Supplemental Materials
for
Inquiry-Based Learning: Inflammation as a Model to Teach Molecular Techniques for Assessing Gene Expression

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Appendix 1: Assessment of learning objectives.

Each Learning Objective is listed below, followed by an explanation of how it is assessed.

1. Students gain experience with molecular and genetic laboratory theory, practice, and experimental design (see specific techniques below).
   Attendance at all lecture and laboratory meetings is required. During these meetings, the conceptual basis of each technique is provided and the procedure for each is laid out and performed. By virtue of being present and conducting the experiments, students gain experience with the techniques. To assess this experience, we require students to complete post-lab worksheets (Appendix 6) and a scientific poster (Guidelines in Appendix 3 and rubric in Appendices 3 and 6). Grades are associated with these assignments. The worksheets ask students about: the question being addressed; the hypothesis; the variables; the controls; the data that was generated and how to present and interpret it; how well the procedure worked; and how the students might extend the question to future directions in light of the data they generated. The poster serves as a visual demonstration of the students’ experiences with each technique.

2. Students research the biological process of inflammation and the inflammatory effects of their assigned mediator.
   During a lecture meeting, students present information about their assigned mediator to their peers. Completion of this task earns the students participation points for the day. In order to complete the poster assignment, students must research inflammation, in general, as well as their specific assigned mediator. A portion of the poster grade relies on the inclusion of primary literature relating to inflammation and the assigned mediator, and another portion of the poster grade depends on integration of that information into the poster and the overall research question and analysis of results (see Appendices 3 and 6 for poster rubric).

3. Students gain experience with experimental design and generate hypotheses regarding predicted experimental results and the effect their assigned mediator will have on cellular processes.
   To assess experimental design and hypotheses, we again use the post-lab worksheets (Appendix 6) and a scientific poster (Guidelines in Appendix 3 and rubric in Appendices 3 and 6). Grades are attached to these items. The worksheets ask students about: the question being addressed; the hypothesis (which varies from group to groups and depends on the information they were able to find regarding their assigned mediator; the variables; the controls; the data that was generated and how to present and interpret it; how well the procedure worked; and how the students might extend the question to future directions in light of the data they generated. The poster allows us to assess the ability of individual
students to synthesize the information they have learned and generated and to discuss it in terms of their original hypotheses.

4. **Students summarize their experience and demonstrate the knowledge they have gained through oral, visual, and written communication.**

   Students complete post lab worksheets associated with each experiment. Students orally present a scientific style poster during a poster session (see Appendix 6 for post lab worksheets and Appendices 3 and 6 for poster rubric).

5. **Students gain experience with the following molecular and genetic laboratory techniques, with emphasis on 1) comprehending their functionality and usefulness, 2) learning typical data presentation format, and 3) performing data interpretation for each method:**

   Mammalian cell tissue culture, ELISA analysis, Mammalian cell transfection, Luciferase assay analysis, Western blot analysis, Protein isolation, Fluorescence microscopy, Real-time quantitative PCR

Students complete the MASE Assessment Test (MAT) (Appendix 5).
Appendix 2: Materials, instrumentation, orders, and recipes.

*MSMU instrumentation is in parentheses. Other instrumentation can be utilized. However, substitutions may necessitate changes to the lab manual and preparation.*

**Required Instrumentation:**
- Plate Reader with a 450nm filter (BioRad 550c)
- Luminometer
- Laminar flow tissue culture hood
- Tissue culture (CO₂) Incubator
- qPCR system (Invitrogen StepOne Plus)
- SDS-PAGE apparatus (Invitrogen XCell SureLock Mini Cell)
- Western transfer apparatus (Invitrogen iBlot)
- Film-developing capability (darkroom with JPI J-33 Automatic Film Processor)
- Tabletop microcentrifuge (for cold spins, we place it in the refrigerator)
- Tabletop centrifuge (Eppendorf Refrigerated 5810R)
- Vortex
- -80°C freezer
- -20°C freezer
- 4°C refrigerator
- Ice machine
- Heat Block
- Plate rocker
- Fluorescence Microscope with DAPI and GFP filters and camera/software for image capture ability
- Large Format poster printer
Orders:

The following is a list of the items that ordered based on our instrumentation and the expectation of 7 laboratory groups with 2-3 students per group. Again, substitutions can be made to align with current instrumentation and equipment holdings.

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<td>I0054-1MG</td>
<td>1</td>
<td>Ca ionophore</td>
<td>$101.00</td>
<td>$101.00</td>
<td>$101.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>Sigma</td>
<td>F4880</td>
<td>1</td>
<td>Fluoromount</td>
<td>$49.17</td>
<td>$49.17</td>
<td>$49.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>Sigma</td>
<td>P2267-50ML</td>
<td>1</td>
<td>Tween 20</td>
<td>$38.10</td>
<td>$38.10</td>
<td>$38.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>Vector Labs, Inc.</td>
<td>L-1110</td>
<td>1</td>
<td>PKh-Phaseolus Vulgaris</td>
<td>$105.00</td>
<td>$105.00</td>
<td>$105.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$4,877.27 Total spent</td>
</tr>
</tbody>
</table>
MASE Daily Prep Notes

The following daily prep sheets include a table of supplies as well as guidelines for preparing reagents, cells, instrumentation. Preparation for this two week laboratory course is divided by day. In each case reagents must be set out the evening prior to each work day. Unless indicated with an asterisk (*), materials may be prepared and aliquot in advance. Following each of the daily lists are specific instructions for seeding cells. In addition, following all of the daily material lists are detailed protocols for preparing reagents and instrumentation. Unless otherwise indicated, 8 aliquots of each reagent were prepared for 7 lab groups of 2-3 students.

In addition to the materials listed below each lab station should be set up with the following materials:

- A set of pipettes (p20, p200 and p1000)
- One set of p20/200 tips and one box of p1000 tips. (On the qPCR day, the autoclaved tips need to be removed and replaced with the nuclease free tips. At the end of the qPCR setup, the remaining nuclease free tips need to be removed and replaced with the autoclave tips.)
- One 500 ml beaker (labeled “Waste”)
- Sharpie
- Microfuge rack
- 15ml/50 ml conical rack
- One 50 ml aliquots of sterile dH2O (autoclaved) manual pipet pump (for 5ml-25ml serological pipets)
- A bag containing sterile serological pipettes (containing an assortment of 5, 10, and 25 ml pipettes).
- 6 Sterile Cell scrapers

For the indicated Experimental Day the following reagents should be prepared:

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Reagent</th>
<th>Volume</th>
<th># aliquots</th>
<th>Storage Cond.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW Cell Media</td>
<td></td>
<td></td>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>Inflammatory mediators (all 6)</td>
<td></td>
<td>2- 50ul aliquots of each</td>
<td>on ice</td>
<td></td>
</tr>
<tr>
<td>24- 10 cm plates seeded with RAW cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer A* (add inhibitor tablet in am)</td>
<td>10 ml</td>
<td>8</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>
RAW cell plating for Western Blotting
Approximately 24 hours prior to their first treatment, seed 8 sets of three 10 cm plates with 4x10^6 cells in 10 ml of RAW cell Media. Incubate at 37°C for 24 hours.

Day 2

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th># aliquots</th>
<th>Storage Cond.</th>
</tr>
</thead>
<tbody>
<tr>
<td>9- 6 well plates of RAW cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimem SF media</td>
<td>5mL</td>
<td>8</td>
<td>4°C</td>
</tr>
<tr>
<td>Lipofectamine</td>
<td>22uL</td>
<td>8</td>
<td>4°C</td>
</tr>
<tr>
<td>NFkB DNA for transfection</td>
<td>depends on</td>
<td></td>
<td>20°C</td>
</tr>
<tr>
<td>RAW cell Media</td>
<td>10mL</td>
<td><strong>20</strong></td>
<td>4°C</td>
</tr>
<tr>
<td>Inflammatory mediators (2 of each)</td>
<td>10uL</td>
<td>as needed</td>
<td></td>
</tr>
<tr>
<td>Saran wrap</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RAW cell plating for Luciferase Assay:
Seed (9)- 6 well tissue culture plates with 2 wells each at 2x10^6 cells/well. Seed these on Day 1 (24 hours prior to transfection) in a final volume of 2 ml/well of Antibiotic-free RAW Cell media.

Day 3

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th># aliquots</th>
<th>Storage Cond.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory mediators (2 of each-all 7)</td>
<td>50uL</td>
<td></td>
<td>as needed</td>
</tr>
<tr>
<td>RAW cell Media</td>
<td>10 mL</td>
<td><strong>20</strong></td>
<td>4°C</td>
</tr>
<tr>
<td>Luciferase Reagent (pre-aliqouted)</td>
<td>1mL</td>
<td></td>
<td>-20C</td>
</tr>
<tr>
<td>Passive Lysis Buffer</td>
<td>600uL</td>
<td></td>
<td>-20C</td>
</tr>
<tr>
<td>96 well ELISA plate 1/station</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saran wrap</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA Capture antibody*</td>
<td>(8) 2ml aliquots</td>
<td></td>
<td>4°C</td>
</tr>
</tbody>
</table>
**RAW cell plating for ELISA:**

On Day 1 (8) 96-well tissue culture plates were seeded with 2x10^5 cells/well in 200ul RAW Cell Media. Only 6 wells of each plate were seeded with RAW 264.7 cells (see diagram below). Plates were incubated approximately 24 hours at 37°C prior to treatment.

In the wells surrounding the cells, add 250ul of sterile 1X PBS to decrease evaporation (see diagram below).

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>PBS</td>
<td>Cells</td>
<td>Cells</td>
<td>Cells</td>
<td>PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>PBS</td>
<td>Cells</td>
<td>Cells</td>
<td>Cells</td>
<td>PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Day 4: Most of the Day 4 – most of these reagents need to be made on Day 4.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th># aliquots</th>
<th>Storage Cond.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection Ab*</td>
<td>(8) 2mL</td>
<td></td>
<td>ice/4°C</td>
</tr>
<tr>
<td>Avidin-HRP*</td>
<td>(8) 2mL</td>
<td></td>
<td>ice/4°C</td>
</tr>
<tr>
<td>Substrate solution</td>
<td>(8) 2mL</td>
<td>aliquot in advance</td>
<td>4°C, dark</td>
</tr>
<tr>
<td>Stop solution</td>
<td>(8) 2mL</td>
<td>aliquot in advance</td>
<td>RT</td>
</tr>
<tr>
<td>Assay diluent</td>
<td>(8) 4mL</td>
<td>aliquot in advance</td>
<td>4°C</td>
</tr>
<tr>
<td>ELISA wash buffer</td>
<td>(8) 500mL</td>
<td>prepare in advance</td>
<td>RT</td>
</tr>
</tbody>
</table>

50mL conical tube 1/station
Paper towels or bench coat–several layers needed next to sinks
Prepare TNF alpha standard curve as described by the manufacturer (eBioscience).
Set out Multichannel micropipettor(s)
Email ELISA analysis spreadsheet to each student (Appendix 10)
Day 5

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th># aliquots</th>
<th>Storage Cond</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M DTT</td>
<td>50uL</td>
<td>-</td>
<td>-20°C</td>
</tr>
<tr>
<td>Protein Assay buffer (made below)</td>
<td>50mL</td>
<td>20</td>
<td>RT</td>
</tr>
<tr>
<td>1X TBST (1mL Tween/L)</td>
<td>500mL</td>
<td>20</td>
<td>RT</td>
</tr>
<tr>
<td>Novex protein ladder</td>
<td>10uL</td>
<td>-</td>
<td>-20°C</td>
</tr>
<tr>
<td>5x Novex Protein Loading dye</td>
<td>100uL</td>
<td>20</td>
<td>4°C</td>
</tr>
<tr>
<td>12% Bis-Tris Protein Gels</td>
<td>1 per group</td>
<td>20</td>
<td>4°C</td>
</tr>
<tr>
<td>1x MOPS Running Buffer</td>
<td>1 liter</td>
<td>-</td>
<td>RT</td>
</tr>
<tr>
<td>Scapelans</td>
<td>-</td>
<td>1 per station</td>
<td></td>
</tr>
<tr>
<td>Ice buckets to thaw samples</td>
<td>-</td>
<td>1 per 2 groups</td>
<td></td>
</tr>
<tr>
<td>Gel Loading tips</td>
<td>-</td>
<td>1 per 2 groups</td>
<td></td>
</tr>
<tr>
<td>2 Power supplies for Protein gels</td>
<td>-</td>
<td>1 per 4 groups</td>
<td></td>
</tr>
<tr>
<td>4 SDS PAGE Electrophoresis Chambers</td>
<td>-</td>
<td>1 per 4 groups</td>
<td></td>
</tr>
<tr>
<td>3 iBlotters and iBlotter supplies to transfer 7 gels</td>
<td>-</td>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>Gel opening spatulas</td>
<td>-</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>1X PBS (sterile)</td>
<td>50mL</td>
<td>-</td>
<td>RT</td>
</tr>
<tr>
<td>Lipofectamine</td>
<td>65uL</td>
<td>-</td>
<td>4°C</td>
</tr>
<tr>
<td>1M Tris pH 7.5 (made above)</td>
<td>200uL</td>
<td>-</td>
<td>RT</td>
</tr>
<tr>
<td>Serum Free DMEM media</td>
<td>1.5mL</td>
<td>-</td>
<td>4°C</td>
</tr>
<tr>
<td>Complete DMEM</td>
<td>30mL</td>
<td>-</td>
<td>4°C</td>
</tr>
<tr>
<td>Ice buckets+ ice</td>
<td>-</td>
<td>1/2 groups</td>
<td>4°C</td>
</tr>
</tbody>
</table>

Western Blotting

**Maintain all Abs at 4°C at all times! Dilute Abs in am of this day**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th># aliquots</th>
<th>Storage Cond</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Milk/1xTBST*</td>
<td>25mL</td>
<td>7</td>
<td>4°C</td>
</tr>
<tr>
<td>1X TBST (1X TBS w/ 1mL Tween/L)</td>
<td>500mL</td>
<td>7</td>
<td>4°C</td>
</tr>
<tr>
<td>Anti-NFkB antibody (1^o Ab)</td>
<td>22uL</td>
<td>-</td>
<td>4°C</td>
</tr>
<tr>
<td>Anti Beta2 microglobulin antibody (1^o Ab)</td>
<td>22uL</td>
<td>-</td>
<td>4°C</td>
</tr>
<tr>
<td>Goat anti Rabbit HRP antibody (2^o Ab)</td>
<td>3uL</td>
<td>-</td>
<td>4°C</td>
</tr>
<tr>
<td>ECL Reagent 1</td>
<td>500uL</td>
<td>4°C</td>
<td></td>
</tr>
<tr>
<td>ECL Reagent 2</td>
<td>500uL</td>
<td>4°C</td>
<td></td>
</tr>
<tr>
<td>Positive control protein – p50 NFkB</td>
<td>7uL</td>
<td>-</td>
<td>4°C</td>
</tr>
<tr>
<td>1M Tris pH 7.5</td>
<td>200uL</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(7) Probing trays (clean pipette box lids)</td>
<td>-</td>
<td>1/station</td>
<td>4°C</td>
</tr>
<tr>
<td>Saran wrap</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4 film cassettes in darkroom</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
**RAW 264.7 cell plating for Fluorescence Microscopy on Day 7:**

For the Fluorescence microscopy experiment, seed 1 - 6 well plate per lab group (we prepared 8 plates- one for each group plus one to spare) 48 hours prior to treatment. Cells are seeded on *Day 5* at 8 x10^5 cells per well (each well contained a sterilized coverslip). Plates were incubated at 37°C for 48 hours prior to inducing inflammation on *Day 7*. Coverslips were sterilized by dipping in ethanol and then flaming (hold with sterilized forceps) in the tissue culture hood.

---

**Day 6**

**qPCR**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th># aliquots</th>
<th>Storage Cond.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>50uL in advance (8)</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>In nuclease free tubes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Veri-Flex Trays (1 per 2 groups)</td>
<td>4</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>7 MicroAmp Tube Holder racks</td>
<td>7</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>8x 3-tube strip</td>
<td>8</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>32x 6-tube strip</td>
<td>32</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>8x 3-cap strip</td>
<td>8</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>32x 6-cap strip</td>
<td>32</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>Cap installing tool</td>
<td>1</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>Untreated cDNA</td>
<td>48ul in advance (7)</td>
<td>-20°C/on ice</td>
<td></td>
</tr>
<tr>
<td>Treated cDNA</td>
<td>48ul in advance (7)</td>
<td>-20°C/on ice</td>
<td></td>
</tr>
<tr>
<td>Incomplete Master Mix</td>
<td>49.5ul day of *(7)</td>
<td>on ice</td>
<td></td>
</tr>
<tr>
<td>In nuclease free tubes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incomplete Master Mix (NFTs)</td>
<td>99ul day of *(28)</td>
<td>on ice</td>
<td></td>
</tr>
<tr>
<td>b-actin Primer/Probe Pairs (PPPs)</td>
<td>15ul in advance (8)</td>
<td>-20°C/on ice</td>
<td></td>
</tr>
<tr>
<td>in NFTs (same for other PPPs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS PPPs</td>
<td>10ul in advance (8)</td>
<td>-20°C/on ice</td>
<td></td>
</tr>
<tr>
<td>NFkB PPPs</td>
<td>10ul in advance (8)</td>
<td>-20°C/on ice</td>
<td></td>
</tr>
<tr>
<td>TNFa PPPs</td>
<td>10ul in advance (8)</td>
<td>-20°C/on ice</td>
<td></td>
</tr>
<tr>
<td>Spray bottles of 70% EtOH</td>
<td></td>
<td></td>
<td>RT</td>
</tr>
<tr>
<td>Gloves</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Email qPCR analysis spreadsheet to each student (Appendix 10)
**Day 7: Fluorescent microscopy**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th># aliquots</th>
<th>Storage Cond</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory mediators (2 aliquots of each)</td>
<td>100uL of each</td>
<td>as needed</td>
<td></td>
</tr>
<tr>
<td>DAPI/Hoescht stain*</td>
<td>2mL</td>
<td>-20°C</td>
<td></td>
</tr>
<tr>
<td>1x TBS (made above)-Tween20</td>
<td>500ul/L</td>
<td>1L</td>
<td>RT</td>
</tr>
<tr>
<td>Fluoromount G</td>
<td>1.5mL</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>Microscope slides</td>
<td>6/station keep dust free</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forceps-1/station</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasture pipettes and bulbs</td>
<td>1 each/station</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tygon tubing</td>
<td>1 length/station</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccum set up</td>
<td>at least 1 per two lab groups needed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescent microscope/camera attachment for visualization</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Week 1 Reagent Preparation:**

**Instrumentation for Week 1:**

- Vortex with multitube/microfuge adaptor – put in the fridge for cold spins
- Microcentrifuge in fridge for cold spins
- Microcentrifuge
- Plate reader with 450nm filter
- Buckets of ice for each lab station
- Mini microcentrifuge for quick spins
- Refrigerated table top centrifuge
- Box of cell scrapers

**Reagent and Instrumentation Preparation for Week 1:**

1. Aliquot 400 ml distilled water into 500 ml bottles. Prepare 8 aliquots.
2. Prepare DNA midipreps of NfkB-GFP, NFkB-Luc and MekK plasmids.
3. Thaw and expand RAW264.7 Cells (murine macrophage cell line, derived from Balb/c mice) from Liquid Nitrogen bullets. We had 7 laboratory groups and prepared 8 sets of plates (an extra in case of mistake):
4. Sterilize and set up Laminar Flow Hoods with:
   - P200 tips, p1000 tips—one box of each
   - P20, P200 and p1000 micropipettors
   - Sharpie pen
   - 2 1L beakers in each hood—one labeled ‘liquid waste’, the other ‘used’ tips
   - Microcentrifuge tube rack and container of autoclaved tubes
5. 500 mL complete DMEM (high glucose) – prepare 4 bottles (1 per hood)
   a. Add 5mL pen-strep
   b. Add 5mL L-glutamine (this may be added already... check label)
   c. Add 50 mL FBS
   d. Use a 500 ml Filter flask (0.45 um) to sterilize the media.
   e. Label, date, Store at 4°C.

- Also, prepare one bottle of Antibiotic Free Raw cell media and one bottle of Serum-free RAW 264.7 cells media. Preparation is identical to that described in step 5 above, but either Pen-strep solution or Serum (FBS) will be eliminated, respectively.

   **Buffer A:**
   - 20mM HEPES buffer
   - 0.1mM EDTA
   - 10mM NaCl
   - 0.1% NP-40
   By combining: 4mL HEPES buffer + 4uL 0.5M EDTA + 400uL 5M NaCl + 200uL NP-40 + 195.4mL dH2O
   a. Prepare 1-ten mL aliquot per lab group and store at 4°C.
   b. *On morning of Day 1—dissolve 1 protease inhibitor tablet into each aliquot
   c. Store at 4°C or on ice until ready to use.

