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Multiplex Immunoassay and Bead Based Multiplex

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1. Introduction

1.1 Background

Protein immunoassays provide information about quantities and forms of endogenous proteins. Uniplex enzyme immunoassays like Elisa have been the workhorse for protein measurement for decades, but they can be laborious and expensive and consume relatively large amounts of specimen. In comparison to the ELISA for a single analyte, multiplex assays offer the possibility of obtaining more reliable quantitative information in a highly parallel analysis. In addition, quantitative multiplexed assays offer the possibility to identify combinations of biomarkers with higher disease specificity than any single established biomarker alone. Because of these reasons, currently one-analysis, many-metabolites, many-diseases approach is receiving more attention than uniplex system with one-analysis, one-metabolite, one-disease.

1.2 Multiplex immunoassay platforms

Presently, antibody-based platforms are the core technology for protein multiplex arrays. Assay formats include suspension arrays (microbead assays) and planar arrays that use traditional immunometric principles.

Planar arrays: MULTI-ARRAY (Meso Scale Discovery), A2 (Beckman Coulter), and FAST Quant (Whatman Schleicher & Schuell BioScience), Searchlight (Aushon Biosystem).

Suspension arrays: Bio-Plex (Bio-Rad Laboratories), FlowCytomix (Bender MedSystems), cytometric bead array (Becton, Dickinson and Company) and the partners of Luminex Corp (XMAP)].

In the first format, different capture antibodies are spotted at defined positions on a 2-dimensional array. In the second, the capture antibodies are conjugated to different populations of microbeads, which can be distinguished by their fluorescence intensity in a flow cytometer.

Optimal assay performance of multiplex immunoassay platforms depend on proprietary information about the antibody pair, the composition of diluents, and the software. The accuracy of quantification for multiplexed immunoassays depends, as with all ELISAs, on the quality of the calibration curves, assay imprecision (CV), recoveries, and assay linearity

(the limits of quantification). Therefore, there is not a single assay platform suitable for all analytes. The selection of a platform or kit should depend on assay sensitivity, the relevant biological concentration to be measured

Platform	MesoScale discovery (MULTI-ARRAY/MULTI-SPOT)	Searchlight	FastQuant (FAST Quant System)
Capture antibody binding surface	Carbon	Plastic	Nitrocellulose
Detection system	Electrochemiluminescent	Biotinylated detector with fluorescent detection	Biotinylated detector with fluorescent detection
Analytes/plex	Up to 10	Up to 24	Up to 10
Image System	CCD camera based	CCD camera based	CCD camera based
Customised array	Yes	Yes	Yes
Kits for designing and building your own assay	Yes	No	No
Reagent company	Waterman Scleicher& Schuell Bioscience	Aushon Biosystem	Waterman Scleicher& Schuell Bioscience
Commercial instrument	Waterman Scleicher& Schuell Bioscience	Aushon Biosystem	Waterman Scleicher& Schuell Bioscience

Table 1. The characteristics of some commercial planar microspot array platforms

Platform	Luminex	Cytometric Bead Array (CBA)
Capture antibody binding surface	Fluorescently tagged beads	Fluorescently labeled beads
Detection system	Biotinylated detector with fluorescent detection	Phycoerythrin conjugated detectors
Analytes/plex	Up to 100	Up to 100
Image System	Luminex XMAP based system	Flow cytometer with dual laser
Customised array	Yes	Yes
Kits for designing and building your own assay	Yes	Yes
Reagent company	Luminex Corp and its partners(many others)	BD Becton,Dickinson and others
Commercial instrument	Lumine(XMAP) and its partners	BD Becton,Dickinson and others

