

RAP publication 2002/22

**A BASIC LABORATORY  
MANUAL**  
for  
**THE SMALL-SCALE PRODUCTION  
AND TESTING OF I-2 NEWCASTLE  
DISEASE VACCINE**

Sally E. Grimes



ISBN 974-7946-26-2

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## **Preface**

The School of Veterinary Science at the University of Queensland has been involved in Newcastle disease virus research and the development of thermostable Newcastle disease vaccines for more than twenty years. The Australian Centre for International Agricultural Research (ACIAR) funded most of this research which was supervised by Peter Spradbrow and involved international collaboration. ACIAR supported the development of the I-2 Newcastle disease vaccine master seed stored at the School of Veterinary Science. The master seed is distributed to collaborating institutions for use in producing thermostable vaccines to control Newcastle disease in village chickens in developing countries.

This manual summarizes the basic laboratory procedures used to produce and test experimental I-2 thermostable Newcastle disease vaccine in the research and training programmes associated with the John Francis Virology Laboratory. The content of the manual is based on an earlier manual written by Peter Spradbrow, Zuhara Bensink and myself. The original manual was prepared for use in a practical laboratory workshop held at the Poultry Reference Laboratory at Onderstepoort in the Republic of South Africa in 1995. The workshop was funded by ACIAR and was the first of a series of workshops that aimed to transfer the technical skills required for the small scale production and testing of Newcastle disease vaccine. The original manual was revised in response to comments from trainees and colleagues and used in several subsequent workshops.

After the second laboratory workshop funded by the Food and Agriculture Organization (FAO) of the United Nations, Denis Hoffmann, Senior Animal Production and Health Officer of the FAO Regional Office for Asia and the Pacific suggested the manual be updated and rewritten for publication on the Animal Production and Health Commission for Asia and the Pacific (APHCA) website. The original content has been expanded and it is hoped the changed format will be easier to follow and translate. Comments are welcome.

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**July 2002**

## Acknowledgements

This project has taken longer than anticipated and I thank Denis Hoffmann for his patience and for giving me the opportunity to write it in the first place.

Special thanks to Peter Spradbrow for ongoing support and generosity with time and knowledge.

Thanks also to the following people who have all contributed to the project and offered support:

Robyn Alders, Mary Young, Joanne Meers, Graham Burgess, Philemon Wambura, Joram Buza, Halifa Msami, Joseph Awuni, S.B. Chamling Rai, Karma Rinzin, Berhan Demeke Meshesha and Denise O'Rourke.

Assistance with the illustrations was provided by Sharka Marvilla of Sharka Designs and Naomi Mason of the University of Queensland.

Photographs were taken by the author and are from a collection kept at the John Francis Virology Laboratory.

## Abbreviations

<b>ACD</b>	Acid citrate dextrose anticoagulant
<b>ACIAR</b>	Australian Centre for International Agricultural Research
<b>EID<sub>50</sub></b>	50 percent embryo infectious dose
<b>ELD<sub>50</sub></b>	50 percent embryo lethal dose
<b>DGV</b>	Dextrose gelatin veronal solution
<b>GMT</b>	Geometric mean titre
<b>HA</b>	Haemagglutination / haemagglutinin
<b>HI</b>	Haemagglutination inhibition
<b>IU</b>	International units
<b>LD<sub>50</sub></b>	50 percent lethal dose
<b>MDT</b>	Mean death time
<b>OIE</b>	Office international des epizooties
<b>PBS</b>	Phosphate buffered saline
<b>PCV</b>	Packed cell volume
<b>PSG</b>	Penicillin Streptomycin Gentamycin antibiotic solution
<b>RBC</b>	Red blood cells
<b>SPF</b>	Specific pathogen free
<b>V4-HR</b>	Heat resistant strain of V4 Newcastle disease virus vaccine
<b>+ ve</b>	Positive
<b>- ve</b>	Negative

# 1. Newcastle disease vaccines: an overview

## Introduction

Chickens are susceptible to many infectious diseases. One of the most important of these is the viral disease known as Newcastle disease, which causes devastating losses in both commercial and village chickens. Reducing losses of large numbers of village chickens to virulent Newcastle disease is an essential first step to improving their productivity. Newcastle disease can be controlled by the use of vaccines. There are many Newcastle disease vaccines suitable for use in commercial chickens. These are available on the international market. The I-2 Newcastle disease vaccine has been developed for local or regional production and use in controlling Newcastle disease in village chickens.

Many Newcastle disease vaccines deteriorate after storage for one or two hours at room temperature. This makes them unsuitable for use in villages where the vaccine may need to be transported for hours or in some cases days at ambient temperature. The I-2 Newcastle disease vaccine is more robust and is known as a thermostable vaccine. Thermostable vaccines still require long-term storage in the refrigerator. However during transportation of the vaccine to the field, the vaccine will not deteriorate as quickly as the traditional vaccines. Evaporative cooling provided by wrapping the vaccine in a damp cloth will be adequate for maintaining the viability of the vaccine during transportation to remote villages. However if it is stored in direct sunlight or allowed to reach high temperatures (above 37°C) for more than a few hours it too will deteriorate and be unsuitable for use as a vaccine.

## Immunity to Newcastle disease virus

Chickens that survive infection with virulent Newcastle disease virus develop a long lasting immunity to further infection with Newcastle disease virus.

The basis of this immunity is:

1. Circulating antibodies.
2. Secreted antibody producing mucosal immunity.
3. Cell mediated immunity.

Newcastle disease virus of low virulence induces similar immune responses without causing severe disease. This is the basis of vaccination.

See **Appendix 2** for more information about Newcastle disease virus.

## Live vaccines

These vaccines are made with virus that is alive and able to infect cells. Strains of virus of low or moderate virulence are used. They mimic natural infection and induce all three immune responses.

## Killed vaccines

The ability of the virus to infect cells has been destroyed by treatment with a chemical, radiation or heat. These vaccines invoke only a circulating antibody response.

## Some vaccine strains of Newcastle disease virus

Strains of Newcastle disease virus have been broadly classified into four pathotypes as follows:

Avirulent Causes no disease

Lentogenic Low virulence, low mortalities, loss of egg production

Mesogenic Moderate virulence, mortalities up to 50 percent, loss of egg production

Velogenic High virulence, severe disease with high mortalities.

(Spradbrow P.B. 1987)

Many strains of Newcastle disease virus other than velogenic strains are used in the production of live vaccines. Eight of these strains are listed in **Table 1**.

**Table 1: Eight strains of Newcastle disease virus used in live vaccines**

Strain	Description
F	Lentogenic. Usually used in young chickens but suitable for use as a vaccine in chickens of all ages.
B1	Lentogenic. Slightly more virulent than F, used as a vaccine in chickens of all ages.
La Sota	Lentogenic. Often causes post vaccination respiratory signs, used as a booster vaccine in flocks vaccinated with F or B1.
V4	Avirulent. Used in chickens of all ages.
V4-HR	Avirulent. Heat Resistant V4, thermostable, used in chickens of all ages.
I-2	Avirulent. Thermostable, used in chickens of all ages.
Mukteswar	Mesogenic. An invasive strain, used as a booster vaccine. Can cause adverse reactions (respiratory distress, loss of weight or drop in egg production and even death) if used in partially immune chickens. Usually administered by injection.
Komarov	Mesogenic. Less pathogenic than Mukteswar, used as booster vaccine. Usually administered by injection.

## **Thermostable Newcastle disease vaccines**

Thermostable Newcastle disease vaccines exhibit a relative resistance to inactivation on exposure to elevated temperatures. Strains of Newcastle disease virus vary in thermostability.

- Thermostable vaccines are prepared from a strain of Newcastle disease virus that retains its ability to infect cells after storage outside a cold chain for a short period of time.
- There are two basic processes used to produce a thermostable Newcastle disease vaccine.
  1. Isolation of naturally occurring thermostable variants of the virus.
  2. Increasing the thermostability of this variant by artificial selection in the laboratory.
- A seed lot system is used to produce the vaccine. The sequential use of a master seed and a working seed minimizes the number of passages to produce a vaccine and maintains the genetic stability of the vaccine virus. The antigenicity and the thermostability of the virus in the master seed should be retained during the two passages that produce the working seed and then the vaccine.

See **Section 13** for more details.



## 2. Basic laboratory skills

### Introduction

Laboratory staff should be familiar with and have practiced the following skills prior to the commencement of Newcastle disease vaccine production. This manual does not contain further details about these skills.

- Aseptic technique.
- Sterilization by autoclaving and hot air of glassware and discarded materials.
- Mixing or stirring by:
  1. Hand
  2. Magnetic stirrer
  3. Vortex mixer
- Measuring pH
- Using centrifuges

### Instructions for using a daybook

The details of all technical procedures carried out to produce and test the I-2 Newcastle disease vaccine must be recorded by the technician in a daybook.

- Use a bound book.
- Sequentially number all the pages.
- Set aside 3 to 4 pages for a “table of contents”.
- Set up an “abbreviations page” at the front of the daybook. List all personalized or uncommon abbreviations.
- Put the date and title of the experiment at the top of each page.
- It is best to use a black pen as this colour photocopies well. Do not use pencil.
- Errors should be ruled through and corrected. Do not use an eraser or cover mistakes with white “liquid paper”.

## Preparation of reagents

The following reagents will be required during the production and testing of the I-2 Newcastle disease vaccine.

- Phosphate Buffered Saline (PBS)
- An anticoagulant. Acid Citrate Dextrose (ACD) or alternatively Alsever's Solution
- Dextrose Gelatin Veronal (DGV). A storage solution for 10 percent washed red blood cells
- An antibiotic solution
- Tryptic soy broth
- Sabouraud agar

Methods for preparing these media and solutions are included in **Appendix 3**.

## Recording details of practical work

Use the following format for recording the details of your practical work.

**Aim:** Outline the purpose of the activity.

**Materials and Methods:** Record enough information that will allow another scientific or technical person to repeat the procedure. If standard procedures are used in a series of experiments, describe them as standard. Examples of such standard procedures are those described in a Laboratory Manual or a previous experiment.

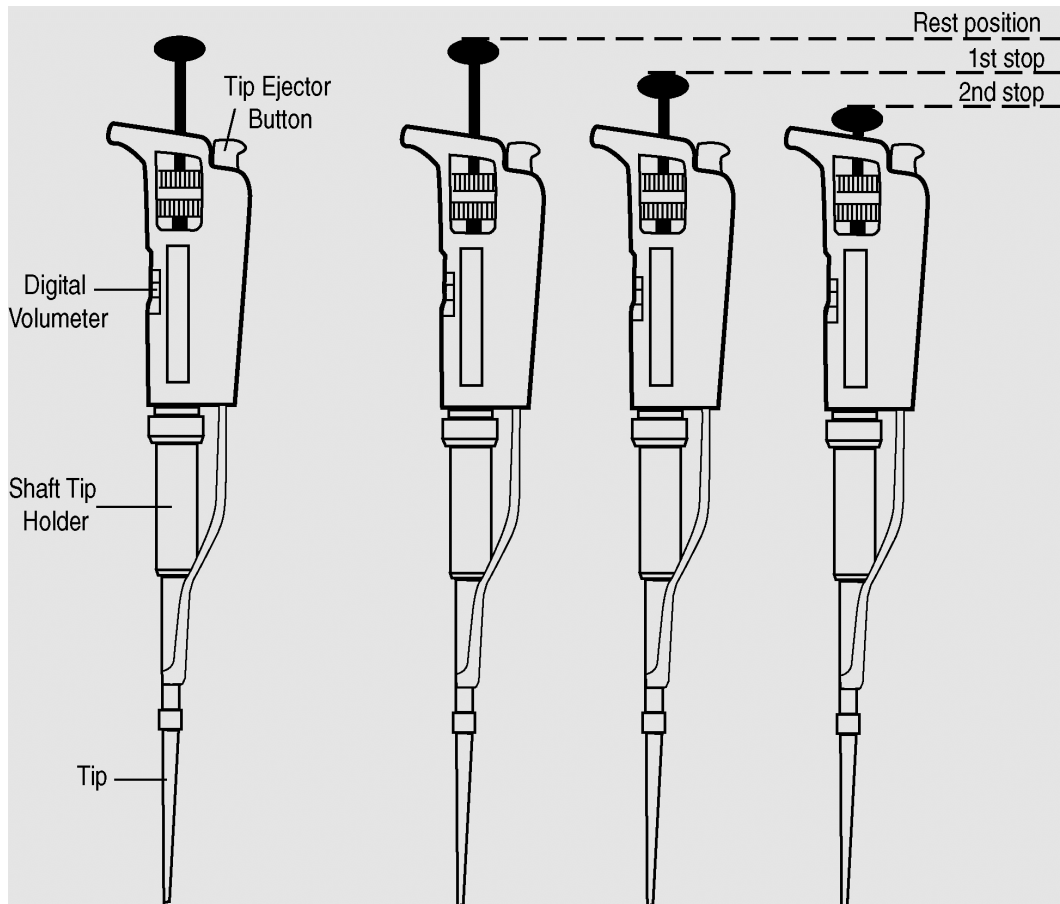
**Results:** Record all the results and observations. Prepare a table for recording data where appropriate.

**Conclusions:** Comment on your results and observations. Make suggestions for further experiments.

## **The care and use of single and multichannel micropipettes**

Manufacturer's instructions are supplied with all micropipettes. Please read and make sure you understand how to operate and care for the pipette. Micropipettes use plastic disposable tips. For ease of use, tips are usually packed into plastic boxes that can be autoclaved. Make sure the tips you are using will fit tightly onto the end of the pipette.

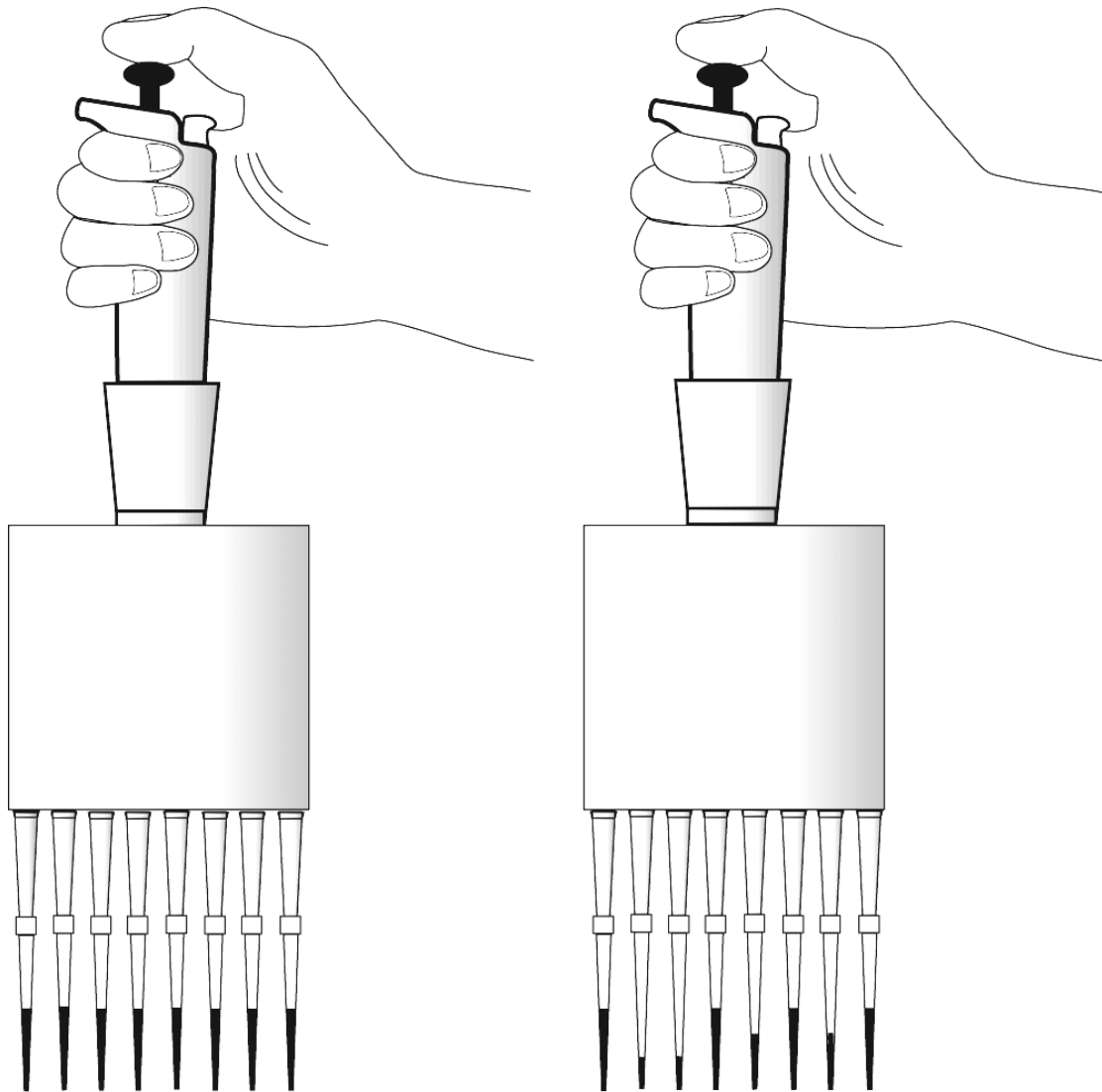
- Treat micropipettes very gently as they are precision instruments.
- Keep upright when in use to prevent liquids running inside the shaft of the pipette.
- Do not leave pipettes lying on the workbench where they can be knocked off and damaged.
- Do not allow pipettes to come into contact with corrosive chemicals.
- Before use, make sure the volume has been correctly set. Adjust the volume before use. Most pipettes have a digital display of volume. Some brands have a micrometer setting, which can be difficult to read.
- Check all tips are securely fitted to pipette.
- Draw liquid up.
- Check that the liquid drawn up has reached the expected level in the tip and there are no air bubbles in the tip. When using multichannel pipettes, check that the volume of liquid is the same in each tip.
- If necessary, expel the liquid and manually tighten the tips onto the pipette.
- Draw up the liquid and check again.



**Figure 1: Single channel micropipette**

**Instructions for pipetting liquids using a micropipette**

1. Micropipettes have 3 positions:
  1. Rest position
  2. First stop
  3. Second stop
2. Fit the tip to the end of the shaft. Press down and twist slightly to ensure an airtight seal.
3. Hold the pipette in a vertical position. Depress the plunger to the first stop. Air equal to the volume of the setting (e.g. 100  $\mu$ L) is displaced.
4. Immerse the tip into the liquid. Release the plunger back to the rest position. Wait a second for liquid to be sucked up into the tip. The volume of liquid in the tip will equal the volume of the setting of the micropipette.
5. Place the tip at an angle ( $10^{\circ}$  to  $45^{\circ}$ ) against the wall of the vessel receiving the liquid, for example a well of a microwell plate. Depress the plunger to the first stop, wait one second, press the plunger to the second stop to expel all the liquid
6. Move the end of the tip away from the liquid. Release the plunger to the rest position.



Correct

Each tip has drawn up an equal volume of liquid.

Incorrect

The volume of liquid drawn up in each tip varies.

**Figure 2: Using a multichannel micropipette**

## **Graduated pipettes**

Graduated pipettes are calibrated and marked with graduation lines that allow the measurement of more than one volume. The volume is read by eye by reading the value indicated on the scale at the bottom of the meniscus. Disposable plastic graduated pipettes are available and are useful for pipetting toxic or viscous substances. Graduated pipettes made of glass can be washed and reused. The pipettes should be plugged with cotton wool on the top and sterilized before use to minimize contamination of fluids being measured.

1 mL and 10 mL graduated pipettes are most commonly used in the laboratory practices described in this manual. Before using graduated pipettes check the volume scale and note:

- Does the pipette empty from full volume to zero or from zero to full volume?
- Is the pipette designed to be emptied by gravity with the tip in contact with the vessel or to be expelled by blowing out with a pipette filler?

## **Pasteur pipettes**

Pasteur pipettes are glass pipettes used to transfer fluids from one place to another. They are not graduated and are therefore not used to measure volumes. Like graduated pipettes they should be plugged with cotton wool and sterilized before use.

## **Pipette fillers**

Pipetting by mouth is not an acceptable laboratory practice. Fluids are drawn up into pipettes using pipette fillers. There are several options. A simple rubber bulb is suitable for a 1 mL pipette. For 10 mL pipettes, use a triple valve rubber bulb, a hand operated pump or an electronic pipette filler.

## **Microwell plates**

Plastic microwell plates are now routinely used in laboratories to perform assays using small volumes of samples and reagents. The plates contain 96 wells arranged in an 8 x 12 format with columns labeled 1 to 12 and rows labeled A to H. Thus each well has a designation for example A 12. Plates are manufactured with different shaped wells suitable for different tests. The wells can be round bottom, flat bottom and V-bottom.

In this manual, the haemagglutination and haemagglutination inhibition tests are carried out in V-bottom microwell plates. Two-fold dilutions can be made across the plate in either orientation that is from A1 to A11, (A12 being a control well) or from A1 to G1 (H1 being a control well).

The microwell plates used at the John Francis Virology laboratory are manufactured by Nalgen Nunc International. The plates cannot be autoclaved and are disinfected after use by soaking overnight in a 2 percent chloride solution. They are then washed and rinsed three times in tap water, followed by rinsing three times in distilled water, dried and reused.

Use of microwell plates is referred to in **Section 10, Section 11 and Appendix 4.**

(For details about the cost and supply of microwell plates visit the Nalgen Nunc website at <http://nuncbrand.com> and look up the product catalogue number 442587.)

### 3 Laboratory safety: an overview

#### Introduction

The person supervising the production and testing of the I-2 thermostable Newcastle disease vaccine is responsible for ensuring that laboratory staff are familiar with the laboratory safety rules and regulations. It then becomes the responsibility of each person involved with the production of the vaccine to work within this framework. Laboratory staff must give due consideration to their own and their colleague's safety. The maintenance of clean and tidy work areas is an important precaution that is easy to implement.

#### Safety manual

Every laboratory should have a safety manual. Ensure all the procedures involved with the production and testing of I-2 Newcastle disease vaccine are covered in the laboratory safety manual.

**This manual does not take the place of a laboratory safety manual**

#### A list of some of the safety issues to be addressed in a laboratory safety manual

- Fire
- First aid
- Safe use of laboratory glassware
- Safety with handling boiling liquids
- Cleaning up spills and broken glass
- Decontamination of benches and waste
- Recycling of infected materials for example, needles and syringes
- Safe use of chemicals

#### A list of basic laboratory rules

- Always wash hands thoroughly with soap on entering and leaving the laboratory.
- Do not eat, drink or smoke in the laboratory.
- Always wear a laboratory coat.
- Label all reagents clearly with contents, date and the initials of the person who prepared the reagent.
- Do not pipette by mouth.
- Dispose of discarded materials as instructed.



## Human infection with Newcastle disease virus

There have been instances recorded of human infection with Newcastle disease virus. On one occasion, allantoic fluid containing virulent Newcastle disease virus splashed into the eye. A mild conjunctivitis resulted. The author is unaware of more serious infections with Newcastle disease virus. It is important to take care when handling live Newcastle disease virus including the I-2 strain. Avoid contact with the virus.

## Websites

More information about basic laboratory safety can be found at the following websites.

- University of Calgary

<http://www.ucalgary.ca/~ucsafety/bulletins/genlab1.htm>

- University of California

<http://envhort.ucdavis.edu/safety/labsafety.html>

- University of Sydney

<http://www.usyd.edu.au/su/ohs/labsafety.html>

- Centers for Disease Control and Prevention, Atlanta, Georgia, USA

<http://www.cdc.gov/od/ohs/manual/labsfty.htm>

- Howard Hughes Medical Institute

The Office of Safety at the Howard Hughes Medical Institute has a useful website with access to Laboratory Chemical Safety Summaries and training materials.

<http://www.hhmi.org/research/labsafe/index.html>

## 4 Incubation of fertile eggs

### Introduction

All strains of Newcastle disease virus grow in embryonated eggs. Embryonated eggs are used for isolating the virus, producing vaccine and producing antigen for serological tests.

### Source of eggs

There is a theoretical risk of contaminating vaccines with pathogens that are transmitted through embryonated eggs. The use of embryos from a Specific Pathogen Free (SPF) flock minimizes this risk. However, in many countries, embryonated eggs from an SPF flock are unavailable. In this case it is practical to use embryonated eggs from a healthy local flock.

When deciding which flock to use to supply the eggs, consider the following points. Seek veterinary advice about the health status of the flock.

- Does the flock appear healthy and free from infectious disease?
- Is there any serological testing performed on the flock?
- What is the vaccination regime used in the flock?
- Is the flock free of *Salmonella pullorum*?
- Are the eggs clean?
- What is the percentage of fertile eggs?
- Is the hatching rate acceptable?
- Are the newly hatched chicks healthy?
- Do not purchase eggs during an outbreak of any disease in the flock supplying the eggs.
- If eggs are purchased from a commercial hatchery at 8 or 9 days old, candle at the hatchery to select eggs with viable embryos. Keep the eggs warm during transport to the vaccine production centre.
- Eggs with white shells are preferable as they are easier to candle.

## **Recording details of egg purchases**

Once a suitable source of embryonated eggs has been located, an order can be placed for the delivery of the eggs. It is useful if the person responsible for placing the orders and receiving the eggs keeps records.

The following information should be recorded in a notebook set aside for this purpose.

- Date when the eggs are ordered and the name of the person who received the order.
- Number and age of the eggs ordered.
- Date and number of the eggs received.
- Colour and appearance of the eggs received.
- Number of eggs damaged during transport.
- Date and number of eggs placed in incubator.
- Number of viable eggs after candling prior to inoculation.

## **Storage and cleaning of eggs**

- Do not buy dirty eggs.
- Eggs that are stained can be disinfected by washing in a warm (37°C) solution of 0.1 percent Chloramin B (benzine sulfonamide sodium salt) or wiped with a 70 percent alcohol solution.
- Fertile eggs that have not been incubated can be purchased. They can then be placed in an incubator when they are delivered. Alternatively, they can be stored for several days in cool conditions (16°C to 18°C) prior to incubation. This may reduce the number of viable embryos, as some embryos may not develop after storage.

