Luminex®

Whitepaper

The Role of Multiplexed Assays in Vaccine Development

Introduction

Vaccine development is a complex and lengthy process, involving multiple stages. The goal of the first stage is to understand the disease burden and pathogenicity of a disease, including all of the variants of the disease-causing organism, and identify the antigens. Next, the preclinical stage of development begins with antigen characterization and purification, in order to develop vaccine candidates. The immunogenicity and safety of the candidates are determined in animal models. Following successful preclinical development, the vaccine enters clinical development, which is divided into three phases. Safety, efficacy and immunogenicity are assessed through the first three clinical trial phases, with increasingly larger numbers of subjects. Once the vaccine has been approved and released, Phase IV trials are conducted to determine any new indications for the vaccine, which could again involve assessment of immunogenicity and efficacy.

Genotyping the Disease-Causing Organism

Successful vaccine development requires a clear and in-depth understanding of the variants of the disease-causing organism and their modes of pathogenesis, as well as the antigens they express. This can be quite a daunting task, as the organism may have a large number of variants. For example, more than 150 genotypes of Human papilloma virus (HPV) have been identified, 18 of which are associated with cervical cancer, and more than 50 of which have been detected in the anogenital region.[1] An assay is needed that can detect all of these variants simultaneously, in order to fully understand how to develop an effective vaccine.

Understanding Immunogenicity

A clear thread across the vaccine development process is the ongoing need for an understanding of the immunogenicity of the vaccine at every stage. Given that multiple variants of the disease-causing organism may express multiple antigenic variants, immunogenicity can become complex. Again, an assay that is capable of distinguishing many antigens and antibodies is a must.

A key to the efficacy of a vaccine is the strength and safety of the immune response. This response involves the expression of multiple cytokines that not only mediate the immune response but also promote inflammation, which could lead to an adverse reaction. The evaluation of multiple cytokines is required for monitoring the immune response.

A Multiplex Assay System for the Vaccine Development Process

Traditional enzyme-linked immunosorbent assays (ELISA) cannot provide the required multiplexing to meet the needs posed by vaccine development. What is required is a reproducible and sensitive assay system that can simultaneously interrogate large numbers of nucleic acid or protein targets per sample, across a large number of samples, in a short time, and easily adapt to the vaccine developer's changing needs.

Rather than low-multiplex ELISA or real-time polymerase chain reaction (PCR) assays, a microsphere-based fluorescent system that utilizes a large number of bead types (as many as 500), each carrying a specific antibody or oligonucleotide, can provide the high multiplex capability required for vaccine development.

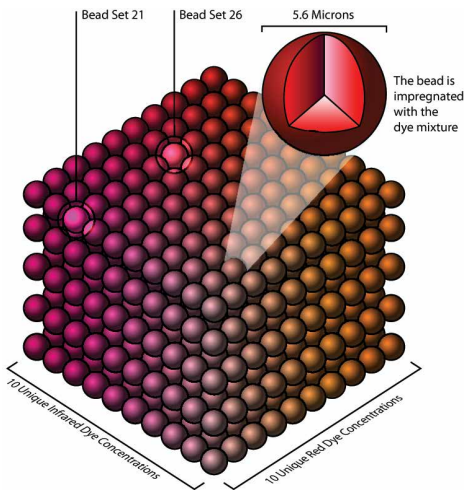

Figure 1. Luminex internally color-codes microspheres with precise concentrations of various florescent dyes, yielding up to 500 distinctly colored bead sets.

In Luminex® xMAP® Technology, a laser or an LED array is used to interrogate a specific mixture of fluorescent dyes in each bead type that is characteristic for that bead type (Figure 1). Once an analyte is bound to the capture ligand on each bead type, a reporter dye is used to quantify the amount of analyte bound. Either flow cytometry fluidics or an imaging device is then used to determine which beads are present and the amount of analyte on each bead.

