A NOVEL IN VITRO ASSAY FOR SCREENING MEDICAL DEVICES

Differentiating Between Contact Dependent and Contact Independent Inflammatory Effects

It is well known that surface contact can activate cells of the myeloid lineage. This has important consequences for the development of coatings for medical devices intended for human transplantation.

Conventional in vitro biological screening may reveal overall inflammatory effects, but does not differentiate between contact dependent and contact independent effects. We have developed an assay to differentiate between such effects.

This assay can rapidly detect inflammatory responses and give information on their nature by determining whether a device is generally pro-inflammatory or if the pro-inflammatory effect is dependant upon cell contact with the device.

Multiple parameters on the nature of the response can be simultaneously measured including cell viability, cytokine production and signal transduction pathways. As such, this assay is both qualitative and quantitative.

Additionaly, this assay permits the rapid screening of multiple prototypes prior to testing in vivo. This reduces the overall cost and likelihood of discovery a pro-inflammatory effect at later stages of testing.

Conferences:

An MD Biosciences representative will be available at the following conferences:

October: BioInterface 2005
October 24 - 26, Minneapolis, MN

November: ACR/ARHP Annual Scientific Meeting
November 12 - 17, San Diego
Booth Number 1323

1 Novel in vitro assay for screening medical devices.
2 COX Activity Assay
3 Allergic Asthma Model
4 In vitro screening for inflammatory activity.
5 ELISA to measure proteolytic degradation of Aggrecan.
6 T and B cell Transfer Model
7 DSS IBD Model
8 Multiplex Assays
The arachidonic acid (AA) cascade produces a variety of factors involved in inflammation including prostaglandins, thromboxanes, leukotrienes and lipoxins. Cyclooxygenase (COX) is a membrane bound enzyme that is responsible for converting AA to PGG₂ and the subsequent reduction of PGG₂ to PGH₂. There are two COX isoforms, COX1 and COX2. COX1 is constitutively expressed and is involved in a number of “housekeeping” functions such as cellular homeostasis, while COX2 is induced in response to multiple stimuli and is involved in inflammation and other pathophysiological processes.

**In vitro** assay for COX activity:
Cells are treated with test compound in the presence or absence of endotoxin and/or cytokine stimulation inducing an inflammatory response. Culture supernatants are assayed for PGE₂ production. PGE₂ production in the absence of cell stimulation is used to assess COX1 activity while PGE₂ production by endotoxin stimulated cells is used to assess COX2 activity. COX gene expression analysis and cell free COX assays can be performed to determine if the compound affects COX transcription, COX enzyme activity or both. Other arachidonic acid pathway components such as sPLA₂ and LTB₄ can also be studied.

**Allergic Asthma Model:**
**Combining the OVA Allergic Asthma Model with an Adoptive Transfer System to Dissect the Role of Antigen Immune Specific Responses.**

Allergic Asthma can be characterized by reversible airway obstruction, elevated levels of IgE, chronic airway inflammation, and airway hyper-responsiveness. The ovalbumin (OVA) induced allergic asthma model is widely used since OVA is a common model antigen. Analysis of this model relies heavily on general indicators of asthma such as histology and bronchoalveolar lavage analysis. While these methods are informative, they give no indication of the role of antigen specific immune responses in the pathology observed.

Furthermore, antigen specific analysis of antibodies is entirely retrospective and provides no information about the developing immune response. By combining the OVA induced allergic asthma model with the adoptive transfer of antigen specific transgenic (Tg) T cells, it is possible to dissect the role of antigen specific immune responses that contribute to pathology.

This combination model allows us to determine not only if a potential therapy is effective in alleviating symptoms, but also where, when, and how it accomplishes this. By adoptive transfer of Tg T cells specific for the model antigen ovalbumin, we can then detect the progress and development of the immune response using a variety of techniques including flow cytometry and immunohistochemistry.