## **Incubation of eggs before inoculation**

Many vaccine production centres will already have large commercial incubators installed. Smaller incubators are available and are suitable for the small-scale production of vaccine.

- Incubation temperature = 38°C to 39°C.
- Humidity should be maintained at 60 to 65 percent. A tray filled with water and placed in the bottom of the incubator is usually sufficient to maintain this level of humidity.
- Place the eggs in the incubator with the air sac on top.
- Eggs should be turned three times a day.

## **Incubation of eggs after inoculation**

Inoculated eggs contain virus and should be placed in a different incubator. Eggs inoculated with virulent strains of Newcastle disease virus should not be incubated in the same incubator as used for eggs inoculated with the avirulent I-2 strain of Newcastle disease virus.

Inoculated eggs are incubated under the same conditions as uninoculated eggs but do **NOT** turn the eggs.

## **Cleaning and decontamination of incubators**

Keep surfaces clean by wiping out with a wet cloth and disinfecting with 70 percent alcohol solution or a non-corrosive disinfectant.

See **Section 16** for more information about cleaning and decontamination.

## 5 Candling eggs

### Introduction

Candling is the process of holding a strong light above or below the egg to observe the embryo. A candling lamp consists of a strong electric bulb covered by a plastic or aluminium container that has a handle and an aperture. The egg is placed against this aperture and illuminated by the light. If you do not have a candling lamp, improvise. Try using a torch.

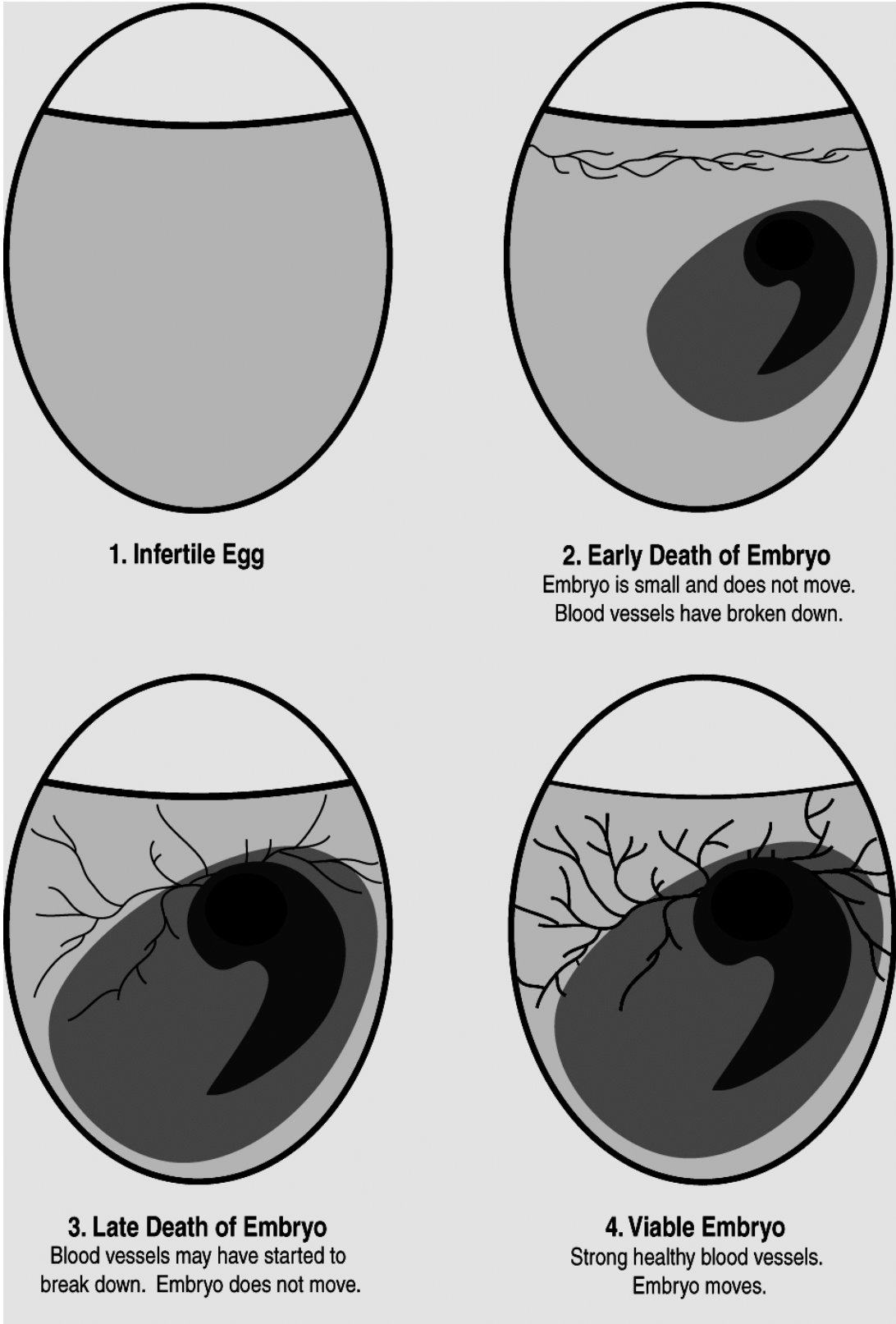
Candling is done in a darkened room or in an area shielded by curtains. **Figure 6** shows the candling booth at the John Francis Virology Laboratory.

### Determining the viability of the embryo

Under the candling lamp, the embryo appears as a dark shadow with the head as a dark spot. Healthy embryos will respond to the light by moving. Sometimes the movement is very sluggish and it can take 30 to 40 seconds for the embryo to move when held under the candling lamp. This indicates the embryo is not healthy and the egg should be discarded.

Look carefully at the blood vessels. They are well defined in a healthy embryo. After an embryo has died, the blood vessels start to break down. They then appear as streaks under the shell when viewed under the candling lamp. Candling will also reveal cracks in the eggshells. Eggs with cracked shells should be discarded.

- **Infertile eggs:** These are easy to detect, as the egg is clear. Discard
- **Early deaths:** The embryo has developed for several days and then died. Candling will reveal a small dark area and disrupted blood vessels. Often deteriorating blood vessels will appear as a dark ring around the egg. Discard.
- **Late Deaths:** These are often difficult to tell apart from a viable embryo at the same stage of development. Look for the absence of movement and the breakdown of the blood vessels. Discard
- **Viable Embryos:** These move in response to the light and have well defined blood vessels. Mark the air sac and the inoculation site and then return the eggs to the incubator ready for inoculation.



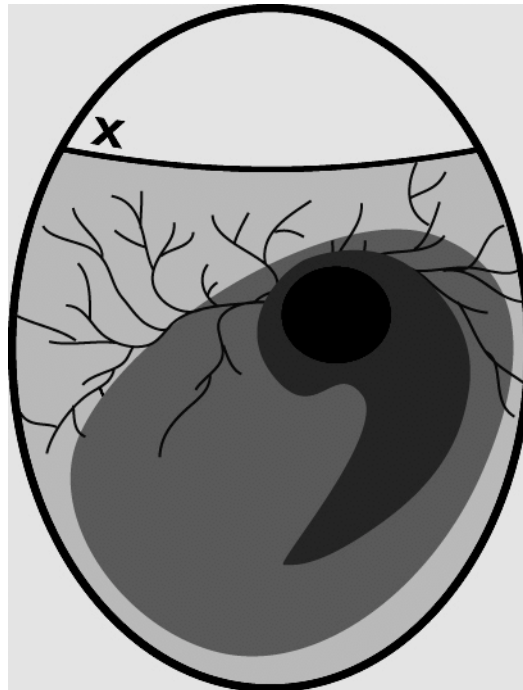
**Figure 3:**  
**Features of embryonated eggs visible during candling**

### Marking the inoculation site:

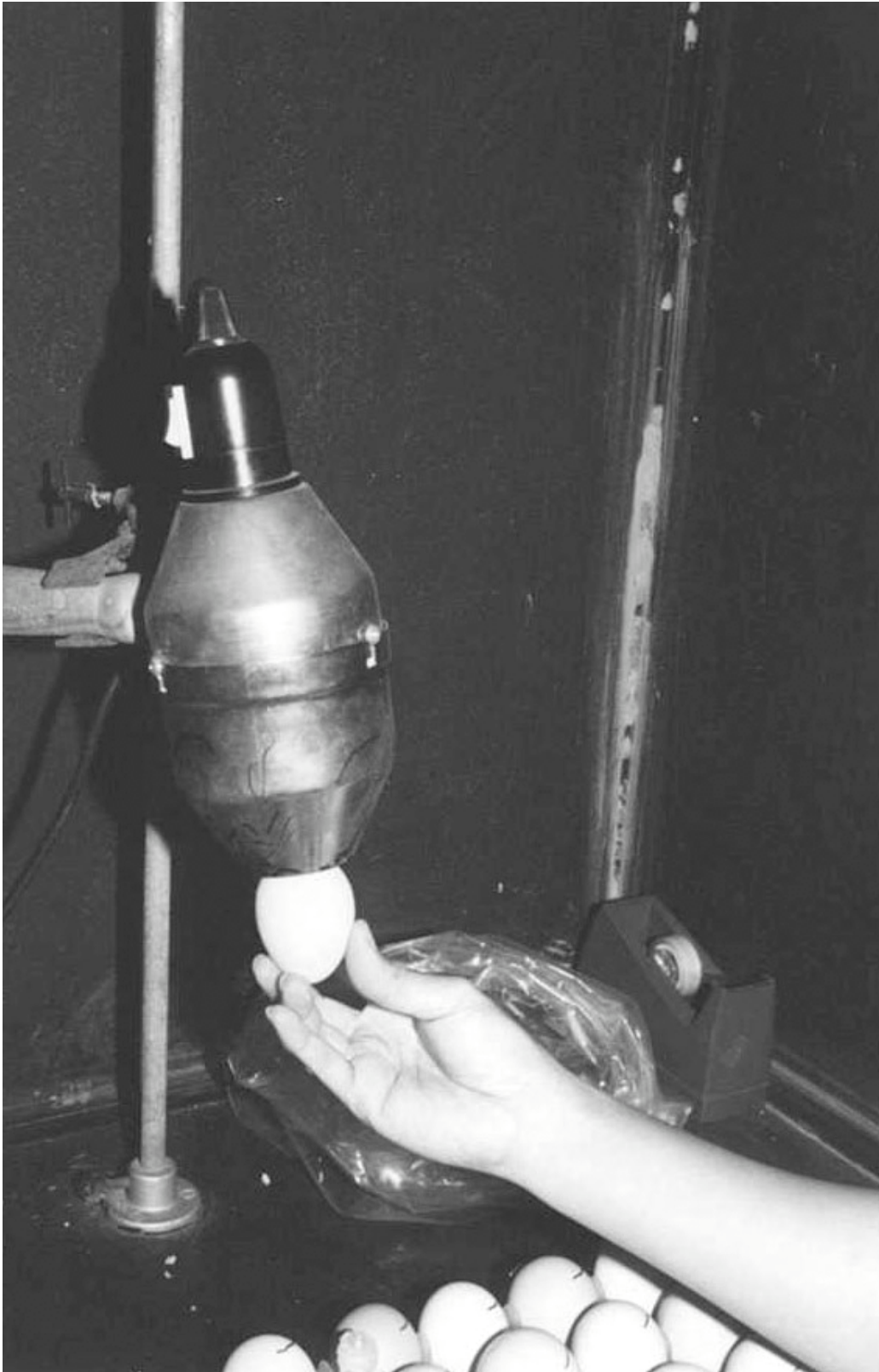
1. Hold the blunt end of the egg against the aperture of the candling lamp and note the position of the head of the embryo.
2. Turn the egg a quarter turn away from the head.
3. Draw a line on the shell marking the edge of the air sac.
4. Draw an **X** approximately 2 mm above this line.
5. The **X** marks the inoculation site.

### Note:

- In some eggs the air sac will have not developed on the blunt end but half way down the egg. These eggs are not suitable for vaccine production. They can be used for inoculation during routine titrations to establish infectivity titres.



**Figure 4: Marking the inoculation site**



**Figure 5: Candling an egg**





**Figure 6: A candling booth**

## **6 Inoculation of embryonated eggs by the allantoic cavity route**

### **Introduction**

The most convenient method of propagating Newcastle disease virus in the laboratory is by the inoculation of the allantoic cavity of embryonated eggs.

All strains of Newcastle disease virus will grow in the cells lining the allantoic cavity. The virus enters these cells where it multiplies. As the cells are disrupted the virus is shed into the allantoic fluid.

Virulent strains of the virus will invade cells beyond the lining of the allantoic cavity and kill the embryo. The time taken for this to occur is the basis of the "Mean Death Time Assays", which indicate the level of virulence.

The avirulent I-2 strain of Newcastle disease virus will not kill embryos inoculated by the allantoic cavity.

Inoculation of the allantoic cavity of embryonated eggs is a technique used in the following procedures:

1. Newcastle disease vaccine production
2. Establishing the infectivity titre of a suspension of Newcastle disease virus.
3. Isolation of Newcastle disease virus from field specimens for laboratory diagnosis.

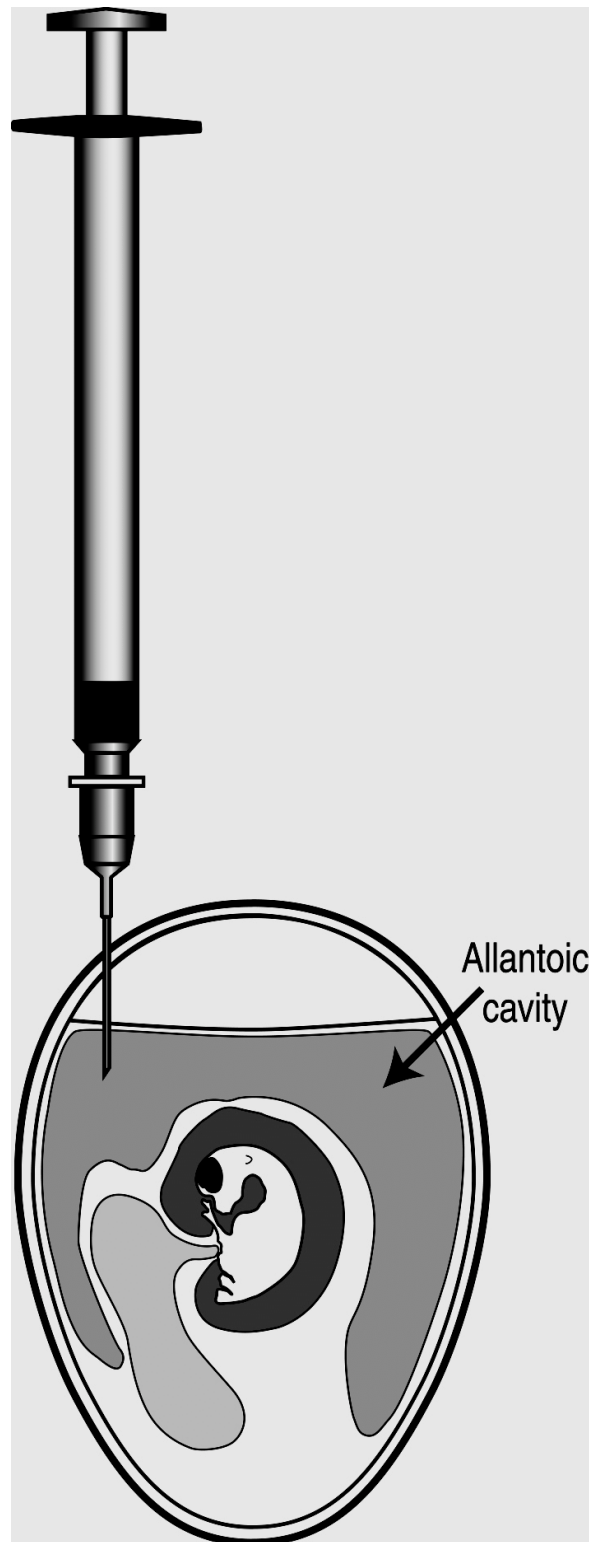
## **Inoculation of the allantoic cavity**

### ***Materials***

- Eggs: 9-day old or 10-day old embryonated eggs. Candle the eggs and mark the inoculation sites as described in **Section 5**. Eggs should be placed in an egg rack with the inoculation site uppermost.
- Egg shell punch.
- Cotton wool.
- A 70 percent alcohol solution in water.
- Syringe 1 mL.
- Needles preferably 25 gauge, 16 mm.
- Stationery tape (also called cello or sticky tape) or melted wax to seal the inoculation site.
- Inoculum. This must be free of microbial contamination.
- Discard tray.

### ***Method***

1. Use cotton wool and 70 percent alcohol to swab the end of the eggs to be inoculated. Allow the alcohol to evaporate.
2. Swab the eggshell punch with 70 percent alcohol solution. Place used cotton wool in discard tray.
3. Pierce a hole in the end of the egg at the marked inoculation site.
4. Attach needle to 1 mL syringe.
5. Draw inoculum into 1 mL syringe.
6. Keeping the needle and syringe vertical, place the needle through the hole in the eggshell. The needle will need to penetrate approximately 16mm into the egg to reach the allantoic cavity.
7. Inject 0.1 mL of inoculum into the egg.
8. Withdraw the needle from the egg.
9. Seal the hole in the shell with stationery tape or melted wax.
10. Discard the used needles and syringes.
11. Place the inoculated eggs into a second incubator. Check the temperature and humidity of incubator.



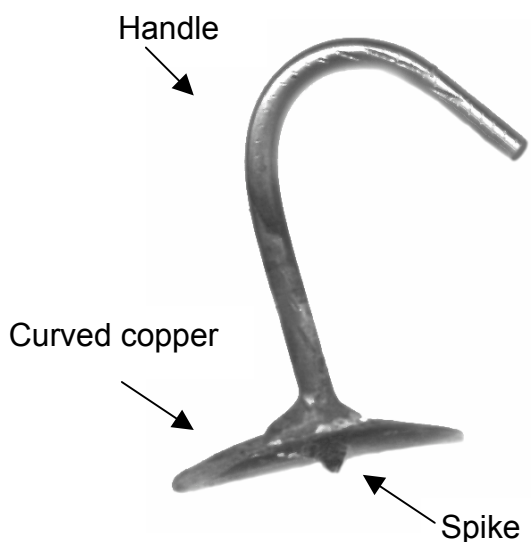
**Figure 7: Inoculation of the allantoic cavity**

## Tools for piercing a hole in the egg shell

A dental drill can be used if it is available. In most laboratories a tool called an eggshell punch can be improvised using materials that are cheap and easy to procure. Two examples are described, the first being the eggshell punch made in the workshop at the John Francis Virology Laboratory.

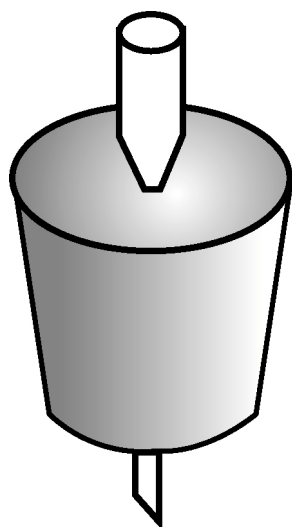
### Example 1:

Materials: A small piece of copper approximately 3 cm by 1 cm.  
A length of 4mm diameter stainless steel rod or steel wire.  
Brazing alloy



A hole is made in the middle of the piece of copper, which is then curved to the shape of an egg. A file is used to create a small spike at the end of the rod or wire, which is then pushed through the hole in the copper until the point extends one mm beyond the copper. The rod or wire is then brazed in place where it passes through the copper. The other end of the steel wire or rod is bent to form a handle.

**Figure 8: Egg shell punch**



### Example 2:

Materials:  
A needle (20 gauge is suitable)  
Rubber stopper

Insert the needle into the rubber stopper so that only 1 mm of the tip is showing. Use and store carefully as the tip of the needle will be very sharp.

**Figure 9: Improvised egg shell punch**

## 7 Collection of blood from chickens

### Introduction

Blood is collected from chickens for two purposes:

1. To obtain serum which will be tested for Newcastle disease virus antibodies, no anticoagulant is required and the blood is allowed to clot. The levels of antibody detected in individual birds and flocks give an indication of the response to a vaccination. It also indicates whether birds have been challenged by field strains of Newcastle disease virus.
2. To obtain red blood cells, the blood is collected into anticoagulant. The cells are washed and used to test for the presence of virus in the haemagglutination test. They are also used in the haemagglutination inhibition test for the presence of antibodies.

It is important that those who bleed chickens use a quick and effective technique. This will develop with practice and by applying the following advice.

- Handle the chickens gently.
- Collect the blood samples quickly.
- Take care not to damage the vein. Damaged veins will result in haematomas being formed.
- Minimize the loss of blood. This minimizes trauma to the chickens and stress to their owners. The owners are then more likely to cooperate by supplying chickens for the collection of blood samples in the future.

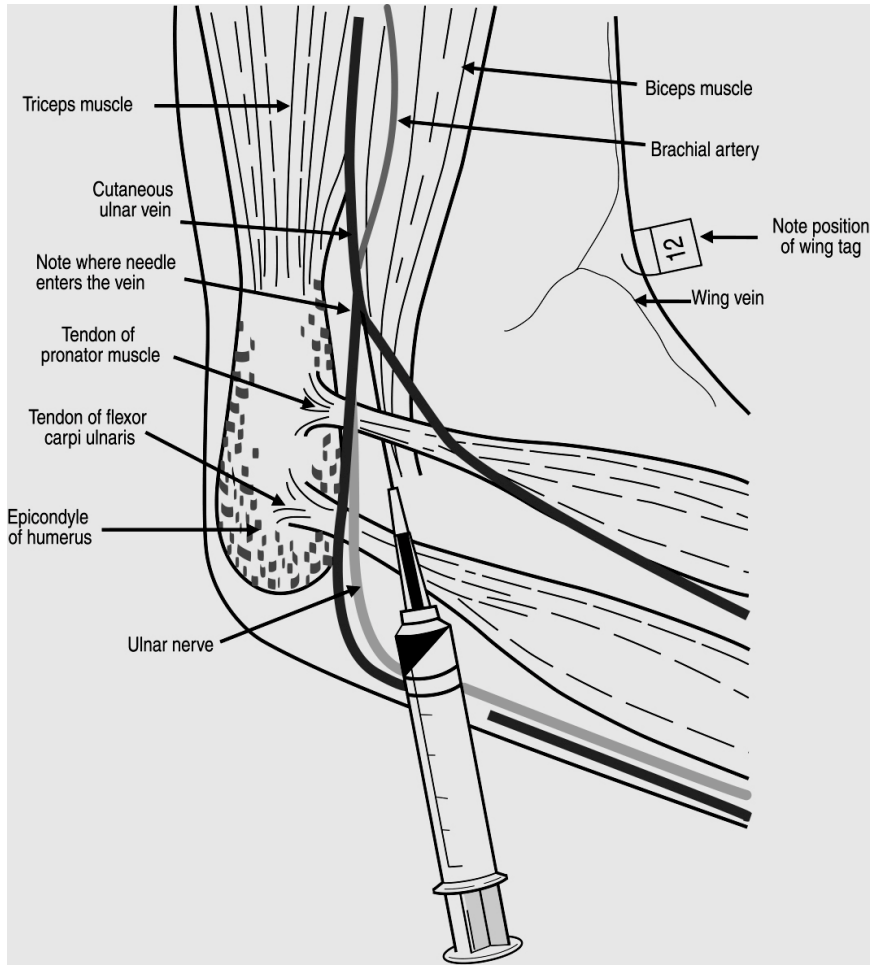
## Wing vein bleeding

### **Materials**

- 2.5 mL syringes
- 25 gauge needles for small chickens
- 23 gauge needles for larger chickens
- Cotton wool
- 70 percent alcohol solution
- Labels or marking pen to label each syringe

### **Method**

1. Ask an assistant to hold the chicken horizontally on its back. The assistant uses one hand to hold the legs and places the other hand under the back to support the chicken.
2. Pull a wing of the chicken out towards you.
3. Note the wing vein, clearly visible running between the biceps and the triceps muscles. The wing vein forms a V (bifurcates). Note the tendon of the pronator muscle that runs across the V.
4. Pluck away any small feathers that obscure the vein.
5. Disinfect the area around the bleeding site by swabbing with 70 percent alcohol.
6. Insert the needle under the tendon. Direct the needle into the wing vein in the direction of the flow of blood. Do not insert the needle too deeply. Keep clear of the ulnar nerve.
7. Once the tip of the needle is in the vein, gently pull the plunger of the syringe. Blood will flow into the syringe. If blood does not flow, release the plunger and make a very slight adjustment to reposition the end of the needle.
8. Be patient and use a gentle suction to withdraw the blood. Chicken veins collapse readily.
9. If a haematoma forms, try bleeding from the other wing.
10. After removing the needle, apply pressure to the vein for a few seconds to discourage further bleeding.
11. Ideally the needle should be removed into a needle disposal container and the cap placed on the end of the syringe to prevent leakage of the serum. However in many places these containers are not available and the cap will be placed over the needle.  
**TAKE CARE!** Do this very carefully to avoid a needle stick injury.
12. Pull the plunger back approximately 1 cm and place the syringe at an angle with the needle end up in a rack facilitate clotting.



**Figure 10: Chicken wing vein bleeding**



## **Bleeding a chicken alone**

The staff at the John Francis Virology Laboratory use the following method for bleeding chickens without an assistant. The method is a modification of the procedure described above and is written for a right-handed person. Left-handed people soon make their own modifications once they start bleeding chickens alone.

1. Sit on a chair. Turn the chicken on its back and place it on your thighs.
2. Arrange the chicken with its head away from you and place the right wing securely between your thighs.
3. Use your left elbow to secure the legs by holding them down onto your left thigh.
4. Place your left forearm across the chicken and use your left hand to spread out the left wing of the chicken.
5. Use your right hand to bleed the chicken as described above.



