CYTOKINE AND CHEMOKINE PROTOCOLS TO DETECT BIOACTIVITY OF RECOMBINANT PROTEINS

Updated 7/1/10

USDA-ARS Beltsville Lunney Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: CCL2 CCL3L1, CCL4, CCL5**	Effective Date: July 2010
Written by	Patricia Boyd	Technical Approval	Joan Lunney
Title	Molecular biologist	Title	Project director

1. OBJECTIVE

To describe the procedure for testing recombinant porcine CCL2 (MCP-1) from *Pichia pastoris* in a standard Boyden chamber with activated monocytes prepared from peripheral blood mononuclear cells (PBMCs).

2. SCOPE

This procedure can be used with activated monocytes from most animal species to assess response to chemokines that are monocyte attractants. This molecule's receptors are CCR5 and CCR2 (3).

3. RESPONSIBILITY

It is the responsibility of the operator to be completely familiar with and display competence in the procedure before performing operations independently. It is the responsibility of the operator's supervisor, in the case of a technician-operator, to ensure competence to operate.

.

4. PROCEDURE

A. PBMC isolation

1. Collect blood in anti-coagulant EDTA
2. Dilute blood 1:2 with Hank's Balanced Salt solution (HBSS) [or HBSS with 0.02M EDTA] in 50 ml sterile tissue culture tube.
3. Underlayer 35-40 ml of diluted blood with 7.5 ml of Ficoll-Hypaque; Centrifuge 30 minutes @ 1200 g at room temp; no brake
4. Collect PBMC layer via 10 ml sterile pipette; dilute $\geq$ 2X with HBSS [or HBSS with 0.02M EDTA]
5. Centrifuge 8 minutes at 700 g at room temp; Discard supernatant
6. Resuspend cells by "ratcheting" pellet to break it up; wash again with HBSS [or HBSS with 0.02M EDTA]; centrifuge 8 minutes at 250-400 g
7. Repeat step 5 & 6 but dilute cells with RPMI. If clumping occurs, filter cells through 100 m nylon mesh before centrifuging.
8. Resuspend final cell pellet in 1-5 ml Blast media
9. Count viable cells by microscopy in Hemacytometer [Dilute 10 µl of cell suspension 1:10 with 90 µL of 0.4% trypan blue]. Calculate cell/ml.
10. Multiply by cells/ml X volume of cells for total cells in tube. Calculate amount of Blast media to add to dilute to give required volume of cells at 4 X 10^6 cells/ml.

B. Collection of monocytes

1. Monocytes are enriched from the PBMCs by isolation on Percoll gradient. Briefly, a 15 ml conical tube is prepared with 4 mls of a 57% Percoll solution, under-layered with an equal volume of an 80% Percoll solution. A 4 ml volume of 25% Percoll solution containing not more than 1 X 10(8) cells is carefully overlaid on the 57% layer. Tubes were centrifuged at 600 X G at 10 C for 25 minutes without brake. The layer of cells between 25% and 57% are collected and washed.(5)

2. Cells will be washed 2 X in RPMI with final resuspension in Happy Harry MO medium. Cells were left in conical laid on its side in a bed of ice overnight. Viability is checked in the morning. [It has always been good.] Cells must be transferred to degassed RPMI with 0.1% BSA at a concentration of 1.7 X 10^6 cells/ml for assay.

C. Preparing the Boyden chamber membrane

The Boyden chamber filter must be precoated with collagen or fibronectin (matrix),

1. Coat PVP free polycarbonate membranes with 80µL collagen or fibronectin (2mg/mL) mixed with 20mL PBS in a Petri dish. Flip a few times as the membrane is a little hydrophobic. Refrigerate overnight.
2. Before use, float membrane over PBS to remove excess matrix. Wash well.
3. Hang up to dry. Use kimwipe to remove excess from edge. Now flip membrane upside down so you create an even distribution of PBS and matrix.

You should be using PVP free membranes. If not, the membranes should be washed in Acetic Acid to strip off the PVP and allow the matrix to adhere better to the membrane.
I put an R on the "right side" with a pencil. I put a drop of nail polish on the wrong side when it is drying. It is very easy to get confused about which side you must scrap later.

D. Preparing the Boyden Chamber.

1. Adjust a variable-volume micropipette with a 1mm tip so that the ejected liquid fills a bottom well. The well will hold 25 to 26 µL. A slight positive meniscus should form when the well is filled; this prevents air bubbles from being trapped when the filter is applied.
2. Orient the bottom plate on a flat surface so that the NP trademark is at the upper left. Warm chemo attractants or control reagents to about 37°C and de-gas them by vortex or vacuum. Fill the bottom wells, completing the 48 wells in no more than 5 minutes, to prevent excessive evaporation.
3. Cut 1mm off the corner of a filter membrane and orient it with the cut corner at the upper left. Lift the filter by the ends with two forceps (use your fingers), hold it evenly over the filled wells, and lower it onto them, allowing the middle portion of the filter to make contact first. The filter position can be adjusted at this point if necessary, but note that too much movement will cause contamination between wells.
4. Apply the silicone gasket with its cut corner at the upper left, then the top plate, aligning its NP trademark with the trademark on the bottom plate. Push the top plate down firmly and do not let go; this helps prevent air bubbles from being drawn in and trapped in the bottom wells. With your free hand, apply and tighten the thumb nuts until finger tight. Do not use pliers or other tools to tighten them.

E. Preparing and Adding Responding Cells

1. In the upper wells the concentration of cells in the suspension should be adjusted so that 50µL contains the desired number of cells for one well. For example, since the exposed filter area for each well is 8mm^2, a suspension of 32,000 cells in 50µL will yield 4,000 cells/mm^2.; 0.6 X 10^6 cells/ml in Blast media. Note: In this strategy the optimum number of cells per mm^2 of filter area is usually the same as the number of pores per mm^2. The recommended 5 micron filter has 4,000 pores per mm^2 and these pores occupy only about 7.85% of the surface area of the filter. The optimum density of cells on this filter would be 4,000 cells per mm^2 of filter area, and only one in 12 cells are likely to settle over a pore. At negative control sites 92% of the cells on the filter would be highly unlikely to arrive at pores at all, and the 8% that started out over pores would be as likely to migrate away from the pore as through it. **This is all theoretical; you find the optimum concentration based on experience. You want enough cells coming through to get a good count but you don't want your background going up. A lot of times you take what you have. You need at least 1 X 10^6cells/ml and you need at least 2 mls to do 40 wells.**
2. Pipette cell suspension into each upper well, adjusting the volume so that the filled wells have a slight positive meniscus. Hold the pipette at a steep angle so that the end of the pipette tip rests against the wall of the chamber just above the filter, and the side of the tip rests against the top rim of the well. Eject the fluid with a rapid motion to dislodge air in the bottom of the well.
3. Check for trapped bubbles in the upper wells. One easy way to do this is to look at the reflections of overhead lights in the meniscuses: a well with an abnormally large positive meniscus usually has a trapped air bubble. To remove any bubbles, suck the well completely dry with a suction line and disposable pipette tip, and then refill it.
4. For most chemotaxis assays the filled chamber is incubated at 37°C in humidified air with 5% CO2. Incubation times vary considerably depending on cell types and chemotactic factors. One good way to determine the optimum incubation time is to use 6 to 12 blind-well chambers (e.g. stock # BW100) set up as negative controls and placed simultaneously in the incubator. Remove one blind-well chamber after a set period (e.g. 30 minutes), and remove the rest sequentially, one every 5-30 minutes. Stain the filters and examine them to see how long stimulated cells have taken to migrate through the filter**, I recommend 1.25 hours (4)**

For more information and strategies for working out incubation times see:
http://www.neuroprobe.com/protocols/info_incubation.html

F. ***Counting the cells:***

Staining Polycarbonate Filters

Implements used in the following procedures are available in an accessory pack (stock # P48AP), or they can be purchased separately (see 48-well chamber accessories).

1. Aspirate fluid from the top wells or empty them by shaking the chamber over a sink or container. **(Before you do this use a hand lens and check for bubbles. This is when you will see your problems)**
2. Remove the thumbnuts while holding down the top plate, and invert the entire chamber onto a paper towel. Grasp the four corners of the top plate (now on the bottom) and push down evenly so that it stays level as it drops to the table. If the gasket should hang up on the post hardware, carefully push it down evenly onto the plate. Take care not to touch the filter, which should be stuck to the gasket. Immerse the remaining plate in cool distilled water. **Unless you want to see what came through. I haven't found this informative yet. These are all monocytes**.
3. The migrated cells are now facing up on the filter—this side of the filter is henceforth referred to as the cell side. Lift up one end of the filter with forceps and catch 1mm of the edge in the large filter clamp. Lift the filter and quickly attach the small filter clamp to the edge of the free end. Keeping the cell side up, wet the underside (non-migrated cell side) of the filter in a dish containing PBS. Do not let the PBS wash over the cell side of the filter.
4. Holding the filter by the large clamp, Have the logo face the right side while you remember which side it is. With the small clamp attached to the other end and hanging free, wipe the cells off the non-migrated cell side of the filter by drawing the filter up over the wiper blade. The blade should first contact the filter just below the jaws of the wide clamp. Use only gentle pressure against the blade, and maintain an angle of about 30° from the vertical for the portion of the filter above the wiper. It is important to complete the wiping carefully and quickly so that the cells will not dry on the filter; drying takes place in 10 to 20 seconds, and will prevent complete removal of the non-migrated cells.
5. Clean the wiper with a Q-Tip, again wet the underside of the filter in PBS, and repeat Step 3. Clean the wiper again, wet the filter a third time in PBS, and repeat Step 3.
6. For granulocytes and monocytes, carefully immerse the filter in methanol, and then place the filter cell-side up on a disposable lint-free towel for air-drying. Rinse all chamber components in cool distilled water. For other kinds of cells, consult the literature for staining techniques.
7. When the filter is dry, clamp the edge of one end with a large filter clamp, weight the other end with a small filter clamp, and stain in Diff-Quik® (available from VWR Scientific Products - www.vwrsp.com) or equivalent dye, according to the manufacturer's instructions. To avoid contaminating the chamber components with stain, it is convenient to have two sets of filter clamps, one for removing the filter from the gasket, and one for staining**. [Alternately you can use Hema-3 from Fisher as I did and follow manufacturer's directions, fix and 2 dyes and wash.**
8. Place the wet filter cell-side up on a 50 x 75mm microscope slide to dry. When the filter is dry, center it on the slide and place a drop of immersion oil on it. The filter is now ready for counting. This can be done at any time because the cells are now fixed, they aren't going anywhere. This gets very messy and the slide sticks to the mount making it impossible to get a good count. I do 3 swipes across the whole field for a count. Use 70% ethanol to clean the bottom of the slide and the mount. You can stand the slide on end and let it drain before counting again.
9. If you are using a template to help locate cell sites on the filter (stock # P48TM or C48TM) modify these steps following the P48TM or C48TM protocol. I use the rubber gasket and a fine tip pencil to mark the bottom of the well. This makes locating the well much easier at 10X magnification. Use 100X with oil. **You must use oil to visualize the cells.**

I strongly recommend reading the suggestions at this web site:
http://www.neuroprobe.com/protocols/info_staining.html
As you are going to need to make choices dependant on what you are measuring.

4. MATERIALS / EQUIPMENT

Hank's Balanced Salt solution (HBSS) or HBSS with 10 mM EDTA and 10mM HEPES.
Blast medium: RPMI with 10% heat-inactivated FCS, gentamycin, 2mMGlutamine, 0.01M HEPES and 2-ME at 50 uM
0.4% Trypan Blue
Ficoll-Hypaque density of 1.08 g/ml
50 ml and 15 ml sterile tissue culture tubes; T75 flasks
Centrifuge capable of spinning at 200 – 1200g
10-200 µl pipettors
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
Neuro Probe AP48 48-well Microchemotaxis Chamber with 5 micron Polycarbonate track-etch (PCTE) membrane and accessory pack.

5. References

Chemotaxis equipment and assays: http://www.neuroprobe.com/protocols/info_design.html

(1) Dillender and Lunney Vet Immunol Immunopathol. 35:301-319, 1993
(2) M Loetscher J. Exp. Med. 184:963-969, 1996
(3) Matsushima et al J Exp Med 169:1485-90, 1989
(4) http://en.wikipedia.org/wiki/CCL2
(5) Solano-Aguilar et al Vet Parasitology. 2010 in submission

6. Materials/Equipment

Hank's Balanced Salt solution (HBSS) or HBSS (Ca, Mg Free), CCL3L1, CCL4, CCL5 with 10 mM EDTA and 10mM HEPES.
Blast media: RPMI with 10% heat-inactivated FCS, gentamicin [50µg/ml], 2mM Glutamine, 0.01M HEPES; 2-ME at 50 uM
Happy Harry MO medium: RPMI 1640 with 10% heat-inactivated normal pig serum (NPS), gentamicin[50µg/ml], 2mMGlutamine, 0.01M HEPES, 4.5gr/L D glucose (add 2.5 gr/L), 1mM Na Pyruvate and 0.1mM Essential amino acids.
Percoll:GE Healthcare Cat#17-0891-01
100% Percoll: To 500 mls of Percoll add 55 mls of 10X PBS. pH to 7.2. A warning, Adding acid to Percoll can make it clump. Stir to pull apart in the cold room over night.
I do 200 ml and 22 mls 10XPBS-CMF pH 7.4, add 0.5 mls sterile 1N HCl and stir.
I make up my dilutions in Happy Harry so your cells need to see Ca and Mg before the Percoll.
25% Percoll: 50 ml 100% and 150 mls media.
57% Percoll: 114 ml 100% and 86 mls media
Skip the 80%, You aren't using that layer. Why throw out expensive reagents? You might want to try 50%. We have seen protocols for that in human.

0.4% Trypan Blue
HEMA-3 staining kit (Fisher)
Ficoll-Hypaque = density of 1.08 g/ml

50 ml and 15 ml sterile tissue culture tubes; T75 flasks
Centrifuge capable of spinning at 200 – 1200g
10-200 µl pipettors
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
Neuro Probe AP48 48-well Microchemotaxis Chamber with 5 micron Polycarbonate track-etch (PCTE) membrane and accessory pack.

7. Safety

All safety precautions when working with biological materials per BARC Laboratory standards should be observed.

USDA-ARS Beltsville Lillehoj Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: CCL20**	Effective Date: January 2008
Written by	Yeong Hong	Technical Approval	Hyun Lillehoj
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Assess bioactivity of recombinant CCL20 (MIP-3α) using a chemotaxis assay for lymphocytes.

2. SCOPE

Chemokine (C-C motif) ligand 20 (CCL20), also known as liver activation regulated chemokine (LARC) and Macrophage Inflammatory Protein-3 (MIP-3α), is a small cytokine belonging to the CC chemokine family. It is strongly chemotactic for lymphocytes and weakly attracts neutrophils. CCL20 is implicated in the formation and function of mucosal lymphoid tissues via chemoattraction of lymphocytes and dendritic cells towards the epithelial cells surrounding these tissues. CCL20 elicits its effects on its target cells by binding and activating the chemokine receptor CCR6. It is expected that this assay will work with lymphocytes from many species.

3. PROCEDURE

A. EXPERIMENT DURATION

2-24 h incubation at 37°C, 5% CO_2 incubator

B. METHOD

1. For chickens, splenic lymphocytes are prepared from spleen by gently flushing through a cell strainer (Becton Dickinson Labware) and centrifugation at 200g/20min using Histopaque-1077 (Sigma) density gradient medium at RT. For other species, PBMC can be obtained as a substitute.
2. 300ul of splenocyte suspension which contains lymphocytes is placed in the upper compartments of the 24-cell microchemotaxis chambers.
3. 500 ul of medium containing 10% FBS or serially diluted recombinant IL-16 is added to the lower chamber well and incubated for 2-24 at 37°C in a 5% CO2 incubator.
4. Transwell is removed and cell migration is quantified by counting the total the number of cells in the lower well.
5. Control migration is establish by assessing the cells stimulated with buffer alone,

4. MATERIALS / EQUIPMENT

Primary lymphocytes
Recombinant CCL20 protein.
Histopaque-1077 (Sigma).
Culture Medium (RPMI-1640 supplemented with 10% FBS).
HTS Transwell®-24 Permeable Supports with 5.0µm Pore Polycarbonate Membrane. The pore size is dependent on the type of cells.

5. REFERENCES

BIOASSAY USING CHICKEN IL-16:
Min W and H Lillehoj. Identification and characterization of chicken interleukin-16 cDNA. Developmental & Comparative Immunology 28:153-162 (2004).

USDA-ARS Beltsville Lunney Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: CXCL9**	Effective Date: February 2008
Written by	Patricia Boyd	Technical Approval	Joan Lunney
Title	Molecular biologist	Title	Project director

1. OBJECTIVE

To describe the procedure for testing recombinant CXCL19 (a.k.a. as MIG = monokine induced by IFNγ) in a standard Boyden chamber with IL-2 stimulated T cells prepared from peripheral blood mononuclear cells (PBMCs).

2. SCOPE

It is expected that this procedure can be used with lymphocytes from most animal species to assess response to chemokines that are T cell attractants. CXCL9's receptor is CXCR3

3. PROCEDURE

A. PBMC ISOLATION

1. Collect blood in anti-coagulant such as heparin (use 10 units/ml blood) or EDTA
2. Dilute blood 1:2 with Hank's Balanced Salt solution (HBSS) [or HBSS with 0.02M EDTA] in 50 ml sterile tissue culture tube.
3. Underlay 35-40 ml of diluted blood with 7.5 ml of Ficoll-Hypaque; Centrifuge 30 minutes @ 1200 g at room temp; no brake
4. Collect PBMC layer via 10 ml sterile pipette; dilute $\geq$ 2X with HBSS [or HBSS with 0.02M EDTA]
5. Centrifuge 8 minutes at 700 g at room temp; Discard supernatant
6. Resuspend cells by "ratcheting" pellet to break it up; wash again with HBSS [or HBSS with 0.02M EDTA]; centrifuge 8 minutes at 250-400 g
7. Repeat step 5 & 6 but dilute cells with RPMI. If clumping occurs, filter cells through 100 m nylon mesh before centrifuging.
8. Resuspend final cell pellet in 1-5 ml Blast medium
9. Count viable cells by microscopy in hemacytometer [Dilute 10 µl of cell suspension 1:10 with 90 µL of 0.4% trypan blue]. Calculate cell/ml.
10. Multiply by cells/ml X volume of cells for total cells in tube. Calculate amount of Blast medium to add to dilute to give required volume of cells at 4 X 10^6 cells/ml.

B. PBMC STIMULATION AND PREPARATION OF STIMULATED T-CELLS

1. Interleukin-2 (IL-2) source: Recombinant swine or human IL-2 can be used. To produce an IL-2 containing supe from our species we stimulate PBMCs at 4 X 10^6 c/ml with Con A [Sigma C-5275] at final 2.5ug/ml, for 48 hours. Supernatants will be collected and spun down, aliquoted and stored at -20.
2. Culture PBMCs at 1-4 X 10^6 cells/ml with IL-2 or 25%ConA supernatants for 10 days to stimulate T-cells in T75 flasks (15-20ml/flask).
3. Cells will be harvested and washed 3 X in RPMI with final resuspension in Blast medium at a concentration of 0.6 X 10^6 cells/ml for the Boyden chamber experiments.