7. Prepare 100 ml Buffer C (Nuclear Lysis Buffer) for Day 1.
   **Buffer C:**
   - 420 mM NaCl
   - 0.2mM EDTA
   - 25 mM Hepes pH 7.6
   - 25% Glycerol
   a. Prepare a single 10 ml aliquot of buffer C for up to 10 lab groups. Store it at 4°C.
   b. *On am of day 1-dissolve 1 protease inhibitor tablet in the 10 ml aliquot.
   c. Make 8- 1mL aliquots in microfuge tubes.
   d. Dispense to students and store on ice.
8. Make 7 L -1x PBS (dilute from 10x stock with ddH2O) 3L will be used for bench aliquots and hood; remaining 4L are for ELISA reagent preparation.
   a. Autoclave to sterilize.
   b. Prepare one aliquot of 100 ml 1x PBS (sterile—label accordingly) per lab group. Place one at each station.
   c. Divide up remaining sterile 1x PBS-place 500 mL aliquot in each hood be sure to maintain sterility!!!
   d. Use remaining 4L for ELISA as indicated.

9. Inflammatory Mediators are used on day 1 and throughout week 2. These should be made and aliquot in advance.
   –Prepare 100 X stock solution of the following inflammatory mediators (use sterile PBS for this unless otherwise indicated by the manufacturer). Prepare 2mL of each reagent and store according to manufacturer recommendations (probably -20°C or -80°C).
     • Lipopolysaccharide (LPS) (2.5 mg/ml) isolated from E. coli - Sigma catalog # L3012-5mg). Re-suspend in 2 ml of 1x PBS to generate a 2.5mg/ml stock solution. Prepare 8-250ul aliquots and store at -20°C
     • IFN gamma (1mg/ml stock stored at -80°C) Dilute 1:100 in 1x PBS/0.1% BSA and filter sterilize. Prepare 8-250ul aliquots; store at -80°C
     • PMA (100ug/ml) (Phorbol Ester or PMA/TPA – Sigma catalog # P8139-1MG) stored in -20°C). Dissolve 1mg (0.001g) in 10 ml PBS to generate a 100ug/ml stock. Prepare 8- 200ul aliquots, store at -20°C
     • PHA-L (1 mg/ml) (Lectin from Phaseolus vulgaris – Sigma catalog # L2769-2MG) Re-suspend 2 mg PHA-L in 2ml sterile 1x PBS. Prepare 8- 250ul aliquots of this, store at -20°C.
     • Homocysteine (Sigma catalog # H4628-10MG – stored at -20)
       Re-suspend 1 gram in 7.39 ml of sterile PBS. This will produce a 1 M stock. Dilute this stock 1:100 by taking 30ul of 1M stock and add 2.97 ml PBS to create 10mM stock. Prepare 8- 200ul aliquots and store them at -20°C.

10. Aliquot plasmids as indicated for transfections. (Day 2)

11. Aliquot Lipofectamine (Day 2 and Day 5) and Optimem (Day 2) as indicated on Daily prep sheets. Both of these are stored at 4°C.

12. Luciferase Reagent (Day 3).
   a. Thaw buffer vial (“Luciferase assay buffer”) to completion.
   b. Add 10ml buffer to vial of Lyophilized substrate (“Luciferase assay substrate”).
   c. Pipette to mix.
d. Make (8) 1mL aliquots in microfuge tubes.
e. Store at -20°C.

13. Passive Lysis Buffer (Day 3) – use "Reporter Lysis Buffer 5X" (stored at RT)
   Dilute 5X Reporter Lysis buffer (stored at RT) to 1x using dH₂O. Prepare one aliquot of 1X Passive Lysis buffer per lab group and store at -20°C.

14. Make 20-10mL aliquots RAW cell media for Day 3– store at 4°C
    Make 20-10mL aliquots RAW cell media for Day 2– store at 4°C.

15. ELISA Reagents: Prepare reagents for the TNF-α ELISA based on manufacturer guidelines. (eBioscience).

**Week 2 Reagent Preparation:**

**Instrumentation for Week 2:**
- 3 iBlotters
- Box of iBlotter (Invitrogen) transfer pads
- (4) PAGE electrophoresis chambers with gel cassette inserts and lids (Invitrogen)
- Power supplies (2)
- Microfuge (2)
- Heat block (2)
- Refrigerated Microfuge
- mini-centrifuge
- Film cassettes-(4)
- Film—place box in dark room
- Rocking platforms (2)
- qPCR instrument
- Table top refrigerated centrifuge

**Reagent and Instrumentation Preparation for Week 2: (the volumes listed are for 7 lab groups)**

**DAY 5 Western Blotting**

1. 3.5 L 1x TBST (TBS-0.05%Tween)
   a. Dilute 20x TBS to 1x—add 0.5ml Tween 20 per 1 L of 1X TBS.
   b. Make (7) 500ml aliquots—one per group and store at RT.

2. (7) developing containers—clean small plastic containers.
3. *(7) aliquots—25ml each of 5% milk in 1x TBST. This must be done the day it will be used.

4. Primary Antibody—NfKB p50 and B2 microglobulin
   a. Make (7) 20 ul aliquots in microfuge tubes. These should be stored at 4°C
   b. Should be placed out on day of experiment and stored on ice.

5. Secondary antibody -- goat anti-rabbit HRP
   a. Make (7) 2 ul aliquots in microfuge tubes. These should be stored at 4°C.
   b. Should be placed out on day of experiment and stored on ice.

6. ECL substrate
   a. Make (7) 0.5ml aliquots of detection solution 1—store at 4°C.
   b. Make (7) 0.5 ml aliquots of detection solution 2- store at 4°C.

   a. Make (7) 7ul aliquots of the positive control lysate.
   b. Store at -20C and then on ice for day of use.

8. 10mL 1M Tris pH 7.5 for Day 5: For each lab group prepare 200 uL aliquots in microfuge tubes labeled “Tris”. These can be stored at room temperature.

9. Protein Assay Buffer for Day 5 - 10mM Tris, 150mM NaCl
   Combine: 5mL 1M Tris stock (pH 7.5-8) + 15mL 5M NaCl stock + 480mL dH2O
   a. Prepare 500 mL of Protein Assay Buffer.
   b. Make a 50 ml aliquot for each lab group and store at RT.

10. Prepare one liter of 1x MOPS Running buffer for Day 5. You will need one liter per lab group.

11. Protein Loading Dye (Invitrogen) (Day 5) - Prepare one 50 ul aliquot for each lab group and store as indicated on the tube.

12. Novex Protein Molecular weight ladder (Day 5)– Prepare one 12 ul aliquot for each lab group and store as indicated.

13. Prepare 500 ml of 3.7% formaldehyde in a 500 ml glass bottle. Store at 4°C.

14. Prepare the Hoescht Stain (a nuclear stain from Invitrogen) (Day 7). **This is a mutagen, so be sure to wear gloves when handling. Prepare a 1000X stock concentration of 10 mg/ml (in dH2O). To treat cells, dilute the 1000X stock concentration. Prepare 20 ml of 1x stock by adding
20 ul of Hoescht (Dapi) Stain) to 20 ml of PBS. This should be done on Day 7 and used immediately or stored at -20°C.

15. Follow qPCR Preparation guidelines below to set up for the qPCR experiment.
qPCR Prep Notes — Here we provide the major details for setting up reagents and tubes for the qPCR experiment. For further details and notes on setting up the qPCR instrument and the computer please refer to Appendix B of the MASE Lab Manual.

qPCR Calculations and Cell Seeding- Prior to MASE Experiment

Determine the amount of cDNA that you need for all the groups and therefore the number of wells that need to be seeded with RAW264.7 cells, treated, and then lysed; and the number of cDNA reactions you need to perform. Once you know the number of wells to seed, seed 2.5 x10^4 cells per well in a 96-well flat-bottom tissue culture plate in 200μl RAW264.7 media and incubated 24 hours at 37°C, 5% CO₂.

After 24 hours, replace the media in each well and treat with inflammatory mediators as indicated in your experiment. Incubate as before for 24 hours.

qPCR with Cells to Cₜ Kit- Prior to MASE Experiment

After 24 hours of treatment, follow the Cells to Cₜ kit protocol (Applied Biosystems). As necessary, refer to the qPCR with Cells to Cₜ Kit protocol for detailed instructions on seeding cells, making lysates, and generating cDNA. It is best to transfer the cDNA from the reaction plate or tube(s) and pool it prior to aliquotting.

Making Labels- Prior to MASE Experiment

To make this lab experiment foolproof, we rely on color coding the reagents using computer generated color coded microfuge tube labels. The qPCR calculations spreadsheet has a function for determining the number of tubes and tube labels you will need. Word document templates are available for making the labels. We use blank label pages (Avery 5667- 80 clear return address labels per page). Use Nuclease-Free tubes throughout this experiment.

Aliquotting cDNA, Primer Probe Pairs, Nuclease-Free Water- Prior to MASE Experiment

cDNA: Each group needs a minimum of 48μl of untreated cDNA and 48μl of their assigned treated cDNA. To be safe and account for pipetting losses, for each group, aliquot 60μl untreated cDNA and 60μl treated cDNA.

PPPs (Primer Probe Pairs, aka TaqMan Gene Expression Assays): Each group needs a minimum of 9.9μl of β-actin PPP and 6.6μl of each of the remaining 3 PPPs. To be safe and account for pipetting losses, for each group, aliquot 15μl β-actin PPP and 10μl of each of the remaining 3 PPPs (iNOS, NFκB, TNFα).

Nuclease-Free Water: Each group needs a minimum of 12μl of Nuclease-Free Water. To be safe and account for pipetting losses, for each group, aliquot 20μl Nuclease-Free Water.
Preparing Ziploc Bags for each lab group:

Incomplete Master Mix tubes: After labeling all of the IMM tubes, separate them into baggies based on IMM #. Instructors can pull from these bags as they prep their IMMs on the day of the experiment.

For each group, generate the following number and length of tube strips and cap strips (using scissors), and use a 50ml conical tube to hold them:

- 1 strip of 3 qPCR tubes
- 4 strips of 6 qPCR tubes
- 1 strip of 3 qPCR caps
- 4 strips of 6 qPCR caps

For each group, label a zip-lock bag and place the following in it:

- 50ml conical tubes of tube and cap strips.
- A 96-well black support rack for the qPCR tubes (you will also use this to spin the tubes in the plate rotor in centrifuge for 1 minute at 1000 rpm at 4°C after the reactions are assembled and prior to placing them in the qPCR instrument).
- Veri-Flex trays for holding the tubes during the qPCR run in StepOnePlus (Applied Biosystems qPCR machine).

Incomplete Master Mix (IMM) Preparation Notes - Day of Experiment

On the day of the qPCR, approximately 30 minutes prior to starting qPCR set up, aliquot the Incomplete Master Mix (IMM) for each group. To do this, determine how much IMM you need to prepare based on how many groups you have. The amount of Nuclease-Free Water added is half as much as the 2x TaqMan Gene Expression Master Mix that you use. For 5 groups:

- 2X Master Mix: 5 * 300μl = 1500μl (half is 750μl)
- Nuclease-Free Water: 5 * 150μl = 750μl (half is 375μl)
- Total: 2250μl (our Nuclease-Free tube maximum volume is either 1.7 or 2ml, so you will need to separate this into two tubes (I suggest half of each reagent into each tube) and then mix them together back and forth a few times).

Using this type of protocol, prepare 5 sets of IMM tubes as described below:

- Into all of the IMM #1 tubes, aliquot 49.5μl (*5 = 247.5μl) of the original IMM
- Into all of the IMM #2-5 tubes, aliquot 99μl (*5 = 495) of the original IMM
- 247.5 + 495 + 495 + 495 + 495 = 2227.5μl
-You should have ~22.5μl left over if you pipet correctly.

-Place the IMMs on ice until you are ready to distribute them to the groups. Each group gets one of each IMM (#s 1-5). The groups can keep them at room temperature as they work on setting up their Complete Master Mixes (CMMs) by adding their primer/probe pairs, aliquotting CMM into the qPCR tubes, and adding cDNA to each tube.

**Primer Probe Pair (PPP) Preparation Notes - Day of Experiment**

-On the day of the qPCR, half an hour before the students are ready to start setting up the qPCR, the PPP tubes should be put on ice to thaw. They should be given a brief spin-down before giving each group a tube labeled “PPP β-actin, PPP iNOS, PPP NFκB, PPP TNFα (4 tubes). -Students will then follow the instructions in the MASE lab manual to set up their tubes. You will have to distribute appropriate cDNAs to each group.

MASE Biotechnology Camp 2012

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A Program sponsored by Mount St. Mary’s University
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Course Syllabus

Instructors:
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Course Format:
The Mount Academy for Scientific Exploration (MASE) Biotechnology Camp is a two week, hands-on, wet lab course designed to provide students with a background in molecular biology and genetics the opportunity to apply their knowledge in an inquiry based experimental research setting.

Course Description:
MASE is designed to simulate the process a molecular biologist would engage in to move a hypothesis from conception to testing. Using current molecular and genetic laboratory techniques, MASE participants will explore hypotheses related to gene expression and regulation of inflammation. The laboratory environment is designed to be inquiry based with the students conducting experiments aimed at addressing questions of biological relevance. Both weeks will focus on the use of various biological assays to address discreet but related questions regarding how gene expression is regulated in response to inflammation. At the end of the two weeks students will have collected multiple pieces of experimental data that they will evaluate, analyze, and interpret. The two week experience culminates with a formal presentation of students’ research findings in a public poster session that follows the format used at scientific meetings.

Course Objectives:
Successful completion of the MASE Biotechnology Camp will result in students having gained substantial experience in developing the following skill sets:
1. Molecular and genetic laboratory theory and practice
2. Independent research on specific scientific questions
3. Hypothesis development and experimental design
4. Scientific written and oral communication

Specific Laboratory Techniques:
MASE participants will gain experience with the following molecular and genetic laboratory techniques:

a. Mammalian cell tissue culture
b. ELISA analysis
c. Mammalian cell transfection
d. Luciferase assay analysis
e. Western blotting analysis
f. Protein Isolation
g. Immunofluorescence
h. Quantitative PCR analysis

Assessment
Quizzes 25%
Poster 50%
Post Lab Questions 25%
**Week 1: A study of gene expression through analysis of promoter activation and protein production.**

*Introduction:*
To address relevant scientific questions, the molecular biologist will often perform multiple distinct assays in order to provide the best support for their conclusions. By approaching the same question in multiple ways the scientist is better able to develop a more complete understanding of the mechanism that accounts for a given observation. In many cases, the investigator must begin by developing or choosing the reagents necessary to test these questions experimentally; this is followed by carrying out a variety of assays, the collective results of which will provide a more complete answer to the hypothesis being addressed. Thus, MASE is designed to simulate the process a molecular biologist would engage in to move a hypothesis from conception to testing.

To do this, our first week will begin with an examination of promoter activation levels in response to cellular exposure to different inflammatory mediators. Utilizing a Luciferase Reporter Gene Assay, we will investigate the effects of molecules that promote inflammation (pro-inflammatory stimuli) through examining transcriptional activation of NFκB, a transcription factor involved in activating the inflammatory response. We will also carry out an immunological assay, Enzyme Linked Immunosorbant Assay (ELISA), to look at cellular expression of pro-inflammatory cytokines. Finally, we will treat cells with pro-inflammatory mediators then collect cellular proteins that will be used in a Western blot analysis to examine changes in NFκB protein levels.

**Pre-Lab Preparation:**

**Day 1:**
1. What macromolecule does Western blot analysis examine?
2. Describe how a Western blot experiment works (what are the molecular principles that make this work)?
3. What is a cell lysate and why is it a critical component in a Western blot experiment?

**Day 2:**
1. Understand the principle of a Lipofectamine-based transfection. How does it work?
2. Provide a brief description of each of the inflammatory agents that will be used in your Experiment 1.3 Part A.
3. Generate a hypothesis regarding TNF-α production in response to the stimulus that was used by your group to treat the cells.
4. Investigate NFκB; what is it, and what is its function?
5. Explain how NFκB leads to gene expression.—ie: what is the role of NFκB, DNA, other proteins, etc. How does this relate to protein production?

Day 3:
1. What is a reporter gene (aka reporter assay)? What are other commonly used reporter assays (not including luciferase)?
2. Understand each of the inflammatory agents to be used in Experiment 1.4 Part B. Generate a hypothesis regarding the effect of your specific stimuli (only the inflammatory stimulus used by your group) on NFκB mediated transcription.
3. Explain why we are assaying tissue culture media, rather than cells, for the presence of TNFα.
4. What is the purpose of testing for TNFα expression?
5. What is the purpose of the ELISA? Explain the first step of the ELISA in terms of antigen and antibody?

Day 4:
1. Investigate the ELISA test. What are some common specific uses for the ELISA (besides assaying tissue culture media for cytokines like TNFα)?
2. Understand the principle of an ELISA. How does it work? What does each step contribute to the final outcome? Can any of the steps be omitted?
3. How could the ELISA approach outlined below be modified to detect the presence of antibodies in a sample rather than antigen? (in 1.6 and 1.7)

Week One Experimental Overview:

<table>
<thead>
<tr>
<th>Day</th>
<th>Experiment</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Day 1 (M)</td>
<td>1.1</td>
<td>Treat cells with pro-inflammatory stimuli then collect cytoplasmic and nuclear lysates later analysis during week 2.</td>
</tr>
<tr>
<td>Day 2 (T)</td>
<td>1.2</td>
<td>Transfection of mammalian cells with Lipofectamine in preparation for Luciferase Assay.</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>Treatment of cells with inflammatory stimuli in preparation for analysis of release of TNFα by ELISA.</td>
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<tr>
<td>Day 3 (W)</td>
<td>1.4</td>
<td>Treatment of cells with inflammatory stimuli for luciferase assay (a.m.); Harvest cell lysates and perform luciferase assay (p.m.)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>Harvest tissue culture supernatants to be analyzed for TNFα content by ELISA.</td>
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</table>
1.6 Coat ELISA plate with capture antibody.

Day 4 (Th) 1.7 Perform ELISA to detect TNFα released by cells treated with inflammatory stimuli.

**What are the Pro-Inflammatory Stimuli and Why are they Useful??**

For more details, see the Review titled *Cytokines and Control of TNFα Gene Expression*:

**Lipopolysaccharide (LPS):** LPS is derived from the cell wall of bacterial cells. Treatment of cells with LPS mimics introduction of bacteria and thus a bacterial infection. One of the first immune responses to bacterial infection is inflammation.

**Interferon Gamma (IFNγ):** IFNγ is an immune response protein produced in response to viral infection and elicits an inflammatory response. Treatment of cells with IFNγ mimics a viral infection.

**Phytohemagglutinin (PHA):** Phytohemagglutinin (PHA), the lectin extract from the red kidney bean (*Phaseolus vulgaris*), contains potent cell agglutinating and mitogenic activities.

**Homocysteine:** A typically non-inflammatory molecule that exerts increased risk of a cardiovascular event through stimulation of inflammatory mediators. In recent years, scientists have identified that inflammation has a critical role in the development of cardiovascular events that may contribute to the plaque formation that is characteristic of Coronary Artery Disease.

**Phorbol Ester (PMA):** Phorbol 12-myristate 13-acetate, commonly known as PMA or TPA, is a polyfunctional diterpene phorbol ester. It is widely used in research as a promoter of tumor growth and inflammation based on its ability to activate the intracellular signaling molecule protein kinase C (PKC)
Day 1: Making Nuclear Lysates for Western Blot Analysis (Exps. 1.1 and 1.2)

**Note: Exp 1.1 has been carried out in advance—begin procedure at Exp 1.2**

Experiment 1.1: Inducing Inflammation - Treatment of RAW Cells with Inflammatory Stimuli

1. Your group will be assigned conditions (*See the chart below) for treating your cells.
2. Obtain one (6) well plate of RAW cells from the incubator.
3. Label the plates with your group name and treatment condition; then label the wells as follows: 0, 6, and 12 hours.
4. This is a time course experiment therefore you will treat each time point independently.
5. **Identify your group’s inflammatory mediator; if you are using two chemicals to treat, be sure you treat the well with both chemicals.**
6. To your **12 hour well (9 am)**, add 20 ul of your assigned chemical.
7. Place the plates back in the incubator.
8. Incubate for **6 hours**.
9. Remove plates, add 20 ul of your assigned chemical to your **6 hour well (3 pm)**.
10. Place the plates back in the incubator.
11. Incubate for **6 hours**.
   a. While incubating begin assembling reagents for experiment 1.2.
   b. Proceed to experiment 1.2 as soon as your final 6 hour incubation has ended.

Assigned Conditions for Stimulation of Inflammation

<table>
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<th>3</th>
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<th>5</th>
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<th>7</th>
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<tbody>
<tr>
<td>Treatment</td>
<td>LPS</td>
<td>IFN-γ</td>
<td>IFN γ +LPS</td>
<td>PMA</td>
<td>PHA</td>
<td>PMA + PHA</td>
<td>Homocysteine</td>
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Isolation of Protein from Cells

We have stimulated an inflammatory response using several different types and combinations of inflammatory stimuli. We will use a Western blotting protocol to analyze the level and location of NfxB expression in RAW cells treated with various inflammatory stimuli over a 12 hour time course. This will allow you to determine whether all inflammatory stimuli utilize the
NfκB pathway to modulate cytokine expression and the inflammatory process. To do this, we will lyse the cells and isolate nuclear and cytoplasmic proteins separately using a protocol that allows us to generate nuclear and cytoplasmic extracts (lysates). These lysates will then be used in a Western blotting analysis that will allow us to specifically detect and visualize NfκB protein.