**Figure 11:**  
**Bleeding a chicken alone, positioning the chicken (1)**



**Figure 12:**  
**Bleeding a chicken alone, positioning the chicken (2)**



**Figure 13:**  
**Bleeding a chicken alone, taking the blood sample**

## 8 Preparation of washed chicken red blood cells

### Introduction

Chickens used for the supply of blood for the preparation of red blood cells should be housed separately from chickens used for other purposes. Usually they are not vaccinated with Newcastle disease vaccine. Blood from vaccinated chickens is acceptable if that is all that is available. Collect blood from more than one chicken. A collection of 1.0 mL from each of three chickens will usually give between 8 to 10 mL of a 10 percent solution of washed red blood cells. If you require a larger volume of washed red blood cells, collect more blood and adjust the volume of anticoagulant accordingly.

There are four steps in the preparation of washed red blood cells.

1. Collection of the blood.
2. Washing the red blood cells.
3. Measuring the packed cell volume.
4. Preparation of a 10 percent suspension of red blood cells for storage.

### Anticoagulant

Blood is collected from the wing veins of three chickens as described in **Section 7**. It is mixed with an anticoagulant. Two anticoagulants are commonly used and easy to prepare:

Acid citrate dextrose (ACD) solution. Allow 1 part ACD to 3 parts blood.

Alsever's solution. Allow 1 part Alsever's to 1 part blood.

Recipes for ACD and Alsever's solution are provided in **Appendix 3**.

## **Two techniques for collection of blood into anticoagulant**

### ***Technique 1***

1. Place 1 mL of ACD or 3 mL of Alsever's solution into a sterile bottle with a lid.
2. Bleed the first chicken and take 1 mL of blood. Immediately remove the needle from the syringe, gently push down the plunger and transfer the blood to the bottle. Replace the lid on the bottle and rotate it gently to mix.
3. Repeat Step 2 and take 1 mL of blood from a second chicken. Transfer the blood to the bottle of blood and anticoagulant and rotate it gently to mix.
4. Repeat Step 2 and take 1 mL of blood from a third chicken. Transfer the blood to the bottle of blood and anticoagulant and rotate it gently to mix.

The bottle now contains either 3 mL of blood mixed with 1 mL of ACD **OR** 3 mL of blood mixed with 3 mL of Alsever's solution.

### ***Technique 2***

1. Have the correct volume of anticoagulant in the syringe before the blood is taken. Allow 0.33 mL of ACD or 1mL of Alsever's solution per mL of blood.
2. Bleed each of three chickens and take 1 mL of blood into the syringe.
3. After collecting the blood from all three chickens, remove the needles from the syringes and pool the blood samples in a sterile bottle with a lid.

## Washing the red blood cells

After collection of the blood into an anticoagulant, the cells are washed and stored. It is best to use dextrose gelatin veronal (DGV) to wash the cells. If you do not have all the reagents to prepare DGV, use phosphate buffered saline (PBS). See **Appendix 3** for the recipes.

**Always treat blood gently to avoid haemolysis.**

**Mix gently with the anticoagulant.**

**Do not squirt the blood through the needle.**

### Materials

- Blood in anticoagulant
- DGV or PBS solution for washing.
- Sterile 20 mL centrifuge tube with a lid or a 20 mL bottle with a lid, to fit the centrifuge bucket.
- Pasteur pipette or 10 mL graduated pipette with pipette filler.

### Method

1. Transfer the blood to a container suitable for centrifugation.
2. Add DGV to fill the container. Mix gently.
3. Centrifuge at 500 *g* for 10 minutes.
4. Use a Pasteur pipette or a 10 mL glass pipette to remove the supernatant. Take care not to disturb the pellet of red blood cells.
5. Repeat Steps 2, 3, and 4 twice.

The cells have now formed a pellet after being washed three times and centrifuged. The next steps are the measurement of the packed cell volume (PCV) and preparation of a 10 percent solution of cells for storage.

## **Preparation of 10 percent red blood cells using a micro-haematocrit centrifuge to measure packed cell volume (PCV)**

### **Materials**

- Washed red blood cells prepared according to method above.
- Micro-haematocrit reader.
- Micro-haematocrit centrifuge.
- Micro-haematocrit capillary tubes to fit the centrifuge and reader.
- Plasticine to seal the ends of the capillary tubes.
- Diluent DGV or PBS.
- 10 mL graduated pipette and pipette filler.

### **Method**

1. Suspended red blood cells in 10 mL of DGV storage solution.
2. Fill 2 micro-haematocrit capillary tubes with the blood.
3. Seal one end of each capillary tube with plasticine.
4. Place the capillary tubes in the micro-haematocrit centrifuge with the sealed ends facing the outside of the centrifuge.
5. Spin for 3 minutes.
6. Remove the capillary tubes.
7. Read the percent packed cell volume using a micro-haematocrit reader.
8. Adjust the volume of the DGV so that it equals the percentage measured. This achieves a final concentration that is equivalent to 10 percent.

**Refer to the instructions provided with the micro-haematocrit centrifuge.**

Example 1. If the packed cell volume measured 12 percent, add 2 mL of storage solution to the 10 mL suspension. The cells will then be suspended in a total volume of 12 mL of storage solution. This will adjust the concentration of the suspension of red blood cells to 10 percent.

Example 2. If the packed cell volume measures less than 10 percent for example 7 percent, centrifuge the suspension at 500 g for 10 minutes. Remove 3 mL from the supernatant to reduce the volume to 7 mL. Take care not to disturb the pellet. This will adjust the concentration of the suspension of red blood cells to 10 percent.

## Instructions for the use of a micro-haematocrit reader

Refer to the instructions provided with the micro-haematocrit reader. This is often written on the back of the reader.

A summary of instructions:

1. Place the capillary tube in the slot so that the base line of the reader lines up with the bottom of the red blood cells in the tube.
2. Move the frame holding the tube so that the top line intersects the top of the liquid in the tube.
3. Use the handle to adjust the middle line to intersect with the top of the red blood cell pellet.
4. Read the percentage packed cell volume. This is the figure where the middle line intersects the scale.

### Note:

- Always look directly down at the reader when lining up the capillary tube with the lines on the reader. This will minimize errors in your readings.

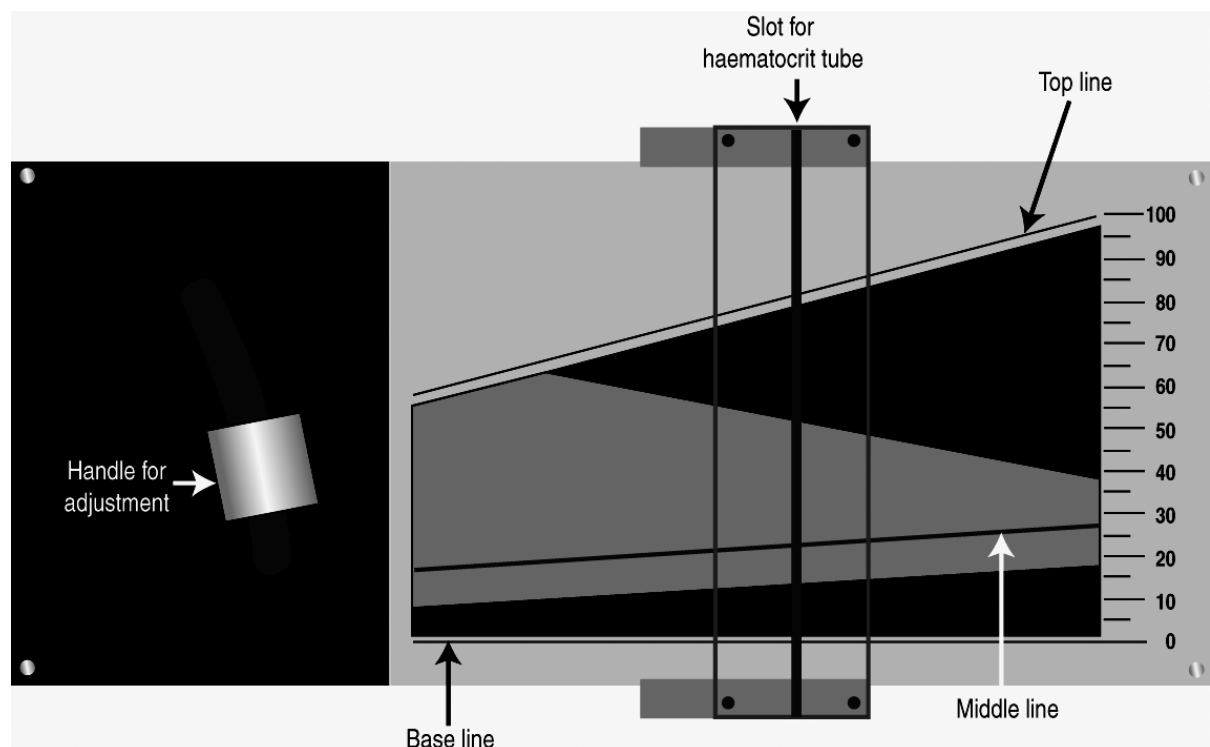


Figure 14: Micro-haematocrit reader

## **Preparation of 10 percent red blood cells without using a micro-haematocrit centrifuge to measure packed cell volume (PCV)**

There are two methods.

1. Use a micropipette or glass pipette to remove one mL of the pellet of packed red blood cells after they have been washed as described above. Add 9 mL of DGV to dilute to a 10 percent suspension.
2. Use a graduated centrifuge tube to prepare the washed red blood cells. Measure the volume of the pellet of washed red blood cells. Calculate the volume of DGV storage solution required to prepare a final suspension of 10 percent. Use a glass pipette to add or remove DGV to achieve the calculated volume.



**Figure 15:**

### **Measuring packed cell volume in a graduated centrifuge tube**

#### **Note:**

- Use of a micro-haematocrit centrifuge and reader will give the most accurate readings. The cells are firmly packed down after centrifugation in the capillary tubes. The use of a micro-haematocrit reader reduces the error in the measurement of packed cell volume.
- It is important to standardize the preparation of 10 percent red blood cells used in the haemagglutination and haemagglutination inhibition tests. This can be achieved by consistently using the same method to measure the packed cell volume each time the preparation is carried out.



### **Storage of 10 percent suspension of red blood cells**

Store the suspension at 4°C. As the cells settle under gravity, the storage solution should remain clear. Any red colour in the storage solution indicates haemolysis of the cells and the suspension is not suitable for use and should be discarded. Normally a 10 percent suspension in DGV can be kept for one week at 4°C.

### **Storage of red blood cells in phosphate buffered saline.**

Red blood cells can be stored as a 10 percent suspension in phosphate buffered saline. These suspensions can be expected to haemolyse after one or two days at 4°C. The red blood cells will settle under gravity. If the storage solution appears pink, the cells have started to lyse and a fresh suspension of washed red blood cells should be prepared.

## 9 Harvesting and storage of allantoic fluid

### Introduction

Allantoic fluid from inoculated eggs will be harvested for three reasons.

1. To prepare I-2 Newcastle disease working seed or vaccine.
2. To use as antigen in the haemagglutination inhibition test.
3. To be tested for the presence or absence of Newcastle disease virus by the haemagglutination test. These results are then used to calculate the infectivity titre of a suspension of virus.

### Minimizing contamination

During the harvesting of allantoic fluid for use as working seed or vaccine, steps must be taken to minimize the risk of exposure of the allantoic fluid to environmental contaminants. The following list contains appropriate steps to be taken.

- Use aseptic technique to harvest the allantoic fluid.
- Harvest the allantoic fluid in a designated room or area of the laboratory if possible. Many laboratories use a laminar flow cabinet for harvesting vaccine. Routine servicing of the cabinet should be carried out to ensure sterile conditions inside the cabinet.
- Remove all materials not associated with the harvesting process from the room or laminar flow cabinet.
- Thoroughly clean the room or cabinet with disinfectant. Irradiation with UV light prior to harvesting vaccine will help kill contaminating organisms.
- Technicians should wear clean laboratory coats, clean hair covers, facemasks and scrub their hands with an antimicrobial soap.

## Harvesting allantoic fluid to prepare working seed, vaccine or antigen

### *Materials*

- Forceps or a small pair of scissors
- Absolute alcohol for flaming forceps
- Cotton wool
- 70 percent alcohol solution in water
- Discard tray
- 50  $\mu$ L micropipette and tips or a wire loop
- Sterile Pasteur pipettes with short blunt ends
- 96 well microwell plate
- 10 percent washed red blood cells
- Sterile containers for receiving the harvested allantoic fluid.

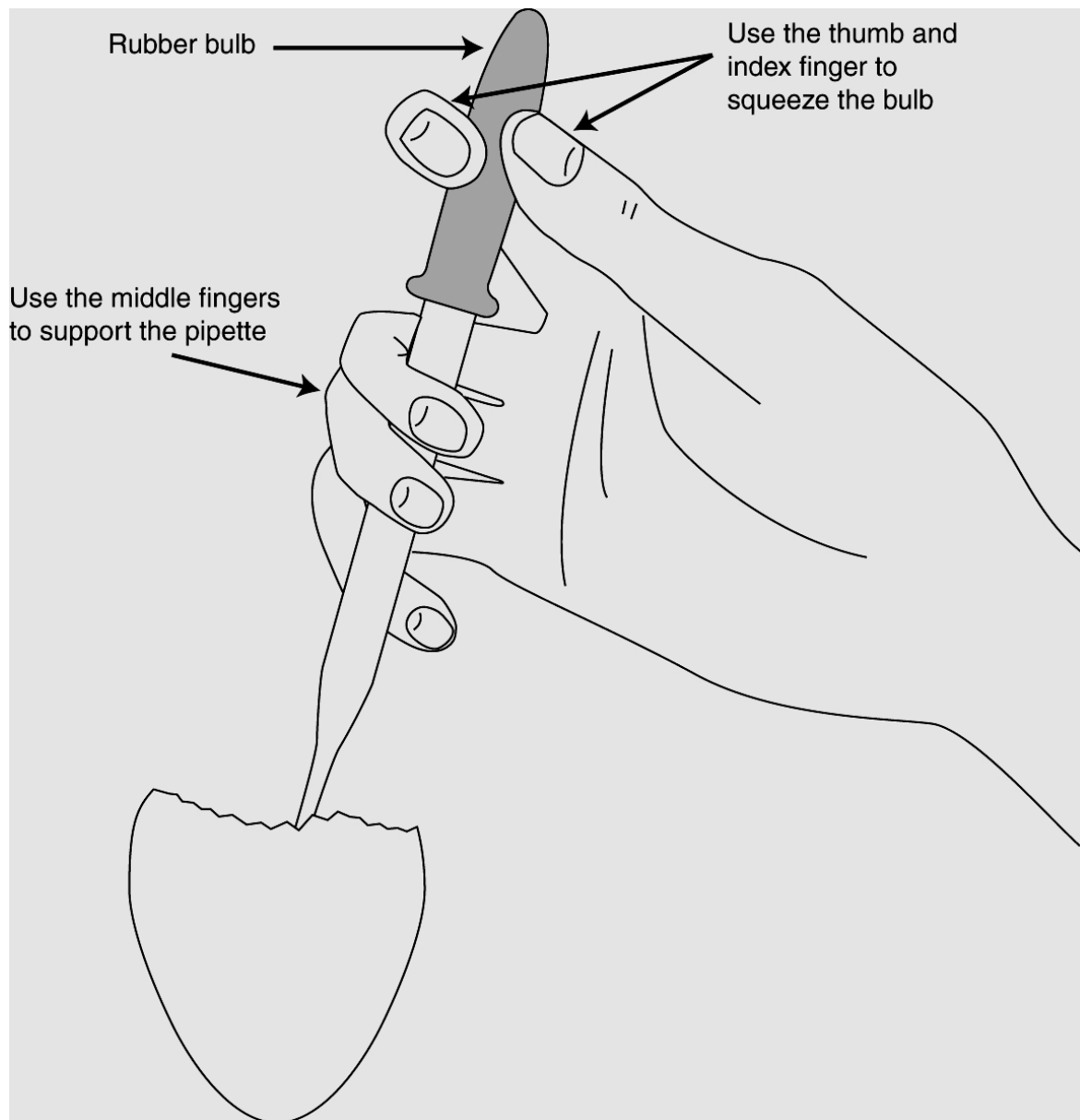
### *Method*

1. Chill eggs at 4°C for at least two hours to kill the embryo and to reduce the contamination of the allantoic fluid with blood during harvesting.
2. Remove stationery tape (if used to seal the eggs) and swab each egg with cotton wool soaked with 70 percent alcohol to disinfect and remove condensation from the shells.
3. Dip the forceps or scissors in disinfectant **OR** if using a Bunsen burner, dip the forceps or scissors in absolute alcohol and flame to sterilize. Remove the eggshell above the air space.
4. Discard embryos that are visibly contaminated.
5. Remove a sample of allantoic fluid from each egg. Use a micropipette and sterile tip, sterile glass pipette or a flamed loop. Test each sample for the presence of Newcastle disease virus by the haemagglutination (HA) test. See **Section 10**.
6. Discard embryos that do not test HA positive for Newcastle disease virus.
7. Use sterile glass Pasteur pipettes to harvest the allantoic fluid from the eggs. The pipettes can be either hand held or used unplugged and connected to a vacuum pump. Collect the fluid into sterile containers. See **Figures 16 and 17**.

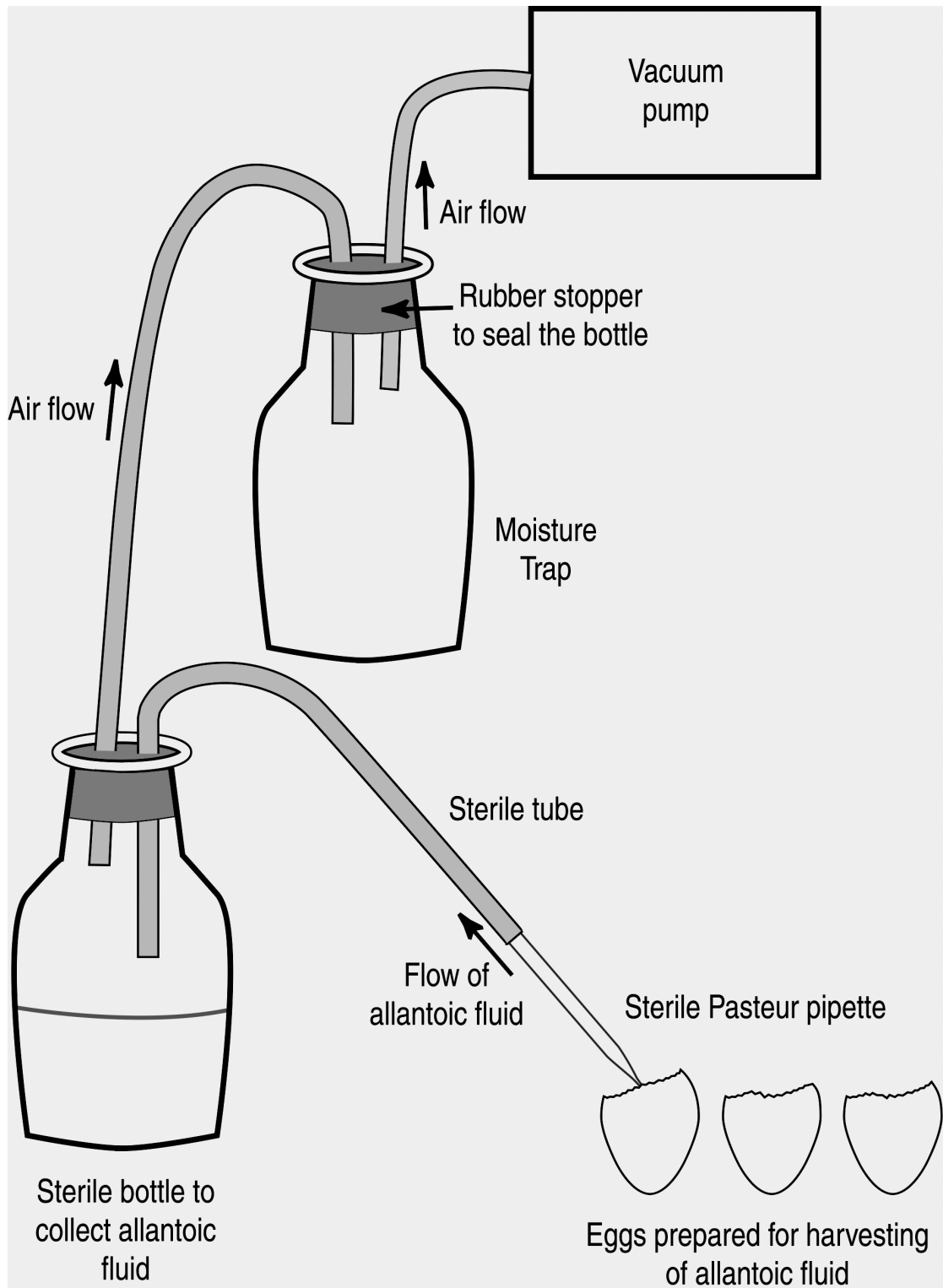
## **Preliminary Quality Control**

This step involves the inoculation of a general purpose broth culture. See **Section 14** for more information about quality control.

1. Test each container for bacterial contamination by inoculating tryptic soy broth with test samples and incubation at 37°C overnight.
2. Centrifuge the samples of allantoic fluid or stand overnight at 4°C to allow particles including red blood cells to settle. The allantoic fluid should appear clear after centrifugation or standing overnight.
3. After 24 hours, read the results of the tests for bacterial contamination.
4. Use aseptic technique to transfer the clear allantoic fluid supernatant from containers that showed no bacterial growth into a sterile container for storage. This step pools the fluid and ensures homogeneity.



**Figure 16:**  
**Harvesting allantoic fluid using a hand held Pasteur pipette**



**Figure 17:**  
**Harvesting allantoic fluid using a vacuum pump**

## Harvesting allantoic fluid to test for presence of haemagglutinin

The following method describes harvesting a small sample of allantoic fluid for testing for the presence of haemagglutinin using the rapid or micro tests described in **Section 10**.

### **Materials**

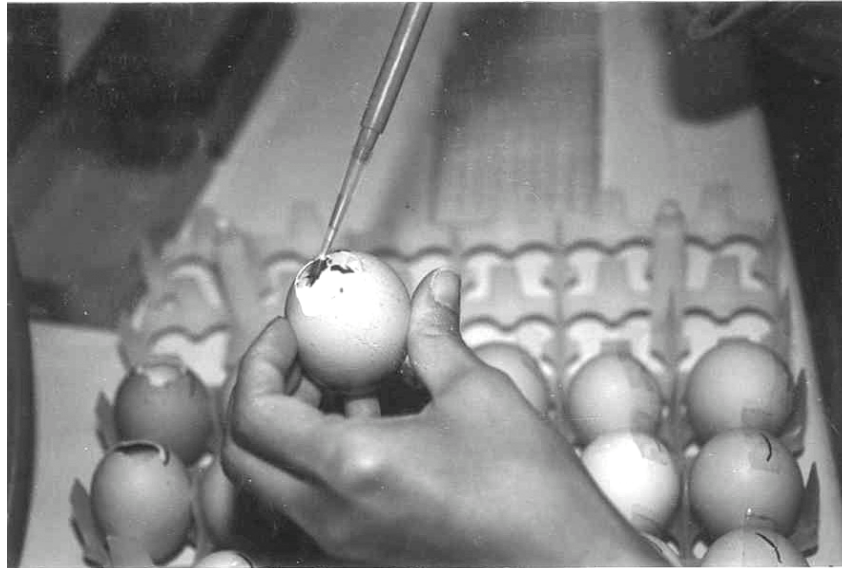
- Forceps or a small pair of scissors
- Absolute alcohol for flaming forceps
- Cotton wool
- 70 percent alcohol solution
- Discard tray
- 50  $\mu$ L micropipette and tips, a wire loop or sterile Pasteur pipettes
- 96 well V-bottom microwell plate and cover or glass slide

### **Method**

1. Chill eggs at 4°C for at least two hours to kill the embryo and to reduce the contamination of the allantoic fluid with blood during harvesting.
2. Remove sticky tape (if used to seal the eggs) and swab each egg with cotton wool soaked with 70 percent alcohol to disinfect and remove condensation from the shells.
3. Dip the forceps or scissors in absolute alcohol and flame to sterilize. Remove the eggshell above the air space.
4. Discard embryos that are visibly contaminated.
5. Remove a sample of allantoic fluid from each egg. Use a micropipette and sterile tip, sterile glass pipette or a flamed loop and dispense the sample according to method being used for the test

### **Note:**

- When harvesting allantoic fluid from each inoculated egg to establish infectivity titres, additional allantoic fluid is usually not harvested for further use. In this case, rigorous steps to minimize contamination are not necessary.



**Figure 18:**

### **Harvesting allantoic fluid for HA test using a micropipette**

#### **Storage of allantoic fluid**

The optimum temperature for storage of allantoic fluid containing live Newcastle disease virus is  $-70^{\circ}\text{C}$ . Storage at  $-20^{\circ}\text{C}$  is not as effective and the infectivity titre will slowly decrease. The action of the freezing and thawing also decreases the infectivity titre of the virus. Allantoic fluid containing Newcastle disease virus has been stored for up to 6 weeks at  $4^{\circ}\text{C}$  without significant loss of titre.

Allantoic fluid will be stored for two purposes.

1. Antigen for use in the haemagglutination inhibition test as described in **Section 11**. Prepare 1 mL aliquots of undiluted allantoic fluid in vials.
2. Preparation of vaccine.

#### **Storage of allantoic fluid at $4^{\circ}\text{C}$ for use as a wet vaccine**

Diluents containing a stabilizing agent are used in the preparation of wet Newcastle disease vaccine. Suitable stabilizing agents are gelatin and skim milk powder. Diluents containing 2 percent gelatin solution or 8 percent skim milk powder in phosphate buffered saline are sterilized prior to use and mixed one part diluent with one part allantoic fluid. A further dilution step in two parts of PSG antibiotic solution will reduce the risk of growth of contaminating bacteria during storage. Trials at John Francis Virology Laboratory have shown that 1 percent gelatin is a superior storage agent to 4 percent skim milk.