Table 2. The characteristics of some commercial suspension bead array platforms

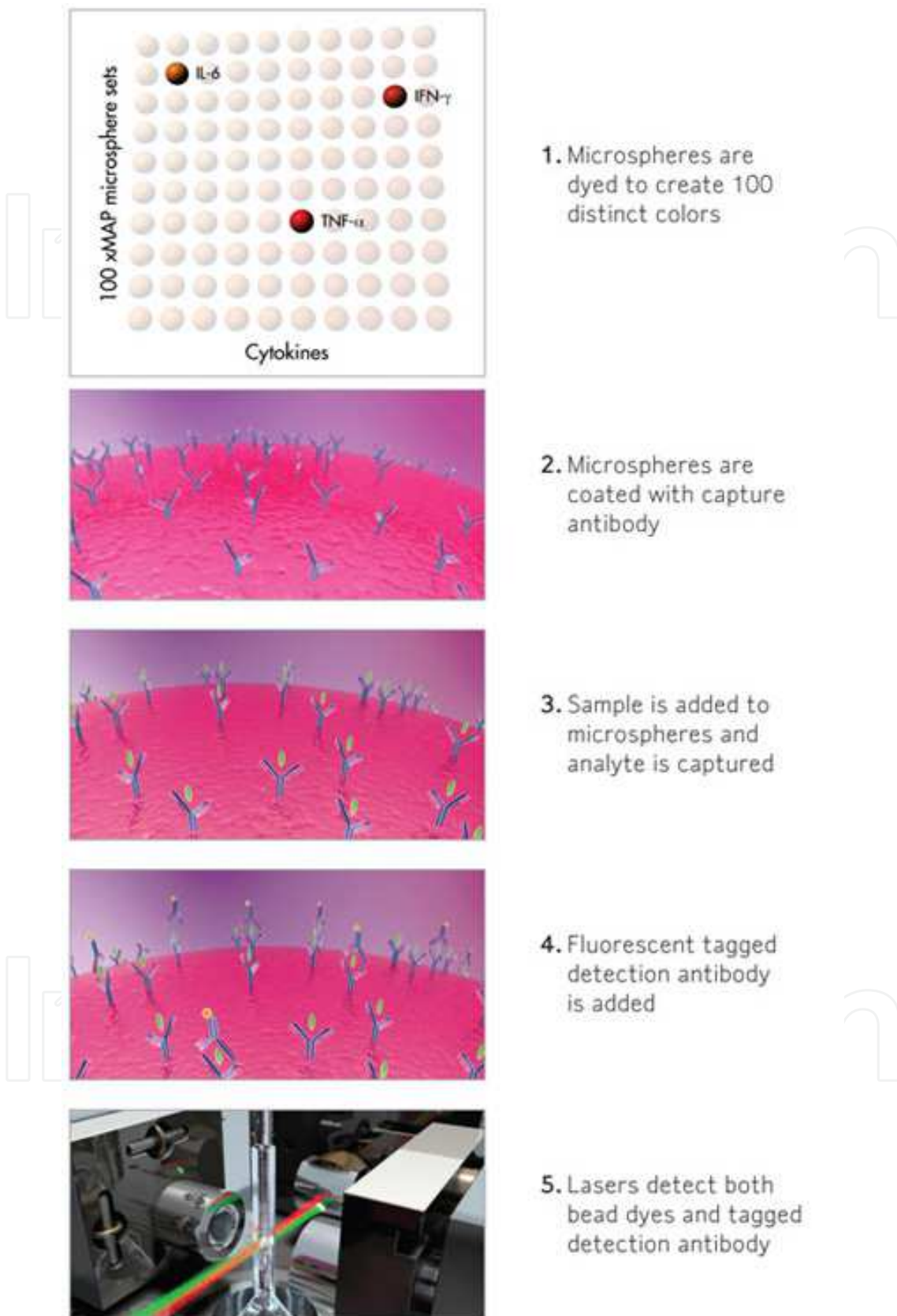


Fig. 1. Process of multiplexed immunodetection using fluorescent-coded beads (www.millipore.com)

2. Bead based multiplex

2.1 Background

In multiplex bead array assays (MBAA), beads of discrete fluorescence intensities and wavelengths provide a capture surface for specific analytes enabling detection of multiple analytes in a single sample. Bead based assays of analyte is an attractive strategy for obtaining large numbers of measurements rapidly. MBAA is an open access system; namely, the parameters to be measured can be determined by the user. The variety of source increases its area of application (nucleic acids, antigens, antibodies, receptors). Since the detection method is based on flow cytometry and this allows repeatability, MBAA performs the quality control of the measurement, simultaneously with the measurement itself.

