

IMMUNOLOGY
MBIO 4010
LAB MANUAL

2006

MBIO 4010 SCHEDULE 2007

Title/Description	Week #	Date
Lab 1: Preparation of Sheep IgG	1	Jan 11
Lab 2: Immunoprecipitation (Part I & II)	2	Jan 18
Lab 2: Immunoprecipitation (Part III & IV)	3	Jan 25
Lab 3: Hemagglutination Assay (Part A)	4	Feb 1
Lab 3: Hemagglutination Assay (Part B)	5	Feb 8
MID-TERM BREAK	6	Feb 15
Lab 4: Enzyme Linked Immunosorbent Assay (ELISA)	7	Feb 22
QUIZ on lab 5 background information/protocol (15 min)	8	Mar 1
Lab 5: Part I: Induction and characterization of a primary cytokine response <i>in vivo</i> . lab starts at 1:45 - be there 5 min early Part II: ELISA precision training. Immunology Department, 6 th floor Basic Medical Science Bldg (Bannatyne Campus)	9	Mar 8
Lab 5: Part III: ELISA ASSAY- Quantitative analysis of IL-13 Response lab starts at 1:45 - be there 5 min early Immunology Department, 6 th floor Basic Medical Science Bldg (Bannatyne Campus)	10	Mar 15
Lab Exam	12	Mar 29

All labs start at 2:30 pm unless otherwise stated.

Lab report	Due dates
Lab 1 report	Jan 25
Lab 2 report	Feb 8
Lab 3 report	Feb 22
Lab 4 Report due	Mar 8
Lab 5 report due	Mar 22

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**Lab 5 & 6 by Dr. Kent Hayglass and Immunology Graduate Students
Location: Immunology Department, 6th floor of Basic Medical Science Bldg.**

INSTRUCTORS

Lab Instructor:
Teaching Assistant:

Dr. L. Cameron
Jyothi Seethuraman

Office: 414B
Lab: 401

Lab Location: 201 Buller

Website:

www.umanitoba.ca/faculties/science/microbiology/staff/cameron/

OR via University of Manitoba Microbiology Homepage:

<http://www.umanitoba.ca/faculties/science/microbiology/labinformation.htm>

Information available at the website: changes/corrections, additional information, data, marks

GENERAL INSTRUCTIONS**REGULATIONS**

1. Lab attendance is compulsory.
2. Must wear a lab coat. Bring a permanent marker.
3. Read lab standard operations procedure before attending lab.
4. Students work in pairs.

EVALUATION

1. The lab is worth 20% of the final course mark; 8% for lab reports and quiz and 12% for lab examination.
2. You must pass the lab to pass the course (10/20%).
3. The lab reports due dates are listed in the schedule. All lab reports must be handed in on due date (by 4:30 pm) or one mark is subtracted for each class day thereafter until all marks are gone. Marked lab reports will be returned to students the next week. A late report will not be accepted after that report has been returned to the class. **Hand in lab reports to slotted drawer in room 204 Buller ONLY (demonstrators will not accept reports).**
4. Lab report value varies depending on the lab, as some lab reports are far more complex than other lab reports. Total marks will be given on each lab report. The marks are added together and divided by total to give a final mark out of 8%.
5. The lab exam takes place during scheduled lab period (refer to schedule for date). The lab exam is 1.5 hours and must be written in pen.
6. Lab report marks are final unless an obvious error in addition of marks has been made. If a student feels they have a legitimate complaint, please direct attention to lab instructor.
7. Approximately two weeks prior to the lab exam, a brief outline of lab exam format and information content will be available on the website.
8. You must notify the lab instructor no later than two school days after the missed lab. A Doctor's certificate is required for a missed lab exam. All deferrals will write the lab exam at a scheduled time set by the instructor. Failure to comply will result in a zero on your lab exam.
9. **Plagiarism (copying another student's lab report (present or previous year) or copying published literature without citing is a violation of University regulations. Refer to the STUDENT DISCIPLINE BY-LAW in your student handbook (rule book) for action taken for plagiarism.**

LAB REPORT PRESENTATION

[Before handing in your report review report to ensure that all information is included. When printing Excel spreadsheets make sure you have selected all information before printing. If you are using text boxes, they must be completely within the selected area or they do not print.]

1. All reports must have an Honesty Declaration attached at end of report. Available as a pdf file on lab website.
2. Lab reports may be done as an individual effort or a group effort by the two students that carried out the experiment. The decision on the number of reports per group is totally dependent on members of the group. This decision may be changed any time during the term. Therefore for each lab report the group has the option to hand in one or two reports exclusive of what has been done before or after that particular report. Indicate on the cover page of the report if the report is a group report or an individual report. If handing in an individual report also include lab partner's name.
3. A reference file is available in the science library (1 hour reserve).
4. If an experiment requires class data for lab report analysis, it will be available on the website.
5. Lab reports must be **typed**. Up to 10% of the mark subtracted for reports not typed. No binders. Stapled left hand corner.
6. On the front page of the report state:
 - Course name and number
 - Experiment number and Title
 - Group #
 - Individual or Group name(s). If handing in an individual report, also include lab partners name.
 - GROUP report or INDIVIDUAL report
 - Date
7. Number pages.
8. Lab reports consist of data presentation, data analysis and possibly questions. The information is to be presented exactly as requested. Number sections the same as the lab manual.
9. Always include a sample of each calculation type.
10. If your group's data is 'not workable' or no data obtained, borrow data from another group and reference. It does not necessarily mean the correct or expected data. If data is not as expected always compare to expected data in analysis.
11. Cite reference in text of lab report and record full reference at end of lab report. When should you cite and reference. The following is a good definition of plagiarism that explains when you should cite a reference. **"The unacknowledged use of another person's work, in the form of original ideas, strategies, and research, as well as another person's writing, in the form of sentences, phases and innovative**

terminology." (Spatt¹, 1983, p.438) This is done by using bracketed reference number that you used when listing references at end of lab report or by bracketing first authors name and date. Quote text unless you paraphrase completely in your own words. But remember, quotes should only be a small part of your work. If you are using the name year system, list the references alphabetically. Some examples are as follows (McMillan² 1997):

Binder V. Hendriksen C, Kreiner S. 1985. Prognosis in Crohn's disease - - based on results from regional patient group from county of Copenhagen. *Gut* 26:146-50.
Danforth DN, editor. 1982. *Obstetrics and gynecology*. 4th ed. Philadelphia: Harper and Row. 1316 p.

Petter JJ. 1965. The lemurs of Madagascar. In: DeVore I, editor. *Primate behavior: field studies of monkeys and apes*. New York: Holt, Rinehart and Winston. p 2920319.
If journal article assessed on the internet, site as journal. However, if available only on the web, reference as follows:

Kingsolver JC, Srygley RB. Experimental analyses of body size, flight and survival in pierid butterflies. *Evol. Ecol. Res.* [serial online] 2000;2:593-612. Available from: Colgate University online catalog. Accessed 2000 Oct 3.

12. Personal or Professional Electronic sources²:

Cite in-text by putting the following in parentheses, author's last name or file name (if no author's name is available) and publication date or the date of access (if no publication date is available).

At the end of report list: author or organization, publication date or date last revised, title of Web site, URL site in angle brackets, and the date accessed.

Cameron, L. 60.344 Microbial Physiology Lab Information

<http://www.umanitoba.ca/faculties/science/microbiology/staff/cameron/60_344.htm>. Accessed 2004 April 12.

Table presentation^a (if format not available on the website)

- Table number and title (legend) presented above the table body.
- Number tables using arabic numbers, even if only one table in a report.
- Include enough information in title to completely describe table, eliminating the necessity to search elsewhere in the lab report to understand information presented in table. Table title starts with an incomplete sentence. Additional complete sentences may be included to adequately describe the table, eg. number of days of colony growth and temperature, media type, microorganism source (this also applies to figures).
- If abbreviations are used in table, indicate what abbreviations mean as a footnote. Other footnotes may be required to clarify material in the table.
- Like information should be in columns making it easier to view the table.
- Data in columns is listed under the centre of each heading. Align decimal points and dashes. If a number value is less than 1 always include zero before the decimal.
- Column or Row headings should be complete and self explanatory. A heading is a separate entity from the title. It cannot be assumed information given in the title is adequate for a heading. The unit of measurement should only be included in the heading, not in column data.
- Group related column headings under larger headings.

¹Spatt, B. (1983). *Writing from Sources*. New York: St. Martin's Press.

²McMillan V.E. 1997. *Writing Papers in the Biological Sciences*. 2nd ed. Boston: Bedford Books: 197 p. and McMillan, V.E. 2001. *Writing Papers in the Biological Sciences*. 3rd ed. Boston: Bedford Books. 123 p.

- If information is the same for each column or row do not include but treat as a footnote.
- Make the table as concise as possible but include all necessary information. For example, when presenting a table of bacteria colony characteristics it is important to state media type, incubation time and temperature as colony characteristics vary depending on these conditions somewhere in the table.
- Tables should be properly set up with a straight edge.

Figure presentation^a

(all graphs must be computer generated - where applicable, required Excel presentation procedure is given in the lab appendix)

- Figures include graphs, diagrams, photographs, films
- Figures are to be numbered separate from tables, using arabic numbers. Include figure number even if only one figure.
- Following the figure number a figure legend should be presented below graph. The figure legend, like the table, starts with an incomplete sentence describing the graph. For example, do not repeat just the labels of the x- and y-axis but present in a descriptive manner. Additional sentences should be included if additional information is required to completely describe figure, for example, abbreviations explanation, any constant experimental conditions, etc.
- All diagrams, photographs, and films are figures and should be completely labelled. For figures of graphs, there is one dependent variable plotted and one or more independent variables plotted. The dependent variable is a function of the independent variable. It is accepted practise to plot the independent variable on the x-axis and the dependent variable on the y-axis. For example the measurement of absorbance (dependent) with increasing concentration of protein (independent). The size of the graph should fit the plot(s). The axis should not necessarily start at zero. Place graph completely within graph grid, this includes axis labels and legend. The overall size of graph should not be too large but should not be so small that information is obscured. Graph must be completely labelled (always include units). Use different symbols for each plot (not different coloured pens) on a graph. If more than one plot explain symbols in legend or in a key included in the body of the graph. Graph plots can be drawn in a number of ways (this depends on the plot): (a) best straight line, (b) join the points with a straight line, and (c) use a curved ruler or french curve.
Note: Do not draw a free hand line.
- Complete label diagram figures.

Note: When writing your lab reports you are frequently requested to present both a table and a figure for a given set of data, similar to keeping a research journal. This is not the accepted practice for papers published in journals or books. Usually either a table or a figure is presented for a given set of data and depending on nature of data; it may only be summarized in the text. How do you make a choice of data presentation? The aim is to effectively and efficiently demonstrate what you want to show, for example, correlations, comparisons, pattern, trends, etc.

^a McMillan, V.E. 1997. Writing Papers in the Biological Sciences. 2nd ed. Boston: Bedford Books. 197 p.

LAB STANDARD OPERATIONS PROCEDURE (SOP)

Bench area: Wash bench area before and after use with AIRx109.

Personal safety: You must wear a lab coat. Wear coat only in the lab, transport separately outside of the lab (in a plastic bag). Wash hands with antibacterial soap before leaving the lab. No eating or drinking in the lab. Use aseptic technique for transfer of bacteria. This is to protect yourself as much as to ensure the purity of your culture. Protect hands with gloves and eyes with glasses when needed. The gloves provided in the lab are to be disposed of in Petri plate containers. . If you forget your lab coat, dark blue lab coats are available in the lab, sign IN/OUT sheet. Long hair must be tied back. Closed toed shoes must be worn.

Biohazards: Know biosafety risk groups. Handle all cultures as potential pathogens. Never mouth pipette. Always use a pro-pipette. If you spill a culture, cover the spill with paper towels. Pour AIRx109 over the towels to saturate. Gather up soaked towels and discard. Wipe area to dryness with fresh paper towels. Wash hands with soap and water. Place cultures on discard trolley. All cultures are autoclaved before disposing. Dispose of ependorf tubes^a in petri plate containers. Dispose of pipetman tips^a in clear plastic lined basins along with glass or plastic Pasteur pipets, broken glassware, glass slides, brittle plastic objects, metal objects^a (not needles or blades). Discard disposable pipettes (1 ml and 10 ml) in yellow plastic pails. Bacteria dilutions may be poured down the sink and the tubes rinsed before placing on the discard trolley. Rinse sink with lots of water.

When handling level 2 microorganisms you must wear disposable gloves, make sure any cuts on your hands are covered with a bandage, and be aware of the possibility of bacteria aerosol when you flame your loop.

^a due to the multi-use nature of the teaching lab, all ependorf tubes, pipetman tips, Pasteur pipets, brittle plastic or metal objects will be treated the same as similar items contaminated with microorganisms.

Animal Tissue: As usual rinse glassware and put on discard trolley. Dispose of any sharp items (tips, Pasteur pipettes, etc) in plastic lined white bucket on discard trolley. Dispose of all other animal tissue contaminated items (paper towels, gloves, microfuge tubes, etc) in Petri plate container. Dilute solutions may be disposed on in the sink with extensive rinsing. Dispose of liquid in animal liquid container on discard trolley. Autoclaved before discarding.

Glassware (unbroken): Remove tape and pen markings (use alcohol) from glassware before placing on discard trolley. Used glassware should be rinsed and placed on the discard trolley. Rinsed test tubes should be placed in tray provided on the discard trolley. Used glass pipettes should be placed in pipette holders.

Petri plate culture and non-sharps solid culture material disposal: use covered plastic containers lined with clear plastic bags for contaminated petri dishes or any bacteria contaminated solid non-sharps material (ependorf tubes, API strips, antibiotic strips, microtitration plates, etc)

Hazardous material disposal: Examples: radioactive material, ethidium bromide, solvents, etc. The lab demonstrator will instruct proper disposal methods for labs that contain hazardous materials. These materials must be disposed of in appropriately labelled containers and disposed via the safety office. Use fumehood when recommended. A MSDS binder available in lab gives information on all hazardous materials used in the lab. Use extreme care with flammable solvents. Alcohol used to flame spread rod should never be positioned within 40 cm of flame. Never put a very hot spread rod into a beaker of alcohol. The alcohol may catch fire. Many of the immunochemicals are preserved in 0.1% Na azide...handle with gloved hands. Handle caustic (acids and bases) solutions with care. Never discard an acid or base greater than one molar down the sink. Discard in labelled glass containers provided. Use lots of water when discard caustic solutions (< 1M). These materials are disposed of through the university safety office. Never pour solvents down the sink (eg. phenol, ether, chloroform, etc). Discard in labelled containers provided.

Sharps disposal: Dispose of all sharps (needles, pipetman tips, etc) in specified container. Dispose of syringe with needle attached - do not take apart. Do not replace the needle cap before disposing (high frequency of accidents occur when replacing cap). Sharp's containers are autoclaved before

disposing.

OR in some labs, **bacterial contaminated slides and pasteur pipette disposal**: Discard slides in designated plastic containers lined with clear plastic bags. This type of container may not be available in all labs. Container is autoclaved before disposing.

Broken glass disposal (not contaminated with bacteria): Dispose of broken glass in labelled plastic containers. Not lined with plastic. Transferred to boxes before discarding.

Know location: Exits, fire extinguisher, eye wash, sink shower, and first aid kit. This information is given in the first pre-lab.

Equipment operation: Know how to operate equipment before use. DO NOT use equipment unless you know exactly how to operate the equipment. The demonstrator is always available to assist. Please follow instructions in appendix for proper clean up of Spectronic 20D. Ensure the spec tubes are thoroughly washed and rinsed with distilled water before replacing in rack upside down as you (hopefully) found the tubes.

Leave your bench area clean All equipment and supplies should be returned to original location.

LABORATORY BIOSAFETY GUIDE

Although no bacteria are used in this lab, both Level 1 & 2 bacteria risk groups are used in this room by other labs. Follow standard operation procedures, SOP (see above).

The University of Manitoba Biosafety Guide (Feb 2000) and Health Canada Laboratory Biosafety Guidelines booklets are available in your lab. Biosafety information is also available at the Health Canada websites:

Guidelines: <http://www.hc-sc.gc.ca/hpb/lcdc/biosafety/docs/index.html>

MSDS (infectious agents): <http://www.hc-sc.gc.ca/hpb/lcdc/biosafety/msds/index.html>

There is no listing of level 1 agents in the guidelines or MSDS pamphlets

Risk group 1 bacteria are low individual and community risk and are unlikely to cause disease in healthy workers.

Risk group 2 bacteria are moderate individual risk and limited community risk. Bacteria in this group can cause human or animal disease but are unlikely to infect healthy laboratory workers. Effective treatment is available. Risk of spreading is limited.

CONTAINMENT LEVEL 1 (UM biosafety guide p. 11)

- microbiology lab with washable walls, countertops and hand wash sink
- established safe laboratory practices (hand washing and disinfection of countertops)
- general WHMIS safety training
- UM lab registration

CONTAINMENT LEVEL 2 (UM biosafety guide p.11)

- all of level 1 specifications
- biosafety permit
- biological safety cabinet (not required)
- biohazard signage
- a written standard operations procedure
- MSDS for the infectious agent

WHMIS

The Workplace Hazardous Materials Information System (WHMIS) is a system for safe management of hazardous materials. WHMIS is legislated by both the federal and provincial governments.