C. PREPARING THE CHAMBER

1. Adjust a variable-volume micropipette with a 1mm tip so that the ejected liquid fills a bottom well. The well will hold 25 to 26 µL. A slight positive meniscus should form when the well is filled; this prevents air bubbles from being trapped when the filter is applied.
2. Orient the bottom plate on a flat surface so that the NP trademark is at the upper left. Warm chemo attractants or control reagents to about 37°C and de-gas them by vortex or vacuum. Fill the bottom wells, completing the 48 wells in no more than 5 minutes, to prevent excessive evaporation.
3. Cut 1mm off the corner of a filter membrane and orient it with the cut corner at the upper left. Lift the filter by the ends with two forceps, hold it evenly over the filled wells, and lower it onto them, allowing the middle portion of the filter to make contact first. The filter position can be adjusted at this point if necessary, but note that too much movement will cause contamination between wells.
4. Apply the silicone gasket with its cut corner at the upper left, then the top plate, aligning its NP trademark with the trademark on the bottom plate. Push the top plate down firmly and do not let go; this helps prevent air

bubbles from being drawn in and trapped in the bottom wells. With your free hand, apply and tighten the thumb nuts until finger tight. Do not use pliers or other tools to tighten them.

5. If you are using an AP48 template (stock # P48TM) to help locate cell sites on the filters, modify these steps according to the P48TM protocol.

D. PREPARING AND ADDING RESPONDING CELLS

1. In the upper wells the concentration of cells in the suspension should be adjusted so that 50µL contains the desired number of cells for one well. For example, since the exposed filter area for each well is 8mm2, a suspension of 32,000 cells in 50µL will yield 4,000 cells/mm2.; 0.6 X 10^6 cells/ml in Blast medium. Note: In this strategy the optimum number of cells per mm2 of filter area is usually the same as the number of pores per mm2. The recommended 5 micron filter has 4,000 pores per mm2 and these pores occupy only about 7.85% of the surface area of the filter. The optimum density of cells on this filter would be 4,000 cells per mm2 of filter area, and only one in 12 cells are likely to settle over a pore. At negative control sites 92% of the cells on the filter would be highly unlikely to arrive at pores at all, and the 8% that started out over pores would be as likely to migrate away from the pore as through it.
2. Pipette cell suspension into each upper well, adjusting the volume so that the filled wells have a slight positive meniscus. Hold the pipette at a steep angle so that the end of the pipette tip rests against the wall of the chamber just above the filter, and the side of the tip rests against the top rim of the well. Eject the fluid with a rapid motion to dislodge air in the bottom of the well.
3. Check for trapped bubbles in the upper wells. One easy way to do this is to look at the reflections of overhead lights in the meniscuses: a well with an abnormally large positive meniscus usually has a trapped air bubble. To remove any bubbles, suck the well completely dry with a suction line and disposable pipette tip, and then refill it.
4. For most chemotaxis assays the filled chamber is incubated at 37°C in humidified air with 5% CO2. Incubation times vary considerably depending on cell types and chemotactic factors. One good way to determine the optimum incubation time is to use 6 to 12 blind-well chambers (e.g. stock # BW100) set up as negative controls and placed simultaneously in the incubator. Remove one blind-well chamber after a set period (e.g. 30 minutes), and remove the rest sequentially, one every 5-30 minutes. Stain the filters and examine them to see how long stimulated cells have taken to migrate through the filter, Our reference for this recommends 1 hour at 37.

For more information and strategies for working out incubation times see:
http://www.neuroprobe.com/protocols/info_incubation.html

E. COUNTING THE CELLS

Non-adherent cells (ones that drop off the bottom of the filter, as do T lymphocytes) must be counted in the bottom of the well, so all of your cells will be in the bottom of that 25 ul well. So resuspend everything and take 10 ul and do a 1:2 dilution in trypan blue and count.

I strongly recommend reading the suggestions at the web site as you are going to need to make choices dependant on what you are measuring.

4. MATERIALS / EQUIPMENT

Hank's Balanced Salt solution (HBSS) or HBSS with 10 mM EDTA and 10mM HEPES.
Blast medium: RPMI with 10% heat-inactivated FCS, gentamycin, 2mMGlutamine, 0.01M HEPES and 2-ME at 50 uM
0.4% Trypan Blue
Ficoll-Hypaque density of 1.08 g/ml
50 ml and 15 ml sterile tissue culture tubes; T75 flasks
Centrifuge capable of spinning at 200 – 1200g
10-200 µl pipettors
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
Neuro Probe AP48 48-well Microchemotaxis Chamber with 5 micron Polycarbonate track-etch (PCTE) membrane

5. REFERENCES:

Chemotaxis equipment and assays: http://www.neuroprobe.com/protocols/info_design.html
Dillender and Lunney Vet Immunol Immunopathol. 35:301-319, 1993
M Loetscher J. Exp. Med. 184:963-969, 1996
Flier J et al, J Invest Dermatol. 113(4):574-8, 1999
Liao F. et al, J. Exp. Med. 182:1301-14, 1995

Click to return to the Table of Contents

USDA-ARS Beltsville Lunney Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: CXCL10**	Effective Date: February 2008
Written by	Patricia Boyd	Technical Approval	Joan Lunney
Title	Molecular biologist	Title	Project director

1. OBJECTIVE

To describe the procedure for testing recombinant CXCL10 (a.k.a. as IP-10) in a standard Boyden chamber with IL-2-stimulated T cells prepared from peripheral blood mononuclear cells (PBMCs).

2. SCOPE

This procedure can be used with lymphocytes from most animal species to assess response to chemokines that are T cell attractants.

3. PROCEDURE

A. PBMC ISOLATION

1. Collect blood in anti-coagulant such as heparin (use 10 units/ml blood) or EDTA
2. Dilute blood 1:2 with Hank's Balanced Salt solution (HBSS) [or HBSS with 0.02M EDTA] in 50 ml sterile tissue culture tube.
3. Underlayer 35-40 ml of diluted blood with 7.5 ml of Ficoll-Hypaque; Centrifuge 30 minutes @ 1200 g at room temp; no brake
4. Collect PBMC layer via 10 ml sterile pipette; dilute $\geq$ 2X with HBSS [or HBSS with 0.02M EDTA]
5. Centrifuge 8 minutes at 700 g at room temp; Discard supernatant
6. Resuspend cells by "ratcheting" pellet to break it up; wash again with HBSS [or HBSS with 0.02M EDTA]; centrifuge 8 minutes at 250-400 g
7. Repeat step 5 & 6 but dilute cells with RPMI. If clumping occurs, filter cells through 100 m nylon mesh before centrifuging.
8. Resuspend final cell pellet in 1-5 ml Blast medium
9. Count viable cells by microscopy in Hemacytometer [Dilute 10 µl of cell suspension 1:10 with 90 µL of 0.4% trypan blue]. Calculate cell/ml.
10. Multiply by cells/ml X volume of cells for total cells in tube. Calculate amount of Blast medium to add to dilute to give required volume of cells at 4 X 10^6 cells/ml.

B. PBMC STIMULATION AND PREPARATION OF STIMULATED T-CELLS

1. Interleukin-2 (IL-2) source: Recombinant swine or human IL-2 can be used. To produce an IL-2 containing supe from our species we stimulate PBMCs at 4 X 10^6 c/ml with ConA [Sigma C-5275] at final 2.5ug/ml, for 48 hours. Supernatants will be collected and spun down, aliquoted and stored at -20.
2. Culture PBMCs at 1-4 X 10^6 cells/ml with IL-2 or 25%ConA supernatants for 10 days to stimulate T-cells in T75 flasks (15-20ml/flask).
3. Cells will be harvested and washed 3 X in RPMI with final resuspension in Blast medium at a concentration of 0.6 X 10^6 cells/ml for the Boyden chamber experiments.

C. PREPARING THE CHAMBER

1. Adjust a variable-volume micropipette with a 1mm tip so that the ejected liquid fills a bottom well. The well will hold 25 to 26 µL. A slight positive meniscus should form when the well is filled; this prevents air bubbles from being trapped when the filter is applied.
2. Orient the bottom plate on a flat surface so that the NP trademark is at the upper left. Warm chemo attractants or control reagents to about 37°C and de-gas them by vortex or vacuum. Fill the bottom wells, completing the 48 wells in no more than 5 minutes, to prevent excessive evaporation.
3. Cut 1mm off the corner of a filter membrane and orient it with the cut corner at the upper left. Lift the filter by the ends with two forceps, hold it evenly over the filled wells, and lower it onto them, allowing the middle portion of the filter to make contact first. The filter position can be adjusted at this point if necessary, but note that too much movement will cause contamination between wells.
4. Apply the silicone gasket with its cut corner at the upper left, then the top plate, aligning its NP trademark with the trademark on the bottom plate. Push the top plate down firmly and do not let go; this helps prevent air

bubbles from being drawn in and trapped in the bottom wells. With your free hand, apply and tighten the thumb nuts until finger tight. Do not use pliers or other tools to tighten them.

5. If you are using an AP48 template (stock # P48TM) to help locate cell sites on the filters, modify these steps according to the P48TM protocol.

D. PREPARING AND ADDING RESPONDING CELLS

1. In the upper wells the concentration of cells in the suspension should be adjusted so that 50µL contains the desired number of cells for one well. For example, since the exposed filter area for each well is 8mm2, a suspension of 32,000 cells in 50µL will yield 4,000 cells/mm2.; 0.6 X 10^6 cells/ml in Blast medium. Note: In this strategy the optimum number of cells per mm2 of filter area is usually the same as the number of pores per mm2. The recommended 5 micron filter has 4,000 pores per mm2 and these pores occupy only about 7.85% of the surface area of the filter. The optimum density of cells on this filter would be 4,000 cells per mm2 of filter area, and only one in 12 cells are likely to settle over a pore. At negative control sites 92% of the cells on the filter would be highly unlikely to arrive at pores at all, and the 8% that started out over pores would be as likely to migrate away from the pore as through it.
2. Pipette cell suspension into each upper well, adjusting the volume so that the filled wells have a slight positive meniscus. Hold the pipette at a steep angle so that the end of the pipette tip rests against the wall of the chamber just above the filter, and the side of the tip rests against the top rim of the well. Eject the fluid with a rapid motion to dislodge air in the bottom of the well.
3. Check for trapped bubbles in the upper wells. One easy way to do this is to look at the reflections of overhead lights in the meniscuses: a well with an abnormally large positive meniscus usually has a trapped air bubble. To remove any bubbles, suck the well completely dry with a suction line and disposable pipette tip, and then refill it.
4. For most chemotaxis assays the filled chamber is incubated at 37°C in humidified air with 5% CO2. Incubation times vary considerably depending on cell types and chemotactic factors. One good way to determine the optimum incubation time is to use 6 to 12 blind-well chambers (e.g. stock # BW100) set up as negative controls and placed simultaneously in the incubator. Remove one blind-well chamber after a set period (e.g. 30 minutes), and remove the rest sequentially, one every 5-30 minutes. Stain the filters and examine them to see how long unstimulated cells have taken to migrate through the filter,

For more information and strategies for working out incubation times see:
http://www.neuroprobe.com/protocols/info_incubation.html

E. COUNTING THE CELLS

Non-adherent cells (ones that drop off the bottom of the filter, as do T lymphocytes) must be counted in the bottom of the well, so all of your cells will be in the bottom of that 25 ul well. So resuspend everything and take 10 ul and do a 1:2 dilution in trypan blue and count.

I strongly recommend reading the suggestions at this web site:
As you are going to need to make choices dependant on what you are measuring.

5. MATERIALS / EQUIPMENT

Hank's Balanced Salt solution (HBSS) or HBSS with 10 mM EDTA and 10mM HEPES.
Blast medium: RPMI with 10% heat-inactivated FCS, gentamycin, 2mMGlutamine, 0.01M HEPES and 2-ME at 50 uM
0.4% Trypan Blue
Ficoll-Hypaque density of 1.08 g/ml
50 ml and 15 ml sterile tissue culture tubes; T75 flasks
Centrifuge capable of spinning at 200 – 1200g
10-200 µl pipettors
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
Neuro Probe AP48 48-well Microchemotaxis Chamber with 5 micron Polycarbonate track-etch (PCTE) membrane

7. REFERENCES

Chemotaxis equipment and assays: http://www.neuroprobe.com/protocols/info_design.html

Dillender and Lunney Vet Immunol Immunopathol. 35:301-319, 1993

M Loetscher J. Exp. Med. 184:963-969, 1996

USDA-ARS Beltsville Lunney Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: CXCL11**	Effective Date: February 2008
Written by	Patricia Boyd	Technical Approval	Joan Lunney
Title	Molecular biologist	Title	Project director

1. OBJECTIVE

To describe the procedure for testing recombinant CXCL10 (a.k.a. I-TAC) in a standard Boyden chamber for its ability to cause chemotaxis of IL-2-stimulated T cells prepared from peripheral blood mononuclear cells (PBMCs).

2. SCOPE

This procedure can be used with lymphocytes from most animal species to assess response to chemokines that are T cell attractants. This molecule should have a higher affinity for its receptor then CXCL10.

3. PROCEDURE

A. PBMC ISOLATION

1. Collect blood in anti-coagulant such as heparin (use 10 units/ml blood) or EDTA
2. Dilute blood 1:2 with Hank's Balanced Salt solution (HBSS) [or HBSS with 0.02M EDTA] in 50 ml sterile tissue culture tube.
3. Underlayer 35-40 ml of diluted blood with 7.5 ml of Ficoll-Hypaque; Centrifuge 30 minutes @ 1200 g at room temp; no brake
4. Collect PBMC layer via 10 ml sterile pipette; dilute $\geq$ 2X with HBSS [or HBSS with 0.02M EDTA]
5. Centrifuge 8 minutes at 700 g at room temp; Discard supernatant
6. Resuspend cells by "ratcheting" pellet to break it up; wash again with HBSS [or HBSS with 0.02M EDTA]; centrifuge 8 minutes at 250-400 g
7. Repeat step 5 & 6 but dilute cells with RPMI. If clumping occurs, filter cells through 100 m nylon mesh before centrifuging.
8. Resuspend final cell pellet in 1-5 ml Blast medium
9. Count viable cells by microscopy in Hemacytometer [Dilute 10 µl of cell suspension 1:10 with 90 µL of 0.4% trypan blue]. Calculate cell/ml.
10. Multiply by cells/ml X volume of cells for total cells in tube. Calculate amount of Blast medium to add to dilute to give required volume of cells at 4 X 10^6 cells/ml.

B. PBMC STIMULATION AND PREPARATION OF STIMULATED T-CELLS

1. Interleukin-2 (IL-2) source: Recombinant swine or human IL-2 can be used. To produce an IL-2 containing supe from our species we stimulate PBMCs at 4 X 10^6 c/ml with ConA [Sigma C-5275] at final 2.5ug/ml, for 48 hours. Supernatants will be collected and spun down, aliquoted and stored at -20.
2. Culture PBMCs at 1-4 X 10^6 cells/ml with IL-2 or 25%ConA supernatants for 10 days to stimulate T-cells in T75 flasks (15-20ml/flask).
3. Cells will be harvested and washed 3 X in RPMI with final resuspension in Blast medium at a concentration of 0.6 X 10^6 cells/ml for the Boyden chamber experiments.

C. PREPARING THE CHAMBER

1. Adjust a variable-volume micropipette with a 1mm tip so that the ejected liquid fills a bottom well. The well will hold 25 to 26 µL. A slight positive meniscus should form when the well is filled; this prevents air bubbles from being trapped when the filter is applied.
2. Orient the bottom plate on a flat surface so that the NP trademark is at the upper left. Warm chemo attractants or control reagents to about 37°C and de-gas them by vortex or vacuum. Fill the bottom wells, completing the 48 wells in no more than 5 minutes, to prevent excessive evaporation.
3. Cut 1mm off the corner of a filter membrane and orient it with the cut corner at the upper left. Lift the filter by the ends with two forceps, hold it evenly over the filled wells, and lower it onto them, allowing the middle portion of the filter to make contact first. The filter position can be adjusted at this point if necessary, but note that too much movement will cause contamination between wells.
4. Apply the silicone gasket with its cut corner at the upper left, then the top plate, aligning its NP trademark with the trademark on the bottom plate. Push the top plate down firmly and do not let go; this helps prevent air

bubbles from being drawn in and trapped in the bottom wells. With your free hand, apply and tighten the thumb nuts until finger tight. Do not use pliers or other tools to tighten them.

5. If you are using an AP48 template (stock # P48TM) to help locate cell sites on the filters, modify these steps according to the P48TM protocol.

D. PREPARING AND ADDING RESPONDING CELLS

1. In the upper wells the concentration of cells in the suspension should be adjusted so that 50µL contains the desired number of cells for one well. For example, since the exposed filter area for each well is 8mm2, a suspension of 32,000 cells in 50µL will yield 4,000 cells/mm2.; 0.6 X 10^6 cells/ml in Blast medium. Note: In this strategy the optimum number of cells per mm2 of filter area is usually the same as the number of pores per mm2. The recommended 5 micron filter has 4,000 pores per mm2 and these pores occupy only about 7.85% of the surface area of the filter. The optimum density of cells on this filter would be 4,000 cells per mm2 of filter area, and only one in 12 cells are likely to settle over a pore. At negative control sites 92% of the cells on the filter would be highly unlikely to arrive at pores at all, and the 8% that started out over pores would be as likely to migrate away from the pore as through it.
    a. Pipette cell suspension into each upper well, adjusting the volume so that the filled wells have a slight positive meniscus. Hold the pipette at a steep angle so that the end of the pipette tip rests against the wall of the chamber just above the filter, and the side of the tip rests against the top rim of the well. Eject the fluid with a rapid motion to dislodge air in the bottom of the well.
2. Check for trapped bubbles in the upper wells. One easy way to do this is to look at the reflections of overhead lights in the meniscuses: a well with an abnormally large positive meniscus usually has a trapped air bubble. To remove any bubbles, suck the well completely dry with a suction line and disposable pipette tip, and then refill it.
3. For most chemotaxis assays the filled chamber is incubated at 37°C in humidified air with 5% CO2. Incubation times vary considerably depending on cell types and chemotactic factors. One good way to determine the optimum incubation time is to use 6 to 12 blind-well chambers (e.g. stock # BW100) set up as negative controls and placed simultaneously in the incubator. Remove one blind-well chamber after a set period (e.g. 30 minutes), and remove the rest sequentially, one every 5-30 minutes. Stain the filters and examine them to see how long unstimulated cells have taken to migrate through the filter.

For more information and strategies for working out incubation times see:
http://www.neuroprobe.com/protocols/info_incubation.html

E. COUNTING THE CELLS

Non-adherent cells (ones that drop off the bottom of the filter, as do T lymphocytes) must be counted in the bottom of the well, so all of your cells will be in the bottom of that 25 ul well. So resuspend everything and take 10 ul and do a 1:2 dilution in trypan blue and count.

4. MATERIALS / EQUIPMENT

Hank's Balanced Salt solution (HBSS) or HBSS with 10 mM EDTA and 10mM HEPES.
Blast medium: RPMI with 10% heat-inactivated FCS, gentamycin, 2mMGlutamine, 0.01M HEPES and 2-ME at 50 uM
0.4% Trypan Blue
Ficoll-Hypaque density of 1.08 g/ml
50 ml and 15 ml sterile tissue culture tubes; T75 flasks
Centrifuge capable of spinning at 200 – 1200g
10-200 µl pipettors
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
Neuro Probe AP48 48-well Microchemotaxis Chamber with 5 micron Polycarbonate track-etch (PCTE) membrane

5. REFERENCES

Chemotaxis equipment and assays: http://www.neuroprobe.com/protocols/info_design.html (I strongly recommend reading the suggestions at this web site as you are going to need to make choices depending on what you are measuring.)

