

## Proteomics applications in biomedical research and diagnosis by luminex in Egypt

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### ABSTRACT

To accurate prognosis, diagnosis and treatment for several diseases such as cancer, diabetes and autoimmune diseases, the results of analysis should be accurate with high precision. Luminex is a one of these technologies that able to give accurate results. So far, luminex is not widely distributed in Egypt or other Arabic countries; however, it is widely used in European countries and United States of America. In Egypt, there are almost 18 instrument distributed all over the country. This instrument is mainly used for protein (proteomics) and nucleic acids analysis. The principle of luminex is to use two lasers that detect micro beads of polystyrene set (5.6  $\mu\text{m}$ ) or beads of magnetic set (6.5  $\mu\text{m}$ ). In this review, we are introducing some new information about luminex technology and its applications in diagnosis and research in different scientific centers in Egypt.

**Keywords:** applications, cancer, Egypt, luminex, multiplexing, proteomics

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### INTRODUCTION

Proteomics is the study of the function of all expressed proteins in the biological system. The number of proteins within the human proteome can be as large as 2million (Anderson et al., 1998). Databases of protein-protein interaction have become an important resource to investigate biological networks and pathways in cells (Hartlet et al., 2005). Multiplexed measurement is used for biological discovery with proteins because of its function is mainly works within networks, pathways, complexes and families. Luminex placed globally with over 10000 instruments.

Luminex offers three different xMAP-based instruments, MAGPIX, Luminex 100/200, and FLEXMAP 3D. MAGPIX is the simplest that performs up to 50 different tests in a single reaction volume. The luminex 200 have ability to perform up to 100 different tests in a single reaction volume while the FLEXMAP 3D is most advanced and versatile multiplexing platform that have the ability to perform up to 500 different tests in a single reaction volume.

### Luminex technology history

The sensitive and rapid quantification of soluble cytokines and other analytes in serum and plasma is becoming increasingly important in the management and study of many diseases. As result, bead-based multiplex immuno assays has developed for measurement of large numbers of analytes in single sample (Elshal *et al.*, 2006). In 1963, Feinberg and Wheeler developed a microspot technique as a means of detecting autoimmune antibody and tissue antigens; hereby the importance of immunoassay miniaturization for diagnostics has been tangible (Feinberg *et al.*, 1963). In 1989, Ekins described microarray multiplex technology principles. He predicted that an immunoassay's limit of detection could be improved by reducing the amount of capture antibody. In addition, it also anticipates that results should be insensitive to the volume of sample as well as the amount of capture antibody added (Ekins *et al.*, 1989). Suspension arrays (microbead assays) in which different the capture antibodies are conjugated to different populations of microbeads, which can be distinguished by their fluorescence intensity in a flow cytometer and the second one is the capture antibodies. Antibodies are spotted at defined positions on a 2-dimensional array (Vostrý M., 2010).

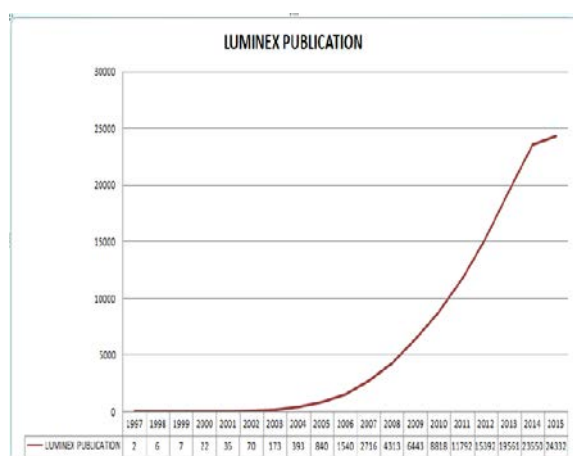


Figure 1: the number of luminex publications from 1997 to 2015.