**Experiment 1.2: Extracting Protein from Cultured Cells**

*NOTE: Proteins are sensitive to temperature change. Samples should remain on ice from step 4 forward. All buffers and reagents should be kept on ice for the duration of the experiment.*

1. **Label (6) eppendorf tubes as follows:**
   a. Label all of them RAW and 6 of them with NE (for nuclear extract) and 6 of them CE (for cytoplasmic extract).
   b. Then label tubes 1-6 as follows:
      1. Tube 1: 0 hour (NE)
      2. Tube 2: 6 hour (NE)
      3. Tube 3: 12 hour (NE)
      4. Tube 4: 0 hour (CE)
      5. Tube 5: 6 hour (CE)
      6. Tube 6: 12 hour (CE)

2. **Prepare 10mls Cytoplasmic Lysis Buffer (buffer A—be sure to label tubes carefully!!) as follows:**
   a. Add 10ml Buffer A to a 15ml tube and
   b. Add one protease inhibitor pellet (Mixture of several protease inhibitors with broad inhibitory specificity. For the inhibition of serine, cysteine, and metalloproteases in bacterial and mammalian cell extracts) to tube A.
   c. Vortex to mix
   d. Place Tube on ice

3. **Student Counselors will prepare 10 mls Nuclear Lysis Buffer (buffer C—be sure to label tubes carefully!!) as follows:**
   a. Add 10 mL of Buffer C to a 15 ml conical.
   b. Add one protease inhibitor pellet to this tube.
   c. Vortex to mix and aliquot 1 ml into each of 10 eppendorf tubes labeled Buffer C.
   d. Give one of these tubes to each of the student groups and have them place these tubes on ice immediately.

4. **For each well, you will carry out the following procedure to lyse the plasma membrane first (using buffer A) followed by the nuclear membranes (Using buffer C) which will allow you to isolate only proteins found in the nucleus (nuclear extracts):**
   a. Remove media.
   b. Add 1 ml of PBS to each well of the plate, swirl to wash.
   c. Remove PBS and discard. (Be sure to remove as much PBS as possible.)
d. Add 1ml PBS to plate.

e. QUICKLY–using a cell scraper, scrape cells to remove from plate, incline plate, transfer cells in PBS to 1.5ml tube (remove as much liquid to tube as possible)

**When all cells are removed from the plate the plastic should look clear.**

f. Centrifuge 15 seconds @ 15,000 rpm (use the refrigerated centrifuge or one in cold box.)

g. Remove as much supernatant as possible without disturbing the pellet.

h. **STOP!! Before moving on... Have your instructor or student counselor check to make sure there is little to no residual PBS in your tube. Residual PBS will impact protein concentration; if it is not properly removed you will not have enough protein to work with.**

i. Add 100 ul Buffer A to the tube.

j. Pipette up and down until the pellet is broken up and the solution looks homogenous.

k. Place tube in ice and incubate for 15 minutes.

l. Following incubation, Centrifuge at 3500 rpm at 4°C for 10 minutes. **(NOTE: MAKE SURE SPEED IS AT 3500 RPM.)**

m. Following the spin you should be able to see a small, opaque pellet at the bottom of each tube; these are the cell nuclei.

n. Remove the supernatant and discard, being careful not to disrupt the nuclear pellet.

o. To the pellet, add 200ul Buffer A, flick to mix

p. Centrifuge for 10 sec at full speed

q. Remove and discard supernatant **(remove as much as possible).**

r. **STOP !!** Again, have one of the counselors or your instructor check to ensure you have removed enough of the supernatant.

s. Add 25 uL of buffer C to each tube and pipette up and down several times (this pellet – the nuclei– will not re-suspend as well as the whole cell pellet did in step j, but you should be able to break up the pellet.)

t. Place tubes on the shaker and vigorously shake for 25 minutes at 4°C.

u. Remove from the shaker and collect the nuclear extracts by centrifuging for 5 min at 15,000 rpm at 4°C.

v. Transfer the supernatant to your reserved pre-labeled tubes labeled NE for nuclear extract. **Be sure you have kept track of the time points and transfer to the appropriate tube** (ie: the 6 hour sample should be transferred to the 6 hour NE tube)

w. Store the nuclear extracts at -80°C until ready to use.
Day 2: Transfection of Mammalian Cells

Ex. 1.3- Introduction of DNA into Cells by Transfection
Ex. 1.4- Induction of Inflammation in Untransfected Cells

Introducing Foreign DNA by Transfection (Exp 1.3)

To begin our studies, we must first introduce our recombinant DNA molecule into the cells. To do this, we will use a reagent called Lipofectamine 2000. Lipofectamine is a cationic lipid that will bind to and surround the DNA. Once the Lipofectamine:DNA complex is formed, the Lipofectamine portion of the complex will interact with the cell membrane and deliver the DNA into the cell. After transfection, we will allow the cells 24 hours to recover and begin expressing the foreign DNA. Our gene of interest is NFκB. NFκB has been shown to be turned on in response to inflammatory stimuli. In this set of experiments, we will introduce an NFκB reporter gene into cells, treat cells with a variety of inflammatory stimuli, and assess how this impacts NFκB mediated transcription. This assessment will rely on the Luciferase Reporter Gene Assay, which is described in Day 3 Ex. 1.5 reading.

A Note about Working with Mammalian Cells

Tissue culture is a generic term used to describe both organ culture and cell culture; the terms are used interchangeably. Cell cultures are derived from either primary tissue explants or cell suspensions. Primary cell cultures typically have a limited life span in culture, while continuous cell lines are abnormal cells and an indefinite life span. In our case, RAW 264.7 cells represent a continuously cultured cell line that was originally derived from a mouse macrophage.

Most cell culture is performed in a laminar air flow hood. These hoods are designed so that the air flows directly at the operator. This air flow protects the cultures from contamination by bacteria, viruses and fungi that may be floating around in the air. Most of these hoods are equipped with a short-wave UV light than can be turned on for a few minutes to sterilize the surfaces of the hood.

- Prior to use, the hood should be turned on for about 10-20 minutes.
- All surfaces of the hood should be wiped down with 70% ethanol before and after each use.

Growth and Maintenance of a Cell Culture

While for our studies we will not be maintaining cells in culture for an extended period of time, we will need to work with and manipulate them over the course of two days. The following guidelines are meant to aid you in the handling of your cell cultures.
- In maintaining or culturing any cell, the goal is to keep them as happy as possible by culturing them in the appropriate growth conditions.
- **Growth** – Cells will initially go through a lag phase that depends on the cell type, the seeding density, media and previous handling. Then they will go into a phase of exponential growth when the cells have become metabolically active. Finally, the cells will enter a stationary growth phase where the cell number remains constant and there is no growth. This is characteristic of a confluent population (the entire surface of the culture dish is covered by cells) and the cells will need to passaged or split at this point.
- **Handling/Passaging Cells** -- Ideally cells are split or passaged when the cell reach a semi-confluent stage. If the cells get too confluent, they may run out of nutrients and you may see a decline in the cell population. Depending on the type of culture, the process of feeding and passaging is variable. Adherent cultures (cells that attach to the bottom surface of the culture dish) are fed by removing the old medium and replacing it with fresh medium. When these cells reach a semi-confluent state they are then removed from the growing surface and diluted into new medium to start a new plate of the cells that is less confluent. The cells can be removed from the growing surface mechanically through the use of cell scraper or chemically by using proteolytic enzymes such as Trypsin in combination with EDTA. Your preferred method depends on which method is less harmful to your cells. Regardless of method, passaging and handling of the cells requires good sterile technique to avoid contamination. Thus, cell plates that will be cultured for any period of time must be handled in a laminar flow hood using only sterile reagents and equipment.

**Induction of Inflammation in Untransfected Cells (Exp 1.4)**

**What will we be testing?**

In this set of experiments (1.4, 1.6, 1.7, 1.8), you will ultimately collect supernatants from untransfected cells that have either been treated or not treated with some type of pro-inflammatory stimulation. To begin today, each group will use a different type of inflammatory stimulus, so that as a class, we are able to investigate many different experimental parameters (see chart below for specific stimuli that will be used, and the groups that will be assigned to these stimuli. Refer to pg 6 for a basic description of each stimulant). The supernatants will be assessed for the presence of the pro-inflammatory cytokine Tumor Necrosis Factor alpha (TNFα) using an ELISA.

**Assigned Conditions for Stimulation of Inflammation**

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<tr>
<td>Treatment</td>
<td>LPS</td>
<td>IFN-γ</td>
<td>IFN-γ +LPS</td>
<td>PMA</td>
<td>PHA</td>
<td>PMA + PHA</td>
<td>Homocysteine</td>
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TNFα is expressed in response to viral or bacterial infection and other inflammatory stimuli such as mutagens or radiation. Macrophages are a primary source of TNFα (1). TNFα evokes numerous responses in immune cells and surrounding tissues, such as expression of ICAM-1 (intercellular adhesion molecule-1) on the surface of epithelial cells to promote adhesion and infiltration of neutrophils and monocytes to the site of inflammation (1,2). TNFα also induces the secretion of proteinases that degrade intercellular matrix proteins and allow macrophages to penetrate tissue to the site of injury or infection (3) and stimulates macrophages to produce other pro-inflammatory cytokines that modulate immune functions. More information on TNFα can be found in the *Tumor Necrosis Factor Alpha* Review by Kevin Tracy.

Although TNFα plays a beneficial role by rapidly activating immune cells and eliciting other cellular responses that protect the host from infection and injury, prolonged or elevated expression of TNFα is associated with a number of pathological conditions, including chronic inflammation (4), septic shock (5), cachexia (3) and autoimmune diseases such as rheumatoid arthritis (6,7). TNFα gene expression must be tightly regulated in an effort to minimize the potentially deleterious effects of TNFα protein. As such, it logically follows that immune cells have developed molecular mechanisms to regulate the inflammatory response initiated by stimuli such as Lipopolysaccharide (LPS), a component of the cell wall of gram negative bacteria (8). This regulation is discussed in greater depth in the Week 2 readings.

**Citations:**

Day 2 Protocols

Experiment 1.3: Lipofectamine Transfection of RAW cells

A. Preparation of Lipofectamine 2000:DNA complexes

*NOTE: All steps should be carried out in the hood using proper sterile technique; before starting, clean and prepare the tissue culture hood as described in the introduction*

1. To a 1.5ml tube-add 100ul Optimem (serum free medium). Label this tube “L” for Lipofectamine. To another 1.5mL tube, add 100uL of Optimem. Label this tube “D” for DNA.
2. To the L tube, add 20uL of lipofectamine 2000 reagent to the tube containing the Optimem media.
   a. *NOTE: Pipette the Lipofectamine 2000 reagent directly into the Optimem (do not let it touch the wall of the tube).*
3. Gently tap the tube to mix.
4. Incubate the Lipofectamine/Optimem mixture for 5 minutes at room temperature.
5. While incubating-pipet your DNA into the D tube.
   a. Add 8 ug of DNA; the volume used will depend on the concentration of your DNA prep (in ug/ul).
   b. Calculate volume needed as follows:
      \[
      \frac{[1\text{ul}]}{[\text{DNA conc in ug}]} \times (8\text{ug}) = \text{volume needed}
      \]
   c. Add the calculated volume to the appropriate tube.
6. Following the 5 minute incubation, add the contents of the D tube to the contents of the L tube. Mix well by pipetting. Incubate for 20 minutes at room temperature.

B. Transfection of Cells

1. Obtain a 6 well plate (with only 2 wells are seeded with cells) from the incubator – you will divide the contents of your L tube between these two wells.
2. Following the 20 minute incubation (in Part A, step 6), you will add half of the contents (consult your instructor to determine the precise _____ ul) of the tube to each of the two wells of RAW cells.
3. Make sure to label each transfected well clearly.
4. Incubate for 4-6 hours at 37 ° C. Replace the media. Incubate overnight at 37 ° C.
Experiment 1.4: Inducing Inflammation in Untransfected Cells for ELISA

Assigned Conditions for Stimulation of Inflammation

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<tr>
<td>Treatment</td>
<td>LPS</td>
<td>IFN-γ</td>
<td>IFN-γ +LPS</td>
<td>PMA</td>
<td>PHA</td>
<td>PMA + PHA</td>
<td>Homocysteine</td>
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1. Obtain one 96-well plate of untransfected RAW cells from the incubator; only 6 wells are seeded with cells (they are labeled on the plate lid and contain pink liquid; the wells surrounding these wells contain PBS ONLY to decrease evaporation of media within the seeded wells) (see diagram below).

2. Label the plate with your group name and treatment condition (from the table above).

   You will treat 3 wells with stimuli and leave 3 wells untreated. Label each well as either “+” or “−” to indicate whether or not the treatment was applied. Three wells must be labeled with “+” and three with “−”.

3. Treat each plate as follows with your assigned in inflammatory mediator:
   a. Use sterile technique in the tissue culture hood for each of the following steps
   b. Use a micropipette to remove the media from the wells; you may remove the media from all wells using the same tip.
   c. Add 200ul of RAW Cell Media to each well; you may add the media to all cells using the same tip.
d. **Check your assigned condition.** To each of your “+” wells, you will add 2ul of your inflammatory mediator (LPS, IFNγ, TPA, etc.); each reagent is made as a 100x stock solution.

   i. **NOTE:** if you are using two chemicals to treat, be sure you treat the well with both chemicals:

   e. Add nothing to the ‘−’ wells

4. Place the plates back in the incubator; incubate at 37°C for approximately 24 hours.
Day 3:

Exp. 1.5- Analysis of NFκB Transcription by Luciferase Reporter Gene Assay

Exp. 1.6- Harvesting Supernatants from Stimulated Cells

Exp. 1.7- Coating the ELISA Plate

Luciferase Reporter Assay (Exp 1.5)

We will use a luciferase reporter assay to assess the level of transcription of the NFκB gene. NFκB has been shown to be turned on in response to inflammatory stimuli. In this set of experiments, we will treat cells with a variety of inflammatory stimuli and assess how this impacts NFκB transcription. The agents used for treatment are the same as those used in Ex. 1.4. Please refer to page 6 to remind yourself of the various stimuli for your group’s assigned conditions.

Reporter assays are commonly used to study gene expression. In a typical reporter assay, the promoter or enhancer element of a gene is cloned upstream of a reporter. The reporter that we will be using is an enzyme called luciferase. The luciferase gene encodes an enzyme that is able to hydrolyze a substrate, producing a reaction that emits light. Emission of this light can be detected by an instrument called a luminometer. Our reporter plasmid contains the enhancer element for the gene NFκB upstream of the luciferase gene sequence. Thus, if NFκB is activated under our experimental conditions, that should drive the expression of the luciferase reporter. This luciferase expression is easily detected by performing a luciferase assay and measuring light emission with a luminometer.

In order to perform a luciferase assay, the reporter plasmid must be transfected into mammalian cells. In this series of protocols, we will be using RAW cells, which are mouse macrophages.

What will we be testing?

In Experiment 1.5, you will collect lysates from cells that have been transfected with the NFκB luciferase reporter, and either treated, or not treated with some type of pro-inflammatory stimulation. Each group will use a different type of inflammatory stimulus, so that as a class, we are able to investigate many different experimental parameters (see chart below for specific stimuli that will be used, and the groups that will be assigned to these stimuli). As a positive control for your experiment, you will be given lysates generated from RAW cells transfected with a plasmid called pFC-MEK. MEKK is a gene that is known to strongly activate NFκB. Thus, expression of MEKK, should yield a strong luciferase signal.
**Harvesting Tissue Culture Supernatants (Exp 1.6)**

When cells are growing in tissue culture, they 1) are affected by their environment and 2) affect their environment. The media and stimuli present in the dish serve to nourish the cells and induce an active response, respectively. Because of the latter, we can harvest the media that cultured, stimulated cells are growing in and assess it for the presence of specific proteins or hormones of interest that have been released from the cell as a response to the stimulus. Today, you will harvest the tissue culture media from the untransfected cells you stimulated yesterday (Ex. 1.4). This media is called the supernatant. Because we want to know how much protein of interest is present in the supernatant a certain time after stimulation, we must take additional steps to separate the supernatant from any cells that may have inadvertently been harvested from the tissue culture dish. Otherwise these carry-over cells may continue to release the protein of interest. This separation is achieved by centrifugation. The supernatants you acquire will be used in Ex. 1.8.

**Coating the ELISA Plate with Capture Antibody (Exp 1.7)**

The Enzyme-Linked ImmunoSorbant Assay (ELISA) is a widely-used method for measuring the concentration of a particular molecule (e.g., a hormone or protein) in a fluid such as serum or cell culture medium (supernatant).

The molecule of interest is detected by proteins called antibodies that have the ability to bind to the molecule of interest specifically; that is, the antibodies used in the assay bind to ONLY the molecule you are interested in detecting and NOT to any other molecule. The molecule that an antibody binds to is called an antigen. If you want to detect a different molecule (i.e., a different antigen), then you have to use a different antibody.

The ELISA test is more fully explained in the Day 4 Ex. 1.8 Reading. For today, you simply need to understand the first step of the ELISA. This first step is the attachment of antibodies to a solid surface, such as the inner bottom surface of a well of a 96-well plate. In Ex. 1.7, you will add your supernatants from Ex. 1.5 to the coated plate. **WHAT WILL THE EFFECT BE?**
Day 3 Protocols

Experiment 1.5 (a.m): Treatment of Transiently Transfected Cells with Inflammatory Stimuli

1. Obtain your plate of transfected cells (exp 1.2 done yesterday) from the 37°C incubator. Label the plate with your treatment condition(s) (from the table above).
2. Treat each plate as follows with your assigned inflammatory mediator:
   a. Use sterile technique in the tissue culture hood for each of the following steps.
   b. Aspirate the media from each well.
   c. Add 2 ml of RAW Cell Media to each well.
   d. To each of your wells, you will add 20 uL of the appropriate inflammatory mediator (LPS, IFNγ, PMA etc.); each reagent is made as a 100x stock solution; if you are using two chemicals to treat, be sure you treat the well with both chemicals.
   e. Place the plates back in the incubator.
3. Incubate at 37°C until it is time to harvest lysates for the luciferase assay (Ex. 1.4 p.m.)

Experiment 1.5 (p.m.): Luciferase Assay of Lysates from Transient Transfection

Part A: Reagent preparation

1. Prepare Luciferase Reagent: Determine amount of luciferase reagent needed. Generally, you use 100 ul per reaction. For example, if you have 2 wells in your transfection experiment, you have 2 reactions. Thus you will need to thaw 2*(100ul) or 0.2 ml of luciferase reagent.

2. Prepare working dilution (1x) of Passive Lysis buffer.
   a. Calculate how much you need based on how many samples you have. Generally you can expect to use 400uL of cell lysis buffer per well.
   b. Stock is 5x. Add 1 volume of 5x Passive Lysis buffer to 4 volumes of distilled water.
   c. Mix well.

3. Collect all supplies needed and organize them at luminometer; this includes: microfuge tubes, p200 pipette, tubes containing lysate, pipette tips, pencil, paper, waste beaker or trash cans

Part B: Preparation of cell lysates. (This can be done on the bench.)

1. Set up 1.5mL eppendorf tubes; you will need one for each sample well.
2. Label tubes with sample number and treatment.
3. Remove media from wells. Wash cells 1 time with 2 mL of 1x PBS. Do not let cells dry out.
4. For each well of a 6 well plate, you will add 400 uL of 1x passive lysis buffer. Use a cell scraper to scrape cells from the bottom of the dish. DO NOT CROSS CONTAMINATE.
5. Collect lysate into a microfuge tube using a p1000 pipet tip. Place lysates on ice.

**Part C: Luciferase Assay and measurement using a luminometer**

1. Turn on luminometer. Set up 4 fresh 1.5mL tubes—you will need one for each of your transfected samples, plus one for a negative control and one for a positive control.
2. Set up a table in your notebook to record the data. Transfer 20uL of each lysate to a clean, appropriately labeled, microcentrifuge tube.
3. To your negative control tube, add 20uL of passive lysis buffer. Please note: you will process each of your samples 1 at a time.
4. Add 100uL of Luciferase Assay II reagent (LARII) to the control tube. Pipette up and down to mix.
5. Quickly take a luminometer reading. Too much of a delay will cause a decay in your luminescent signal.
6. Record the reading.
7. Repeat steps 5-7 for each sample. Following the negative control, you should have 3 more samples to process.
   A. The lysate from your transfected cells that were treated with your inflammatory mediator
   B. The lysate from your transfected cells NOT treated with any inflammatory mediator.
   C. A positive control lysate (MEKK).