**Traditional Analysis:**
- Measurement by cell counting of eosinophils, macrophages, and neutrophils in bronchoalveolar lavage fluid (BAL).
- Measurement of cytokines in BAL.
- Histology of lung sections
- Total and antigen specific IgG/E

**Antigen-Specific Analysis:**
- Flow cytometry of BAL for proportion of Tg T cells
- Flow cytometry of draining lymph node for proportion of Tg T cells.
- Immunohistochemistry of lungs and lymph nodes for Tg T cells using light microscopy or quantitatively by laser scanning cytometry (LSC).
**In Vitro Assays for Inflammatory Activity**

**Cell Systems**

MD Biosciences uses a number of primary and immortalized cell lines to assay test compounds for their effect on inflammatory mediator production, signal transduction pathway activation, transcription factor activity, gene expression and other specialized assays. Using these in vitro assays, compounds can be rapidly and cost effectively screened for activity prior to more expensive and time consuming in vivo analysis.

Partial listing of cell lines and primary cell systems:
- Peripheral Blood Mononuclear Cells
- T-Cells
- B-cells
- Macrophages
- Epithelial Cells
- Fibroblasts
- Synoviocytes
- Chondrocytes

**Assays**

Depending on the cell system used, a variety of downstream readouts can be analyzed for human, mouse, and rat.

**Production of Inflammatory mediators**

**Cytokines & Chemoikines**
- GM-CSF
- IFN-α, -β
- Interleukins 1-17, IL-1RA, IL-2R, IL-6R
- TNF-α
- ENA-78
- Eotaxin
- IP-10
- GRO-α, -β
- MCP-1
- MIG
- MMP-1α, -1β, -3α
- Rantes

**Arachidonic Acid cascade**
- PGE₂
- LTB₄
- sPLA₂

**Matrix Metalloproteinases**
- MMP-1, -2, -3, -7, -8, -9, -13

**Signal transduction pathway activation**
- Detection of phosphorylated forms of Akt, JNK, p38 MAPK, ERK 1/2, IkBα, p53, p70S6K, STAT1

**Transcription Factor Activity**
- Detection of transcription factors NFκB, AP-2, CREB, EGR, HIF, NF-1, NFAT, PPAR, SRE, YY-1 by DNA binding assay.

**Gene Expression:**
- Cytokines
- Chemokines
- COX1, COX2

**Multiplex Assays:**
- Cytokines
- Chemokines
- Growth factors
- MMPs

**Specialized assays:**

**Osteoclast resorption/differentiation:**
Compounds are incubated with differentiated osteoclasts (bone resorption assay) or osteoclast precursors (osteoclast differentiation assay) in the presence of human bone particles. The release of collagen peptides is used to determine osteoclast activity.

**Chondrocyte assays:**

Primary chondrocytes incubated in 2D or 3D cultures are stimulated with pro-inflammatory cytokines to mimic chronic inflammation in the presence or absence of test compound. The stimulated cells are assayed for proteoglycan synthesis and production of inflammatory mediators such as PGE₂ and NO. The induction of MMPs may also be determined. Thus, one can determine if a compound affects the cytokine-stimulated response of cultured chondrocytes.

**Custom protocols:**

MD Biosciences specializes in customizing protocols based on individual client needs. Contact us with your project objective and we will customize a study based on your specific requirements.

**Inflammatory Disease Models**

**Rheumatoid Arthritis:**
- CIA model
  - 42 days
  - Induction w/collagen on day 0 and collagen boost on day 21
- mAb model
  - 11 days
  - Induction w/mAb on day 0 with LPS boost on day 3

**Allergic Asthma:**
- OVA model
  - 25 days
  - Combined with adoptive transfer system

**Multiple Sclerosis:**
- EAE model
  - 21 days
  - Induction with PLP

**IBD:**
- DSS model
  - 7 - 14 days
  - Induction with DSS in drinking water.

**T cell B cell Transfer Model**
- 14 days
  - Know more about when, where and how a compound alters the immune system
ELISA to Measure the Proteolytic Degradation of Aggrecan: Hallmark in the Pathology of Arthritis

Aggrecan is a large aggregating proteoglycan of articular cartilage [1], making up 10% of the dry weight. It is responsible for hydrating cartilage giving it compressibility and resilience during joint loading, thereby playing a major role in the normal function of cartilage. Depletion of glycosaminoglycan bearing aggrecan fragments from articular cartilage is one of the earliest events in cartilage destruction.