Depending upon the instrument used, up to 500 bead types can be used in each well of a 96- or 384-well plate, thus generating a very high throughput assessment of a large number of protein or oligonucleotide targets. This microsphere "liquid array" assay system is unique in its ability to provide both high-throughput and high-content data, and the ligands can be readily changed to meet the needs of the project. Multiplexing reduces cost and labor, and preserves precious sample.

Genotyping Assays for Disease Causing Organisms

The ability to genotype the variants of the organism and determine their association with the severity of the disease is essential to understanding the etiology of the disease and determining which variant(s) to direct the vaccine against. During clinical trials, genotyping from subject specimens is necessary to determine the effectiveness of the vaccine. These steps in vaccine development provide an ideal application for xMAP Technology.

For example, multiple vaccines have been developed against the Human papilloma virus (HPV). To determine the efficacy of the vaccine, a very large number of HPV genotypes associated with a high risk of cervical cancer must be differentiated. Oligonucleotide-based microsphere multiplex assays have been developed to accomplish this daunting task.

The MPTS123 PCR assay utilizes three type-specific PCRs (designated MPTS1, MPTS2, and MPTS3) that generate small biotinylated amplicons of 55 to 139 bp from the HPV E6 region of the viral genome specific for 16 HPV genotypes.[1] The mixture of biotinylated PCR products derived from a sample was added to a microsphere bead set containing immobilized HPV probes for all of the genotype-specific amplicons. The mixture was then heated to denature the double-stranded PCR products and allow subsequent hybridization under stringent conditions. After stringent washing to remove unbound PCR product, the beads were incubated with streptavidin-conjugated R-phycoerythrin (SAPE) to detect all of the bound amplicons. This assay has been used to identify significantly more genotoypes in cervical biopsy specimens than the PCR DNA enzyme immunoassay (DEIA) LIPA system, including types 16, 35, 39, 45, 58, and 59.[1]

The digene® HPV Genotyping LQ test uses a similar PCR and microsphere-based amplicon hybridization assay to differentiate 18 HPV types using amplification of specific sequences on the L1 viral genome region.[2] In 219 specimens found to be negative by a competitive assay, the LQ test still detected at least one of the high-risk genotypes in 5.5% of the specimens.

A third PCR based genotyping assay based on xMAP Technology, known as HSL-PCR, has been used for the analysis of all known wart-associated HPV types.[3] It has also been used to accurately identify HPV types in surface swabs of cutaneous warts.[4]

Immunogenicity: Generating Effective Antibodies

The efficacy of a vaccine is dependent on its ability to stimulate the production of antibodies that can neutralize the infectious agent. Since multiple variants of the disease-causing agent may be involved, the simultaneous detection and quantitation of multiple antibodies is essential. The xMAP Technology is ideally suited to this task, and in fact xMAP multiplex antibody assays have played a key role in the development of HPV vaccines.

One of the two HPV vaccines on the market, Gardasil®, is a quadrivalent vaccine designed to solicit an antibody response to HPV Types 6, 11, 16, and 18. This vaccine utilizes a virus-like particle (VLP) to generate the immune response. In order to evaluate the effectiveness of the vaccine, a competitive Luminex immunoassay (cLIA) was developed that employs four VLPs, each specific to a different HPV type, bound to microspheres that are used in a competitive Luminex immunoassay, to quantitate antibody titers in treated subjects.[5,6] Known, type-specific dye-labeled neutralizing antibodies for the four HPV types compete with patient serum antibodies for binding to the immobilized VLPs. Relative inhibition is compared to a pooled reference serum to quantitate the antibody titers.