**Experiment 1.6: Harvesting Supernatants for Assessment of TNFα production by ELISA**

Obtain your group’s 96 well plate, stimulated yesterday in Exp. 1.3. These have now incubated for 24 hours:
   a. Remove plate from incubator and perform the following steps on the bench.
   b. Label 6 sterile Eppendorf tubes with your group name and “+”; label 6 Eppendorf tubes with your group name and “−”(you will use 3 of each now and the remaining tubes after you spin).
c. Use a micropipettor to remove all liquid from each seeded well and transfer it to an appropriately labeled (+ or −) Eppendorf tube; you must use a new tip for each well.

d. Spin tubes for 5 minutes at 1000 rpm in a benchtop microfuge to separate media from any cells that may also have been transferred from the wells.

e. Use a micropipettor to remove 150 ul of media from each tube; be sure NOT to touch the tip to the pellet of cells at the bottom of the tube; transfer this 150 ul to an appropriately labeled (+ or −) fresh sterile Eppendorf tube; you must use a new tip for each sample/tube.

f. Place labeled tubes in a rack at 4°C to be assayed for the presence of TNFα on Day 4.

g. Discard plate in biohazard waste bag.

**Experiment 1.7: Assaying TNFα production by ELISA (Coating the Plate with Capture Antibody)**

1. Obtain a 96-well ELISA plate; label it with your group name
2. Obtain a 1.5ml tube of Capture Antibody diluted in Coating Buffer; the tube contains 1.3ml of reagent
3. Using a micropipette, add 100ul of diluted Capture Antibody (Cap Ab) to each well in Column 1 and Column 2 (see diagram below); you may use the same tip for each well. This antibody is specific for TNFα.

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</table>
4. Cover the plate with Saran Wrap.
5. Place plate at 4°C overnight; this plate will be used on Day 4 to assay your culture supernatants for the presence of TNFα.
**Day 4: Analysis of Secreted TNF-alpha Using ELISA (Exp. 1.8)**

**Enzyme-Linked ImmunoSorbant Assay (ELISA)**

We will use an ELISA to assess the level of TNF$\alpha$ released by cells stimulated with various inflammatory mediators. A description of these mediators was provided in the previous section. In this set of experiments, we will treat cells with a variety of inflammatory stimuli, harvest tissue culture supernatants, and assay those supernatants for the presence of TNF$\alpha$ by ELISA.

**What will we be testing?**

In this set of experiments (1.3, 1.5, 1.6, 1.7), you have collected supernatants (tissue culture media) from cells that have been either treated or not treated with some type of pro-inflammatory stimulation. Each group used a different type of inflammatory stimulus, so that as a class, we are able to investigate many different experimental parameters (Refer to Chart in Ex. 1.3 for specific stimuli that were used, and the groups assigned to those stimuli). Now, you will assess whether specific stimuli induced the production and release of a cytokine called Tumor Necrosis Factor alpha (TNF$\alpha$) into the tissue culture supernatant.

The Enzyme-Linked ImmunoSorbant Assay (ELISA) is a widely-used method for measuring the concentration of a particular molecule (e.g., a hormone or protein) in a fluid such as serum or cell culture medium (supernatant).

The molecule is detected by proteins called antibodies that have the ability to bind to the molecule of interest specifically; that is, the antibodies used in the assay bind to ONLY the molecule (antigen) you are interested in detecting and NOT to any other molecule. If you want to detect a different molecule (antigen), then you have to use a different antibody.

The ELISA test requires:

- the antibodies be fixed to a solid surface, such as the inner bottom surface of a well of a 96-well plate;
- a sample that you want to assay for the presence of a particular antigen;
- a preparation of the same antibodies and a way to couple them to an enzyme. The enzyme must be one that produces a colored product from a colorless substrate (e.g., HorseRadish Peroxidase (HRP)). Sometimes this is achieved by coupling the antibodies to a molecule called Biotin and coupling the enzyme (HRP) to a molecule called Avidin. Biotin binds Avidin with strong affinity;
- a substrate for the enzyme.
- by adding these antibodies, your samples, and the enzyme in a specific order, a sandwich of sorts is built. See Figure 1 on the next page.
**Basic Outline of the Steps of the ELISA Test:**

1. The wells are filled with the capture antibody. The antibodies will adhere to the plastic and become immobilized. Wash.
2. The wells are filled with the antigen solution to be assayed (tissue culture media). Any antigen molecules present bind to the immobilized antibody molecules. Wash.
3. The antibody-biotin conjugate is added to the reaction mixture. The antibody part of the conjugate binds to any antigen molecules that were bound previously, creating an antibody-antigen-antibody "sandwich". Wash.

4. The avidin-enzyme conjugate is added to the reaction mixture. The avidin part of the conjugate binds to any biotin molecules that were bound previously. Wash.

5. After washing away any unbound conjugate, the substrate solution is added.

6. After a set interval, the reaction is stopped (e.g., by adding 1N H₂SO₄) and the concentration of colored product formed is measured in a spectrophotometer. The intensity of color (optical density or O.D.) is directly proportional to the concentration of bound antigen.
Day 4 Protocol

Experiment 1.8: Assaying Tissue Culture Supernatants for TNF-alpha by ELISA

Steps 1-10 have been done for you...begin at step 11

1. Retrieve your coated plate (Exp. 1.6 done yesterday) from 4°C
2. Remove the Saran Wrap
3. Over a tub, quickly turn the plate over, and with a flicking motion, remove the liquid from the wells; blot the plate on bench paper or a paper towel to remove additional liquid
4. At your bench station, use a micropipettor to add 250ul Wash Buffer to each well in Column 1 and Column 2; you may use the same tip for each well
5. Over a tub, quickly turn the plate over, and with a flicking motion, remove the liquid from the wells; blot the plate on bench paper or a paper towel to remove additional liquid
6. Repeat Steps 4 and 5 FOUR MORE times (5 times total)
7. Obtain a tube of Assay Diluent
8. Using a micropipettor, add 200ul of Assay Diluent to each well; you may use the same tip for all wells
9. Cover the plate with Saran Wrap
10. Incubate plate at room temperature for 1 hour
11. After the incubation, over a tub, quickly turn the plate over, and with a flicking motion, remove the liquid from the wells; blot the plate on bench paper or a paper towel to remove additional liquid
12. Repeat Steps 4 and 5 FIVE times
13. Obtain a set of Eppendorf tubes containing a range of TNFα concentrations; these will be used to generate your Standard Curve
   a. 1000pg/ml = 1ng/ml
   b. 500pg/ml
   c. 250pg/ml
   d. 125pg/ml
   e. 62.5pg/ml
   f. 31.25pg/ml
   g. 15.63pg/ml
   h. 7.8pg/ml
14. Using a micropipettor, add 100ul of each standard to the appropriate wells as shown in the diagram below
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15. Using a micropipettor, add 100ul of each of your samples to the appropriate wells as shown in the diagram above. You will just use 2 of your (+) samples and 2 of your (-) samples.

16. Add 100ul of Assay Diluent to Well 2E (labeled “BLANK”).

17. Cover the plate in Saran Wrap.

18. Incubate 2 hours at Room Temperature.

19. After the incubation, remove the Saran Wrap; over a tub, quickly turn the plate over, and with a flicking motion, remove the liquid from the wells; blot the plate on bench paper or a paper towel to remove additional liquid.

20. Repeat Steps 4 and 5 FIVE times.


22. Using a micropipette, add 100ul of Detection Antibody to each well in Column 1 and Column 2; you may use the same tip for each well.

23. Cover the plate in Saran Wrap.

24. Incubate 1 hour at Room Temperature.

25. After the incubation, remove the saran wrap; over a tub, quickly turn the plate over, and with a flicking motion, remove the liquid from the wells; blot the plate on bench paper or a paper towel to remove additional liquid.

26. Repeat Steps 4 and 5 FIVE times.

27. Obtain a tube of Avidin-HRP.
28. Using a micropipette, add 100ul of Avidin-HRP to each well in Column 1 and Column 2; you may use the same tip for each well.
29. Cover plate with saran wrap and incubate 30 minutes at Room Temperature.
30. After the incubation, remove the saran wrap; over a sink, quickly turn the plate over, and with a flicking motion, remove the liquid from the wells; blot the plate on bench paper or a paper towel to remove additional liquid.
31. Repeat Steps 4 and 5 SEVEN times.
32. Obtain a tube of Substrate Solution.
33. Using a micropipette, add 100ul of Substrate Solution to each well in Column 1 and Column 2; you may use the same tip for each well.
34. Incubate the plate at Room Temperature for 15 minutes.
35. After the incubation, obtain a tube of Stop Solution.
36. Using a micropipette, add 50ul of Stop Solution to each well in Column 1 and Column 2; you may use the same tip for each well.
37. Read plate at 450nm in ELISA plate reader.
38. Input data in Spreadsheet emailed to you. (Also a copy of this information can be found on in Appendix C.)
39. Format and print graph (be sure to save this work for your poster).
**Week 2: Analyzing NfκB Localization and Expression in Response to Inflammatory Stimuli**

*Introduction*

The ultimate goal of a scientist in engineering recombinant DNA is to produce a reagent that will be used to address questions regarding how a gene of interest functions. Last week we used a luciferase reporter plasmid containing NFκB to examine activation of the NFκB gene in response to inflammatory stimuli. This week we will continue to focus on understanding how our gene of interest, the regulatory molecule NFκB, mediates the inflammatory response by examining what happens to the NFκB protein as a result of inflammatory stimulation.

Last week we also investigated, by ELISA, the production of Tumor Necrosis Factor alpha (TNFα) by cells in response to inflammatory stimuli. Macrophages are a primary source of the TNFα pro-inflammatory cytokine, which is expressed in response to viral or bacterial infection and other inflammatory stimuli such as mutagens or radiation. TNFα evokes numerous responses in immune cells and surrounding tissues, such as expression of ICAM-1 (intercellular adhesion molecule-1) on the surface of epithelial cells to promote adhesion and infiltration of neutrophils and monocytes to the site of inflammation (1,2). TNFα also induces the secretion of proteinases that degrade intercellular matrix proteins and allow macrophages to penetrate tissue to the site of injury or infection (3) and stimulates macrophages to produce other pro-inflammatory cytokines that modulate immune functions. More information on TNFα can be found in the *Tumor Necrosis Factor Alpha* Review by Kevin Tracy.

Although TNFα plays a beneficial role by rapidly activating immune cells and eliciting other cellular responses that protect the host from infection and injury, prolonged or elevated expression of TNFα is associated with a number of pathological conditions, including chronic inflammation (4), septic shock (5), cachexia (3) and autoimmune diseases such as rheumatoid arthritis (6,7). The potentially deleterious effects of TNFα necessitates that there must be a mechanism of maintaining stringent control over its expression. As such, it logically follows that immune cells have developed molecular mechanisms to regulate the inflammatory response initiated by stimuli such as Lipopolysaccharide (LPS), a component of the cell wall of gram negative bacteria (8). For example, if we examine LPS mediated TNFα expression, a key mediator of many inflammatory responses, it is clear that after approximately 2 hours a gradual accumulation of NF-κB p50 homodimers occurs. NF-κB p50 and p65 are members of the Rel family of transcription factors. NF-κB p50 lacks transcriptional activation domains and is capable of inhibiting transcription of target genes, while NF-κB p50:p65 heterodimers serve to activate transcription of target genes (9). p50 dimers accumulate with delayed kinetics in macrophages following LPS stimulation and preferentially bind to three κB elements in the murine TNF–α promoter (κB1, κB2a, and κB3). Binding of p50 dimers appears to play a key role in attenuating TNFα gene transcription (8,10,11). See the Review titled *Molecular Mechanism in Tolerance to LPS* (by Ziegler-Heitbrock) for more details on the process of attenuation and the role of NFκB in mediating the inflammatory response.
**How does Expression of an Exogenous Gene Occur?**

Expression proceeds essentially in the same manner that occurs naturally in a eukaryotic cell. Recombinant DNA technology has provided us with methods for studying the structure and function of numerous gene products (i.e.: proteins). A eukaryotic gene such as NF-κB can be expressed in both animal and bacterial cells by cloning the gene of interest into the appropriate expression vector. In addition to the ampicillin resistance gene, origin of replication, and a multiple cloning site present in most vectors, an expression vector such as pcDNA will also contain a viral or eukaryotic promoter and an RNA polymerase binding sequence located immediately before the cloning site for your gene of interest. Upon introduction of the recombinant DNA molecule to the cell, these sequences allow RNA polymerase to bind to the plasmid and initiate transcription and translation of your gene in an effort to produce a functional protein.

**How does the recombinant DNA molecule get into the cell?**

In bacteria, we use the process of transformation to introduce foreign (recombinant) DNA into the cell. This process requires that bacterial cells be treated with chemicals to make the cell membrane porous. Once treated, the DNA is added and the cells absorb it from the environment. When working with mammalian cells, such as the RAW 264.7 cells we will be using, rather than making the cell membranes porous, we will complex our recombinant DNA with wither a positively charged moiety or an amphipathic lipid based molecule that will interact by fusing with the cell membrane and, thus, deliver the DNA onto the cell. Once inside the cell, the recombinant DNA will undergo the normal cellular processes of transcription and translation to produce the protein product, NF-κB.

**What will we be testing?**

As mentioned above, the involvement of NF-κB in modulating pro-inflammatory pathways is well established. So we will examine the specific signals and molecules involved in triggering translocation and release NF-κB from macrophages using the RAW 264.7 macrophage cell line. To address this question, we will treat RAW 264.7 cells, a murine macrophage cell line, with various compounds that are known to initiate an inflammatory response. Then, we will use Western Blotting and fluorescence microscopy to determine the effect of treatment on the location and expression level of NF-κB within the RAW cells.

Pro-inflammatory compounds being examined (see the Review titled *Cytokines and Control of TNFα Gene Expression* for more details):
Lipopolysaccharide (LPS): LPS is derived from the cell wall of bacterial cells. Treatment of cells with LPS mimics introduction of bacteria and thus a bacterial infection. One of the first immune responses to bacterial infection is inflammation.

Interferon Gamma (IFNγ): IFNγ is an immune response protein produced in response to viral infection and elicits an inflammatory response. Treatment of cells with IFNγ mimics a viral infection.

Phytohemagglutinin (PHA): Phytohemagglutinin (PHA), the lectin extract from the red kidney bean (*Phaseolus vulgaris*), contains potent cell agglutinating and mitogenic activities.

Homocysteine: A typically non-inflammatory molecule that exerts increased risk of a cardiovascular event through stimulation of inflammatory mediators. In recent years, scientists have identified that inflammation has a critical role in the development of cardiovascular events that may contribute to the plaque formation that is characteristic of Coronary Artery Disease.
**Pre-lab preparation:**

*Again, carry out research using the internet or the appendix materials provided to answer the following questions:*

**Day 5:**

1. What is transfection and why is it useful in this experiment?
2. Research inflammation and NfκB. Work on developing a basic understanding of inflammatory processes. What role does NfκB play in regulating inflammation under inflammatory conditions?

**Day 6:**

1. Based on your understanding of inflammation and the function of NfκB, develop a hypothesis and predicted outcome for your experiment.

**Day 7:**

1. If treatment with pro-inflammatory compounds results in NfκB translocation, what does this suggest regarding the effect of the specific compound on the ability of NfκB to function as a pro-inflammatory molecule?
2. If treatment with pro-inflammatory compounds does not result in NfκB translocation, what does this suggest regarding the effect of the specific compound on the ability of NfκB to function as a pro-inflammatory molecule?
Week Two: Experimental Overview

Day 5 (M)  2.1 Western blotting Part I: SDS-PAGE gel electrophoresis of lysates made on Day 1
           2.2 Western blotting Part II: Transfer of Protein and Probing for NfκB expression
           2.3 Transfect RAW cells with NfκB-GFP plasmid for analysis of Nfκb localization by fluorescence microscopy.

Day 6 (T)  2.4 Quantitative PCR (qPCR) analysis of transcriptional changes (mRNA expression) in response to inflammatory stimuli

Day 7 (W)  2.5 Inducing Inflammation with LPS to observe translocation
           2.6 Fluorescent Microscopy: Visualizing NfκB-GFP expression and translocation in a living cells

Day 8 (TH) Data Analysis and Poster Preparation (Powerpoint files must be delivered to the Media center by 2:00 pm.)

Day 9 (F)  Poster Presentation (9:00-11:00 am)

Citations:
DAY 5:
Western Blot Analysis of NFκB Expression and Introduction of Foreign DNA by Transfection

When performing a Western blot analysis, it is critical to determine how much protein is present in each of your sample extracts. This is necessary because we will be making a comparison between the cytoplasmic and nuclear expression of NFκB over a 12 hour time course. In order to compare these samples directly, we will need a method to determine how much protein was present in each of our lysates. To do this we will examine the levels of separate protein, β₂-microglobulin.

β₂-microglobulin is considered a ‘house-keeping’ gene. In short, this means that the amount of β₂-microglobulin that is expressed will not be affected by the various treatments we have subjected the cells to. Because expression of this gene is independent of the various stimuli, we can use it as a means to quantify the amount of protein that was run in each sample. This is known as ‘normalizing’. We will use a software package called ‘Image J’ to do this analysis. Using a serious of calculations, Image J will determine how much protein was loaded into each well. We can then use this information to calculate and quantify differences in protein expression between different samples. Your instructors will provide more information on this when you move into the analysis phase.

Western Blotting (Exps. 2.1 and 2.2)

Cells contain thousands of different proteins; however, for our experiment, we are only interested in examining a single protein, NFκB. Because cells contain so many proteins, specialized methods have been developed to allow scientists to isolate and visualize a single protein among the thousands present. Western blotting or immunoblotting is a commonly used technique to detect a specific protein within a complex cell lysate. Western blotting utilizes polyacrylamide gel electrophoresis (SDS-PAGE) to denature and separate proteins based on mass. The proteins are then transferred or immobilized onto a membrane or support matrix (typically nitrocellulose). Once transferred, antibodies are used to probe or detect the presence of the protein of interest (also known as an antigen). Because antibodies are specific for a particular antigen, probing the membrane results in detection of only the protein one is interested in.

For our experiment, the antigen will be NFκB. We will detect the presence of NFκB by using an antibody that specifically recognizes this protein (this is the primary antibody). Using an antibody alone would not allow us to see what we are looking for. Therefore, a second (secondary) antibody, that is able to detect and bind to the primary antibody is used. The
secondary antibody is conjugated with an enzyme (ie: the antibody has an enzyme attached to it). Upon addition of the enzyme substrate, the enzymatic reaction product emits light that can be detected using x-ray film. Thus, at the end of the experiment we will have a piece of film showing a band where NfκB p50 has migrated to on the original gel. To confirm that we are in fact detecting NfκB specifically, we will also run a positive control of purified NfκB protein. In addition we will use the protein β2-microglobulin as a standard for the amount of protein added to each well. This will allow us to compare NfκB p50 in all samples, ensuring that differences in NfκB protein are not due to random loading variations.

**Experiment 2.1: Protein Analysis-Western Blotting Part I**

- **NOTE: Each group will have 8 samples to load onto the gel: mass marker, positive control, 0 hour, 6 hour, 12 hour cytoplasmic extracts and 0 hour, 6 hour, 12 hour nuclear extracts. You will load one gel with both your nuclear and cytoplasmic extract samples. Because we can run two gels per electrophoresis chamber, two groups will share one chamber.**

**Sample Preparation and SDS-PAGE Electrophoresis**

1. Remove the gel cassette from the package. Each cassette contains a smaller plate, larger plate and a 10 tooth comb.
   - **i.** Remove the white tape along at the bottom of the gel.
     - **1. Have your instructor check to confirm that the tape has been removed properly.**
2. Carefully, remove the comb from the gel and rinse the gel with water
   - **i.** Flick the gel to remove water from the wells
   - **ii.** Place 2 gel cassettes adjacent to one another in a single electrophoresis chamber such that the smaller (shorter) plates are facing towards the inside of the apparatus.
     - **1. Have your instructor check to confirm that the gels are seated properly**
3. Slowly fill the internal compartment of the electrophoresis chamber with 1x Running buffer until it reaches about 0.5 cm above the shorter plate.
4. Fill the external compartment with the same buffer until it is approximately, 0.5 inches from the top
   - **i.** Have your instructor check to confirm you have added enough buffer for the gel to run properly
5. Use a p200 pipette to rinse out the wells using the buffer in the chamber. To do this, simply place the tip at the top of each gel, between the plates and pipette up and down a few times
6. Prepare your protein samples as follows:
   - **i.** Label (8) 1.5ml eppendorf tubes
1. Tube 1: ‘Marker’
2. Tube 2: ‘Control’
3. Tube 3: ‘0 hour CE’
4. Tube 4: ‘6 hour CE’
5. Tube 5: ‘12 hour CE’
6. Tube 6: ‘0 hour NE’
7. Tube 7: ‘6 hour NE’
8. Tube 8: ‘12 hour NE’

ii. To the marker tube, add 10ul kaleidescope marker; set this tube aside

iii. To your control tube add:
    1. 5ul NfκB protein
    2. 15ul protein buffer
    3. 5ul loading dye

7. For tubes 3-8, add:
   i. To each tube: Add 24ul of the appropriate sample—NOTE: be sure to double check that you are adding the correct sample to each tube (ie: you are adding the 0 hour cytoplasmic extract to the ‘0 hour CE’ tube).
   ii. Add 8 ul of loading dye to each tube
   iii. Flick tubes gently to mix

8. When prepared, place all tubes, except the marker, in a 100⁰C heat block.
9. Heat for 5 min.
10. After heating, remove samples and centrifuge all three for 10 seconds at max speed to collect sample at the bottom of the tube.