The literature on suspension immunoassay technology dates back to the 1970s when Horan and Wheelless mentioned in a review

some of the various applications of flow cytometry that antigen attached micro-particles could form a solid support for the capture of blood-borne antibodies. Moreover, by adopting differentially sized microspheres distinguishable by their light scatter properties, several analytes may be detected simultaneously. This approach improved in the 1980s and 1990s to include DNA and lipids (Nolan *et al.*, 2006). The suspension format includes platforms such as Luminex<sup>TM</sup>, cytometric bead arrays and bio-PlexPro<sup>TM</sup> whereby high-affinity capture ligands are immobilized discretely on fluorescently activated plastic microbeads and mixed with the sample in liquid phase (Tighe *et al.*, 2015). In the late 1990's, scientists at Luminex<sup>®</sup> Corporation designed the first specific flow cytometer for multiplexed microbead analysis. The corporation began the commercial production of its first generation system in 1997. FDA approved the first suspension array in 2007 chiefly for allergies, autoimmune or infectious diseases (Ellington *et al.*, 2010). In 2015, xMAP technology enabled the research reported in 24304 peer-reviewed publications, while there were just two publications in 1997 (Figure 2).

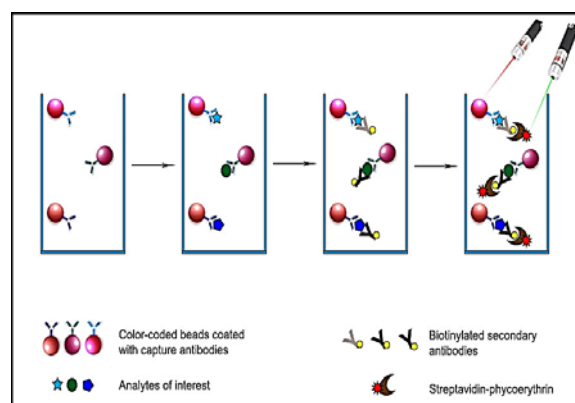


Figure 2: Luminex immunoassay principle for detecting protein analytes in biological fluids

### Principle of xMAP technology:

The luminex is a relatively novel multi-analyte profiling (xMAP) bioassay based on an antigen-antibody interaction, flow cytometry, lasers and digital signal processing technology (Ranjan *et al.*, 2015). Currently, the xMAP technology uses up to 500 (500-plex) distinct sets of either paramagnetic (6.5  $\mu\text{m}$ ) or

polystyrene (5.6  $\mu\text{m}$ ) microsphere beads, allowing up to 500 different analytes to be detected and quantified simultaneously in a single reaction vessel of a 96-well microplate-format assay. As shown in Figure 2, the beads are internally coded with red and infrared fluorophores of different fluorescent intensities and each spectrally addressed bead is given a unique number, or bead region, allowing the differentiation of one bead from another. The surface of the beads allows a covalent coupling of various molecules specific to a number of biological assays, including immunoassays (proteomic assays), nucleic acid assays, enzyme activity assays or receptor-ligand assays (Reslova *et al.*, 2017).



Figure 3: ??????

A capture sandwich format is commonly used for xMAP protein assays where each bead set can be coated with specific monoclonal antibodies specific to a particular antigen, allowing the capture of specific analytes from a test sample. A biotinylated secondary antibody cocktail then streptavidin-R-phycoerythrin (PE) conjugate are added for the subsequent detection (Angeloni *et al.*, 2016).

The resuspended beads are analyzed using a dual-laser flow-based detection instrument, such as the Luminex 100/200™, Luminex FLEXMAP3D®, the equivalent Bio-Rad®BioPlex®, or the Luminex/Bio-Rad MAGPIX® analyzers (R&D Systems). The red laser (635 nm) excites the internal dyes of the microspheres. The emitted light can be detected at wavelengths of 660 nm (red) and 730 nm (infrared), thus the beads can be classified into sets according to their spectral signature and the analytes can be identified

concomitantly. If the analytes of interest are present, the second green laser (525-532 nm) excites the reporter fluorophore (PE) and the magnitude of the PE-derived signal is detected at 576 nm, which is directly proportional to the amount of the bound analytes (Lachmann *et al.*, 2013). Each bead set must be detected at least 100 times in order to further validate the results (Glais *et al.*, 2017).



Figure 4: ???????

#### Applications of luminex technology

Multiplexing luminex test has quickly become standard in research, pharmaceutical and clinical laboratories. Luminex xMAP technology provides more data from each sample of plasma, serum or tissue culture supernatant. This technology is highly desirable to laboratories due to its speed, reducing time, versatility, accuracy, flexibility and reproducibility. It could increase the predictability of early drug development and identifies non-invasive biomarkers of toxicity or efficacy. Over the past 10 years, tremendous progress has been made in the evolution and amelioration of genomic and proteomic technologies for the recognition of novel drug targets and molecular signatures related to clinically important disease states, disease subsets, or differential responses to therapies. In addition, the evolution of high-throughput technologies for genomic and proteomic analysis has ushered in a novel era of

biomarker discovery, which has thus far yielded numerous new markers, many of which maintain an undefined pathophysiological significance and limited practical clinical application (Trojanowski *et al.*, 2010; Brooks, 2012; Vanmassenhove *et al.*, 2013).