Dillender and Lunney Vet Immunol Immunopathol. 35:301-319, 1993

M Loetscher J. Exp. Med. 184:963-969, 1996

Cole et al., J. Exp. Med. 187: 2009-2021, 1998

University of Kentucky Horohov Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: GM-CSF**	Effective Date: January 2008
Written by	Catherine Merant	Technical Approval	David Horohov
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) by assessing its ability to stimulate growth of adherent cells from PBMC.

2. SCOPE

Principle: monocyte proliferation in response to GM-CSF.

3. PROCEDURE

A. PREPARATION OF CELLS

1. Prepare PBMC by centrifugation on ficoll-hypaque (specific gravity of 1.077).
2. Count the PBMC and resuspend them at $10 \cdot 10^6$ cells/ ml cRPMI.
3. Prepare a culture plate which will later contain the series of wells described in #B1 for each GM-CSF sample to test.
4. Add 200 µl of cell suspension to these wells.
5. Let the monocytes adhere for 4 hours.
6. Remove the non-adherent cells and wash the wells 3 times in warm PBS.

B. GM-CSF BIOASSAY

1. Distribute 100 µl of the following medium:
2. CPMed alone
3. CPMed containing a reference GM-CSF (if available; can also be diluted 2x, 4x… to set-up a curve)
4. CPMed containing the new rGM-CSF.
5. Add 100 µl cRPMI to all the wells.
6. Incubate cells for 48 hours at 37°C, 5% CO_2 in a humidified incubator.
7. Add 10 µl/ well of 5 mg/ml MTT solution to the plate and incubate for 4 hours.
8. Add 50 µl/ well of MTT Lysing Solution to the plate and incubate overnight.
9. Read plate at 570-650 nm.
10. Graph standard curve (if available) and analyze data.

4. MATERIALS / EQUIPMENT

Blood in heparin or in CPD/ ACD
Ficoll-hypaque
Complete RPMI Medium (cRPMI): RPMI-1640 supplemented with 10 % FBS (or FES if preferred), 1 % penicillin-streptomycin- L-glutamine and 0.1 % 2-mercaptoethanol
Cytokine production medium (CPMed)
MTT solution (Sigma Cat. No. M5655) 5 mg/ml stock in PBS kept at room temperature (protect from light)
MTT Lysing Solution 20% SDS/ 50% DMF
96-well flat-bottom culture plate

5. REFERENCES

Chen BD, Clark CR, Chou TH. Granulocyte/macrophage colony-stimulating factor stimulates monocyte and tissue macrophage proliferation and enhances their responsiveness to macrophage colony-stimulating factor. Blood 1988, 71, 997-1002.

FOR EQUINE GM-CSF USING Tf-1 CELLS:
Mauel, S, F Steinbach, and H. Ludwig Monocyte-derived dendritic cells from horses differ from dendritic cells of humans and mice. 2006. Immunology 117: 463–473.

University of Kentucky Horohov Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: GM-CSF alternate**	Effective Date: March 2008
Written by	Catherine Merant	Technical Approval	David Horohov
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure the bioactivity of recombinant Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) by using a cell line TF1.

2. SCOPE

Principle: assess the ability of GM-CSF to induce the proliferation of the human erythroleukemia cell lineTF1.

3. PROCEDURE

A. PREPARATION OF CELLS

1. Wash TF1 cells 3 times with RPMI-1640.
2. Count the TF1 cells and resuspend them at 100,000 / ml cRPMI-GM-CSF.

B. GM-CSF BIOASSAY

1. Prepare a culture plate with the following wells (100 µl/ well):
    a. CPMed alone
    b. CPMed with human GM-CSF at 200 U/ml (can be diluted 2x, 4x… to set-up a curve)
    c. CPMed containing the new GM-CSF.
2. Add 100 µl of cell suspension to each well.
3. Incubate cells for 60 to 72 hours at 37°C, 5% CO_2 in a humidified incubator.
4. Add 10 µl/well of 5 mg/ml MTT solution to the plate and incubate for 4 hours.
5. Add 50 µl/well of MTT Lysing Solution to the plate and incubate overnight.
6. Read plate at 570-650nm.
7. Graph standard curve (if available) and analyze data.

4. MATERIALS / EQUIPMENT

TF1 erythroleukemia human cell line
Recombinant human GM-CSF of known activity: e.g. SIGMA Cat. No. G-5035
Complete RPMI-GM-CSF Medium (cRPMI-GM-CSF): RPMI-1640 supplemented with 10 % FBS, 1 % penicillin-streptomycin- L-glutamine and 0.1 % 2-mercaptoethanol + 100 U/ml human GM-CSF
96-well flat-bottom culture plate
Cytokine production medium (CPMed)
MTT solution (Sigma Cat. No. M5655) 5 mg/ml stock in PBS kept at room temperature (protect from light)
MTT Lysing Solution 20% SDS/ 50% DMF

5. REFERENCES

Kitamura T, Tojo A, Kuwaki T, Chiba S, Miyazono K, Urabe A, Takaku F. Identification and analysis of human erythropoietin receptors on a factor-dependent cell line, TF-1. Blood. 1989, 73, 375-380.

Vecchione A, Catchpole B, D'Mello F, Kanellos T, Hamblin A. Modulating immune responses with dendritic cells: an attainable goal in veterinary medicine? 2002. Vet Immunol Immunopathol.87,215- 21
Steinbach F, Stark R, Ibrahim S, Gawad EA, Ludwig H, Walter J, Commandeur U, Mauel S. Molecular cloning and characterization of markers and cytokines for equid myeloid cells. Vet Immunol Immunopathol. 2005, 108, 227-36.

University of Massachusetts			
Standard Operating Procedure for US-VIRN		**Protocol title: IFN-α/β or IFN-tau**	Effective Date: January 2008
Written by	Cynthia Baldwin	Technical Approval	Carolyn Herzig
Title	Project Director	Title	Res. Assist.

1. OBJECTIVE

To evaluate bioactivity of recombinant IFN-α or IFN-β or IFN-tau using a bioassay that depends on stimulation of the transfected Madin Darby Bovine Kidney (MBDK) cell line.

2. SCOPE

This assay measures bioactivity of type I IFNs (IFNα's or IFNβ's) by their ability to stimulate the cells through their receptor resulting in activation of the reporter gene (MxCAT).

3. PROCEDURE

A. METHOD

1. Seed transfected MBDK cells in tissue culture plates at 1 X 10^6cell/3.5cm well and with 2 ml of culture medium.
2. Culture for 24 hr, discard medium and replace with the samples under test using dilutions to establish endpoints.
3. Recombinant human type I IFN can be used as a positive control and titrated to get a standard curve. Set up cells in at least duplicates.
4. Culture at least another 24 hr. and determine CAT expression by commercial ELISA kit (Roche Biochemicals) by lysing all cells with 500ul TritonX-100 for 20 min.
5. 200ul of the lysate was used per well of the ELISA and manufacturer's instructions followed.

B. CELL LINE

This cell line may be available for Dr. B. Charleston upon request with appropriate MTA signed with the Institute of Animal Health, Compton, UK..

4. REFERENCES

Fray MD, Mann GE, Charleston B. Validation of an Mx/CAT reporter gene assay for the quantification of bovine type-I interferon. J Immunol Methods. 2001 Mar 1;249(1-2):235-44.
PMID: 11226480 [PubMed - indexed for MEDLINE]

University of Massachusetts			
Standard Operating Procedure for US-VIRN		**Protocol title: IFN-γ**	Effective Date: January 2008
Written by	Cynthia Baldwin	Technical Approval	Carolyn Herzig
Title	Project Director	Title	Res. Assist.

1. OBJECTIVE

To evaluate bioactivity of recombinant IFN-γ using a bioassay that depends on stimulation of monocytes/macrophages to produce nitric oxide production.

2. SCOPE

This assay measures bioactivity of IFN-γ by its ability to activate monocytes or macrophages to produce nitric oxide using any cell population such as splenocytes or peripheral blood mononuclear cells (PBMC) that contain them. Here we give it using PBMC. Measuring nitric oxide (NO) by the Griess reaction is a very simple colormetric reaction.

3. PROCEDURE

A. ISOLATION OF PBMCs

1. Collect blood in anti-coagulant such as heparin (use 10 units/ml blood)
2. Dilute blood 1:2 with Hank's Balanced Salt solution (HBSS with heparin)
3. Place 20 ml of Ficoll-Hypaque in 50 ml sterile tissue culture tube
4. Slowly layer over 30 ml of diluted blood onto Ficoll-Hypaque
5. Depending on clarity of separation of PBMCs from red blood cells
   - Centrifuge 35 – 50 minutes @ 800 g (room temp)
6. Collect PBMC layer via 10 ml sterile pipette
7. Dilute $\geq$ 2X with HBSS
8. Centrifuge 10 minutes at 400 g at room temp
9. Discard supernatant
10. Resuspend cells in 100 ML HBSS then top up tube with HBSS to 45 ml final volume
11. Centrifuge 10 minutes at 250 g
12. Repeat step 10 & 11
13. Discard supernatant
14. Resuspend cell pellet in 5 ml cRPMI and repipette several times to break up pellet
15. Pipette up 20 µL of suspension
16. Dilute 20 µl of cell suspension 1:10 with 180 µL of 0.4% trypan blue in the well of a Microtiter plate using multiple repipetting
17. Load Hemacytometer (from the pipettor)
18. Count viable cells by microscopy
19. Using the tube from step 14, dilute to 5 X 10^6 cells/ml with RPMI
    - From hemacytometer count determine how many cells in 5 ml
    - Multiply by cells/ml X volume of cells for total cells in tube
    - Calculate amount of medium to add

B. NITRIC OXIDE REACTION

1. Add 100 µL from the 5X10^6 dilution cells to wells of a 96 well flat-bottom sterile Microtiter plate
2. To the first 4 wells add 100 µL medium (cRPMI)
3. To the next 4 wells add 100 µL recombinant human TNF-α at 300 Units/ml
4. Make serial dilutions at dilutions of 1:3.33 of the "test IFNγ cytokine" from 1:1, 1:3.33, 1:10, 1:33.3 etc. The number of dilutions depends upon the operator.
5. The cytokine IFNγ is then added to 4 wells for each dilution alone. To a replicate set of wells add the various dilutions of the IFNγ plus to all wells add the rHuTNFα at 300Units/ml
6. Culture @ 37^{o}C in a humidified 5% CO_2 incubator
7. After 3 days (72 hr) of culture collect 50ul of supernatant from each well (can be done with multichannel pipette) and transfer to new 96 well plate to assess for nitric oxide in Griess reaction.

C. GRIESS REACTION

1. To the wells with culture supernatant, add 200ul of Griess reagent (1% sulfanilamide, 0.1%

naphthylethylenediamine dihydrochloride, 2.5% H_2PO_4).
2. Incubate for 10 minutes
3. Read absorbance at 540 nm using a spectrophotometer.
4. A standard curve can be generated using 1.5-200 uM $NaNO_2$.

D. EXPECTED RESULTS
IFNγ is expected to be active for maximal nitric oxide production in range of 5-500 units/ml when combined with HurIFN-α.

4. MATERIALS / EQUIPMENT

Hank's Balanced Salt solution (HBSS)(with 2 units/ml heparin)
Ficoll-Hypaque
50 ml sterile tissue culture tubes
Centrifuge capable of spinning at 200 – 800g
10-200 µl pipettors ^{O}C
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
96 well microtiter plates
RPMI with 10% heat-inactivate FCS, gentamycin and 2-ME at 50 uM
0.4% Trypan Blue
Commercial recombinant human TNFα
Humidified CO_2 incubator
sulfanilamide
naphthylethylenediamine dihydrochloride
H_2PO_4
$NaNO_2$
Spectrophotometer

5. REFERENCES

FOR USE WITH BOVINE CELLS:
Goff, W.L., W.C. Johnson, C.R. Wyatt and C.W. Cluff. 1996. Assessment of bovine mononuclear phagocytes and neutrophils for induced L-arginine-dependent nitric oxide production. Vet Immunol Immunopathol. 55:45-62.

GRIESS REACTION:
Ding, A.H., C.F. Nathan, and D.J. Strehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritonaeal macrophages: comparison of activating cytokines and evidence for independent production. J. Immunol. 141:2407-2412.

University of Kentucky Horohov Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-1**	Effective Date: January 2008
Written by	Catherine Merant	Technical Approval	David Horohov
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant IL-1 by its ability to stimulate mouse D10 cell line.

2. SCOPE

Principle: D10 mouse cell line proliferation. D10 cells have been shown to respond to IL-1 from cattle (Winstanley and Eckersal, 1992), pigs (Asai et al., 1993) and fish (Hong et al., 2001)

3. PROCEDURE

A. PREPARATION OF CELLS

1. Wash D10 cells 3 times with RPMI-1640.
2. Count the D10 cells and resuspend them at $8 \cdot 10^6$ cells/ ml cRPMI.

B. IL-1 BIOASSAY

1. Prepare a culture plate with the following wells (100 µl/ well):
2. CPMed alone
3. CPMed with a reference IL-1 (if available; can also be diluted 2x, 4x… to set-up a curve)
4. CPMed containing the new rIL-1.
5. Add 50 µl of Culture Medium containing 8 µg ConA/ ml to each well of the 96-well assay plate.
6. Add 50 µl of cell suspension to each well.
7. Incubate cells for 48 hours at 37°C, 5% CO_2 in a humidified incubator.
8. Add 10 µl/well of 5 mg/ml MTT solution to the plate and incubate for 4 hours.
9. Add 50 µl/well of MTT Lysing Solution to the plate and incubate overnight.
10. Read plate at 570-650nm.
11. Graph standard curve (if available) and analyze data.

4. MATERIALS / EQUIPMENT

D10.G4.1 mouse cell line (Cat. No. TIB-224, ATCC)
Complete RPMI Medium (cRPMI): RPMI-1640 supplemented with 10 % FBS (or FES if preferred), 1 % penicillin-streptomycin- L-glutamine and 0.1 % 2-mercaptoethanol
96-well flat-bottom culture plate
MTT solution (Sigma Cat. No. M5655) 5 mg/ml stock in PBS kept at room temperature (protect from light)
MTT Lysing Solution 20% SDS/ 50% DMF

5. REFERENCES

e-Bioscience cytokine bioassays, http://www.ebioscience.com/ebioscience/appls/BAC.htm

Bromander A, Holmgren J and Lycke N. Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro. J. Immunol. 1991, 146, 2908-2914.

FOR SWINE CELLS:
Asai T, Okada M, Ono M, Irisawa T, Mori Y, Yokomizo Y, Sato S. Increased levels of tumor necrosis factor and interleukin 1 in bronchoalveolar lavage fluids from pigs infected with Mycoplasma hyopneumoniae. Vet Immunol Immunopathol. 1993, 38, 253-260.

Hong S, Zou J, Collet B, Bols NC, Secombes CJ. Analysis and characterisation of IL-1beta processing in rainbow trout, Oncorhynchus mykiss. Fish Shellfish Immunol. 2004, 16, 453-459.

FOR BOVINE CELLS:
Winstanley FP, Eckersall PD. Bioassay of bovine interleukin-1-like activity. Res Vet Sci. 1992 52, 273-276.

University of Massachusetts			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-2**	Effective Date: January 2008
Written by	Cynthia Baldwin	Technical Approval	Carolyn Herzig
Title	Project Director	Title	Res. Assist.

1. OBJECTIVE

Evaluate lymphocyte proliferation in response to recombinant IL-2.

2. SCOPE

This procedure can be used with lymphocytes from most animal species to assess response to cytokines that are T cell growth factors. A nonradioactive component for assessing proliferation can be substituted.

3. PROCEDURE

A. ISOLATION OF PBMCs

1. Collect blood in anti-coagulant such as heparin (use 10 units/ml blood)
2. Dilute blood 1:2 with Hank's Balanced Salt solution (HBSS with heparin)
3. Place 20 ml of Ficoll-Hypaque in 50 ml sterile tissue culture tube
4. Slowly layer over 30 ml of diluted blood onto Ficoll-Hypaque
5. Depending on clarity of separation of PBMCs from red blood cells. Centrifuge 35 – 50 minutes @ 800 g (room temp)
6. Collect PBMC layer via 10 ml sterile pipette
7. Dilute $\geq$ 2X with HBSS
8. Centrifuge 10 minutes at 400 g at room temp
9. Discard supernatant
10. Resuspend cells in 100 ML HBSS then top up tube with HBSS to 45 ml final volume
11. Centrifuge 10 minutes at 250 g
12. Repeat step 10 & 11
13. Discard supernatant
14. Resuspend cell pellet in 5 ml cRPMI and repipette several times to break up pellet
15. Pipette up 20 µL of suspension
16. Dilute 20 µl of cell suspension 1:10 with 180 µL of 0.4% trypan blue in the well of a Microtiter plate using multiple repipetting
17. Load Hemacytometer (from the pipettor)
18. Count viable cells by microscopy
19. Using the tube from step 14, dilute to 5 X 10^6 cells/ml with RPMI.
    - From hemacytometer count determine how many cells in 5 ml
    - Multiply by cells/ml X volume of cells for total cells in tube
    - Calculate amount of medium to add
20. Add 100 µL from the 5X10^6 dilution cells to 36 or more wells per animal (to a 96 well flat-bottom sterile Microtiter plate)
21. To the first 4 wells add 100 µL medium (cRPMI)
22. To the next 4 wells add 100 µL commercial recombinant human IL-2 as positive control
23. To the next 4 wells add 100 µL mitogen (ConA as positive control to show cells can proliferate)
24. Make serial dilutions at dilutions of 1:3.33 of the "test cytokine" from 1:1, 1:3.33, 1:10, 1:33.3 etc. The number of dilutions depends upon the operator. Generally a cytokine such as IL-2 is active in range of 1 unit/ml to maximum activity at 50 units/ml. Since units are based on bioactivity a simple set of serial dilutions may have to suffice for these purposes.
25. Culture @ 37^{o}C in a humidified 5% CO_2 incubator
26. After 3 days of culture assess for proliferation.

EVALUATE LYMPHOCYTE PROLIFERATION

1. Add Tritiated thymidine (3H-thymidine) to remainder in microtiter plate wells (20µci/well in 50 µl cRPMI)
2. Culture 18 hours @ 37^{o}C humidified 5% CO_2 incubator
3. Harvest cultures with Cell Harvester (refer to SOP for operation of your model)
4. After manipulating according to Cell Harvester SOP, add appropriate scintillant (5 ml/tube)

Click to return to the Table of Contents

5. Count incorporated 3H-thymidine in the scintillation counter (counts beta radiation)

5. MATERIALS / EQUIPMENT

Hank's Balanced Salt solution (HBSS)(with 2 units.ml heparin)
Ficoll-Hypaque
50 ml sterile tissue culture tubes
Centrifuge capable of spinning at 200 – 800g
10-200 µl pipettors ^{O}C
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
96 well microtiter plates
RPMI with 10% heat-inactivate FCS, gentamycin and 2-ME at 50 uM
0.4% Trypan Blue
Commercial recombinant human IL-2
Mitogen (Concanavallin A) @ 1 µg/ml
Tritiated Thymidine (3H-thymidine)
Humidified CO_2 incubator
Cell Harvester
Scintillation Counter

6. SAFETY

Any operators performing this procedure must have Radiation Safety Training.

7. REFERENCES

Olsen, SC, Stevens MG. Effects of recombinant human cytokines on mitogen-induced bovine PBMC proliferation. Cytokine 1993 5, 498-505

University of Massachusetts			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-4**	Effective Date: January 2008
Written by	Cynthia Baldwin	Technical Approval	Carolyn Herzig
Title	Project Director	Title	Res. Assist.