(Bensink Z. and P. Spradbrow, 1999)

For more details see **Section 13**: The production of I-2 Newcastle disease vaccine.



# 10 Haemagglutination test

## Introduction

All strains of Newcastle disease virus will agglutinate chicken red blood cells. This is the result of the haemagglutinin part of the haemagglutinin/neuraminidase viral protein binding to receptors on the membrane of red blood cells. The linking together of the red blood cells by the viral particles results in clumping. This clumping is known as haemagglutination.

Haemagglutination is visible macroscopically and is the basis of haemagglutination tests to detect the presence of viral particles. The test does not discriminate between viral particles that are infectious and particles that are degraded and no longer able to infect cells. Both can cause the agglutination of red blood cells.

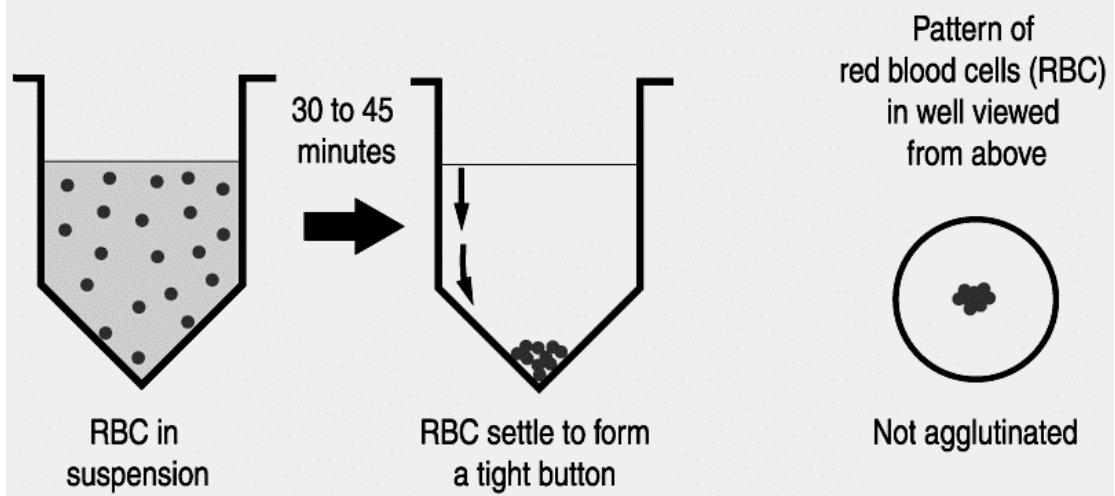
Note that some other viruses and some bacteria will also agglutinate chicken red blood cells. To demonstrate that the haemagglutinating agent is Newcastle disease virus, it is necessary to use a specific Newcastle disease virus antiserum to inhibit the haemagglutinating activity.

Substances that agglutinate red blood cells are referred to as haemagglutinins.

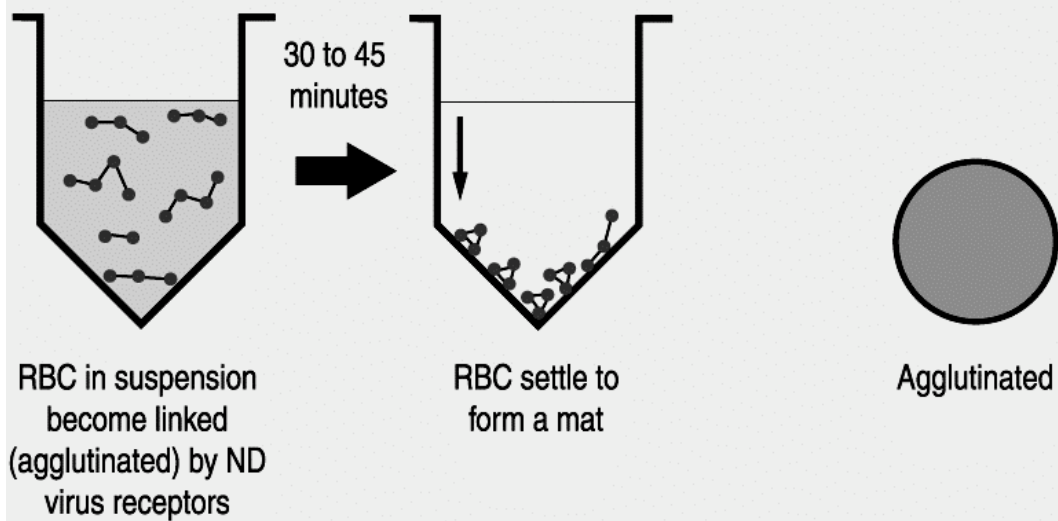
## Note:

- The abbreviation **HA** is used in this manual for haemagglutinin and haemagglutination.
- Two tests are described, the rapid test which takes one minute and the micro test which takes 45 minutes.

**A. Negative control well (no haemagglutinin)**



**B. Positive control well (contains haemagglutinin)**



**Figure 19:  
Principle of the haemagglutination test**

## **Red blood cell control in the haemagglutination test**

Every time a haemagglutination test is carried out, it is necessary to test the settling pattern of the suspension of red blood cells. This involves mixing diluent with red blood cells and allowing the cells to settle.

1. Dispense diluent.
2. Add red blood cells and mix by gently shaking.
3. Allow the red blood cells to settle and observe the pattern.
4. Observe if the cells have a normal settling pattern and there is no auto-agglutination. This will be a distinct button of cells in the micro test and an even suspension with no signs of clumping in the rapid test.

### **Note:**

- The diluent used for haemagglutination tests in this manual is PBS.
- There should be no signs of haemolysis in the red blood cell suspension. If there are signs of haemolysis, a fresh suspension must be prepared.
- There should not be any sign of auto-agglutination in the red blood cell control. If an agglutination pattern is observed, discard the suspension of red blood cells. Prepare a fresh suspension and test again.

## **Control allantoic fluid samples**

Negative and positive control samples are tested in both the rapid and micro haemagglutination tests to ensure the validity of the test.

Negative control allantoic fluid is harvested from 14-day old embryonated eggs that have not been inoculated with Newcastle disease virus. It should always test negative for the presence of haemagglutinins. There should not be any sign of haemagglutination.

Positive control allantoic fluid is known to contain a high infectivity titre of Newcastle disease virus. It should always test positive for the presence of haemagglutinins. Haemagglutination should be visible.

## Rapid haemagglutination test

This test can determine the presence of a haemagglutinating agent in one minute. If testing many samples at the same time, it is necessary to test the negative and positive control samples only once.

### **Materials**

- Clean glass microscope slide or a clean white ceramic tile.
- 10 percent suspension of washed chicken red blood cells. See **Section 8**.
- Micropipette and tips, glass Pasteur pipette or a wire loop.
- PBS.
- Negative and positive control allantoic fluid samples.
- Sample to be tested for the presence of Newcastle disease virus, for example allantoic fluid.

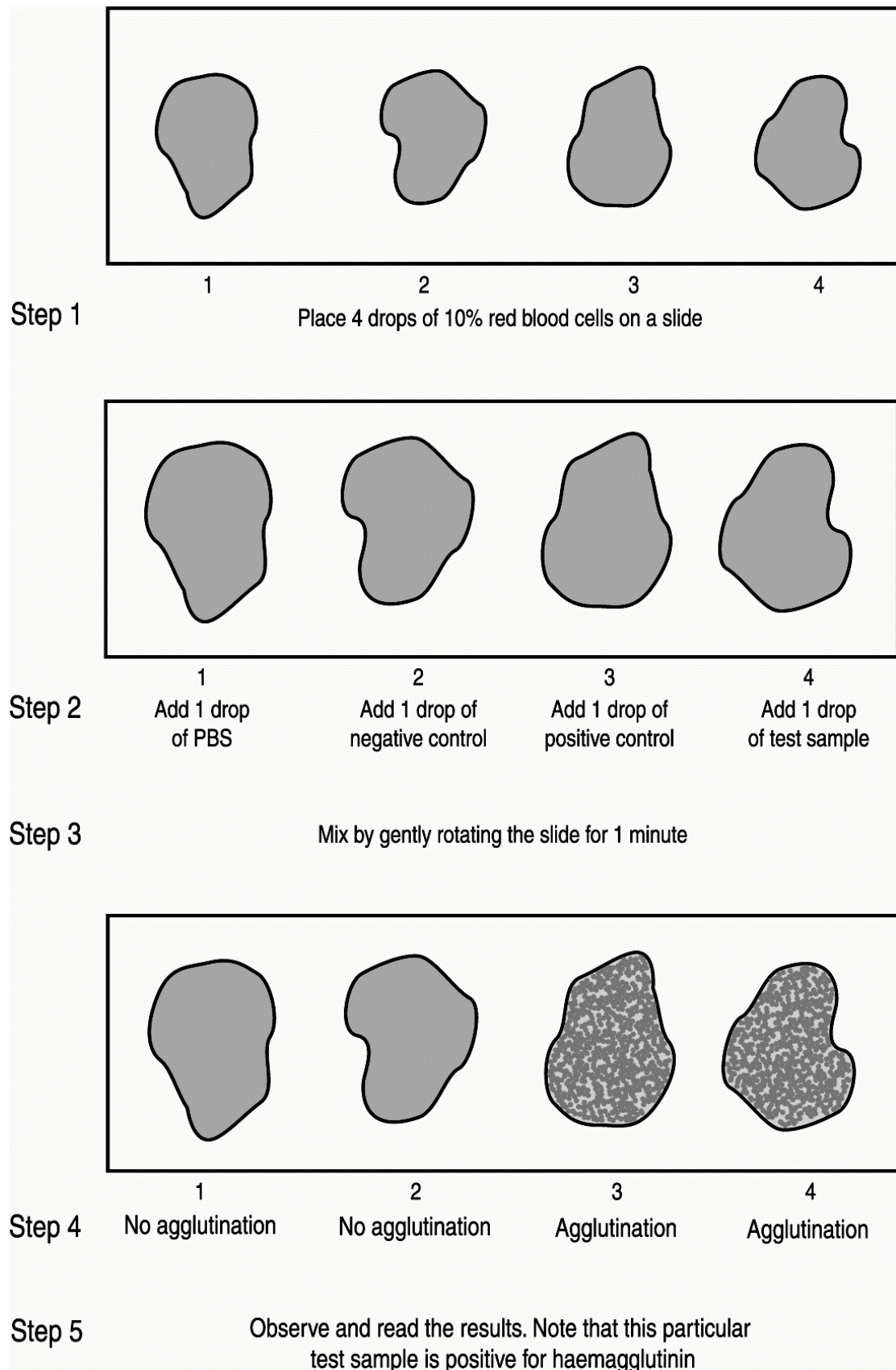
### **Method**

1. Place 4 separate drops of 10 percent chicken red blood cells onto a glass slide or a white tile.
2. To each drop of blood, add one drop of the control and test samples as follows. Use separate tips, pipettes or a flamed loop to dispense each sample.

Drop 1	PBS
Drop 2	Negative control allantoic fluid (no haemagglutinin)
Drop 3	Positive control allantoic fluid (contains haemagglutinin)
Drop 4	Unknown sample to be tested
3. Mix by rotating the slide or tile for one minute.
4. Observe and record results. Compare results of the test samples with the control samples.

### **Results**

- ❖ Agglutinated red blood cells in suspension have a clumped appearance distinct from non-agglutinated red blood cells.
- ❖ The red blood cells mixed with the positive control allantoic fluid will clump within one minute.
- ❖ The red blood cells mixed with the PBS and negative control allantoic fluid remain as an even suspension and do not clump.
- ❖ Judge the results of the test sample by comparison with the positive and negative controls.
- ❖ The PBS and negative allantoic fluid controls are used to detect clumping of the red blood cells in the absence of virus. This is unlikely to occur. If it does occur, the test is invalid.



**Figure 20: Rapid haemagglutination test**

## Micro haemagglutination test in a V-bottom microwell plate

This method is convenient when testing allantoic fluid from a large number of embryonated eggs for the presence or absence of haemagglutinin. A 1 percent solution of red blood cells is used. The cells settle faster in V-bottom plates and there is a better contrast between positive and negative results than observed in U-bottom plates. The method for preparing eggs for and harvesting of allantoic fluid for this test is described in detail in **Section 9**.

### **Materials**

- Inoculated eggs, chilled for at least 2 hours, preferably overnight
- Negative and positive control samples
- V-bottom microwell plate and lid
- Micropipette and tips to measure 50  $\mu\text{L}$
- 1 percent suspension of red blood cells
- 70 percent alcohol solution
- Cotton wool
- Forceps and/or small scissors
- Absolute alcohol
- Discard tray
- Microwell plate recording sheet. See Appendix 9

### **Method**

1. Fill in the details of samples being tested on a recording sheet. Samples and controls will be distributed into the wells as indicated on this sheet.
2. Use a micropipette to remove 50  $\mu\text{L}$  of allantoic fluid from each egg and dispense into a well of the microwell plate. Use a separate tip for each sample.
3. Include negative and positive control allantoic fluid samples on one of the plates.
4. Dispense 50  $\mu\text{L}$  of PBS into two wells. These wells will be the red blood cells controls for auto-agglutination.
5. Add 25  $\mu\text{L}$  of 1 percent red blood cells to each well.
6. Gently tap sides of the plate to mix. Place a cover on the plate.
7. Allow the plate to stand for 45 minutes at room temperature.
8. Observe and record the results.

## Results

- ❖ The settling patterns of single and agglutinated red blood cells are different. Single cells roll down the sides of the V-bottom well and settle as a sharp button. Agglutinated cells do not roll down the sides of the well to form a button. Instead they settle as a diffuse film.
- ❖ Negative HA result = a sharp button
- ❖ Positive HA result = a diffuse film
- ❖ Red blood cell control = a sharp button
- ❖ Mark the HA results on microwell recording sheet.

See **Appendix 10** for an example completed microwell plate recording sheet.

**Table 2: Summary of Haemagglutination tests**

Tests	Result	Interpretation
Rapid HA, Micro HA	Positive	Presence of viral particles that may or may not be infectious.
Rapid HA, Micro HA	Negative	Absence of viral particles or presence of viral particles in levels too low to detect.

## **Titration to establish haemagglutinin (HA) titre of a suspension of virus**

The haemagglutination test is used to quantify the amount of Newcastle disease virus in a suspension. This is done by carrying out two-fold serial dilutions of the viral suspension in a microwell plate and then testing to determine an end point. This result can then be used to determine the amount of haemagglutinin in the suspension and is expressed as a HA titre.

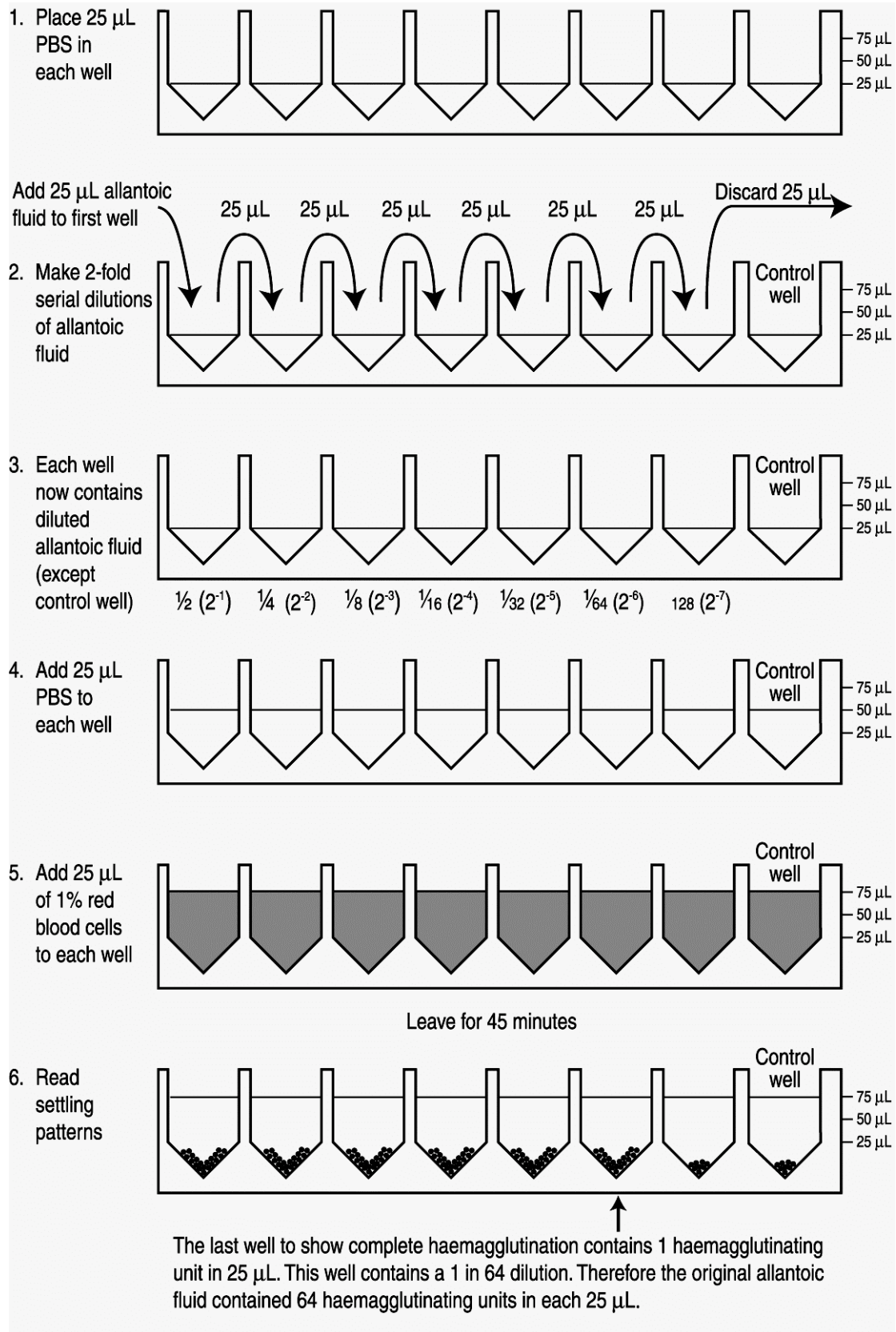
### **Materials**

- 96 well V-bottom microwell plate and cover
- 25  $\mu\text{L}$  single and multi-channel micropipettes and tips
- PBS
- 1 percent chicken red blood cells
- Sample to be titrated
- Reagent troughs
- Microwell plate recording sheet. See Appendix 9.

### **Method**

1. Record on recording sheets how samples will be dispensed in microwell plate.
2. Dispense 25  $\mu\text{L}$  of PBS into each well of the microwell plate.
3. Place 25  $\mu\text{L}$  of test samples in first well of each row of column 1. Samples can be tested in duplicate or triplicate if necessary.
4. Use a multichannel pipette to carry out two-fold serial dilutions across the plate until Column 11. See **Appendix 4** for instructions on carrying out two-fold serial dilutions.
5. Add 25  $\mu\text{L}$  of PBS to each well.
6. Add 25  $\mu\text{L}$  of 1 percent red blood cells to each well including Column 12. The wells in this column are control wells that contain only PBS and red blood cells.
7. Gently tap sides of the plate to mix. Place a cover on the plate.
8. Allow the plate to stand for 45 minutes at room temperature.
9. Read and record the results in each well. All the control wells should be HA negative.
10. **HA negative:** A sharp button of red blood cells at the bottom of the V-bottom well.
11. **HA positive:** A hazy film of red blood cells, no button or a very a small button of red blood cells at the bottom of the V-bottom well.
12. Identify the end point. This will be the last well to show complete haemagglutination and contains one haemagglutinating unit.





**Figure 21:**  
**Titration to determine HA titre of allantoic fluid sample**

### **Definition of one HA unit**

One HA unit in the haemagglutinin titration is the minimum amount of virus that will cause complete agglutination of the red blood cells. The last well that shows complete agglutination is the well that contains one HA unit.

### **Calculation of the HA titre of the test sample**

The HA titre is the reciprocal of the dilution that produces one HA unit.

Example of HA titration shown in **Figure 21**.

A 1 in 64 (1/64) dilution contains 1 HA unit.

- ❖ The HA titre of the test sample is therefore the reciprocal of  $1/64 = 64 = 2^6$
- ❖ The titre of the suspension of Newcastle disease virus can be expressed as 64 or  $2^6$  HA units in 25  $\mu\text{L}$ .

# 11 Serology

## Introduction

The presence of antibodies to Newcastle disease virus in chickens is detected by serological testing. The results of these tests are used for three purposes.

1. To assess the efficacy of Newcastle disease vaccine in laboratory and field trials.
2. To assess the level of Newcastle disease virus antibodies in the field.
3. Serum known to contain antibodies to Newcastle disease virus is used to confirm the presence of Newcastle disease virus in a test sample of allantoic fluid. Such a sample would be obtained during the isolation of virulent Newcastle disease virus. See **Section 15**.

There are two assays commonly used to carry out serological testing for Newcastle disease virus antibodies.

1. Haemagglutination inhibition (HI) test. The HI test is a convenient and commonly used assay that requires cheap reagents and is read by eye.
2. ELISA (Enzyme linked immunosorbent assay). This is a colourimetric assay and requires the use of a sophisticated instrument to read the optical density of the reactions. ELISA kits for Newcastle disease virus antibody detection are prepared and sold commercially. Detailed instructions are supplied with the kits. They are usually quite expensive.

In this manual a protocol for the HI test based on the test described by Allan and Gough will be used for serological testing. (Allan and Gough, 1974 a.)

## **Preparation of serum**

Serum samples are collected for testing for the presence of antibodies to Newcastle disease virus. Blood is collected as described in **Section 7**. The blood forms a clot in the syringe in a few minutes. Once the blood clots, the syringes of blood can be kept with the needle upright to prevent serum filling the needle cap. The serum will separate from the clot within a few hours at room temperature or in approximately 2 hours at 37°C. Storage at 37°C will help the serum separate from the clot.

### ***Materials***

- Syringes with blood samples.
- Sterile glass Pasteur pipettes.
- Microfuge tubes.
- Storage tubes. (1.8 mL Nunc cryotubes are ideal but microfuge tubes are a cheaper alternative.)
- Sharps container and discard bag for biological waste.

### ***Method***

1. Remove the plunger from the syringe. Transfer the serum to a microfuge tube by pouring or using a glass Pasteur pipette.
2. Dispose of needles, syringes, clots and pipettes in appropriate containers.
3. Often the serum will contain red blood cells. Centrifuge for 30 seconds in a microfuge centrifuge or allow to settle under gravity overnight at 4°C to pellet the cells. Do not freeze the samples at this step. Freezing will lyse the red blood cells.
4. Transfer clear serum to a second tube.
5. Remember to label each tube after the transfer of the serum. This ensures the serology results can be applied to individual birds and groups.

### **Storage of Serum**

- Store ampoules of serum at -20°C.
- Storage at 4°C is acceptable for a short period, up to 2 weeks.

## Notes on serum samples

- Samples collected in the field may end up at room temperature overnight and often do not separate well.
- It has been noted at the John Francis Virology Laboratory that in some samples the serum does not separate from the clot. In these cases, the whole clot is centrifuged. This usually results in a small amount of serum separating.
- It is important that the serum samples are clear. Pink samples contain lysed red blood cells. When pink samples are tested observe both test and control samples carefully to determine effect of the colour on the results of the test.
- Pink coloured samples can affect results of an ELISA, which is a colourimetric assay.

## An overview of the Haemagglutination Inhibition (HI) test

- Antibody response to the haemagglutinin protein in the Newcastle disease virus envelope can be measured by the HI test.
- When serum containing these antibodies is mixed with Newcastle disease virus, the antibodies bind to the haemagglutinin protein in the envelope of the virus. This blocks the haemagglutinin protein from binding with the receptor site on chicken red blood cells.
- Thus the haemagglutination reaction between the virus and the red blood cells is inhibited.
- By performing two-fold serial dilutions on the serum prior to testing, the concentration of the serum antibodies can be expressed as an HI titre to the log base 2.

## Standardization of the HI test

It is important that there is correlation between the results of tests carried out by different technicians and in different laboratories. For this reason HI tests should be standardized both within a laboratory and between laboratories.

Standardization is achieved by following a standard protocol. This will include:

- Using a standard 4 HA units of Newcastle disease virus antigen.
- Using standard positive anti-serum and negative serum.
- Including a serum control for each test serum to detect the presence of non-specific agglutinins.
- Using a standard 1 percent dilution of red blood cells.

## **The use of control serum in the HI test**

- Positive and negative control sera are tested to avoid errors in the interpretation of the results of the HI test. Inconsistencies in the results of the HI test may be caused by variations in reagents and procedures.

Examples of possible variations include:

- The source and exact percentage by volume of the red blood cells.
- The exact amount of antigen used in the HI test.
- Accuracy of dilutions.
- Temperature.
- Time allowed for the antibody/antigen reactions to occur.
- Quality of test serum samples (haemolysed serum samples may be responsible for non-specific reactions)

Two standard sera are used.

1. A standard negative control serum known to contain no antibodies to the Newcastle disease virus. It has no HI titre and does not agglutinate chicken red blood cells.
2. A standard positive control serum also known as a standard anti-serum. The HI titre of the anti-serum will have been established by repeated titration.

## **Variability in HI titres of standard positive serum**

It is sometimes observed that the standard HI titre of the standard anti-serum tested on the same day with the same antigen preparation and protocol may vary. A difference in HI titre of one dilution that is one log base 2 ( $2^1$ ) can be regarded as due to random errors and an inherent variability in biological responses. However if the titre of the standard positive serum differs by more than one dilution or log base 2, then the test is invalidated. In this case, fresh antigen must be prepared and tested and the HI test repeated.

## Three categories of standard sera

1. **International standard reference serum.** Certain laboratories prepare reference serum to a very high standard. Standard reference Anti-Newcastle disease serum is available for purchase. It is used as a reference serum in the preparation of national and laboratory standard sera.