Aggrecan monomers consist of a 250 kDa core protein and three globular domains, G1, G2, and G3 [2]. With the attachment of a chondroitin sulfide (CS) chain at the C-terminus and a keratin sulfide (KS) chain at the N-terminus, the monomer exists as a 1000-2000 kDa molecule. It is retained within the collagen network as an aggregate by interaction through the G1 domain and hyaluron, resulting in a large aggregate containing up to 100 aggrecan monomers, which is weaved into the collagen network [1, 3].

Proteolytic cleavage of its interglobulin domain (IGD) results in release of aggrecan fragments from tissue, which eventually leads to loss of joint function. This cleavage has been attributed to metalloprotease activity. Members of the matrix metalloprotease (MMP) family that are present in cartilage (MMP-2, -3, -7, -8, -9, -13, and -14) are capable of degrading aggrecan between the Asn341 and Phe324 amino acids within the IGD, while members of the ADAMTS family (ADAMTS4 and ADAMTS-5/11 referred to as aggrecanase-1 and -2 respectively) are capable of degrading aggrecan at the Glu373 and Ala374 amino acids [4 - 7]. In addition, ADAMTS4 also cleaves the relevant aggrecanase sites within the CS2 domain [4]. The major portion of aggrecan released from tissue appears to be cleaved by aggrecanases [2], and this release eventually leads to loss of joint function in diseases such as rheumatoid arthritis and osteoarthritis.

Enzymatic activity of aggrecanases has been analyzed with isolated aggrecan preparations, recombinant aggrecan fragments, and a 41-residue peptide immobilized onto streptavidin-coated microplates. An ELISA method for aggrecanase activity provides an improved and ready-to-use method for sensitive determination of aggrecanase activity and can be used to screen and characterize aggrecan inhibitors.

Aggrecanase Activity Assay

Aggrecanase Module: Proteolysis of aggrecan-IGD by aggrecanase. A recombinant fragment of human aggrecan-IGD is first digested with aggrecanase and proteolytic cleavage releases an aggrecan peptide with the N-terminal sequence ARGSVIL (ARGSVIL Peptide). Samples of unknown aggrecanase activity would also be incubated with aggrecanase and the amount of ARGSVIL-peptide would be compared to the recombinant aggrecan-IGD standard.

ELISA Module: Aggrecan peptide ELISA. The ARGSVIL-peptide resulting from the proteolytic degradation from the recombinant aggrecan-IGD and the unknown aggrecanase in the sample is then quantified with two monoclonal antibodies using an ELISA format. The amount of ARGSVIL-peptide measured from both proteolytic degradations is correlated to the ARGSVIL-peptide standard provided to determine the amount of aggrecanase activity in the sample.

Protocol for Testing potential Aggrecanase Inhibitors using the Aggrecanase Activity Assay:

1. Prepare the diluted ADAMTS-4 standard from stock solution.
2. Prepare reaction mixture: 10 µL aggrecan-IGD
   - Pefabloc (inhibitor)*
   - 'x' µL inhibitor test sample
   Bring to 95 µL with reaction buffer
3. Preheat reaction mixture to 37°C.
4. Start reaction by adding 5 µL ADAMTS-4.
5. Incubate for 15 min. at 37°C.
6. Stop reaction with 150 µL EDTA solution
7. Assay 100 µL of reaction for ARGSVIL-peptide by ELISA as described above.

*Pefabloc is a serine protease inhibitor used to inhibit proteases found in the test sample and does not affect aggrecanase activity. If the test sample is thought to be free of protease activity, Pefabloc can be excluded. Depending on the mode of action of an inhibitor, a ADAMTS-4/test inhibitor pre-incubation step (30 min at 37°C) may be necessary. After the pre incubation, the reaction will be started with the addition of aggrecan-IGD.