1. OBJECTIVE

To describe the procedure for isolation of peripheral blood mononuclear cells (PBMC) from heparinzed whole blood and evaluate for lymphocyte proliferation in response to recombinant IL-4.

2. SCOPE

This procedure can be used with lymphocytes from most animal species to assess response to cytokines that are T cell growth factors. A nonradioactive component for assessing proliferation can be substituted.

3. PROCEDURE

A. ISOLATION OF PBMCs

1. Collect blood in anti-coagulant such as heparin (use 10 units/ml blood)
2. Dilute blood 1:2 with Hank's Balanced Salt solution (HBSS with heparin)
3. Place 20 ml of Ficoll-Hypaque in 50 ml sterile tissue culture tube
4. Slowly layer over 30 ml of diluted blood onto Ficoll-Hypaque
5. Depending on clarity of separation of PBMCs from red blood cells. Centrifuge 35 – 50 minutes @ 800 g (room temp)
6. Collect PBMC layer via 10 ml sterile pipette
7. Dilute $\geq$ 2X with HBSS
8. Centrifuge 10 minutes at 400 g at room temp
9. Discard supernatant
10. Resuspend cells in 100 ML HBSS then top up tube with HBSS to 45 ml final volume
11. Centrifuge 10 minutes at 250 g
12. Repeat step 10 & 11
13. Discard supernatant
14. Resuspend cell pellet in 5 ml cRPMI and repipette several times to break up pellet
15. Pipette up 20 µL of suspension
16. Dilute 20 µl of cell suspension 1:10 with 180 µL of 0.4% trypan blue in the well of a Microtiter plate using multiple repipetting
17. Load Hemacytometer (from the pipettor)
18. Count viable cells by microscopy
19. Using the tube from step 14, dilute to 5 X 10^6 cells/ml with RPMI
20. From hemacytometer count determine how many cells in 5 ml
21. Multiply by cells/ml X volume of cells for total cells in tube
22. Calculate amount of medium to add
23. Add 100 µL from the 5X10^6 dilution cells to 36 or more wells per animal (to a 96 well flat-bottom sterile Microtiter plate)
24. To the first 4 wells add 100 µL medium (cRPMI)
25. To the next 4 wells add 100 µL commercial recombinant human IL-4 as positive control
26. To the next 4 wells add 100 µL mitogen (ConA as positive control to show cells can proliferate)
27. Make serial dilutions at dilutions of 1:3.33 of the "test cytokine" from 1:1, 1:3.33, 1:10, 1:33.3 etc. The number of dilutions depends upon the operator. Since units are based on bioactivity a simple set of serial dilutions may have to suffice for these purposes.
28. Culture @ 37^{o}C in a humidified 5% CO_2 incubator
29. After 3 days of culture assess for proliferation.

B. EVALUATE LYMPHOCYTE PROLIFERATION

1. Add Tritiated thymidine (3H-thymidine) to remainder in microtiter plate wells (20µci/well in 50 µl cRPMI)
2. Culture 18 hours @ 37^{o}C humidified 5% CO_2 incubator
3. Harvest cultures with Cell Harvester (refer to SOP for operation of your model)
4. After manipulating according to Cell Harvester SOP, add appropriate scintillant (5 ml/tube)

5. Count incorporated 3H-thymidine in the scintillation counter (counts beta radiation)

4. MATERIALS / EQUIPMENT

Hank's Balanced Salt solution (HBSS)(with 2 units.ml heparin)
Ficoll-Hypaque
50 ml sterile tissue culture tubes
Centrifuge capable of spinning at 200 – 800g
10-200 µl pipettors ^{O}C
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
96 well microtiter plates
RPMI with 10% heat-inactivate FCS, gentamycin and 2-ME at 50 uM
0.4% Trypan Blue
Commercial recombinant human IL-2
Mitogen (Concanavallin A) @ 1 µg/ml
Tritiated Thymidine (3H-thymidine)
Humidified CO_2 incubator
Cell Harvester
Scintillation Counter

5. SAFETY

All safety precautions when working with biological materials per accepted laboratory standards should be observed. Any operators performing this procedure must have Radiation Safety Training.

6. REFERENCES

Olsen, SC, Stevens MG. Effects of recombinant human cytokines on mitogen-induced bovine PBMC proliferation. Cytokine 1993 5, 498-505.

University of Kentucky Horohov Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-5**	Effective Date: January 2008
Written by	Catherine Merant	Technical Approval	David Horohov
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant IL-5 using a human cell line TF1.

2. SCOPE

Principle: TF1 human cell line proliferation. This cell line has been shown to respond to equine recombinant IL-5 (R&D Systems) using the method described by Kitamura et al. (1989).

3. PROCEDURE

A. PREPARATION OF CELLS

Wash TF1 cells 3 times with RPMI-1640.

Count the TF1 cells and resuspend them at 100,000 / ml cRPMI-GM-CSF.

B. IL-5 BIOASSAY

1. Prepare a culture plate with the following wells (100 µl/ well):
    a. CPMed alone
    b. CPMed with a reference IL-5 (if available; can also be diluted 2x, 4x… to set-up a curve)
    c. CPMed containing the new rIL-5.
2. Add 100 µl of cell suspension to each well.
3. Incubate cells for 60 to 72 hours at 37°C, 5% CO_2 in a humidified incubator.
4. Add 10 µl/well of 5 mg/ml MTT solution to the plate and incubate for 4 hours.
5. Add 50 µl/well of MTT Lysing Solution to the plate and incubate overnight.
6. Read plate at 570-650nm.
7. Graph standard curve (if available) and analyze data.

4. MATERIALS / EQUIPMENT

TF-1 erythroleukemia human cell line (Cat. No. CRL-2003, ATCC)

Complete RPMI-GM-CSF Medium (cRPMI-GM-CSF): RPMI-1640 supplemented with 10 % FBS, 1 % penicillin-streptomycin- L-glutamine and 0.1 % 2-mercaptoethanol + 100 U/ml GM-CSF

96-well flat-bottom culture plate

MTT solution (Sigma Cat. No. M5655) 5 mg/ml stock in PBS kept at room temperature (protect from light)

MTT Lysing Solution 20% SDS/ 50% DMF

5. REFERENCES

e-Bioscience cytokine bioassays, http://www.ebioscience.com/ebioscience/appls/BAC.htm

Kitamura T, Tojo A, Kuwaki T, Chiba S, Miyazono K, Urabe A, Takaku F. Identification and analysis of human erythropoietin receptors on a factor-dependent cell line, TF-1. Blood. 1989, 73, 375-380.

R&D Systems. Recombinant Equine IL-5: specifications and use. Catalog Number 2470-EL. 2/28/2005

USDA-ARS Beltsville Lunney Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-6**	Effective Date: January 2008
Written by	Patricia Boyd	Technical Approval	Joan Lunney
Title	Molecular Biologist	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant IL-6 using the mouse B9 cells which are sensitive to "human, rat, rabbit, pig, and mouse IL-6" according to the Current Protocols in Immunology reference. B9 cells have also been used successfully for chicken, equine, and bovine IL-6.

2. SCOPE

IL-6 is scored as hybridoma growth factor according to the extent to which an IL-6-containing sample can be diluted and continue to support B9 cell proliferation. B9 is a mouse B cell hybridoma line that requires IL-6 for survival and proliferation. The cells are cultured in a series of microwells containing increasing dilutions (decreasing concentrations) of the sample to be assayed. After incubation the cultures are pulsed with [3H] thymidine and the amount of radioactivity incorporated into the DNA is measured. Alternatively, proliferation can be assessed using a tetrazolium salt (MTT), which is modified by mitochondrial enzymes in proportion to cell number. Either measurement reflects the amount of biologically active IL-6 present in the sample. This assay is not the only IL-6 assay but it is straight forward and it measures biologically active IL-6 as well as IL-13 using murine B9 hybridoma cells. It fits in with other assays as a final readout of bioactivity, e.g.for IL-17. [These B9 cell also respond to IL-4 but only at 1000 fold higher concentrations and only to murine IL-4.] There are three components to this assay; 1) the p388D1 cells to produce murine IL-6 for maintenance of the B9 cells, 2) method to induce high titer IL-6 supe, and 3) use of B-9 cells, that are IL-6 dependant, for testing of stimulated culture supernatants.

The P388D1 cell line is available from ATCC and will produce murine IL-6 for the maintenance and reference standard for the B-9 line.

3. RESPONSIBILITY

The B-9 cells are available for the US VIRN from Dr. Joan Lunney's lab. This is with the permission of Dr. Lucien Aarden in the Netherlands. The B-9 cell line is not currently available from ATCC or Dr. Aarden.

4. PROCEDURE

This protocol was adapted from *Current protocols in immunology* Unit 6.61 Supplement 17

A. BIOASSAY OF IL6-CONTAINING CULTURE SUPERNATANTS

1. Count the number of B9 cells in an aliquot removed from the stock culture flask in the log phase of growth. For each 96 well microtitre plate in the assay, transfer 2 x 10^5 cells from the flask to a sterile 15ml screw cap tube.
2. Centrifuge the cells 5 minutes in a tabletop centrifuge at 180 x g, 4^o C, and resuspend in 10 ml complete RPMI-10. Repeat twice and resuspend the cells in complete RPMI-10 at 2 x 10^4 cells/ml. Approximately 10 ml of cells are needed for each 96 well microtitre plate.
3. Add 100 ul of washed cells (2 x 10^4 cells/ml) to each well of a 96 well microtitre plate. This step is facilitated by using a 12 channel pipettor and a sterile V-bottom reservoir tray to hold the washed cells.
4. Add 100ul of 2-fold serial dilutions of the test sample to each well (reserving two or three rows of the plate for IL-6 standards). Add each sample dilution in triplicate wells to control for variation. In order to determine the background response, include a row of wells that receive 100ul complete RPMI-10 medium only. Each well will have a final volume of 200ul and a final concentration of 1 x 10^4 cells/ml (2000 cells per well). If any samples are known or suspected to possess high titers of IL-6, several two fold serial dilutions of the sample should be prepared and assayed. The object is to find an initial dilution that will fall into the effective range of the assay.
    a. When attempting to quantitate IL-6 activity, two or three rows of the plate should be reserved for a standard that contains a known level of IL-6 activity. This allows for the normalization of the interassay variation. Because there is currently no standard unit forIL-6 activity, a standard must be prepared in the user's laboratory.
5. Incubate the plates 72 hours in a humidified 37^o C, 5% CO_2 incubator.
6. Add [^{3}H] thymidine and incubate plates 4 hr at 37oC. Harvest the cells, determine [^{3}H] thymidine incorporation by liquid scintillation counting, and quantitate IL-6 activity by calculating HGF U/ml against a know standard

wither it is recombinant mouse Il-6 or a P388D1 supe. To assess proliferation using the nonradioactive colorimetric MTT assay, use the normal methodology with the following exceptions: add 50 ul of 10% (v/v) Triton X-100/0.5 M HCL and incubate overnight at room temperature in the dark to allow maximal color development; read plates at 540 nm. Radioactive and nonradioactive methods for quantitating DNA should provide similar results.

B. B9 CELL MAINTENANCE

1. Prepare B9 culture medium by supplementing complete RPMI-10 with human or mouse IL-6 at a level of 50 to 100 hybridoma growth factor (HGF) U/ml. Until the activity (in HGF U/ml) of the stock supply of IL-6 is established, several different concentrations of IL-6 should be used to maintain cells. If IL-6 containing supernatant is used, try 0.1%, 1.0%, and 10%. If recombinant IL-6 is used, try 100 and 500 pg/ml. Choose the lowest concentration at which maximal growth is obtained.
2. Subculture B9 cells by transferring 2.5 x 10^5 cells to a fresh 25-cm^2 tissue culture flask containing 5 ml of B9 culture medium. The culture will be in the log phase of growth between days 3 and 5. If more cells are needed, the culture can be scaled up accordingly.
3. When the culture reaches a density of 1 x 10^6 cell/ml (4 to 5 days), repeat step 2.
4. Thaw a fresh vial of cells when the background response from the control wells in the Basic Protocol begins to rise (approximately every 60 days). When the B9 cells are first received, a 100 ml flask of cells should be grown. When the cell density reaches 5-8 x 10^5 cells/ml, 10-20 vials of cells should be cryopreserved. Centrifuge the cells 5 minutes at 180 x g, 4^oC. Resuspend at 3-5 x 10^6 cells/ml in 10% DMSO/90% FBS and aliquot into freezing vials. Immediately place the vials in a -70^oC freezer overnight and transfer to liquid nitrogen storage the next day.

C. B9 MAINTENANCE BY CULTURE IN 25% P388D1 SUPERNATANT

1. The murine macrophage cell line P388D1 can be induced to produce a supernatant containing murine IL-6. This supernatant can then be used to maintain the B-9 cell line and as a source of IL-6 standard.
2. Murine macrophage cell line P388D1 (ATCC #TIB 63)
3. RPMI 1640 with 5% FBS or DMEM with 10 or 5 % FBS and serum free RPMI 1640.

D. PRODUCTION OF P388D1 SUPERNATANT

1. To maintain P388D1 cells, grow the cells as an adherent cell culture in RPMI with 5% FBS.
2. These should not be trypsinized. When the density is high enough transfer the free cells to a new large flask and plate at 1 X 10(5)cells/ml. Use a large flask.
3. When these cells are confluent replace medium with serum free RPMI. After 5 days collect supernant. You must spin down as you will have a lot of debri. Collect all supernants and freeze. When you have collected enough, sterile filter and aliquot in 12.5 ml aliquots to freeze. These can be stored at -20C and used to make the 25% supe containing medium.
4. In the mean time start another large flask to make additional supe. You can not reuse the starved cells.
5. To freeze this line scrape, count and freeze in 10%DMSO. Treat line as a normal adherent cell line.

5. MATERIALS / EQUIPMENT

B9 cells in log phase of growth (see B-9 cell maintenance)
Complete RPMI-10 medium
IL-6 Standard
[^{3}H] thymidine or MTT
96 well flat bottomed microtiter plates
15 ml screw cap tubes, sterile
12 channel pipettor (50- to 250- ul adjustable volume
V-bottom reservoir trays, sterile

Additional reagents and equipment for counting and harvesting cells and determination of DNA synthesis by [^{3}H] thymidine incorporation or MTT colorimetric assay

Human or mouse IL-6 stock: IL-6 containing supernatant from cell culture (see P388D1 protocol)
B9 cells (for source, see above paragraph)
10% dimethyl sulfoxide (DMSO)/90% heat inactivated FBS
25-cm^2 tissue culture flask

6. REFERENCES

Current protocols in immunology, Unit 6.61 Supplement 17

Kingfisher Biotech			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-6**	Effective Date: January 2008
Written by	Joanna Labresh	Technical Approval	Carrie Stefans
Title	Project Director	Title	Research. Associate

1. OBJECTIVE

To evaluate bioactivity of recombinant IL-6 using a cell proliferation assay with B9 cell line. The B9 cells have been used in bioassays for IL-6 of human, bovine, horse, pig, dog, rat, mouse and seal origin.

2. SCOPE

The IL-6 bioassay is based on a cell proliferation assay using the B9 cell line, a murine B cell hybridoma. In this assay, the stimulatory effect of IL-6 is measured directly using the following method: MTS (a tetrazolium salt) is added to the culture medium and is bioreduced by LDH, which is found in metabolically active cells, into a colored end product, formazan, that is soluble in the culture media. The quantity of formazan, as measured by the amount of absorbance at 490 nm, is directly proportional to the number of living cells in culture. Therefore, looking at test results represented as optical density (O.D.)- 490 nm versus [IL-6], increasing proliferation is expected from increasing concentrations of IL-6, under optimal growth conditions. This assay can be used to test human, bovine, equine, porcine, canine, rat, and mouse IL-6.

3. PROCEDURE

A. NDICATOR CELL CULTURE

1. Grow B9 cells in B9 Maintenance Medium (Appendix B). The cells are grown in T25 flasks, incubated in a horizontal position, using 10 ml B9 Maintenance Medium per flask. The cells are transferred using a 1:3 - 1:4 dilution 2 - 3 times per week, or diluted to 2.0 x 10^4 cells/ml when a density of 2.0 x 10^5 cells/ml is reached.
2. Maintain cultures between 1,000 - 900,000 cells/ml; 5% CO2;37°C. [The B cell hybridoma B9 has been cloned from the parent line B13.29 and is highly dependent on IL-6 for growth. Cells also proliferate in response to high concentrations (approximately 1000 pg/ml) of murine IL-4 and human IL-13. For routine culture, cells are grown in 10-100 units/ml IL-6. An IL-6 assay can be carried out in a flat bottom microtiter plate in 200µl. 5000 B9 cells/well are cultured and labelled at 68-72 hours with 7.4kBq 3H-Thymidine. Samples to be tested are titrated and related to a standard preparation; 1U/ml is the concentration that leads to half-maximal 3H-Thymidine incorporation.]

B. PROCEDURE

1. 48 hours following the last cell transfer, pool the cells from all flasks into a 50 ml conical centrifuge tube. Bring the volume up to 50 ml with B9 Assay Medium (Appendix B) and spin down at 250 x g for 10 minutes at room temperature.
2. Resuspend the cells with 50 ml B9 Assay Medium and spin down at 250 x g for 10 minutes at room temperature.
3. Resuspend the cells in 10 ml B9 Assay Media.
4. In a separate tube, mix 20 µl of B9 cells with 20 µl 0.4% Trypan Blue (a 1:1 ratio).
5. Count the cells with a hemocytometer. Count at least 100 cells: the live cells are clear (Trypan Blue exclusion), while the dead cells appear blue. The viability of the cells should be higher than 95%.
6. Dilute the cells in B9 Assay Medium to 1.0 x 10^5 cells/ml.
7. Aliquot the cells into a 96-flat well plate using a density of 5000 cells/well (i.e. 50 µl/well of 1.0 x 10^5 cells/ml)

C. POSITIVE CONTROL AND SAMPLE PREPARATION AND ADDITION

1. For a positive control, use a recombinant human IL-6 sample that has a known biological activity in this assay. Perform a 2-fold dilution series using B9 Assay Medium with the known recombinant human IL-6 protein and sample starting at 4 ng/ml and ending at 4 pg/ml. Include a negative control (*i.e.* Assay medium only (0 pg/ml)). In the assay the final concentrations for the dilutions will be 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004, 0.002 ng/ml.
2. When testing a lyophilized protein, reconstitute the recombinant IL-6 with sterile filtered NanoPure water to a final concentration of 100 µg/ml.

3. Add 50 µl/well of the positive control and sample dilutions to the 96-flat well microplate containing the B9 cells. Each dilution should be repeated in three or more replicates. Include a set of replicates for negative controls (*i.e.* cells with B9 Assay Medium only and 0 pg/ml recombinant protein).
4. Incubate the plates for 48 hours at 37°C in a humidified 5% CO_2 incubator.
5. Add 20 µl/well of MTS/PMS solution (20:1) and incubate the plates for 1 - 6 hours at 37°C in a humidified 5% CO_2 incubator.
6. Read the O.D. (optical density) on a 96-well plate reader at 490 nm after 1 hour and every hour for 2 - 6 hours to determine the optimal readings (*i.e.* the highest ratio of O.D.s recorded for maximal proliferation/proliferation at 0 ng/ml recombinant IL-6.
7. After the last reading, dispose of all cells, plates and used reagents in a biohazard container.

D. CALCULATION OF RESULTS
Plotting the OD versus log [IL-6] will give a sigmoidal curve.

The ED_{50}, to be calculated from the linear portion of the graph, represents 50% proliferative response relative to the maximal proliferative response obtained in the linear portion of the graph.

