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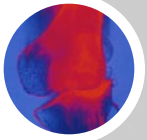

- Inflammatory diseases
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TARGET inflammation

an applications newsletter for inflammatory research

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

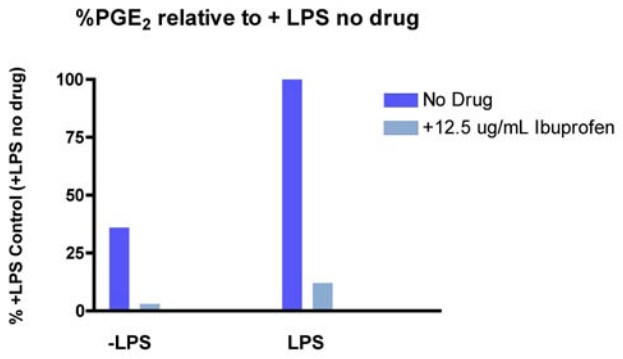
November:
ACR/ARHP Annual Scientific Meeting
November 12 - 17, San Diego
Booth Number 1323

COX Activity Assay

The arachidonic adic (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 PGG_2 and the subsequent reduction of PGG_2 to PGH_2. 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 processess.

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 PGE_2 production. PGE_2 production in the absence of cell stimulation is used to assess COX1 activity while PGE_2 production by endotoxin stumulated 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 $sPLA_2$ and LTB_4 can also be studied.

This model is compatible with multiple cell types including mononuclear cells, macrophage cell lines and epithelial cell lines. ●



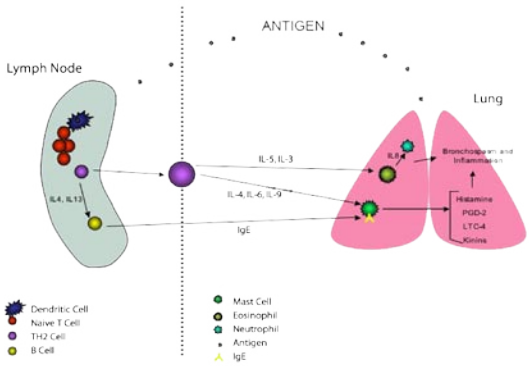
Human peripheral blood mononuclear cells were incubated with LPS in the presence of ibuprofen. Cell culture supernatants were assayed for PGE_2 by ELISA.

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
- MIP-1α, -1β, -3α
- Rantes

Arachidonic Acid cascade

- PGE_2
- LTB_4
- $sPLA_2$

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, IκBα, 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 resportion 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 the production of inflammatory mediators such as PGE_2 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

Mulitple 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

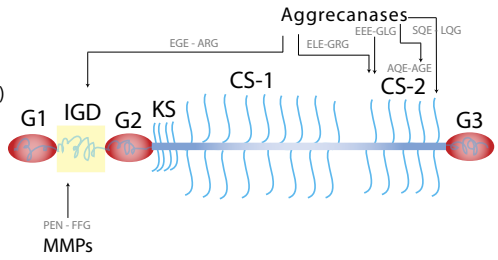
- 14 days
- Know more about when, where and how a compound alters the immune system

ELISA to Measure the Proteolytic Degradation of Aggrecan: Hallmark in the Pathology of Arthritis

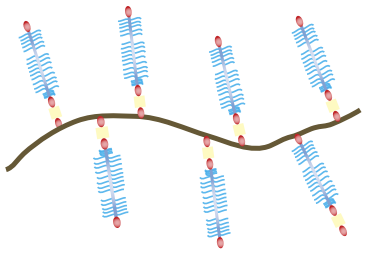
Aggrecan is a large aggregating proteoglycan of articular cartilage [1], making up 10% of the dry weight. It is responsible for hydrating cartilage giving it compressibility and resilience during joint loading, thereby playing a major role in the normal function of cartilage. Depletion of glycosaminoglycan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



A) aggrecan monomer showing proteolytic cleavage sites



B) aggregate showing aggrecan monomers bound to hyaluronic acid.

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
   10 µL 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. ●

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1. Knudsen, C.B. and Knudsen, W. (2001) Seminars Cell & Developm. Biol. 12, 69.
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4. Tortorella, M.D. *et al.* (2000) J. Biol. Chem. 275:18566.
5. Tortorella, M.D. *et al.* (2002) Matrix Biology 21:499.
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7. Malfait, A.-M. et al. (2002) J. Biol. Chem. 277:22201.

Research Tools:

ELISAs and Activity Assays

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

Recombinant & Natural Matrix Metalloproteinases (MMPs)

Protein	Catalog #	Species	Source	Size(s)
ADAMTS1, truncated His-tagged	5028002	human	Sf	5/100 µg
ADAMTS4 (aggrecanase 1), truncated His-tagged	5028001	human	Sf	5/100 µg
Aggrecan interglobulin domain	5028003	human	E. coli	100/500 µg
MMP-2 (gelatinase A)	5028013	human	Sf 9	10/200 µg
MMP-9 (gelatinase B) monomer	5028012	human	natural human blood	10/200 µg
MMP-13 (procollagenase-3)	5028014	human	Sf 9	10/200 µg
MMP-13 (collagenase-3) catalytic domain	5028015	human	E. coli	10/200 µg
MMP-14 (MT1-MMP) catalytic domain	5028004	human	E. coli	10/200 µg
MMP-14 prodomain-catalytic domain	5028005	human	E. coli	10/200 µg
MMP-14 prodomain-catalytic domain	5028006	human	E. coli	10/200 µg
MMP-14 hemopexin domain	5028007	human	E. coli	20/200 µg
MMP-15 (MT2-MMP) catalytic domain	5028008	human	E. coli	10/200 µg
MMP-15 hemopexin domain	5028009	human	E. coli	20/200 µg
MMP-16 (MT3-MMP) catalytic domain	5028010	human	E. coli	10/200 µg
MMP-17 (MT4-MMP)	5028011	human	E. coli	10/200 µg
MMP-24 (MT5-MMP) catalytic domain	5028016	human	E. coli	10/200 µg