The MBAA readers allow analysis of up to 100 reactions or assays per well. This remarkable capability allows incorporating a large number of assays, such as DNA, receptor-ligand, Immunoassay and enzyme in life science research, drug discovery as well as diagnostic areas.

2.2 The principle of measurement

These systems depend on measurement of fractional antibody occupancy using two different labels: one labeling the “capture” antibody, the second labeling a “detection antibody”, selected to react either with occupied or unoccupied sites on the “capture” antibody. The ratio of signals emitted by the two labeled antibodies reveals the analyte concentration to which the capture antibody has been exposed. An array of capture antibodies, each labeled with the same fluorescent label, is scanned (by a laser), and the fluorescent signal ratio emitted from each discrete antibody couplet in the array is measured.

2.3 Summary of principles

1. Color-coded beads, pre-coated with analyte-specific capture antibody for the molecule of interest are added.
2. Analyte-specific antibodies capture the analyte of interest. Biotinylated detection antibodies specific to the analyte of interest are added and form an antibody-antigen sandwich.
3. Phycoerythrin (PE)-conjugated Streptavidin is added.
4. The beads are read by a dual-laser flow-based detection instrument which excites the internal dyes marking the beads set and a second laser excites PE, the fluorescent dye on the reporter molecule (www.luminexcorp.com).

The system is capable of measuring potentially up to 100 analytes simultaneously in a small sample volume (25–50 μ L).

2.4 Advantages of the system

1. **Speed/High Throughput** – Because each microsphere serves as an individual test, a large number of different assays can be performed and analyzed simultaneously
2. **Versatility** – Bead based multiplex system can perform assays in several different formats, including nucleic acids and antigen-antibody binding, along with enzyme, receptor-ligand and other protein interactions

3. **Flexibility** – The technology can be customized for the user's specific needs based upon their analytes of interest
4. **Accuracy** – The technology generates real-time analysis and accurate quantification of the biological interactions (www.luminexcorp.com).

Advantages of this approach also include specimen conservation, limited sample handling, and decreased time and cost. However, it is difficult to optimize assay format for each protein, to select common dilution factors and to establish reliable quality control algorithms.

Characteristics	Antibody-based
Principle	Antibody-antigen interaction
Required reagents/information	Specific antibody pairs
Quantification basis	flowcytometer and others
Multiplexicity	1-100
Clinical diagnostic test	Yes
Sample enrichment	No
Sensitivity	>sub ng/mL-pg/mL
Reproducibility	Excellent
Assay development	Time and resource demanding
Consumable	High demand
Robustness	Yes
Throughput	High
Matrix effect	Yes
Sample manipulation	No
Automation	Yes
History	>15 years

Table 3. The characteristics of MBAA

2.5 Comparison with other technologies and assays

The assay which is most often compared to MBAA is the enzyme-linked immunosorbent assay (ELISA). ELISAs, in general, use a similar immobilized antibody to capture a soluble ligand, with subsequent detection of the captured ligand by a second antibody. There are, however, several substantial differences between MBAA and ELISAs. For example, MBAA uses fluorescence as a detection system where ELISAs use enzyme amplification of a colorimetric substrate. MBAA captures ligands onto spherical beads in suspension while ELISAs generally rely upon flat surfaces in 96-well plates. Most importantly, MBAA techniques, by their very nature, are multiplexed and therefore may be subject to any perturbations that arise from analyzing multiple ligands simultaneously, such as cross-reactivities. By contrast, ELISA methodologies generally study one analyte at a time, and thus avoid any concerns arising from multiplexing.

Multiplex system presents additional Quality control (QC) challenges compared to uniplex analyses. The failure of 1 constituent assay to meet QC specifications results in rejection of results for all assays on the panel. Samples failing QC specifications should be retested using the same measurement system, because substitution of a uniplex assay may introduce bias

due to differences in assay format. However, the probability of all assays simultaneously meeting QC specifications is much lower than the probability of a uniplex test passing QC. At present, reference guidelines for multiplex QC programs are under development by the Food and Drug Administration (FDA).