Under WHMIS legislation, laboratories are considered to be a workplace, and students are workers. By law, all workers must be familiar with the basic elements of the WHMIS system.

The WHMIS program includes:

1. Cautionary labels on containers of controlled products. Consumer products, explosives, cosmetics, drugs and foods, radioactive materials, and pest control products are regulated separately, under different legislation.
2. Provision of a Material Safety Data Sheet (MSDS) for each controlled product.
3. A worker education program

1. A. SUPPLIER LABELS

Controlled products must have a label of prescribed design which includes the following information:

PRODUCT IDENTIFIER - trade name or chemical name
SUPPLIER IDENTIFIER - supplier's name and address
MSDS REFERENCE - usually, "See MSDS supplied"
HAZARD SYMBOL - (see illustration on next page)
RISK PHRASES - describes nature of hazards
PRECAUTIONARY MEASURES
FIRST AID MEASURES

B. WORKPLACE LABELS

All material dispensed in a workplace container must be labelled with the **Product Name**, **Precautionary Measures** (simplified) and **Reference to Availability of MSDS**.

2. MSDS












Individual course MSDS are located in a binder in your lab (Room 201 binder located in 204). The main MSDS binders are located in the Microbiology preparation room, 307/309 Buller. MSDS are also available on the local area computer network (see your demonstrator, if necessary).

The MSDS will provide: relevant technical information on the substance, chemical hazard data, control measures, accident prevention information, handling, storage and disposal procedures, and emergency procedures to follow in the event of an accident.

3. SAFETY

The Laboratory Supervisor will provide information on the location and use of safety equipment, and emergency procedures.



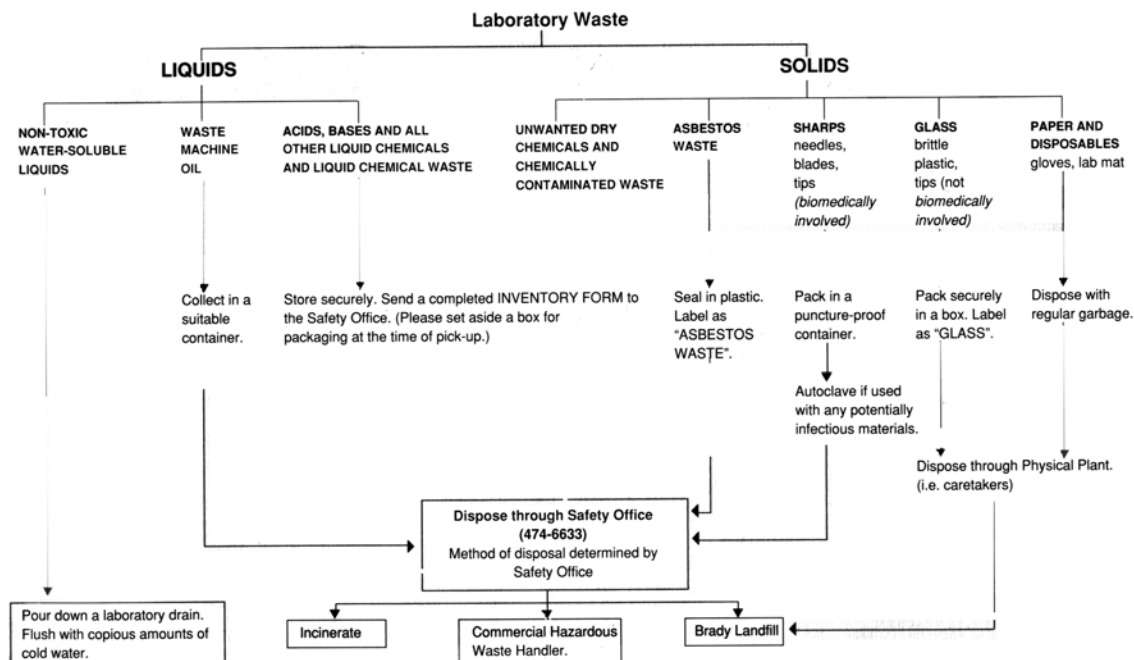
HAZARD CLASSIFICATION	SYMBOL	WORKPLACE LABELS	MATERIAL SAFETY DATA SHEET
CLASS A - COMPRESSED GAS CLASS B - FLAMMABLE AND COMBUSTIBLE MATERIAL: 1. FLAMMABLE GAS 2. FLAMMABLE LIQUID 3. COMBUSTIBLE LIQUID 4. FLAMMABLE SOLID 5. FLAMMABLE AEROSOL 6. REACTIVE FLAMMABLE MATERIAL	     	<p>These labels are applied at the workplace on controlled products or containers of controlled products when</p> <ol style="list-style-type: none"> The original supplier label is missing or becomes unreadable, The material is decanted or transferred from the supplier's container to another container, The material is produced at the workplace for use within the workplace. <p>Workplace labels will include the following information</p> <ol style="list-style-type: none"> Name/Identity of the Product Safe Handling Information - refers to any precautions that must be followed to minimize the risks of adverse health effect or injury. This also includes any personal protective equipment (PPE) e.g., type of gloves, eye or respiratory protection to be worn or other controls to be used through statements or pictograms (symbols) Reference to the MSDS. 	<p>A MATERIAL SAFETY DATA SHEET (valid for three years) will include information relating to each of the following categories</p> <ol style="list-style-type: none"> Product Identification and Use Hazardous Ingredients Physical Data Fire and Explosion Data Reactivity Data Toxicological Properties Preventive Measures First Aid Measures Date and Source of MSDS <p>and any other hazard information of which the Supplier is aware or ought reasonably to be aware.</p>
CLASS C - OXIDIZING MATERIAL CLASS D - POISONOUS AND INFECTIOUS MATERIAL 1. MATERIALS CAUSING IMMEDIATE AND SERIOUS TOXIC EFFECTS 2. MATERIALS CAUSING OTHER TOXIC EFFECTS 3. BIOHAZARDOUS INFECTIOUS MATERIAL CLASS E - CORROSIVE MATERIAL CLASS F - DANGEROUSLY REACTIVE MATERIAL	    	<p>SUPPLIER LABELS</p> <p>Supplier labels on a controlled product must be in English and French and include the following information</p> <ol style="list-style-type: none"> Product Identifier Hazard Symbols Risk Phrases Precautionary Measures First Aid Measures Supplier Identifier Reference to the availability of a Material Safety Data Sheet <p>This type of border (shown) is the indicator that the label identifies a controlled product.</p>	<p>FIRE / MEDICAL EMERGENCIES</p> <p>555 from 474, 789, 975, 977 exchanges or #555 from a cell phone (MTS or AT&T) OR 911 from other exchanges. If 911 is called you must also call Campus Security at 474-9341</p> <p>CHEMICAL / BIOHAZARDS EMERGENCIES 474-6633</p> <p>(8:30 am - 4:30 Mon. to Fri.). If busy or after hours, call 555</p>
<p>For further information on WHMIS, contact the ENVIRONMENTAL HEALTH AND SAFETY OFFICE at 474-6633.</p>			

MANITOBA WORKPLACE HEALTH HAZARD REGULATION 53/88 REQUIRES THAT A COMPLETE AND CURRENT CHEMICAL INVENTORY IS MAINTAINED AT ALL TIMES.



The University of Manitoba

WASTE DISPOSAL CHART FOR LABORATORIES



NO CHEMICALS ARE TO BE LEFT FOR THE UNIVERSITY OF MANITOBA CUSTODIAL STAFF. Empty reagent bottles are to be rinsed and have the labels de-faced. All potentially infectious materials (biomedically involved) must be autoclaved or de-activated using a chemical sterilizing agent prior to disposal. Animal carcasses are to be incinerated. Radioisotope users should consult the University of Manitoba "Waste Disposal Chart For Radioisotope Users".

THE ABOVE CHART IS A GUIDE, MORE INFORMATION IS AVAILABLE THROUGH THE OCCUPATIONAL HEALTH & SAFETY OFFICE (474-6633).

LAB 1 PREPARATION OF SHEEP IgG

OBJECT

The object of this experiment is to isolate and partially purify IgG from whole sheep serum. The resulting crude IgG preparation will be analyzed in the immunodiffusion lab.

INTRODUCTION

Since serum contains two major types of protein, globulins and the albumins it is a good source of immunoglobulins. Especially serum IgG, as it more abundant than IgM, IgA, IgD or IgE. Three purification techniques are used to isolated IgG from sheep serum, salt precipitation, anion exchange chromatography and dialysis. Immunoglobulins are 'tough' compared to other proteins, not easily degraded and retain antigenic properties even when exposed to heat, extreme pH values (2 to 11) for a short period of time, precipitation, ionic strength variation and even tolerate low concentrations of detergent and urea.

Sodium sulfate precipitation

Sodium sulfate precipitation is the most widely used technique for the preparation of immunoglobulin from whole serum. As the salt concentration of the medium is raised, there is an interference with the interaction of water molecules with charged polar groups on protein molecules. The protein molecules become less hydrophilic permitting greater hydrophobic interaction between protein molecules. Eventually the proteins become insoluble. The salt concentration differs at which each protein precipitates, with overlap between similar proteins. When preparing the crude immunoglobulin fraction, unwanted serum proteins stay in solution while immunoglobulins (mostly gamma globulin with some contaminating beta globulin) are precipitated out. The protein concentration in the medium influences the precipitation limits of the protein and affects the co-precipitation limits of the protein. Therefore, when precipitating serum, sodium sulfate is added slowly to avoid high local concentrations which would decrease the specificity of the precipitation.

Ion exchange chromatography

Protein molecules bind electrostatically onto an ion exchange matrix that carries an opposite charge to the protein. IgG can be purified by using the anion exchange resin, DEAE cellulose. Cellulose is the support for diethylaminoethyl groups which are positively charged. By using appropriate equilibration conditions (pH and ionic strength), DEAE cellulose will bind all the serum proteins (negatively charged) except IgG (positively charged). Therefore IgG is separated in the void volume. Contaminating serum components in the IgG preparation depends upon the ratio of ion exchanger to serum. The greater the amount of DEAE cellulose added per ml of serum the greater the purity of the IgG. However, this leads to lose of IgG. For this lab, 5 g of DEAE cellulose will be used per ml serum to obtain a sample that is reasonably pure with a good yield.

Dialysis

Dialysis separates proteins based on the size of the dialysis pores. Depending on the purchased pore size molecules larger than the dialysis membrane pore size are contained inside the dialysis tubing while all smaller molecules equilibrate across the dialysis membrane.

Measurement of proteins

Protein concentration of crude IgG is estimated by UV light absorption. Most proteins absorb ultraviolet light maximally at 280 nm because they contain tryptophan, tyrosine, and phenylalanine residues. Within certain concentration ranges, protein solutions obey the Beer-Lambert law; $A = Kcl$. Where A is the absorbance (optical density) of the light absorbing substance, K is the proportionality constant (dependent on the chemical nature of light absorbing solution) for IgG = $1.5 \text{ ml mg}^{-1} \text{ cm}^{-1}$, c is the concentration of the substance in the solution (mg/ml) and l is 1 cm (pathway of solution UV light passes through).

REFERENCES (for preparation of lab manual - not required by students)

(1) DeToma, F.J. and A.B. MacDonald 1987. Experimental Immunology: A Guidebook. Isolation of Immunoglobulin G (IgG) from Rabbit Serum p 7-8.

- (2) Hudson, L. and F.C. Hay 1980. *Practical Immunology*, 2nd edition, Blackwell Scientific Publications, London 359 p.
 (3) Johnstone, A. and R. Thorpe (1987) *Immunochemistry in Practise*, 2nd edition. Blackwell Scientific Publications, Boston 306 p. (p.48-52).

PROCEDURE

Week 1

Part I: Preparation of Sheep Serum

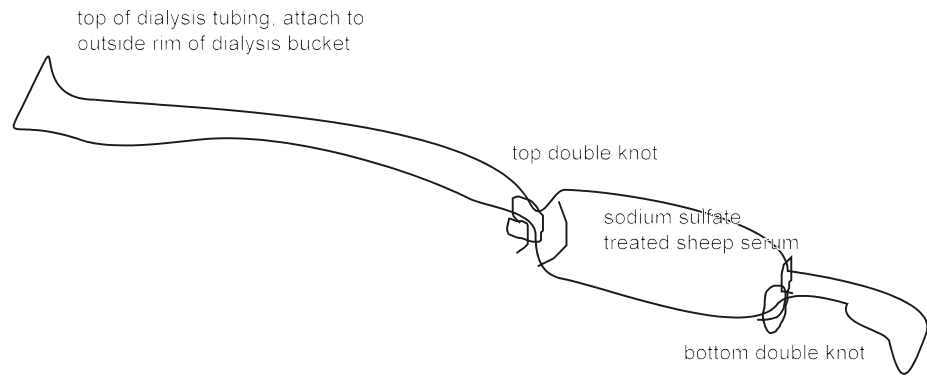
1. Allow fresh blood to stand at room temperature for 1 hour.
2. Store blood overnight at 4°C to allow clot formation.
3. Pour off serum, the majority of clot should remain in bottom of flask.
4. Centrifuge at 3000 rpm for 10 min. Transfer supernatant to new centrifuge tube and repeat centrifugation. If supernatant is not cleared of contaminating red blood cells, repeat centrifugation until homogeneous supernatant obtained; serum. Note: The serum usually has a slight pink tinge due to presence of hemoglobin from lysed red blood cells.

Part II: Crude IgG Preparation by Sodium Sulfate Fractionation (3)

Student lab starts here:

1. Obtain a screw capped centrifuge tube containing 11 ml heat treated sheep serum from student cold box. Remove 1 ml to a small beaker and place on ice (for Part III of this experiment). Place remaining 10 ml in a 25°C waterbath for 10 min.
 *Pre-treated serum: The complement system had been inactivated by incubating the serum at 56°C for 30 minutes. The serum was centrifuged at 10000 rpm for 30 min at 4°C. The low temperature is important as serum lipids are soluble above 4°C. The cold serum was quickly filtered through a small swatch of glass wool placed in a funnel to remove the insoluble lipids. The lipid-free serum should be clear and colourless.
2. Add 1.8 g sodium sulfate (~18% w/v solution). Use a spatula to help dissolve sodium sulfate.
 w = weight, v = volume
3. Centrifuge at 10000 rpm for 15 min at 25°C. The temperature is important as other proteins will precipitate at lower temperatures. Empty supernatant into a container labelled 'albumin fraction' located at the front bench. The supernatant contains the crude albumin required for a later experiment.
4. Estimate the volume of the pellet. Add distilled water to 10 ml. Completely resuspend pellet using a plastic spatula. Add 1.4 g sodium sulfate powder to make ~14% (w/v) solution.
5. Repeat step 3. Using a plastic spatula, completely resuspend pellet in 3 ml distilled water.

6. Remove a length of dialysis tubing from PBS buffer. See diagram for details. Rinse the inside of the tubing with distilled water using a squirt bottle. Hold over sink while doing this. Use your fingers to remove excess water. Tie two knots together at one end. Pop open one end of the dialysis tubing and fill tubing with your sodium sulfate precipitated sheep IgG



Dialysis tubing set up:

1. Tie the first double knot about 5 cm from one end.
2. Using a 10 ml pipette add serum.
3. Use your fingers to smooth down open end of tubing - serum should take up full inner volume of tube.
4. Tie the second double knot just above serum - okay to trap a small air bubble. The serum area inside the dialysis tubing should be taut. The top open end should be much longer than the bottom. Secure this end to the top outside rim of the 4 liter dialysis bucket with masking tape labelled with group # and names. Groups should attach their dialysis sample evenly spaced around the top outside rim of the bucket.

sample. Tie the open end with two knots. Label and attach to dialysis bucket as described in diagram. Dialyse overnight against 4 litres PBS, pH 7.2 at 4°C. Dialysis tube sample must be submerged in buffer. Next day change PBS and dialyse another 2 hours.

7. Put on disposable gloves. Rinse gloves with distilled water. Collect sample by cutting the bottom knot off the tubing while holding over a small beaker. Squeeze out the sample. Using a pipetman dispense crude IgG in 2 ml aliquots in small plastic vials (located below glassware). Label vials with names/group # and contents. Store in MBI0 4010 student tray in the -20°C freezer in room 201.
8. Next week during lab determine protein concentration.

Part III: Batch Preparation of Sheep IgG with DEAE Cellulose

A. Equilibration of DEAE Cellulose

1. Add 100 g DE52 DEAE cellulose to 550 ml 0.01 M phosphate buffer, pH 8.0 in a one liter beaker while slowly stirring.
2. Titrate with 1.0 M HCl back to pH 8.0.
3. Let the slurry settle for 30 min. Carefully decant or aspirate off the supernatant and fines (small particles that do not settle out of the supernatant in 30 min). Add 0.1 M phosphate buffer, pH 6.6 to fill flask. Mix gently and again let the slurry settle for 30 min. Remove supernatant.
4. Repeat wash once more but pour the slurry into a Buchner funnel using 2 layer of Whatman No. 1 filter paper. Apply suction for 30 sec leaving a damp cake of cellulose.