References:

### Research Tools:

#### ELISAs and Activity Assays

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Catalog #</th>
<th>Species</th>
<th>Sensitivity</th>
<th>Range</th>
<th>Sample Size</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecanase Activity</td>
<td>ACT-AGG.96</td>
<td>human</td>
<td>&lt; 0.025nM</td>
<td>0.024 - 4 nM</td>
<td>100 µL</td>
<td>4 hrs</td>
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<tr>
<td>Sensitivity Aggrecanase Activity</td>
<td>SEN-AGG.96</td>
<td>human</td>
<td>2 pM</td>
<td>0.022 - 100 pM</td>
<td>100 µL</td>
<td>4 hrs</td>
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<tr>
<td>Animal Cartilage Oligomeric Matrix Protein (COMP) ELISA</td>
<td>A-COMP96</td>
<td>rat/mouse/sheep/bovine/canine</td>
<td>&lt;0.2 U/L</td>
<td>up to 0.9 U/L</td>
<td>100 µL</td>
<td>3.5 hrs</td>
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<tr>
<td>active MMP-13</td>
<td>ACT-MMP13.96</td>
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<td>7 pg/mL</td>
<td>32 - 2000 pg/mL</td>
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<td>pro-MMP-13</td>
<td>PRO-MMP13.96</td>
<td>human</td>
<td>4 pg/mL</td>
<td>16 - 1000 pg/mL</td>
<td>100 µL</td>
<td>4.5 hrs</td>
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#### Recombinant & Natural Matrix Metalloproteinases (MMPs)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Catalog #</th>
<th>Species</th>
<th>Source</th>
<th>Size(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS1, truncated His-tagged</td>
<td>5028002</td>
<td>human</td>
<td>Sf</td>
<td>5/100 µg</td>
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<tr>
<td>ADAMTS4 (aggrecanase 1), truncated His-tagged</td>
<td>5028001</td>
<td>human</td>
<td>Sf</td>
<td>5/100 µg</td>
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<tr>
<td>Aggrecan interglobulin domain</td>
<td>5028003</td>
<td>human</td>
<td>E. coli</td>
<td>100/500 µg</td>
</tr>
<tr>
<td>MMP-2 (gelatinase A)</td>
<td>5028013</td>
<td>human</td>
<td>Sf 9</td>
<td>10/200 µg</td>
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<tr>
<td>MMP-9 (gelatinase B) monomer</td>
<td>5028012</td>
<td>human</td>
<td>natural human blood</td>
<td>10/200 µg</td>
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<tr>
<td>MMP-13 (procollagenase-3)</td>
<td>5028014</td>
<td>human</td>
<td>Sf 9</td>
<td>10/200 µg</td>
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<tr>
<td>MMP-13 (collagenase-3) catalytic domain</td>
<td>5028015</td>
<td>human</td>
<td>E. coli</td>
<td>10/200 µg</td>
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<tr>
<td>MMP-14 (MT1-MMP) catalytic domain</td>
<td>5028004</td>
<td>human</td>
<td>E. coli</td>
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<td>MMP-14 prodomain-catalytic domain</td>
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<td>human</td>
<td>E. coli</td>
<td>10/200 µg</td>
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<tr>
<td>MMP-14 prodomain-catalytic domain</td>
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</tr>
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<td>MMP-14 hemopexin domain</td>
<td>5028007</td>
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<td>E. coli</td>
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<td>MMP-15 (MT2-MMP) catalytic domain</td>
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<td>human</td>
<td>E. coli</td>
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<td>MMP-15 hemopexin domain</td>
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<td>human</td>
<td>E. coli</td>
<td>20/200 µg</td>
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<td>MMP-16 (MT3-MMP) catalytic domain</td>
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<td>human</td>
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<td>MMP-24 (MT5-MMP) catalytic domain</td>
<td>5028016</td>
<td>human</td>
<td>E. coli</td>
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#### Antibodies to MMPs