4. MATERIALS / EQUIPMENT

DESCRIPTION	*SUPPLIER*	*CATALOG #*
Murine B9 Cells (Appendix A)	ECACC	96080128
B9 Maintenance Media	Appendix B	
B9 Assay Media	Appendix B	
Recombinant Human IL-6	Pierce/Endogen	RIL610
MTS/PMS Solution (Cell Titer 96 Aqueous®)	Promega	G5421
0.4% Trypan Blue	Gibco BRL	15250-061
96-well Flat Bottom Microplate	Corning /Costar	3595
Tissue Culture Flasks (T25)	Corning	10-126-39
Round Bottom Microplates	Corning/Costar	3790

B9 MAINTENANCE MEDIA:
- RPMI 1640 supplemented with:
- 4mM L-Glutamine
- 5% FBS (Fetal bovine serum)
- 50 µM β-ME (beta-mercaptoethanol)
- 100 U/ml Penicillin
- 100 □g/ml Streptomycin
- 50 pg/ml Recombinant human IL-6

B9 ASSAY MEDIA:
- RPMI 1640 supplemented with:
- 4mM L-Glutamine
- 5% FBS (Fetal bovine serum)
- 50 µM β-ME (beta-mercaptoethanol)
- 100 U/ml Penicillin
- 100 ug/ml Streptomycin

B9: MOUSE B CELL LYMPHOBLASTOID SPLENIC CELL LINE:
IMDM or RPMI 1640 + 2mM Glutamine + 50µM 2-Mercaptoethanol (2ME) + 50pg/ml recombinant human IL-6 (diluted and stored in PBS containing 1% BSA) + 5% FBS (no Glutamine required with IMDM).

5. SAFETY

Recombinant IL-6 and MTS/PMS solution are toxic. Use proper precautions when handling these materials.

6. REFERENCES

Aarden, L. A. *et al*. (1987) Eur. J. Immunol. **17**:1411.

Bernard, C *et al.* (1998) Hypertension **31**:1350.

Helle, M *et al*. (1988) Eur. J. Immunol. **18**: 1535.

Marshall, J.S. *et al.* (1996) J. Clin. Invest. **97**:1122.

http://www.promega.com/tbs/tb245/tb245.pdf. Cell Titer 96 Aqueous One Solution Cell Proliferation Assay.

FOR B9 CELL LINE :
Eur J Immunol 1987;17:1411

Eur J Immunol 1988;18:1535

USDA-ARS Beltsville Lunney Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-7**	Effective Date: February 2008
Written by	Patricia Boyd	Technical Approval	Joan Lunney
Title	Molecular biologist	Title	Project director

1. OBJECTIVE

To describe the procedure for testing recombinant IL-7 in a standard proliferation assay with the necessary PHA stimulated T-cells

2. SCOPE

This procedure can be used with lymphocytes from most animal species to assess response to the cytokine IL-7. A nonradioactive component for assessing proliferation can be substituted.

3. PROCEDURE

T-cell stimulation:

A. PBMC isolation

1. Collect blood in anti-coagulant such as heparin (use 10 units/ml blood) or EDTA
2. Dilute blood 1:2 with Hank's Balanced Salt solution (HBSS) [or HBSS with 0.02M EDTA] in 50 ml sterile tissue culture tube.
3. Underlayer 35-40 ml of diluted blood with 7.5 ml of Ficoll-Hypaque; Centrifuge 30 minutes @ 1200 g at room temp; no brake
4. Collect PBMC layer via 10 ml sterile pipette; dilute $\geq$ 2X with HBSS [or HBSS with 0.02M EDTA]
5. Centrifuge 8 minutes at 700 g at room temp; Discard supernatant
6. Resuspend cells by "ratcheting" pellet to break it up; wash again with HBSS [or HBSS with 0.02M EDTA]; centrifuge 8 minutes at 250-400 g
7. Repeat step 5 & 6 but dilute cells with RPMI. If clumping occurs, filter cells through 100 m nylon mesh before centrifuging.
8. Resuspend final cell pellet in 1-5 ml Blast medium
9. Count viable cells by microscopy in Hemacytometer [Dilute 10 µl of cell suspension 1:10 with 90 µL of 0.4% trypan blue]. Calculate cell/ml.
10. Multiply by cells/ml X volume of cells for total cells in tube. Calculate amount of Blast medium to add to dilute to give required volume of cells at 4 X 10^6 cells/ml.

B. PBMC stimulation with IL-7 supernatants

1. Prestimulation of PBMC
    a. Culture PBMCs at 1-4 X 10^6 cells/ml with PHA (Sigma L 9132) at 20 ug/ml for 2 days to stimulate T-cells in T75 flasks (15-20ml/flask).
    b. Cells will be harvested and washed 3 X in RPMI with final resuspension in Blast medium at a concentration of 4 X 10^6 cells/ml of a 96 well plate.

C. Stimulation of T cell blasts

1. Culture 1-4 X 10^5 cells/well in a 96 well plate with serial diluted recombinant IL-7, 0.2ng/ml to 10 ng/ml. Use human recombinant IL-7 as a positive control. Porcine is more similar to human than mouse and mouse IL-7 works in human assays.
2. Add 50 µl/well of the positive control and sample dilutions to the 96-flat well microplate containing the stimulated T cells. Each dilution should be repeated in three or more replicates. Include a set of replicates for negative controls (*i.e.* cells with Medium only and 0 ng/ml recombinant protein).
3. Incubate the plates for 72 hours at 37°C in a humidified 5% CO_2 incubator.
4. Add 20 µl/well of proliferation dye MTS/PMS solution (20:1) and incubate the plates for 1 - 6 hours at 37°C in a humidified 5% CO_2 incubator.
5. Read the O.D. (optical density) on a 96-well plate reader at 490 nm after 1 hour and every hour for 2 - 6 hours to determine the optimal readings.
6. After the last reading, dispose of all cells, plates and used reagents in a biohazard container.

C. CALCULATION OF RESULTS

1. Plotting the OD versus log [IL-7] will give a sigmoidal curve.

2. The ED_{50}, (effective dose) to be calculated from the linear portion of the graph, represents 50% proliferative response relative to the maximal proliferative response obtained in the linear portion of the graph.

5. MATERIALS / EQUIPMENT

Hank's Balanced Salt solution (HBSS) or HBSS with 10 mM EDTA and 10mM HEPES.
Blast media: RPMI with 10% heat-inactivated FCS, gentamycin, 2mMGlutamine, 0.01M HEPES and 2-ME at 50 uM
0.4% Trypan Blue
Ficoll-Hypaque density of 1.08 g/ml
50 ml and 15 ml sterile tissue culture tubes; T75 flasks
Centrifuge capable of spinning at 200 – 1200g
10-200 µl pipettors
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
MTS/PMS solution Promega G5421
Reader for 490 nm.
GRAPHING SOFTWARE.

6. SAFETY

All safety precautions when working with biological materials per BARC Laboratory standards should be observed. MTS/PMS solution are toxic. Use proper precautions when handling these materials.

7. REFERENCES

Armitage R J et al J Immunol. 144:938-941 1990

USDA-ARS Beltsville Lunney Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-8**	Effective Date: January 2008
Written by	Patricia Boyd	Technical Approval	Joan Lunney
Title	Molecular Biologist	Title	Project director

1. OBJECTIVE

To evaluate bioactivity of recombinant IL-8 by a neutrophil chemotaxis assay.

2. SCOPE

The assay relies on the basic Boyden chamber, which can be purchased from Neuro Probe or Millipore. It is a set of holes on bottom of which a filter with particular sized pores sits. You put the compound to be tested in the well below the filter. You put the filter apparatus over the well with some manipulation to prevent air being trapped; you need a good interface. You add your counted cells and medium +/- controls in triplicate to the individual wells. You wait the appropriate amount of time, usually between 1 to 2 hours, and you separate the apparatus and count what went through or what migrated and stuck to the other side of the filter. This will depend on cell type. Simple. Tedious. Time consuming. But highly accurate if appropriate controls are run.

3. PROCEDURE

A. SUMMARY

1. Prepare cells appropriate to the compound being tested.
2. Dilute and arrange compounds being tested, i.e., +/- controls in wells.
3. Overlay the filter apparatus assuring no bubbles are trapped in meniscus.
4. Add medium and controls with cells being tested.
5. Incubate at 37 in 5%CO2 for 1 to 2 hours.
6. Remove remaining cells from top of filter with swab, remove filter apparatus. If cells are adherent, like macrophages, follow manufacturers' directions for fixing and staining. If cells are non adherent like T-cells or neutrophils, count what has migrated to the bottom of the well. Three counts for each data point will give decent statistics.

B. EXPERIMENTAL SET UP

You will need a negative control, a positive chemokine control, your unknown and a chemotaxis control. It is strongly recommend you see this web site http://www.neuroprobe.com/protocols/info_controls.html for a description of these controls. We strongly recommend having three wells per assay point to provide statistically relevant data; this is why you will need a larger number of wells than seems obvious at first.

C. PREPARING THE CHAMBER

1. Adjust a variable-volume micropipette with a 1mm tip so that the ejected liquid fills a bottom well. The well will hold 25 to 26 µL. A slight positive meniscus should form when the well is filled; this prevents air bubbles from being trapped when the filter is applied.
2. Orient the bottom plate on a flat surface so that the NP trademark is at the upper left. Warm chemo-attractants or control reagents to about 37°C and de-gas them by vortex or vacuum. Fill the bottom wells, completing the 48 wells in no more than 5 minutes, to prevent excessive evaporation.
3. Cut 1mm off the corner of a filter membrane and orient it with the cut corner at the upper left. Lift the filter by the ends with two forceps, hold it evenly over the filled wells, and lower it onto them, allowing the middle portion of the filter to make contact first. The filter position can be adjusted at this point if necessary, but note that too much movement will cause contamination between wells.
4. Apply the silicone gasket with its cut corner at the upper left, then the top plate, aligning its NP trademark with the trademark on the bottom plate. Push the top plate down firmly and do not let go; this helps prevent air bubbles from being drawn in and trapped in the bottom wells. With your free hand, apply and tighten the thumb nuts until finger tight. Do not use pliers or other tools to tighten them.
5. If you are using an AP48 template (stock # P48TM) to help locate cell sites on the filters, modify these steps according to the P48TM protocol.

D. NEUTROPHIL ISOLATION

Keep everything on ice as you go through this.

1. Original Neutrophil enrichment: Collect whole blood in citrate. Spin at 1000 g (2000 rpm) 15', 4 C, no brake, in a 50 ml conical. Buffy coat lymphocytes (white cell layer) will be right on top of the Erythrocyte (RBC) layer. Remove all of plasma and the lymphocyte layer down to ~ 7 mls in the bottom. Add 30 mls of ice cold PBS at pH 7.2 and mix gently30-60 seconds.
2. Neutrophil isolation: Underlay 7 mls of Ficoll-hypaque [at 1.08 density] Spin at 1,800rpm at RT for 45 minutes, no brake. Remove everything but the lower red layer containing the neutrophils and RBCs. [This should be <10 ml.]
3. Lysing remaining RBC: To this lower red layer add 2 volumes off ice cold 0.2% NaCl and mix gently for exactly 1 min. Immediately add half the original volume of lower red layer volume of cold 3.7% NaCl solution to restore isotonicity.
4. Final Resuspension and washing of neutrophils: Spin down lyzed cells at 1000rpm (250 g) for 6 minute. Repeat lysis if high RBC contamination. Wash pellet twice with 20 mls of HBSS. Resuspend in HBSS or test medium, do cell count and viability.

E. PREPARING AND ADDING RESPONDING CELLS

1. In the upper wells the concentration of cells in the suspension should be adjusted so that 50µL contains the desired number of cells for one well. For example, since the exposed filter area for each well is 8mm2, a suspension of 32,000 cells in 50µL will yield 4,000 cells/mm2.
    - In this strategy the optimum number of cells per mm2 of filter area is usually the same as the number of pores per mm2. A 5 micron filter has 4,000 pores per mm2 and these pores occupy only about 7.85% of the surface area of the filter. The optimum density of cells on this filter would be 4,000 cells per mm2 of filter area, and only one in 12 cells are likely to settle over a pore. At negative control sites 92% of the cells on the filter would be highly unlikely to arrive at pores at all, and the 8% that started out over pores would be as likely to migrate away from the pore as through it.
2. Pipette cell suspension into each upper well, adjusting the volume so that the filled wells have a slight positive meniscus. Hold the pipette at a steep angle so that the end of the pipette tip rests against the wall of the chamber just above the filter, and the side of the tip rests against the top rim of the well. Eject the fluid with a rapid motion to dislodge air in the bottom of the well.
3. Check for trapped bubbles in the upper wells. One easy way to do this is to look at the reflections of overhead lights in the meniscuses: a well with an abnormally large positive meniscus usually has a trapped air bubble. To remove any bubbles, suck the well completely dry with a suction line and disposable pipette tip, and then refill it.
4. For most chemotaxis assays the filled chamber is incubated at 37°C in humidified air with 5% CO2. Incubation times vary considerably depending on cell types and chemotactic factors. [For neutrophils, incubate at 37 in 5% CO2 for 1 to 2 hours.] One good way to determine the optimum incubation time is to use 6 to 12 blind-well chambers (e.g. stock # BW100) set up as negative controls and placed simultaneously in the incubator. Remove one blind-well chamber after a set period (e.g. 30 minutes), and remove the rest sequentially, one every 5 minutes. Stain the filters and examine them to see how long unstimulated cells have taken to migrate through the filter.
5. Counting the cells: Non-adherent cells (ones that drop off the bottom of the filter, as do neutrophils) must be counted in the bottom of the well. So all of your cells will be in the bottom of that 25 ul well. Resuspend everything and take 10 ul and do a 1:2 dilution in trypan blue and count. Adherent cells require more detailed work.

5. MATERIALS / EQUIPMENT

A Nuero Probe 48 well or Millipore apparatus will be prepared according to the manufacturers' directions. You use 3 or 5 micron pores in a Polycarbonate track-etch (PCTE) for neutrophils.

6. COMMENTS

Many chemokines will use this type of chemotaxis assay. For each chemokine a different cell type will be needed. For the majority of these assays some stimulation or separation of PBMC should yield the appropriate cell type (neutrophils or monoctyes). It is important to have a known positive control for this assay, preferably a chemokine.

7. REFERENCES

For more information and strategies for working out incubation times see:
http://www.neuroprobe.com/protocols/info_incubation.html

IL-8 (Black)

University of Massachusetts Black Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-8**	Effective Date: January 2008
Written by	John Loftus	Technical Approval	Sam Black
Title	Research Assistant	Title	Project Director

1. OBJECTIVE

Bioassay for recombinant IL-8 by a neutrophil chemotaxis assay.

2. SCOPE

This assay uses neutrophil chemotaxis to determine IL-8 bioactivity. This has been successfully used for both equine and bovine rIL-8 and is expected to work similarly with neutrophils from all species.

3. PROCEDURE

A. ISOLATION OF NEUTROPHILS

1. Blood is collected via jugular venipuncture using 21g, one-inch needles into 60ml syringes with 3.2% sodium citrate (1:10 dilution, 0.32% final concentration).
2. 20 ml of whole blood is put into a 50 ml centrifuge tube with the addition of 20 ml 3% dextran to get rid of RBCs by rouleaux facilitation for 20-30 min at 4ºC if this works for your species (e.g. horses) otherwise use high speed centrifugation to pellet RBC and WBC will float on top.
3. The leukocyte-rich plasma layer is aspirated and transferred to a new 50 ml centrifuge tube and centrifuged at 1000 rpm (250 x g) at 4°C for 10 min (with brake).
4. Remove the supernatant and re-suspend the cell pellet in 40 ml 0.9% NaCl. Layer cell suspension over 10 ml of ficoll-hypaque (sterile removal).
5. Centrifuge for 40 min at 1400 (300 x g) rpm at 4°C without brake.
6. Aspirate and discard saline and ficoll-hypaque layers using vacuum collector (can aspirate with serological pipette, but vacuum aspiration reduces mononuclear contamination), making sure to remove adherent mononuclear cells at the ficoll hypaque-saline interface.
7. Re-suspend the cell pellet in 1-2 ml of DPBS. Layer 1 ml over 9ml of 90% Percoll in a Beckman ultracentrifuge tube.
8. Centrifuge at 24,000 rpm (60,650 x g) in the Beckman L8-60M ultracentrifuge (type 70.1 Ti rotor) at 4°C for 40 min.
9. Aspirate and discard mononuclear layer first with a 1 1/2, 21 gauge needle (monocytes on top, neutrophils in the middle and erythrocytes on bottom). Collect the Neutrophil band. Re-suspend to 12-14 ml with DPBS and centrifuge at 1400 rpm (400 x g) with brake for 6 min at 4°C.
10. Re-suspend in 1 ml of appropriate medium (i.e. RPMI 1640). Count and assess cell viability in hemocytometer using the second of two 1:10 dilutions (final 1:100 dilution) into 0.04% (v:v) trypan blue dye.

B. CHEMOTAXIS ASSAY

1. Supplement RPMI 1640 medium (without phenol red) with 100 ng/ml of equine recombinant IL-8 and dispense in volumes of 27 μl into the bottom wells of a 48-well chemotaxis chamber, assuring a positive meniscus.
2. Insert a new polycarbonate membrane (5 μm pore size) with forceps, place gasket over membrane, then install top chamber unit. Hand-tighten only.
3. Instill purified neutrophils (see neutrophil purification protocols) at a concentration of $1x10^6$ cells/ml in ice-cold RPMI (+/- potential inhibitors) into the wells of the top chamber using a volume of 52 μl/well, and insuring a negative meniscus to indicate the well was free of trapped air.
4. Incubate the chamber at 37°C in a humidified incubator with a 5% CO_2 atmosphere, generally for 1 hour, but duration can be adjusted for each experiment.
5. Disassemble the chemotaxis apparatus, and count cells in the bottom well using trypan blue to determine viability. Quantify response to IL-8 by determining the percent of total cells loaded that had migrated to the lower chamber. With above volumes and cell concentrations, the average number of cells per quadrant in the hemocytometer is equivalent to percent responding cells of total cells loaded.

C. NOTES

1. Loading volumes are critical! Loading the precise volume into the top and bottom wells assures the correct meniscus, to confirm that bubbles are not present in the wells. Load wells quickly, with the pipette tip in

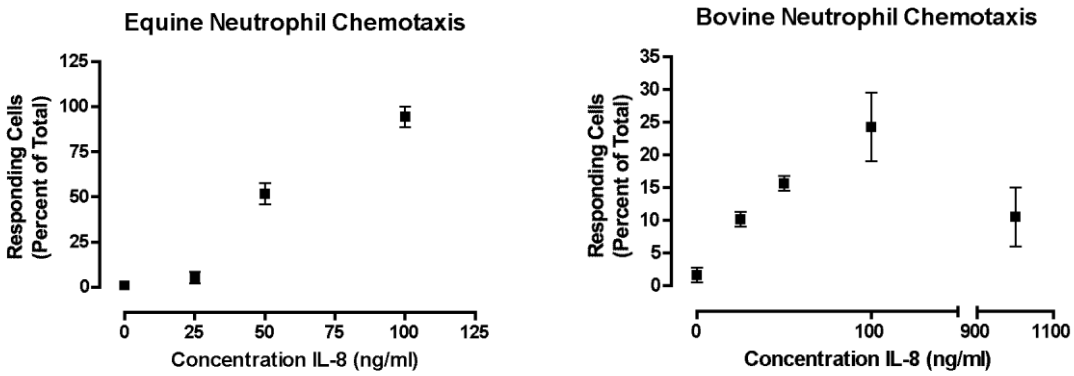
contact with the side of the well.