STUDENT LAB STARTS HERE for batch preparation of sheep IgG with DEAE cellulose

B. Preparation of sheep IgG

1. Add 3 ml distilled water that has been prechilled on ice to the 1 ml heat treated sheep serum that you previously placed in a small beaker. Mix and return beaker to ice bucket. Add 5 g wet weight DEAE cellulose (also at 4°C). Swirl or use spatula to mix.
Note: Distilled water is added to reduce the ionic concentration of the serum.
2. Incubate the mixture on ice for 1 hour. Mix thoroughly but gently every 10 min.
3. Place 2 layers of Whatman No. 1 filters in the Buchner filter. Use PBS to wet filter (approximately 1 ml). Attach funnel to sidearm flask. Suction the slurry of serum and DEAE cellulose for 30 sec.
4. Using a Pasteur pipette, transfer all effluent in a plastic vial (purified IgG). Label completely with names (group #) and sample type (be sure to specify DEAE cellulose purified sheep IgG). Store in the -20°C freezer in room 201. Discard cellulose.
5. Next week determine protein concentration.

Week 2

Determination of the protein concentration

Repeat the following procedure for both IgG samples

1. Prepare dilution in lab before taking to spectrophotometer room (313 Buller). Add 1 ml of PBS, pH 7.2 to an eppendorf tube. Next add 5 μ l sheep IgG. Mix.
2. Using a 1 ml pipetman or a Pasteur pipette transfer your entire sample to a quartz cuvette (1 cm pathway). Measure absorbance at 280 nm. The reference for spectrophotometer is PBS, pH 7.2. Why are you using a quartz cuvette, not glass or plastic? Hint: sunglasses. If you have not used the spec4000 before make sure the TA assists you.

LAB REPORT

Data Presentation and Analysis.

- 1.0 1. Determine the concentration of crude IgG (mg/ml) for both samples; Na₂SO₄ precipitation and DEAE cellulose ion exchange. Include a sample calculation.
- 1.5 2. Outline in detail how to prepare 500 ml 0.1 M phosphate buffer, pH 6.6 using NaH₂PO₄·H₂O (F.W. 138) and Na₂HPO₄ (F.W. 142).
3. Catilho³ et al (2002) investigated IgG purification methods focussing on efficiency and large scale production of IgG from monoclonal Ab supernatant.
- 1.5 a) Name the two procedures that are combined. State the best material for each. State why Catilho et al (2002) consider it the best method.
- 0.5 b) 0.1 M phosphate/phosphoric acid pH 3 and pH 7.4 are used in the procedure. State function of each.
- 0.5 c) What is the purpose of figures 6-8.

5.0

³Castilho, LR, Anspach, FB, Deckwer, W-D. 2002. Comparison of affinity membranes for the purification of immunoglobulins. J. Membrane Science 207: 253-264. via UM NetDoc Science Direct, UM eJournal, etc. Link on lab website.

LAB 2 IMMUNOPRECIPITATION

OBJECT

The object of this experiment is to learn the immunoprecipitation techniques; double immunodiffusion, immunoelectrophoresis, single radial immunodiffusion and rocket immunoelectrophoresis all based on the principle that a precipitin line forms in gel at antigen/antibody optimal ratio (equivalence).

INTRODUCTION

The precipitation reaction occurs when a soluble antigen reacts with a homologous antibody to form immunocomplexes. The structures and solubilities of immune complexes are dependent on the natures and relative amounts of the reacting antigens and antibodies, and also on the numbers of combining sites on each. Antigens may have one to numerous antibody-binding sites, on the other hand, IgG antibodies are bivalent having 2 antigen-binding sites. Repeated antigen-antibody linkages can result in large insoluble complexes at antibody optimal ratio (AOR). The AOR is the ideal antibody/antigen ratio for formation of insoluble immune complexes (equivalence). When the number of antigen particles is much higher than the number of antibody molecules, many antibody binding sites on the antigen will remain empty. The complexes that are formed are small and soluble, not visible with the naked eye. When number of antibody molecules is much higher than the number of antigen molecules, there is not enough antigen to form cross-linkages. Again the complexes that are formed are small and soluble. Refer to the figure 4.

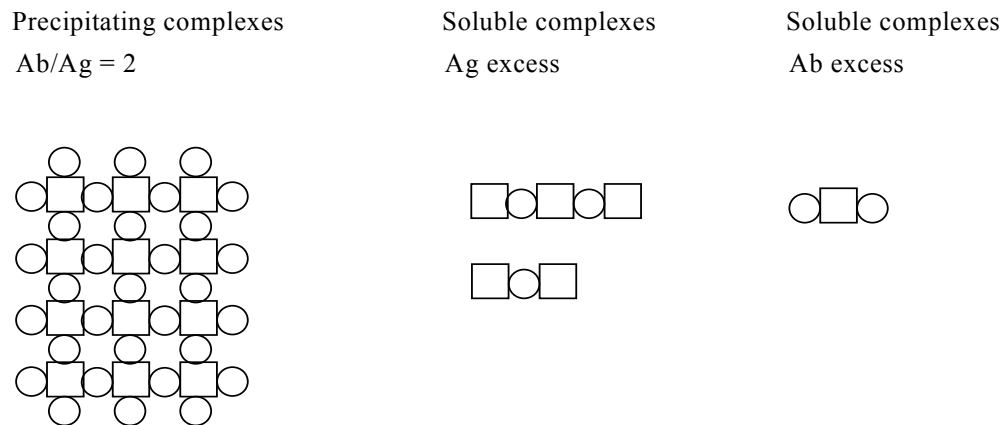


Figure 4. Schematic complexes of antigen and bivalent antibody

□ Ag, ○ Ab.

The antigens used in this experiment is sheep IgG and bovine albumin.

A visible antigen-antibody precipitation reaction occurs in agarose gel at AOR (optimal antigen/antibody ratio) or equivalence. A single diffusing antibody meeting its cognate antigen will produce a single line of precipitation. A number of currently used techniques make use of this principle: double immunodiffusion, single radial diffusion, and immunoelectrophoresis.

Double Immunodiffusion (The Ouchterlony Technique)

This procedure was developed by Ouchterlony (1966). Wells are cut in a gel (agar or agarose); one well contains the antibody and the other well contains the antigens. Antigens and antibodies diffuse towards each other at rates that increase in proportion to their concentrations in the well but decrease in proportion to their sizes. They form a line of precipitation (precipitin line) where they meet at equivalence. Generally the agar does not interfere with the diffusion of the two species. The initial formation of the precipitin line moves the equilibrium between the antibody and antigen towards

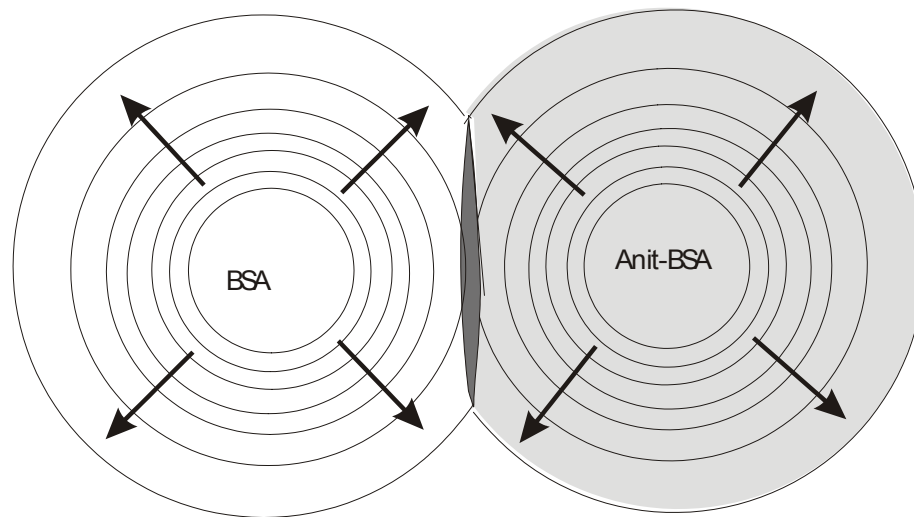


Figure 5. Mechanism of precipitation formation in the Ouchterlony Technique

formation of more precipitation - increasing the flow of reactants into the zone of precipitation, figure 5. It also acts as a barrier across which neither components can pass.

The double diffusion technique has many applications:

1. Determine the homogeneity of antigen-antibody complexes.
2. Immunodiffusion can also be used to follow the purification of an antigenic mixture.
3. Determine the minimum number of antibody-antigen complexes present.
4. Determine whether a given antigen shares structural characteristics (cross-reacts) with other molecules of interest.

This technique has the advantage that several antigens can be compared around a single well of antibody. There are three basic patterns of precipitation as shown figure 6.

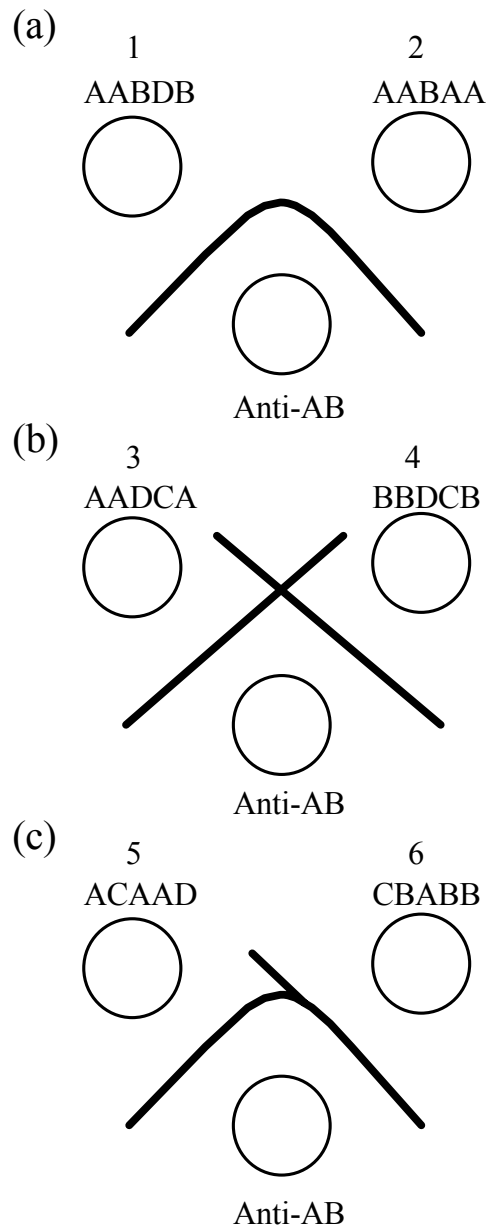


Figure 6. Three basic patterns of precipitation for double immunodiffusion, (a) identity, (b) non-identity, and (c) partial identity.

A-D represent antigenic determinants present within a series of protein molecules, 1-6, used as antigens. An antiserum has been raised against two of these determinants, A and B. Note, the detection is purely qualitative and, in addition, does not give any information on the other determinants present. (a) Reaction of Identity: The precipitation lines have fused completely showing the presence of identical determinants in antigens 1 and 2.

(b) Reaction of Non-identity: The two precipitation lines cross without any interaction. Proteins 3 and 4 do not share any determinants detectable by the antiserum.

(c) Reaction of Partial Identity: The two precipitation lines have fused, but in addition a spur has formed towards the well containing protein 5. Therefore, proteins 5 and 6 share some common determinants but protein 6 has additional unique determinants detected by the antiserum. Note: If there were unique determinants in both antigens, this would give rise to two spurs.

Variation in results of immunodiffusion can occur. This variation is sometimes due to the animals used for immunization. For example, two rabbits immunized with the same pool of antigen (human IgG), can produce antisera that can behave differently towards a combination of antigens. Antisera prepared by immunizing one rabbit recognized IgG1, IgG2, IgG3, and IgG4 but can only recognize IgG1, IgG2, and IgG4 when prepared in another rabbit.

Single Radial Immunodiffusion (SRID)

The single radial immunodiffusion technique is used to measure the concentration of a particular substance in solution that is mixed with other substances when appropriate antiserum is available. In this technique, which was developed by Mancini, a gel (usually agarose) is prepared that contains antiserum. A sample containing the antigen is placed in a well cut in the gel and allowed to diffuse out. As the antigen meets its cognate antibody a visible precipitate is formed as a halo around the well. As more antigen diffuses into the equivalence zone, there is now excess antigen and some of the precipitin in this area dissolves. The antigen diffuses further outwards until concentration drops to that necessary for equivalence, and is reprecipitated by the antibody. This precipitation, partial redissolving, reprecipitation continues until antigen is too low to redissolve the rim of the precipitin halo. The precipitin halo stops increasing in diameter.

There is a linear relationship between the amount of antigen and diameter of the halo. Because the actual diameter of the halo is also a function of temperature and other factors, SRID experiments always include a set of known varying concentration of the antigen. A standard curve is plotted to determine the concentration of unknown sample.

Electroimmunoassay (Rocket Immunelectrophoresis) is very similar to SRID except it has the added dimension of electrophoresis. Again the antibody is incorporated into the gel (pH 8.6 buffered; generally immunoglobulins do not have any charge at this pH, i.e., isoelectric point) and sample of unknown concentration along with standard antigen of varying concentration is subjected to electrophoresis. Precipitin line formation is theoretically the same as SRID but under electrical current a rocket is formed, figure 7. Rocket immunelectrophoresis is a rapid method to determine antigen or antibody concentration.

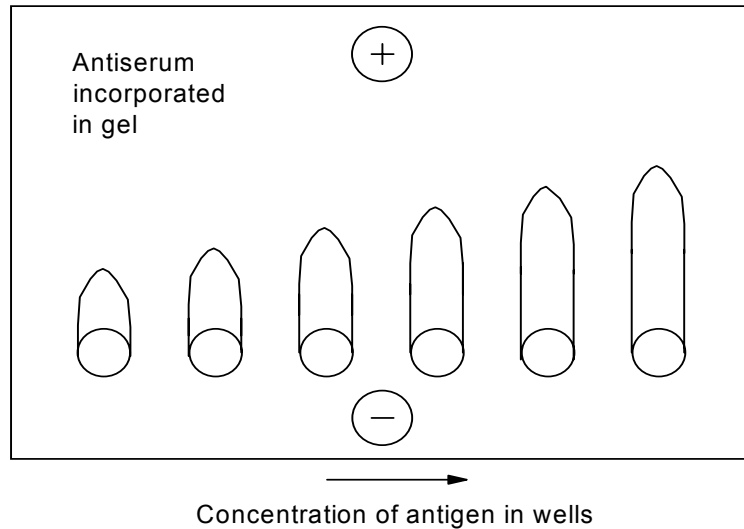


Figure 7. Diagram of rocket immunoelectrophoresis.

The heights of the rockets increase in proportion to the initial amount of antigens in well. Therefore a standard curve can be constructed and the concentration of the unknown sample is read off the curve.

Immunoelectrophoresis

Some antigen mixtures are too complex to analyze by double diffusion. To study complex antigen mixtures the technique of immunoelectrophoresis was developed by Grabar and Williams (1953). This technique involves the placing of the antigen in a gel well and subjecting it to electrophoresis, figure 8.

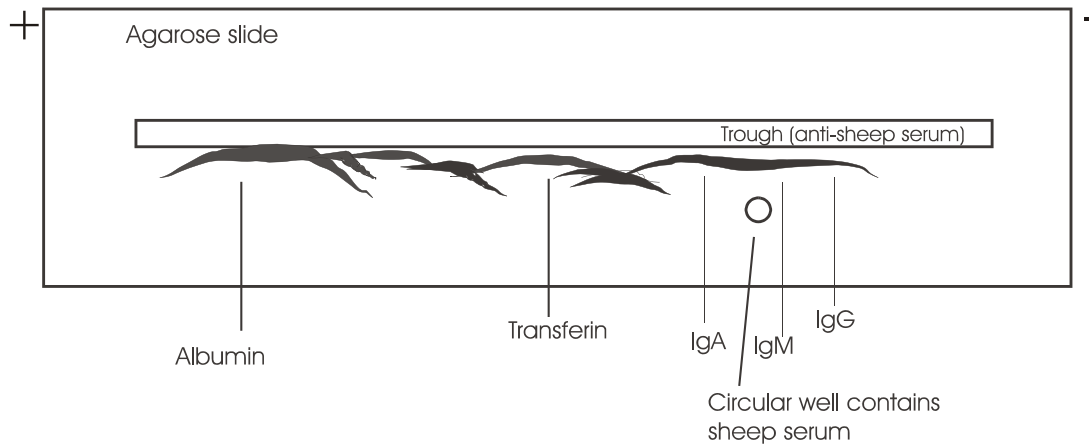


Figure 8. Immunoelectrophoresis precipitin pattern of sheep serum proteins. Before electrophoresis sheep serum is placed in the well and after electrophoresis anti-sheep serum is placed in the trough. + = positive electrode; - = negative electrode

The antibody to one or more of the antigens in the mixture is loaded into a long well parallel to the electrophoretically separated antigens. The antibody is allowed to diffuse outward and precipitin arcs form when meet cognate antigen.

When comparing immunoelectrophoresis with double diffusion, immunoelectrophoresis has several advantages: less chance of precipitin arcs overlapping, characterizes antigen movement in electrical field, and shape of arcs are of clinical significance. The serum of individuals with suspected disease can be compared to normal humans quickly by using immunoelectrophoresis.

REFERENCE

Kimball J.W. 1986. *Introduction To Immunology*, 2nd edition, Macmillan Publishing Company, New York. Chapter 4.