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalog #</th>
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<th>Type</th>
<th>Size(s)</th>
<th>Application</th>
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</thead>
<tbody>
<tr>
<td>aggrecan N-terminal sequence of ARGSVIL</td>
<td>1028023</td>
<td>human</td>
<td>mouse IgG monoclonal</td>
<td>100 µL</td>
<td>WB</td>
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<tr>
<td>Collagenase-3 (MMP-13)</td>
<td>2028019</td>
<td>human</td>
<td>rabbit IgG polyclonal</td>
<td>100 µL</td>
<td>WB, ELISA</td>
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<tr>
<td>Collagenase-3 (latent &amp; human)</td>
<td>1028020</td>
<td>human</td>
<td>mouse IgG monoclonal</td>
<td>100 µL</td>
<td>WB, ELISA</td>
</tr>
<tr>
<td>pro-collagenase-3</td>
<td>1028021</td>
<td>human</td>
<td>mouse IgG monoclonal</td>
<td>100 µL</td>
<td>WB, ELISA</td>
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<tr>
<td>pro-collagenase-3</td>
<td>1028022</td>
<td>human</td>
<td>mouse IgG monoclonal</td>
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<td>Staining or detection</td>
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<td>MMP-14</td>
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<td>rabbit IgG polyclonal</td>
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<td>WB, ELISA</td>
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<tr>
<td>MMP-15</td>
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<td>rabbit IgG polyclonal</td>
<td>100 µL</td>
<td>WB, ELISA</td>
</tr>
</tbody>
</table>

For research use only. Not for use in diagnostic procedures.
The immune system is comprised of two ‘arms’: the innate response and the adaptive immune response. The innate response is a rapid and relatively non-specific response that controls infection until the adaptive response takes over. It is comprised of inflammatory cells along with other cells, such as dendritic cells, which communicate with the adaptive immune response.

The adaptive response on the other hand is a highly specific response with its main components being T and B cells. Many inflammatory diseases, as well as representative models of those diseases, contain a T and/or B cell component. Examples include Arthritis, Asthma, IBD, Multiple Sclerosis and their representative animal disease models. An effective immunomodulator for these diseases would therefore alter the adaptive immune response; however, there are a large number of cellular interactions with possible target sites within the adaptive immune response. In addition, it is very difficult to track these cellular interactions in vivo due to the low frequency of antigen specific cells.

**Principle of the T and B cell Transfer Model**

In order to overcome the difficulty of tracking cellular interactions in vivo, the precursor frequency of T and B cells are artificially increased.

T cell Receptor and B cell receptor transgenic animals that recognize one antigen are used and a small number of trackable transgenic lymphocytes are adoptively transferred into naive recipients.

**Advantages of the T and B Cell Model:**

The T and B cell model allows the sequential dissection of each of the points on the immune response pathway, enabling determinations of the exact target site of the immunomodulator.

This ability to track and dissect the immune response pathway provides the following advantages:

- Examine the response behind events such as protective immunity, allergy, graft rejection and vaccination
- Elucidation of mode of action and target potential immunomodulators
- Interactions occur in defined anatomical niche (tissue vs lymph node)
- Determination of target site of an immunomodulator indicates potential effectiveness in other mouse models thereby saving time and money. Prevents random screening of efficacy in a range of animal models.
- Allows optimization of therapeutic timing since requirements for the dosing regime can be known.

Additionally, results can be obtained rapidly, often within 2 weeks, enabling informed forward planning.

To read more about the T and B cell model, log onto www.mdbiosciences.com and check out our white paper.
**Inflammatory Bowel Disease**

Inflammatory Bowel Disease (IBD) is a common chronic gastrointestinal disorder whose incidence occurs in up to 100,000 cases per year depending on the geographical location. IBD is an umbrella term that covers a range of diseases including ulcerative colitis (UC) and Crohn’s disease. In UC, crypt abscess are observed along with ulceration of the mucosa and sub mucosa within the large intestine, while Crohn’s disease presents with transmural lymphocyte aggregates and small lesions of the mucosa. Although the mechanisms that cause IBD remain undefined, there is an increasing awareness that a range of immunological parameters probably contribute to the pathology (1).

