assay has since been used in combination with other screening methods to enhance detection of undeclared allergens in other foodstuffs, including imported garlic.[22] The multi-antibody antigenic profiling ability of the assay was also uniquely used by Cho et al. to determine cross-reactivity patterns and helped distinguish between legumes and tree nut contaminants.[23]

Meanwhile, Levin et al. partnered with R&D Systems Custom Services (a division of Bio-Techne, MN, USA) to manufacture a custom assay to quantify glypican-1 in serum and plasma.[24] Glypican-1 is a biomarker that could facilitate early detection and diagnosis of prostate cancer, but a lack of dependable immunoassays has prohibited its use. Current methods to diagnose prostate cancer have a low specificity, causing men to undergo unnecessary and invasive prostate biopsies. The custom assay for glypican-1 used MagPlex® Microspheres coupled with a MIL-38 capture antibody and biotinylated 3G5 detection antibody. The resulting GPC-1 Luminex assay enabled precise quantification of circulating human glypican-1 in both serum and plasma and demonstrated a clear difference in glypican-1 levels between prostate cancer patients and non-cancer patients with normal and benign

prostatic hyperplasia, validating its potential as an effective biomarker for early prostate cancer detection.[24]

Utilization of Kits to replace or enhance Western blot analysis

Several studies investigating different aspects of cancer have utilized ready-made kits with xMAP Technology. For example, a study by Gallo et al. used multiplexing to explain the mechanism of the antitumor activity of zoledronic acid on estrogen receptor (ER)-positive breast cancer.[25] The study investigated the effects of the drug on the interaction between breast cancer cells and mesenchymal stem cells. They utilized a **Bio-Plex® Cytokine array system** to examine the expression of cytokines and angiogenic factors in mesenchymal stem cells grown in the presence or absence of zoledronic acid. They additionally examined the effects of zoledronic acid on the activation of signaling proteins using a **Bio-Plex® phosphoprotein array** and quantified results with the **Bio-Plex® phosphoprotein array** and **Bio-Plex® Manager Software 5.0**. Multiplexing produced similar results to Western blotting and enabled the conclusion that zoledronic acid may fight tumors by inhibiting migration of mesenchymal stem cells and blocking their secretion of several factors that facilitate breast cancer progression.[25]

In a similar study, Crucero et al. harnessed the high throughput ability of multiplexing to simultaneously look at potential therapeutic targets for glioblastomas.[26] Glioblastomas are among the most common tumors in the central nervous system and are most often lethal. The cancer is molecularly complex and traditional biochemical methods like Western blot and ELISA often overlook essential aspects of the disease. The study examined the expression levels of several signal transduction molecules using glioblastoma cells and patient-derived cells under different conditions on a **Luminex® 200™** platform using **MILLIPLEX® MAPmates** for total ERK ½, JNK, P70S6K, IκBα, p38, cAMP response element-binding protein (CREB), and glyceraldehyde 3-phosphate dehydrogenase total proteins. They also assessed MEK inhibition by MEK inhibitor effects on phosphorylation of ERK 1/2 using a **MILLIPLEX® 8-plex Multi-Pathway Signaling kit** for phosphoproteins. Multiplex analysis produced similar results to Western blotting, but the authors remarked on how the high throughput nature of the multiplex platform is useful for brain tumor diagnosis and the discovery of new therapeutic targets. Furthermore, combining multiplexing with other technologies like genomics, proteomics, and metabolomics could enhance tumor analysis for differences between similar tumors or even unique aspects present in an individual tumor. The study led to the identification of the PI3K pathway as a potential target for glioblastoma treatment.[26]

In another study examining pathways involved in cancer, Lee et al. used multiplexing to explore how chemokine ligand 7 (CCL7) enhances colon cancer progression. The researchers generated cells overexpressing CCL7 and confirmed its secretion with microsphere-based **ProcartaPlex™ immunoassays** with a **Luminex 200™ analyzer** and Western blot analysis. These cell lines were then

used in the discovery of interactions between CCL7 and CC chemokine receptor 3 and the ERK and JNK signaling pathway to enhance cellular proliferation, invasion, and migration in colon cancer. These findings were verified in mouse models and provide potential therapeutic strategies to prevent metastasis in colon cancer.[27]

In a further example of the power of multiplexing, Marangoni et al. examined the effectiveness of **BioPlex™ 2200 Syphilis IgG** and **BioPlex™ 2200 Syphilis IgM** tests as options for first-line reverse-sequence screening to diagnose the sexually transmitted disease syphilis. They directly compared the effectives of the test against Western blot analysis using a large selection of blood donor sera, syphilis patient sera, and sera from patients with potentially interfering conditions. The **BioPlex™ 2200 Syphilis IgG** was about 10% less specific than Western blot analysis but had a comparable sensitivity. Conversely, the **BioPlex™ 2200 Syphilis IgM** test was about 5% less specific and 15% less sensitive than Western blot analysis. The authors concluded that the **BioPlex™ 2200 Syphilis IgG** assay could be effective for high-volume screening of samples while the **BioPlex™ 2200 Syphilis IgM** assay could be an efficient addition to IgG testing to identify active syphilis infections. Both assays enabled high throughput analysis with easy automation, making diagnoses more achievable in developing nations.[28]

Lawrence et al. used multiplexing to examine the toxic environmental contaminant tributyltin (TBT) widely used for different household, agricultural and industrial purposes around the world. The study looked at impact of exposure to tributyltin on cytokine levels in mouse serum using a **MILLIPLEX® Map Mouse Cytokine/Chemokine Magnetic Bead Panel** and a **MAGPIX® instrument** with **xPONENT®** multiplex software for analysis. Their results were confirmed by Western blot and illustrated that TBT exposure has a clear impact on the secretion of inflammatory cytokines [29]

Meanwhile, Scotece et al. combined multiplexing with Western blot analysis to examine nefsfatin-1, a satiety-inducing adipokine involved in regulating energy balance. They investigated the adipokine's localization in human and murine chondrocytes and the protein's effect on pro-inflammatory cytokines expression. A **Bio-Plex Pro™ Mouse Cytokine Assay** combined with **Bio-Plex Manager™** software helped evaluate the effect of nesfatin-1 on various inflammatory cytokines. Results from the assay were comparable to Western blot analysis and helped demonstrate that nesfatin-1 induced pro-inflammatory agents.[30]

Summary

The ability to evaluate up to 500 analytes simultaneously in a single well is critical to a variety of scientific fields. Custom multiplex assays have been used to replace or enhance Western blot analysis in the clinical diagnosis of conditions like HIV, Lyme disease, or roundworm infections. Custom multiplex assays have also enhanced Western blot analysis of biomarkers, inspired the creation of the DigiWest® assay, and enabled screening for allergen contaminants in foodstuffs. Meanwhile, the existence of over 1400 kits with xMAP Technology has helped

replace or enhance Western blot analysis. Specifically, kits manufactured by **Bio-Rad**, **MILLIPORE SIGMA**, and **Thermo Fisher Scientific** have led to multiplex analysis of different types of cancers, sexually-transmitted diseases, environmental contaminants, and adipokines. These collective multiplex approaches offer many benefits over classical Western blots, including a reduced cost, increased sensitivity, quicker results, high throughput capability, better reproducibility, and small volumes when sample is limited.

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