In theory, the MBAA platform may also provide a wider dynamic range than conventional ELISA methods because of the greater linear range of fluorescence intensity compared with absorbance. Detection limits of MBAA (luminex) and ELISA are different. A comparison of detection limits of some test for the Luminex assays and the ELISAs is shown in Table 4. Data Supplement (available at <http://www.clinchem.org/content/vol51/issue7/>) Disadvantage of MBAA are time consuming in assay development, lacking antibody pairs for new biomarkers and limited commercial multiplex assay kits.

Multiplex panels	Analytes	Luminex	ELISA	ELISA manufacturer
Linco Endocrine	Leptin	0.13 µg/L	1.39 µg/L	Linco
	Insulin	0.31 mIU/L	0.14 mIU/L	Alpco
	C-peptide	0.037 µg/L	0.132 µg/L	Linco
Biosource Chemokine	MCP-1	2.35 ng/L	2.57 ng/L	R&D
	Eotaxin	4.37 ng/L	3.80 ng/L	R&D
Upstate Chemokine	MCP-1	20.5 ng/L	2.57 ng/L	R&D
	Eotaxin	27.72 Eotaxin	3.80 ng/L	R&D
Linco Cytokine	TNF-α	1.61 ng/L	0.092 ng/L	R&D
	IL-8	2.52 ng/L	0.26 ng/L	Biosource
Upstate Cytokine	TNF-α	0.065 ng/L	0.092 ng/L	R&D
	IL-8	0.70 ng/L	0.26 ng/L	Biosource
	IL-6	0.75 ng/L	0.068 ng/L	R&D
R&D Cytokine	TNF-α	1.53 ng/L	0.092 ng/L	R&D
	IL-6	0.977 ng/L	0.068 ng/L	R&D

Table 4. Comparison of detection limits for the Luminex multiplexed assays and the individual ELISAs.

Protein microarray kits, which use capture antibodies and detection antibodies in a multiplex fashion similar to MBAA, should also be considered as a competing technology which is most commonly used for simultaneous determination of multiple proteins in a biological fluid. The technique uses primary antibodies as the immobilized probe on a solid surface, and protein antigens labeled with fluorophores with or without bound secondary antibodies are recognized and detected. However, binding of antibodies and antigens to a solid support can cause denaturation or drying of proteins. MBAA provides multiplexing in a solution phase and thus is particularly flexible and nondestructive for protein analysis. Also protein microarray assay are relatively new, are not widely accepted as a 'gold standard' for clinical use, and also may be of limited sensitivity.

2.6 Areas of application

Since a disease can result from various reasons and it may include functional disorders of multiple genes, a multi analyte analysis is necessary for diagnostic purposes. The

technology called omic and multiplex system allows a fast and systematic detection of the effects of micro molecules in different molecular and cellular contexts. In this respect, it can be used for diagnosing multi-reason/multi-gene diseases, performing a comprehensive disease management and investigating complex cellular functions. In addition, it provides a wide range of application area for research area because of its plasticity which allows the researchers to perform various studies.

2.7 Clinical utility

Despite the introduction of hundreds of multiplexed protein immunoassays to the research market in recent years, only a limited number have been cleared by the FDA for clinical use, an observation that illustrates the complexity of constructing robust arrays. Antibody-based multiplexed assay (and commercial instruments and kits) have a relatively long history with over 15 years of development and optimization. However, most commercial multiplex assays are developed for research laboratories and nonclinical tests; only a limited number of multiplex assays are approved by FDA for clinical testing. For example FDA-cleared planar protein multiplex arrays consist primarily of the lateral flow immunoassays used for point-of-care evaluation. For example, the Triage® Cardio Profiler® 4-plex measures troponin-I, creatine kinase-MB, myoglobin, and brain natriuretic peptide (BNP) to assist with evaluation of chest pain using a portable lateral flow platform. At present, suspension immunoassay is the prevailing technology for FDA-cleared multiplex protein measurements, especially for testing antibodies in the serum of patients with allergies or autoimmune or infectious diseases in clinical laboratories.