The National Institute for Biological Standards and Control (NIBSC) in the United Kingdom can supply an international standard reference serum. The potency of the international reference serum has been determined by the supplier and is expressed in International Units (IU) per ampoule of freeze-dried serum.

**Contact**      Email [enquiries@nibsc.ac.uk](mailto:enquiries@nibsc.ac.uk)  
Tel      44 (0) 1707 654753  
Fax      44 (0) 1707 646730  
Website      <http://www.nibsc.ac.uk>

2. **National standard serum** is prepared by a national laboratory and distributed to collaborating laboratories as required. This serum is prepared by mixing sera with known HI titres. A series of HI tests are carried out to establish the HI titre of the national standard. This is compared with the HI titre of the international standard reference serum if available. The national standard serum is then stored in multiple aliquots and distributed to collaborating laboratories within a country.

3. **Laboratory standard serum** is prepared by some laboratories in order to conserve supplies of national standard serum. The laboratory standard is prepared by mixing serum samples with known HI titres. Comparative HI testing of the laboratory standard and the national standard is used to establish the HI titre of the laboratory standard.

## Preparation of national and laboratory standard sera

Each laboratory will require a supply of negative and positive control serum in order to carry out HI tests on serum samples.

## Collection of HI negative serum

Collect serum from chickens that have had no exposure to Newcastle disease virus. The serum should show no inhibition of viral haemagglutination activity when tested by the HI test. It is often difficult to find serum without any HI activity. In this case use serum with low HI titres of  $2^1$ .

## Collection of HI positive serum

There may be stored serum that is suitable for this purpose. If not, vaccinate several chickens with 1-2 Newcastle disease vaccine. Two weeks after the primary vaccination, give a booster vaccination. Two weeks later, take a serum sample from each chicken and test the HI titre. Collect extra serum from chickens with a titre of  $2^5$  or above. The volume of blood collected from each chicken depends on the size of the chicken. Pool all the serum samples with HI titres of  $2^5$  or above and test the HI titre of the pooled serum.

## Comparative HI testing of international reference and national standard sera

Information supplied by NIBSC in 2001 indicated their international standard reference contained 320 IU per ampoule.

The serum can be reconstituted in PBS. A suitable volume of PBS would be 2 mL, which would give 4 IU in the 25  $\mu$ L test sample. Refer to instructions provided by the manufacturer.

Use the standard HI test described in this section to determine the titre of the international reference standard and the national standard.

- Test both samples in triplicate on the same microwell plate.
- Carry out a series of at least three tests on different days.

Once the HI titre of the international standard serum and the national standard serum have been established, prepare a series of two-fold dilutions of the serum that are spread around the endpoint used to establish the HI titres. Titrate the dilutions that range from complete inhibition to no inhibition as an additional check on the HI titres. The results of testing these dilutions will confirm the HI titre of the undiluted serum.

**Example** of confirming HI titre of standard samples by testing a series of dilutions of the sample.

- Repeated testing of the pooled serum established the HI titre at  $2^5$ .
- Prepare 1/8, 1/16, 1/32 and 1/64 dilutions of the pooled serum.
- Test each dilution for HI titre.
- Confirmatory results: The results tabled confirm that the HI titre of the serum used to prepare the dilutions had a titre of  $2^5$ .



**Table 3: Results confirming HI titre of serum = 2<sup>5</sup>**

Serum dilution	HI titre
1/8	2 <sup>2</sup>
1/16	2 <sup>1</sup>
1/32	- ve
1/64	- ve

The test results will give a HI titre for both the International reference serum and the national laboratory serum being tested. The HI titre for 4 IU of the International reference serum can be used to determine the number of IU in the national reference serum.

**Example**

The standard HI test of 25 µL of the international reference serum containing 4 IU and has a HI titre of 8 (2<sup>3</sup>). The national serum tested HI titre of 64 (2<sup>6</sup>).

How many IU does the national serum contain?

4 IU in a sample with a HI titre of 8.

How many (?) IU in a sample with a HI titre of 64.

$$? = \frac{4 \times 64}{8} = 32 \text{ IU}$$

**Note:**

- Not all laboratories use the same protocol for testing HI titre and IU are independent of the test system used.
- Serological results of samples expressed in IU should give equivalent results when tested by different systems.
- By comparing the HI titre of the standard national serum with the HI titre of the international reference serum enables you to express the results of HI tests in IU. However it is not very often that you will be required to express the results of HI testing in IU.
- Most publications of Newcastle disease serology results describe the assay used and express HI titres to log base 2.

## **Preparing a laboratory standard serum**

Each laboratory should receive a national standard serum from a central laboratory. The HI titre and activity in International units of the serum will have been determined by rigorous testing as described above. To conserve the supply of national standard serum some laboratories will decide to prepare a secondary laboratory standard serum.

Collect positive serum as described above. Do comparative testing of the laboratory and national standards. Test each sample in triplicate on the same microwell plate. Repeat several times and analyze HI titres for both samples. Record the HI titre for the laboratory standard.

Store 1 mL aliquots of the laboratory standard at  $-20^{\circ}\text{C}$ . This standard is used every time the HI test is carried out and should always show the same HI titre when tested with the 4 HA units of antigen.

## **Preparing a national standard serum without an international reference serum**

Many laboratories will not be able to obtain an international reference serum with which to compare their national standard serum. In this case, more rigorous HI testing of the pooled HI positive serum samples will be required to establish the HI titre of the standard serum. It is suggested that the serum is tested up to ten times. Use freshly prepared 4 HA units of antigen for each test and carry out the test on different days if possible. The results will be a range of HI titres. The most frequently occurring titre can be considered the HI titre of the standard serum. All future HI tests carried out on the standard serum should give this titre.

## **Storage of standard serum**

Once the positive and negative standard sera have been tested, aliquots can be prepared, labeled and stored frozen. Storage at  $-70^{\circ}\text{C}$  is optimal (but  $-20^{\circ}\text{C}$  is adequate). Do not thaw and refreeze the samples frequently. Representative samples should be thawed and tested to confirm that there is no loss of titre in the storage process.

## **Preparation of Newcastle disease virus antigen for use in HI tests**

Antigen is prepared by inoculating embryonated eggs with a sample of Newcastle disease virus and harvesting the allantoic fluid four days later. Part of the first batch of I-2 Newcastle disease vaccine prepared from the I-2 working seed can be set aside for storage as antigen. A volume of 50 mL to 100 mL is adequate for a large number of tests. Centrifuge the sample at 1 200 *g* to clarify and remove any contaminating red blood cells. Store the antigen in one mL aliquots at  $-20^{\circ}\text{C}$ .

See **Sections 6 and 9**.

## Preparation of 4HA units of Newcastle disease virus antigen

The standard amount of Newcastle disease virus used in the haemagglutination inhibition (HI) test is 4HA units. It is necessary to prepare and test a suspension of Newcastle disease virus containing 4HA units in order to carry out the HI test. This involves a series of following steps.

1. Titrate the stored suspension of virus to be used as the antigen in the HI test. See **Section 10** Calculate the HA titre.
2. Calculate the dilution factor required to produce 4 HA units. A simple way is to divide the HA titre by 4.
3. Apply the dilution factor and dilute the original suspension of antigen in PBS to produce an adequate volume of 4HA antigen to carry out the HI test. Allow 2.5 mL for each microwell plate.
4. Titrate the diluted (4HA) suspension of virus. This is a back titration to check the diluted antigen contains 4 HA units.
5. Read HA titre. It should equal 4HA units. If not adjust the dilution and titrate again.
6. Use the 4HA unit dilution of antigen in an HI test to test the standard positive and negative serum. The HI titre of the laboratory standard positive serum should equal the predetermined titre.

## Interpretation

- The results of the back titration of the diluted antigen and the HI titre of the standard positive are both used to confirm the antigen has been diluted to a concentration equivalent to the standard 4 HA units.
- If the HI titre of the positive control serum is less than the standard titre, the antigen is too concentrated. Prepare a new dilution and test again.
- Conversely if the HI titre of the positive control serum is too high the antigen is too dilute. Prepare a new dilution and test again.

## Worked example

### Preparation of 4HA units of antigen for HI test in 10 microwell plates:

- The HA titre of the antigen was tested according to the protocol described above. The HA titre = 128
- Calculation of dilution factor to prepare 4 HA units:  $128/4 = 32$
- Calculation of volume of 4HA unit dilution of antigen required:  
Allow 2.5 mL per plate; total volume required =  $10 \times 2.5 \text{ mL} = 25 \text{ mL}$   
Apply dilution factor =  $25 \text{ mL}/32 = 0.781 \text{ mL} = 781 \mu\text{L}$
- Preparation of the diluted antigen: mix 781  $\mu\text{L}$  of original virus suspension with 24.219 mL of diluent. PBS is a suitable diluent.

**Note:** In this case it would be easiest to prepare 32 mL of the 4HA antigen. This would use 1 mL of the original suspension diluted in 31 mL of PBS.

## Haemagglutination inhibition

### Materials

- Thawed serum samples in racks
- V-bottom microwell plates and covers
- PBS
- 1 percent washed red blood cells
- V-bottom reagent trough
- 25  $\mu\text{L}$  single and multichannel pipettes and tips
- Microwell plate recording sheet.
- Newcastle disease virus antigen diluted to 4 HA units per 25  $\mu\text{L}$
- Standard positive and negative serum

### Method

1. Fill in recording sheets to record how samples will be dispensed into microwell plates.
2. Calculate the number of plates required and number each plate.
3. Dispense 25  $\mu\text{L}$  of PBS into each well of the plates.
4. Shake each serum sample and dispense 25  $\mu\text{L}$  into the first well and the last (control) well of a row of a microwell plate.
5. Use a multichannel pipette to make two-fold serial dilutions along the row until the second last well from the end. The last well is the serum control. Do not dilute this well. See **Appendix 4** for instructions on carrying out two-fold serial dilutions.
6. Add 25  $\mu\text{L}$  of the 4HA dilution of antigen to each well excluding the control wells in the last column. See **Section 10** for preparation of 4HA units of antigen
7. Gently tap the sides of the microwell plates to mix the reagents. Cover plates with a lid. Allow to stand for 30 minutes at room temperature.
8. Add 25  $\mu\text{L}$  of 1 percent washed red blood cells to each well including the control wells in the last column.
9. Gently tap the sides of the microwell plates to mix the reagents. Cover the plates with a lid. Allow to stand at room temperature for 45 minutes.
10. Read the settling patterns for each serum sample. Read the control serum well first then read the patterns in the other wells.
11. Record the pattern observed in each well on a microwell plate recording sheet. Determine the endpoint. This is the point where there is complete inhibition of haemagglutination.
12. Record the antibody level for each serum sample. This is expressed as a log base 2. For convenience, the titre is often recorded as just the log index. For example a titre of  $2^6$  would be recorded as 6.

## **Interpretation of results**

- ❖ In the wells where antibodies are present there will be haemagglutination inhibition. The red blood cells will settle as a button.
- ❖ In the wells where antibodies are absent, the red blood cells will agglutinate.
- ❖ The end point of the titration is the well that shows complete haemagglutination inhibition. Sometimes it is not easy to determine. Look at the size of the button as an indication of the degree of haemagglutination inhibition. Use the control well as a point of comparison. Be consistent in determining the endpoint.

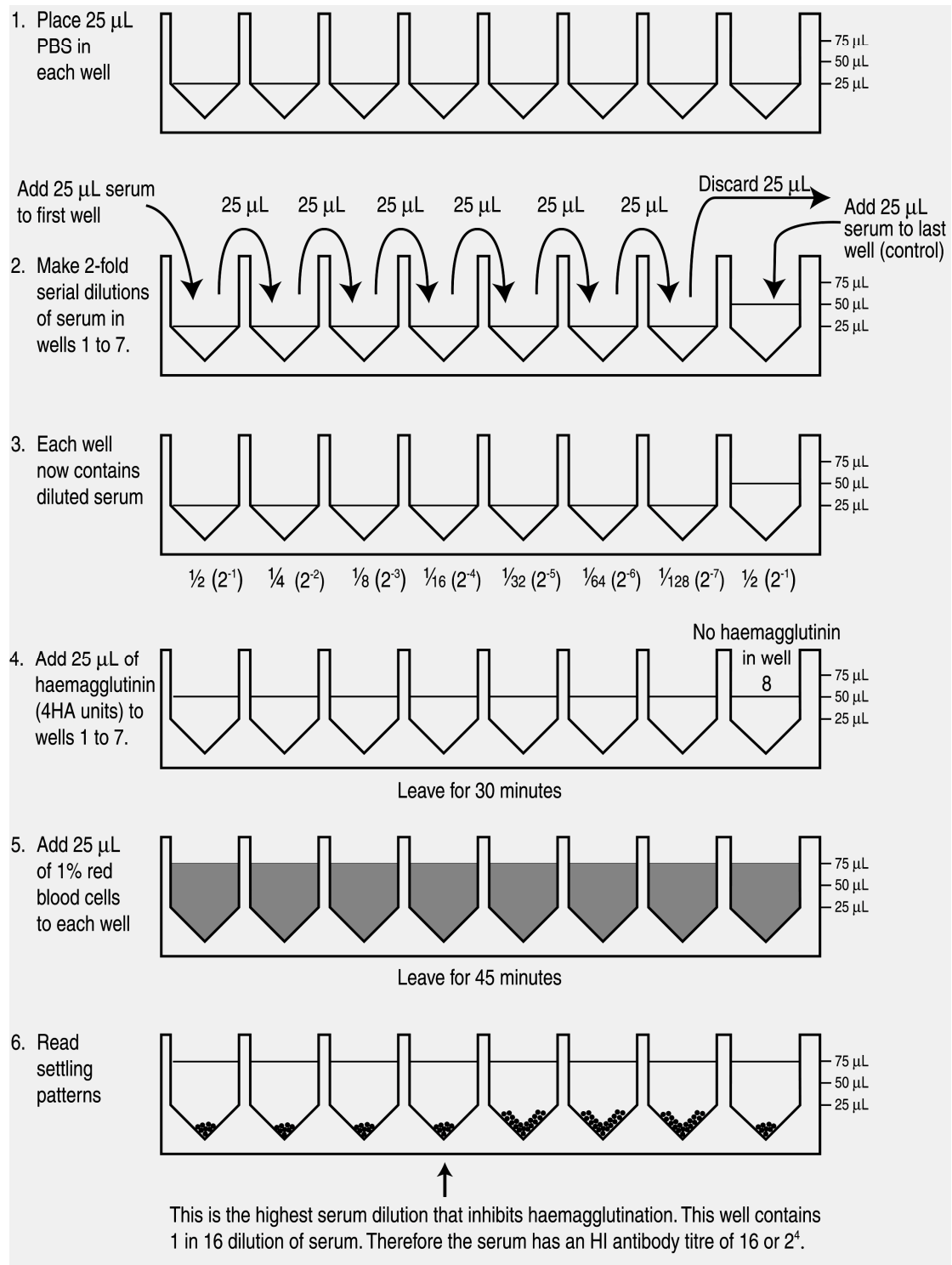
## **Elution**

The neuraminidase enzyme present in the virus particle will eventually break the bond between the virus and red blood cells. This process is called elution.

When elution occurs, the red blood cells are no longer agglutinated. They roll down the side of V-bottom microwell plates to resemble the negative settling pattern, a tight button.

Some Newcastle disease virus strains elute more rapidly and the test must be read before this occurs.

Usually elution takes longer than 45 minutes. A control well with virus and red blood cells is useful to determine elution time.



**Figure 22: Titration to determine HI titre**

## **Non-specific inhibitors**

Some sera may contain substances other than antibodies that inhibit viral haemagglutinin. These substances are described as non-specific inhibitors and are rarely observed with chicken serum and Newcastle disease.

## **Natural agglutinins**

Some chicken sera contain substances that will agglutinate chicken red blood cells. The settling pattern of the agglutinated cells is similar to that produced by Newcastle disease virus. These natural agglutinins are present in low concentration. The control serum well will indicate the presence of natural agglutinins.

## **Adsorption of natural agglutinins**

If natural agglutinins are present in a serum sample, they can be removed by adsorption with chicken red blood cells. The serum can then be retested in the HI test.

### ***Materials***

- Suspension of 10 percent washed chicken red blood cells.
- Microfuge (Eppendorf) tube
- Micropipette, 200  $\mu\text{L}$  to 1 000  $\mu\text{L}$  and tips
- Discard tray
- Microfuge centrifuge
- Serum samples

### ***Method***

1. Place 200  $\mu\text{L}$  of the 10 percent washed red blood cells into a microfuge tube.
2. Centrifuge for 15 seconds.
3. Remove the supernatant.
4. Shake the serum sample and remove 500  $\mu\text{L}$  of serum. Some samples may contain less volume. Use a new tip for each sample.
5. Add the serum to the red blood cells in the microfuge tube. Mix gently.
6. Stand for not less than 30 minutes at 4°C.
7. Centrifuge for 15 seconds.
8. Remove the serum (supernatant) immediately and transfer to a clean microfuge tube.
9. Store the adsorbed sera at 4°C overnight or at -20°C for longer periods.

## 12 Titrating Newcastle disease virus for infectivity

### Introduction

The aim of the titration is to measure the concentration of infectious Newcastle disease virus in a suspension. Examples of suspensions of Newcastle disease virus that will need to be titrated are wet vaccine and freeze dried vaccine that has been reconstituted in diluent.

### Measuring the concentration of Newcastle disease virus in a suspension

#### An overview

- The concentration of Newcastle disease virus in a suspension is expressed as an **Infectivity Titre**. The **Infectivity Titre** is established by carrying out a **titration**.
- The unit of measurement of infectivity of avirulent Newcastle disease virus is the **50 percent Embryo Infectious Dose or EID<sub>50</sub>**. One EID<sub>50</sub> unit is the amount of virus that will infect 50 percent of inoculated eggs.
- The unit of measurement of infectivity of virulent Newcastle disease virus is the **50 percent Lethal Dose or LD<sub>50</sub>**. If the assay is carried out in embryonated eggs, it is **50 percent Embryo Lethal Dose or ELD<sub>50</sub>**.
- One **LD<sub>50</sub>** is the amount of infectious virus that will cause the death of 50 percent of inoculated chickens.
- One **ELD<sub>50</sub>** is the amount of infectious virus that will cause the death of 50 percent of inoculated embryonated eggs.
- The **Infectivity Titre** of a suspension of Newcastle disease virus is the number of infectious units of virus per unit volume, usually expressed per mL.
- To determine the **Infectivity Titre** of a suspension of I-2 Newcastle disease virus, a series of ten-fold dilutions are carried out on the suspension. Five embryonated eggs are inoculated with each dilution. After incubation for 4 days, the HA test is used to determine whether or not the virus has infected and multiplied in each of the eggs. This data is used to calculate the **Infectivity Titre**.
- An adaptation of the mathematical technique devised by **Reed and Muench** is used to calculate the dilution of the suspension of virus being tested that produced the end point. The end point contains one unit of infectivity (1 EID<sub>50</sub>). This dilution is then used to calculate the **Infectivity Titre**. (Reed and Muench, 1938.)



## Measuring the concentration of I-2 Newcastle disease virus in a suspension

### *Method*

1. Carry out ten-fold serial dilutions of the test suspension of virus. See **Appendix 5** Materials and Method are below. The range of dilutions required will be determined by the estimated infectivity titre of the suspension in each 0.1 mL of inoculum.
2. Estimate the infectivity titre in the suspension by considering previous titrations and storage conditions. Is the titre is likely to have dropped during storage and if so by how much? The range of dilutions should include at least two ten-fold dilutions above and below the dilution expected to contain the end point. If the titre is unknown, dilute from  $10^{-1}$  to  $10^{-10}$ .
3. Inoculate 5 eggs with each dilution. Use a separate needle and syringe for each dilution. If needles and syringes are in short supply and will be used to inoculate more than one set of eggs, it is very important to start with the most dilute sample. See **Section 6** for Materials and Method for inoculation of eggs. Usually inoculation of 30 eggs is sufficient to determine the endpoint.
4. Incubate eggs for 4 days at 38°C according to instructions in **Section 4**.
5. After 4 days incubation, harvest allantoic fluid from each egg and test for haemagglutinin to determine the presence or absence of Newcastle disease virus. See **Sections 9 and 10** for Materials and Method.
6. Tabulate the results in the daybook.
7. A description of the application of the Reed and Muench mathematical technique to calculate the infectivity titre of the original suspension is included in this section. A recording sheet is provided in **Appendix 10**.

## Ten-fold serial dilutions of a suspension of I-2 Newcastle disease virus

### Overview

- 100  $\mu\text{L}$  of a Newcastle disease virus suspension mixed with 900  $\mu\text{L}$  of diluent will be a 1 in 10 or  $10^{-1}$  dilution. This will be one-tenth the concentration of the original suspension.
- 100  $\mu\text{L}$  of the 1 in 10 or  $10^{-1}$  dilution mixed with 900  $\mu\text{L}$  of diluent will be a 1 in 100 or  $10^{-2}$  dilution.
- By repeating these ten-fold dilutions in series, the original suspension can be diluted to a point where there is no longer enough virus present to infect embryonated eggs.
- See **Appendix 5** for detailed instructions in carrying out ten-fold serial dilutions.

### Materials

- Sterile diluent. Use the standard antibiotic solution, PSG.
- Sterile glass tubes in a rack for carrying out the dilutions. It is preferable if the tubes are fitted with lids.
- One mL micropipette and tips for dispensing diluent.
- 100  $\mu\text{L}$  micropipette and tips.
- Vortex mixer.
- Samples to be titrated.

### Method

1. Place required number of dilution tubes in the rack.
2. Label the dilution tubes in sequential order. Each tube must be clearly labeled with the dilution factor of the original suspension. For example, to prepare a series of dilutions from  $10^{-1}$  to  $10^{-4}$ ; the labels would read  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ .
3. Dispense 900  $\mu\text{L}$  of diluent into each tube.
4. Take 100  $\mu\text{L}$  of the virus suspension and transfer to the first tube labeled  $10^{-1}$ . Discard the tip.
5. Mix well using the vortex mixer or by hand.
6. Use a clean tip to transfer 100  $\mu\text{L}$  of the diluted virus suspension from the tube labeled  $10^{-1}$  to the next tube in the series. Discard the tip.
7. Mix well using the vortex mixer or by hand.
8. Repeat steps 6 and 7 until the final dilution is reached.

**Use a new pipette tip for each dilution**

**Mix well after each dilution**

**Errors made in virus dilutions will result in errors in the calculation of infectivity titres.**

## Application of the Reed and Muench mathematical technique to calculate 50 percent embryo infectious dose (EID<sub>50</sub>)

The method described above uses a titration of the infectivity of a test virus suspension to quantify the amount of infectious virus in the suspension. The end point of the titration is used to calculate the infectivity titre of the original suspension of virus. The Reed and Muench mathematical technique is used to calculate this end point from the results of the HA test on each of the inoculated eggs. A formula is used to calculate an index (proportionate distance) that is then applied to the appropriate dilution. The infectivity titre is expressed as EID<sub>50</sub> per mL.

Use the table provided in **Appendix 10** as a working sheet.

### Instructions

Tabulate the results of the HA test as follows:

- **Dilution of inoculum:** Include the range of dilutions from 100 percent infection to 0 percent infection. This will be the range 5 out of 5 eggs showing HA positive to 5 out of 5 eggs being HA negative.
- For each dilution, record the **Number of eggs infected (HA +ve)** and the **Number of eggs not infected (HA -ve)** by the inoculum. An extract from the working sheet is reproduced for clarification.

**Table 4: Extract from Reed Muench working sheet (1)**

Dilution of Inoculum	Number of eggs infected (HA +ve)	Number of eggs not infected (HA -ve)
	↑	↓
	↑	↓
	↑	↓
	↑	↓
	↑	↓

- These figures are then accumulated by addition as indicated by the arrows on the recording sheet.
- From these accumulated numbers, the percentage of infected eggs for each dilution is calculated.
- See extract from working sheet on next page.

**Table 5: Extract from Reed Muench working sheet (2)**

Number of eggs infected (HA +ve)	Number of eggs not infected (HA -ve)	Accumulated numbers			Percentage infected
		Infected (A)	Not infected (B)	Total (A+B)	$A/(A+B) \times 100$
↑	↓				
↑	↓				
↑	↓				
↑	↓				
↑	↓				

At this step, the “**Percentage infected**” column may include the figure of 50 percent. In this case, the dilution in this row has produced the 50 percent end point and contains one EID<sub>50</sub> in the inoculum. If the figure of 50 percent has not been calculated it lies between two dilutions. The Reed Muench calculation is then used to establish the dilution by calculating an index. The index is then added to the dilution that produced the percentage infected immediately above 50 percent.

The Reed and Muench formula to calculate the index is:

$$\text{Index} = \frac{(\% \text{ infected at dilution immediately above } 50\%) - 50\%}{(\% \text{ infected at dilution immediately above } 50\%) - (\% \text{ infected at dilution immediately below } 50\%)}$$

- The index is applied to the dilution that produced the percentage of infection immediately above 50 percent.
- We now have the dilution that theoretically produced one EID<sub>50</sub> unit.
- The reciprocal of this dilution is the number of EID<sub>50</sub> in the 0.1 mL volume of inoculum inoculated into each egg. Thus ten times this number will be the number of EID<sub>50</sub> in 1 mL of inoculum.

### Worked example

The titration of a suspension of I-2 Newcastle disease wet vaccine stored at 4°C for 4 weeks.

The Materials and Method were according to **Section 12** of this Laboratory Manual.

Results of HA tests of allantoic fluid from each of the inoculated eggs are recorded in the table below.

**Table 6: Extract from completed Reed Muench working sheet (1)**

Dilution of Inoculum	Number of eggs infected (HA +ve)		Number of eggs not infected (HA -ve)		Accumulated numbers	
					Infected (A)	Not infected (B)
10 <sup>-7</sup>	5	↑	0	↓	11	0
10 <sup>-8</sup>	4	↑	1	↓	6	1
10 <sup>-9</sup>	2	↑	3	↓	2	4
10 <sup>-10</sup>	0	↑	5	↓	0	9

This data is used to calculate the percentage infected. The percentage infected is in the table below.