**In vitro assays**

The migration and activation of leukocytes into the intestinal mucosa contributes to the chronic intestinal inflammation observed in inflammatory bowel diseases. Pro-inflammatory cytokines and chemokines mediate these events. It has recently been demonstrated that a distinct set of chemokines is upregulated in colon tissue isolated from IBD patients (Puleston et al., 2005 Aliment Pharmacol Ther 21, 109-120). Furthermore, cytokine stimulated epithelial cell lines were found to display a similar chemokine response.

To determine the effect of a test compound on the cytokine stimulated epithelial cell chemokine response, human colon adenocarcinoma cells are exposed to TNF-α in the presence and absence of test compound. Culture supernatants are collected and assayed for inflammatory mediators such as PGE₂, LTB₄, IL-8, GRO-α, GRO-β and MIP-3α.

**In vivo animal model**

There are a large number of mouse models of IBD which mimic, to a greater or lesser extent, the pathology of IBD (2). One of the oldest and most representative model is the DSS model (3), which involves administering dextran sodium sulphate via the drinking water to mice over a period of 7-10 days.

**Analysis of the Model:**

Prior to termination, disease is principally assessed by individual weight analysis; however other techniques are available such as the presence of blood in stools and haematocrit measurement.

At the termination of the study measurements include:

- Colon length and weight
- Histological measurement by haematoxylin and eosin staining and assessment using a standard scoring protocol
- Crypt mitotic activity
- Myeloperoxidase testing

**In Development: Using MRI to assess intestinal pathology in situ.**

MD Biosciences is currently developing a method of assessing intestinal pathology in situ using MRI capabilities. The major advantage of using the MRI technique to study inflammatory diseases such as IBD is that it offers the potential to scan live animals and observe pathological changes in the colon in real-time in vivo. The onset and progression of intestinal inflammation can therefore be studied for each individual animal over a period of time thus reducing the number of animals required for an experiment but increasing the amount of data generated per animal in a study. At the moment, it is only possible to observe these changes post-mortem using histological techniques, and this only gives a snapshot of disease progression.

**References:**


www.mdbiosciences.com
Multiplex assays are becoming increasingly popular due to the convenience of assaying multiple analytes per well in the same amount of time that it takes to run a traditional ELISA assay.

Benefits of using a multiplex assay:
- Small sample size - 50 µL
- Multiple analytes per well
- Ability to customize desired analytes
- Sensitivity of 10 pg/mL offers relevant measurements
- Precise, accurate and reproducible results that you can count on
- Low background and high specificity so your results withstand scrutiny

Multiplex Technology
The multiplex system utilizes a 5.6 micron bead/microsphere which is internally dyed with red and infrared fluorophores. Different intensities of these two dyes are used allowing each bead set to have a unique spectral signature. Capture reagents such as antibodies are then coated onto the surface of the beads, which are then incubated with samples and pre-mixed standards. After the incubation with the antibody-coated beads, a second pre-mixed biotinylated antibody is added for detection, followed by the addition of substrate. Fluidics technology based on the principle of flow cytometry causes the suspended beads to line up singly before they are passed through the detection chamber. As each bead individually passes through the detection chamber, a red laser excites both the internal red and infrared dyes classifying the analyte, while a green laser excites any orange fluorescence associated with the binding of that analyte.

Bringing this technology into your lab will offer you many advantages such as saving time and reducing the volume needed of samples, however, the cost of implementing this technology may be a challenge. MD Biosciences now offers the xMAP® Technology as part of our in vitro testing capabilities, making it possible to take advantage of the technology without implementing the associated costs. You can choose from a large panel of human, rat, or mouse analytes available and customize the analytes that you want to test in one single sample:

- Cytokines
- Chemokines
- Th1/Th2
- Growth factors
- MMPs
- Kinase/Phosphorylated proteins

To see a full listing of analytes currently available, visit our web site at www.mdbiosciences.com or send us an e-mail at info-us@mdbiosciences.com.

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