#### **Practices of multiplex technology in cancer research**

Recently, the proteomic era has emerged after the completion of human genome sequencing project. All over the world, cancer is known as a complicated disease and need a huge effort to find new treatments. Therefore, cancer provides a fertile environment to study proteomic applications. A great deal of scientific effort focus on research for the identification of biomarkers for cancer and their future use as diagnostic for early detection and validating clinical trials of cancer drugs. Most investigations of biomarkers focus on tyrosine kinases and their phosphorylation states as well as cytokines as an immune-mediated tumor eradication using genetically engineered receptors to activate T cells (Hanash *et al.*, 2011).

Traditional enzyme-linked immunosorbent assay (ELISA) cannot provide the required multiplexing to meet cancer researcher's needs. Therefore, cancer research trails need a reproducible and sensitive assay system that can simultaneously interrogate large numbers of nucleic acid or protein targets per sample, large number of samples, in a short time, and easily adapt to researchers changing needs. Comparing with single-plex ELISA or low-multiplex real-time PCR assays, a microsphere-based fluorescent system that utilizes a large number of bead types, each carrying a specific antibody or oligonucleotide, can provide high multiplex capability required for cancer research (Carretero *et al.*, 2010).

#### **Luminex in cancer research**

In luminex system, different fluorescent and spectrally resolvable beads coated with distinct antibodies to various cytokines are incubated with biological sample. Applications in breast cancer (BC) have included cytokine profiling on serum of patients to generate a cytokine signature of HERs-based vaccination (Debqanzada *et al.*, 2007). Another study was

done on 27 patients achieving pathological complete or partial responses compared with 16 patients demonstrating no response. Serum levels of epidermal growth factor receptor (EGFR), soluble Fas ligand, migration inhibitory factor and matrix metalloproteinase-2 were shown to be higher in responders than in non-responders (Nolen *et al.*, 2008). Another study investigated that serum concentration of EGFR, soluble CD40 ligand and proapolipoprotein A1 levels were increased in BC patients (Kim *et al.*, 2009). On the other hand, 27 cytokines were profiled in 78 breast tumor interstitial fluid (TIF) samples, 43 normal interstitial fluid (NIF) samples and 25 matched serum samples obtained from BC patients with luminex xMAP multiplex technology. Eleven cytokines exhibited significantly higher levels in the TIF samples compared with the NIF samples: interleukin (IL)-7, IL-10, fibroblast growth factor-2, IL-13, interferon (IFN) $\gamma$ -inducible protein (IP-10), IL-1 receptor antagonist (IL-1RA), platelet-derived growth factor (PDGF)- $\beta$ , IL-1 $\beta$ , chemokine ligand 5 (RANTES) vascular endothelial growth factor, and IL-12 (Espinoza *et al.*, 2016).

Lung cancer is the leading cause of cancer deaths in the United States, with more than 190,000 cases per year (Bigbee *et al.*, 2012). Sensitive and specific lung cancer biomarkers, measured in noninvasively collected biospecimens such as serum, could help guide clinical decision making regarding the level of lung cancer risk.

Multiplexed serum immunoassays were performed using the Luminex Corporation xMAP technology facilitates quantitation of up to 100 soluble analytes in a single sample. Wide ranges of 70 cancer-associated serum biomarkers were analyzed in samples from lung cancer patients and matched controls. Regarding this concept, 8 biomarkers: prolactin, TTR, thrombospondin-1, E-selectin, C-C motif chemokine 5 (CCL5, RANTES), macrophage MIF, plasminogen activator inhibitor 1 (PAI-1 and SERPINE 1), and receptor tyrosine-protein kinase erb B-2 were established for lung cancer diagnosis with SN of 02.9% and SP of 87.5% (Kaneko *et al.*, 1996). Other investigations established the



advantage of Luminex technique with multiplex a panel of 14 tumor-associated autoantigens in detecting lung cancer patients. For instance, p53 autoantibody was detected in 15% of cancer patients (Mack *et al.*, 2000; Sangrajrang *et al.*, 2003). Autoantibody to NY-ESO-1, BIRC, and p62 were also detected in lung cancer patients with 20%, 19.5% and 18.8% SN, respectively (Tureci *et al.*, 2006; Ma *et al.*, 2010; Liu *et al.*, 2011). Recently, exploiting the immune response to tumors provides a unique opportunity for developing new tools for the serological detection of cancer as well as a lead for therapy (Jia *et al.*, 2014).