The other platforms for commercial multiplex diagnostic tests include Luminex (www.luminex.com), Triage system (www.Biosite.com), Evidence (www.Randox.com), Vidas (www.biomerieux-diagnostics.com), Planner arrays (www.VBC-genomics), Whatman (www.whatman.com) and bead array (www.bioarrays.com). At present, suspension immunoassay is the prevailing technology for FDA-cleared multiplex protein measurements, especially for testing antibodies in the serum of patients with allergies or autoimmune or infectious diseases in clinical laboratories. Most of the currently available multiplex immunoassays have been designed to quantify the concentrations of various cytokines. The recent development of spectrally-distinguishable fluorescent beads (Luminex) (Kellar and Iannone, 2002) has resulted in the widespread use of antigen-coupled beads for monitoring antibodies in sera by flow cytometry. These bead arrays have been adapted for serologic screening of antigens and have been described for up to ten antigens for HIV (Opalka et al., 2004), HPV (Opalka et al., 2003; Dias et al., 2005), Epstein-Barr virus (Klutts et al., 2004; Binnicker et al., 2008; Gu et al., 2008), B. anthracis (Biagini et al., 2004; Biagini et al., 2005), Influenza (Drummond et al., 2008) and M.tuberculosis (Khan et al., 2008).

The results from a growing number of research studies, demonstrate that multiplex technology may be useful in clinical research to measure a large number of analytes to examine the association with a clinical phenotype and the effects of therapeutic interventions, and that this technology may be particularly useful when sample volume is limited, such as in large epidemiologic studies and clinical trials. Clinical applications must follow establishment of globally accepted calibration standards, performance criteria, and QC programs.