PROCEDURE

Week 2

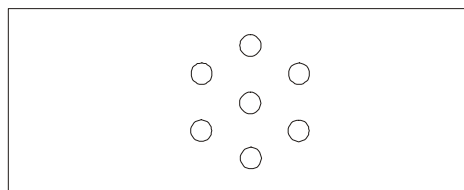
Part I: Double Immunodiffusion

1. Slides are pre-coated with an 1% agarose in distilled water to hold the final agarose gel in place. This is done by first washing a glass microscope slide in ethanol and burning off excess alcohol to give residue free slides. The clean dry slides are then placed on top of glass pipettes to raise the slide off the bench surface. The agarose is dissolved by heating while stirring, then cooled to 55°C. The warm 1% agarose solution is then pipetted on the slides, adding just enough to cover the entire surface of the slide. The slide is air dried completely. A thin almost invisible plastic-like layer is formed. The side with the pen mark is the underside. The slides can be stored at room temperature in a dust free environment until required.

Student Lab starts here:

(Three groups should work together when preparing the slides, but not experiment. Each group (pair) requires two slides for double immunodiffusion or one if put two punch patterns on one slide)

2. Prepare 100 ml 1% solution of agarose in Tris-tricine buffer (three groups work together). Heat with gentle stirring on a heater/stirrer. Cool to ~ 65°C.
3. Align two 1 ml glass pipettes on a level bench top approximately 3 cm apart and tape to bench. Pipette approximately 5 to 6 ml of molten agarose (2 slides per group, allow a few extra for errors) onto each pre-coated glass microscope slide. Take care to prevent agarose from running off the edge of the slide. Make sure agarose goes out to all edges. Allow the gel to harden for 10 min (turns opaque).
4. Use Bio Rad template (**6 holes surrounding centre hole - see pattern below**) for punching holes. Use water aspirators. Connect to punch attachment via tubing. Have a beaker of distilled water nearby. Using grid punch ONE pattern per slide. You may punch two



patterns per slide and load all samples on one slide. Turn on water aspirator. Set slide in punch hole grid. Put end of punch attached through grid perpendicular to slide. Touch bottom of slide, then remove punch attachment. Put the end of punch attachment in distilled water for about 5 sec to wash agarose through tubing and aspirator. Repeat for all remaining required holes and slides.

5. The antiserum is placed in the centre well and the antigens are placed in the surrounding 6 wells. To give the best precipitin lines with little background staining, the concentration of antigen and antiserum should be 1 mg/ml protein or less. If spillage or a mistake occurs when filling the wells the slides can be rinsed in distilled water and reused. Excess water can be removed from well by the capillary action of a paper wick. Add 10 μ l per well of the following antigens and antibodies.

Slide I (1) Anti-sheep whole serum (commercial) - centre well
 (2) Antigens - surrounding wells
 (LOAD WELLS CLOCKWISE IN THE FOLLOWING ORDER **ONLY**)
 a) pure sheep albumin (1 mg/ml stock) (commercial)
 b) crude sheep albumin (student and demonstrator prepared)
 c) crude sheep IgG (student prepared by sodium salt precipitation)
 d) pure sheep IgG (1 mg/ml stock) (commercial)
 e) whole sheep serum (demonstrator prepared)
 f) crude sheep IgG (student prepared with DEAE cellulose)

Slide II (1) Anti-sheep IgG (commercial) - centre well
 (2) Antigens - surrounding wells as slide I

6. Place the slide in a humid chamber and incubate overnight at room temperature. A humid chamber is prepared by covering the bottom of a petri plate with moist paper towels and placing an unused glass slide on the moist paper towels. Place immunodiffusion slide on glass slide in humid chamber and seal the lid of the plate with masking tape.
7. Next day, make a diagram of the wells and precipitin lines of each slide. On the diagram, indicate the contents of each well, including central one.

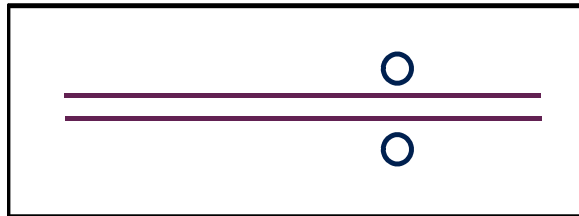
Note: it is important that you make a drawing prior to staining as crude preparations often containing other proteins that hide the precipitin lines.

8. Drying of Gel:
 a) Rinse the slide briefly with PBS, pH 7.2 and then rinse briefly with distilled water to wash salt from the slide.
 b) Cover the slide first with a piece of good quality filter paper and next with 2-3 cm of paper towels. Next place a sheet of glass on top of the paper towels and then weigh down with a 250 ml or 500 ml beaker of water.
 c) Allow to dry overnight or weekend. Drying of the slide may take longer depending on room temperature and humidity.
 d) After the slide is dry, remove the paper towels and filter paper. The gel must be completely dry before staining.
9. Staining of Gel: The dried slides are stained to help distinguish precipitin lines, to allow the slide to be stored indefinitely and to make photography easier. The slide is stained with the protein dye, Coomassie blue. Covered containers are provided for the different solutions used in staining procedure. In these containers a stirring bar is placed below slides so that the solutions are stirred.
 a) Place the slide in a container of 0.5% (w/v) Coomassie brilliant blue R in 20% (v/v) acetic acid and 30% (v/v) ethanol for 2 to 10 min. **DO NOT DISCARD COOMASSIE BLUE STAIN** (the same stain is used by all groups).
 b) Destain in two changes of the same solution without the dye (2 destaining containers will be available). The destaining time will depend on the speed at which the background dye is removed, destaining time may take overnight. Change destain solution if deep blue.
 c) Allow slide to air dry. Do not discard slide. Hand in dried and stained slide with lab report.
 d) Draw stained precipitin lines. Hopefully the pattern is the same as unstained. If more bands are present, definitely include. Do not include diffuse background stained proteins.

Part II: Immunoelectrophoresis

Four groups should work together when preparing the slides, but not experiment. Each group (pair) requires ONE slide for immunoelectrophoresis. Make a few extra for unforeseen problems.

1. Use the same 1% agarose in Tris-tricine and slide set up to make immunoelectrophoresis slide as used for double immunodiffusion slide.
2. Pipette approximately 5 to 6 ml of molten agarose (prepare 2 slides per group to allow for error) onto each pre-coated slide. Take care to prevent agarose from running off the edge of the slide. Make sure agarose goes out to all edges. Allow the gel to harden for 10 min (turns opaque).
3. Use Bio Rad template to punch two holes and make trough. Use water aspirators. Connect to punch attachment via tubing. Have a beaker of distilled water nearby. Using grid punch for immunoelectrophoresis, see diagram. Turn on water aspirator. Set slide in punch hole grid. First punch two holes by putting the end of punch attached through grid perpendicular to slide. Touch bottom of slide, then remove punch attachment. Put the end of punch attachment in distilled water for about 5 sec to wash agarose through tubing and aspirator. Using the trough tool run down the trough grid, do not remove the trough agarose yet. The trough should be approximately 0.5 cm from each end of the slide.
4. Fill one well with 10 μ l sheep IgG (prepared by sodium salt precipitation) and the other well with 10 μ l sheep IgG (prepared by DEAE cellulose ion exchange) serum. Position the glass slide in the electrophoresis unit such that the wells are closed to the negative electrode. Running buffer is Tris-tricine, pH 8.6 buffer. Electrophoresis for 1h 15 min at 240 volts. Refer to appendix for principle of electrophoresis.
5. When the electrophoresis is complete, use a pasteur pipette to remove agarose strip from the trough. Fill the trough with 60 μ l antiserum (anti-sheep whole serum prepared in rabbit).



6. Store the slides in a humid chamber at room temperature overnight.
7. Make a labelled drawing of slide. Refer to lab introduction for expected location of IgG.
8. Dry and stain slide as outlined in double diffusion section. Hand in dried stained slide with lab report.

Week 3

Part III: Single Radial Immunodiffusion (SRID)

In this experiment the concentration of IgG in normal sheep serum will be determined. The slide has been prepared similar to that described for immunodiffusion except antiserum (rabbit anti-sheep IgG) is added to melted, cooled agarose before pouring slides. The hole punch pattern consists of a single row of evenly spaced wells down the centre of the slide; 6 wells, 4 wells for standard and 2 wells for sample.

1. Sheep IgG standard preparation: Dilute the 10 mg/ml sheep IgG stock to 1 mg/ml (10 μ l stock + 90 μ l distilled water), 3 mg/ml (30 μ l stock + 70 μ l distilled water), 5 mg/ml (50 μ l stock + 50 μ l distilled water), and 7 mg/ml (70 stock + 30 μ l distilled water).
2. Gel loading: Load 5 μ l sheep IgG standards in the first 4 wells; 1 mg/ml, 3 mg/ml, 5 mg/ml, and 7 mg/ml respectively. In the remaining 2 wells load 5 μ l undiluted DEAE cellulose crude sheep IgG and 5 μ l 1/5 diluted sodium salt precipitation crude sheep IgG. Prepare sample dilution in total volume of 100 μ l using distilled water.
3. Place slide in a humid chamber, cover, and incubate at room temperature overnight.
4. Measure the diameter of the halos to the nearest 0.1 mm (best before staining if possible). Stain gel slides only if precipitin halos are not visible and measure diameter of halos.
5. Dry the slide. Start drying slide Friday, as the slide can dry for an extended period of time, once dry stable.
6. Hand in dried slide with lab report.

Part IV: Rocket Immunoelectrophoresis

In this experiment the concentration of albumin in bovine serum will be determined. Bovine serum albumin (BSA) is used to demonstrate rocket immunoelectrophoresis instead of sheep serum albumin as anti-sheep albumin is not readily available. The slide has been prepared similar to that described for immunodiffusion except rabbit anti-BSA (anti-bovine serum albumin) is added to melted, cooled agarose before pouring slides. The hole punch pattern consists of a single row of evenly spaced wells 1/3 distance from one end.

1. BSA standard preparation: Dilute the 1 mg/ml BSA stock to 0.05 mg/ml (5 μ l stock + 95 μ l distilled water), 0.1 mg/ml (10 μ l stock + 90 μ l distilled water) and 0.5 mg/ml (50 μ l stock + 50 μ l distilled water).
2. Gel loading: Load 5 μ l BSA standards in the first 4 wells; 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml respectively. In the remaining 2 wells load 5 μ l 1/2 dilution and 5 μ l 10^{-1} dilution of bovine serum. Prepare bovine serum dilutions in a total volume of 100 μ l using distilled water.
3. Transfer the slide to the electrophoresis chamber (well at negative electrode - black). Cover ends of gel with wick. Gel electrophoresis buffer is tris-tricine. Carry out electrophoresis for 60 min at 240 volts.
4. Immediately following electrophoresis, measure and record the heights of the "rockets" and make a drawing of them. Note if rockets are not visible, stain before measuring the heights of the rockets.
5. Dry and stain gel (if necessary) as described for double immunodiffusion. Hand in dried rocket immunoelectrophoresis slide with lab report.
Note: Drying of slide can be done on Thursday or Friday and left over the weekend or mid-term break. If you are not drying the slide until Friday, put the slide at 4°C in a humid chamber overnight.

LAB 2 REPORT

Data Presentation and Analysis

Dried slides are to be scotch taped to a sheet of paper and attached to the back of your report. Under each slide label experiment section. This is not a figure, therefore complete labelling is not necessary. Be sure to scotch tape the entire edge of each slide to prevent breakage. If individual reports, just state partners with slides. No marks allotted but marks subtracted if not attached to one of the group member's report.

Part I: Double Immunodiffusion

- 2 1. Include labelled figure of slide I and II double diffusion (stained or unstained - best diagram or combination of two).
Hand in dried stained slides.
- 2 2. Include precipitin reaction data sheets (available on website in Word format, save and open in Word to record requested information). Referring to immunoelectrophoresis results will assist you in answering expected precipitin reaction for your crude preps.

Center Well: Anti-sheep whole serum		
Adjacent Antigens in Surrounding Wells	Precipitin Reaction ^c	
	Observed	Expected
pure ^a sheep albumin: crude ^b sheep albumin		
crude sheep albumin: crude sheep IgG (sodium sulfate fractionation)		
*crude sheep IgG (sodium sulfate fractionation): pure sheep IgG		
pure sheep IgG: whole sheep serum		
whole sheep serum: crude sheep IgG (DEAE cellulose)		
crude sheep IgG (DEAE cellulose): pure sheep albumin		

Center Well: Anti-sheep IgG		
Adjacent Antigens in Surrounding Wells	Precipitin Reaction	
	Observed	Expected
pure ^a sheep albumin: crude ^b sheep albumin		
crude sheep albumin: crude sheep IgG (sodium sulfate fractionation)		
crude sheep IgG (sodium sulfate fractionation): pure sheep IgG		
*pure sheep IgG: whole sheep serum		
whole sheep serum: crude sheep IgG (DEAE cellulose)		
crude sheep IgG (DEAE cellulose): pure sheep albumin		

^a purchased pure chemical

^b crude albumin fraction collected in the lab

^c a precipitin reaction is what happens between two adjacent antigen (outside wells) with respect to centre antibody. If one well does not contain an antigen that is recognize by the centre antibody then there is NO REACTION.

- 1 3. Analyse the expected precipitin reaction for reactions footnoted with (*) in above table.

Part II: Immuno-electrophoresis

- 1 4. State the approximate number of precipitin bands present in each of your sheep IgG preps (sodium sulfate and DEAE cellulose prepared in lab 1). State the relative position of IgG with respect to electrophoresis charge orientation. Explain why immuno-electrophoresis results help you to answer the previous question (#3).

Part III: Single Radial Immunodiffusion (SRID)

- 2 5. Include a completed spreadsheet (Excel (available on website). Include all requested information. The standard curve is IgG (μg) (y-axis) vs halo diameter (x-axis). Even though halo diameter is the variable and should be plotted on the x-axis, it is plotted on the y-axis to make calculation of regression formula easier. See lab 2 appendix for Excel functions and graph presentation procedure.
- 0.5 6. Explain why the concentration of your sheep IgG samples (sodium sulfate and DEAE cellulose) are expected to be lower when determined by SRID as compared to absorbance at 280 nm/Beer-Lambert formula. Explanation must include specific reference to SRID experiment.

Part IV: Rocket Immuno-electrophoresis

- 1.5 7. Include a completed spreadsheet (Excel - available on website). Include all requested information. The standard curve is BSA (μg) (y-axis) vs rocket height (x-axis). Even though rocket height is the variable and should be plotted on the x-axis, it is plotted on the y-axis to make calculation of regression formula easier. See lab 2 appendix for Excel functions and graph presentation procedure.
- Note: Two different volumes of bovine serum were loaded, only choose data that is located within the standard curve range, omit even if close to range limits. If no ideal data is available, use the best sample data you have. In reality each volume should be loaded in duplicate or triplicate but the size of the slide limits this.

Lab 2 Appendix

EXCEL OPERATIONS AND STANDARD CURVE PLOT METHODS FOR MBIO 4010.

Procedures may vary depending on excel version. There are numerous ways to use Excel, only one described below. HINT: Right click on whatever you want to change and select appropriate item from the pull down menu.

Excel spreadsheet file contains two worksheets (SRID and Rocket), select using tab on bottom of page.

It may be easier to do required numerical calculation in a text box, either Excel or Word, then insert where requested on Excel spreadsheet.

Formula and Function calculations

Put the cursor in the cell where you want to enter formula or function. Type in formula or select function button

In the spreadsheet just set up formula for one cell then copy and paste for similar calculations.

Cell Formulae Notations:

Remember to use correct mathematical brackets in your formulae.

start equation with an = sign, do not leave spaces between entries

* multiply

/ divide

+ add

- minus

^power or use the POWER function

FUNCTIONS

When you are using a function in a formula always put the information after the function in brackets.

EXP this is the exponential applied to the base e. The constant e is equal to 2.718... , the base of the natural logarithm.

Example of regression equation cell formulae entries:

For exponential regression equation $y = 3.7613e^{0.1524x}$, the Excel cell formula is

=3.7613*EXP(D10*0.1524) where D10 is the cell containing either halo diameter or rocket height of sample

For polynomial regression equation $y = 0.1361x^2 + 0.0232x + 4.331$, the Excel cell formula is

=0.1361*(D10^2)+(0.0232*D10)+4.331

MS EXCEL STANDARD CURVE CHARTS for MBIO 4010 lab 2

Select Insert pull down menu.

Select chart - (standard types), select (XY) Scatter chart type. Defaults to only markers present. Click next.

Put cursor in data range box, click. Make sure there is no default information in box - delete if present as Excel will often automatically select data. Use the mouse to click and hold down to select y-axis data (mass). Column should select - just check.

Select series tab. Series 1 plot is highlighted. Put the cursor in x values box, click. Again make sure there is no information in this box - delete if present. Use the mouse to click and hold down to select x-axis (data halo diameter or rocket height).

Note: Normally the x-axis is the known value, in our case protein mass and the y-axis is the measured parameter (diameter or height). Here the axes have been reversed to make it is to solve regression equation, ie solve for y. You may reverse the axes if you are familiar with solving quadratic equations.

Click next.

Chart Options menu appears. Under default titles tab, enter title and axes labels. Unfortunately the title defaults to the top of the graph. After you are finished setting up graph, just select and move below graph. Mostly likely you will need to move the graph up. May require resizing, font size change, etc. Click the legend tab. Remove check mark from legend - not required as only one plot.