11. Load samples as follows:
    i. Load 10 ul of molecular mass marker
    ii. Load 25ul of control
    iii. Skip a lane
    iv. Load 30 ul of 0 hour CE
    v. Load 30 ul of 6 hour CE
    vi. Load 30 ul of 12 hour CE
    vii. Load 30 ul of 0 hour NE
    viii. Load 30 ul of 6 hour NE
    ix. Load 30ul of 12 hour NE

12. When both groups have loaded their gels, place the electrophoresis cell lid in position and connect the cables from the electrophoresis cell unit to the power supply.
    i. Be sure that you have connected red to red (positive) and black to black (negative).

13. Set the voltage to 200 volts and push the run button.
    i. Your gel should run for about 1 hour or until the bromophenol blue (dye front) in the samples has migrated to within 1.0 cm of the bottom of the gel. (Do NOT let the gel run farther as this will result in loss of your samples.)

14. Once your dye front has reached the bottom of the gel, turn off the power supply
Experiment 2.2: Protein Analysis-Western Blotting Part II—Transfer and Probing

**ElectroTransfer to Nitrocellulose**

1. Wear gloves for all steps
2. Obtain a cathode and anode transfer pack, and sponge pack (You will need one pack of each per TWO groups)
3. Carefully open the anode pack, and label your nitrocellulose with your group name—Keep the stack in the plastic tray
   a. STOP!! Check with your instructor to be sure that you are labeling the nitrocellulose and not filter paper
4. Open the lid of the I blot instrument
5. Place the Anode Stack (still in the tray) on the blotting surface. Align it with the Gel Barriers on the right.
6. Disassemble your gel by using the prying tool to separate the two plastic plates (your instructor will demonstrate this)
1. Place the pre-run gel(s) on the transfer membrane of the Anode Stack with the molecular weight ladder on the left—be sure your nitrocellulose is labeled.
   a. NOTE: two groups will transfer using the same transfer stack
   b. STOP!! Have your instructor check to be sure your set up is correct.
2. Place the pre-soaked (in deionized water) iBlot® Filter Paper on the pre-run gel and remove air bubbles as demonstrated by your instructor.
3. Unseal the Cathode Stack—Discard the red plastic tray.
4. Place the Cathode Stack over the pre-soaked Filter paper with the electrode (copper) side facing up and aligned to the right edge.
5. Remove air bubbles as demonstrated for step 8
6. Place the Disposable Sponge with the metal contact on the upper right corner of the lid.
7. Close the lid and secure the latch. The red light should be on indicating a closed circuit.
8. Select Program 2
   a. the display should read P2 7:00 minutes
9. Press the Start/Stop button, the red light should change to green.
10. Current automatically shuts off at the end of each run. The end of transfer is indicated by beeping sounds, and a flashing red light and digital display.
11. Press and release the Start/Stop button; the light should turn to a steady red.
   a. Once the transfer is complete, you may remove the nitrocellulose membrane from the gel. Place the membrane on a piece of Saran Wrap. Make a small mark by each of the marker bands on your blot with a ball point pen.
12. Transfer nitrocellulose to a container of distilled water. Leave membrane soaking until you are ready to blot.
Western Blot Probing (Excerpt from the Life Technologies/Invitrogen Website):

**Invitrogen: detection methods and the iblot dry blotting system:**

The final step in Western Blot Analysis is the detection of proteins. During detection, the protein of interest on the membrane is probed by a specific antibody linked to a reporter enzyme that interacts with a specific substrate creating a colorimetric or chemiluminescent reaction. The antibody detection step can be performed in one or two steps, the latter being the more commonly used. The two-step process uses an antigen-specific primary antibody that binds to the protein of interest, and a reporter enzyme conjugated secondary antibody that binds to the primary antibody. The reporter enzyme attached to the secondary antibody is frequently alkaline phosphatase (AP) or horseradish peroxidase (HRP). The reporter enzyme reacts with a chemiluminescent substrate and this reaction product produces luminescent (light) signal proportionate to the amount of antibody bound protein. In the chemiluminescent assay, the image is captured on photographic film. The result can be used for qualitative or semi-quantitative analysis. We will detect the light emission by exposing our blot to X-ray film. This will require that we work in the dark room to both expose and develop the film.

**Probing for NfκB using the iblot dry blot system**

**IMPORTANT:** Do NOT perform immunodetection on dry membranes. Verify that membranes are wet before performing detection. Your membrane should be soaking in dH2O.

NOTE: we will probe two gels at the same time - set up side by side

**Step I: Blocking**

1. Collect a bottom and top blotting stack
2. Collect the necessary solutions:
   a. Antibody Diluent Solution (blocking solution)
   b. Primary Antibody Solution
   c. Secondary Antibody Solution
3. Obtain a white **antibody matrix** (this is NOT your nitrocellulose)
4. Place the white antibody matrix on a piece of Saran wrap as shown below:
5. To block the membrane, apply 3.5ml of Antibody Diluent Solution containing the Additive evenly on the nitrocellulose using a clean pipette (see photo above).
6. Open the iBlot® Western Detection Bottom Stack. Leave the stack in the plastic tray.
7. Place the plastic tray containing the Bottom Stack directly on the blotting surface.
8. Align the tray with the gel barrier on the right.
   a. The alignment guide on the left of the blotting surface should be visible and not hidden under the rim of the tray. If the alignment guide is not visible, the Stack tray is not properly positioned, and may fail to make the proper electrical connection.
9. Use forceps to place the pre-wetted nitrocellulose on the Bottom Stack with the protein side facing up (see the image below).

10. Remove any bubbles by rolling a serological pipette back and forth (using even pressure) across the nitrocellulose surface.
    Note: The pipette is used several times throughout this protocol, and should be washed between each step.
11. Use forceps to place the antibody matrix soaked with Antibody Diluent Solution (Step 5) onto the membrane (see image below).
12. Remove any bubbles by rolling the assembly using a clean pipette.
13. Open the iBlot® Western Detection Top Stack. **Keep the red plastic tray.**
14. Remove the Top Stack from the tray, and place it over the white matrix with the electrode side facing up (see image below).

15. Remove any bubbles by rolling as above.
16. Position the iBlot® Disposable Sponge so the metal contact is at the upper right corner of the lid.
17. Close the lid and secure the latch. The red light is on indicating a closed circuit.
18. **Select program P9.**
   a. This program is a 3-step program for the iBlot® Western Detection protocol. The complete program runs for 8 minutes, and cannot be modified.
19. Press the Start/Stop Button. The red light changes to green. **Do not turn off the iBlot® Device, or change programs at any step during program P9.**
Step II: Probing with Primary Antibody

1. While the iBlot® Device runs, obtain a new white antibody matrix sheet,
2. Place the antibody matrix on a clean piece of saran wrap
3. Apply 3.5 ml of the primary antibody solution evenly across the matrix
4. The first (blocking) step ends after 2 minutes, and is indicated by beeping, and a flashing green light. Open the lid, leaving the sponge in place.
   a. Two flashing horizontal bars appear between the program number and the time in the display.
   b. **DO NOT CHANGE THE PROGRAM OR TOUCH ANY BUTTONS** until instructed to do so!!!
5. Remove the Top Stack and set it aside in the red tray for re-use in the next step.
6. Discard the used blocking solution matrix.
7. Use forceps to place the new matrix containing the primary antibody onto the membrane.
8. Remove any bubbles by rolling as before
9. Return the Top Stack to its position over the matrix (copper side up).
10. Remove any air bubbles by rolling.
11. Close the lid and secure the latch.
12. Press the Start/Stop Button to start the second (primary antibody) step.
   a. The two horizontal bars should stop flashing.

Step III: Adding Secondary Antibody

1. While the iBlot® Device runs, obtain a fresh, white antibody matrix
2. Apply 7 ml of the secondary antibody solution
3. At the end of the primary antibody step, the machine will beep to indicate this step is finished, and the green light will flash
   a. **AGAIN DO NOT TOUCH ANY BUTTONS OR SETTINGS**
4. Open the lid, leaving the sponge in place.
   a. **Three flashing horizontal bars appear in the display** between the program number and the time in the display.
7. Remove the Top Stack and set it aside in the red tray for re-use in the next step.
8. Discard the used primary antibody matrix
9. Use forceps to place the new matrix containing the secondary antibody onto the membrane.
10. Remove any bubbles by rolling as before
11. Return the Top Stack to its position over the matrix (copper side up).
12. Remove any air bubbles by rolling.
13. Close the lid and secure the latch.
14. Press the Start/Stop Button to start the third (secondary antibody) step.
15. The three horizontal bars should stop flashing.
16. While the probe is running, add 20ml of wash solution to a small tray.
17. At the end of three minutes, the machine will beep indicating the end of the run
18. Open the lid, discard everything, **except your nitrocellulose membrane**
19. Place the nitrocellulose membrane in a tray containing 20ml of wash solution
20. Proceed to the detection section

**Step IV: Detection of protein using chemiluminescence**

1. Pour off wash solution that membrane is soaking in (do not discard the membrane!).
2. Add 20 ml of fresh wash solution
3. Place the tray on a rocker and allow the membrane to wash for 5 minutes.
4. After the 5 minute wash, discard the solution.
5. Repeat steps 2-5, two more times.
6. After the final wash, discard the wash solution.
7. Add 20 ml of dH2O, rock briefly by hand then discard the dH2O.
8. Repeat step 7 one time

**STOP!!** Your instructor will tell you when to proceed to the dark room to complete the detection procedure. When dismissed, take your membrane, still in the water, to the dark room.

**Completing the detection: The following steps will be carried out in the dark room**

Obtain a film cassette, a piece of saran wrap, and scotch tape before starting.

9. Place the membrane with **protein-side facing up** on a sheet of Transparency plastic.
   a. **Do not allow the membrane to dry out.**
10. Cover the membrane with 3 mL of Chemiluminescent Substrate.
    a. **Do not touch the membrane surface while adding the substrate.**
    b. **Make sure the membrane is evenly covered for the duration of the reaction.**
11. Allow the reaction to develop for 5 minutes.
12. Blot any excess Chemiluminescent Substrate solution from the membrane by touching one corner of the membrane on filter paper with protein-side facing up.
    a. **Do not allow the membrane to dry out.**
13. Place the nitrocellulose membrane on a piece of plastic wrap and wrap so that it is completely sealed.
14. Place wrapped membrane into film cassette so that two groups can use the same cassette.
15. Scotch tape so that the membrane will not move.
16. Expose an X-ray film as directed by your instructor.
17. Develop film as directed.
18. When finished, your film will be scanned into the computer and emailed to you. You will use this file on Day 6 to quantitate protein expression levels.
Transfecting cells to examine subcellular localization (Exp 2.3)

To look at the location of the protein we are interested in, we will transfect the cells with the gene for NfκB (similar to what we did last week); however, in this case, the NfκB gene is linked up with a second gene, green fluorescent protein (GFP), that emits green fluorescent light. Using this fusion of two genes, we will be able visualize where in the cell NfκB is located.

As a first step in this experiment, we must introduce our recombinant DNA molecule into the cells. When we did this last week, we used Lipofectamine 2000. You’ll recall that Lipofectamine is a cationic lipid that will bind to and surround the DNA. Once the Lipofectamine:DNA complex is formed, the Lipofectamine portion of the complex will interact with the cell membrane and deliver the DNA into the cell. After transfection, we will allow the cells 24 hours to recover and begin expressing the foreign DNA.

In this week’s experiments we will use the transfected cells to answer a new question. Our first transfection was used in a luciferase assay as a means to measure activation of the NFκB gene (gene expression) in response to inflammatory stimuli. This week we will use the transfected cells to examine the behavior of this protein following exposure to the inflammatory stimuli; specifically, we would like to be able to see where NfκB normally exists in the cell and what might happen to it after we treat the cells with pro-inflammatory stimuli. In doing this experiment, we expect to visualize the subcellular location of the NFκB protein before and after induction of inflammation.

Reminders about Sterile Technique and Tissue Culture:
Regardless of method, passaging and handling of the cells requires good sterile technique to avoid contamination. Thus, cell plates that will be cultured for any period of time must be handled in a laminar flow hood wearing gloves and using only sterile reagents and equipment.
Experiment 2.3: Transfection of cells with NfκB-GFP Plasmid

Preparation of Lipofectamine 2000:DNA complexes

*NOTE: All steps should be carried out in the hood using proper sterile technique; before starting, clean and prepare the tissue culture hood as described in the introduction*

*For this transfection we will preparing two sets of L (lipofectamine) tubes and D (DNA) tubes. We will designate them D1/D2 and L1/L2, respectively. The L1/D1 tubes will correspond to the NfκB-GFP transfection and the L2/D2 tubes will correspond to the Control plasmid transfection.*

1. Prepare two 1.5ml tubes: add 300ul Optimem (serum free medium) to each tube. Label them tubes “L1” and “L2” for Lipofectamine. Then you will need to prepare two more 1.5mL tubes, with 300uL of Optimem. Label these tubes “D1” and “D2” for DNA.
2. To each L tube, add 30uL of Lipofectamine 2000 reagent to the tube containing the Optimem media.
   a. *NOTE: Pipette the Lipofectamine 2000 reagent directly into the Optimem (do not let it touch the wall of the tube).*
3. Gently tap the tube to mix.
4. Incubate the Lipofectamine/Optimem mixture for 5 minutes at room temperature.
5. While incubating-pipet the appropriate amount of DNA into tubes D1 and D2.
   a. Add 8 ug of NfκB-GFP DNA into tube D1 and 6 ug of control DNA into tube D2; the volume used will depend on the concentration of your DNA prep (in ug/ul).
   b. Calculate volume needed as follows:
      \[
      \frac{[1\text{ul}]}{[\text{DNA Conc in ug}]} \times 8\text{ug} = \text{volume needed}
      \]
   c. Add calculated volume to the appropriate tube (as describe in step 5a).
6. Following the 5 minute incubation of the L tubes (step 4 above), add the contents of the D1 and D2 tube to the contents of the L1 and L2 tube, respectively. Mix each well by pipetting. Incubate for 20 minutes at room temperature.

Proceed to transfection procedure beginning on the next page.
Transfection of Cells

7. During the incubation, obtain a 6 well plate (your 6 well plate that was seeded with 1.4 x 10^6 cells per well 24 hrs prior to transfection)

8. Label the plate carefully as follows (also see diagram below):
   a. The top row should be labeled “NfkB -GFP” and the bottom row should be labeled “Control”.
   b. Label the two left most wells (top and bottom) 1 and 3; the two right most wells (top and bottom) should be labeled 2 and 4
   c. Label wells 1 and 3 “0 hour”
   d. Label wells 2 and 4 “5 hour”

9. Following the 20 minute incubation (in step 6 above), you will add **110ul of tube L1 to each of the wells labeled 1 and 2**. You will add **110ul of tube L2 to each of the wells labeled 3 and 4**.

10. Incubate for 4-6 hours at 37°C. Replace the media with 3 ml of Complete RAW Cell Medium.

11. Incubate overnight at 37°C and then the cells are ready to be treated with inflammatory stimuli.
DAY 6:

Quantitative Polymerase Chain Reaction (qPCR)

qPCR to determine effect of inflammatory mediators on expression of β-actin, iNOS, NFκB, and TNFα (Exp 2.4)

Vocabulary

PCR (including melt/denature, anneal, extend/extension/elongation)
qPCR (aka real-time PCR; may or may not involve a reverse transcriptase step prior to the qPCR reaction)
target/transcript
housekeeping gene
β-actin, iNOS, NFκB, TNFα
mRNA
gene expression
lysate
DNase I
reverse transcriptase
RT reaction
Oligo dT primer
cDNA
cycle threshold (C_t), ΔC_t (delta/change-in cycle threshold), and ΔΔC_t (change-in the change-in C_t)
Primer/Probe Pairs (Taqman probes)
quencher
Incomplete Master Mix
Complete Master Mix
threshold
normalization (relative gene expression analysis using housekeeping genes)
negative control
UNG enzyme (optional)

Video

(ignore SYBR Green explanations; focus on Taqman instead)

Link to “New to qPCR” Handbook

(focus on pages 3-15, 18-21, 34-37, 43-45; SKIP 1-step qRT-PCR, Standard curve, Correlation coefficient, Dynamic range, Absolute quantification, Melting curve, DNA-binding dyes, Primer-based detection systems, Melting curve analysis, Multiplex real-time PCR, Real-time PCR instrument calibration, Exogenous normalizers )
What is qPCR?

For additional info, please visit http://qpcr.community.appliedbiosystems.com/community/qpcr/blog/2012/04/19/what-is-real-time-pcr

Real-Time PCR—also called quantitative polymerase chain reaction (qPCR)—is one of the most powerful and sensitive gene analysis techniques available and is used for a broad range of applications including quantitative gene expression analysis, genotyping, SNP analysis, pathogen detection, drug target validation and for measuring RNA interference. Frequently, real-time polymerase chain reaction is combined with reverse transcription to quantify messenger RNA (mRNA) in cells.

Real-Time PCR Steps:

To perform Real-Time PCR, start with a basic PCR mix (containing DNA polymerase, dNTPs, magnesium, buffer, primers, template, and add fluorescent labels to the PCR mix. A light source in the Real-Time PCR instrument then excites the fluorescence and a camera captures the fluorescent signals. As amplification proceeds, the fluorescence accumulation is captured by the instrument after every cycle and is translated into a Real-Time PCR graph.

Image of a qPCR graph:

With Real-Time PCR, There are Three Amplification Stages:

- Exponential
- Linear
- Plateau

In the Exponential phase the reagents are in abundance, and the PCR product doubles every cycle. In the Linear phase the reagents begin to run out, and the PCR reaction slows down. In the Plateau phase the reagents are depleted, and the PCR reaction stops. Real-Time PCR focuses on the Exponential phase because it provides the most precise and accurate data for quantitation (quantification).

Within the Exponential Phase Two Values are Calculated:

- Threshold
- $C_T$ Value

The threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The PCR cycle at which the sample reaches this level is called the Cycle Threshold, $C_T$. The $C_T$ value is used in downstream quantification analyses and data crunching.

Advantages of Real-Time PCR Over Traditional PCR:
First, it is more sensitive, which allows detection of low copy targets with greater confidence. It is also more quantitative which provides easy access to highly quantitative data. Real-Time PCR is faster. No gels are needed, which saves time and effort. Because Real-Time PCR does not involve ethidium bromide or radioactivity, it is much safer than traditional PCR.

Real-Time PCR Systems from Life Technologies use Two Main Fluorescence Detection Formats:

- SYBR Green Dye
- TaqMan Probes

The SYBR fluorescence format uses a dye called SYBR Green, which binds non-specifically to double-stranded DNA. The DNA dye complex emits green light which is recorded by the Real-Time PCR instrument. For SYBR Green detection it is important to run a Melting Curve analysis following Real-Time PCR to ensure that the desired amplicon was detected. The inflection point on the curve indicates the melting point of the amplicon. Contaminating DNA or primer dimers would show up as additional peaks separate from the desired amplicon peak.

The TaqMan probe fluorescence format uses 1) two primers, 2) a probe with a fluorescent reporter dye and a quencher, 3) target DNA, and 4) Taq DNA polymerase.

Probes:
The design of the probe is key. The Taqman probe is an oligonucleotide that contains a fluorescent reporter dye bound to the 5’ end and a quencher on the 3’ end. The probe is designed to bind to the target DNA sequence. While the dye and quencher are intact, there is no fluorescence. As the polymerase elongates the product, it is able to cleave the probe, thereby separating the reporter from its quencher, and allowing the reporter to fluoresce. This fluorescent signal is captured by a light detector within the Real-Time PCR instrument. All Real-Time PCR formats detect fluorescence in real-time, and use the $C_T$ value to perform quantitation.
A schematic of the primers and Taqman probe annealing to the DNA template:

http://www.google.com/imgres?imgurl=http://www.vetscite.org/gfx/08_00/fig1_leute.gif&imgrefurl=http://www.vetscite.org/issue1/tools/txt_leut_0800.htm&h=220&w=387&sz=5&tbnid=Lj-42HgVuGsM:&tbnh=75&tbnw=132&prev=/search%3Fq%3Danimation%2BqPCR%26tbm%3Disch%26tbo%3D3o&zoom=1&q=animation+qPCR&docid=_0iRgqtukWNVxM&hl=en&sa=X&ei=HrOxT77PBEPUBMAM&ved=0CJIBEPUBMAM&dur=2992

Summary of the qPCR Experiment:
What was set up prior to today’s experiment:

RAW264.7 cells were seeded in 96-well tissue culture-treated plates. 24 hours post-seeding, media was aspirated and fresh media was added. Cells were left untreated or were treated with various inflammatory mediators for 24 hours. At this point, media and inflammatory mediators were aspirated, and the cells were rinsed with sterile 1X PBS. Cells were lysed in the wells for 5 minutes using Cells to C Kit Lysis Buffer + DNase I (to degrade DNA) (by Ambion/Life Technologies). The lysis reaction was stopped using the Stop Solution from the same kit. Lysates were combined with Reverse Transcriptase (RTase) and Reverse Transcription Buffer from the same kit. The Reverse Transcription reaction was carried out in a thermal cycler using the following program: 60min at 37°C, 5min at 95°C. Resulting cDNA was stored at -20°C until being provided to MASE students.