APPLICATION	AVAILABLE KITS*	COMPANY
Allergy Testing	Alternaria (Mold) (h), Bermuda Grass (h), Cat Dander (h), Egg White (h), Milk (h), Mite Pternoyssinus (h), Mountain Cedar (h), Short Ragweed (h), Timothy Grass (h), Wheat (food) (h)	ImTech (h)
Autoimmune	ASCA (h), beta-2 Microglobulin (h,m), Centromere B (h), Chromatin (h), DNA (h), ENA Profile 4 (SSA, SSB, Sm, ENA Profile 5 SSA, SSB, Sm, RNP, Scl-70) (h), ENA Profile 6 (SSA, SSB, Sm, RNP, Scl-70, Jo-1) (h), Gliadin A (h), Gliadin G (h), Histone (h), Histone H1 (h), Histone H2A (h), Histone H2B (h), Histone H3 (h), Histone H4 (h), HSP-27 pS82 (G), HSP-27 Total (G), HSP-32 (h), HSP-65 (h), HSP-71 (h), HSP-90 a (h), HSP-90 b (h), Jo-1 (h), PCNA (h,m), PR3 (h), PR3 (cANCA) (m), RF (h), Ribosomal P (h,m), RNP (h,m), RNP-A (h), RNP-C (h), SCF (h,m), Scl-70 (h,m), Serum Amyloid P (h), SLE Profile 8 (SSA, SSB, Sm, RNP, Scl-70, Jo-1, Ribosome-P, chromatin) (h), Sm (G) (h), Smith (h,m), SSA (h,m), SSB (h,m), Streptolysin O (h), TG (h), TPO (h,m), Transglutaminase A (h), Transglutaminase G (h)	RBM(h,m)
Cancer Markers	Alpha Fetoprotein (h), Cancer Antigen 125 (h), Carcinoembryonic Antigen (h), PSA, Free (h)	RBM(h)
Cardiac Markers	Creatine Kinase-MB (h), Endothelin-1 (m), PAP (h), SGOT (h,m), TIMP-1 (h,m)	RBM(h,m)
Cytokine	Abeta 40 (h), Abeta 42 (h), BDNF (h), DR-5 (h), EGF (h,m), ENA-78 (h), Eotaxin (h,m), Fatty Acid Binding Protein (h), FGF-basic (h,m), G-CSF (h,m), GCP-2 (m), GM-CSF (h,m,rt), GRO alpha (h), GRO-KC (rt), HGF (h,m), I-TAC (h), ICAM-1 (h), IFN-alpha (h), IFN-gamma (h,m,rt), IL-10 (h,m,rt), IL-11 (m), IL-12 (h,m), IL-12 p40 (h,m), IL-12 p40/p70 (m) (rt), IL-12 p70 (h,m,rt), IL-13 (h,m), IL-15 (h,m), IL-16 (h), IL-17 (h,m), IL-18 (rt), IL-1alpha (h,m,rt), IL-1beta (h,m,rt), IL-1ra (h), IL-1ra/IL-1F3 (h), IL-2 (h,m,rt), IL-3 (h,m), IL-4 (h,m,rt), IL-5 (h,m,rt), IL-6 (h,m,rt), IL-7 (h,m), IL-8 (h), IL-9 (m), IP-10 (h,m), JE/MCP-1 (m), KC (m), KC/GROa (m), LIF (m), IL-8 (h), IL-9 (m), IP-10 (h,m), JE/MCP-1 (m), KC (m), KC/GROa (m), LIF (m), MCP-3(h,m), MCP-5 (m)	B-R(m); Bios (h,m,rt); Linco (h,m,rt); RD(h,m); UP(h,m), RBM (h,m)
Endocrine ACTH (h), Adiponectin (h,m), Amylin (m) (rt) (h), C-Peptide (h), Calcitonin (h), CRF Linco	ACTH (h), Adiponectin (h,m), Amylin (m) (rt) (h), C-Peptide (h), Calcitonin (h), CRF(h), FGF-9 (m), FSH (h), GH (h), GLP-1 (h,m,rt), Glucagon (m) (rt) (h), Growth Hormone (h,m), Insulin (h,m,rt), Leptin (h,m,rt), LH (h), Lipoprotein (a) (h), PAI-1(active) (h), PAI-1 (total) (h,m), Prolactin (h), Resistin (h,m,rt), T3 (h), T4 (h), TBG (h), Thyroglobulin (h), TSH (h)	Linco (h,m,rt); RBM (h,m)
Gene Expression	IL6R(h), ACTB (h), BAD (h), BAK1 (BAK) (h), BCL2 (h), BCL2L1 (BCL-XL) (h), CDKN1A (CDKN1) (h), CFLAR (CFLIP) (h), CSF2 (h), GAPD (h), IFN-gamma (h), IL-1 beta (h), IL-10 (h), IL-2 (h), IL-6 (h), IL-8 (h), NFKB2 (h), NFKBIA (NFKIA) (h), NKFB1 (h), PPIB (h), Ptk2B (RAFTK) (h), RELA (h), RELB (h), TNF (h), TNFAIP3 (A20) (h), TNFRSF6 (FAS) (h), TNFSF6 (FASL) (h), VEGF (h)	Bios (h); MBio (h)
MMP	MMP-1 (h), MMP-12 (h), MMP-13 (h), MMP-2 (h), MMP-3 (h), MMP-7 (h), MMP-8(h), MMP-9 (h)	RD (h); Bios (h)
Genotyping	FlexMAP™ (G), Mitochondrial DNA Screening (h), Tag-It™ Mutation Detection Kit(G), Y-SNP Identification (h)	Bio (h), Mira(h), TmBio (h)