#### miRNA by Luminex

Several studies suggested that miRNAs may play an important role in the development of lung cancer and its pathogenesis (Eder and Scherr, 2005; Hammond, 2006). Wang *et al.* (2012) performed the analysis of xMAP with oligoribonucleic acid references to detect the expression of miR-21, miR, miR-31, miR-222, miR-145 and miR-40 NSCLC cancer tissues and adjacent normal tissues by xMAP bead based suspension array. The Luminex xMAP array for detecting miRNA allows a smaller sample size (only 2  $\mu$ l), no sample amplification, fast detector, high throughput, and flexible combination of multiple detection targets. In comparison with RT-PCR, the intrassay and interassay coefficients of variation were lower in xMAP than in RT-PCR. xMAP proved cheaper and more flexible in detecting multiple miRNAs of one sample

In routine work concepts, multiplex panel of blood biomarkers relied 95.3% SN and 99.4% SP for the detection of ovarian cancer (Visintin *et al.*, 2008). Moreover, an ovarian cancer marker panel including leptin, prolactin, osteopontin, insulin-like growth factor II (IGFII), macrophage migration inhibitory factor and CA125 was tested for multiplex measurements. All levels of tumor markers were significantly higher in cancer group compared with the control (Ulcova *et al.*, 2009). Another investigation was done for clinical validation of biomarkers for pancreatic cancer (Park *et al.*, 2017). Four proteins: apolipoprotein A-IV, apolipoprotein CIII, insulin-like growth factor

binding protein 2 and tissue inhibitor of metalloproteinase 1 were significantly altered in pancreatic cancer in both the discovery and validation phase. Luminex-based clinical assay was developed and validated for measurement serum IgM specific to BG-Atri as predictive biomarker (Lucas *et al.*, 2017).

#### Multiplex cytokine as biomarkers for occupational exposure assessment.

Inflammation is an adaptive response involving soluble mediators and specialized immune cells that is triggered in response to infection, trauma, ischemia, toxicants, or other forms of injury (Medzhitov, 2008). For example, engineering nanomaterials (ENMs) may act as inducers and stimulate the production of inflammatory mediators by innate immune cells (Bhattacharya *et al.*, 2013). Studying the panel of inorganic ENMs, Wiemann *et al.* (2016) reported that an *in vitro* alveolar macrophage assay was predictive of short-term inhalation toxicity of ENMs.

Adverse health effects can result from occupational exposure to dust, fine particles, or toxic substances. Accumulating evidence demonstrates that particulate air pollutants can cause both pulmonary and airway inflammation. Peripheral blood of individuals exposed during 10 and 20 years to coal fired power plants assessed for inflammatory cytokine profile using xMAP technology (Luminex<sup>®</sup> 200TMxMAP). The changes of the *in vivo* and *ex vivo* inflammatory cytokine profile exhibit early statistically significant differences in exposed groups compared to control (Codorean *et al.*, 2011). The effects of organic compounds e.g. polycyclic aromatic hydrocarbon (PAHs) and carbon black particles together or human THP-1-derived macrophages result in changes in secretion of IL-8 (Goulaswe *et al.*, 2008). On the other hand, pulmonary endothelial cells exposed to aqueous cigarette smoke extract and ambient ultrafine particles gives rise to much higher expression of interleukin 6 (IL-6) than the both exposure agents alone (Mo *et al.*, 2012). Co-exposure with fullerene with by products to human THP-1-derived macrophages resulted in secretion of pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  (Lehto *et al.*, 2014).

The ELISA technique had a minimum detectable concentration (MDC) of 0.06 µg/ml in human serum samples. The reliable detection limit (RDL) was 0.09 µg/ml, while the dynamic range was 0.06 to 1.7 µg/ml. In addition, its diagnostic specificity was 94.2% for clinically verified cases of anthrax. On the other hand, Luminex xMAP technology exhibited dynamic range 0.006-6.8 µg/ml, with SP value 100%.