What you will do today:

Each group will perform qPCR for 4 targets/transcripts (β-actin, iNOS, NFκB, TNFα), on cDNA from 1 untreated cell sample and 1 treated cell sample, with 3 replicates of each of those 2 samples and 4 targets; additionally, each group will set up 3 reactions that lack cDNA; therefore, each group will set up a total of 27 reactions (rxns).

First, each group will receive 5 tubes of Incomplete Master Mix that lack cDNA and Primer/Probe Pairs, as well as 4 tubes of Primer/Probe Pairs.

* Incomplete Master Mix will be prepared in advance and will be given to each group, pre-distributed into 5 separate tubes:

  Tube 1: β-actin for Negative control (3 rxns: all with water in place of cDNA)
  Tube 2: β-actin for Normalization (6 rxns: 3 containing cDNA from untreated cells and 3 containing cDNA from treated cells)
  Tube 3: iNOS (6 rxns: as above)
  Tube 4: NFκB (6 rxns: as above)
  Tube 5: TNFα (6 rxns: as above)

Second, each group will add the appropriate Primer/Probe Pair to each Incomplete Master Mix to make a Complete Master Mix.

Third, each group will distribute the contents of each Complete Master Mix into 3 or 6 individual tubes, seated in a Tube Holder rack to protect the bottom of each tube.
These tubes are connected to one another in strips of 3 or 6. Tube strips should be kept in a Tube Holder rack that is in the format of a 96-well qPCR reaction plate. These tubes are tiny, so it’s best to leave the strip in the Tube Holder rack as you pipet. Using tubes rather than plates will allow 2 groups to place their tubes into the qPCR instrument at the same time.

**Fourth**, each group will transfer the appropriate cDNA sample to individual qPCR reaction tubes.

**Finally**, each group will cap each tube strip with a cap strip to seal the contents within. Tubes in Tube Holder racks will be centrifuged for 5 minutes at 1000rpm to move all liquid to tube bottoms. The tubes will be transferred to a Veri-Flex tray and can then be placed in the qPCR instrument and processed using the following thermal cycling program, or at 4°C freezer to be run when the instrument is available:

2min at 50°C; 10min at 95°C; (15sec at 95°C; 1min at 60°C) x 40

These steps correspond to, respectively:
UNG enzyme activation to prevent carryover contamination; activation of hot-start Taq polymerase; 40 cycles of melting, primer/probe annealing and product elongation (during which the fluorescent signal generated by cleaving the fluorescent tag away from the quencher is measured during each cycle)
Summary of the qPCR Experiment, continued

Each group will have 5 strips of qPCR reaction tubes that should be placed in a Tube Holder rack and loaded with reactions as follows. A-H and 1-12 refer to specific locations on the Tube Holder rack. Do NOT deviate from these placement instructions!

<table>
<thead>
<tr>
<th>GROUPS 1, 3, 5, 7</th>
<th>GROUPS 2, 4, 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
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<td>2</td>
<td>8</td>
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<td>3</td>
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<td>6</td>
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<td><strong>A</strong> Un</td>
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<td><strong>B</strong> Un</td>
<td>Un</td>
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<td><strong>C</strong> Un</td>
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<tr>
<td><strong>D</strong> Un</td>
<td>Un</td>
</tr>
<tr>
<td><strong>E</strong> Un</td>
<td>Trtd</td>
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<tr>
<td><strong>F</strong></td>
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<td><strong>G</strong></td>
<td></td>
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<tr>
<td><strong>H</strong></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Color</th>
<th>Primer/Probe Pair</th>
<th>cDNA (4µl/tube)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red</strong></td>
<td>β-actin</td>
<td><strong>None; 4µl water to each of 3 tubes</strong></td>
</tr>
<tr>
<td><strong>Orange</strong></td>
<td>β-actin</td>
<td>3 tubes Un; 3 tubes Trtd</td>
</tr>
<tr>
<td><strong>Yellow</strong></td>
<td>iNOS</td>
<td>3 tubes Un; 3 tubes Trtd</td>
</tr>
<tr>
<td><strong>Green</strong></td>
<td>NFκB</td>
<td>3 tubes Un; 3 tubes Trtd</td>
</tr>
<tr>
<td><strong>Blue</strong></td>
<td>TNFα</td>
<td>3 tubes Un; 3 tubes Trtd</td>
</tr>
</tbody>
</table>

Un = cDNA from untreated cells
Trtd = cDNA from cells treated with inflammatory mediator for 24 hours

The qPCR instrument is a Step One Plus from Applied Biosystems/Life Technologies. It is nicknamed Sophia. The computer it is connected to is nicknamed Seamus. Each pair of 2 groups (1-2; 3-4; 5-6; 7) has a file preloaded onto Seamus and Sophia that must be opened in order to run their qPCR experiment. The file has the plate/tube setup and run method setup already completed.

Important note to counselors, professors, and students: The next plate/experiment cannot be run until the previous experiment has been downloaded from Sophia to Seamus or from Sophia to a USB drive.
Exp 2.4: qPCR

Warnings to the students:

RNA is extremely sensitive to degradation by enzymes that we carry on our skin. Therefore, it is IMPERATIVE that you decontaminate work area and pipets; wear gloves & do not touch yourself; do not lean over tubes. Your instructors will provide further instruction on this.

Have a professor or counselor check your tubes between each major step. If you do not use proper pipetting technique, you will waste $1000s. Pipet with care! Check volumes in lab manual and on pipettors twice! You are advised to divide the work among members of the group such that each group has someone reading the protocol and checking off what has been completed and someone pipetting.

1. Obtain 5 tubes of Incomplete Master Mix that lack cDNA and Primer/Probe Pairs (tubes labeled 1-5) and 4 tubes of Primer/Probe Pairs (tubes labeled with “PPP” and gene names). You may keep these at room temperature.

   Tube 1: β-actin for Negative control (this tube should have half as much volume as Tubes 2-5)
   Tube 2: β-actin for Normalization (relative gene expression analysis using a housekeeping gene)
   Tube 3: iNOS
   Tube 4: NFκB
   Tube 5: TNFα

2. Add the appropriate Primer/Probe Pair (PPP) to each Incomplete Master Mix to make a Complete Master Mix. Use a fresh tip EVERY time. To do this, follow the instructions for Tubes 1-5, below:

   Tube 1: β-actin for Negative control-
   add 3.3μl of β-actin Primer/Probe Pair (PPP)

   Tube 2: β-actin for Normalization-
   add 6.6μl of β-actin PPP

   Tube 3: iNOS-
   add 6.6μl of iNOS PPP

   Tube 4: NFκB-
   add 6.6μl of NFκB PPP
Tube 5: TNFα - add 6.6μl of TNFα PPP

3. Distribute the contents of each Complete Master Mix into either 3 or 6 individual tubes of a 3- or 6-tube qPCR reaction tube strips, seated in a Tube Holder rack to protect the bottom of each tube. **To do this, add 16μl of the appropriate Complete Master Mix (from Tubes 1-5) to each tube.** You can use the same tip for all of the “A” tubes, a new tip for all of the “B” tubes, a new tip for all of the “C” tubes, etc. Follow the plate layout diagram below. A-H and 1-12 refer to specific locations on the Tube Holder rack.

<table>
<thead>
<tr>
<th></th>
<th>1 or 7</th>
<th>2 or 8</th>
<th>3 or 9</th>
<th>4 or 10</th>
<th>5 or 11</th>
<th>6 or 12</th>
<th>Complete Master Mix Tube #</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>β-actin</td>
<td>β-actin</td>
<td>β-actin</td>
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<td>1</td>
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<tr>
<td>B</td>
<td>β-actin</td>
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<td>β-actin</td>
<td>β-actin</td>
<td>β-actin</td>
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<td>C</td>
<td>iNOS</td>
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<td>iNOS</td>
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<td>iNOS</td>
<td>iNOS</td>
<td>3</td>
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<tr>
<td>D</td>
<td>NFκB</td>
<td>NFκB</td>
<td>NFκB</td>
<td>NFκB</td>
<td>NFκB</td>
<td>NFκB</td>
<td>4</td>
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<tr>
<td>E</td>
<td>TNFα</td>
<td>TNFα</td>
<td>TNFα</td>
<td>TNFα</td>
<td>TNFα</td>
<td>TNFα</td>
<td>5</td>
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</table>

4a. Transfer 4μl sterile nuclease-free water to tubes A1, 2, 3 OR A7, 8, 9 in the Tube Holder rack. Use a fresh tip EVERY time.

4b. Obtain 1 tube of cDNA from Untreated cells and 1 tube of cDNA from Treated cells. Transfer 4μl of the appropriate cDNA to each tube, **as directed below.** Use a fresh tip EVERY time. Follow the plate layout diagram below:

<table>
<thead>
<tr>
<th></th>
<th>1 or 7</th>
<th>2 or 8</th>
<th>3 or 9</th>
<th>4 or 10</th>
<th>5 or 11</th>
<th>6 or 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>H2O</td>
<td>H2O</td>
<td>H2O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Un</td>
<td>Un</td>
<td>Un</td>
<td>Trtd</td>
<td>Trtd</td>
<td>Trtd</td>
</tr>
<tr>
<td>C</td>
<td>Un</td>
<td>Un</td>
<td>Un</td>
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<td>D</td>
<td>Un</td>
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</tbody>
</table>

Un = cDNA from untreated cells
Trtd = cDNA from cells treated with inflammatory mediator for 24 hours
Protocol- qPCR Experiment, continued

5a. Obtain a 3-cap or 6-cap strip, as needed, and cap the strips of qPCR reaction tubes that are seated in the Tube Holder rack. Professors will come by with the cap-installing tool to ensure that caps are snug.

5b. A professor or counselor will combine Tube Holder racks from 2 groups, such that the Tube Holder rack matches the diagram below. Each combination Tube Holder rack will then be centrifuged (with a balanced rotor) for 5 minutes at 1000 rpm to move all liquid to tube bottoms. The 2 groups sharing the Tube Holder rack should write their group #s on a piece of lab tape and stick it to the Tube Holder rack so the counselors will know which file to open on Sophia and Seamus for each Tube Holder rack. DO NOT WRITE ON THE TUBES THEMSELVES!

<table>
<thead>
<tr>
<th>GROUPS 1, 3, 5, 7</th>
<th>GROUPS 2, 4, 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5 6</td>
<td>7 8 9 10 11 12</td>
</tr>
<tr>
<td>A Un Un Un Trtd</td>
<td>Un Un Un Trtd Trtd Trtd</td>
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<tr>
<td>B Un Un Un Trtd</td>
<td>Un Un Un Trtd Trtd Trtd</td>
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<tr>
<td>C Un Un Un Trtd</td>
<td>Un Un Un Trtd Trtd Trtd</td>
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<tr>
<td>D Un Un Un Trtd</td>
<td>Un Un Un Trtd Trtd Trtd</td>
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<td>E Un Un Un Trtd</td>
<td>Un Un Un Trtd Trtd Trtd</td>
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<table>
<thead>
<tr>
<th>Color</th>
<th>Primer/Probe Pair</th>
<th>cDNA (4μl/tube)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>β-actin</td>
<td><strong>None; 4μl water to each of 3 tubes</strong></td>
</tr>
<tr>
<td>*</td>
<td>β-actin</td>
<td>3 tubes Un; 3 tubes Trtd</td>
</tr>
<tr>
<td>**</td>
<td>iNOS</td>
<td>3 tubes Un; 3 tubes Trtd</td>
</tr>
<tr>
<td>**</td>
<td>NFκB</td>
<td>3 tubes Un; 3 tubes Trtd</td>
</tr>
<tr>
<td>**</td>
<td>TNFα</td>
<td>3 tubes Un; 3 tubes Trtd</td>
</tr>
</tbody>
</table>

Un = cDNA from untreated cells  
Trtd = cDNA from cells treated with inflammatory mediator for 24 hours

5c. Transfer tubes to a Veri-Flex Tray. Place tubes (in a Veri-Flex Tray) at 4°C. Professors and counselors will run the tubes as time allows.

6. There are 2 options for running each Tray (Sophia alone or Sophia with Seamus). There are two options for how to download data from Sophia to Seamus (with USB or without USB). Information regarding how these options work can be found in the appendix at the end of the manual.
Protocol- qPCR Data Analysis

1. Look at your qPCR graph and table. Your 3 Non-Template Control (NTC) wells in which you attempted to amplify β-actin should each have curves that remain below the threshold. If they do not, there is a strong chance that you contaminated one or more of your reagents. If you get a C_T value for these tubes, please see your professor.

2. Identify the C_T value for each sample well.

3. You will be emailed an Excel spreadsheet to help you analyze the qPCR data. All you have to do is input the C_T data into the Part 1 table, and the rest of the parts change based on the table in Part 1.

The Excel calculations allow you to:
- Determine the average C_T of all 3 replicates for each sample (untreated and treated) and target (β-actin, iNOS, NFkB, TNFα).
- Determine the standard deviation of each of your 8 average C_T values.
- Determine the ΔC_T for each untreated target by subtracting the untreated β-actin C_T value from the untreated target C_T value. To determine the +/- standard deviation = s, use the following equation, where s_1 is the standard deviation of the untreated gene of interest replicates and s_2 is the standard deviation of the untreated housekeeping gene replicates.

\[ s = \sqrt{(s_1^2 + s_2^2)} \]

- Determine the ΔC_T for each treated target by subtracting the treated β-actin C_T value from the treated target C_T value. To determine the +/- standard deviation = s, use the following equation, where s_1 is the standard deviation of the treated gene of interest replicates and s_2 is the standard deviation of the treated housekeeping gene replicates.

\[ s = \sqrt{(s_1^2 + s_2^2)} \]

- Determine the ΔΔC_T by subtracting the housekeeping (β-actin) ΔCt from the target (iNOS, NFkB, TNFα) ΔCt +/- standard deviation.

-Ultimately determine if the difference in expression in your treated samples compared to your untreated samples for each target gene (iNOS, NFkB, TNFα).
DAY 6: Data Analysis (Afternoon)

This afternoon will be dedicated to data analysis. Yesterday, you ran a western blot to examine NfκB protein levels. Today, you performed a qPCR analysis to determine how inflammatory stimuli impact the transcription of various genes involved in the inflammatory response. For both of these assays, the experimental protocol is only half the job. To complete the experiment we must analyze the data and determine what it means. This afternoon will dedicated to this analysis.

First, your instructors will go over how to analyze and interpret qPCR data. As this is a complicated process, we have not included details here.

You will also need to analyze your western results. To this, we need to normalize the amount of protein that was loaded into the gel for each sample. A description of why this is necessary can be found under the background information for experiment 2.1. To normalize, we will use a software package called Image J. Directions for using this analysis package are below. Your instructors will also be available to help and answer any questions you may have.

Directions for Quantitating a Western blot – Using ImageJ Software

2. Your image files have been saved as Tiff files.
3. Start the Image J software
4. In Image J, Open your film image file using File>Open.
5. The gel analysis routine requires the image to be a gray-scale image. The simplest method to convert to grayscale is to go to Image>Type>8-bit.
6. Choose the Rectangular Selections tool from the ImageJ toolbar. Draw a rectangle around the first lane. Be sure to make this large enough to fit around your largest band. This will ensure proper quantitation of your samples.
7. After drawing the rectangle over the first lane, press the 1 key or go to Analyze>Gels>Select First Lane to set the rectangle in place. The first lane will now be highlighted and have a 1 in the middle of it.
8. Use your mouse to click and hold in the middle of the rectangle on the first lane and drag it over to the next lane. Center the rectangle over the lane from left to right. Then press 2 or go to Analyze>Gels>Select Next Lane to set the rectangle in place over the 2nd lane. A 2 will appear in the lane when the rectangle is placed.
9. Repeat step 6 for each subsequent lane on the gel, pressing 2 each time to set the rectangle in place.
10. After you have set the rectangle in place over the last lane, press 3 or go to Analyze>Gels>Plot Lanes to draw a profile plot of each lane.

11. The profile plot represents the relative density of the contents of the rectangle over each band. The rectangles are arranged top to bottom on the profile plot.

12. Images of real western blots or gels will have some background signal, so it is necessary to close off the bottom of the peak in order to measure its size.

13. Choose the Straight Line Selection Tool from the ImageJ toolbar. For each peak that you want to analyze in the profile plot, draw a line to close off the peak. This step requires some subjective judgement on your part. Decide where the peak ends and the background noise begins.

14. When each peak is closed off at the base with the Straight Line selection tool, select the Wand tool from the ImageJ toolbar. With the Wand tool, click inside the first peak. Repeat this for each peak as you go down the profile plot. For each highlighted peak, measurements should appear in the Results window.

15. When all the peaks have been highlighted, go to Analyze>Gels>Label Peaks. This labels each peak with its size, expressed as a percentage of the total size of all the highlighted peaks.

16. The values from the Results window can be moved to an Excel spreadsheet by selecting Edit>Copy All in the Results window. Paste the Values into Excel.

17. You should complete steps 4-16 for the NfkB bands and then repeat for the β2-microglobulin bands.

   a. Paste the B2 microglobin data into a separate set of cells on the same spreadsheet.

18. To determine the normalized NfkB values, first determine the relative densities for both NfkB and β2-microglobulin. Divide each Percent by the control percent (in our case this will be the 0 hr timepoint. You will do this for both NfkB and β2-microglobulin. Normalized values for NfkB can then be determined by dividing the relative density for Nfkβ by the relative density for β2-microglobulin.

19. The normalized values for NfkB expression can be graphed (Fold Increase on the Y-axis, time on the X-axis).
DAY 7: Analysis of Protein Expression and Fluorescent Microscopy

Today will be our last day of experiments. We will examine the subcellular localization of NfκB using fluorescence microscopy. In order to have a record of our results we will use a camera to take photographs of our microscope images. Once the images are captured, they will be emailed to you for use in your poster. Fluorescence imaging is somewhat time-consuming. Therefore, this morning we will treat and fix your cells. This afternoon, individual groups will collect images of their experiment. If you are not working on your experiment, you and your group members should be using the time to do data analysis and prepare posters. Instructors will be available to help with the data analysis and to answer any questions.

Examining the effect of Inflammatory Mediators on NfκB function (Exps 2.4 and 2.5)

As outlined above, our goal is to determine what impact various inflammatory stimuli (LPS, IFNγ, PMA, etc.) have on NfκB function. More specifically we will be examining the following question: **Do any of the known inflammatory mediators impact NfκB location within the cell (i.e.: can we observe translocation of NfκB from the nucleus to the cytoplasm in Raw 264.7 cells following treatment)?** The experiment will proceed in two phases; first you will treat your cells with the appropriate inflammatory mediator. Following treatment, you will begin the staining process to visualize the location of NfκB protein within the cell.

The various treatment conditions will initiate the process of inflammation in our cells. Our primary focus is to understand how NfκB behaves in response to inflammation. Since **NfκB expression in the nucleus peaks at 4-6 hours for our experiment we will** examine untreated cells (0 hour) in comparison to cells treated for 3 and 5 hours following treatment with various inflammatory stimuli.

Since these cells were transfected with an NfκB-GFP plasmid, we can take advantage of Green Fluorescent Protein (GFP) expression as our indicator of the location of NfκB within the RAW cells following treatment with your specific inflammatory stimuli. Under the appropriate wavelength of light, the GFP will fluoresce and be visualized as a green light within the cell. This technique known as fluorescence microscopy allows for the visualization of proteins based on their fluorescent light emission.
Experiment 2.5: Inducing Inflammation (Steps 1-7 have been completed for you; begin at exp 2.6)

1. Your group will be assigned conditions for treating your cells
2. Obtain one (6) well plate that contains both control transfected and NFκB-GFP transfected cells from the incubator.
3. Label the plates with your group name, treatment condition, and transfected or untransfected and 0 hour and 5 hour. You will treat both the control and NFκB-GFP cells for 5 hours with your assigned inflammatory stimuli.
4. Treat each plate as follows with the appropriate dose of your inflammatory mediator (LPS, IFNγ, PHA, PMA etc.); if you are using two chemicals to treat, be sure you treat the well with both chemicals:
   a. To each of your 5 hour wells, add 20 ul of your assigned chemical.
5. Place the plates back in the incubator.
6. Incubate for 5 hours.
7. Proceed to experiment 2.5 as soon as this 5 hour incubation has ended.