Click next. Keep the default setting (graph in data page).

Click finish.

Right click any empty area on graph area. Select format plot area. Change the background color to white. Click OK.

Put the cursor on any one of the plot markers. Right click. Select Add Trendline - Under the default Type tab you may select any trendline that will give you a good fit trendline eg polynomial regression with order 2 or exponential regression line. Linear regression trendline is acceptable if the R-squared value is acceptable >0.95 .

Select the Options tab. Select display equation on chart. Select display R-squared value on chart. Press OK. If required, move the equation so completely visible. Move the graph to specified area on data sheet.

Print to fit portrait page.

*

R- squared value

The value is between 0 and 1. The R squared value is an indicator of how closely the estimated values used to draw the linear regression Trendline correspond to the actual data (line is close to all points).

The linear regression trendline is most reliable when R value is close to 1 - both polynomial, order 2 and exponential should be close to 1.

LAB 3

HEMAGGLUTINATION ASSAY

OBJECT

To determine the titre of antiserum using hemagglutination assays; direct hemagglutination (lectin) and passive hemagglutination.

INTRODUCTION

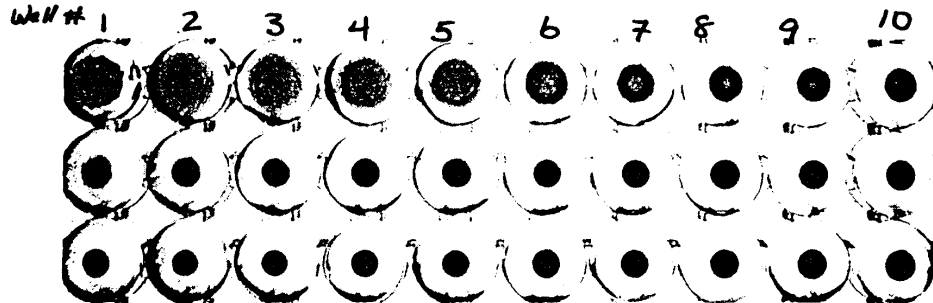
In this experiment sheep red blood cells, which contain large amounts of hemoglobin, can serve as carriers of antigens. The inert carrier particles can be composed of numerous substances other than red blood cells, for example latex beads. The red blood cells increase the sensitivity of the antibody-antigen reaction as it can be coated with a number of different types of antigens. Exposure of the antigen-coated sheep red blood cells to an appropriate antiserum will cause cross linking or agglutination of the carrier particles thereby decreasing the amounts of antigen and antibody required to insure that the immune complexes are visible to the naked eye. **The titre of an antibody containing solution is the reciprocal of the greatest dilution of the solution that still has a strong detectable amount of antibody.** For example, refer to figure 10, well 5 would be the well you would select to calculate the titre as mat size is constant well 1 through 5.

Red blood cells have different affinities for different antigens, that is, some stick better than others. Some glycoproteins and proteins will effectively adsorb to red blood cells without the tanning step and cause agglutination of red blood cells (direct hemagglutination). Lectin is a glycoprotein which binds and cross-links red blood cell via specific surface carbohydrate determinants (glycoproteins). But for most proteins, the red blood cells must first be made sticky by 'tanning' - treating cells with tannic acid.

There are several steps in the procedure that affect the sensitivity and specificity of the test. The concentration of sheep red blood cells and antigen to be coupled is definitely significant. With increased red cell concentration there is decreased sensitivity of test. Excess of antigen may result in nonspecific agglutination. Serum which has been inactivated and absorbed with sheep erythrocytes is used to resuspend tanned sRBC and to serially dilute antiserum. This prevents nonspecific agglutination due to complement or heterophile antibodies present in the serum. Heterophile antibodies cross react with antigens between species. It is good practise to use fresh sheep's blood which helps overcome variables that may occur (reproducibility and occasionally nonspecificity).

Briefly, one carries out a passive hemagglutination assay by making twofold serial dilutions of the test antiserum (Ab) in the wells of a microtitration plate. Next, one adds the tanned and antigen-coated red blood cells. In the wells where antibody is present in sufficient concentration to cross-link the cells, they will settle to the bottom as a circular, red 'carpet' - a positive hemagglutination reaction. Refer to the following diagram for interpretation of results. The reaction is strongly positive in the first 5 wells. Wells 6 - 9 show increasingly weak reactions. The solid button of settled red blood cells in well 10, second, and third row are negative reactions.

A direct hemagglutination assay is similar to a passive hemagglutination assay except the sRBC



tanning and antigen coating steps can be eliminated.

Hemagglutination, particularly passive hemagglutination, is a sensitive method for detecting small amounts of antibody - as little as $0.05 \mu\text{g/ml}$ - and is often used to 'titre' antiserum (Ab).

REFERENCES

Kimball J.W. 1986. *Introduction To Immunology*, 2nd edition, Macmillan Publishing Company, New York. Chapter 5.

Roitt, I., Brostoff, J., & D. Male 1985. *Immunology*, C.V. Mosby Co., Toronto. p.25.3

PROCEDURE

Week 4

A. PASSIVE HEMAGGLUTINATION RED BLOOD CELLS PREPARATION

Part I and Part II step 1. has been done by the demonstrator prior to the start of the lab.

Part I: Collection and Washing the sheep red blood cells (sRBC)

1. Sheep blood is collected directly from the sheep into sterile Alsever solution with a final dilution of 1:1 sheep blood to Alsever solution.
2. The sheep red blood cells (sRBC) are centrifuged at 3000 rpm for 10 minutes. Carefully aspirate off the supernatant as the pellet is very loose. Keep cells cold throughout washing procedure.
3. Resuspend cells by adding PBS, pH 7.2 to original volume. Centrifuge at 3000 rpm for 5 minutes. Aspirate off supernatant. Repeat twice more.
4. Resuspend cells at a final concentration of 20% sRBC with PBS, pH 7.2 - 3 ml required per group. Keep the cells on ice or at 4°C.

Part II: Preparation of Normal Serum Diluent (NSD)

1. Incubate the serum (0.5 ml per group) in a 56°C water bath for 30 minutes. This treatment inactivates the complement system, which may cause the antigen-coated cells to burst when exposed to the serum. This step has been done prior to start of lab.
Note: The control serum used to prepare normal serum diluent should come from the host animal used to prepare test antiserum (in our case rabbit).

Student lab starts here.

Each student is supplied with a large plastic centrifuge tube containing 3 ml washed 20% sRBC.

2. Absorb 0.5 ml heat-treated normal rabbit serum (already aliquoted in a plastic vial) by adding 2 ml washed 20% sRBC.
3. Incubate the mixture at room temperature for 30 minutes.
4. Divide the solution between 2 eppendorf tubes. Sediment the sRBC by microfuging 2 min at room temperature. Draw off the serum using a pasteur pipette to eppendorf tubes. To remove remaining sheep red blood cells, centrifuge for 1 min at room temperature and transfer serum to clean eppendorf tubes.. Keep the serum on ice or at 4°C. Note: In the process of absorption, the serum has been diluted fivefold (1:5).
5. Just before using dilute 20-fold (Part IV-step 4).

Part III: Preparation of the Tanned sRBC using the Remaining washed sRBC

1. Add 7 ml PBS, pH 7.2 to the remaining 1 ml 20% sRBC in the screw capped centrifuge tube.
2. Then add 0.3 ml of 0.1% tannic acid solution.
3. Mix, and incubate in a 37°C waterbath for 10 min.
4. Centrifuge for 10 min at 4000 rpm at 4°C and discard the supernatant fraction. Wash the sRBC once with 7.5 ml of pH 7.2 PBS. Centrifuge as above for 5 minutes and again discard the supernatant fraction. The sRBC are now tanned. Resuspend the cells in 7.5 ml physiological saline (0.88% NaCl). Keep cells on ice or store at 4°C.

Part IV: Coating the Tanned sRBC

1. Add 7.5 ml PBS, pH 6.4 to the 7.5 ml tanned cells prepared in Part III.
2. Add 15 ml of 0.2% BSA (bovine serum albumin prepared in PBS, pH 6.4) and incubate at room temperature for 15 min, shaking occasionally.
3. Centrifuge at 4000 rpm for 10 min at 4°C and discard the supernatant.
4. Dilute inactivated and absorbed normal serum (prepared in Part II) 20-fold by mixing 1.5 ml serum with 28.5 ml PBS, pH 7.2...this is called NSD, normal serum diluent.
Note: If you do not have quite 1.5 ml absorbed normal serum, dilute what you have proportionally 20-fold.
5. Wash the cells with 20 ml normal serum diluent. Again centrifuge at 4000 rpm for 10 min at 4°C. Discard the supernatant. Washing the cells with NSD is necessary to maintain stability - otherwise the cells may agglutinate even if no antiserum was present.
6. Resuspend the cells in 0.5 ml to 1 ml NSD depending on the concentration of the cells. The cells are now ready for assay. For this lab, the cells will be stored in small plastic vials at 4°C until next week. (Coated tanned sRBC can be stored at 4C for several weeks.)

Week 5**B. HEMAGGLUTINATION ASSAYS****Part 1: The Passive Hemagglutination Assay**

Take care NOT to create bubbles when adding assay components. Once the bubbles have formed, they are very difficult to remove - A TIME CONSUMING PROCESS.

1. Place 20 μ l NSD on the bottom of wells 1-24 (use two rows of microtitration place).
2. Add 20 μ l anti-BSA to the first well, mix by pipetting solution up and down the tip about 5-8 times. Remove 20 μ l from well 1 and transfer to well 2, again mix and transfer 20 μ l to well 3. Repeat this serial dilution until reach well 23. Do not transfer 20 μ l to well 24 but discard. Well 24 is the control.
3. Gently resuspend tanned sheep red blood cells. Add 20 μ l tanned BSA coated sRBC to each well and mix by stirring with the end of the pipette tip. Start with the control and work from the highest dilution to lowest dilution so that one tip can be used. It is important that the sRBC-antisera mixture cover the entire bottom of well with no bubbles. The majority of bubbles can be removed using one or two syringe needles.
4. Seal the plates with saran wrap and label. Incubate at room temperature. Read the microtitration plate the next day.
Note: Microtitration plates can be read easily from the underside.

Part II: Direct Hemagglutination

1. Place 20 μ l PBS, pH 7.2 on the bottom of wells 1-24 (use two rows of microtitration place).
2. Add 20 μ l lectin* to the first well, mix by pipetting solution up and down the tip about 5-8 times. Remove 20 μ l from well 1 and transfer to well 2, again mix and transfer 20 μ l to well 3. Repeat this serial dilution until reach well 23. Do not transfer 20 μ l to well 24 but discard. Well 24 is the control.
Lectin: *Helix ponatia*, 0.2 mg/ml
3. Gently resuspend sheep red blood cells. Add 20 μ l 3% sRBC to each well and mix by stirring with the end of the pipette tip. Again start with the control and work from the highest dilution to lowest dilution so that one tip can be used.
4. Seal the plates with saran wrap and label. Incubate at room temperature. Read the microtitration plate the next day.

LAB 3 REPORT (Lab report format available on lab website as a Word document.)**Data Presentation and Analysis**

- 2.5 1. Record hemagglutination titration results for anti-BSA and lectin, *Helix ponatia* in table provide. **Indicate titre well** in table with an asterisk or highlight. Determine the titre of anti-BSA and lectin. Include a sample calculation below table.

Table 1. Determination of anti-BSA and lectin, <i>Helix ponatia</i> sheep red blood cell hemagglutination titre.					
agglutination interpretation ^a				agglutination interpretation ^a	
well #	anti-BSA	lectin, <i>Helix ponatia</i>	well #	anti-BSA	lectin, <i>Helix ponatia</i>
1			13		
2			14		
3			15		
4			16		
5			17		
6			18		
7			19		
8			20		
9			21		
10			22		
11			23		
12			24 CONTROL		
			Titre ^b		

^a Interpretation of agglutination results (settling patterns): Positive: Varying degrees of mat formation (+ -> +++++). Negative: A clearly defined button in the bottom of the tube (-).

*titre well

^bTiter is the reciprocal of the highest dilution of serum showing ++ mat formation.

Sample Calculation.

- 0.5 2. Determine the concentration of lectin, *Helix ponatia* at titre (include calculation).

continued on next page . . .

Question

1. Hull-Jackson et al (2006)⁴ investigated the commercial latex agglutination assay for serological diagnosis of Leptospirosis. Although the microscopic agglutination test (MAT) is the best method to identify the disease it is time consuming and requires expert technicians. An accurate rapid test that is easy to use is required.

- 0.5 a) What causes Leptosirosis and what is it?
- 1.0 b) Is the rapid latex assay an indirect agglutination assay? Justify your answer.
- 1.5 c) Describe the latex agglutination procedure (Organon Teknika BV, manufacturers) that meets the need for serological diagnosis of leptospirosis using acute phase sera in resource poor countries.

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⁴Hull-Jackson, C, Glass, MB, Ari, MD, Bragg, SL, Branch, SL, Whittington, CU, Edwards, CN, Levett, PN. 2006. Evaluation of a commercial latex agglutination assay for serological diagnosis of Leptospirosis. J. Clin. Micro. 44: 1853-1855. [ASM journal, use UM NetDoc on campus or log-on off campus, ejournal as recent article.]

LAB 4 Introduction to the ELISA (Enzyme Linked Immunosorbant Assay)

INTRODUCTION

The enzyme linked immunosorbent assay is a sensitive assay that is simple, versatile, and reliable. ELISA procedure may also be referred to as the EIA system (enzyme immunoassay). It can be adapted to obtain the titers of either antigen or antibody samples. As the sensitivity of any immunoassay depends on the ability of the system to detect and to amplify any reaction between cognate antigen and antibody, the ELISA is a particularly sensitive assay because the detection and amplification is enzyme dependent. Possible enzymes used in the ELISA system are alkaline phosphatase (this lab), beta-galactosidase and horseradish peroxidase. Each has a high turnover number (rapid conversion of chromogenic substrate to coloured product) resulting in high sensitivity. In addition, the ELISA results can be measured quantitatively by means of a spectrophotometer allowing quick clinical analysis.

Antigen titre can be determined by the sandwich method, figure 11. First, the antigen-specific antibody is adsorbed to an immobile surface such as the wells of a microtiter plate. The plates are washed to remove excess unbound antibody. Cognate antigen is added and binds to antibody. Excess unbound antigen is washed off and enzyme-conjugated antibody against the antigen is added binding still exposed determinants on the antigen. Excess unbound enzyme-conjugated antibody is washed away, and chromogenic substrate added. Colour develops in wells containing the bound antigen. The absorbance of the solution in each well is proportional to the amount of bound antigen in it.

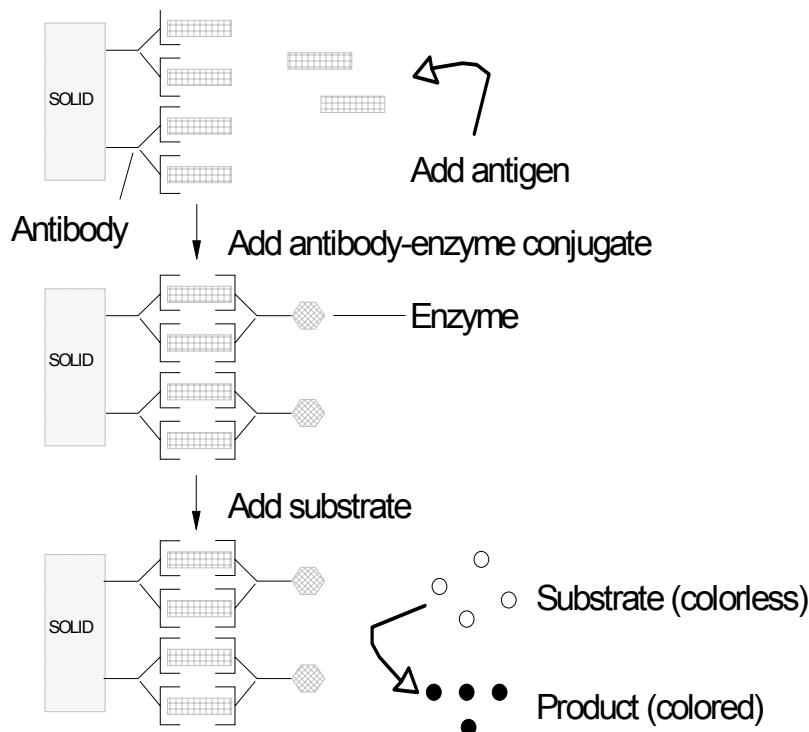


Figure 11. Enzyme Linked Immunosorbent assay - Sandwich method.

Antibody titre can be obtained by a modification of the enzyme linked immunoassay, indirect enzyme immunoassay method, figure 12. First, the antigen is adsorbed to an immobile surface such as the wells of a microtiter plate. The excess unbound antigen is washed off. Dilutions of test antibody is added to each immobilized antigen sample and allowed to bind to the antigen. The unbound antibody is washed away. This is followed by addition of a secondary antibody which is directed against the test antibody. The secondary antibody is conjugated to an enzyme, for our experiment, alkaline phosphatase. This enzyme catalyses the hydrolysis of ρ -nitrophenyl phosphate (a colourless substrate) to phosphate and ρ -nitrophenol (yellow at alkaline pH). Again, the excess of this secondary conjugated antibody is washed away. Chromogenic substrate is added and colour develops which is directly proportional to the amount of bound antibody.