The Multiplex Competitive Assay for HPV Immunogenicity

This assay has been used extensively in the evaluation of the Gardasil vaccine and other applications. For example, in a randomized population of young women that received the vaccine, vaccine-induced HPV antibody levels were 12 to 26 fold higher than those in non-immunized women. The response reached a plateau and remained stable through the end of the three-year study.[7] A clinical trial of women aged 24-45 years also demonstrated the safety, immunogenicity, and efficacy of the quadrivalent vaccine, using the cLIA method,[8] and a third study utilized this assay to prove efficacy in a group of HIV-infected children 7 to 12 years old.[9] These are just a few of the many studies that have been completed using the cLIA format.

The vaccine has also been shown to be immunogenic in HIV-1 infected men at risk for anal cancer from HPV, using the cLIA method.[10] Infection by HPV can lead to anogenital warts, and several types of oral and anogenital cancers in men. Using the cLIA, it was shown that at month 36, 88.9%, 94%, 97.9%, and 57% of subjects treated with the quadrivalent vaccine were still seropositive for HPV-6, -11, -16, and -18, respectively.[11] This is an example of a Phase IV clinical trial for additional indications for the vaccine.

In a study to determine the relationship between seropositivity to HPV16 following natural infection and risk of subsequent HPV16 infection, the cLIA was shown to capture a more specific measure of protective immunity than the ELISA or secreted alkaline phosphatase protein neutralization assay (SEAP-NA).[12]

Direct Assays for HPV Immunogenicity

Direct binding IgG assays can also be used to measure antibody levels to the HPV VLPs. These assays are sensitive, reproducible, simple to perform, and amenable to high-throughput testing.[13] The VLP for each HPV subtype is bound to the microspheres in the same fashion as the cLIA competitive format, but then serum

antibodies are measured by direct binding to the VLP's and quantitated using a phycoerythrin-conujugated anti-IgG antibody. A direct binding assay specific for the HPV 6, 11, 16, 18, 31, 33, 45, 52, and 58 VLP types has been developed and used to provide detection of serum antibody levels to these nine HPV types that was approximately 5-fold more sensitive than the cLIA previously used by the same research group.[13]

A direct assay has also been developed for HPV 16, 18, 31, 33, 45, 52, and 58 and used to measure changes in seroprevalence between two nationwide surveillance studies in the Netherlands, conducted 10 years apart.[14] An increase in HPV16 seroprevalence was measured across the 10 year period, which could be due to changes in sexual behavior, particularly in age of sexual debut (Figure 2).

HIV Immunogenicity

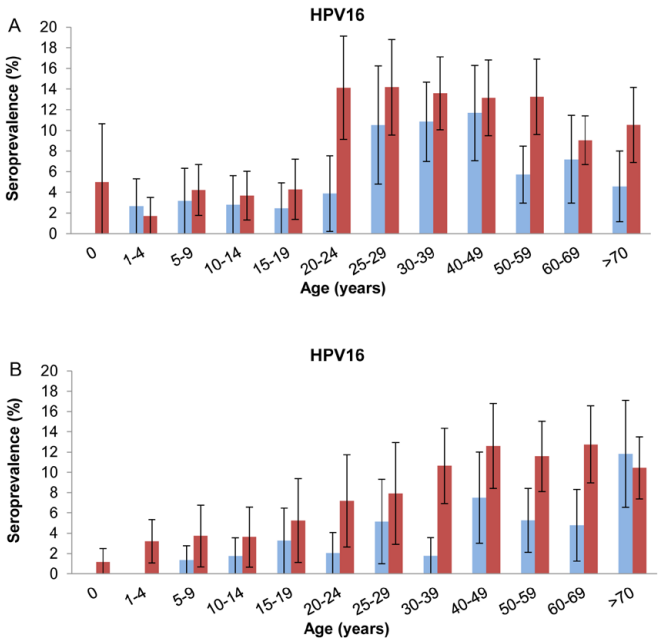

Figure 2. Comparison of trends in HPV16 antibody seroprevalence. Seroprevalence among women (A) and men (B) in the 1995-1996 (blue bars) and 2006-2007 (red bars) surveys.
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xMAP Technology has also been used to develop custom tests for direct measurement of antibody levels in response to HIV infection and vaccine candidates. One assay was used to characterize antibody responses in HIV-infected individuals to the ectodomain of the HIV-1 gp41 envelope glycoprotein.[15] The antigen panel included intact recombinant gp41, the fusion peptide region, the polar region, the N-heptad region, and the C-heptad region as well as overlapping epitopes in the 2F5 and 4E10 monoclonal antibody-binding regions. The results revealed a broad pattern of immune responses against the test antigens.