Experiment 2.6: DAPI stain, fixing, and Fluorescent Microscopy

You will begin this experiment immediately following step 8 above—do not delay as this is a timed experiment!! The zero time point represents your untreated control well. You will be fixing and staining all six wells on your plate (transfected and untransfected).

You will visualize the location of NfkB based on the presence of the Green Fluorescent Protein. We will use a fluorescent microscope to examine and photograph your cells. To help determine the location of the green fluorescence, you will counterstain the cells with a nuclear stain called DAPI, a blue-fluorescent nucleic acid stain that preferentially stains dsDNA. DAPI is a popular nuclear counterstain for use in multicolor fluorescent techniques. It’s blue fluorescence stands out in vivid contrast to green, yellow, or red fluorescent probes of other structures.

15. Remove media from each well.
16. Add 1 ml of 3.7% Formaldehyde to each cover slip.
17. Incubate for 30 min. at RT.
18. Following the incubation, remove the formaldehyde.
19. Add 2 ml PBS to each cover slip (BE GENTLE); swirl gently to wash.
20. Tilt the plate and Remove the PBS from each well (be sure that you removed as much of the supernatant as possible). Repeat wash.
21. Add approximately 300 ul of the prepared DAPI stain solution directly on top of each coverslip. Do this in every well.
22. Incubate the coverslips for 5 minutes at room temperature.
23. Remove the DAPI stain.
24. Wash the coverslip by GENTLY adding 2 mL of PBS to the coverslip. Be sure to add the PBS to the side of the well so that the RAW cell monolayer on the coverslip is not disrupted.
25. Remove the PBS and repeat step 10 two more times.
26. Remove coverslips from well using forceps and mount the cover slips on the appropriately labeled microscope slide (see mounting instructions below).
27. To mount your coverslip:
   a. Obtain one slide for each of your coverslips and label with your group name, whether the coverslip contains control or NfκB transfected cells, and the time of treatment
   b. Add 250 ul of Fluoromount G to a slide
   c. Use forceps to transfer the coverslip onto the Fluoromount G treated slide.
      **BE VERY CAREFUL. Coverslips are delicate and break easily!!!**
   d. You will invert the cover slip so that you are placing it cell side down.
28. An instructor or counselor will aid you in viewing the slides using a fluorescence microscope with appropriate filters. You will take photos of both time points for both DAPI and Green Fluorescent Protein.
29. Then you can merge your DAPI and Green Fluorescent photos. **Include the merged photos for zero and 5 hour time points for your posters.**
Appendix A

Poster Preparation and Grading Guidelines
Guidelines for Preparing your Poster

Overview of Content and Organization of Your Poster Presentation:
You will work with your lab partner to present your research findings accumulated over the 2-week MASE experience in a poster format. We will be looking for a clear statement of the problem, a well organized presentation that includes the sections mentioned below, as well as a clear understanding of the data and how it relates to your question/topic.

In your presentation, be sure to address the following components

- Title
- Introduction
- Research question or hypothesis
- Methodology used in the experimental data used to address your question/hypothesis
- Properly organized and described data
- Summary of Results
- Conclusion based on the data presented

Preparing Your Poster:

Each poster should contain following:

Title – This should be a creative and descriptive title.
- Detailed statement that concisely and accurately describes the purpose of your experiment.
- There should be an author byline below the title.

Introduction -- Your introduction should be written in paragraph form and should include the following:
- Rational for Importance of the study
- Necessary background information to understand the study
- Description of the Experiment
- Basis or Rational for the Hypothesis
- Statement of Hypothesis

Methods – Overview of the methods used. This should be in the format of a flow diagram, illustrating the steps taken to address the research question.

Results/Data Analysis – this section may include graphs, flow diagrams, pictures or tables.

- Written results section: For each experimental question asked, you must include:
  - Recap the Question
  - Overview of your experimental method or approach
  - Detailed description of your data
  - Conclusion or trend of your results

- Formatted Data
  - All results must have a figure legend that describes what is being displayed in the figure, table or diagram. This should be your own words.
Graphs should be done using Excel and MUST include the following:

- Axis label
- Key (What is being represented by each line or bar in the graph)
- Figure Legend (that begins with the title of your figure, followed by 2-4 sentences that clearly and accurately describes what is being illustrated by the figure)

Conclusions – bulleted conclusion statements based on the results presented in the last section and other information on serological and genetic tests in the literature. This should relate to your title and question.

References – You should have separate reference or bibliography section on your poster. All of your figures and text should be cited with a superscript and then the references listed in this section.

Designing your Poster

You may find the following website helpful to you when you are preparing your poster: http://www.swarthmore.edu/NatSci/cpurrin1/posteradvice.htm. Also, look around the hallways as many posters from our college students are hanging up and can serve as good examples. You will prepare your poster using PowerPoint and the large format printer in the media center.

**Note: When designing your poster keep in mind that you are creating figures and text boxes that are easy to read and interpret. So make sure you view them at 100% size to ensure they are readable from a couple feet away.

How to prepare a large format poster:

Setting up your poster in PowerPoint

To print your poster using the printer in the Media center you’ll need to have 2 slides in PowerPoint each measuring 42” wide and 21” tall. You’ll need to design the poster so it can be split in half longitudinally, printed, and then taped together.

In the Page Setup menu, under slides sized for select custom. Set the width for 42 inches and the height for 21 inches. Select landscape for slide orientation. Use 2 slides, one for the top half of your presentation, and one for the bottom half.
Grading Criteria for the Poster Project

Your grade for the poster project will be based on 3 components:

Content 35%
Design 35%
Performance during poster session 30%

POSTER CONTENT: Breakdown of percentage value of each component

Accuracy (5 pts)
• Does the material in the poster accurately reflect the laboratory experiments, purpose, and goals?
• Are there errors of fact or interpretation in the poster?

Clarity (5 pts)
• How easy is it to understand the material in the poster?
• How well are the main points of the poster (the thesis) expressed?
• How well does the body of the poster convey a clear hypothesis and goals?

Depth (5 pts)
• Is the information in the poster of sufficient depth?

Literature Cited—referencing (5 pts)
• Is the material in the poster (text and graphics) sufficiently and properly cited?
• Is the Literature Cited section complete, accurate, and formatted properly?

Literature Cited—sources (5 pts)
• Does the poster demonstrate use of primary sources beyond the lab manual (this may include those provided in the manual)?
• Are the sources used high-quality and appropriate sources?

Integration (5 pts)
• How well is the material from the various sources integrated throughout the text? (i.e., the poster should not consist of individual summaries of the primary sources or rely solely on the language and information found in the lab manual)
Conclusions (5 pts)

• How well has the material been synthesized to reach conclusions?
• How complete, accurate, and thoughtful are the conclusions?
• Does the poster suggest what research should be done next in the field?

TOTAL SCORE CONTENT (max score = 35 pts)

POSTER DESIGN:

Overall layout and appearance (10 pts)

• Is the poster visually appealing and pleasing?
• Is the poster inviting to the audience?
• Does the poster look neat and professional?

Logical flow and organization (10 pts)

• How well-organized and clear is the layout of the different sections in the poster?
• Is the flow of the material in the poster easily evident? (Can the audience easily move from one section to the next?)

Readability (5 pts)

• How easy is it for the audience to read the text of the poster? (consider type and size of font)

Graphics (5 pts)

• Are sufficient and appropriate graphics used in the poster?
• Are the graphics easy to understand and properly captioned and/or referenced?
• Do the images have appropriate size and resolution?

Grammar, spelling, and syntax (5 pts)

• Is the text free of grammatical and spelling errors?
• Is the text well-written with clear, concise, and precise language?

TOTAL SCORE DESIGN (max score = 35 pts)
PERFORMANCE DURING THE POSTER SESSION:

Command of the material (15 pts) 

• How well does the student understand the material in the poster?
• How well can the student convey his/her understanding of the material in the poster?
• How concisely and clearly can the student summarize the main points of the poster?
• How well does the student utilize the poster when summarizing the material or answering questions?

Ability to answer questions (15 pts)

• How clearly, completely, and accurately can the student answer questions regarding the poster?
• How well does the student think on his/her feet in responding to questions?
• To what extent does the student exhibit poise during the poster session?
• How does the student handle it when he/she doesn’t know the answer to a question?

TOTAL SCORE PERFORMANCE (max score = 30pts)

PERCENTAGE GRADE FOR POSTER
Appendix B

Supplemental Information on qPCR runs and Data Collection
qPCR represents a sophisticated, computer assisted analysis that provides a method by which changes in transcription can be assessed by quantitating changes in mRNA levels. To do this, we utilize a qPCR instrument (affectionately name ‘Sophia’) and a computer software package (named ‘Seamus’). Below, you will find some technical information regarding the different approaches we can use to carry out qPCR experiments.

Running the experiment using the instrument only - Sophia alone:

-Turn-on Sophia (switch on left back)
-After Sophia boots, the “main menu” screen appears
-The drawer should have a one inch blue band visible. If so, the drawer can be opened and the Veri-Flex Tray can be placed in the base
-Close the drawer gently; you will feel it click into place.
-The upper left button on the “main menu” touchscreen is “browse/new experiments”
-Select this
-Select the MASE2012 folder
-Touch the appropriate file to select it
-Then touch “start run”
-The experiment parameters appear. Select “start run now”
-The drawer will raise and the blue band will disappear
-The run will commence
-After the run is over, if the drawer is still raised, select “abort run” (the stop square button), and then press “OK” that you are sure you want to cancel/abort the run. The drawer will lower, and the blue band will reappear. You can remove your tray and store it at -20°C for future use.

Running the experiment using the instrument and software- Seamus with Sophia:

-Turn-on Sophia (switch on left back)
-Wake Seamus
-Ctrl-alt-del to log in
-Click “OK”
-User name: INSTR-ADMIN
-Password: INSTR-ADMIN
-Click “OK”
-double click on StepOne Software v2.2.2 icon
-User name: guest
-Click “Log in as guest” or “OK”
-Seamus should connect to Sophia
-Click on “open”
-Navigate to desktop
-Select “MASE 2012” folder
- Select appropriate file (Groups 1 and 2, 3 and 4, 5 and 6, or 7)
- Click “Open”
- Click “assign targets and samples” tab
- Ensure that your tubes are in the correct order based on the template
- Click “run” tab in the leftmost “experiment menu”
- Select “start run”
- The run will commence
- After the run is over, if the drawer is still raised, select “abort run” (the stop square button), and then press “OK” that you are sure you want to cancel/abort the run. The drawer will lower, and the blue band will reappear. You can remove your tray and store it at -20°C for future use.

**Downloading Data to a USB drive:**

- When the run is completed, insert the USB drive into the port on the upper front left of Sophia
- On the “main menu” screen, the upper right touch screen button should be “collect results”
- Select this option
- Downloading the data to the USB will only take a minute or two
- Then, when it gives you permission, remove the USB and insert it into Seamus. Open the USB folder and copy and paste the experiment to the MASE folder on the desktop of Seamus. This should overwrite the pre-run file.
- If the file is open, close it and either use Sophia or Seamus to open the next file to run the next set of tubes.

**Downloading Data- without USB:**

- This option can take several minutes to complete
- With the Step One Software open and the file open, too; on the top ribbon of the screen on Seamus, select “download experiment from instrument”.
- Seamus will search Sophia for files to download.
- Seamus will ask you where to save the experiment file to
- Browse for the “MASE 2012” folder on the desktop and select it
- Then hit “download experiment”.

7. If Seamus is hooked up to a printer, data can be printed for each group so that they may perform analysis and determine \( \Delta C_T \) and \( \Delta \Delta C_T \) between untreated and treated samples and normalize to the housekeeping gene (determine relative gene expression using a housekeeping gene). Alternately, one can generate a report for each file and either print it or save it to the Desktop and then to a USB directly to the students or from USB to email.

**Hints for analyzing data using the software package (analysis with Seamus):**

- When the runs are done, each group should receive a printout or email PDF of their data
- Open your file on Seamus
- The files should be 13 kb to start and around 2000-8000kb after the run
- Open your experiment
- On the left side of the screen on Seamus “experiment menu”, select “analysis”.
- Select “amplification plot” to see the curves of C<sub>T</sub> versus cycle.
- On the right half of the screen, select “view plate layout” to look at the wells and see if there are any alerts you should check.
- If there are no curves on the graph on the left, try highlighting all the wells on the plate in “view plate layout” on the right.
- To look at just one group, just highlight those wells (same if you just want to look at a single target).
- You can right click on any graph and save it as a JPEG then off-load it to a flash drive.
- Select “view well table” on the right side.
- This will show you the calculated C<sub>T</sub>, delta C<sub>T</sub>, and delta delta C<sub>T</sub> (but I don’t think I have the file setup right to give the correct delta delta C<sub>T</sub>).
- If you go to “file” and “print report” then select “print preview”, a report will be generated (Or there is a “print report” option on the top ribbon).
- Then you can print the report, or save the report file to the desktop and offload it to a USB for each group.
- This will give each group their C<sub>T</sub> values for each well that they can plug into the spreadsheet to calculate delta C<sub>T</sub> and delta delta C<sub>T</sub> values.
- When the options for how to generate the report pop up, select “amplification plot delta run vs cycle.”
Appendix 4: Teaching notes (including daily flow and reference list).

Daily Flow

Day 1 Morning: We begin with orientation and introductions. Rules and regulations, goals of the module, and evaluation of student work are discussed. Days 1 and 2 are lecture-heavy, but after that point, more time is spent in the lab. Day 1 lecture includes a primer on inflammation and discussion of mammalian tissue culture and transfection. Students form small groups of two to three, and groups are assigned specific inflammatory mediator(s). Students are asked to begin researching their assigned mediator(s) so that they may present their findings to their peers the following afternoon. Reading for Day 2 (cytokine review article and Lab Manual (LM) Day 2) is assigned.

Day 1 Afternoon: Students harvest nuclear lysates from RAW264.7 cells that were treated with the mediators for 0, 6, or 12 hours. In the interest of time, our lab aides begin treatment of the cells the night before and early in the morning on Day 1. This way, the students can just harvest their lysates in the afternoon on Day 1. Cells are treated with the mediators multiple times during the module, so students do get a chance to perform treatments themselves.

Day 2 Morning: There is a quiz on cytokines, inflammation, transfection, and plasmids. The lecture includes information on plasmids, transfection, and specifics of the pNFκB-Luc reporter plasmid and luciferase reporter gene assay.

Day 2 Afternoon: Students perform their first tissue culture and sterile technique, and they must be monitored carefully. An additional lecture in the afternoon introduces the concept of the ELISA in preparation for Day 3. Students present their research on their designated mediator(s). Using this information, they develop hypotheses for the ELISA and Luciferase Assay experiments. Day 3 reading is assigned (LM Day 3).

Day 3 Morning: Due to timing constraints, students first treat their cells with the mediators for the Luciferase assay. This is followed by a quiz on ELISAs and Reporter assays. The Day 4 reading is assigned (LM Day 4), and a short review lecture on TNFα and macrophages is conducted.

Day 4: The quiz covers TNFα and macrophages. Most students have not produced a scientific poster in the required format, so guidelines for poster generation are provided. Students are asked to begin working on their posters immediately and daily as they obtain each piece of data. Reading for Day 5 is assigned (LM Day 5 and NFκB review articles) along with post-lab questions (due Day 5) for the Luciferase reporter assay and the ELISA. The ELISA includes multiple lengthy incubation periods, during which additional
information about NFκB subunits p50 and p65 is presented. A spreadsheet for analysis of the ELISA data (Appendix 10) is emailed to students.

Day 5 Morning: The post-lab assignment is collected. During the hour long electrophoresis the western blot procedure is explained.

Day 5 Afternoon: In the interest of time, probing of western blots is initiated by lab aides. Students pick up the blotting procedure when they are finished with their transfections. Day 6 reading is assigned (LM qPCR section). As soon as blots are developed and imaged, image files are emailed to students so they may initiate work preparing western blot figures and data analysis.

Day 6 Morning: We begin with the qPCR quiz, followed by a qPCR lecture.

Day 6 Afternoon: qPCR data as well as a qPCR analysis spreadsheet (Appendix 10) are emailed to students so they may begin data analysis. Western blot data is also analyzed. It works well to break the group in half such that some students work on qPCR data with one faculty while the other students work on western data with another faculty. Day 7 reading is assigned (LM Day 7).

Day 7 Morning: Students are quizzed on NFκB, cytoplasm to nucleus translocation, and fluorescence microscopy. Again, in the interest of time, lab aides treat cells with the mediators in the early morning. Instructors recap of NFκB activity.

Day 7 Afternoon: Students finish any remaining analyses. When fluorescence microscopy data are acquired, images are emailed to students.

Day 8: Students complete their posters by noon and spend the afternoon preparing oral poster presentations. They also work together or separately to complete post-lab questions on qPCR, western blot, and fluorescence microscopy; these are due on Day 9.

Day 9 Morning: Students complete the MASE Assessment Tool (30 minutes) and then participate in an hour-long poster session.

Reference List:

Appendix 5: MASE Assessment Tool (MAT).

MASE Assessment

Name: ________________________________________

(Parts 1-2 are handed out first. Upon completing and turning in Parts 1 and 2, students receive Part 3)

Part 1. Usefulness/functionality of various techniques

1. Which of the following techniques utilize antibodies? **Circle all that apply.**
   a. Luciferase assay
   b. ELISA
   c. Western blot analysis
   d. Restriction enzyme analysis
   e. Transfection
   f. qPCR (also known as real-time PCR)

2. Which of the following techniques are used to detect either the presence of a protein or the activity of a protein? **Circle all that apply.**
   a. Luciferase assay
   b. ELISA
   c. Western blot analysis
   d. Fluorescent microscopy
   e. qPCR (also known as real-time PCR)

3. Which of the following techniques are used to analyze transcription but **NOT** translation? **Circle all that apply.**
   a. Luciferase assay
   b. ELISA
   c. Western blot analysis
   d. Fluorescent microscopy
   e. Transfection
   f. qPCR (also known as real-time PCR)
4. Which of the following techniques utilize an enzyme? **Circle all that apply.**

   a. Luciferase assay
   b. ELISA
   c. Western blot analysis
   d. Fluorescent microscopy
   e. Transfection
   f. qPCR

5. Which of the following techniques can provide information about the specific location (compartment/organelle) of a protein inside a cell? **Circle all that apply.**

   a. Luciferase assay
   b. ELISA
   c. Western blot analysis
   d. Fluorescent microscopy
   e. Transformation
   f. qPCR

6. Define transfection, and state the value of transfection in ONE of the experiments you conducted during MASE.

   ________________________________________________________________
   ________________________________________________________________
   ________________________________________________________________

7. Retrospective question - Prior to MASE, did you possess the knowledge to correctly answer any of the above questions (1-6)? If yes, please state which ones.

   ________________________________________________________________
   ________________________________________________________________
   ________________________________________________________________
   ________________________________________________________________
   ________________________________________________________________
   ________________________________________________________________
Part 2. Data format and presentation

8. The data shown below were generated by which **ONE** of the following techniques?
   a. Luciferase assay
   b. ELISA
   c. Western blot analysis
   d. Fluorescent microscopy
   e. Transfection
   f. qPCR

![Graph](image1.png)

9. The data shown below were generated by which **ONE** of the following techniques?
   a. Luciferase assay
   b. ELISA
   c. Western blot analysis
   d. Fluorescent microscopy
   e. Transfection
   f. qPCR

![Graph](image2.png)
10. The data shown below were generated by which one of the following techniques?

   a. Luciferase assay
   b. ELISA
   c. Western blot analysis
   d. Fluorescent microscopy
   e. Transfection
   f. qPCR

11. The data shown below were generated by which of the following techniques? Circle all that apply.

   a. Luciferase assay
   b. ELISA
   c. Western blot analysis
   d. Fluorescent microscopy
   e. Restriction enzyme analysis
   f. qPCR
12. The data shown here were generated by which ONE of the following techniques?

a. Luciferase assay  
b. ELISA  
c. Western blot analysis  
d. Fluorescent microscopy  
e. Transfection  
f. qPCR

13. Retrospective question - Prior to MASE, did you possess the knowledge to correctly answer any of the above questions (8-12)? If yes, please state which ones.
Part 3. Data Analysis and Interpretation. Please neatly write a 2-3 sentence answer for each question.

(This part is to be handed out and completed after Parts 1 and 2 have been turned in.)

14. qPCR- According to the data shown below, which gene demonstrates the greatest change in expression between treated and untreated conditions? In your answer, please refer to specific data from the table.

Greatest change:

Evidence:

<table>
<thead>
<tr>
<th></th>
<th>β-actin untreated</th>
<th>β-actin treated</th>
<th>iNOS untreated</th>
<th>iNOS treated</th>
<th>NFκB untreated</th>
<th>NFκB treated</th>
<th>TNFα untreated</th>
<th>TNFα treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt; (average)</td>
<td>19.67</td>
<td>21</td>
<td>33.33</td>
<td>26.67</td>
<td>30.33</td>
<td>30</td>
<td>35.5</td>
<td>25.83</td>
</tr>
<tr>
<td>ΔΔC&lt;sub&gt;T&lt;/sub&gt; (absolute values)</td>
<td>8</td>
<td>+/-1.15</td>
<td>1.67</td>
<td>+/- 1.41</td>
<td>+/-1.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(average +/- standard deviation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
15. ELISA- According to the data shown below, what is the effect of the mediator? In your answer, please refer to specific data from the figure.