2. Can adjust concentration of IL-8 for testing dose responses. For equine and bovine assays, concentrations of 25, 50, 75 and 100 ng/ml of IL-8 give clear dose response results.
3. Perform all steps prior to incubation using aseptic technique to reduce endotoxin contamination.

4. MATERIALS / EQUIPMENT

Chemotaxis chamber: Neuroprobe, Gaithersburg MD. Cat. #AP48.
Pipettes and pipette tips
RPMI 1640 Medium
Forceps
Humidified Incubator
Trypan blue (0.04%) and hemocytometer

5. TYPICAL RESULTS



Note the decrease in chemotaxis at high concentrations of bovine IL-8. This result is expected, as engagement of the low affinity IL-8 receptor reduces chemotactic activity.

University of Kentucky Horohov Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-10**	Effective Date: January 2008
Written by	Catherine Merant	Technical Approval	David Horohov
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure recombinant IL-10's bioactivity by its ability to inhibit IFNγ mRNA expression.

2. SCOPE

Principle: inhibition of the IFN- messenger RNA in PBMC cultured with recombinant human IL-12 or as a resultof activation of cells with the mitogen ConA which induces IFN-γ mRNA. Here we have written the protocol for ConA but rHuIL-12 can be substituted if it works for your species of indicator PBMC. This has been shown to work with bovine PBMC when secretion of IFNγ protein was measured.

3. PROCEDURE

A. PREPARATION OF CELLS

1. Prepare PBMC by centrifugation on ficoll-hypaque (d= 1.077).
2. Count the PBMC and resuspend them at $4 \cdot 10^6$ cells/ ml cRPMI.

B. IL-10 BIOASSAY

1. Prepare a culture plate with following wells (100 µl/ well):
    a. CPMed alone
    b. CPMed containing mitogen (Con A at 20 µg/ml)
    c. CPMed containing ConA and a reference rIL-10 (if available; can also be diluted 2x, 4x… to set-up a curve)
    d. CPMed containing ConA and the new rIL-10
2. Add 100 µl of cell suspension to these wells.
3. Incubate cells for 24 hours at 37°C, 5% CO_2 in a humidified incubator.
4. Transfer the cells into Eppendorf tubes.
5. Spin the cells at 1500 g and resuspend them in 100 l RNA STAT. Samples can be conserved STAT at -70°C.
6. Extract the RNA and reverse-transcribe them (RT).
7. Perform a real-time-PCR on the IFN- gene and on a housekeeping gene (e.g. -GUS).

4. MATERIALS / EQUIPMENT

Blood in heparin or in CPD/ ACD
Ficoll-hypaque
Complete RPMI Medium (cRPMI): RPMI-1640 supplemented with 10 % FBS (or FES if preferred), 1 % penicillin-streptomycin- L-glutamine and 0.1 % 2-mercaptoethanol
Cytokine production medium (CPMed)
Concanavalin A (ConA)
96-well flat-bottom culture plate
RNA STAT 60

5. REFERENCES

Hillyer LM and Woodward B. Interleukin-10 concentration determined by sandwich enzyme-linked immunosorbent assay is unrepresentative of bioactivity in murine blood. Am J Physiol Regul Integr Comp Physiol 2003, 285, R1514–R1519.

USDA-ARS Beltsville Lillehoj Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-12 p35**	Effective Date: January 2008
Written by	Yeong Hong	Technical Approval	Hyun Lillehoj
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of IL-12 p35 by its ability to stimulate IFNγ release by T cells and in turn the ability of IFNγ to stimulate nitric oxide release. An alternative protocol of the ability of IL-12 to stimulate lymphocyte proliferation is also suggested.

2. SCOPE

This gene encodes a subunit of IL-12 cytokine that acts on T and natural killer cells, and has a broad array of biological activities. The cytokine is a disulfide-linked heterodimer composed of the 35-kD subunit encoded by this gene, and a 40-kD subunit that is a member of the cytokine receptor family. This cytokine is required for the T-cell-independent induction of interferon (IFN)-γ and is important for the differentiation of both Th1 and Th2 cells. The responses of lymphocytes to this cytokine are mediated by the activator of transcription protein, STAT4. Nitric oxide synthase 2A (NOS2A/NOS2) is required for the signaling process of this cytokine.

3. PROCEDURE

A. EXPERIMENT DURATION

18-48 hr incubation at 37°C, 5% CO_2 incubator following 1 hr assay preparation.

B. NITRIC OXIDE ASSAY FOR THE INDUCTION OF IFN-γ BY IL-12.

1. Chicken primary spleen cells (splenocytes) are placed in triplicate in a 96-well plate at a density of 0.5 x10^6 cells/well in 100 ul.
2. To each well, 50 ul of serial diluted recombinant IL-12p35, IL-12p40, IL-12p70 protein or irrelevant control protein is added.
3. 48h after incubation, supernatants (75 ul) are collected and analyze for the presence of biologically active IFN-γ.
4. For IFN-γ bioassay, 100 ul of 1.5 x 10^6/ml macrophage cells are incubated with 75 ul of the supernatant containing IL-12 for 24 h at 37°C, 5% CO_2 incubator.
5. Activation of macrophages by IFN-γ is measured by the nitrite accumulation in the culture supernatants using the Griess assay (Ding et al., 1988).

C. ASSAY FOR SPLEEN CELL PROLIFERATION BY IL-12.

1. After removing 75 ul of the supernatants (see A.3 above), 50 ul of medium and 25 ul [*methyl*-^{3}H] thymidine (Perkin-Elmer, Boston, MA) were added to each well in the 96-well plate.
2. Incubate for 18-20 h at 41°C in 5% CO_2 and the incorporated radioactivity was counted using an LKB Betaplate beta counter (LKB Instruments, Gaithersburg, MD).

4. MATERIALS / EQUIPMENT

Primary spleen cells or spleen cell line. Established macrophage cell line is also used, e.g., HD11 in chickens.
Culture Medium (RPMI-1640 supplemented with 10% FBS)
96-well flat-bottom culture plate (Costar Cat. No. 3595)
Recombinant IL-12p35, IL-12p40 or IL-12p70 protein.
NO2/NO3 Assay Kit (For poultry, we use NK05-10 from Dojindo molecular technology Inc, MD).

5. REFERENCES

Degen WG et al. Identification and Molecular Cloning of Functional Chicken IL-12. The Journal of Immunology 172: 4371–4380 (2004).

Ding AH et al. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol. 141:2407 (1988).

University of Massachusetts			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-13**	Effective Date: January 2008
Written by	Cynthia Baldwin	Technical Approval	Carolyn Herzig
Title	Project Director	Title	Res. Assist.

1. OBJECTIVE

To evaluate recombinant IL-13 for bioactivity by upregulation of MHC class II molecules on either B cells or monocyte-derived macrophages.

2. SCOPE

While IL-13 from some species including dog (see Yang reference) stimulates the proliferation of TF-1 cells, the IL-13 from other species including swine does not. Increased expression of class II major histocompatibility complex (MHC) molecules has been shown to occur with bovine B cells (see Trigona reference) and porcine monocyte-derived dendritic cells (MoDC) (see Bautista reference).

3. PROCEDURE

1. Viable peripheral blood mononuclear cells (PBMC) or splenocytes are isolated (see IL-2 protocol for PBMC) and enumerated using trypan blue exclusion and microscopy.
2. If desired, the population can be enriched for monocytes/macrophages by adherence to plastic by incubating on plastic (flasks or multi-well plates) for 2 hr at 37C and decanting non-adherent cells. Adherent cells can be obtained by scraping with a rubber policeman or using trypsin-EDTA to distribute into other culture vessels.
3. Cells are incubated at a concentration of about 10^6 cells/ml with complete tissue culture medium alone (negative control), or with recombinant GM-CSF (human GM-CSF works for many species if your particular species' GM-CSF is not available) either alone (negative control) and with GM-CSF and the rIL-13 together. Both cytokines have to be titrated in a block titration to determine optimal concentrations.
4. Cells are incubated at 37C for 5-7 days and removed from plastic vessel by gently scraping if interested in MoDC or simply collecting the non-adherent B cells. If adherent cells are collected they need to be kept on ice throughout to avoid re-attachment to plastic.
5. Cells are stained by immunofluorescence using an anti-MHC class II antibody and evaluated by flow cytometry being sure to keep cells cold throughout to avoid adherence to the plastic tubes in which they are suspended for flow cytometric evaluation using standard techniques.
6. An increase in MHC class II above that achieved with medium control cultures or GM-CSF alone is expected for bioactive IL-13.

4. MATERIALS / EQUIPMENT

Hank's Balanced Salt solution (HBSS)(with 2 units.ml heparin)
Ficoll-Hypaque
50 ml sterile tissue culture tubes
Centrifuge capable of spinning at 200 – 800g
10-200 µl pipettors ^{O}C
Hemacytometer with appropriate cover slip
Microscope with up to 400X resolution
Multiwell plastic culture plates
RPMI with 10% heat-inactivate FCS, gentamycin and 2-ME at 50 uM (complete culture medium)
0.4% Trypan Blue
Commercial recombinant human GM-CSF or recombinant for your species if available
Humidified CO_2 incubator
Rubber policeman
Flow cytometer
Flow cytometer staining medium (RPMI with 2% horse serum and sodium azide)
Anti-MHC class II monoclonal antibody specific for MHC class of your species

5. REFERENCES

Bautista, E.M., C. Nfon, G.S. Ferman and W.T. Golde. 2007. IL-13 replaces Il-4 in development of monocyte derived dendritic cells (MoDC) of swine. Vet Immunol Immunopathol 115:56-67.

Click to return to the Table of Contents

Trigona, W.L., A. Hirano, W.C. Brown and D.M. Estes. 1999. Immunoregulatory roles of IL-13 in cattle. J. Inter Cyto Res 19:1317-24.

Yang, S., K.L. Boroughs, and M.J. McDermott. 2000. Canine IL-13: molecular cloning of full-length cDNA and expression of biologically active recombinant protein. J Inter Cyto Res 20:779-85.

University of Kentucky Horohov Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-15**	Effective Date: January 2008
Written by	Catherine Merant	Technical Approval	David Horohov
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure recombinant IL-15's bioactivity by its ability to stimulate proliferation of PBMC.

2. SCOPE

Principle: PBMC proliferation in response to IL-15 will likely be successful for any species.

3. PROCEDURE

A. PREPARATION OF CELLS

Prepare PBMC by centrifugation on ficoll-hypaque (d=1.077).
Count the PBMC and resuspend them at $4 \cdot 10^6$ cells/ ml cRPMI.

B. IL-15 BIOASSAY

1. Prepare a culture plate with the following wells (100 µl/ well):
    a. CPMed alone
    b. CPMed with 20 □g ConA/ ml
    c. CPMed containing a reference rIL-15 such as human IL-15 (if available; can also be diluted 2x, 4x… to set-up a curve)
    d. CPMed containing the new rIL-15
2. Add 100 µl of cell suspension to these wells.
3. Incubate cells for 48 hours at 37°C, 5% CO_2 in a humidified incubator.
4. Add 10 µl/ well of 5 mg/ml MTT solution to the plate and incubate for 4 hours.
5. Add 50 µl/ well of MTT Lysing Solution to the plate and incubate overnight.
6. Read plate at 570-650 nm.
7. Graph standard curve (if available) and analyze data.

4. MATERIALS / EQUIPMENT

Blood in heparin or in CPD/ ACD
Ficoll-hypaque
Complete RPMI Medium (cRPMI): RPMI-1640 supplemented with 10 % FBS (or FES if preferred), 1 % penicillin-streptomycin- L-glutamine and 0.1 % 2-mercaptoethanol
Cytokine production medium (CPMed)
ConA (will be 20 µg/ ml in CPMed)
MTT solution (Sigma Cat. No. M5655) 5 mg/ml stock in PBS kept at room temperature (protect from light)
MTT Lysing Solution 20% SDS/ 50% DMF
96-well flat-bottom culture plate

5. REFERENCES

Burton JD, Bamford RN, Peters C, Grantt AJ, Kurys G, Goldman CK, Brennan J, Roessler E, And. Waldmann TA. A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. J. Immunol. 91, 4935-4939.

USDA-ARS Beltsville Lillehoj Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-16**	Effective Date: January 2008
Written by	Yeong Hong	Technical Approval	Hyun Lillehoj
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant IL-16 in a T cell chemotaxis assay.

2. SCOPE

Interleukin 16 (IL-16) is an inflammatory cytokine that is released by a variety of cells (including lymphocytes and some epithelial cells) and has been characterized as a chemoattractant for certain immune cells expressing the cell surface molecule CD4. IL-16 was originally described as a factor that could attract activated T cells in humans and was previously called lymphocyte chemoattractant factor (LCF). Since then, this interleukin has been shown to recruit and activate many other cells expressing the CD4 molecule, including monocytes, eosinophils, and dendritic cells.

3. PROCEDURE

A. EXPERIMENT DURATION: 2 - 24h incubation at 37°C, 5% CO_2 incubator

B. METHODS

1. Prepare primary splenic lymphocytes prepared by gently flushing through a cell strainer (Becton Dickinson Labware) and purified using the Histopaque-1077 (Sigma) density gradient medium by centrifuging at 200g/20min at RT.
2. Place 300ul of T-cell suspension in the upper compartments of the 24-cell microchemotaxis chambers .
3. Add 500 ul of medium containing 10% FBS or serially diluted recombinant IL-16 to the lower chamber well and incubate for 2-24 at 37°C, 5% CO_2 incubator.
4. Remove the Transwell and quantify cell migration by counting the total the number of cells in the lower well.
5. Control migration is established by the number of cells stimulated with buffer alone.

4. MATERIALS / EQUIPMENT

This protocol was modified Boyden chemotaxis chamber technique (Cruikshank et al., 1987)
Primary splenic lymphocytes (5.0 x 10^6 cells/ml)
Recombinant IL-16 protein
Culture Medium (RPMI-1640 supplemented with 10% FBS)
24 well Transwell chamber (5.0 um pore size, Costar)
CytoSelectTM 24-well cell migration assay (Cell Biolab Inc) is available

5. REFERENCES

Krug N. et al. Interleukin 16 and T-cell Chemoattractant Activity in Bronchoalveolar Lavage 24 Hours after Allergen Challenge in Asthma Am. J. Respir. Crit. Care Med. 162: 105-111 (2000).

Min W and H Lillehoj. Identification and characterization of chicken interleukin-16 cDNA. Developmental & Comparative Immunology 28:153-162 (2004).

Cruikshank, W. W., J. S. Berman, A. C. Theodore, J. Bernardo, and D. M. Center. Lymphokine activation of T41 T lymphocytes and monocytes. J. Immunol. 138:3817–3823 (1987).

USDA-ARS Beltsville Lunney Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-17**	Effective Date: January 2008
Written by	Patricia Boyd	Technical Approval	Joan Lunney
Title	Molecular Biologist	Title	Project director

1. OBJECTIVE

To assess bioactivity of recombinant IL-17 to induce expression of IL-6 or IL-8 in primary cells or cell lines.

2. SCOPE

Recombinant IL-17 has been expressed and bioassayed for chicken, swine, and bovine but not horse. Swine used swine splenocytes, chicken used chicken embryonic fibroblasts, and bovine used primary culture of bovine mammary epithelial cells (MECs).

The mouse NIH/3T3 fibroblast cells is available from ATCC; for other species it is likely epithelial cells or fibroblasts will be responsive to IL-17. For swine we will plan to stimulate PBMCs or IPEC cells with our recombinant swine IL-17 and then measure IL-6 production on murine B6 myeloma cells. [Alternately an IL-6 ELISA can be used if available.]

3. PROCEDURE

Adapted from *Current protocols in immunology (CPiI)* Unit 6.25 Supplement 41 pg 3.

A. CULTURE 3T3 CELLS (FOR MURINE IL-17)

1. Grow Murine 3T3 cells to confluence in DMEM supplemented with penicillin, streptomycin, L-Glutamine, and 10% FBS (DMEM/FBS) in a 175 cm^2 flask.
2. To passage confluent cells, remove cell culture supernatant and rise cell monolayer with PBS. Add 0.05 % trypsin/ 0.53 mM EDTA to cover cell layer, e.g., 2.5 ml for a 175 cm2 flask, and incubate for 5 minutes at 37C to detach cell monolayer from flask.
3. Add 20 ml of DMEM/FBS to cell suspension.
4. Dilute cell suspension 1:5 to 1:10 into a new 175 cm2 flask with DMEM/FBS to a final volume of 25 ml per 175 cm2 flask.

B. BIOASSAY IL-17

1. For IL-17 bioassay, trypsinize cells as in step 2.
2. Resuspend cells at 2.5 x 10^5 cells/ml in DMEM/FBS.
3. Plate 2 ml per well (5 x 10^5 cells/well) into a 6 well tissue culture dish.
4. Incubate overnight at 37oC to allow cells to adhere.
5. On the following day, replace medium with serial dilution of test and control IL-17 culture supernatants diluted in DMEM/FBS. Include DMEM/FBS alone as a negative control.

Addition of known amounts of recombinant IL-17 can be used as a positive control.

6. Incubate cells overnight at 37oC.
7. Harvest culture supernatant, clarify as described in Basic Protocol, step 6 annotation.
8. Assay for IL-6 production (see IL-6 bio assay).

Supernatants can be stored for several weeks at -70C and assayed at a later date.

4. MATERIALS / EQUIPMENT

Murine NIH3T3 cells (ATCC #CRL-1658 = CCL-163™)
Dulbecco's modified Eagle Medium (DMEM) supplemented with Penicillin, streptomycin, L-glutamine (see recipe) and 10% FBS (DMEM/FBS)
PBS
0.05% (w/v) trypsin in 0.53 mM EDTA
Serial dilutions of IL-17 culture supernatant
175 cm2 flask
6 wells tissue culture dish
Additional reagents and equipment for measuring IL-6 (see IL-6 protocol)
Deionized, distilled water in all recipes and protocol steps.

PENICILLIN/STREPTOMYCIN/L-GLUTAMINE, 100X

10,000 U/ml penicillin G sodium
10,000 ug/ml streptomycin sulfate
29.2 mg/ml L-glutamine
10 mM sodium citrate
0.14 % (w/v) NaCl

5. COMMENTS

UNCLEAR IF THESE WOULD WORK FOR OTHER SPECIES

6. REFERENCES

Min W, Lillehoj HS. 2002. Isolation and characterization of chicken interleukin-17 cDNA. J Interferon Cytokine Res. 22:1123-8.

EFFECT ON SWINE SPLENOCYTES:
Katoh S, Kitazawa H, Shimosato T, Tohno M, Kawai Y, Saito T. 2004. Cloning and characterization of Swine interleukin-17, preferentially expressed in the intestines. J Interferon Cytokine Res. 24(9):553-9.

EFFECT ON PRIMARY BOVINE MAMMARY GLAND CELL:
Riollet C, Mutuel D, Duonor-Cérutti M, Rainard P. 2006. Determination and characterization of bovine interleukin-17 cDNA. J Interferon Cytokine Res. 26(3):141-9.

USDA-ARS Beltsville Lillehoj Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-17D**	Effective Date: January 2008
Written by	Yeong Hong	Technical Approval	Hyun Lillehoj
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Assess bioactivity of recombinant IL-17D by assessing mRNA expression of other cytokines using quantitative real-time RT-PCR.