**Table 7: Extract from completed Reed Muench working sheet (2)**

Dilutions	Number of eggs infected (HA +ve)		Number of eggs not infected (HA -ve)		Accumulated numbers			Percentage infected A/(A+B) x 100
					Infected (A)	Not infected (B)	Total number tested (A+B)	
10 <sup>-7</sup>	5	↑	0	↓	11	0	11	11/11 = 100%
10 <sup>-8</sup>	4	↑	1	↓	6	1	7	6/7 = 86%
10 <sup>-9</sup>	2	↑	3	↓	2	4	6	2/6 = 33%
10 <sup>-10</sup>	0	↑	5	↓	0	9	9	0/9 = 0%

From this data, the percentage figures entered in the Reed and Muench formula are as follows:

- Dilution of 10<sup>-8</sup> % infected at dilution immediately above 50%  
= **86 percent**
- Dilution of 10<sup>-9</sup> % infected at dilution immediately below 50%  
= **33 percent**

## Calculation of the index

$$\text{Index} = \frac{(\% \text{ infected at dilution immediately above } 50\%) - 50\%}{(\% \text{ infected at dilution immediately above } 50\%) - (\% \text{ infected at dilution immediately below } 50\%)}$$

$$\text{Index} = (86\% - 50\%) \div (86\% - 33\%)$$

$$\text{Index} = 36 \div 53 = 0.7$$

The index is then applied to the dilution that produced the percentage infected immediately above 50 percent. In this example this is the  $10^{-8}$  dilution. The index of 0.7 is applied to this dilution.

In this worked example the dilution that provided the 50 percent infection of eggs or 1 EID<sub>50</sub> is  $10^{-8.7}$ .

The reciprocal of this dilution is the amount virus contained in the 0.1 mL of the original suspension =  $10^{8.7}$  EID<sub>50</sub>/0.1 mL

$$= 10^{9.7} \text{ EID}_{50} / \text{mL}$$

The infectivity titre of I-2 wet vaccine stored at 4<sup>0</sup>C has been calculated to be  $10^{9.7}$  EID<sub>50</sub>/mL.

See **Appendix 10** for an example of a completed work sheet.

## **13 The production of I-2 Newcastle disease vaccine**

### **The seedlot system**

Preparation of vaccines by a seedlot system reduces the chance of genetic alterations occurring during continuous passages in eggs. With each passage, there is the possibility of genetic alterations to the virus. These alterations may affect virulence, antigenicity and the yield of vaccine. A selected isolate is used to produce a volume of virus that comprises the master seed, which is divided into aliquots and stored. The master seed is usually freeze dried. An aliquot of master seed is used to produce a volume of working seed, which is again divided into aliquots and stored. An aliquot of the working seed is then used to produce a batch of vaccine. All batches of vaccine are then only two passages removed from the master seed.

### **Preparation of the I-2 Newcastle disease vaccine master seed**

The I-2 Newcastle disease vaccine master seed was prepared at the John Francis Virology Laboratory, School of Veterinary Science, University of Queensland. The I-2 strain is an avirulent Australian Newcastle disease isolate. It was chosen for its antigenicity and thermostability. The master seed stock of virus was derived from parent stock that had survived at 56<sup>0</sup>C for thirty minutes.

The I-2 master seed was propagated in embryonated chicken eggs obtained from a minimal disease flock. Rigorous testing of the master seed revealed no evidence of contamination with aerobic or anaerobic bacteria, mycoplasmas, fungi or viruses other than Newcastle disease virus. The allantoic fluid was diluted 1:1 with a 10 percent skim milk solution and freeze dried in glass ampoules.

Experimental I-2 vaccine produced from the I-2 master seed was further tested for thermostability. The vaccine's safety and immunogenicity in chickens were tested. It was used to vaccinate a simulated village chicken flock. As clutches of chicks were produced by the flock, they were vaccinated. The flock maintained adequate antibody levels for 9 months with HI titres apparently boosted by the vaccine virus excreted by the chicks.

The I-2 vaccine administered by eye drop protected village chickens from a local velogenic challenge strain in both laboratory and village trails in several countries including Vietnam.

The I-2 Newcastle disease vaccine is now being manufactured in many countries for use in controlling Newcastle disease in village chickens.

**References:** Tu, Phuc, et al., 1998.

Bensink, Z. and P. Spradbrow, 1999.

## Supply and transport of the I-2 master seed

The I-2 Newcastle disease master seed virus is stored at the School of Veterinary Science at the University of Queensland. It is supplied to Vaccine Production Laboratories in developing countries to produce vaccine for use in programmes to control Newcastle disease virus in village chickens.

For more information contact the Head of the School of Veterinary Science at [hosvetsci@uq.edu.au](mailto:hosvetsci@uq.edu.au)

To keep the cost of transport at a reasonable level, the master seed is transported by non refrigerated airfreight, usually with DHL World-wide Express. Three ampoules of freeze dried master seed are packed into a HAZPAK packaging system that has been specially designed for transport of biological materials. Dry ice or chiller packs are added to keep the vaccine cool for as long as possible. However the vaccine usually reaches ambient temperature before delivery. It is anticipated that during transport the infectivity titre of the master seed may be reduced. It is therefore necessary to titrate the vaccine on arrival to establish the infectivity titre.

An information sheet is supplied with the master seed. The sheet includes instructions for reconstitution, titration and storage of the master seed.

### Note:

- The ampoules of master seed are best stored at  $-70^{\circ}\text{C}$ .
- Use aseptic technique to open one vial of the freeze dried I-2 master seed and resuspend the contents in 1 mL sterile water.
- Further dilute with phosphate buffered saline or antibiotic solution (PSG) to 20 mL.
- Prepare one mL aliquots of the reconstituted master seed.
- Titrate one ampoule (see **Section 12**), label and store the remaining aliquots at  $-70^{\circ}\text{C}$ .



## Production of the I-2 Newcastle disease vaccine working seed

1. Calculate the volume of diluted master seed required to inoculate the eggs with 0.1 mL per egg.
2. Remove and thaw an aliquot of reconstituted master seed from the -70°C freezer. Further dilute the thawed vaccine to ensure each egg receives at least  $10^{2.0}$  EID<sub>50</sub> preferably  $10^{3.0}$  EID<sub>50</sub> per 0.1 mL of inoculum. Label unused reconstituted master seed with the date of thawing and return to freezer for future use. Remember thawing and freezing can reduce the infectivity titre. Retitration of a sample returned to the freezer will reveal any loss of infectivity titre during the freeze/thaw.
3. Candle eggs and mark the inoculation site according to the method in **Section 5**.
4. Swab the inoculation site with 70 percent alcohol and inoculate 0.1 mL of the diluted I-2 master seed into the allantoic cavity of each embryonated egg according to the method described in **Section 6**.
5. Place eggs in incubator. Check the temperature and humidity.
6. After 24 hours of incubation candle the eggs. Discard any dead embryos.
7. After 4 days of incubation, remove the eggs from the incubator and chill for at least 2 hours, preferably overnight.
8. Test each egg for haemagglutinin activity. See **Section 10**.
9. Discard embryos that do not test positive for the virus or are visibly contaminated.
10. Harvest the allantoic fluid from the eggs into sterile containers preferably that can be centrifuged. See **Section 9**.
11. Test for gross bacterial contamination. See **Section 14**.
12. Centrifuge or stand overnight at 4°C to clarify the allantoic fluid.
13. Pool allantoic fluid to ensure homogeneity.
14. Prepare 1 mL aliquots in sterile containers.
15. Clearly label the aliquots with contents and date.
16. Store at -70°C.
17. Titrate the working seed to establish infectivity titre. See **Section 12**.

## Production of I-2 Newcastle disease vaccine

As with the production of the working seed, a reliable source of healthy embryonated eggs is necessary. Estimates of the quantity of vaccine required will determine the number of eggs to be inoculated with the working seed. Large eggs will yield up to 10 mL of allantoic fluid. Each mL should contain in excess of  $10^{9.0}$  EID<sub>50</sub> (about 1 000 doses of vaccine).

It is recommended that each egg is inoculated with at least  $10^{4.5}$  EID<sub>50</sub> of the working seed in a volume of 0.1 mL (Allan, et al. 1978). This is a dilution of 1 in 10 000 of working seed containing  $\geq 10^{9.5}$  EID<sub>50</sub> of virus per mL. Thus a 1 mL aliquot of working seed will be adequate for inoculating 100 000 eggs.

1. Remove an aliquot of I-2 working seed and thaw.
2. Dilute with antibiotic solution or PBS to give an adequate volume to inoculate each egg with 0.1 mL.
3. Swab inoculation site with 70 percent alcohol and proceed with egg inoculation according to Section 6.
4. Place the eggs in an incubator for 4 days. See Section 4.
5. Candle the eggs after one day. See Section 5. Discard any dead eggs.
6. After four days, remove the eggs from the incubator, chill and harvest the allantoic fluid according to the method in **Section 9**.

## Quality control of the I-2 Newcastle disease vaccine

See **Section 15**.

## Protective diluents for wet vaccine

Dilution in either a 2 percent gelatin solution or 4 percent skim milk is recommended. (Bensink and Spradbrow, 1999.)

At the John Francis Virology Laboratory, the standard wet I-2 Newcastle disease vaccine has been diluted as follows:

- Mix one part allantoic fluid with one part 2 percent gelatin solution and two parts antibiotic solution. This gives a 0.5 percent solution of gelatin in which the infectivity titre of the virus remains high after storage at 4°C for eight weeks. (Unpublished results of experiments at the John Francis Virology Laboratory.)
- Both the gelatin and skim milk solutions can be prepared in distilled water or phosphate buffered saline and sterilized by autoclaving.

## **Freeze drying of the I-2 Newcastle disease vaccine**

It is beyond the scope of this manual to describe the freeze drying process. Vaccine production centres with freeze drying facilities will have detailed instructions on how to manage and operate the facility. Staff trained to maintain and use the freeze drying equipment will be required to supervise this operation.

## **Labeling individual vials of vaccine**

Labels designed to fit even very small vials can be prepared on a computer using a basic word processing programme. The label should include the following information:

- I-2 thermostable Newcastle disease vaccine
- Place of production of vaccine
- Number of doses
- Batch number and expiry date
- Recommended storage conditions
- For veterinary use only

## **Preparing a leaflet to distribute with the vaccine**

After the experimental I-2 vaccine has been tested in the laboratory and in the field, commercial vaccine will be produced for distribution and sale to farmers. A leaflet describing the vaccine and instructions for its use should be prepared. As well as repeating the information on the labels, the leaflet would include the following additional information:

- A description of the composition of the vaccine.
- Properties of the I-2 Newcastle disease virus strain.
- More details of dose and administration of the vaccine.
- More details about the production of the vaccine.

**Note:** The manufacturer determines the expiry date of vaccine after a consideration of the viral content of the vaccine, and the rate of heat inactivation under local conditions.

## **More information about labels and leaflets**

Refer to the Principles of Veterinary Production section of the OIE website for guidelines. [http://www.oie.int/eng/normes/MANUAL/A\\_00017.htm](http://www.oie.int/eng/normes/MANUAL/A_00017.htm)

**(Further reading about Newcastle disease vaccine production is advised.)**

**References:** Mowat, N. and M. Rweyemamu, 1997.

Allan, W.H.; Lancaster J.E., et al., 1978.

## 14 Quality control: an overview

### Introduction

Published guidelines for the production of veterinary vaccines will include rigorous 'quality control' to ensure a very high standard of vaccine is produced. Vaccine production laboratories that decide to produce the I-2 thermostable Newcastle disease vaccine will need to prepare their own in-house protocol for 'quality control' based on these guidelines.

There is always a risk of a vaccine becoming contaminated with extraneous microorganisms. Before the production of the I-2 Newcastle disease vaccine commences, an assessment of these risks should be carried out. A strategy to minimize the potential for contamination can then be developed. The I-2 vaccine has been developed for use in village chickens and must be of low cost. The capacity of the vaccine production laboratory to carry out a large number of quality control tests should be considered. There will inevitably be a compromise between the certainty with which the vaccine can be considered free of contaminants and the need for a cheap, locally produced thermostable Newcastle disease vaccine. Such a vaccine must be suitable for use in programmes that aim to control Newcastle disease in village chickens.

Testing for gross bacterial contamination of the vaccine should take place as soon as the allantoic fluid is harvested. This will involve inoculation and incubation of a suitable broth with the vaccine. If the broth is free of gross bacterial contamination after overnight incubation, the vaccine can then be divided into aliquots and stored as a wet vaccine or it can be freeze dried. Further testing of the stored vaccine can then be carried out before the vaccine is distributed for use in village chickens. As the level at which testing is carried out in each vaccine production laboratory will vary, this manual will deal with the basic tests used on batches of experimental vaccine. This section has been prepared after discussions with veterinarians involved in the production of the I-2 vaccine in Mozambique, Bhutan and Myanmar.

The following publications have been used as references and are recommended reading:

ASEAN (1998) **Manual of ASEAN Standards for Animal Vaccines**, Livestock Publication Series No 2A.

Allan, W.H. and J.E. Lancaster (1978) **Newcastle Disease Vaccines, Their Production and Use**, FAO Animal Production and Health Series No 10, 1978.

Office International des Epizooties (OIE) **Manual of Standards for Diagnostic Tests and Vaccines, 2000**.

Website [http://www.oie.int/eng/normes/mmanual/A\\_summry.htm](http://www.oie.int/eng/normes/mmanual/A_summry.htm)

Mowat, N. and M. Rweyemamu (1997) **Vaccine Manual: The Production and Quality Control of Veterinary Vaccines for Use in Developing Countries**, FAO Animal Health and Production Series No 35.

## Sources of contamination

There are two possible sources of contamination with microorganisms:

1. Environmental contamination during the production of the vaccine. This risk is minimized by the use of aseptic technique, sterilized materials where appropriate, rigorous cleaning and disinfecting of all surfaces and equipment.
2. Vertical transmission through the eggs. By using embryonated eggs from a healthy flock, this risk is minimized. See **Section 4**.

## Testing for bacterial contamination

Correct storage and preparation of media is essential for all the following tests.

### Testing for aerobic contaminants

Use a general purpose broth culture medium that supports the growth of most likely contaminants for example Tryptic soy broth or nutrient broth.

1. Inoculate 1 mL of the wet vaccine or reconstituted freeze dried vaccine into 9 mL of broth.
2. Incubate at 30°C to 37°C for 24 hours.
3. Record results

### Interpretation

- ❖ Gross contamination after 24 hours, retest in twice the number of cultures. If contamination is evident after the second culture, discard the vaccine

### Testing for anaerobic contaminants

Many anaerobic bacteria have fastidious growth requirements. Using aseptic technique will minimize environmental contamination by anaerobes during the harvesting of the allantoic fluid. Protocols published by FAO, ASEAN and the OIE detail procedures for testing Newcastle disease vaccine for the presence of anaerobic bacteria.

## Testing for extraneous viruses

There is a range of tests available for detecting the presence of extraneous viruses. Deciding which pathogens to test for and which test to use is beyond the scope of this manual. Testing for extraneous viruses is also likely to be beyond the capacity of many vaccine production centres. In these centres, the risk of including extraneous viruses in vaccine is minimized by using embryonated eggs from healthy flocks free of viral infections.

The OIE Manual of Standards for Diagnostic Tests and Vaccines, 2000 recommends reading the purity test procedures for avian vaccines published in the European Pharmacopoeia.

Look up <http://pharmacos.eudra.org/F2/eudralex/vol-7/B/7BIm3a.pdf>.

## Testing for *Salmonella* species

### **Materials**

Liquid media:

- Selenite broth
- Tetrathionate broth

Solid Media

- MacConkey agar
- Salmonella-Shigella agar
- Brilliant Green agar
- Desoxycholate citrate agar
- XLD agar

Detailed information about preparing and using these media is available on the Oxoid website at <http://www.oxoid.com/uk/index.asp>

### **Method**

1. Inoculate 9 mL of one of the liquid media with 1 mL of vaccine
2. Incubate at 37°C for 18 to 24 hours
3. Inoculate a loopful of incubated broth onto one of the solid media.
4. Incubate 18 to 24 hours. Observe. If no *Salmonella* growth is detected, incubate again for 18 to 24 hours.
5. Discard the vaccine, if *Salmonella* are detected.

## Testing for *Mycoplasma* species

Several *Mycoplasma* species are transmitted through the egg. Embryonated eggs used to produce Newcastle disease vaccine should ideally be collected from a flock free of pathogenic *Mycoplasma* species. If the flock cannot be verified free of *Mycoplasma*, the vaccine should be tested for their presence. They are fastidious organisms and require specialized expertise to culture and identify. A commonly used media and protocol should be used to determine whether the vaccine is contaminated with *Mycoplasma*.

## Testing for contamination with fungi

Sabouraud dextrose agar is used to cultivate fungi. Chloramphenicol at 0.05 g/L can be added to inhibit bacteria. Inoculate the agar with 100 mL of vaccine and spread over the plate. Inoculate a control plate with diluent only. Incubate at 25°C to 30°C for one week. Observe daily and record results. Growth on the control plate invalidates the test, which should then be repeated. Vaccine should be free of fungal contamination.

## Further information about bacteriological media

Difco Laboratories and Oxoid are international manufacturers and suppliers of culture media.

Visit the Oxoid website at <http://www.oxoid.com/uk/index.asp>

This website contains detailed product information including formulas, techniques and material safety data sheets and is very informative.

Difco Laboratories have combined with BD Biosciences.

Visit their website at <http://www.bd.com/industrail/difco/nmce.html>

See **Appendix 3** for recipes for TSB and Sabouraud agar

## Testing virus content of vaccine

The virus content is a measure of the amount of infective Newcastle disease virus in the vaccine. This is important to know to ensure that vaccinated chickens receive enough virus to induce a protective immunity.

To measure the infectivity titre of wet vaccine or reconstituted freeze dried vaccine, follow the procedure described in **Section 12**.

## Summary of eye drop size and vaccine dose

- The recommended route of inoculation is eye drop.
- The standard dose for the I-2 Newcastle disease vaccine is  $10^{6.0}$  EID<sub>50</sub> per chicken.
- The size and number of eye-drops delivered may vary.
- The drop size is usually in the range of 10  $\mu$ L to 50  $\mu$ L.
- For a 10  $\mu$ L eye-drop to contain one standard vaccine dose, the undiluted vaccine must contain  $\geq 10^{9.0}$  EID<sub>50</sub>/mL.
- For a 50  $\mu$ L eye-drop to contain one standard vaccine dose, the undiluted vaccine must contain  $\geq 10^{8.3}$  EID<sub>50</sub>/mL.

## Safety test in young chicks

This test involves giving susceptible chickens ten doses of vaccine. The formal test described by OIE uses one-day-old SPF chicks. In many circumstances, it will be necessary to use commercial chickens and they may be older than one day old. In this case collect serum and test for HI titres to make sure they do not have maternal antibodies to Newcastle disease virus.

- Use two groups of chickens, a minimum of ten chickens per group.
- Take a serum sample from the chickens.
- Use the HI test to test for antibodies to determine susceptibility.
- Use only chickens that test negative for Newcastle disease antibodies, preferably SPF chickens.
- Administer ten doses ( $10^{7.0}$  EID<sub>50</sub>) by eye-drop of the I-2 vaccine to each chick in the treatment group.
- Administer diluent only by eye-drop to each chick in the control group.
- Observe daily for 21 days for clinical signs.

## Interpretation

- ❖ If any serious clinical signs or deaths attributable to the vaccine are observed, discard the vaccine.



## **Efficacy test of vaccine**

This test involves vaccination of susceptible chickens and measuring their antibody response to the vaccine. As in the safety test a source of susceptible chickens must be found and bled. Test the serum samples for the presence of antibodies to Newcastle disease virus. Suitable chickens will have HI titres of  $2^1$  or less.

An adequate antibody response to vaccine can be measured in two ways:

1. Testing serum collected from vaccinated chickens.
2. Challenging vaccinated chickens with a virulent strain of Newcastle disease virus.

In 1974, Allan and Gough published trials that showed an HI titre of  $2^3$  indicated the birds would be protected against fatal Newcastle disease if challenged. At some laboratories including the John Francis Virology Laboratory, challenge with virulent Newcastle disease virus is not carried out and serological testing as described by Allan and Gough is regarded as sufficient for testing vaccine efficacy.

Most laboratories already producing other strains of Newcastle disease vaccine will have protocols in place for testing the vaccine according to international protocols. These protocols can be applied to testing I-2 Newcastle disease vaccine.

## **Serological testing of efficacy of I-2 Newcastle disease vaccine**

- Divide the chickens into two groups of approximately ten chickens per group. House the groups separately.
- Vaccinate the chickens in one group with a standard vaccine dose of  $10^{6.0}$  EID<sub>50</sub>.
- The other group is the unvaccinated control group.
- Bleed the chickens in both groups two weeks after vaccination.
- Test the serum for antibodies using the HI test.
- HI titres of  $2^3$  and above are considered protective.

**Reference:** Allan, W.H. and R.E. Gough, 1974b.

**See Section 7:** The collection of blood from chickens.

**See Section 11:** Serology.

Further testing of the efficacy of the vaccine may be carried out by challenging both groups of chickens with virulent Newcastle disease virus.

## Outline of a protocol for testing I-2 Newcastle disease vaccine efficacy by challenge with virulent Newcastle disease virus

Further reading of the references listed at the beginning of the section will be required before challenge trails can be designed and successfully carried out.

Following is an outline only, based on information contained in the references.

- Select at least twenty chickens with antibody levels of  $2^1$  or less.
- Divide the chickens into two groups, a control group and a treatment group.
- House the two groups separately and arrange for two attendants to look after the chickens, one attendant for each group of chickens.
- Implement reasonable biosecurity measures to minimize the risk of uncontrolled introduction of Newcastle disease virus to either of the groups.
- The control group will not be vaccinated.
- The treatment group will receive one vaccine dose per bird.
- Bleed both groups at weekly intervals.
- Test the serum to measure the HI antibody response of the chickens to vaccination and to indicate levels of protection.
- Challenge the chickens with virulent Newcastle disease virus when HI titres of vaccinated birds are  $\geq 2^3$  indicating a protective antibody response. This will be 2 to 3 weeks after vaccination. See **Section 15** for a method for the isolation of virulent Newcastle disease virus.
- Make daily observations of the chickens and record the results.
- Collect serum from the surviving chickens after 2 weeks and determine HI antibody titres. See **Section 11**.

## Challenging chickens with virulent Newcastle disease virus

The two groups of birds can be housed together for the challenge. The challenge virus can be introduced in more than one way.

- Eye drop or intranasal infection of all the birds.
- Eye drop or intranasal infection of a few susceptible birds that are then placed with the flock. This allows the virus to spread by contact.
- Intramuscular injection.
- OIE Manual of Standards for Diagnostic Tests and Vaccines 2000 recommends a challenge dose is  $10^{5.0}$  LD<sub>50</sub>.
- Allan, W.H. and J.E. Lancaster (1978) "Newcastle Disease Vaccines, Their Production and Use" recommends a challenge dose of  $10^{6.0}$  LD<sub>50</sub>.

## Interpretation of results

- ❖ At least 90 percent of the vaccinated chickens should survive the challenge and show no clinical signs of the Newcastle disease.
- ❖ All unvaccinated control chickens should die of Newcastle disease.

## Notes on recording results of Newcastle disease vaccine efficacy trials

These trials can be laboratory or village based trials. Prepare tables to summarize the data collected. This can be done on a computer or tables can be hand drawn in the daybook.

Most of the data collected will be the results of serological haemagglutination inhibition (HI) tests. These results are usually expressed as HI titres to the log base 2.

Useful statistics calculated for each group of chickens are the geometric mean titre (GMT), number of chickens per group (n) and the percentage of the group having a protective HI titre. A protective titre is a titre that is greater than or equal to  $2^3$ .

Chicken identification (usually numbers of wing tags or leg bands) must also be recorded.

See **Appendix 6** for instructions for calculating GMT.

## 15 Isolation of virulent Newcastle disease virus

### Introduction

Virulent Newcastle disease virus is used in challenge trials of vaccinated chickens to test the efficacy or potency of the I-2 Newcastle disease vaccine. There are many strains of Newcastle disease virus and they vary in pathogenicity. This is reflected in the severity of disease in chickens infected with isolated Newcastle disease virus strains.

Virulent stains of Newcastle disease virus are not stored or used in the research projects at the John Francis Virology Laboratory at the University of Queensland. This information and procedures in this section are based primarily on two sources.

1. "Manual of Standards for Diagnostic Tests and Vaccines" published by the Office International des epizooties (OIE) at [http://www.oie.int/eng/normes/MANUAL/A\\_00036.htm](http://www.oie.int/eng/normes/MANUAL/A_00036.htm) (Sometimes this address sometimes opens up the OIE homepage only. In this case look under OIE publications.)
2. "Newcastle Disease" edited by D.J. Alexander, 1988.

There are three designations used to describe Newcastle disease virus strains that cause disease.

1. Velogenic
2. Mesogenic
3. Lentogenic

A fourth designation, "avirulent" has been introduced and includes strains that do not cause disease (Spradbrow, P.B., 1987).

These designations reflect the severity of the disease and are used to describe Newcastle disease pathotypes. In the laboratory, the designations are applied to strains that are grouped according to chicken embryo mortality. This is measured by the mean death time (MDT) of embryos inoculated with the virus by the allantoic cavity route. The MDT is the mean time in hours for the minimal lethal dose to kill inoculated embryos.