Antibodies to MMPs

Antibody	Catalog #	Species	Type	Size(s)	Application
aggrecan N-terminal sequence of ARGSVIL	1028023	human	mouse IgG monoclonal	100 µL	WB
Collagenase-3 (MMP-13)	2028019	human	rabbit IgG polyclonal	100 µL	WB, ELISA
Collagenase-3 (latent & human)	1028020	human	mouse IgG monoclonal	100 µL	WB, ELISA
pro-collagenase-3	1028021	human	mouse IgG monoclonal	100 µL	WB, ELISA
pro-collagenase-3	1028022	human	mouse IgG monoclonal	100 µL	Staining or detection
MMP-14	2028017	-	rabbit IgG polyclonal	100 µL	WB, ELISA
MMP-15	2028018	-	rabbit IgG polyclonal	100 µL	WB, ELISA

For research use only. Not for use in diagnostic procedures.

T Cell Antibodies

Stable and selective markers for determining immune status and progress of disease.

Woon, Chan et al., (2001) J. Immunol. 167: 1238.

Mouse anti-human ST2L Antibody

- Catalog # 101002
  101002F (FITC)
  101002B (biotin)
- Description:
  IgG1 affinity purified monoclonal antibody as a marker for human CD4+, CD8+, Th1/Th2, Tc1/Tc2, NK1/NK2, T1/ST2, and ST2L; Clone B4E6.
- Applications:
  Flow cytometry, FACS, Cell sorting, IHC

Rat anti-mouse T1/ST2 Antibody

- Catalog # 101001
  101001F (FITC)
- Description:
  Affinity purified monoclonal antibody, Clone DJ8.
- Applications:
  Flow Cytometry, IP, IHC

IL-18 Receptor Antibody

- Catalog # 201006
- Description:
  Anti-human IL-18R IgG1 affinity purified polyclonal antibody.
- Applications:
  IHC, cytoplasmic staining, western blot, flow cytometry

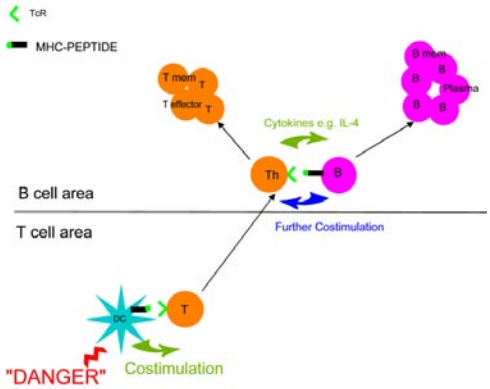
The T and B Cell Transfer Model

The Advantage of Predicting a Compounds Potential Role in Inflammatory Diseases

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, Muliple 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 PGE_2, LTB_4, 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

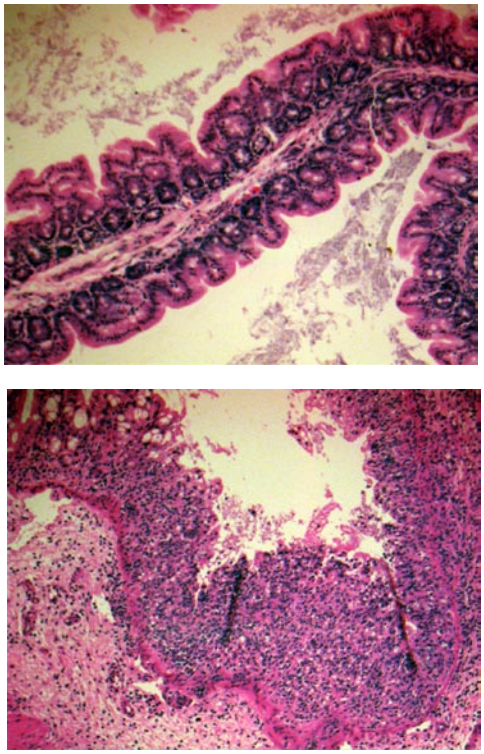


Figure 1: Top - Normal Gut, Bottom - Diseased Gut

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. ●

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Multiplex Testing Services

Quantify Multiple Analytes per Well using the Luminex xMAP® Technology

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:

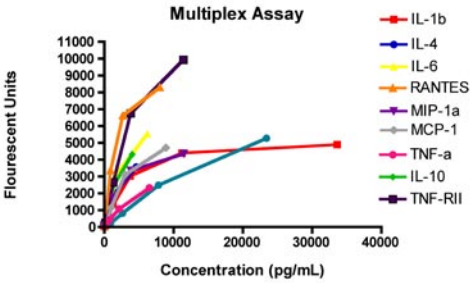
- small sample size - 50 µL
- multiple analytes per well
- ability to customize desired analytes
- sensitivity of 10 pg/mL offers relevant measurements
- precise, accurate and reproducible results that you can count on
- low background and high specificity so your results withstand scrutiny

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. Fuidics 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



Standard curves from a multiplex assay where 10 analytes were measured simultaneously in a single sample well.

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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mdbiosciences.
1000 Westgate Dr, Suite 162
St Paul, MN 55114

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