Direct assays for HIV antigens have also been used to determine immunogenicity for two vaccines in Phase I clinical trials. An xMAP assay for antibodies to gp140 envelope proteins induced by a replication-defective adenovirus Type5 HIV vaccine was used to demonstrate that the vaccine induced antibodies against at least one HIV-1 Env antigen in all recipients.[16] Microspheres coupled to recombinant proteins originating from clade C/ZA were used in an xMAP assay to determine immunogenicity induced by three doses of Advax DNA vaccine plus one dose of recombinant modified vaccinia virus Ankara (Group A), or three doses of the Ankara vaccine alone (Group B). Antibody responses were dramatically better in Group B than Group A. However, only weak neutralizing antibodies were induced, confirming several other studies).[17]

Malaria Immunogenicity

A multiplex xMAP assay for 10 malaria antigens was used to screen children under the age of 6 in Ghana to identify antigens which induce antibodies that correlate with protection from *P. falciparum* malaria. The data suggest that a multivalent vaccine using several antigens would be the most effective.[18] A second assay was used in a clinical trial of the RTS,S vaccine to measure the levels of four surface antigens of the R29 laboratory strain. Unfortunately, the vaccine had only a modest effect.[19]

Personalized Peptide Vaccination for Cancer

Promising results for cancer vaccination have been obtained with a personalize peptide vaccine approach. It involves challenging pre-vaccination patient blood with a large number of human leukocyte antigen (HLA) peptides. Those peptides that produce an immune reaction are then used to immunize patients in clinical trials. The xMAP multiplex technology is used to measure the resultant level of antibodies to the peptides. This approach has shown promising immunogenicity in patients with advanced malignant glioma,[20] refractory small cell lung cancer,[21] and castration-resistant prostate cancer,[22] as well as in patients with other types of cancer.

Cytokine Response as a Measure of Immunogenicity

The cytokine response to a vaccine is a widely used measure of immunogenicity during vaccine development, and xMAP Technology has become the assay platform of choice for measuring cytokine responses. Several highly multiplexed cytokine assays based on xMAP Technology are available commercially and are currently being used in all phases of vaccine development. For example, an assay for as many as 22 cytokines has been used as part of a novel approach for the elucidation of CD4+ T cell specificity in response to large pathogens that enabled the identification of immunoprevalent antigens following smallpox vaccination.[23]

Multiplex microsphere-based cytokine assays have also been used to measure immunogenicity elicited by the use of nanoparticles made of the coat protein of papaya mosaic virus as an adjuvant to improve the immune response to the trivalent inactivated flu vaccine.[24] Use of this adjuvant in mice and ferrets increased the magnitude and breadth of the humoral immune response to NP and HA antigens (Figure 3).

Such multiplex cytokine assays have also been used to evaluate the use of flagellin expression as an adjuvant in a *Lactobacillus acidophilus* strain used for vaccine delivery and expressing the HIV-1 Gag protein.[25] Enhanced production of some cytokines helped to confirm the adjuvant effect of the flagellin protein.