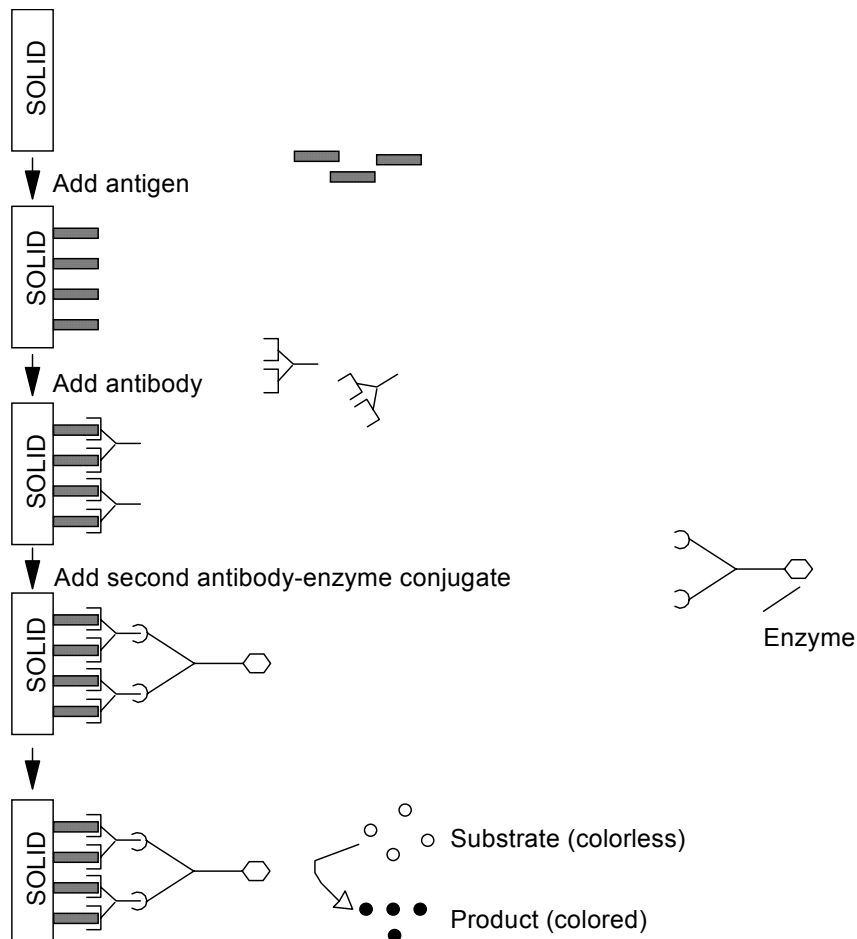


Figure 12. The indirect method of enzyme linked immunosorbent assay.

OBJECT

The object of this experiment is to determine the titre of antiserum by the indirect EIA method.

OUTLINE

1. Bovine IgG (antigen) is adsorbed to each well of a microtitration plate.
2. The plates is washed to remove excess bovine IgG.
3. Serial dilutions of rabbit anti-bovine IgG are added to the wells.
4. The plate is washed to remove unbound rabbit anti-bovine IgG.
5. The goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) is added to the wells.
6. The plate is washed to remove unbound conjugate.
7. The enzyme substrate, ρ -nitrophenyl phosphate (ρ -NPP) is added to the wells.
8. The bound enzyme cleaves the substrate to produce ρ -nitrophenol which is a yellow colour. The amount of ρ -nitrophenol equals amount of antibody present.

Week 7

PROCEDURE

1. The microtitration plates have been coated with bovine IgG prior to the start of the class lab as follows: 200 μ l of 10 μ g/ml bovine IgG prepared in coating buffer was added to each well, covered with plastic wrap, incubated at room temperature for 1 hour, and then stored overnight at 4°C (or until lab period).

Only the first 6 columns are coated. Check this when you first get your microtitration plate. You cannot use wells that are not coated.

Class lab starts with step 2.

2. Invert the microtitration plate and dump the liquid out of the wells.
3. Wash the wells by filling them with PBS-Tween solution. This is done quickly using a plastic squeeze bottle. Allow the plate to sit for 30 sec and then dump PBS-Tween. Repeat this step twice more.
4. Experiment is performed in triplicate (see 96 well microtitration plate diagram).
 - a) Place 100 μ l PBS-Tween in all highlighted wells. Wells A1, A3 and A5 are triplicate background wells.
 - b) Fill well B1, B3 and B5 with 100 μ l original anti-bovine IgG (2-fold dilution or 1/2).
 - c) Serial dilutions: Transfer 100 μ l from well B1 to well C1(1/2 or 2-fold dilution). Mix, repeat 2-fold dilution until well H2, ie., going from well B1 through to H1, then up to A2 and ending 2-fold dilution at well H2 Discard the 100 μ l extra from the last well. You should end with 100 μ l in each well when you are finished. Repeat anti-bovine IgG 2-fold dilution starting at well B3 and B5. This gives you triplicate anti-bovine IgG 2-fold dilutions.
5. Cover the plate with plastic wrap and incubate the plate for 30 min at 37°C.
6. Wash the wells as described in step 2 and 3.

96-well Microtitration Plate

	Trial 1		Trial 2		Trial 3							
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank		blank		blank							
B												
C												
D												
E												
F												
G												
H												

7. To all wells used, add 100 μ l goat anti-rabbit IgG conjugated with alkaline phosphatase (AP) (1:1000 dilution in PBS-Tween-BSA). Cover the plate with plastic wrap and incubate at 37°C for 30 min.
8. Wash the wells as described in step 2 and 3.
9. Add 100 μ l substrate to each well containing blank, sample or dilution. Do not touch the well or well solution with tip, just drop in the 100 μ l substrate. DO NOT create bubbles. The ELISA reader will give spurious results if bubbles are present.
10. Cover the plate with plastic wrap and incubate the plate for 30 min at 37°C. If there is insufficient colour development after 30 min at 37°C, put plate at 4°C and again read the next day - generally there is sufficient color development to read same day.
11. Remove covering from microtitration plate. Wipe bottom of plate with alcohol soaked cloth to remove fingerprints. Read absorbance of each well in ELSIA Reader microtitration plate spectrophotometer set in Dr. Butler's lab. Collect printout.

ELISA READER procedure:

- (a) The reader should be turned on 30 min prior to assay.
- (b) Select **SOFT Max** icon. Software used to analyze microtitration plate results.
- (c) If the program is not already set up, go to **Set Up, Instrument**, put in the following parameters: endpoint L1, nm =405, automix, select OK.
- (d) Go to **Control** in the pull down menu, select **read plate**. Asks for file name, just cancel.
- (e) The spectrophotometer moves the plate into the reader, reads absorbance, and returns plate.
- (f) Select **File, print Window**. The printer prints out data as a table of optical density (absorbance) identical to plate layout.

REFERENCES (used to write experiment and introduction, not required)

- Kimball J.W. 1986. *Introduction To Immunology*, 2nd edition, Macmillan Publishing Company, New York. Chapter 5.
 Kuby, J. 1997. *Immunology*. 3rd ed. New York: W. H. Freeman and Company.
 Sharon, J. 1998. *Basic Immunology*. Baltimore: Williams & Wilkins.

LAB 4 REPORT

Data Presentation and Analysis

- 1 1. Append completely labelled ELISA reader printout set up as a figure. In title include dilution information: original dilution of anti-bovine IgG and dilution series type with respect to wells. Dilution factor is the reciprocal of the dilution in each well.
- 4 2. Include a completed Excel spreadsheet of ELISA data analysis. Excel spreadsheet available on website. Include all requested information. Place graph in spreadsheet as requested plotting bovine IgF absorbance (405 nm) (y-axis) vs reciprocal of Ab dilution. See Lab 4 appendix for Excel operations and graph set up. When plotting data include only a few values once the plot has levelled off. This allows you to graph the relevant part of the data. Definition of antibody titer⁵: reciprocal of the antibody dilution that is the closest (less dilute) match to the average background absorbance +3x average SD.

Question

- 2 1. Hong et al⁶ (2003) used a PCR-Enzyme-Linked Immunosorbent Assay for the rapid detection of *Salmonella enterica* on poultry carcasses. Present a completely label schematic diagram of the ELISA assay similar to the schematic diagrams in your lab manual (molecular level). Be specific when labelling your diagram. State appearance of positive and negative result relating to object of assay. Hand drawn is acceptable.

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⁵Birmingham, N, Payankulam, S, Thanesorakul, S, Stefura, B, Hayglass, K, Gangur, V. 2003. An ELISA-based method for measurement of food-specific IgE antibody in mouse serum: an alternative to the passive cutaneous anaphylaxis assay. *J. Immunol. Meth.* 275: 89-98. UM NetDoc via Science Direct. OR Available through UM ejournals, just search the *J Immunol Methods*. <http://www.umanitoba.ca/libraries/online/ejournals/>

⁶Hong, Y, Berrange, ME, Liu, T, Hofacre, CL, Sanchez, S, Wang, L, Maurer, JJ. 2003. Rapid Detection of *Campylobacter coli*, *C. jejuni*, and *Salmonella enterica* on Poultry Carcasses by Using PCR-Enzyme-Linked-Immunosorbent Assay. *Appl. Environ. Micro.* 69: 3492-3499. [ASM journal, linked on website]

Lab 4 Appendix

Microsoft EXCEL Operations

There are a variety of methods to carry out Excel operations . Only one way is described here. Make use of copy and paste, once you have designed a formula, just copy and paste for like calculations.

See lab 2 appendix for review of cell formula entry.

AVERAGE

Put the cursor in the cell where you want to record average. Select paste function button (f_{*}), then statistics (or defaults to most recently used), then AVERAGE. Or use pull down menu - select Insert, function, statistics, then AVERAGE. Click OK. A pop-up menu appear, use or just move out of the way if necessary. Using your mouse click the first or last cell of data set. Hold down button and scroll down to the last or first cell in the data set. Release button. Click OK on pop-up menu or press enter. The average value appears in your selected cell. Repeat for remaining data sets.

STDEV (standard deviation).

Put the cursor in the cell where you want to record standard deviation. Select paste function button (f_{*}), then statistics (or defaults to most recently used), then STDEV. Or use pull down menu - select Insert, function, statistics, then STDEV. Click OK. A pop-up menu appears, use or move out of the way if necessary. Using your mouse click the first or last cell of data set. Hold down button and scroll down to the last or first cell in the data set. Release button. Click OK on pop-up menu. The standard deviation value appears in your selected cell. Repeat for remaining data sets.

MS EXCEL Anti-bovine IgG STANDARD CURVE CHART

Select Insert pull down menu.

Select chart - (standard types), select (XY) Scatter chart type. Defaults to only markers present. Select scatter with data points joined by straight lines. Click next.

Put cursor in data range box, click. Make sure there is no default information in box - delete if present as Excel will often automatically select data. Use the mouse to click and hold down to select y-axis data (average absorbance for all anti-bovine IgG dilutions - do not include background). Column should select - just check.

Select series tab. Series 1 plot is highlighted. Put the cursor in x values box, click. Again make sure there is no information in this box - delete if present. Use the mouse to click and hold down to select x-axis data (reciprocal of dilutions - do not include background). Click next.

Chart Options menu appears. Under default titles tab, enter title and axes labels. Unfortunately the title defaults to the top of the graph. After you are finished setting up graph, just select and move below graph. Mostly likely you will need to move the graph up. May require resizing, font size change, etc. Select Legend tab. Remove check from show legend.

Click next. Keep the default setting (graph in data page).

Click finish.

Right click any empty area on graph area. Select format plot area. Change the background color to white. Click OK.

Insert STANDARD DEVIATION bars for average absorbance data: Right click plot to bring up menu. Click FORMAT DATA SERIES. Select Y ERROR BARS tab. Select display BOTH. Select CUSTOM. Put the cursor in the + box. On the spreadsheet put cursor on first entry of standard deviations. Holding mouse button down, drag cursor to last entry. Release mouse button. Range is now entered in the box. Put the cursor in the - box. Repeat the same standard deviation data selection. A standard deviation bar length is equal to + and - standard deviation, ie 2x SD. Click OK. Standard deviation bar appear on plot.

Add drawn background lines:

If the drawing Toolbar is not present, right click the main Toolbar. Check Drawing, the drawing Toolbar should appear. Select line. On graph hold down mouse button at left y-axis average

background value and draw a horizontal straight line to right y-axis. Release button. When selected you can edit the line however you want using buttons on drawing toolbar. Repeat above to draw a linear horizontal line for average background $+3x$ SD. Using the text box in the drawing Toolbar label each line.

Move the graph to specified area on data sheet.

Print graph, data and calculations to fit landscape page.

LAB 5 INDUCTION AND ANALYSIS OF CYTOKINE RESPONSES BY EXOGENOUS ANTIGEN IMMUNIZATION

by Dr. Kent HayGlass and Graduate students in Immunology

Purpose: The objective of this lab is threefold.

(i) to induce and restimulate a cytokine response via immunization of mice with ovalbumin.

(ii) to assess intra-assay variability of pipette technique.

(iii) to quantify the intensity of the IL-13 response induced following immunization with OVA and microscope examination of immune cells participating in inflammation.

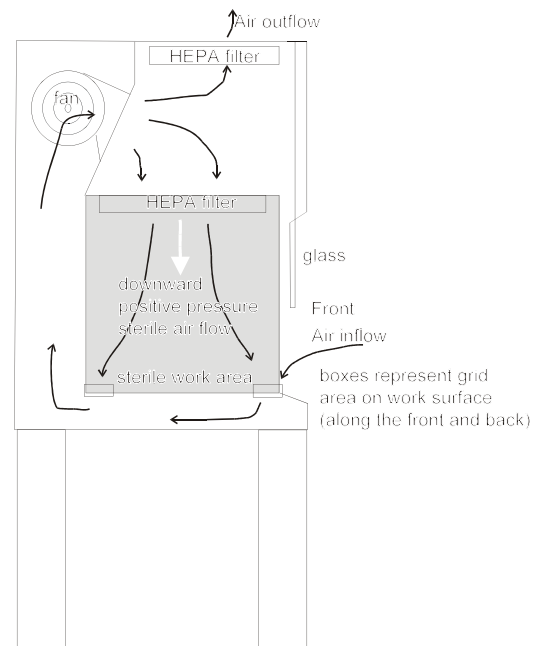
Background

Ovalbumin is a protein antigen that is widely used in immunologic studies, largely because it stimulates strong (readily quantified) immune responses *in vivo* and is readily obtained in pure form. Interestingly, it is also one of the two most clinically significant food allergens in human neonates. OVA immunization under the conditions used here stimulates CD4 T cell activation (hence proliferation and cytokine production) as well as B cell activation (hence IgE and IgG antibody production). Today we will assess the intensity of the IL-13 cytokine response, a key cytokine in allergic diseases. Numerous other immune activities are also stimulated (proliferation, IL-2, IFN γ , antibody synthesis, etc) but given the time constraints, we will only examine IL-13 synthesis.

Biosafety cabinet

The class II safety cabinet⁷ is designed to protect both you and the environment from exposure to biohazards and to protect samples from contamination during experiment. Refer to diagram for operation. Air inside the cabinet moves in a circular motion. As you sit at the front of the cabinet air is drawn from outside downward through the front grid of work area. Do not put anything on the grid or remove any sterile item towards you past this grid (no longer sterile area). Refer to diagram, you will see that air next moves below and up the back of the cabinet drawn by a fan at the top of the safety cabinet. The percentage of the air either recycled or exhausted through the top depends on the make of the safety cabinet. Regardless, the air moving down onto the sterile work space or out into the environment goes through a HEPA filter (sterilizes air). As the sterile air moves downward over the work space it is drawn out through both the front grid and a back grid where it again it drawn up the back of the cabinet by the fan. The cycle is repeated. The positive pressure of sterile air keeps the work space sterile.

Biological Safety Cabinet
(arrows indicate air movement)



ELISA assay

This is a widely used capture sandwich ELISA. The basic principle behind it is:

- Anti-IL13 antibodies (the “capture” reagent) are used to coat the ELISA plate. These bind to the polystyrene that has been electrostatically charged to increase its ability to bind any protein used to coat it.
- A blocking solution (BSA here, sometimes skim milk powder or other sources of unrelated proteins) is used to bind any sites on the polystyrene not filled by the capture Ab to reduce non-specific (background) signal.
- The standard (recombinant IL-13 of known concentration) or samples (the complex mixture of tissue culture proteins which includes any IL-13 generated following OVA or Con A stimulation) are added.

⁷http://www.escoglobal.com/Biotech/PDF/safecab_introduction.pdf

The IL-13 is captured by the capture antibody, all other molecules are washed out. A titration (rather than a single sample concentration) is used to increase precision.

·A “development” anti-IL13 antibody is added to each well. This antibody must recognize a different epitope than that bound by the capture Ab. This Ab is usually conjugated with an enzyme or (more commonly for ELISAs sensitive enough to detect the pg to ng levels of cytokine produced in most antigen stimulated immune responses) is **conjugated with biotin to enhance sensitivity**.

·Streptavidin (or avidin) conjugated enzyme is added to each well. Avidin forms an irreversible chemical bond with biotin (hence the anti-IL13 development Ab). Because several biotin molecules are present on each development antibody, this amplifies the signal, leading to increased sensitivity. The most commonly used avidin-enzyme conjugates are alkaline phosphatase or horseradish peroxidase. We use alkaline phosphatase in this experiment.