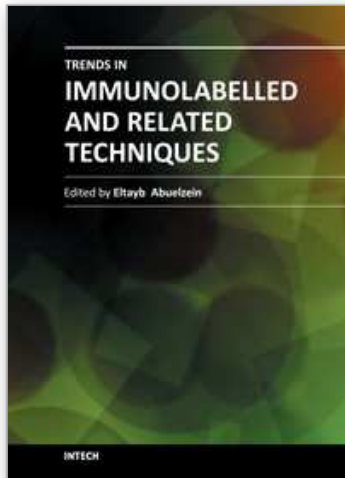
APPLICATION	AVAILABLE KITS*	COMPANY
Infectious Disease	Adenovirus (h,m), Bordetella pertussis (h), Campylobacter jejuni (h), Chlamydia pneumoniae (h), Chlamydia trachomatis (h), Cholera Toxin (h), Cholera Toxin b (h), Clostridium piliforme (Tyzzer's) (m), Cytomegalovirus (h,m), Diphtheria Toxin (h), Ectromelia virus (m), EDIM (Epidemic diarrhea of infant mice) (m), Encephalitozoon cuniculi (m), Epstein-Barr EA (h), Epstein-Barr NA (h), Epstein-Barr VCA (h), HBV Core (h), HBV Envelope (h), HBV Surface (Ad) (h), HBV Surface (Ay) (h), HCV Core (h), HCV NS3 (h), HCV NS4 (h), HCV NS5 (h), Helicobacter pylori (h), Hepatitis A (h), Hepatitis D (h), HEV orf2 3KD (h), HEV orf2 6KD (h), HEV orf3 3KD (h), HIV-1 gp120 (h), HIV-1 gp41 (h), HIV-1 p24 (h), HPV (h), HSV-1 gD (h), HSV-1/2 (h), HSV-2 gG (h), HTLV-1/2 (h), Influenza A (h), Influenza A H3N2 (h), Influenza B (h), Leishmania donovani (h), Lyme disease (h), Lymphocytic choriomeningitis virus (m), M. pneumoniae (h), M. tuberculosis (h), Minute virus (m), Mumps (h), Mycoplasma pulmonis (m), Parainfluenza 1 (h), Parainfluenza 2 (h), Parainfluenza 3 (h), Parvovirus (m), Pneumonia virus of mice (m)	RBM (h,m)
Isotyping	IgA (h,m), IgE (h,m), IgG1 (m), IgG2alpha (m), IgG2beta (m), IgG3 (m), IgM (h,m), light chain (kappa or gamma) (m)	UP (h,m); RBM(h,m)
Metabolic Markers	Apolipoprotein A-1 (m), Apolipoprotein A-I (h), Apolipoprotein A-II (h), Apolipoprotein B (h), Apolipoprotein C-II (h), Apolipoprotein C-III (h), Apolipoprotein E (h), beta-2 Glycoprotein (h,m), Collagen Type 1 (h), Collagen Type 2(h), Collagen Type 4 (h), Collagen Type 6 (h), Glutathione S-Transferase (h,m), Pancreatic Islet Cells (h), tTG (Celiac Disease) (h)	RBM (h,m), Linco
Tissue Typing	HLA Class I and II (h), HLA Class I Single Antigen Antibody, Group 1 (h), HLA Class I Single Antigen Antibody, Group 2 (h), PRA Class I (h), PRA Class I and II (h), PRA Class II (h), SSO Class I HLA-A (h), SSO Class I HLA-B (h), SSO Class I HLA-C(h), SSO Class II DP (h), SSO Class II DQB1 (h), SSO Class II DRB1 (h), SSO Class II DRB3,4,5 (h)	Lambda (h)
Kinase Phosphorylated Protein	Akt (G), Akt (Ser473) (G), Akt (total) (G), Akt/PKB (total) (G), Akt/PKBpS473 (G), ATF2 (Thr71) (G), ATF2 (total) (G), CREB (pS133) (G), CREB (Total) (G), Erk 1/2(pTpY185/187) (G), Erk 1/2 (Total) (G), Erk-2 (G), Erk1 (Thr202/Tyr204) (G), Erk1/2 (Thr202/Tyr204, Thr185/Tyr187) (G), Erk2 (Thr185/Tyr187) (G), Erk2 (total) (G),GSK 3beta (pS9) (G), GSK-3a/b (Ser21/Ser9) (G), GSK-3beta (G), IGF 1R	UP (G), Bios (G)
Transcription Factors-Nuclear Receptors	AP-2 (G), CREB (G), EGR (h), HIF-1 (h), NF-1 (h), NFAT (h), NFkB Gene Family(h), PPAR (h), SRE (h), YY1 (h)	Bios (h), MBio (h)

* Human (h), mouse (m): rat (rt): general (G), ImmuneTech (ImTech); Rules Based Medicine (RBM); BioRad (B-R); BioSource (Bios); Linco Research (Linco); Qiagen; R&D Systems (RD); and Upstate Group (UP); Marligen Biosciences (MBio), MiraBio (Mira); Tm BioScience (TmBio); One Lambda (LAMBDA).

Table 5. Application area and available kits

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Trends in Immunolabelled and Related Techniques

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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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