Vaccination with an allogenic gene-modified tumor line

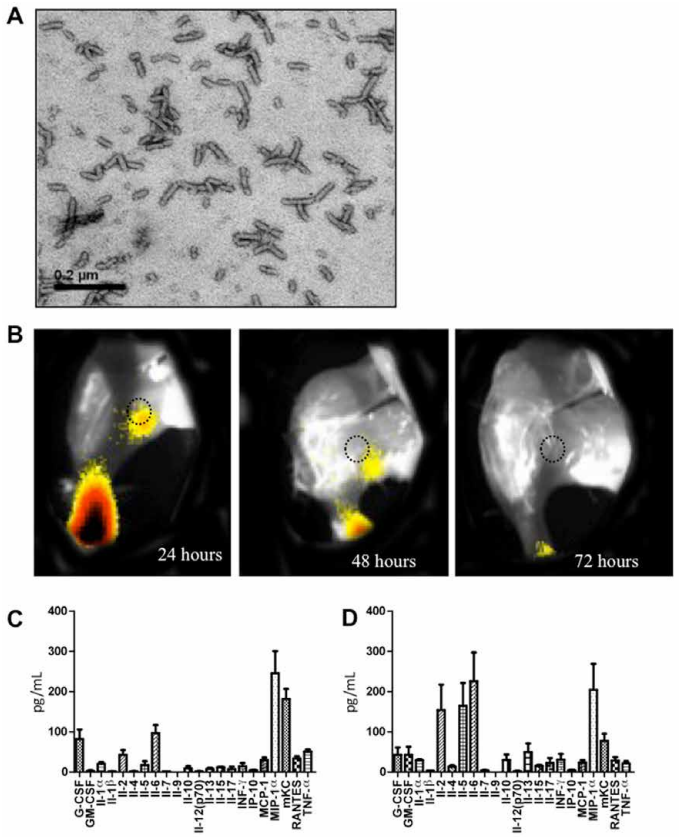

Figure 3. PapMV nanoparticles and the secretion of TH1/TH2 cytokines.
A. Observation of adjuvant PapMV nanoparticles by electron microscopy. Bar 0.2 mm.
B. In vivo imaging of fluorescently labeled PapMV nanoparticles. Data are presented as pseudocolor images indicating fluorescence (Alexa@680) intensity, with a gradation from red (more intense) to yellow, superimposed over gray-scale reference photographs of the left inferior member of the treated mouse. Images were taken at 24, 48 and 72 h post-injection. The proximal popliteal lymph node is indicated with a dotted circle. At 24 h, a strong signal is detected in the foot pad of the animal where the fluorescent protein was injected.
C,D. Cytokine/chemokine profile of splenocytes reactivated with PapMV nanoparticles (100 mg/ml) isolated after one (C) or two (D) subcutaneous immunizations.

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demonstrated feasibility and safety in a Phase 1 clinical trial, and substantial disease stabilization was observed in most patients. An effort was then made to determine the immune responses responsible for the effect. Using a multiplex xMAP assay, it was shown that IFN-γ, IL-2, IL-4, and IL-5 and IL-10 levels increased after multiple stimulation of PBMCs, while IL-1β, IL-6, IL-8, and TNF-α levels decreased.[26]

Several species of adenoviruses are currently being evaluated as vaccine candidates in clinical trials, but little is known about the innate immunity that each species might induce. A study done of three adenovirus serotypes (Ad35, 26, and 48) using an xMAP multiplex cytokine assay showed that the cytokine responses triggered by these species were substantially greater than those induced by Ad5 in rhesus monkeys.[27] All three of these species utilize CD46 as their primary cellular receptor, rather than the CAR receptor utilized by Ad5. Assessment of the impact of these profoundly different cytokine responses on vaccines utilizing the Ad 35, 26, and 48 serotypes will be required for successful vaccine development.

Luminex xMAP® Technology

Luminex provides the only flexible and open multiplexing technology used by several market leaders to provide assays for both gene and protein expression. 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. xMAP Technology combines advanced fluidics, optics, and digital signal processing with proprietary microsphere technology. Featuring a flexible, open-architecture design, xMAP Technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately. Focused, flexible multiplexing of 1 to 500 analytes meets the needs of a wide variety of applications, including genotyping, protein expression profiling and gene expression profiling.