**Table 8: Designations of virulence of Newcastle disease virus**

<b>Designation</b>	<b>Virulence in Chickens</b>	<b>Approximate MDT</b>
<b>Velogenic</b>	Highly virulent strains that kill a high percentage of chickens	Less than 60 hours
<b>Mesogenic</b>	Moderately virulent strains that cause serious disease, some deaths and loss of egg production	Between 60 and 90 hours
<b>Lentogenic</b>	Strains of low virulence that cause respiratory disease especially in young chicks and loss of egg production.	More than 90 hours
<b>Avirulent</b>	Does not cause disease	Does not kill embryos

## **Overview**

- Newcastle disease virus isolates are replicated by inoculation in the allantoic cavity of ten-day old embryonated eggs.
- The virus grows in the cells of the allantoic membrane that lines the allantoic cavity. It is shed from these cells into the allantoic fluid, which can then be harvested.
- **Velogenic** and **Mesogenic** strains will grow in the cells of the three layers of the allantoic membrane. These strains rapidly infect the cells of the embryo itself. This causes the death of the embryo in less than four days of incubation.
- Some **Lentogenic** strains do not cross the allantoic membrane and consequently the embryo remains alive after four days of incubation.
- An indication of the virulence of the isolates can be derived from observing whether embryos inoculated with and isolate die and if they do, how long it takes.
- Protocols for establishing the **mean death time (MDT)** of embryos are available. They require that eggs are free of maternal antibody which influences the death time of embryos inoculated with mesogenic and velogenic isolates. For the purposes of preparation of a challenge strain for experimental purposes approximate mean death times are an adequate indication of virulence.
- Other procedures for determining pathogenicity are the intracerebral pathogenicity index and the intravenous pathogenicity index. Protocols for these tests are not included in this manual.
- A local velogenic strain of Newcastle disease must be used to challenge chickens to test the efficacy of Newcastle disease vaccine. The velogenic strain must be isolated from a chicken that shows clinical signs of Newcastle disease or a carcass where the cause of death has been diagnosed as Newcastle disease.

## **Isolation of virulent Newcastle disease virus in the laboratory**

Isolation of Newcastle disease virus from field samples should not be carried out in the vaccine production laboratory as this would introduce a risk of virulent Newcastle disease virus contaminating the vaccine. If a separate facility is not available, a separate room should be set up with an incubator used only for eggs used to isolate Newcastle disease virus from field samples. Make sure the incubator is carefully cleaned and disinfected after use.

### **Field samples**

Use aseptic technique to collect a sample of brain, liver, spleen or marrow from a long bone from a bird showing clinical signs of Newcastle disease or a carcass from which Newcastle disease has been diagnosed.

### **Detailed protocol**

Use the following Materials and Method to prepare a protocol for use in your own laboratory. The protocol will be a detailed step by step description of the procedures involved in isolating virulent Newcastle disease virus in your laboratory. Further reading is recommended.

### **Materials**

- Sterilized glass homogenizer, mortar and pestle or needle and syringe.
- Centrifuge tubes and centrifuge. A microfuge is suitable.
- 0.45  $\mu\text{m}$  filter (not essential).
- 10-day-old embryonated eggs. If possible, purchase the eggs from a layer flock free of high levels of Newcastle disease virus antibodies.
- Egg incubator.
- Candling lamp.
- Antibiotic solution (PSG)
- Materials for haemagglutination test. See **Section10**.

## Isolation of virulent Newcastle disease virus in the laboratory

### *Method*

1. Use aseptic technique to homogenize the sample with 2 to 3 mL of PSG buffered antibiotic solution.
2. Transfer the homogenized sample to an appropriate tube and centrifuge.
3. Filter the supernatant through a 0.45 µm filter if available.
4. Candle, mark and disinfect the inoculation site of 10 ten-day old embryonated eggs. Use five eggs per sample. See **Section 5**.
5. Inoculate the allantoic cavity of the embryonated eggs with 0.1 mL of the supernatant. See **Section 6**.
6. Place the inoculated eggs in the incubator.
7. Candle eggs daily post inoculation. Record deaths and note the time it takes for the embryo to die. Deaths within the first day are regarded as non-specific and discounted. Discard these embryos. Deaths on subsequent days are likely to be due to Newcastle disease virus.
8. Harvest some allantoic fluid from each dead egg and check for the presence of haemagglutinin. A positive result is an indication of the presence of Newcastle disease virus. Live eggs are unlikely to contain virulent Newcastle disease virus. **See Section 10**
9. Use aseptic technique to harvest allantoic fluid from those eggs with positive HA. Dispense fluid into centrifuge tubes.
10. Centrifuge the harvested allantoic fluid, pool and then prepare aliquots of the supernatant. Label clearly and store at -20°C.
11. Confirm the allantoic fluid collected contains Newcastle disease virus. This is done by using harvested allantoic fluid as antigen to test for haemagglutination inhibition (HI) standard Newcastle disease virus antiserum. See the following part.
12. This allantoic fluid must be passaged again in embryonated eggs to establish mean death time.

## Confirmation of identity of Newcastle disease virus

The haemagglutination test is not specific for Newcastle disease virus and other viruses will agglutinate red blood cells. Therefore a sample of allantoic fluid testing positive for haemagglutinin will need further testing to confirm the presence of Newcastle disease virus. The presence of Newcastle disease virus in the sample is confirmed by using the haemagglutination inhibition (HI) test.

- Harvested allantoic fluid that tests positive for haemagglutinin is titrated for HA titre and diluted to 4HA units in 25 µL. See **Section 10**.
- The allantoic fluid is used as the antigen in an HI test of the standard positive serum containing antibodies to Newcastle disease virus and of the standard negative serum that does not contain antibodies to Newcastle disease virus.
- If the standard positive inhibits haemagglutination in the test allantoic fluid sample, this result will confirm the presence of Newcastle disease virus in the test sample. There should be no inhibition by the negative control serum.
- See **Section 11** for details of HI test.

## Mean death time (MDT) in eggs

The following protocol to establish the MDT is taken from the protocol published by Office International des Epizooties. It is available from the OIE website at [http://www.oie.int/eng/normes/mmanual/A\\_00036.htm](http://www.oie.int/eng/normes/mmanual/A_00036.htm)

- Dilute fresh infective allantoic fluid in sterile saline to give a ten-fold dilution series between  $10^{-6}$  and  $10^{-9}$ .
- For each dilution, inoculate 0.1 mL into the allantoic cavity of each of five 9-day-old or 10-day-old embryonated SPF chicken eggs and incubate at 37°C.
- Retain the remaining virus dilutions at 4°C and inoculate another five eggs with 0.1 mL of each dilution 8 hours later and place at 37°C. This staggers the time of inoculation.
- Each egg is examined twice daily for 7 days and the times of any embryo deaths are recorded.
- The minimum lethal dose is the highest virus dilution that causes all the embryos inoculated with that dilution to die.
- The MDT is the mean time in hours for the minimum lethal dose to kill embryos.



## **Use of non-SPF embryonated eggs**

Eggs from flocks vaccinated with Newcastle disease vaccine can be used to grow Newcastle disease virus. Eggs from these flocks will contain varying levels of antibodies in their yolks. These antibodies are derived from the hen. As the embryos develop, the maternal antibodies enter the blood. Virulent strains of Newcastle disease virus might not kill the embryos if there are high levels of antibodies. However the virus will grow in the cells lining the allantoic cavity, where there are no antibodies and is then shed into the allantoic fluid. The haemagglutination test, described in **Section 10**, will detect the presence of Newcastle disease virus in the allantoic fluid.

The presence of antibodies in eggs used for the isolation of Newcastle disease virus would be expected to increase the mean death time. Therefore when non SPF eggs are used to propagate the virus, the mean death times as described in Table 3 are not a reliable indication of pathotypes. In this situation, the pathotype of an isolate would have to be established by inoculation of susceptible chickens.

## **Measuring the concentration of virulent Newcastle disease virus in a suspension**

For measuring the 50 percent Embryo Lethal Dose or ELD<sub>50</sub>, carry out ten-fold serial dilutions and inoculate ten-day old embryonated eggs according to the Material and Methods in **Section 12**. Virulent virus will kill the embryos and the end point will be determined by the death of the embryos. Calculate the 50 percent endpoint using the Reed Muench method as described in **Section 12**.

This manual does not include a protocol for measuring the LD<sub>50</sub> or 50 percent lethal dose in susceptible chickens. Further reading of books describing virology laboratory techniques will be required for details of the materials and method for carrying out this infectivity assay.

## Testing the virulence of a challenge isolate

1. Find a source of chickens that are likely to be free of Newcastle disease virus antibodies. Take serum samples and test for HI antibodies. See **Section 11**. Select chickens that are free of Newcastle disease virus antibodies. If antibodies are detected, titres of 8 ( $2^3$ ) and higher are considered protective so select chickens with titres of less than 8 for challenge.
2. House a small group of these chickens in isolation and take quarantine measures to avoid spread of virulent Newcastle disease from the chickens after infection.
3. Remove one of the aliquots of the stored allantoic fluid containing virulent Newcastle disease virus. Challenge some of the chickens by eye drop or intramuscular injection with undiluted allantoic fluid.
4. Do not infect the remainder of the birds. Their response will indicate whether contact spread between the birds occurs.
5. Monitor the birds. Infection by eye-drop with virulent Newcastle disease virus will cause clinical signs within three days and mortality in about five to seven days. The response to contact infection will become evident a few days later.
6. Maintain uninfected control chickens from the same source in a separate room. They should remain free of Newcastle disease.

**TAKE CARE** not to contaminate your vaccine or the working seed with the Newcastle disease virus challenge strain. Label all samples clearly, store separately and preferably carry out bench work in a separate section of the laboratory.

**Disinfect materials and benches after handling the virus!**

## 16 Cleaning and decontamination

### Introduction

Appropriate chemical disinfectants must be used for cleaning equipment, materials and work surfaces. All laboratory wastes must be assigned to a category and placed in clearly labeled bins from where the waste will be disposed of appropriately. This is an important area of laboratory management and is covered only in a superficial manner in this manual. Further reading is recommended.

### Alcohol

A 70 percent volume/volume solution of alcohol diluted with water is useful for wiping down benches and disinfecting the outside of eggs before inoculation and harvesting of allantoic fluid. The addition of 2 percent iodine will increase the effectiveness of this solution.

**Note that 70 percent solution of alcohol is flammable!**

### Chlorine

There are several chlorine compounds that are used as disinfectants. Sodium hypochlorite (NaOCl) or household bleach is readily available and cheap. Soaking overnight in a 2 percent solution of chlorine is useful for disinfecting plastic materials for example V-bottom microwell plates.

Note that commercial bleach contains 12 to 14 percent hypochlorite when manufactured but this concentration deteriorates with time. Powdered chlorine-based disinfectants are available.

Follow the manufacturer's instructions for the recommended weight of powder to dissolve in a volume of water.

**Note that chlorine damages fabric and corrodes many metals!**

### Wide spectrum hospital grade disinfectants

There is a range of products in this category. Check availability and price. Pearson Medol is a product that is widely used at the University of Queensland. The active ingredient is 4-chloro-3, 5-xyleneol.

A 2 percent solution is used for wiping down benches, cleaning incubators and disinfecting contaminated objects such as plastic egg racks and discarded tips.

## Formaldehyde

Formaldehyde is available as a 37 to 40 percent aqueous solution known as formalin. Paraformaldehyde is a solid polymerized form of formaldehyde. Formaldehyde is a potential carcinogen, causes burns and the vapour irritates the eyes and respiratory tract. It must be handled with caution and only used as a disinfectant when there are no suitable alternatives.

Formaldehyde vapour is useful for fumigating egg incubators and animal houses. Eggs suspected of being contaminated with bacteria can be fumigated prior to incubation. There are two methods for generating the vapour.

1. Heating 91 percent paraformaldehyde at the rate of 5 grams per cubic metre of space in an electric evaporator. An electric frying pan on a low temperature setting is suitable.
2. Adding 35 mL of formalin (40 percent formaldehyde) to 10 g potassium permanganate per cubic metre of space.

### Note:

- Prior to fumigation, seal all openings and wet the surfaces.
- Wear protective clothing, gloves, mask and safety glasses.
- Beware of splashing as the reagents react. The reaction generates heat.
- Avoid inhalation of the fumes.
- Do not carry out this procedure alone.
- Allow 24 hours for the vapour to penetrate all the surfaces.

**Always read the instructions before using disinfectants and cleaning reagents!**

## 17 Frequently asked questions

### 1. Does it matter if you harvest amniotic fluid as well as allantoic fluid?

No. Ideally, you should try to harvest only allantoic fluid. Amniotic fluid does not contain the I-2 Newcastle disease virus and will reduce the concentration of virus in the harvest. Take care not to rupture the amniotic membrane.

### 2. Does it matter if the fluid that is harvested contains red blood cells?

It is best to avoid contamination with red blood cells. Chill the eggs at 4<sup>0</sup>C for at least 2 hours or preferably overnight before harvesting to kill the embryos. Chilling also reduces the bleeding should the vessels rupture during harvesting of the allantoic fluid. Any red blood cells that are present should be removed by centrifugation and harvesting the supernatant using aseptic technique.

### 3. Can maternal antibodies in the embryos reduce the titre of the I-2 Newcastle disease vaccine harvest?

No. Avirulent strains of Newcastle disease virus such as V4 and I-2 inoculated into the allantoic cavity are confined in the cavity. They do not cross the allantoic membrane and do not enter the embryo. Maternal antibody is confined to the yolk sac until about 14 days of incubation and then enters the embryo. Virus harvested in allantoic fluid at 14 days will not have been exposed to antibody.

### 4. Is the haemagglutination test specific for Newcastle disease virus?

No. Many viruses will agglutinate chicken red blood cells. A specific identification requires a haemagglutination inhibition test with specific anti-Newcastle disease virus antiserum.

### 5. Can you give chickens too much I-2 Newcastle disease vaccine?

I-2 Newcastle disease vaccine is a safe, avirulent vaccine tolerated at high doses. If you have excess vaccine, it is better to increase the dose of vaccine than to discard a portion of the vaccine. The I-2 vaccine was shown to be safe when day old SPF chickens were given 100 times the standard dose by eye drop in a trial at the John Francis Virology Laboratory.

**6. Can red blood cells from vaccinated donor chickens that have antibodies to Newcastle disease virus be used in the haemagglutination-inhibition test?**

Yes. The cells will be washed free of antibody. It is recommended to pool red blood cells from at least three donor chickens to guard against individual birds whose cells may react in an unusual way. Remember to always use a red blood cell control when doing the haemagglutination inhibition test. See **Section 11**.

**7. Can you use the same incubator for preparation of vaccine and isolation of Newcastle disease virus from field samples?**

No. There would be too much risk of unrecognized contamination of vaccine with the field virus.

**8. How do I clean the incubator?**

Remove all solid debris with a damp cloth. Then swab all surfaces with a disinfectant or 70 percent alcohol. See **Section 16** for instructions for fumigation with formaldehyde.

**9. If an embryo is dead, do I collect the allantoic fluid?**

The I-2 strain of Newcastle disease virus will rarely kill embryos when inoculated into the allantoic cavity. Dead embryos inoculated with I-2 are likely to have been killed by bacterial contamination and the embryo should be discarded. If you are working with more virulent strains of Newcastle disease virus, the virus will kill embryos. In this case allantoic fluid from these eggs will be collected for testing. See **Section 15**.

**10. What do the standard positive and negative sera do?**

They ensure that your tests are standard and producing similar results each time you perform them. They can also ensure standard results between different laboratories. See **Section 11** for more details.

**11. What does passaging the Newcastle disease virus through eggs achieve?**

The virus is passaged through eggs by the inoculation of the allantoic cavity to increase the quantity of virus. A small amount of the I-2 Newcastle disease virus seed material produces a lot of vaccine. However, the number of passages must be kept as small as possible, or some characteristics of the vaccine may alter. This especially applies to thermostable Newcastle disease vaccines that have been selected for heat-resistance. This property of the virus may be lost during subsequent passaging in embryonated eggs without further heat selection.

**12. Why is it necessary to change the micropipette tip when carrying out ten-fold serial dilutions but not when carrying out two-fold serial dilutions?**

In this manual, two-fold serial dilutions are used in quantitative assays to indicate the concentration of Newcastle disease virus (HA test) or Newcastle disease virus antibodies (HI test) in each dilution. These data is then used to calculate a titre to the log base 2. In both assays, the small quantities of the antibodies or virus carried over on tips between the two-fold dilutions will not greatly influence the result of the titres.

In this manual, ten-fold dilutions are used in quantal assays to titrate viral infectivity. Each dilution is inoculated into eggs and a single viral particle is sufficient to infect the embryo. Infected embryos will give a positive result when the allantoic fluid is tested for the presence or absence of virus after incubation for four days. For this reason even a small number of infective virus particles that are carried over on the pipette tip will falsify the results. The infectivity titres calculated from these results will be erroneously high. By using clean tips for each dilution, this error will not be introduced into the assay.

## 18 Glossary

### Definitions of terms as used in this manual

<b>Absorption</b>	The movement of a fluid or a dissolved substance across a membrane.
<b>Adsorption</b>	The process whereby a layer of molecules, atoms or ions attach to the surface of a red blood cells.
<b>Agglutination</b>	A specific reaction whereby viruses link together microscopic foreign particles such as red blood cells. This causes visible clumping.
<b>Antibody</b>	A protein produced by the immune system in response to an antigen. The antigen can be an infectious agent for example Newcastle disease virus.
<b>Antigen</b>	Any substance, usually a protein, that causes an antibody response when introduced into the body. Antigens also react with antibodies in serological tests.
<b>Bifurcates</b>	Divides into two branches.
<b>Biosecurity</b>	Precautions taken to minimize the risk of introducing an infectious agent into a population.
<b>Elution</b>	The detachment of virus from the receptors on the surface of red blood cells.
<b>50% Embryo Infectious Dose (EID<sub>50</sub>)</b>	Reciprocal of that dilution of virus per unit volume that results in <u>infection</u> of 50 percent of the inoculated embryos. See Section 13
<b>50% Embryo Lethal Dose (ELD<sub>50</sub>)</b>	Reciprocal of that dilution of virus per unit volume that results in the <u>death</u> of 50 percent of inoculated embryos.



<b>Endpoint</b>	The point in an assay where the highest dilution of the substance being tested produces a positive result.
<b>Haemagglutination (HA)</b>	The agglutination or clumping of red blood cells by virus. A diffuse film is formed on the well of a microwell plate as the virus-red blood cell complex settles under gravity.
<b>Haemagglutination Inhibition (HI)</b>	Mixing a serum sample with Newcastle disease virus antigen resulting in specific binding between anti-Newcastle disease virus antibodies in the serum and the Newcastle disease virus. The binding sites on the virus particles that attach to red blood cells are no longer free for binding and haemagglutination is therefore inhibited. The red blood cells roll to the bottom of the V-bottom wells of microwell plates and form a distinct button.
<b>Haemagglutination Inhibition Titre</b>	The reciprocal of the highest or last two-fold dilution where haemagglutination inhibition is detected.
<b>Haemolysis</b>	The destruction of red blood cells and release of haemoglobin.
<b>Hyperaemia</b>	Increased blood supply to a part of a body. The blood remains in vessels and does not haemorrhage.
<b>Isotonic</b>	A solution that causes no change in the state or tone of cells for example red blood cells when they are stored in DGV storage solution.
<b>I-2 Master seed</b>	A collection of uniform aliquots of the I-2 strain of Newcastle disease virus from which all other seed passages are derived.

<b>I-2 Working seed</b>	A collection of uniform aliquots of the I-2 strain of Newcastle disease virus produced by passage of the I-2 master seed. Used to prepare the I-2 Newcastle disease vaccine.
<b>Labile</b>	Unstable and readily changes.
<b>50% Lethal dose (LD<sub>50</sub>)</b>	Reciprocal of that dilution of virus per unit volume that results in the <u>death</u> of 50 percent of inoculated susceptible chickens.
<b>Lysis</b>	Disintegration.
<b>Lentogenic</b>	Low virulence.
<b>Mesogenic</b>	Moderate virulence.
<b>Mean death time (MDT)</b>	The time taken for a minimal lethal dose of Newcastle disease virus to kill chicken embryos.
<b>Passage</b>	The subculture of Newcastle disease virus in embryonated eggs.
<b>Pathotype</b>	A term used to designate virulence of isolates of Newcastle disease virus.
<b>Proteases</b>	Enzymes that digest proteins.
<b>Pleomorphism</b>	Occurring in many shapes.
<b>Seed lot</b>	A term used in vaccine production to describe a system where the original master seed virus is multiplied, harvested and stored in aliquots. This is referred to as the working seed. These aliquots are then multiplied to produce vaccine. This system minimizes the number of passages of virus needed to produce vaccine and reduces the risk of genetic change and of contamination.

<b>Thermolabile</b>	Sensitive to and changed by heat.
<b>Thermostable</b>	Exhibiting a relative resistance to inactivation on exposure to elevated temperatures.
<b>Titration</b>	A procedure used to measure the concentration of a substance in a solution.
<b>Velogenic</b>	High virulence.

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Spradbrow, P.B. (1987). In Newcastle Disease in Poultry. A new food pellet vaccine. J.W. Copland editor. Canberra, ACIAR: pp 12-18 Newcastle Disease - An Overview.

## Further Reading

### ACIAR publications

ACIAR has published a series of publications relating to the control of Newcastle disease in village chickens. A detailed laboratory manual will be published in the near future. For information about ACIAR publications visit the ACIAR website at <http://www.aciar.gov.au>

Two of these publications that can be accessed and downloaded from the ACIAR website are:

Alders, R. and P. Spradbrow, (2001) Controlling Newcastle Disease in Village Chickens, A Field Manual. ACIAR Monograph No 82, Australian Centre for International Agricultural Research, Canberra.

Alders, R., dos Anjos F., Bagnol B., Fumo A., Mata B. and Young M., (2002) Controlling Newcastle Disease in Village Chickens, A Training Manual. ACIAR Monograph No 86, Australian Centre for International Agricultural Research, Canberra.

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Reed, R.D.D. Holmes et al, (1998). Practical Skills in Biomolecular Sciences, Longman. Harlow, England ISBN 0582298261 (June 2002).

Seidman, L.A. and Moore, C.J. (2000) Basic Laboratory Methods for Biotechnology, Textbook and Laboratory Reference. Upper Saddle River, New Jersey, Prentice-Hall ISBN: 0-13-795535-9 A comprehensive reference and textbook. Especially useful are the units on maths, preparation of laboratory solutions and general safety in the laboratory.

Burleson F.G., T.M. Chambers and D.L. Wiedbrauk (1992) Virology: a laboratory manual. Academic Press San Diego, Sydney ISBN 0121447308 Useful chapters on quantal virus assays including protocols for calculating LD<sub>50</sub> and ELD<sub>50</sub>.

ASEAN 1998 Livestock Publication Series No 2, Public Information Unit, the ASEAN Secretariat, 70A Jl. Sisingamangaraja, Jakarta 12110, Indonesia.

Fax 6221 7398234, 7243504

ASEAN homepage: <http://www.aseansec.org/>

Email [public@ASEAN.or.id](mailto:public@ASEAN.or.id)

Titles:            Manual of ASEAN Standards for Animal Vaccines  
                         Manual of ASEAN Rules and Proceedings for the Registration of Animal Vaccines  
                         Manual of ASEAN Accreditation Criteria for Animal Vaccine Testing Laboratories  
                         Manual of ASEAN code of practice for the Commercial Storage, Transportation and Handling of Animal Vaccines

## Useful Websites

**Office International des Epizooties.** An intergovernmental organization with 158 member countries that collects veterinary scientific information.

Home page: <http://www.oie.int/>

The OIE publishes a Manual of Standards for Diagnostic Tests and Vaccine. This is recommended reading and can be downloaded at

[http://www.int/eng/notmes/mmanual/a\\_summry.htm](http://www.int/eng/notmes/mmanual/a_summry.htm)

**Rural poultry in developing countries.** A website sponsored by the Australian Centre for International Agricultural Research and the University of Queensland <http://www.vsap.uq.edu.au/ruralpoultry>

### Websites with information about general laboratory safety

- University of Calgary  
<http://www.ucalgary.ca/~ucsafety/bulletins/genlab1.htm>
- University of California  
<http://envhort.ucdavis.edu/safety/labsafety.html>
- University of Sydney  
<http://www.usyd.edu.au/su/ohs/labsafety.html>
- Centers for Disease Control and Prevention, Atlanta, Georgia, USA  
<http://www.cdc.gov/od/ohs/manual/labsfty.htm>
- Howard Hughes Medical Institute  
<http://www.hhmi.org/research/labsafe/index.html>

### The European Pharmacopoeia

- Procedures for avian vaccines  
<http://pharmacos.eudra.org/F2/eudralex/vol-7/B/7Blm3a.pdf>

### Information about bacteriological media

- Oxoid website  
<http://www.oxid.com/uk/index.asp>
- Difco Laboratories have combined with BD Biosciences  
<http://www.bd.com/industrail/difco/nmce.html>

# Appendix 1

## Newcastle disease: an overview

- Newcastle disease is a severe clinical manifestation of infection with Newcastle disease virus. The disease is seen mainly in chickens.
- In chickens, Newcastle disease often causes high or total mortality in a flock. Chickens may die without showing any clinical signs of infection.

### Common clinical signs of acute infection

- Greenish blood-stained diarrhoea
- Dehydration
- Tremor
- Torticollis (abnormal position and twisting of the neck, limited movements of the head)
- Paralysis
- Respiratory distress
- Loss of appetite

### Common post-mortem findings

- Hyperaemia and congestion in respiratory tract
- Serous or catarrhal exudates in larynx and trachea
- Thickened air sacs, may contain yellow exudates
- Haemorrhagic lesions in digestive tract: with some necrosis especially in proventriculus
- Haemorrhages in the intestinal lymphoid nodules and caecal tonsils
- Enlarged spleen

### Diagnosis

- Presumptive: Based on clinical signs and post mortem findings
- Unequivocal: Isolation of virulent Newcastle disease virus from samples for example blood, bone, spleen, lungs
- Sero-diagnosis: Monitoring of antibody levels in acute and convalescent serum samples.