Effect:

Evidence:

16. Luciferase Assay- According to the data shown below, what is the effect of the mediator? In your answer, please refer to specific data from the figure.

Effect:

Evidence:
17. Western blot analysis- According to the data shown below, what is the overall effect of the mediator? In your answer please refer to specific data from the figure.

Effect:

Evidence:

<table>
<thead>
<tr>
<th>Nuclear Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr + Mediator</td>
</tr>
<tr>
<td>p50 NFκB</td>
</tr>
<tr>
<td>β-2 microglobulin</td>
</tr>
</tbody>
</table>

18. Fluorescent microscopy- According to the data shown here, what is the effect of the mediator after 2 hours of treatment? In your answer please refer to specific data from the figure.

Effect:

Evidence:

<table>
<thead>
<tr>
<th>Empty vector-GFP</th>
<th>Protein X-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>untreated</td>
</tr>
<tr>
<td>1</td>
<td>a</td>
</tr>
<tr>
<td>2</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>c</td>
</tr>
<tr>
<td>4</td>
<td>d</td>
</tr>
<tr>
<td>5</td>
<td>e</td>
</tr>
<tr>
<td>6</td>
<td>f</td>
</tr>
</tbody>
</table>

- GFP only
- DAPI only
- GFP merged with DAPI
Appendix 6: Quizzes and post-lab worksheets.

Quizzes 1-5 are shown below. All quizzes were graded out of 10 points. In each case the answer is indicated in bold.

**MASE Quiz 1**

1. Cytokines are:
   a. Chemicals released by bacteria during an infection
   b. **Important regulatory molecules that help govern the immune response**
   c. Only released when a person is sick
   d. Only produced by specialized glands

2. Which of the following occur as part of the inflammatory response?
   a. Vasodilation
   b. Swelling (edema)
   c. Increased blood vessel permeability
   d. Release of cytokines
   e. **All of the above**

3. Which of the following is NOT a general characteristic of inflammation?
   a. Pain
   b. Heat
   c. **Bruising**
   d. Redness
   e. Swelling

4. Plasmids are:
   a. **Small circular pieces of DNA that can carry genes**
   b. Part of the bacterial chromosome
   c. Only able to be taken up by mammalian cells
   d. Only able to be taken up by bacterial cells

5. The transfer of plasmids into mammalian cells is called:
   a. Transformation
   b. **Transfection**
   c. Lipofectamine
   d. Immortalization
MASE Quiz 2

1. During a positive luciferase reporter assay, which of the following is observed?
   a. Bioluminescence
   b. Fluorescence
   c. Both bioluminescence and fluorescence

2. Luciferase is:
   a. An enzyme that produces light given the proper substrate
   b. A substrate needed for a bioluminescent reaction
   c. An enzyme involved in the inflammatory response
   d. A cytokine that activates mouse macrophages

3. In the transfection that you did yesterday, the plasmid that you used contained the luciferase gene downstream of the ______ enhancer.
   a. TNF alpha
   b. NFkB
   c. Lipopolysaccaride
   d. Interferon gamma

4. Reporter assays are commonly used to:
   a. Assess whether a gene of interest is transcribed and translated
   b. **Assess only whether a gene of interest is transcribed**
   c. Determine how much of a substance is secreted into cell culture media
   d. Determine the viability of cells

5. Proteins that bind to promoter elements of genes to drive production of the messenger RNA are called:
   a. Transcription factors
   b. Restriction enzymes
   c. Ligases
   d. Introns

6. In the ELISA that you will perform, you are assaying for the presence of what molecule?
   a. NFkB
   b. **TNF alpha**
   c. Lipopolysaccaride
   d. Luciferase
   e. Interferon gamma
7. The **FIRST** step of the ELISA is to:
   
   a. Add the media from your treated cells  
   b. Add the avidin-coupled enzyme  
   c. **Add the capture antibody**  
   d. Add the substrate

8. The color change in an ELISA is ______ proportional to the amount of the substance for which you are assaying.
   
   a. Directly  
   b. Indirectly

9. The **LAST** step of the ELISA is to:
   
   a. Add the media from your treated cells  
   b. Add the avidin-coupled enzyme  
   c. Add the capture antibody  
   d. **Add the substrate**

10. The ELISA has many wash steps. These are important for:
    
    a. Amplifying the signal, such that a strong reading is obtained from the spectrophotometer  
    b. Allowing the biotin-streptavidin interaction to occur  
    c. **Reducing background caused by nonspecific carryover of reagents from one step to the next**

**MASE Quiz 3**

1. TNF-alpha is a:
   
   a. Transcription factor  
   b. Polymerase  
   c. **Cytokine**  
   d. Amino acid

2. TNF-alpha is:
   
   a. Produced by macrophages only  
   b. Produced by macrophages and other white blood cells  
   c. **Produced by many cell types in the human body, including macrophages**

3. After the TNF-alpha protein is produced, where does it go to mediate its biological effects?
   
   a. To the nucleus  
   b. To the cytoplasm
c.  **Outside of the cell**

4. TNF-alpha binds to two receptors: TNFR1 and TRNF2. Both receptors bind TNF-alpha with high affinity (meaning that the binding is strong), but the kinetics are different. TNFR1 binds to TNF-alpha:

   a. **Reversibly (meaning it repeatedly binds and unbinds)**
   b. Irreversibly (meaning it stays bound)

5. Binding of TNF-alpha to the TNFR (TNF-alpha receptor) launches a signaling cascade, ultimately resulting in the transcription of NFkB only and no other target genes.

   a. True
   b. False

6. Macrophages are immune cells that work by:

   a. Secreting antibodies
   b. **Phagocytosis**
   c. Agglutination (clumping cells together)

7. Where are macrophages located in the body?

   a. Blood
   b. Liver
   c. Lungs
   d. Gastrointestinal tract
   e. **All of the above**

8. Macrophages:

   a. Secrete cytokines
   b. Respond to cytokines
   c. **Both secrete and respond to cytokines**

9. TNF-alpha is primarily considered to be a:

   a. **Pro-inflammatory cytokine**
   b. Anti-inflammatory cytokine

10. Many diseases are marked by chronic inflammation, including rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, and sepsis. As a potential therapy, what class of molecules is currently being used in clinical trials to treat these chronic inflammatory disorders?

    a. TNF-alpha agonists (drugs that mimic TNF-alpha)
    b. **TNF-antagonists (drugs that block TNF-alpha)**
MASE Quiz 4

1. A basic PCR reaction mix contains all of the following EXCEPT:
   a. dNTPs
   b. Primers
   c. Template
   d. Calcium
   e. Magnesium

2. A real time (quantitative) PCR reaction mix is different from a traditional PCR mix in that it contains:
   a. Radioisotopes
   b. Primers
   c. Fluorescent labels
   d. dNTPs
   e. Template DNA

3. Real time (quantitative) PCR is measured during which phase of the PCR run:
   a. Exponential phase
   b. Linear phase
   c. Plateau phase

4. In which phase of the real time (quantitative) PCR reaction do the reagents begin to run out and the reaction slows down?
   a. Exponential phase
   b. Linear phase
   c. Plateau phase

5. The point at which a real time (quantitative) PCR reaction reaches a signal higher than background is called:
   a. Threshold
   b. Plateau
   c. Linear
   d. Amplicon peak

6. Which detection chemistry is more specific to the desired amplicon?
   a. SYBR Green
   b. TaqMan

7. The TaqMan probe format uses all of the following EXCEPT:
   a. Reverse transcriptase
After PCR, 2 primers, template DNA, Taq DNA polymerase, probe with a fluorescent reporter and quencher, and 8. When is new fluorescence emitted from the reaction?

a. During the melting phase when the 2 DNA strands separate
b. During the annealing phase when the TaqMan probe binds to the template DNA
c. During the extension phase when the TaqMan probe is cleaved from the template
d. All of the above

9. Which gene will you be using as a “housekeeping gene” for normalization purposes?

a. iNOS
b. TNF-alpha
c. NFkB
d. β-actin

10. A gene that is transcribed at a HIGHER level than another gene would have:

a. A higher cycle threshold ($C_T$)
b. A lower cycle threshold ($C_T$)

**MASE Quiz 5**

1. NFkB expression is:

a. Ubiquitous (meaning that all or nearly all cell types express it)
b. Limited (only certain specific cell types express it)

2. IKK is a:

a. Transcription factor
b. Kinase
c. Inhibitor of NFkB

3. The NFkB proteins bind DNA as:

a. Monomers
b. Dimers
c. Trimers
d. Large protein complexes

4. The name of the protein that sequesters NFkB in the cytoplasm in unstimulated cells is:
a. IKK  

b. IKB  

c. RelA  

d. p50

5. NFKB dimers composed of p50/p50 subunits:
   a. Activate transcription of target genes  
   b. Repress transcription of target genes

6. A pro-inflammatory stimulus capable of activating NFkB would do which of the following FIRST:
   a. Cause NFkB to translocate to the nucleus  
   b. Cause NFkB to bind to the promoter sequence of a target gene  
   c. Cause phosphorylation of IKB  
   d. Cause activation of IKK

7. NFkB has negative feedback control, wherein activation of NFkB ultimately causes more NFkB to be localized in the cytosol where it is inactive.
   a. True  
   b. False

8. The IKB proteins all contain a common domain essential for their function. This domain is:
   a. An ankyrin repeat  
   b. A nuclear localization signal  
   c. A DNA binding domain  
   d. A kinase domain

9. Suppose you had a drug that increased expression of IKB. What do you postulate would be the downstream effect on TNF-alpha expression?
   a. TNF-alpha expression would increase  
   b. TNF-alpha expression would decrease

10. Post-translational modifications of NFkB including ubiquitination, phosphorylation, hydroxylation and acetylation all affect NFkB function as a transcription factor.
   a. True  
   b. False
Post Lab Worksheets:

**Week 1: Data Analysis Worksheet – ELISA Experiment**  
(1 point each = 10 points total)

1. State the experimental question that you are addressing in this experiment.

2. State your inflammatory stimuli and hypothesis that you developed regarding how the treatment with inflammatory stimuli affected your independent variable.

3. Explain the rationale for your hypotheses; that is, why does your hypothesis make sense?

4. What is the independent variable (the variable being manipulated)? What are the levels of the independent variable? What is your control treatment?

5. What is the dependent variable (the variable that you are measuring)? What procedure did you use to quantify your measurement of the dependent variable? Briefly explain the specifics of the procedure. What were you directly measuring in this assay?

6. Identify your standardized variables (the variables that must remain the same throughout your experiment), and describe how you standardized them.

7. Produce a figure that best illustrates the data collected in this experiment. Be sure to include a figure legend (caption) that describes how the data was obtained.

8. Summarize your ELISA data (be sure to include numerical values in your summary) and provide a statement of how your data relates to your hypothesis. Does your data support your hypothesis? What does that mean in terms of the question that you were asking?

9. If you were to do the experiment again, are there any modifications that you would make? Explain.

10. Based on the results from this experiment, discuss the NEXT experiment that you would design?
Week 1: Data Analysis Worksheet- Luciferase Assay
(1 point each = 10 points total)

1. State the experimental question that you are addressing in this experiment.

2. State your inflammatory stimuli and hypothesis that you developed regarding how the treatment with inflammatory stimuli affected your independent variable.

3. Explain the rationale for your hypotheses; that is, why does your hypothesis make sense?

4. What is the independent variable (the variable being manipulated)? What are the levels of the independent variable? What is your control treatment?

5. What is the dependent variable (the variable that you are measuring)? What procedure did you use to quantify your measurement of the dependent variable? Briefly explain the specifics of the procedure. What were you directly measuring in this assay?

6. Identify your standardized variables (the variables that must remain the same throughout your experiment), and describe how you standardized them.

7. Produce a figure that best illustrates the data collected in this experiment. Be sure to include a figure legend (caption) that describes how the data was obtained.

8. Summarize your luciferase data (be sure to include numerical values in your summary) and provide a statement of how your data relates to your hypothesis. Does your data support your hypothesis? What does that mean in terms of the question that you were asking?

9. If you were to do the experiment again, are there any modifications that you would make? Explain.

10. Based on the results from this experiment, discuss the NEXT experiment that you would design?
Week 2: Data Analysis Worksheet- Gene Expression (Western Blot and qPCR)

(2 points each = 20 points total)

*Note: In this worksheet many of your responses you will need to provide two answers – one for the Western Blot Experiment and one for the qPCR Experiment.

1. State the experimental question that you are addressing in this experiment.

2. State your inflammatory stimuli and hypothesis that you developed regarding how the treatment with inflammatory stimuli affected your independent variable.

3. Explain the rationale for your hypotheses; that is, why does your hypothesis make sense?

4. What is the independent variable (the variable being manipulated)? What are the levels of the independent variable? What is your control treatment?

5. What is the dependent variable (the variable that you are measuring)? What procedure did you use to quantify your measurement of the dependent variable? Briefly explain the specifics of the procedure. What were you directly measuring in this assay?

6. Identify your standardized variables (the variables that must remain the same throughout your experiment), and describe how you standardized them.

7. Produce a figure that best illustrates the western blot data and a 2nd figure that best illustrates your qPCR data collected in this experiment. Be sure to include figure legends (captions) below each figure that describe how the data was obtained.

8. Summarize your Western and qPCR data (be sure to include numerical values in your summary) and provide a statement of how your data relates to your hypothesis. Does your data support your hypothesis? What does that mean in terms of the question that you were asking?

9. If you were to do the experiment again, are there any modifications that you would make? Explain.

10. Based on the results from this experiment, discuss the NEXT experiment that you would design?
Week 2: Data Analysis Worksheet - Fluorescence Microscopy
(1 point each = 10 points total)

1. State the experimental question that you are addressing in this experiment.

2. State your inflammatory stimuli and hypothesis that you developed regarding how the treatment with inflammatory stimuli affected your independent variable.

3. Explain the rationale for your hypotheses; that is, why does your hypothesis make sense?

4. What is the independent variable (the variable being manipulated)? What are the levels of the independent variable? What is your control treatment?

5. What is the dependent variable (the variable that you are measuring)? What procedure did you use to quantify your measurement of the dependent variable? Briefly explain the specifics of the procedure. What were you directly measuring in this assay?

6. Identify your standardized variables (the variables that must remain the same throughout your experiment), and describe how you standardized them.

7. Produce a figure that best illustrates the data collected in this experiment. Be sure to include a figure legend (caption) that describes how the data was obtained.

8. Summarize your fluorescence microscopy and provide a statement of how your data relates to your hypothesis. Does your data support your hypothesis? What does that mean in terms of the question that you were asking?

9. If you were to do the experiment again, are there any modifications that you would make? Explain.

10. Based on the results from this experiment, discuss the NEXT experiment that you would design?
Appendix 7: Student evaluation of MASE.

2012 MASE Student Evaluation

Scale: 5 = Excellent; 4 = Very Good; 3 = Satisfactory; 2 = Poor; 1 = Unsatisfactory; 0 = N/A

1. The instructor made the objectives of the course clear.
2. The instructor organized the course to achieve the objectives.
3. The instructor was generally well prepared for class.
4. The instructor responded to questions adequately.
5. The instructor communicated clearly and effectively.
6. The lab was well organized and pertinent to the class.
7. The textbook explained things very clearly and was used effectively.
8. The instructor showed concern for the needs of individual students.
9. Overall, rate this instructor.
10. The level of difficulty of the course was: (5 = too hard; 3 = just right; 1 = too easy)

Open ended questions:

11. What did you like about the instructor and the course?
12. What did you dislike about the instructor and the course?
13. Please give any additional constructive comments.
### Appendix 8: Results from 2011 and 2012 cohorts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Luciferase Assay (fold change in NFkB promoter activity in treated cells compared to untreated cells)</th>
<th>ELISA (fold change in TNF-alpha concentration in treated cells compared to untreated cells)</th>
<th>Fluorescence Microscopy (presence of NFkB in the nucleus following treatment with mediator)</th>
<th>qPCR (fold change in gene expression in treated cells compared to untreated cells; less than 2 fold difference cannot be reliably considered a change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>No change</td>
<td>Increase ~30 fold</td>
<td>Yes</td>
<td>iNOS increases 35-60,000 fold; TNFa increases ~10 fold; NFkB does not change</td>
</tr>
<tr>
<td>IFN-g</td>
<td>Increase ~2 fold</td>
<td>Increase ~18 fold</td>
<td>Yes</td>
<td>iNOS increases 6-25,000 fold; TNFa increases ~10 fold; NFkB does not change</td>
</tr>
<tr>
<td>LPS + IFN-g</td>
<td>Increase ~2 fold</td>
<td>Increase ~28 fold</td>
<td>Yes</td>
<td>iNOS increases 60-80,000 fold; TNFa</td>
</tr>
<tr>
<td>Condition</td>
<td>Change</td>
<td>Increase</td>
<td>Decrease</td>
<td>iNOS</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------</td>
<td>----------</td>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>PMA</td>
<td>Decrease ~2 fold</td>
<td>Increase ~2 fold</td>
<td>No</td>
<td>iNOS increases 2-3 fold; TNFa increases 1-2 fold; NFkB increases 1-2 fold</td>
</tr>
<tr>
<td>PHA</td>
<td>No change</td>
<td>Increase ~5 fold</td>
<td>Yes</td>
<td>iNOS increases 12-20,000 fold; TNFa increases 1-2 fold; NFkB does not change</td>
</tr>
<tr>
<td>PMA + PHA</td>
<td>Increase ~2 fold</td>
<td>Increase ~4 fold</td>
<td>Yes</td>
<td>iNOS increases 3 fold; TNFa increases 1-2 fold; NFkB increases 1-2 fold</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>Decrease ~2 fold</td>
<td>Decrease ~2 fold</td>
<td>No</td>
<td>iNOS increase 1-2 fold; TNFa increases 1-2 fold; NFkB does not change</td>
</tr>
</tbody>
</table>
Appendix 9: Assessment tool data part 3.

**Question 14 - qPCR.** According to the data shown below, which gene demonstrates the greatest change in expression between treated and untreated conditions? In your answer, please refer to specific data from the table.

**Correct** - 85.7% identified TNFα and provided ΔΔCT evidence. **Incorrect** - The remaining students chose NFkB and cited the highest standard deviation value.

**Question 15 - ELISA.** According to the data shown below, what is the effect of the mediator? In your answer, please refer to specific data from the figure.

**Correct** - 90.5% stated that the mediator increases TNFα levels and provided TNFα concentration changes as evidence. **Partially Correct** - The remaining students stated the mediator increases inflammation through the function of NFkB and cited the increased TNFα concentration (while not completely incorrect, this answer fails to address the direct effect of the mediator and instead states an implication of the data).

**Question 16 - Luciferase assay.** According to the data shown below, what is the effect of the mediator? In your answer, please refer to specific data from the figure.

**Correct** - 52.4% stated the mediator increases NFkB transcription and cited increased relative light units. 9.5% stated the mediator increases transcription of the luciferase gene and cited increased relative light units. 14.3% stated the mediator increases light generation and cited increased relative light units. **Partially Correct (state an implication rather than direct effect of mediator)** - 14.3% stated the mediator stimulates inflammation via NFkB and cited increased relative light units as evidence. 4.8% stated the mediator increases inflammation and cited increased relative light units. **Incorrect** - 4.8% stated the mediator increases the substrate and cited increased relative light units.

**Question 17 - Western blot.** According to the data shown below, what is the overall effect of the mediator? In your answer please refer to specific data from the figure.

**Correct** - 71.4% stated the mediator caused increased NFkB levels in the nucleus because band intensity increased from 0-6 hours. 14.3% stated the mediator increased production of NFkB p50 because band intensity increased from 0-6 hours. **Incorrect** - 9.5% stated the mediator decreased NFkB because intensity of the NFkB bands was not as great as the β-2 microglobulin (loading control) bands. 4.8% stated the mediator had no effect because the NFkB band intensity does not change between 6 and 12 hours.

**Question 18 - Fluorescence microscopy.** According to the data shown here, what is the effect of the mediator after 2 hours of treatment? In your answer please refer to specific data from the figure.

**Correct** - 38.1% stated the mediator caused the protein to move to the cytoplasm and cited movement of green fluorescence from the nucleus to the cytoplasm. **Partially Correct** - 42.9% stated the protein was only present in the cytoplasm and cited green fluorescence in the cytoplasm. **Incorrect** - 9.5% stated the mediator had no effect because there was no green fluorescence in the nucleus. 9.5% focused on the nuclear DAPI staining rather than the GFP-Protein X fusion protein.
Appendix 10: Spreadsheets for qPCR and ELISA data analysis.

For ELISA and qPCR, we have developed Microsoft Excel spreadsheets that allow students to plug-in their data and easily analyze it using the embedded formulas. Please contact the corresponding author, and the files will be emailed to you.