·Substrate for the enzyme is added to each well. For alkaline phosphatase, this is PNPP (p-nitrophenyl phosphate). It is colorless. Its cleavage product is yellow. Wells with larger amounts of IL-13 in the sample will have bound more anti-IL13 development antibody, hence enzyme, so more substrate will be converted, leading to a more intense yellow color in those wells.

·By comparing the intensity of color in sample wells with that generated in the standard curve by known concentrations of rIL-13, it is possible to determine how much IL-13 is present in your sample.

Essential definitions:

Wash medium: RPMI* plus 5% calf serum as a source of protein RPMI was developed by Moore et al⁸ at Roswell Park Memorial Institute. RPMI medium uses a bicarbonate buffering system, with added amino acids and vitamins. When adequately supplemented the media supports the growth of many types of cell culture.

Culture medium: RPMI plus 10% fetal calf serum, antibiotics, L-glutamine and 2-mercaptoethanol.

Counting medium: RPMI plus 15% fetal calf serum

⁸Moore, GE, Woods, LK. 1976. Culture media for human cells - RPMI 1630, RPMI 1634, RPMI 1640 and GEM 1717. Tissue Culture Association Manual, 3: 503-508.

Procedure

Week 8

There is a quiz on lab 5 information and protocol the week before the lab.

Week 9

Students do not attend MBIO 4010 lecture before each of the downtown labs. Notes from Dr. Court's lectures will be given to you.

Be on time - at least 5 minutes before lab starts at 1:45 on the 6th floor of the Basic Medical Science Bldg (Immunology Department). Bring your lab coat.

Part I: Induction and characterization of a primary cytokine response in vivo.

Steps done in advance for you.

1. Prepare 2 μ g ovalbumin (OVA) in 2 mg alum adjuvant.
2. Immunize each C57Bl/6 mouse (an inbred strain that is a high responder to this antigen) intraperitoneally at day 0.
3. At day 5 post immunization (which we have previously determined to be the time of the peak primary cytokine response), we sacrifice mice and sterilely remove spleen.

STUDENT LAB START HERE

Students carry out this procedure working in pairs. Each group will set up their own culture plate. EVERYTHING MUST BE DONE WITH STERILE EQUIPMENT AND TECHNIQUE - work in assigned biohazard safety cabinet.

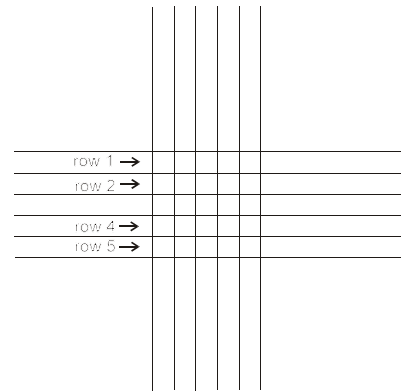
1. Put on latex gloves. As a pair, prepare a single cell suspension from the half spleen you are given, using the homogenizer:
 - a. Open foil on tissue culture grinder such that you do not touch the inside . Place the foil sterile side up on safety cabinet work space. Use opened foil as a sterile surface to place grinder.
 - b. Take plunger out and place on foil. Each group will be given a sterile tube containing $\frac{1}{2}$ spleen suspended in tissue culture medium buffer. Flick tube to remove spleen from bottom of tube then quickly pour into grinder tube (so spleen does not remain in the tube after the liquid is transferred. If it does, return medium to tube and repeat so as to transfer the spleen.
 - c. Insert plunger all the way to the bottom, and mash with a back and forth sideways motion about 8-10 times then lift plunger up about 4 cm and push down as far as the plunger will go with reasonable force. Repeat this process for about 30 seconds. There should be no "chunks" of spleen remaining.
2. While one person is making the single cell suspension, the other member of the pair should work with the foil covered sterile beaker containing a funnel lined with nylon small pore mesh and forceps. Use the sterile forceps to place the funnel on top of your original tube the spleen came in or use a clean tube (sterile). Once this is set up, the person making the single cell



suspension should pour the entire contents of the grinder back through the funnel into the tube to remove remaining particle matter such as connective tissue.

- Top up to ~ 10 ml with wash medium. Centrifuge 3 minutes at 1400- 2000 rpm at room temperature. Speed depends on the centrifuge you are assigned.
- In hood, pour off supernatant into waste beaker, cap, resuspend cells by vigorously flicking pellet and turning tube sideways, add wash medium to ~ 5 ml and centrifuge again.
- Gently pour off supernatant to the waste container, resuspend cells by vigorously flicking pellet and turning the tube sideways, add culture medium to ~ 5ml. From this suspension, accurately remove 50 μ l cell suspension for counting, transferring to 96 well non-sterile "counting plate".

- Add 50 μ l counting medium, 50 μ l acetic acid (to lyse RBC) and 50 μ l trypan blue (to differentiate live (white) from dead cells (blue)) in that order. Mix gently with P200. This dilutes your cells 4-fold or 1/4.



- Each hemocytometer holds two samples. The hemocytometer is stored in EtOH. Remove and clean all dye off the hemocytometer and coverslip with a kimwipe. Transfer ~10 μ l of solution to V-shaped groove of the hemocytometer*, cover lengthwise with cover slip and allow sample to settle for 1 minute then count. After using hemocytometer and cover slip return to EtOH.

Row 1, magnification of row 1 above. Example of microorganism distribution (22 microorganisms)

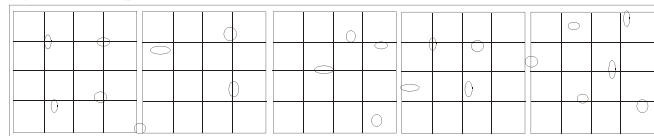


Figure . Hemocytometer counting area.

- Count non-blue cells in squares only. Read only 50% of cells on lines or just make sure you do not read the same cell twice. Refer to diagram. Count the number of viable cells in row 1, 2, 4 & 5. Skip row three.
 Method 1. Divide by 4 to determine the average cell count per row (5 squares double lined squares). Multiply by 2 and divide by 10 to obtain cell concentration (million cells/ml). This calculation has already taken into consideration the 1/4 dilution of cells made prior to counting.
 Method 2. Add cell count of all four rows to get the total number of cells in 20 squares. Divide by 20 to obtain the number of cells in one double line square. Multiply by 4 to take into account the cell dilution prior to counting. Then multiply this value by 1ml/(0.02 ml x 0.02 ml x 0.01 ml) to determine the number of cells in 1 ml.
 The volume of one double lined hemocytometer square is 0.02 cm x 0.02 cm x 0.01 cm. Remember 1 ml = 1 cubic cm.
 Note: Method 2 has been included to explain the calculation rational.
- Each group should find that they have ~10- 20 million cells/ml. Calculate the volume required to obtain 7 million cells. Each group will then put 7 million cells into each of six wells of a 48 well culture plate.
 eg. if you have 15 million cells/ml then you require $7/15 \times 1 \text{ ml} = 0.46 \text{ ml}$ of your cell suspension should be added to each well.

10. While one partner is counting cells to determine the cell concentration, the other should be **SETTING UP THE CULTURE PLATE.**
Culture plate should be labelled with your group's initials on the bottom of plate as well as the top lid to avoid later confusion. Two groups will share a culture plate as you will need only 6 wells per group and a plate contains 48 wells.
11. Prior to adding counted cells, add antigen volumes as indicated below.
GROUP
A: No stimulus (negative control for cytokine response). 100 μ l of culture medium/well in first two wells.

B: Concanavalin A (Con A) (add 100 μ l of 1000 μ g/ml Con A stock to each of 2 wells)
Positive control for cytokine response. Con A is a lectin protein with mitogenic activity isolated from Jack bean (*Canavalia ensiformis*). Con A is a non-specific inducer of B and T cell proliferation, consequently cytokine (IL-13).

C: OVA (add 100 μ l of 3000 μ g/ml OVA stock to final two wells) Experimental group.
12. Add the required volume of your cells from the immunized mouse to obtain 7 million cells/well. This is likely some volume between 0.25 and 0.7 ml of the counted cell suspension.
13. Finally, add culture medium to bring total volume of all wells up to 1 ml total.
14. Place labelled plates in incubator at 37°C, 5% CO₂ (for pH balance). We will harvest supernatant for you at 96 hours (the time of peak production for IL-13) and store at -20°C until next week for you to use in quantitating the IL-13 concentrations obtained in your cultures.

Part II: ELISA technique precision training.

EACH INDIVIDUAL (NOT GROUP) DOES THIS PART OF THE EXPERIMENT

This comparison of 8 vs 2 identical samples will demonstrate the impact of having a larger number of replicates on your precision.

BACKGROUND: “Expired” ELISA solution is a solution from a previous ELISA containing intense colour. It is useful to generate a reading in the ELISA plate reader and allow you to determine how precisely you can reproducibly measure volumes.

Diagram of 96 well ELISA plate design and the specific wells used (shaded).

	50 µl repeats		100 µl repeats		triplicate 2-fold dilutions							
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank											
B												
C												
D												
E												
F												
G												
H												

1. Column 1: Do not add anything to well A1 - background (blank) well. Pipet seven 50 µl volumes of pre-prepared expired ELISA solution into each well (B1 to H1).
2. In column 2, pipet two 50 µl volumes of expired ELISA solution in the top two wells (A2 & B2).
3. In column 3, pipet eight 100 µl volumes of expired ELISA solution into each well (wells A3 to H3).
4. In column 4, pipet two 100 µl volumes of expired ELISA solution in the top two wells (A4 & B4).
5. In columns 5-7, add 50 µl of dilution buffer to all 24 wells (three columns of 8 wells each). Then pipet 50 µl of expired ELISA solution into the top three wells (A row). Working on one column at a time carry out 2-fold dilutions. Mix carefully (do not create bubbles). Transfer 50 µl from A well to B well (½ or 2-fold dilution). Mix, repeat 2-fold dilution until come to H well. Discard the 50 µl extra from the last well. You should end with 50 µl in each well when you are finished. This titration will provide information on your capacity to carry out “identical” titration such as you will use in next week’s ELISA.
6. Using ELISA plate reader and assistance of a demonstrator, determine the absorbance values for each of your data sets.
7. The Softmax software (demonstration provided) of the ELISA plate reader provides coefficient of variation “CV” (your variability in dispensing and in titrating samples) data.

The teaching assistant will assist with this and generate a print out of your findings during the lab. *Sample data from this experiment is attached. It demonstrates the plate design and typical values. For your ELISA to be useful next week, your CV in measuring solutions must be less than 10%, better if less than 3%. Should be less than 3% especially when doing 2-fold serial dilutions because this amount of error is added at each step ie cumulative.*

$$CV = (\text{sample standard deviation}/\text{sample mean}) \times 100$$

CV is used instead of SD (standard deviation) to neutralize different sizes of variable values. This is accomplished by dividing the sample standard deviation by the sample mean. That is, coefficients of variation are comparable across data sets with dramatically different means.

Week 10

Possible Demonstrations:

- (i) Confocal fluorescent microscope.
- (ii) Immune Cells participating in Inflammation.

Part III: ELISA ASSAY Quantitative analysis of IL-13 Response

Sterile technique is not required for this laboratory.

1. ELISA plate (one/group) is coated for you with a predetermined optimal concentration of anti-IL-13 (3 $\mu\text{g}/\text{ml}$), then incubated overnight at 4°C.
2. Plates are blocked with BSA solution for you for ~1 hour, then washed.
3. The plate must be set up exactly as shown in the following diagram of a 96 well ELISA plate (unless otherwise specified by the TA).
- 4.

	Standard IL-13i	Non- stimulated	Con A stimulated	OVA stimulated								
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank											
B												
C												
D												
E												
F												
G												
H												

note: samples added to shaded area

4.

Each group is given four eppendorf tubes to set up ELISA plate:

Standard IL-13	labeled red
Supernatant from Non-stimulated cells	labeled #1
Supernatant from Con A stimulated cells	labeled #2
Supernatant from OVA stimulated cells	labeled #3

5. Add samples and buffer to shaded area of ELISA plate as described below (see diagram):

Standard IL-13 standard (use either column 1 or 7):

- a) Do not add anything to well A1 or A7 (blanks).
- b) Add 50 μ l standard IL-13 to well B1 or B7 (No dilution).
- c) Add 50 μ l buffer to wells C1 to H1 or C7 to H7.
- d) Add 50 μ l standard IL-13 to well C1 or C7. Mix gently. Do not create bubbles. Transfer 50 μ l to well D1 or D7 (2-fold dilution). Mix. Repeat 2-fold dilutions through to well H1 or H7. Discard 50 μ l from this well.

This will generate a standard curve of 1000, 500, 250, 125, 62.5, 31.25, 15.6 pg/ml.

Note: The range of the standard curve may vary - refer to ELISA printout for IL-13 standard range.

Non-stimulated cell supernatant (use either column 2 or 8):

- b) Add 50 μ l non-stimulated cell supernatant to well A2 or A8 (No dilution).
- c) Add 50 μ l buffer to wells B2 to D2 or B8 to D8.
- d) Add 50 μ l non-stimulated cell supernatant to well B2 or B8. Mix gently. Do not create bubbles. Mix. Transfer 50 μ l to well C2 or C8 (2nd 2-fold dilution). Mix. Transfer 50 μ l to well D2 or D8 (3rd 2-fold dilution). Discard 50 μ l from this well.

Con A stimulated cell supernatant (either column 3 or 9):

Do titration series as for non-stimulated cell supernatant, but in columns 3 or 9.

OVA stimulated cell supernatant (either column 4 or 10):

Do titration series as for non-stimulated cell supernatant, but in columns 4 or 10.

6. Cover plate with Saran wrap and incubate at 37°C for 40 minutes.
7. Wash using plate washer. Ensure plate is properly settle into rack to avoid damaging the machine. The washer is preset to wash 4 times. Afterwards, shake upside down by tapping 3 times on paper towels.
8. Add 50 μ l/well of development biotin-anti-IL-13 Ab (300 ng/ml, prediluted for you). Incubate 40 minutes at 37°C. Wash. Bang dry on paper towels.
9. Add 50 μ l of prediluted Streptavidin-alkaline phosphatase, incubate 30 minutes at 37°C. Wash. Bang dry on paper towels.
10. Add 50 μ l of PNPP solution (careful not to create bubbles) and incubate at room temperature or 37°C for 30 min.
11. Read at 30 to 45 minutes using pre-designed template. Obtain hard copy of your absorbance data.

Lab 5 Report (Report format available as a Word document, save before entering requested information - acceptable if only use 2b Table.)

Part II ELISA technique precision training

This section of the report must be done by each student even if you are handing in a group report.

- 1.0 1. a) Append printout of intra-assay variability data (if a group report, attach a copy of printout for each student). Completely label microtitration plate.
- 0.5 b) Highlight all values greater than acceptable range. Comment on your overall accuracy with respect to acceptable range.
- 1.0 c) In your lab you carried out an experiment to assess intra-assay variability of pipette technique. State how **intra-** assay variability differs from **inter-**assay variability?

Part III: ELISA ASSAY Quantitative analysis of IL-13 Response

This section of the report may be done individually or as a group.

- 1.0 2. a) Append ELISA printout. Completely label microtitration plate data.
- 1.5 b) Record requested information in the following table (available as a Word document on lab website). All information is given on ELISA printout. Only calculation(s) that may be required is averaging IL-13 concentration. On ELISA printout highlight adjusted result values for IL-13 concentration (pg/ml) selected.

Table 1. Quantitative analysis of the cytokine, IL-13 in mice spleen by ELISA assay.		
Absorbance range of the linear ^a part of your standard curve:		
supernatant sample	IL-13 concentration ^b (pg/ml) value(s)	mean IL-13 concentration ^b (pg/ml)
Non-stimulated cells		
Con A stimulated cells		
OVA stimulated cells		

^aExponential increase in absorbance relative to concentration. Concentration range (pg/ml) and 2bsorbance values are located in standard curve table.

^bUse only absorbance values that fit only the linear (ie. exponential) part of the curve. No calculations other than averaging (if more than one value) are required as adjusted value(s) given on ELISA printout. Make sure you understand that the concentration value has been multiplied by the dilution factor. If no values fit on the linear part of the curve, state this.

- 1.0 3. a) Does your group's experimental data agree with expected results. Explain why or why not.
- 0.5 b)What IL-13 production response would you predict in supernatant from ovalbumin stimulated cells for a mouse immunized with dead measles virus? Explain why.
- 1 4. Dr. Hayglass's lab⁹ investigated the prevention of allergen-specific, Th2-biased immune responses. In addition to doing similar cytokine ELISA assay they also used ELISA to determine IgG concentration. Present a completely label schematic diagram of the ELISA assay for IgG2c. Diagram should be similar to the schematic diagrams in your lab manual (molecular level). Be specific (isotype IgG2c) when labelling your diagram. Hand drawn is acceptable.

⁹Lewkowich, IP, Rempel, JD, HayGlass, K. 2005. Prevention of allergen-specific, Th2-biased immune responses in vivo: Role of increased IL-12 and IL-18 responsiveness. J. Immunol. 175: 4956-4962. Linked on lab website or search Highwire or UM NetDoc.