2. SCOPE

Interleukin-17 (IL-17) was originally described as a cytokine secreted exclusively by activated memory T cells and induced fibroblasts to secrete other cytokines involved in proinflammatory or hematopoietic processes, such as IL-6, IL-8 and granulocyte-colony stimulating factor (G-CSF) (Yao et al., 1995). A number of homologous proteins comprising an IL-17 family have been identified, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. The IL-17D gene appears to be most homologous to IL-17B and was preferentially expressed in resting $CD4^+$ T cells, skeletal muscle, brain, pancreas, heart, lung and adipose tissue (Starnes et al., 2002). Human IL-17D stimulated vascular endothelial cells to secrete IL-6, IL-8 and GM-CSF (Starnes et al., 2002). This effect is has been suggested to be mediated through NF-κB activation, as was shown for IL-17A and IL-17E.

3. PROCEDURE

A. EXPERIMENT DURATION

24 hour incubation at 37°C, CO_2 incubator.

B. METHOD

1. Embryonic fibroblast cell line is cultured in 6-well plates at 1.0 x 10^6 cells/well in DMEM (Sigma) supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C, CO_2 incubator.
2. The cells are treat with serial dilution of IL-17D recombinant protein or an equivalent amount of the control protein for 24 h.
3. After 24h, total RNA is extracted with Trizol and cDNA is synthesized.
4. Quantitative RT-PCR performed for IL-6, IL-8, GM-CSF and GAPDH gene expression.

4. MATERIALS / EQUIPMENT

Fibroblast cell line
Culture Medium (Dulbecco's Modified Eagle's medium supplemented with 10% FBS)
6-well culture plate
Recombinant IL-17D protein.
StrataScript First-Strand synthesis system (Stratagene)
Brilliant SYBR Green QPCR Master mix (Stratagene)
DNase I (Ambion)
Trizol (Invitrogen)

5. REFERENCES

Starnes, T., Broxmeyer, H.E., Robertson, M.J., Hromas, R. Cutting edge: IL-17D, a novel member of the IL-17 family, stimulates cytokine production and inhibits hemopoiesis. J. Immunol. 169, 642-646 (2002).

Yao, Z., Fanslow, W.C., Seldin, M.F., Rousseau, A.M., Painter, S.L., Comeau, M.R., Cohen, J.I., Spriggs, M.K. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity 3, 811-821 (1995).

USDA-ARS Beltsville Lillehoj Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-18**	Effective Date: January 2008
Written by	Yeong Hong	Technical Approval	Hyun Lillehoj
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant IL-18 by its ability to induce IFN-γ and nitric oxide.

2. SCOPE

Interleukin-18 (IL-18) is a pro-inflammatory cytokine which belongs to the IL-1 superfamily and is produced by macrophages and other cells. IL-18 shares biologic activities with IL-12 to induce cell-mediated immunity following activation by microbial products such as lipopolysaccharide (LPS). After stimulation with IL-18, natural killer (NK) cells and certain T cells release interferon-γ (IFN-γ) or type II IFN which activates the macrophages or other cells. The gene encoding this non-glycosylated protein of 24 kDa has been identified originally as IGIF (IFN-gamma inducing factor). It encodes an inducer of IFN-γ production by T-cells (Okamura et al, 1995) and natural killer cells (Tsutsui et al, 1996) and is a more potent inducer than IL-12.

3. PROCEDURE

A. EXPERIMENT DURATION

24-48 hour incubation at 37°C, 5% CO_2 incubator

B. METHODS

A. IFN-γ induction in response to IL-18 treatment

1. Cells are incubated with three different concentrations of recombinant IL-18 (125, 62.5, and 31.25 ng/mL) at 37°C in 5% CO_2 incubator. Irrelevant control protein serves as a negative control.
2. After 24 or 48h, total RNA was extracted from cells with Trizol and Northern blot or quantitative RT-PCR performed for IFN-γ and GAPDH gene.

C. NITRIC OXIDE inducing test

1. Cells are seeded at a density of 2.5 x 10^6 cells/mL in 96 well plates (100 ul/well) and treated with the indicated concentration of recombinant IL-18 (125, 62.5, and 31.25 ng/mL).
2. After 48h, the supernatants are harvested and add to macrophage cells to measure IFN-γ -induced NO synthesis using the Griess assay (Ding et al., 1988).

4. MATERIALS / EQUIPMENT

Indicator cell line: KG-1, HL60 in human or mouse, B19-2D8 or HD11 in chickens
Culture Medium (RPMI-1640 supplemented with 10% FBS)
96-well flat-bottom culture plate (Costar Cat. No. 3595)
NO2/NO3 Assay Kit (We use NK05-10 from the Dojindo molecular technology Inc, MD)
Trizol (Invitrogen)

5. REFERENCES

Okamura H et al., Nature 378: 88-91 (1995).

Taniguchi M et al., J of Immunological Methods 217: 97-102 (1998).

Taniguchi M et al., J of Immunological Methods 206(1-2): 107-113 (1997).

Tsutsui H et al., J of Immunology 157: 3967-3973 (1996).

Ding AH et al., J Immunol. 141:2407 (1988).

University of Kentucky Horohov Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: IL-23**	Effective Date: January 2008
Written by	Catherine Merant	Technical Approval	David Horohov
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant IL-23.

2. SCOPE

Principle: induction of IL-17 mRNA by IL-23.

3. PROCEDURE

A. PREPARATION OF CELLS

Prepare PBMC by centrifugation on ficoll hypaque (d= 1.077).
Count the PBMC and resuspend them at $4 \cdot 10^6$ cells/ ml cRPMI.

B. BIOASSAY

1. Prepare a culture plate with the following wells (100 µl/ well):
    a. CPMed alone
    b. CPMed containing 5 ng/ml PMA
    c. CPMed containing 5 ng/ml PMA and a reference rIL-23 (if available; can also be diluted 2x, 4x… to set-up a curve)
    d. CPMed containing 5 ng/ml PMA and the new rIL-23
2. Add 100 µl of cell suspension to these wells.
3. Incubate cells for 24 hours at 37°C, 5% CO_2 in a humidified incubator.
4. Transfer the cells into Eppendorf tubes.
5. Spin the cells at 1500 g and resuspend them in 100 □l RNA STAT. Samples can be conserved STAT at -70°C.
6. Extract the RNA and reverse transcribe-it (RT).
7. Perform a real-time-PCR on the IFN-□ gene and on a housekeeping gene (e.g. □-GUS).

4. MATERIALS / EQUIPMENT

Blood in heparin or in CPD/ ACD
Ficoll hypaque
Complete RPMI Medium (cRPMI): RPMI-1640 supplemented with 10 % FBS (or FETAL EQUINE SERUM if preferred), 1 % penicillin- streptomycin- L-glutamine and 0.1 % 2-mercaptoethanol
Cytokine production medium (CPMed)
Phorbol myristate acetate (PMA)
96-well flat-bottom culture plate
RNA STAT 60

5. REFERENCES

Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem. 2003, 278, 1910-1914.

USDA-ARS Beltsville Lillehoj Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: LITAF**	Effective Date: January 2008
Written by	Yeong Hong	Technical Approval	Hyun Lillehoj
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant LITAF using a cytotoxicity assay and/or Q-RT-PCR

2. SCOPE

A novel transcription factor, named LPS-induced TNF-α factor (LITAF), was cloned and characterized in human, mouse and chicken. LITAF mRNA was mostly expressed in lymphoid tissues including peripheral blood leukocytes, lymph nodes, spleen and non-lymphoid organs, especially in placenta and liver. Its induction was dependent on LPS activation of THP-1 cells; hence, the name LPS-induced TNF-alpha factor (LITAF). In mammals, LITAF induce TNF-α and the inhibition of LITAF mRNA expression in THP-1 cells resulted in a reduction of TNF-transcripts.

3. PROCEDURE

A. CYTOTOXICITY ASSAY

Experiment Duration: 24-48 hour incubation at 37°C, 5% CO_2 incubator

1. 100ul of cell line suspension (1 x 10^5 cells/well) is placed in flat-bottomed 96 well plate in triplicates.
2. Incubate with serial diluted recombinant LITAF protein at 37°C, 5% CO_2 incubator for 48h. Irrelevant protein is served as a control.
3. Cell numbers are determined with WST-8 cell counting kit according to the manufacturer's protocol.
4. O.D. is measured at 450nm.

B. QUANTITATIVE REAL-TIME RT-PCR FOR TNFSF15

1. 100ul of cell line suspension (1 x 10^5 cells/well) is placed in flat-bottomed 96 well plate.
2. Cells are incubated with serially diluted recombinant LITAF protein for 24h and harvested for total RNA extraction.
3. cDNA is synthesize and TNF mRNA level is determined using Q-RT-PCR.

4. MATERIALS / EQUIPMENT

Chicken tumor cell lines (HTC, HD11, LSCC-RP9 et al.)
Recombinant LITAF protein
culture Medium (RPMI-1640 supplemented with 10% FBS)
96 well flat-bottomed microtiter plate (Corning Costar)
WST-8 cell counting kit (Dojindo Molecular Technologies, Gaithersburg, MD)
StrataScript First-Strand synthesis system (Stratagene)
Brilliant SYBR Green QPCR Master mix (Stratagene)
DNase I (Ambion)
Trizol (Invitrogen)

5. REFERENCES

Myokai F et al. A novel lipopolysaccharide-induced transcription factor regulating tumor necrosis factor alpha gene expression: molecular cloning, sequencing, chracterization, and chromosomal assignment. PNAS 96: 4518-23 (1999).

Hong YH, HS Lillehoj, SH Lee, DW Park, EP Lillehoj, Molecular cloning and characterization of chicken lipopolysaccharide-induced TNF-α factor (LITAF) . Developmental & Comparative Immunology. 30:919-929 (2006).

USDA-ARS Beltsville Lillehoj Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: XCL1 (Lymphotactin)**	Effective Date: January 2008
Written by	Yeong Hong	Technical Approval	Hyun Lillehoj
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant Lymphotactin (XCL1) in a chemotaxis assay using T cells.

2. SCOPE

Chemokine (C motif) ligand (XCL1) is a small chemokine belonging to the XC chemokine family that is also known as lymphotactin. It is found in high levels in spleen, thymus, intestine and peripheral blood leukocytes, and at lower levels in lung, prostate gland and ovary. Cellular sources for XCL1 include activated thymic and peripheral blood $CD8^+$ T cells. This chemokine attracts T cells. In humans, XCL1 is closely related to another chemokine called XCL2, whose gene is found at the same locus on chromosome 1. XCL1 induces its chemotactic function by binding to a chemokine receptor called XCR1.

3. PROCEDURE

A. EXPERIMENT DURATION

2-24 h incubation at 37°C, 5% CO_2 incubator

B. METHOD

1. Splenic lymphocytes are prepared from spleen by gently flushing through a cell strainer (Becton Dickinson Labware) and centrifuged at 200g/20min on Histopaque-1077 (Sigma) density gradient medium at RT.
2. 300ul of T-cell suspension is placed in the upper compartments of the 24-cell microchemotaxis chambers.
3. 500 ul of medium containing 10% FBS or serially diluted recombinant XCL1 is added to the lower chamber well and incubate for 2-24 at 37°C, 5% CO_2 incubator.
4. Transwell is removed and cell migration is quantified by counting the total the number of cells in the lower well.
5. Control migration is assessed by counting cells stimulated with buffer alone,

4. MATERIALS / EQUIPMENT

Primary lymphocytes
Recombinant lymphotactin protein.
Histopaque-1077 (Sigma).
Culture Medium (RPMI-1640 supplemented with 10% FBS).
HTS Transwell®-24 Permeable Supports with 5.0µm Pore Polycarbonate Membrane. The pore size is dependent on the type of cells.

5. REFERENCES

Min W and H Lillehoj. Identification and characterization of chicken interleukin-16 cDNA. Developmental & Comparative Immunology 28:153-162 (2004).

University of Kentucky Horohov Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: TGF-β**	Effective Date: January 2008
Written by	Catherine Merant	Technical Approval	David Horohov
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant TGF-beta by its ability to stimulate proliferation of the mink CCL64 cell line.

2. SCOPE

Principle: induction of proliferation of CCL64 cell line.. This cell line has been shown to respond to bovine (Adler et al., 1995), porcine (Gupta et al., 1998) and equine (Chesters et al., 2000) TGF- β.

3. PROCEDURE

A. PREPARATION OF CELLS

1. Trypsinize the CCL64 mink lung epithelial cells: discard supernatant and rinse cell monolayer with PBS. Add 0.05 % trypsin/ 0.02 % EDTA to cover cell layer, and incubate for 2 minutes at 37°C. Look at the cells in the microscope. If they are still adherent, leave the trypsin/ EDTA for 1 more minute or until they are detached.
2. Resuspend the CCL64 cells in cDMEM.
3. Centrifuge them at 500 g for 3 min and resuspend them in assay medium at 10^6 cells/ ml

B. TGF BIOASSAY

1. Prepare a culture plate which will later contain the series of wells described in #4 for each rTGF-β sample to test:
2. Add 100 µl of cell suspension to these wells.
3. Incubate cells for 1 hour at 37°C, 5% CO_2 in a humidified incubator.
4. Distribute 60 µl of the following media:
5. CPMed alone
6. CPMed containing a reference rTGF-β (if available; can also be diluted 2x, 4x… to set-up a curve)
7. CPMed containing the new rTGF-β
8. Incubate cells for 20 hours at 37°C, 5% CO_2 in a humidified incubator.
9. Add 10 µl/ well of 5 mg/ml MTT solution to the plate and incubate for 4 hours.
10. Add 50 µl/ well of MTT Lysing Solution to the plate and incubate overnight.
11. Read plate at 570-650 nm.
12. Graph standard curve (if available) and analyze data.

4. MATERIALS / EQUIPMENT

Sub-confluent Mv1Lu mink lung epithelial cells (Cat. No. CCL-64, American Type Culture Collection, Rockville, MD)
Trypsin 0.05% w/v / EDTA 0.02% w/v
Dulbecco's Modified Eagle's medium-F12 medium containing 10% fetal bovine serum (FBS) and 1 % penicillin-streptomycin (cDMEM)
Assay medium: minimal essential medium, 0.2% FBS, 10 mM Hepes
Cytokine production medium (CPMed)
MTT solution (Sigma Cat. No. M5655) 5 mg/ml stock in PBS kept at room temperature (protect from light)
MTT Lysing Solution 20% SDS/ 50% DMF
96-well flat-bottom culture plate

5. REFERENCES

Adler H, Frech B, Thöny M, Pfister H, Peterhans E, Jungi TW. Inducible nitric oxide synthase in cattle. Differential cytokine regulation of nitric oxide synthase in bovine and murine macrophages. J Immunol. 1995, 154, 4710-4718.

Chesters PM, Hughes A, Edington N. Equid herpesvirus 1: platelets and alveolar macrophages are potential sources of activated TGF-B1 in the horse. Vet Immunol Immunopathol. 2000, 75, 71-79

Gupta A, Dekaney CM, Bazer FW, Madrigal MM, Jaeger LA. Beta transforming growth factors (TGFbeta) at the porcine conceptus-maternal interface. Part II: uterine TGFbeta bioactivity and expression of immunoreactive TGFbetas (TGFbeta1, TGFbeta2, and TGFbeta3) and their receptors (type I and type II). Biol Reprod. 1998, 59, 911-7.

Kingfisher Biotech			
Standard Operating Procedure for US-VIRN		**Protocol title: Porcine TNFα**	Effective Date: January 2008
Written by	Joanna Labresh	Technical Approval	Carrie Stefans
Title	Project Director	Title	Research. Associate

1. OBJECTIVE

Measure cytotoxicity of PORCINE TNFα (which cannot be measured in other conventional WEHI cell line cytotoxicity assay) using the porcine kidney cell line.

2. SCOPE

The TNFα bioassay is based on the cytotoxicity induced by TNFα on the porcine kidney cell line PK(15). In this assay, the cytotoxic effect of TNFα is measured indirectly using the following method: MTS (a tetrazolium salt) is added to the culture medium and is bioreduced by LDH, which is found in metabolically active cells, into a colored end product, formazan, that is soluble in the culture media. The quantity of formazan, as measured by the amount of absorbance at 490nm, is directly proportional to the number of living cells in culture. Therefore, looking at test results represented as %Cytotoxicity versus. [TNFα] we expect to see more cytotoxicity corresponding to a higher concentration of TNFα. (MTS/PMS are from Promega's Kit.)

3. PROCEDURE

A. CELL CULTURE

1. Grow PK(15) cells (ATCC# CCL-33) in PK(15) Maintenance Medium (Appendix B). The cells are grown in T25 flasks, incubated in a horizontal position, using 10 ml PK(15) Maintenance Medium per flask and are transferred using a 1:2 - 1:6 dilution two or three times per week. For details of culturing and sub-culturing conditions see Appendix A.
2. Note: The cells need to be confluent prior to use in this assay.
3. On the day of the assay, pipet out the PK(15) Maintenance Medium from the flask and discard in a waste container.
4. Add 2 ml Trypsin-EDTA to each flask. Swirl to ensure even coverage on the growth surface of the flask.
5. Incubate at 25°C for approximately 5 - 15 minutes at room temperature or until cells start to detach.
6. Add 8 ml of PK(15) Maintenance Medium and rinse, by pipetting, the growth surface.
7. Pool the cells from all flasks in a 50 ml conical centrifuge tube and spin down at 250 x g for 10 minutes.
8. Resuspend the cells in 10 ml PK(15) Maintenance Media.
9. In a separate tube, mix 20 µl of PK(15) cells with 20 µl 0.4% Trypan Blue (a 1:1 ratio).
10. Count the cells in a haemocytometer. Count at least 100 cells. The live cells are clear-Trypan Blue exclusion, while the dead cells look blue. The viability of the cells used in the assay should be higher than 95%.
11. Dilute the cells in PK(15) Maintenance Medium to 2.5 x 10^5 cells/ml.
12. Aliquot the cells into 96-flat well microplates at a density of 2.5 x 10^4 cells/well (*i.e.* 100 µl/well of 2.5 x 10^5 cells/ml).
13. Incubate the cells for 24 hours at 37°C in a humidified 5% CO_2 incubator.
14. Resuspend the Actinomycin D to a final concentration of 1mg/ml using DMSO. The stock solution should be stored at 2 - 8°C and brought to room temperature (22 -25°C) prior to diluting. On the day of the assay dilute the stock solution of Actinomycin D to 3 □g/ml using PK(15) Assay Media.
15. Remove medium from wells and wash the wells with 100 µl/well PK(15) Maintenance Media.
16. Add 50 □l/well Actinomycin D (3 µg/ml) in PK(15) Assay Medium to the empty wells.
17. Incubate the plate containing cells with Actinomycin D for 2 hours at 37°C in a humidified 5% CO_2 incubator.

B. POSITIVE CONTROL AND SAMPLE PREPARATION AND ADDITION

1. For a positive control, use a recombinant porcine TNF sample that has a known biological activity in this assay. Perform a 10-fold dilution series using PK(15) Assay Medium with the porcine TNF□ recombinant protein positive control starting at 200 ng/ml and ending at 0.0002 ng/ml. In the assay the final concentrations for the dilutions will be: 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng/ml.
2. When testing a lyophilized protein, reconstitute the recombinant TNFα protein using 200 µl sterile filtered NanoPure water to a final concentration of 100 µg/ml.
3. Perform a 10-fold dilution series using PK(15) Assay Medium starting at 200 ng/ml and ending at 0.0002 ng/ml. Final concentrations for the dilutions will be: 100, 10, 1, 0.1, 0.01, 0.001 and

0.0001 ng/ml.