All of the microsphere bead assays described in this white paper were developed using xMAP Technology. The open architecture of the system made it feasible for the researchers to create their own antibody assessment and genotyping assays, or obtain commercially available kits. An ever-expanding menu of assays for other applications is also available from Luminex and its commercial partners.

A Sampling of Institutions using Luminex xMAP® Technology for Vaccine Research & Development

Beth-Israel Deaconess Hospital, Boston, USA

Department of Virology, Ludwig Institute for Cancer Research, Sao Paolo, Brazil

Division of Cancer Epidemiology and Genetics, National Cancer Institute, USA

Duke Human Vaccine Institute, Duke University

Guangxi Center for Disease Prevention and Control, Guangxi, China

Infectious Disease Research Center, Laval University, Quebec City, Canada

Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, The Netherlands

Malaria Vaccine Development Branch, National Institute of Allergy and Infectious Disease, USA

Merck Research Laboratories, Wayne, PA, USA

Netherlands Vaccine Institute

Novartis Vaccines & Diagnostics GmbH, Marburg Germany

Pfizer Vaccine Research

United States National Institutes of Allergy and Infectious Disease

Vaccine Trial Center, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

REFERENCES

1. van Alewijk D, Kleter B, Vent M, et. al. A human papilloma virus testing algorithm comprising a combination of the L1 broad-spectrum Spf(10) Pcr assay and a novel E6 high-risk multiplex type-specific genotyping Pcr assay. J Clin Microbiol 2013;51:1171-8.
2. Geraets D, Lenselink C, Bekkers R, et. al. universal human papillomavirus genotyping by the digene Hpv genotyping Rh and Lq tests. J Clin Virol 2011;50:276-280.
3. de Koning MNC, ter Schegget J, Eekhof J, et. al. Evaluation of a novel broad-spectrum PCR-multiplex genotyping assay for identification of cutaneous wart-associated human papillomavirus types. J Clin Microbiol 2010;48:1706-11.
4. de Koning MNC, Khoe LV, Eekhof JAH, et. al. Lesional Hpv types of cutaneous warts can be reliably identified by surface swabs. J Clin Virol 2011;52:84-7.
5. Opalka D, Lachman C, MacMullen S, et. al. Simultaneous quantitation of antibodies to neutralizing epitopes on virus-like particles for human papillomavirus types 6, 11, 16, and 18 by a multiplexed Luminex assay 2. Clin Diagn Lab Immunol 2003;10:108-15.
6. Dias D, Van Doren J, Schlottmann S, et. al. Optimization and validation of a multiplexed Luminex assay to quantify antibodies to neutralizing epitopes on human papillomaviruses 6, 11,16, and 18. Clin Diagn Lab Immunol 2005;12:959-69.
7. Villa L, Ault K, Giuliano A, et. al. Immunologic responses following administration of a vaccine targeting human papillomavirus types 6, 11, 16, and 18. Vaccine 2006;24:5571-83.
8. Munoz N, Manalastas R, Pitisuttihum P, et. al. Safety, immunogenicity, and efficacy of quadrivalent HPV (types 6, 11, 16, 18) recombinant vaccine in adult women between 24 and 45 years of age: a randomized, double-blind trial. Lancet 2009;373:1921-2.
9. Levin M, Moscicki A, Song L, et. al. Safety and immunogenicity of a quadrivalent human papillomavirus (types 6, 11, 16, and 18) vaccine in Hiv-infected children 7 to 12 years old. J Acquir Immune Defic Syndr 2010;55:197-204.
10. Wilkin T, Lee JY, Lensing SY, et. al. Safety and immunogenicity of the quadrivalent human papillomavirus vaccine in HIV-1-infected men. *J Infect Dis*. 2010;202:1246-53.