### **Toxiroteomics by luminex**

Proteomics in Toxicoproteomics can increase the speed and sensitivity of toxicological screening by identifying protein markers related to toxicants, i.e. the discovered full range of proteins in a biological sample can be identified, this allows an individual protein or group of proteins to be associated with a disease/toxicity and their subsequent use as biomarkers. Proteomics studies have already provided insights into the mechanisms of action of a wide range of substances, from metals to peroxisome proliferators (Kennedy, 2002). Investigation studies may help to identify new molecular targets for toxicants or provide novel insights into mechanisms of action. The belief that a specific group or class of compounds will induce specific patterns of protein expression changes provides a basis for the application of proteomics to predictive toxicology. Such patterns or fingerprints could be used, for example, to screen novel compounds and to study structure-activity relationships (SARs) within a group. Barbara *et al.* (2004) mentioned that luminex technology could be used in proteomics examination for its potential in discovery of new biomarkers and toxicity signatures, in mapping serum, plasma, and other biofluid proteomes, and in parallel proteomic and transcriptomic studies. A number of emerging technologies such as Luminex have made it possible to measure several proteins in a single sample in a reliable and rapid way, in multi-well plates, such that large numbers of samples can be analyzed and run in a high throughput manner (Lock 2010). Luminex® technology expands the range of analytes measured in a single sample to as many as 27 by using microspheres in solution phase as the solid support for trapping

antibodies. These assays are high-throughput and offer detection levels as low as the femtomolar range, but they are currently unsuitable for development of a bedside assay of toxicity.

Test assays for four proteins associated with oxidative stress (transferrin, NFκB, HSP60 and HSP70) were developed using this methodology and then multiplexed. Further investigation of the effect of HSP70 on the HSP60 assay allowed development of a combined model for both proteins and the successful incorporation of the fourth assay into the multiplex (Lowe *et al.*, 2003). Development of these assays facilitates the future implementation of multiplexed LabMAP™ technology in predictive toxicity screens.

### **Future of Luminex in Egypt**

In this review, we attempt to capture recent developments in the application of genomic and proteomic technologies include luminex bead-based. By discussing examples covering a diversity of its using in many fields. Multiplex confers abundant features over broad biological assays as well as raised adequacy at a reduced expense, greater output per sample volume ratios and higher throughput predicating more resolute detailed diagnostics and facilitating personalized medicine. It represents flexible multiplexing of 1 to 500 analytes that meet the needs of a wide variety of applications. Multiplex confers abundant features over broad biological assays as well as raised adequacy at a reduced expense, greater output per sample volume ratios and higher throughput predicating more resolute detailed diagnostics and facilitating personalized medicine (Patrick *et al.*, 2015).

Luminex xMAP Technology is immensely used for nucleic acid applications, involving single nucleotide polymorphism (SNP) genotyping, molecular genetics, molecular detection for infectious diseases, microRNA profiling. It is a potentially powerful tool for use in proteomic biomarker profiling for toxicity prediction, unlike conventional technologies that can only measure one or a few biomarkers. Researchers have the capability to easily scale up or down the number of biomarkers measured and to

customize assays. Numerous serological immunoassays have been described for multiplexed, simultaneous measurement of antibodies to a variety of pathogens and to monitor vaccine effectiveness. Multiplex serological assays are very beneficial for deciding the immunoreactive epitopes on antigens and may help recognize candidates for vaccine development. Several researchers in rapid screening for Cancer detection assays have applied luminex xMAP. Challenges for diagnostic clinics include awareness of proteome complexity in clinical samples, the effects of high-abundance proteins, such as albumin, that could mask detection of other and low abundance disease proteins or biomarkers, (Lea *et al.*, 2009). Standardized approaches to sample collection and preparation, new analytical techniques and novel algorithms for bio-statistical analysis will facilitate release of the great potential of clinical multiplex diagnostic proteomics.

Kits for different samples e.g. human, mouse, rat, canine, monkey and porcine are available to cover various model organisms, with permanently growing number of covered analytes and species. Furthermore, custom assays can be built by antibody coupling to magnetic beads and Luminex Corp. provides support for their customers in custom assay development. Eventually, Clinical multiplex diagnostic proteomics considered to be the application of proteomic technology to improve a patient's clinical outcomes. Therefore, we expected that Luminex Technology will growing rapidly in all universities and medical centers in Egypt.

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