4. Add 50 µl/well for each of the positive control and sample dilutions to the 96-flat well microplates containing the PK(15) cells. Each dilution should be repeated in three or more replicates.
5. Note: Two sets of 3 wells each (if triplicates are chosen) should contain medium only. One set will represent the negative control or 0% cytotoxicity and the other set will be used as a 100% cytotoxicity control (see Step 6 for details).
6. Incubate the cells with the recombinant protein and Actinomycin D for 16 - 18 hours at 37°C in a humidified 5% CO_2 incubator.
7. 15 minutes prior to the addition of the MTS/PMS solution (Step 7), remove the medium from 3 of the "Medium only" wells and replace it with an equal volume of sterile water. These wells represent the cells that have experienced 100% cytotoxicity.
8. Add 20 µl/well of MTS/PMS solution (mixed per kit instructions) and incubate the plates for 1 - 6 hours at 37°C in a humidified 5% CO_2 incubator.
9. Read the O.D. (optical density) on a 96-well plate reader at 490nm after 1 hour and every hour for 2 - 6 hours to determine the optimal readings (*i.e.* the highest ratio between 0 - 100% cytotoxicity).
10. After the last reading, dispose of all cells, plates and used reagents in a biohazard container.

C. CALCULATION OF % CYTOTOXICITY

The %cytotoxicity is calculated according to the formula:

$$\%Cytotoxicity=100x[(A_0-A_{100})-(A_s-A_{100})]/(A_0-A_{100})$$

A_0=optical density of the 0ng/ml control (0%cytotoxicity)

A_{100}=optical density of the 100%cytotoxicity control

A_s = optical density of the standard or sample

Plotting the % Cytotoxicity versus log[TNF□] will give a sigmoidal curve.

The ED_{50}, to be calculated from the linear portion of the graph, represents 50% cytotoxicity relative to the maximal cytotoxic activity of the linear portion of the graph obtained in the assay.

4. MATERIALS / EQUIPMENT

Description	Supplier	Catalog #
Porcine PK(15)	ATCC (Appendix A)	CCL-33
Actinomycin D	Sigma	A9415
Trypsin-EDTA	Sigma	T4049
MTS/PMS solution (Cell Titer 96 Aqueous®)	Promega	G5421
Cell culture flasks	Corning	10-126-39
96-well flat bottom microplate	Corning /Costar	3595
PK(15) Maintenance Media	See Appendix B	
PK(15) Assay Media	See Appendix B	
0.4% Trypan Blue	Gibco BRL	15250-061

PK(15) MAINTENANCE MEDIA

Eagle's MEM/EBSS with 2 mM L-glutamine supplemented with:
2 mM L-glutamine (For a total of 4mM L-glutamine?)
0.1 mM MEM/NEAA (Minimal Essential Medium/Non-Essential Amino Acids)
1 mM Sodium pyruvate
1.5 g/L sodium bicarbonate
10% FCS (Fetal calf serum)

PK(15) ASSAY MEDIUM

IMDM with 4 mM L-Glutamine with HEPES (HyClone) containing
5 g/L Peptone (meat)

5. SAFETY

TNFα, Actinomycin D and MTS/PMS solution are toxic. Use proper precautions when handling these materials.

6. REFERENCES

Bertoni, G. *et al*. (1993) Improved bioassay for the detection of porcine tumor necrosis factor using a homologous cell line: PK(15). J. Immunol. Methods 160: 267.

7. APPENDIX A

From ATCC on-line catalog:

ATCC Number:	CCL-33		Price:	$224.00	
Designation:	PK(15)		Depositors:	Cutter Laboratories, Inc.	
Biosafety Level:	1		Shipped:	frozen	
Medium & Serum:	CCL-33"See Propagation		Growth Properties:	adherent	
Organism:	*Sus scrofa* (pig)		Morphology:	epithelial	
Tissue:	kidney; normal				
Cellular Products:	plasminogen activator; keratin				
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.				

Related Cell Culture Products

Comments:	The presence of a porcine papovavirus in PK(15) cells has been reported in cells obtained from multiple sources including the ATCC. [53398] [53399] The Foreign Animal Disease Diagnostic Laboratory of the US Department of Agriculture has determined that ATCC CCL-33 is not infected with Hog cholera virus or African swine fever virus, and uses this line to screen for those viruses. [5588] The cell line harbors an endogenous C-type retrovirus. [26185] [53399] [56104] The cells are positive for porcine circovirus (PCV) antigens. The cells are positive for keratin by immunoperoxidase staining.	
Virus Susceptibility:	hog cholera; African swine fever; vesicular exanthema of swine; foot and mouth disease (FMDV); vesicular stomatitis (Indiana); vaccinia; reovirus 2, 3; adenovirus 4, 5; coxsackievirus B2, B3, B4, B5, B6	
Virus Resist:	poliovirus 2	
Reverse Transcript:	positive	
Age:	adult	
Passage submitted to the ATCC:	129	
Propagation:CCL-33"	ATCC complete growth medium: Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10% Temperature: 37.0 C	

Subculturing:	Rinse the cell sheet 2 times with fresh 0.25% trypsin, 0.03% EDTA solution, remove trypsin and allow the culture to stand at room temperature for 5 to 10 minutes. Add fresh medium, aspirate and dispense into new flasks.	
Freeze Medium:	culture medium 95%; DMSO, 5%	
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium) - ATCC 30-2003 recommended serum - ATCC 30-2020	
References:	1. 222988: Dulac GC , Afshar A . Porcine circovirus antigens in PK-15 cell line (ATCC CCL-33) and evidence of antibodies to circovirus in Canadian pigs. Can. J. Vet. Res. 53: 431-433, 1989. PubMed: 2686830 2. 26184: Pirtle EC , Woods LK . Cytogenetic alterations in swine kidney cells persistently infected with hog cholera virus and propagated with and without antiserum in the medium. Am. J. Vet. Res. 29: 153-164, 1968. PubMed: 4965860 3. 26185: Armstrong JA , et al. C-type virus particles in pig kidney cell lines. J. Gen. Virol. 10: 195-198, 1971. PubMed: 4324256 4. 53398: Newman JT , Smith KO . Characteristics of a swine papovavirus. Infect. Immun. 5: 961-967, 1972. PubMed: 4344097 5. 53399: Tumilowicz JJ , et al. Concurrent replication of a papovavirus and a C-type virus in the CCL 33 porcine cell line. In Vitro 15: 922-928, 1979. PubMed: 232060 6. 56104: Todaro GJ , et al. Characterization of a type C virus released from the porcine cell line PK(15). Virology 58: 65-74, 1974. PubMed: 4132403 7. 5588: James A House, personal communication	

Kingfisher Biotech			
Standard Operating Procedure for US-VIRN		**Protocol title: TNF-α**	Effective Date: January 2008
Written by	Joanna Labresh	Technical Approval	Carrie Stefans
Title	Project Director	Title	Research. Associate

1. OBJECTIVE

Measure the cytotoxic ability of recombinant TNFα against the TNF-sensitive mouse cell line WEHI-13VAR in the presence of actinomycin D. This assay will cross-react with TNFα from the following species: human, mouse, rat, bovine, equine, and ovine. Porcine TNFα can NOT be tested using this assay.

2. SCOPE

The TNFα bioassay is based on the cytotoxicity induced by TNFα on the mouse WEHI-13VAR cell line. In this assay, the cytotoxic effect of TNFα is measured indirectly using the following method: MTS (a tetrazolium salt) is added to the culture medium and is bioreduced by LDH, which is found in metabolically active cells, into a colored end product, formazan, that is soluble in the culture media. The quantity of formazan, as measured by the amount of absorbance at 490nm, is directly proportional to the number of living cells in culture. Therefore, looking at test results represented as %Cytotoxicity vs. [TNFα] we expect to see more cytotoxicity corresponding to a higher concentration of TNF.

3. PROCEDURE

A. CELL CULTURE

1. Grow WEHI-13VAR cells (ATCC# CRL-2148) in WEHI-13VAR Maintenance Medium (Appendix B). The cells are grown in T25 flasks, incubated in a horizontal position, using 10 ml WEHI-13VAR Maintenance Medium per flask and are transferred using a 1:20 dilution twice a week. For details of culturing and sub-culturing conditions use Appendix A.
   Note: The cells can be transferred at a lower dilution than 1:20 for at least 24 hours prior to the assay, if needed sooner. The cells do not need to be confluent, but they should not be denser than one layer per flask prior to use in this assay.
2. On the day of the assay, pipet out the WEHI-13VAR Maintenance Medium from the flask and discard in a waste container.
3. Add 2 ml Trypsin-EDTA to each flask. Swirl to ensure even coverage on the growth surface of the flask.
4. Incubate at 25°C for approximately 3 minutes or until cells start to detach.
5. Add 8 ml of WEHI-13VAR Maintenance Medium and rinse, by pipetting, the growth surface.
6. Pool the cells from all flasks in a 50 ml conical centrifuge tube and spin down at 250 x g for 10 minutes
7. Resuspend the cells in 10 ml WEHI-13VAR Maintenance Media.
8. In a separate tube, mix 20 µl of WEHI-13VAR cells with 20 µl 0.4% Trypan Blue (a 1:1 ratio).
9. Count the cells in a haemocytometer. Count at least 100 cells. The live cells are clear-Trypan Blue exclusion, while the dead cells look blue. The viability of the cells used in the assay should be higher than 95%.
10. Dilute the cells in WEHI-13VAR Maintenance Medium to 5×10^5 cells/ml.
11. Aliquot the cells into 96-flat well microplates at a density of 5×10^4 cells/well (*i.e.* 100 µl/well of 5×10^5 cells/ml).
12. Allow the cells to adhere for 6 - 7 hours at 37°C in a humidified 5% CO_2 incubator.
13. Remove medium from wells and wash the wells with 100 µl/well WEHI-13VAR Maintenance Media.
14. Add 50 µl fresh WEHI-13VAR Maintenance Medium to each well.

B. POSITIVE CONTROL AND SAMPLE PREPARATION AND ADDITION

1. For a positive control, use a recombinant mouse TNF sample that has a known biological activity in this assay. Perform a 10-fold dilution series using WEHI-13VAR Assay Medium (Appendix B) with the mouse TNF recombinant protein starting at 300 ng/ml and ending at 0.0003 ng/ml. In the assay the final concentrations for the dilutions will be: 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng/ml.
2. When testing a lyophilized protein, reconstitute the recombinant TNF protein using 200 µl sterile filtered NanoPure water to a final concentration of 100 µg/ml.
3. Perform a 10-fold dilution series using WEHI-13VAR Assay Medium starting at 300 ng/ml and ending at 0.0003 ng/ml. Final concentrations for the dilutions will be: 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng/ml.

4. Add 50 µl/well for each of the positive control and sample dilutions to the 96-flat well microplates containing the WEHI-13VAR cells. Each dilution should be repeated in three or more replicates.
   Note: Two sets of 3 wells each (if triplicates are chosen) should contain medium only. One set will represent the negative control or 0% cytotoxicity and the other set will be used as a 100% cytotoxicity control (see Step 10 for details).
5. Resuspend the Actinomycin D to a final concentration of 1 mg/ml using DMSO. The stock solution should be stored at 2 - 8°C and brought to room temperature (22 - 25°C) prior to diluting. On the day of the assay dilute the stock solution of Actinomycin D to 3 µg/ml using WEHI-13VAR Maintenance Media.
6. Add 50 µl Actinomycin D (3 µg/ml) to each well bringing its final concentration to 1ug/ml. The total volume per well now equals 150 µl/well and the concentrations of the proteins have been diluted 1:3 yielding the desired final concentrations: 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001ng/ml.
7. Incubate the cells, with the recombinant protein and Actinomycin D, 16 - 20 hours at 37°C in a humidified 5% CO_2 incubator.
8. 15 minutes prior to the addition of the MTS/PMS solution (Step 11), remove the medium from 3 of the "Medium only" wells and replace it with an equal volume of sterile water. These wells represent the cells that have experienced 100% cytotoxicity.
9. Add 20 µl/well of MTS/PMS solution (mixed per kit instructions) and incubate the plates for 1 - 4 hours at 37°C in a humidified 5% CO_2 incubator.
10. Read the O.D. (optical density) on a 96-well plate reader at 490 nm after 1 hour and every hour for 2 - 4 hours to determine the optimal readings (*i.e.* the highest ratio between 0 - 100% cytotoxicity).
11. After the last reading, dispose of all cells, plates and used reagents in a biohazard container.

C. CALCULATION OF % CYTOTOXICITY

The %cytotoxicity is calculated according to the formula:

$$\%Cytotoxicity=100x[(A_0-A_{100})-(A_s-A_{100})]/(A_0-A_{100})$$

A_0=optical density of the 0ng/ml control (0%cytotoxicity)

A_{100}=optical density of the 100%cytotoxicity control

A_s = optical density of the standard or sample

Plotting the % Cytotoxicity versus log[TNFα] will give a sigmoidal curve.

The ED_{50}, to be calculated from the linear portion of the graph, represents 50% cytotoxicity relative to the maximal cytotoxic activity of the linear portion of the graph obtained in the assay.

5. MATERIALS / EQUIPMENT

Description	Supplier	Catalog #
Mouse WEHI-13VAR cells	ATCC (Appendix A)	CRL-2148
Actinomycin D	Sigma	A9415
Trypsin-EDTA	Sigma	T4049
MTS/PMS solution (Cell Titer 96 Aqueous®)	Promega	G5421
Cell culture flasks	Corning	10-126-39
96-well flat bottom microplate	Corning /Costar	3595
WEHI-13VAR Maintenance Media	See Appendix B	
WEHI-13VAR Assay Media	See Appendix B	
0.4% Trypan Blue	Gibco BRL	15250-061

WEHI-13VAR MAINTENANCE MEDIUM

RPMI 1640 supplemented with:

- 4 mM L-glutamine
- 10 mM HEPES
- 1 mM Sodium pyruvate
- g/L glucose
- 1.5 g/L sodium bicarbonate
- 10% FCS (Fetal calf serum)
- 100 U/ml Penicillin
- 100 µg/ml Streptomycin

WEHI-13VAR ASSAY MEDIUM

RPMI 1640 supplemented with:

- 4 mM L-Glutamine
- 25 mM HEPES
- 1 mM sodium pyruvate
- 10% FCS (Fetal calf serum)
- 1% Non-essential amino acids
- 1:166.6 (v/v) Insulin-Transferrin-Selenium
- 50 μM β-ME (beta-mercaptoethanol)
- 100 U/ml Penicillin
- 100 □g/ml Streptomycin
- 1.25 □g/ml Gentamycin

6. SAFETY

TNFα, Actinomycin D and MTS/PMS solution are toxic. Use proper precautions when handling these materials.

7. REFERENCES

Kitani, H. *et al*. (2002) Infect. Immun. **70**:2210

Khabar, K.S. *et al*. (1995) Immunol. Lett. **46**:107.

ATCC Number:	CRL-2148	Price:	$179.00
Designation:	WEHI-13VAR	Depositors:	JA Armstrong
Biosafety Level:	1	Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	mixed, adherent and suspension
Organism:	*Mus musculus* (mouse)	Morphology:	fibroblast
Tissue:	fibrosarcoma		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Comments:	WEHI-13VAR, is a variant of WEHI 164 clone 13 which has lost its sensitivity to Tumor Necrosis Factor (TNF) in the absence of actinomycin D. The WEHI-13VAR cell line was found to maintain its high sensitivity to TNF when the assay was performed in the presence of 500 ng/ml actinomycin D. This line provides a stable and highly sensitive bioassay system to detect and measure mouse and human natural and recombinant tumor necrosis factors (TNF alpha and lymphotoxin. It is more sensitive to TNF alpha and lymphotoxin than L929 (ATCC CCL-1) or WEHI 164 (ATCC CRL-1751).		
Strain:	BALB/c		
Propagation:	ATCC complete growth medium: RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10% Temperature: 37.0 C		

Subculturing:	Protocol: 1. Remove culture medium to a centrifuge tube. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 - 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. To remove trypsin-EDTA solution, transfer cell suspension to the centrifuge tube with the medium and cells from step #1 and spin at approximately 125 x g for 5 - 10 minutes. 6. Discard supernatant and resuspend cells in fresh growth medium. Add appropriate aliquots of cell suspension to new culture vessels. 7. Place culture vessels in incubators at 37°C. Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:6 is recommended Medium Renewal: Twice per week
Freeze Medium:	Complete growth medium supplemented with 5% (v/v) DMSO
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium) - ATCC 30-2001 recommended serum - ATCC 30-2020
References:	1. 22472: Espevik T , Nissen-Meyer J . A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J. Immunol. Methods 95: 99-105, 1986. PubMed: 3782828 2. 22786: Abu-Khabar KS , et al. Type I interferons (IFN-alpha and -beta) suppress cytotoxin (tumor necrosis factor-alpha and lymphotoxin) production by mitogen-stimulated human peripheral blood mononuclear cell. J. Leukocyte Biol. 52: 165-173, 1992. PubMed: 1506772 3. 24378: Khabar KS , et al. WEHI-13VAR: a stable and sensitive variant of WEHI 164 clone 13 fibrosarcoma for tumor necrosis factor bioassay. Immunol. Lett. 46: 107-110, 1995. PubMed: 7590904

USDA-ARS Beltsville Lillehoj Lab			
Standard Operating Procedure for US-VIRN		**Protocol title: TNFSF15 (TL1A)**	Effective Date: January 2008
Written by	Yeong Hong	Technical Approval	Hyun Lillehoj
Title	Post-doctoral Research Associate	Title	Project director

1. OBJECTIVE

Measure bioactivity of recombinant TNFSF15 (TL1A) using a cytotoxicity assay and a variety of cell lines or primary cells.

2. SCOPE

TNFSF and TNFSFR proteins are abundantly expressed in the immune system and are critically involved in the differentiation, proliferation and apoptosis of immune cells. Several chicken TNFSF members are homologous to their mammalian counterparts. In particular, the chicken TNFSF15 gene is similar to mammalian TL1A and the long form of mammalian vascular endothelial growth inhibitor (VEGI). Chicken TNFSF15 mediate cytotoxicity in vitro against murine fibroblast, primary chicken cell culture or CHCC-OU2 (Takimoto et al., 2005, Park et al., 2007).

3. PROCEDURE

Experiment Duration: 48 hour incubation at 37°C, 5% CO_2 incubator

1. 100ul of cell line suspensions (5 x 10^3 or 1 x 10^5 cells/well) are placed in flat-bottomed 96 well plate in triplicates.
2. Incubate with serially diluted recombinant TNFSF15 protein for 48h at 37°C, 5% CO_2 incubator. Irrelevant protein serves as a control.
3. WST-8 cell counting kit is used according to the manufacturer's protocol to measure cell viability by reading O.D. at 450nm.

4. MATERIALS / EQUIPMENT

Chicken tumor cell lines (HTC, CHCC-OU2, LSCC-RP9 et al.)
Recombinant LITAF protein
Culture Medium (DMEM or RPMI-1640 supplemented with 10% FBS)
96 well flat-bottomed microtiter plate (Corning Costar)
WST-8 cell counting kit (Dojindo Molecular Technologies, Gaithersburg, MD)

5. REFERENCES

Park SS, HS Lillehoj, YH Hong, SL Lee. Functional characterization of TNFSF15 induced by lipopolysaccharide and Eimeria infection. Developmental & Comparative Immunology 31:934-944 (2004).

Takimoto T, K Takahasshi, K Sato, Y Akiba. Molecular cloning and functional characterization of chicken TL1A. Dev. Comp. Immunol. 29(10): 895-905 (2005).