11. Machalek DA, Poynten M, Jin F, et. al. Anal human papillomavirus infection and associated neoplastic lesions in men who have sex with men: a systematic review and meta-analysis. Lancet Oncol 2012 May;13(5):487-500.
12. Lin SW, Ghosh A, Porras C, et. al. HPV16 seropositivity and subsequent HPV16 infection risk in a naturally infected population: comparison of serological assays. PLoS One 2013; 8:e53067.10.1371/journal.pone.0053067
13. Opalka D, Matys K, Bojczuk P, et. al. Multiplexed serologic assay for nine anogenital human papillomavirus types. Clin Vaccine Immunol 2010;17:818-27.
14. Scherpenisse M, Mollers M, Schepp RM, et. al. Changes in antibody seroprevalence of seven high-risk Hpv types between nationwide surveillance studies from 1995-96 and 2006-07 in the Netherlands. PloS One 2012;7:U327 -4.
15. Opalka D, Pessi A, Bianchi E, et. al. Analysis of the Hiv-1 Gp41 specific immune response using a multiplexed antibody detection assay. J Immunol Methods 2004;287:49-65.
16. Peiperl L, Morgan C, Moodie Z, et. al. Safety and immunogenicity of a replication-defective adenovirus type 5 Hiv vaccine in Ad5-seronegative persons: A randomized clinical trial (Hvtn 054). PLoS One 2010;5:U99-U113.
17. Hayes P, Gilmour J, von Lieven A, et. al. (2013) Safety and immunogenicity of DNA prime and modified vaccinia ankara virus-Hiv subtype C vaccine boost in healthy adults. Clin Vaccine Immunol 2013;20:397 - 408.
18. Dodoo D, Atuguba F, Bosomprah S, et. al. Antibody levels to multiple Malaria vaccine candidate antigens in relation to clinical Malaria episodes in children in the Kasena-Nankana District of Northern Ghana. Malaria Journal 2011;10:U1-U9.
19. Campo JJ, Dobano C, Sacarlal J, et. al. Impact of the Rts,S Malaria vaccine candidate on naturally acquired antibody responses to multiple asexual blood stage antigens. PLoS One 2011;6:U381-9.
20. Yajima N, Yamanaka R, Mine T, et. al. Immunologic evaluation of personalized peptide vaccination for patients with advanced malignant Glioma. Clin Cancer Res 2005;11:5900-11.
21. Terazaki Y, Yoshiyama K, Matsueda S, et. al. Immunological evaluation of personalized peptide vaccination in refractory small cell lung cancer. Cancer Science 2012;103:638-44.
22. Uemura K, Miyoshi Y, Kawahara T, et. al. Prognostic value of a computer-aided diagnosis system involving bone scans among men treated with docetaxel for metastatic castration-resistant prostate cancer. *BMC Cancer*. 2016;16:109.
23. Judkowski V, Bunying A, Ge F, et. al. Gm-Csf production allows the identification of immunoprevalent antigens recognized by human Cd4+ T cells following smallpox vaccination. PLoS One 2011;6:U127.
24. Savard C, Guerin A, Drouin K, et. al. Improvement of the trivalent inactivated flu vaccine using Papmv nanoparticles. PLoS One 2011;6:U314 - U323.
25. Kajikawa A, Zhang L, Long JL, et. al. Construction and immunological evaluation of dual cell surface display of Hiv-1 Gag and Salmonella Enterica Serovar Typhimurium Flic in Lactobacillus Acidophilus for vaccine delivery. Clin Vaccine Immunol 2012;19:1374-81.
26. Pohla H, Buchner A, Stadlbauer B, et. al. High immune response rates and decreased frequencies of regulatory T cells in metastatic renal cell carcinoma patients after tumor cell vaccination. Molecular Medicine 2012;18:1499-1508.
27. Teigler JE, Iampietro MJ, Barouch DH. Vaccination with adenovirus serotypes 35, 26, and 48 elicits higher levels of innate cytokine responses than adenovirus serotype 5 in rhesus monkeys. J Virol 2012;86:9590-8.