7.5

APPENDIX**SOLUTION PREPARATION PROCEDURE**

eg. How do you prepare 300 ml Alsever's solution (pH 6.1) containing 6.15 g glucose, 2.4 g sodium citrate, 1.26 g NaCl per 300 ml?

1. Put less than 300 ml (~250 ml) distilled water in a beaker with a stirring bar. Whenever the solvent is not give, assume it is distilled water. Put on a stirrer and start to stir.
2. Slowly add 6.15 g glucose. Stir until completely dissolved. Then slowly add 2.4 g sodium citrate. Again stir until completely dissolved. Next slowly add 1.26 g NaCl. Stir until dissolved.
3. Adjust the pH to 6.1. (You do not need to specify the acid or base added unless given in question.) You must adjust the pH before bring to the final volume.
4. Bring to a final volume of 300 ml with distilled water.

SOLUTIONS**Preparation of Sheep IgG**

PBS, pH 7.2 (phosphate buffered saline, pH 7.2): 8.77 g NaCl, 10.1 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5.1 g KH_2PO_4 to 500 ml distilled water, check pH and bring volume up to 1 liter.

10x PBS, pH 7.2: as above only 10x reagents/liter

phosphate buffer, pH 8.0: Prepare 0.5 M stocks of mono-basic and di-basic phosphate. Add 69.0 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (F.W. 138) to final volume 1 liter distilled water and 71.0 g Na_2HPO_4 (F.W. 142) to final volume 1 liter distilled water. Mix the two solutions to obtain the required pH, pH 8.0, then adjust to the required molarity (0.1 M).

Immuno-electrophoresis

Tris-tricine Immuno-electrophoresis Buffer: 30.4 g Trisma, 19.4 g tricine, 0.42 g calcium lactate, pH to 8.6 and distilled water to 4 liters.

Hemagglutination Assay

Alsever's solution: 6.15 g glucose, 2.4 g sodium citrate, 1.26 g NaCl per 300 ml. Adjust pH to 6.1 with 10% acetic acid prior to bring up to volume (300 ml). Filter sterilize. Add to an equal volume of fresh blood.

Physiological saline: 8.77 g NaCl/liter

0.1% (w/v) tannic acid in PBS, pH 7.2: prepare fresh daily

Diluent: 1:20 dilution of absorbed normal rabbit serum, which has already been diluted 1:5. Use PBS, pH 7.2 for the dilution.

ELISA

0.05 M carbonate-bicarbonate buffer, pH 9.6 (coating buffer): Dissolve 1.59 g Na_2CO_3 , 2.93 g NaHCO_3 , and 0.2 g NaN_3 in 500 ml distilled water. Bring to approximately 6 mM polyethylene glycol (PEG, MW 6000-8000) by adding 42 ml 1 M PEG. Adjust to pH 9.6, bring to 1 liter with distilled water.

PBS-Tween buffer, pH 7.4: Dissolve 8.77 g NaCl, 5.1 g KH_2PO_4 , and 10.05 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in 500 ml distilled water, check pH, then bring to 1 liter. Add 0.05% Tween.

PBS-Tween-BSA buffer: as PBS-Tween buffer, pH 7.4 with 1 mg/ml BSA added.

p-nitrophenyl phosphate (p-NPP) substrate (1 mg/ml) in 10% diethanolamine buffer, pH 9.8: 10 ml diethanolamine and 90 ml distilled water. Adjust to pH 9.8 with 1 N HCl. Dissolve 100 mg pNPP, to a final volume 100 ml 10% diethanolamine buffer, pH 9.8.

enzyme-antibody conjugate: Dilute (1:1000) in PBS-Tween-BSA buffer at room temperature immediately before use.

CONFOCAL FLUORESCENT MICROSCOPE DEMONSTRATION

“Visualizing early intracellular signaling events in lymphocyte activation using confocal fluorescent microscopy.”

by Dr. Aaron Marshall
Assistant Professor, Department of Immunology, University of Manitoba

Room 608 Basic Medical Science Bldg
Groups of 4-5 students
Demonstration 20-30 min

Microscope experiment:

- Observation of a live culture of B lymphocytes transfected with a fluorescent “reporter” protein or loaded with a fluorescent “reporter dye”.
- Explanation of how the confocal microscope can take digital optical sections through the sample that can be displayed on the computer screen through different optical planes.
- Explanation of how the fluorescent “reporters” allow visualization of intracellular signaling events during lymphocyte activation by either (1) increasing or decreasing fluorescent intensity or (2) changing their location within the cell.
- Demonstration of lymphocyte activation - anti-IgM (antibody that mimics the real antigen) will be added to the antigen receptor while continuously imaging on the computer screen. Within 1-3 minutes a change in fluorescent intensity or distribution should be observed.
- While replaying the sequence of captured digital images, the significance of what is being observed will be discussed. For example, observation of the earliest response the lymphocyte makes to antigenic stimulation, part of the cascade of events changing the cellular activity dramatically from a quiescent/resting state to an active response. The cells produce more copies, differentiate to become effector cells, antibody production, etc.

OPERATION OF FLOOR MODEL CENTRIFUGES

Note: If procedure varies depending on centrifuge manufacturer a step by step operation procedure is usually located on or nearby the centrifuge or the teaching assistant will help you.

HITACHI HIGH SPEED HIMAC REFRIGERATED CENTRIFUGE

- to select or change settings the CHECK button must first be pressed (light on). The light stays on for 16 sec. When the light is off you can no longer select, change setting or carry out any operation, just press check button again and continue.
- When the centrifuge is turned on and the CHECK button is not pressed. The centrifuge displays real time parameters.

OPERATION

Centrifuge tubes should be balanced by scale by adding or removing appropriate solution from one of the tubes.

1. Turn power switch on. The indicators on the control panel are illuminated. The door lock is released.
2. Open door. If required set the rotor gently in position and close door. Turn the rotor lightly by hand to check that the rotor is correctly set. Remove the rotor lid and place balanced tubes opposite each other in rotor. You cannot run the centrifuge with an odd number of tubes.
SCREW ON LID.
3. Call up memory code number or enter parameters.
Call up pre-programmed memory code number: Press CHECK button, MEMORY button, memory code number, and CALL button. Each memory code number consists of a specified set of operation parameter (see sheet on centrifuge cover). See below for a list of operation parameters and how to set and store operation parameters.
OR
Real time operation (enter original parameters): see setting of operation parameters below.
4. After the parameters are set make sure the check light is still on. If not, press the CHECK button.
5. Press the START button. The rotor starts running. The start lamp begins flashing. The timer starts to count down.
6. The timer counts down to zero or press the STOP button. The rotor begins to decelerate. The stop light begins flashing.
7. The rotor stops. The stop light stops flashing. A buzzer sound occurs. The door lock is released.
8. Unscrew rotor lid and remove tubes. If required, use tweezers to help remove tubes. Wipe out rotor if spills occur. DO NOT SCREW ON THE LID just place on top of the rotor.
9. Close centrifuge lid and turn off power.

PIPETMAN OPERATION

In your lab, you have available three different pipetmen depending on the lab. If you look at the top of the plunger it states the size of the pipetman.

P20 measures accurately from 2 μ l to 20 μ l.

P200 measures accurately from 20 μ l to 200 μ l.

P1000 measures accurately from 100 μ l to 1000 μ l.

Never turn the pipetman above the maximum volume; 20 μ l for P20, 200 μ l for P200, and 1000 μ l for P1000 as this breaks the pipetman. The scale on the pipettor is read different for each type - refer to Figure 5 for an example of how to read the scale.

(Excerpted from Gilson pipetman operation manual.)

1. Setting the volume: The required volume is set on the digital volumeter by turning the knurled adjustment ring (Figure 3-2A). The volumeter display is read from top to bottom in μ l for P20 and P200 and ml for P1000 (Figure 3-2).
2. Place a disposable tip on the shaft of the Pipetman. Press on firmly with a slight twisting motion to ensure an airtight seal. Depress the push-button to the first positive stop (Fig. 3-3A). While holding the Pipetman vertical, immerse the tip 2-4 mm into the sample liquid. Release the push-button slowly to draw up the sample (Fig. 3-3B). Wait 1 to 2 seconds, then withdraw the tip from the sample.
3. To dispense the sample, place the tip end at a 10-45° angle against the inside wall of the vessel and depress the push-button SMOOTHLY to the first stop (Fig 3-3C). Wait 1 to 2 seconds and then depress the push-button completely to expel any residual liquid (Fig. 3-3D). With the push-button fully depressed, carefully withdraw the Pipetman, sliding the tip along the inside wall of the tube. Release the push-button. Remove the used tip by depressing the tip ejector button (Figure 3-1F).

pipetman operation

ELECTROPHORESIS

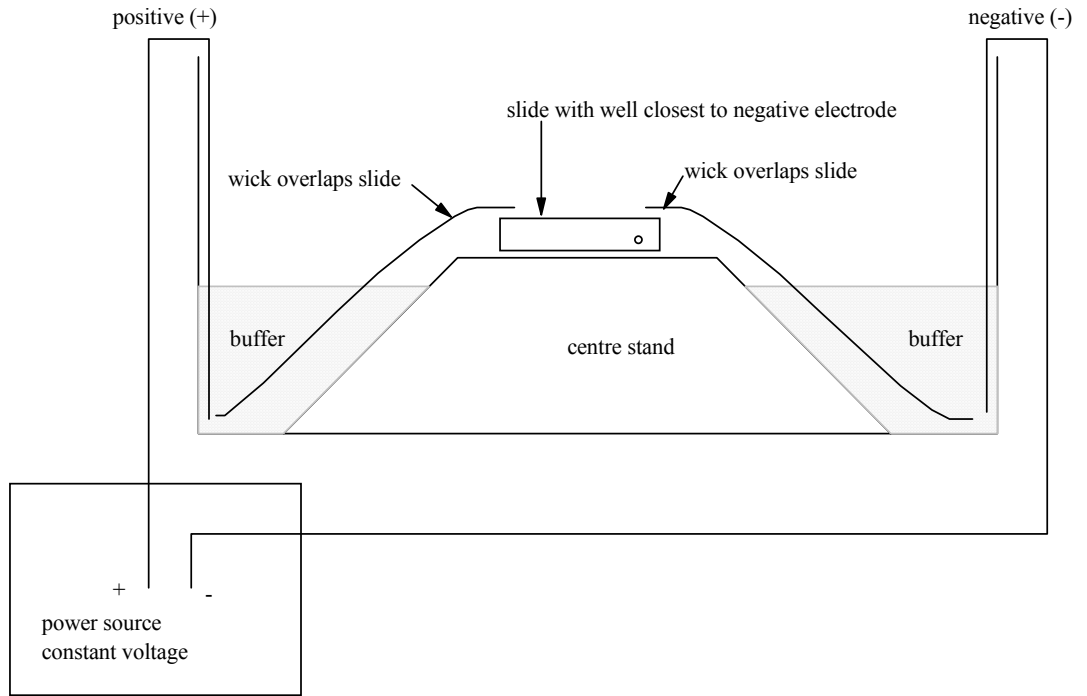
Electrophoresis is based on the theory that complex molecules often carry a net positive or negative charge. If the molecule does not have a charge it is unable to move in an electrical field with first being complexed with charged compound.

Proteins can be separated from each other by electrophoresis because they have different net charges. Each protein has a characteristic isoelectric point (pH at which the number of positively charged amino acids is equal to the number of negatively charged amino acids). The magnitude of the net charge on a protein molecule depends upon the magnitude of the difference between its pI and the pH of the electrophoretic buffer. For example, if electrophoresis is carried out in a pH 8.6 buffer, albumin will be strongly negatively charged as its pI is 4.7 whereas, gamma globulins will not be as negatively charged as they have a pI of 7.2. When electrophoresis is carried out in a 8.6 buffer, the albumin will migrate much more rapidly towards the positive electrode than the gamma globulins resulting in a separation of these proteins.

An electrophoresis apparatus is composed of four main components; electrodes, the buffer chamber, power supply, and matrix that supports the components allowing separation, figure 9. The electrodes are usually made of inert non-conducting material such as platinum or carbon. There are two buffer chambers, one for the positive electrode and one for the negative electrode. Electrophoresis is carried out using a constant voltage (caution as may be a problem of overheating when using constant voltage). Applying Ohm's law $I = V/R$ (I = current, V = voltage, and R = resistance) the power pack compensates for an increase in resistance by increasing the current which leads to overheating. **THE ELECTROPHORESIS UNIT IS DANGEROUS** when using constant voltage - if a student comes into contact with the supporting medium and is well grounded the resistance goes down and the power pack compensates by increasing the current the results of which may be **DEADLY** to the student. Stay away from electrophoresis apparatus when they are in operation.

The buffer system used should buffer well at the required pH and have low ionic strength (low conductivity - does not migrate in an electrical field) as a high ionic strength buffer would increase current leading to overheating. The second requirement, of low ionic strength, interferes with the buffering capacity of the buffer therefore should only be used once or twice.

Immuno-electrophoresis System



FINAL LAB EXAM: Microbiology MBIO 4010 IMMUNOLOGY
DATE: SAMPLE PAGE: 1 of 2 TIME: 1.5 h
INSTRUCTOR: Dr. L. Cameron
Student Name: _____ Student Number: _____

Write exam in PEN only.

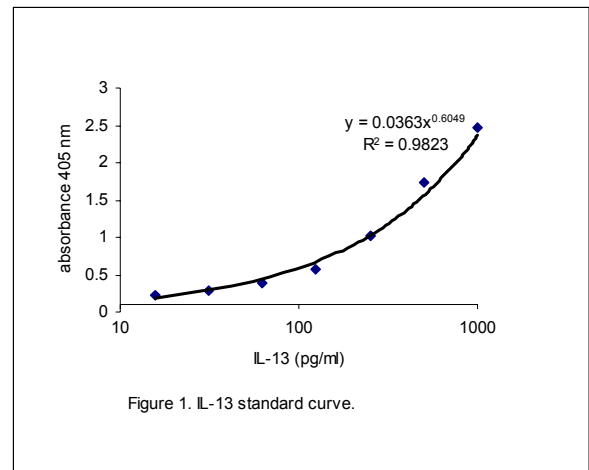
Answer all QUESTIONS on EXAM PAPER. Spacing of questions has been compressed. Questions pertaining to lab report questions have been omitted as they vary from year to year but still responsible for information in questions. Sample exam is longer than final lab exam to give a wide range of sample questions.

- 6 1. Briefly answer each of the following questions.
- Outline procedure to prepare 200 ml of 0.1% tannic acid (w/v)?
w= weight, v = volume
 - Define hemagglutination antibody titre.
 - Define ELISA antibody titer as determined by Hayglass et al (2003). Note: same as you did in the lab.
 - Compare the sensitivity of the ELISA and hemagglutination assay.
 - Prior to starting the floor model refrigerated centrifuge, outline centrifugation procedure.
 - In rocket immunoelectrophoresis, antibody is added to the agarose and the concentration of the antigen is determined. Since both are proteins, how is it possible to get rocket formation during electrophoresis?
- 10 2. Explain the function of the following reagents or procedures used in your immunology lab.
- Na₂SO₄ solution
 - precoating immunodiffusion slides with 1% agarose prepared in distilled water
 - 0.1% tannic acid solution
 - normal serum diluent
 - PBS-Tween
 - Tris-tricine buffer
 - DEAE cellulose
 - wick in immunoelectrophoresis
 - red blood cells in hemagglutination assay
 - PBS-Tween-BSA buffer
- 2 3. Determine the concentration at titre for lectin, *Helix ponatia* using the following data. The original concentration of *Helix ponatia* is 0.5 mg/ml. Two-fold serial dilutions in wells 2 through 24: diffuse carpet of sheep red blood cells wells 2 through 8, weak reaction wells 9 and 10, and solid button well 11 to 24.
- 2 4. Outline an experiment to determine if your crude sheep IgG you purified in the immunology lab contains impurities and to tentatively identify major impurities.

CONTINUED ON PAGE 2...

FINAL LAB EXAM: Microbiology MBIO 4010 IMMUNOLOGY
DATE: April 4, 1996 PAGE: 2 of 2 TIME: 1.5 h
INSTRUCTOR: Dr. L. Cameron

- 2 5. a) Explain the mechanism of precipitin line formation.
- 3 b) Diagram the precipitin lines obtained for a double immunodiffusion plate set up as in your lab, centre well anti-sera, surrounded by 5 antigenic wells using the following data. Completely label diagram.
 Centre Well: anti-rabbit whole serum
 Outside 5 wells in the following order:
 1) rabbit whole serum
 2) rabbit IgG (pure)
 3) rabbit IgG prepared by sodium salt precipitation
 4) rabbit albumin, supernatant from rabbit IgG prepared by sodium salt precipitation
 5) rabbit albumin (pure)
- 3 6. In lab you did two ELISA experiments, how do they differ and why? Answer must include a schematic diagram at the molecular level.
- 1 7. Define inter-assay variability. Give a practical example.
- 1 8. Assume you performed a cytokine assay exactly as performed in your lab. The following is a printout of the standard curve. What approximate absorbance range must an experimental sample be within?



- 1 9. You use ovalbumin twice in your cytokine lab. State how and explain why.
- 1 10. Determine the volume (ml) of mouse spleen cells required to add 7 million cells to each culture plate well for hemocytometer count results:
 Row 1, 45; row 2, 43; row 4 39; row 5, 42. The cells were diluted 1/4 before counting. The volume of each cell is (0.02 cm by 0.02 cm by 0.01 cm)