## Appendix 2

# Newcastle disease virus: classification and important viral proteins

### Classification of the virus

- Family: *Paramyxoviridae*
- Subfamily: *Paramyxovirinae*
- Genus: *Rubulavirus*

### Morphology of the virus

- Spherical shape although pleomorphism is common
- 100 to 300 nanometres in diameter
- Enveloped

### Important viral proteins

Two proteins of Newcastle disease virus are inserted in the envelope. See **Figure 23**. They are the haemagglutinin/neuraminidase protein and the fusion protein. These two proteins are important in determining the virulence of the virus and how the virus infects host cells.

### The haemagglutinin/neuraminidase protein

This protein has two sections that are of interest.

1. The haemagglutinin section. This is an attachment protein and binds to receptors on the outside of the membrane of host cells including red blood cells. Attachment of the virus to red blood cells is an important property used in the laboratory to detect the presence of the virus and to detect antibodies to the virus.
2. The neuraminidase section is the active site of an enzyme that aids in the release of the virus from the membrane of host cells. The activity of this enzyme affects the time taken for the virus to elute from red blood cells.

### The fusion protein

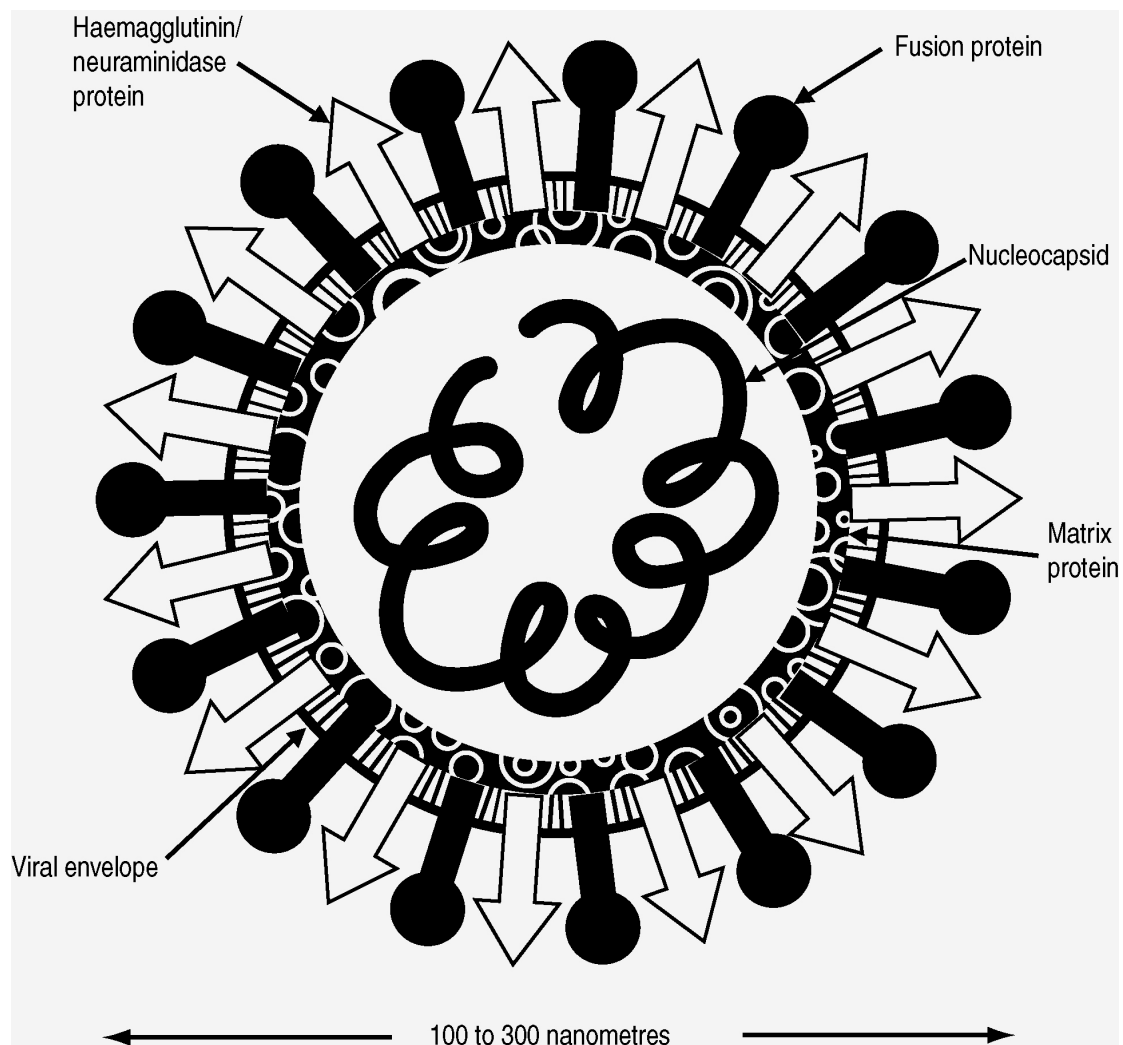
- This protein fuses the virus envelope to the membrane of the host cell. This allows penetration of the host cell by the viral genome.
- In order for fusion to occur, the shape of the native fusion protein must be changed. This change happens when a host cell protease cleaves or cuts the protein at a specific cleavage site. After this has happened, the fusion protein is activated and can now fuse to the membrane of the cell.
- The sequence of the amino acids around the cleavage site determines the range of proteases that can activate cleavage of the protein. This sequence therefore determines the virulence.



## Molecular analysis of the Newcastle disease virus genome

A link between the amino acid sequence of the cleavage site of the fusion protein and virulence of Newcastle disease virus isolates has been established. Molecular techniques are now used to indicate the virulence of new isolates of Newcastle disease virus. This involves the use of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and sequencing of the DNA product. The amino acid sequence of the viral protein can then be deduced and analyzed.

The complete viral genome of the I-2 strain of Newcastle disease virus has now been sequenced. (Personal communication: Philemon Wambura.)



**Figure 23:**  
**Diagrammatic representation of Newcastle disease virus**

## Appendix 3

### Media and solutions

#### 1. Buffer solution

##### Phosphate buffered saline (PBS)

##### Recipe to prepare five litres of PBS

###### Reagents

- Sodium chloride NaCl 40.0g
- Potassium chloride KCl 1.0g
- Potassium dihydrogen phosphate anhydrous  $\text{KH}_2\text{PO}_4$  1.0g
- Disodium hydrogen phosphate anhydrous  $\text{Na}_2\text{HPO}_4$  4.6g
- OR Disodium hydrogen phosphate dihydrous  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  5.75g
- OR Disodium hydrogen phosphate dodecahydrous  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  11.6g
- Distilled water to make up to 5L

###### Method

1. Weigh out the reagents and place in a 5 L conical flask.
2. Add distilled water to make 5 L. Mix well.
3. Check pH. Adjust to pH 7.3 to 7.4.
4. Pour into storage bottles.
5. Autoclave at  $121^\circ\text{C}$  for 15 minutes. Use a slow exhaust.
6. Allow to cool, then tighten the lids and label the bottles.

## Ten times (10 x) stock solution

If you require large volumes of PBS in your laboratory, you may find it useful to prepare a stock solution that is ten times the strength of the working solution used in the laboratory procedures.

This is called a ten times stock solution, usually labeled by the abbreviation “10 x stock solution”. Before use, prepare the “working solution” by making a one in ten dilution of the stock solution.

To prepare 1 L of working solution, mix 100 mL of the stock solution with 900 mL of distilled water.

Three recipes for the stock solution are provided for total volumes of 1 L, 2 L and 5 L. Choose the recipe for the volume best suited for your laboratory’s requirements.

Remember 5 L of the stock solution is enough to prepare 50 L of the working solution. This may be far more than required for the next few months. 2 L of the stock solution may be an adequate supply of the stock solution.

## Reagents for varying volumes of ten times stock solution of PBS

Reagents	Total volume		
	1 Litre	2 Litres	5 Litres
NaCl	80.0g	160.0g	400.0g
KCl	2.0g	4.0g	10.0g
KH <sub>2</sub> PO <sub>4</sub>	2.0g	4.0g	10.0g
One of the following			
Na <sub>2</sub> HPO <sub>4</sub> anhydrous	9.2g	18.4g	45.9g
<b>OR</b> Na <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	11.5g	23.0g	57.5g
<b>OR</b> Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	23.1g	46.1g	115.7g

### Method

1. Weigh out reagents for the selected total volume of PBS.
2. Mix with distilled water to the required volume. Adjust pH to 7.3.
3. Dispense into sterile glass bottles, sterilize by autoclaving at 121°C for 15 minutes. Use a slow exhaust.
4. When cool, tighten lids and label.

## 2. Storage solution for washed red blood cells

### Dextrose gelatin veronal (DGV)

#### Reagents

- Barbitone (diethylbarbituric acid)  
 $(C_2H_5)_2C.CO.NH.CO.NH.CO$  0.6g
- Gelatin 0.6g
- Barbitone sodium  
 $(C_2H_5)_2C.CO.NH.C(ONa).N.CO$  0.3g
- Either Calcium chloride ¶ entimete  $CaCl_2.2H_2O$  0.03g  
**OR** Calcium chloride anhydrous  $CaCl_2$  0.02g
- Magnesium sulphate  $MgSO_4.7H_2O$  0.12g
- Sodium chloride  $NaCl$  8.5g
- D-Glucose  $C_6H_{12}O$  10.0g
- Distilled water to make up to 1 L

#### Method

1. Add the barbitone and gelatin to 250 mL of distilled water. Heat to dissolve.
2. Add remainder of the ingredients to 600 mL of distilled water and dissolve.
3. Mix these two solutions in a conical flask.
4. Make up to a total volume of 1 L with distilled water.
5. Pour into 100 mL bottles for storage.
6. Autoclave or pressure cook at 116°C for 10 minutes. Use a slow exhaust.
7. Allow to cool, then tighten the lids and label the bottles.
8. Store in the refrigerator.

### 3. Anticoagulants

#### Acid Citrate Dextrose (ACD)

##### Reagents

- Citric Acid  $C(OH)(COOH)(CH_2.COOH)_2.H_2O$  4.0g
- Sodium Citrate  $Na_3C_6H_5O_7.2H_2O$  11.3g
- D-Glucose  $C_6H_{12}O_6$  11.0g

##### Method

1. Weigh out reagents into a conical flask.
2. Dissolve in 300 mL of distilled water.
3. Make up to 500 mL with distilled water.
4. Dispense into 100 mL bottles and put on lids. Do not tighten.
5. Sterilize by autoclaving at 116°C for 10 minutes. Use a slow exhaust
6. Allow to cool, then tighten the lids and label the bottles.
7. Store in the refrigerator.

#### Alsever's Solution

##### Reagents

- Citric acid  $C(OH)(COOH)(CH_2.COOH)_2.H_2O$  0.055g
- Sodium Citrate  $Na_3C_6H_5O_7.2H_2O$  0.8g
- D-Glucose  $C_6H_{12}O_6$  2.05g
- Sodium chloride  $NaCl$  0.42g
- Distilled water to make up to 100 mL

##### Method

1. Weigh out reagents into a conical flask.
2. Dissolve of distilled water and make up to 100 mL.
3. Dispense into sterile 10 mL bottles.
4. Sterilize by autoclaving at 116°C for 10 minutes. Use slow exhaust.
5. Allow to cool, then tighten the lids and label the bottles.
6. Store in the refrigerator.

#### 4. Antibiotic Solution

The recipe given is for a solution of Penicillin, Streptomycin and Gentamycin dissolved in PBS. In this manual, this solution is given the abbreviation PSG.

Benzyl penicillin (Penicillin G) is a broad range antibiotic active against Gram positive and Gram negative aerobic cocci and most spirochaetes. It comes in various forms, which vary in solubility. Potency may be expressed in International Units (IU) rather than mg.

Streptomycin is an antibiotic effective against Gram negative bacteria.

Gentamycin is a broad spectrum antibiotic mainly affecting Gram negative aerobes. It is stable at higher temperatures. If Gentamycin is difficult to obtain it can be omitted from the solution.

#### Reagents

- Benzyl penicillin 6 g
- Streptomycin 500 mg
- Gentamycin 250 mg
- Sterile PBS 1 L

#### Method

1. Dissolve reagents in approximately 800 mL of PBS.
2. Make up to 1 L with PBS.
3. Cold sterilize by passing solution through a 0.2 micron filter. Dispense into 100 mL sterile glass bottles, lid and label.

#### DO NOT AUTOCLAVE!

If a system for cold sterilization under vacuum does not exist in your laboratory, make sure sterile PBS and aseptic technique are used to minimize the risk of contamination.

## 5. Bacterial culture media

### Tryptic Soy Broth (TSB)

This is a general purpose broth medium prepared for the cultivation of fastidious and non fastidious organisms.

#### Materials for preparing 1L of TSB

- 30 g dehydrated TSB medium
- 1 L distilled or deionized water
- Sterile 20 mL glass bottles with lids.

#### *Method*

1. Dissolve media in 1 L of water.
2. Warm slightly to dissolve completely.
3. Dispense 9 mL aliquots into the glass bottles.
4. Sterilize in the autoclave at 121°C for 15 minutes.

### Sabouraud dextrose agar

Sabouraud dextrose agar is recommended for cultivating fungi. It is readily available and is supplied by Oxoid and Difco with instructions for its preparation. Chloramphenicol is a bacterial inhibitor that can be added.

The following recipe has been supplied by the Diagnostic Bacteriology Laboratory at the School of Veterinary Science, University of Queensland.

#### Materials

- Sabouraud dextrose agar 65 g
- Oxoid agar No. 1 or Difco Bacto agar 5 g
- Chloramphenicol (optional) 0.05 g dissolved in 10 mL of 95 percent ethanol.
- 1 L distilled water
- Sterile Petri dishes

#### *Method*

1. Suspend the agar in the distilled water. Heat to boiling point.
2. Add the chloramphenicol mixture to the agar. Stir thoroughly.
3. Dispense into 100 mL bottles.
4. Sterilize by autoclaving at 115°C for 10 minutes. Allow to cool to 50°C.
5. Pour into sterile Petri dishes. Allow approximately 25 mL for a 90 mm Petri dish.
6. Incubate plates at 37°C overnight and check for growth of bacterial contaminants.
7. Store at 4°C.

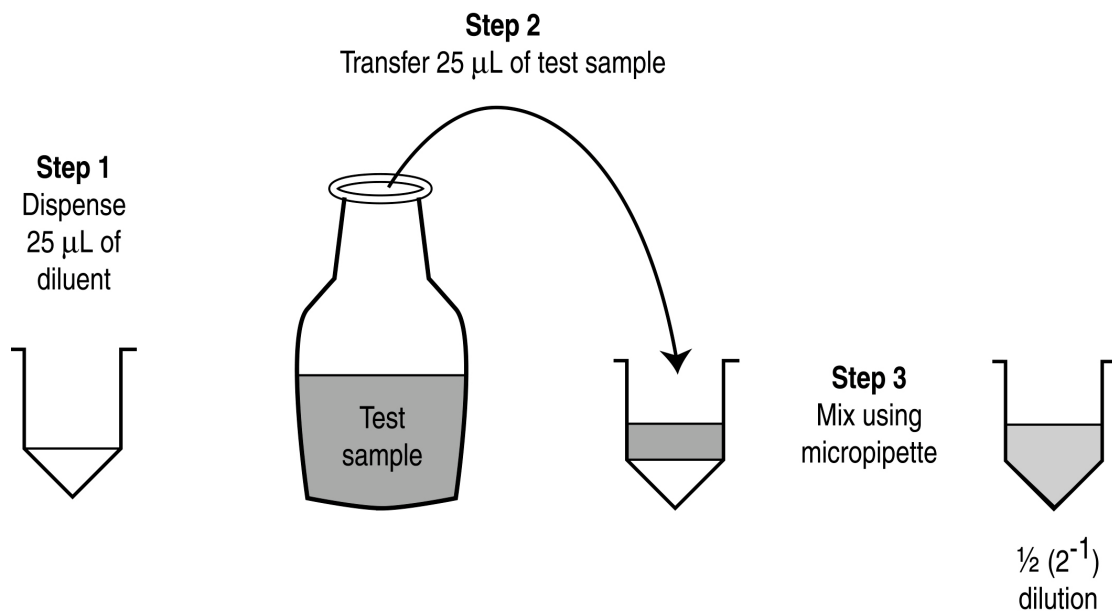
## Appendix 4

### Two-fold serial dilutions

A two-fold dilution reduces the concentration of a solution by a factor of two that is reduces the original concentration by one half. A series of two-fold dilutions is described as two-fold serial dilutions. In this manual, two-fold serial dilutions are carried out in small volumes in microwell plates. They are used in both the haemagglutination and haemagglutination inhibition tests to establish titres of the test samples.

#### A two-fold dilution

- Step 1. Use the micropipette to dispense 25  $\mu\text{L}$  of PBS diluent to the first well.
- Step 2. Use the micropipette to transfer 25  $\mu\text{L}$  of the test solution to the first well.
- Step 3. Use the micropipette to mix by drawing up the liquid and expelling it again. Carry out this action twice.
- Step 4. The well now contains 25  $\mu\text{L}$  of the original test solution diluted by one half in a total volume of 50  $\mu\text{L}$ .

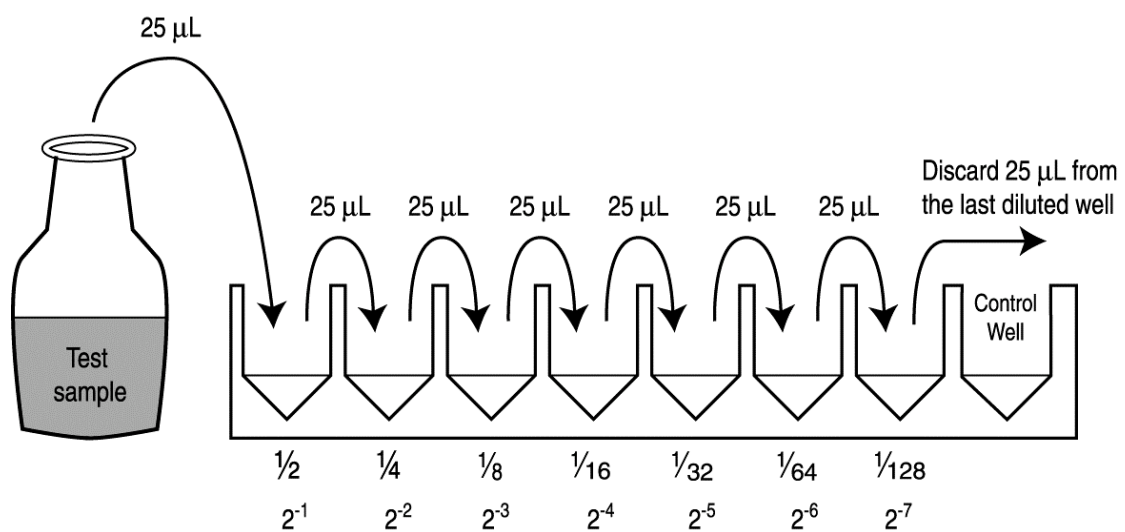


**Figure 24: A two-fold dilution**



## Two-fold serial dilutions

- Step 1. Use the micropipette to dispense 25  $\mu\text{L}$  of the PBS diluent to all the wells across a row of a microwell plate.
- Step 2. Use the micropipette to transfer 25  $\mu\text{L}$  of the test solution to the first well and mix. This is the first two-fold dilution.
- Step 3. Use the micropipette with the same tip to carry out a second two-fold dilution.
- Step 4. Continue the series of two-fold dilutions until the second last well of the microwell plate. The last well is used as a control well in the haemagglutination and haemagglutination inhibition tests.



**Figure 25: Two-fold serial dilutions**

## Appendix 5

### Ten-fold serial dilutions

A ten-fold dilution reduces the concentration of a solution or a suspension of virus by a factor of ten that is to one-tenth the original concentration. A series of ten-fold dilutions is described as ten-fold serial dilutions. In this manual, ten-fold serial dilutions are used in titrations of a suspension of Newcastle disease virus to establish the infectivity titre. They are carried out in small sterile test tubes. These tubes are usually made of glass and it is preferable if they have fitted lids to minimize the risk of contamination during the dilution.

#### A ten-fold dilution

- Step 1. Use a micropipette to dispense 900  $\mu\text{L}$  of the diluent to glass tube.
- Step 2. Use a micropipette to transfer 100  $\mu\text{L}$  of the test solution to the first well. Discard the tip.
- Step 3. Mix by shaking by hand or using a vortex mixer.
- Step 4. The well now contains 100  $\mu\text{L}$  of the original test solution diluted by one tenth in a total volume of 1000  $\mu\text{L}$ .

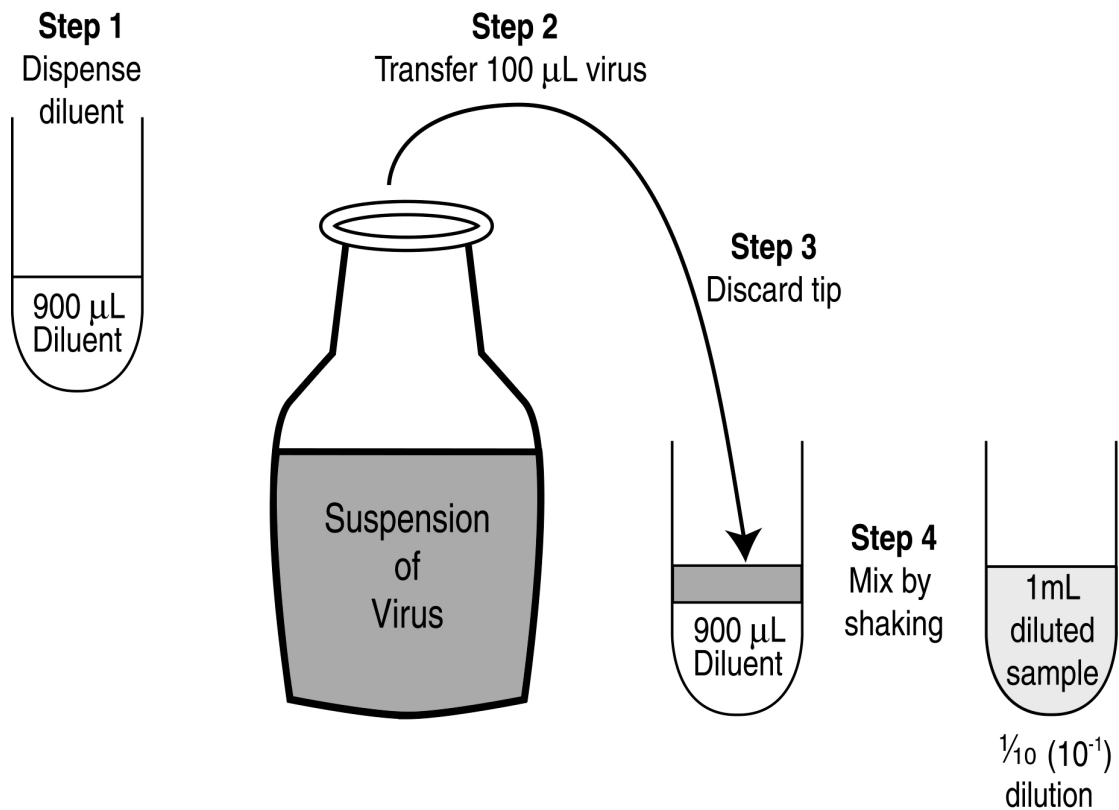
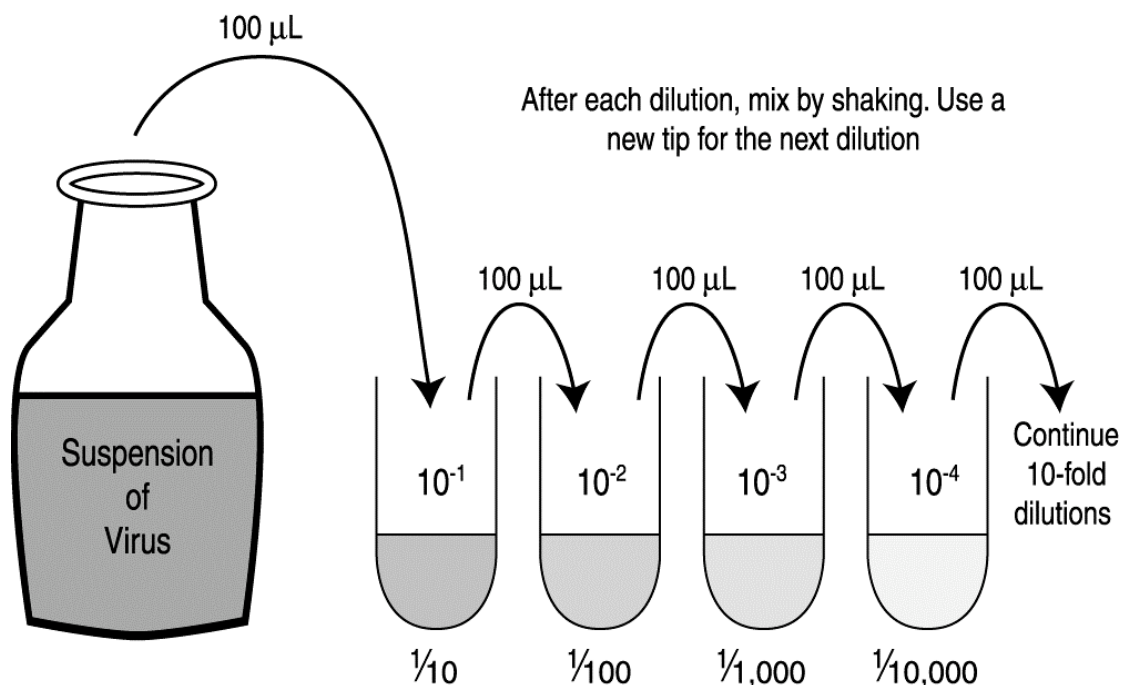


Figure 26: A ten-fold dilution

## Ten-fold serial dilutions

- Step 1. Set up the sterilized glass test tubes in a rack. Label each tube clearly to indicate the dilution of its contents after the ten-fold serial dilution has been carried out.
- Step 2. Use a micropipette to dispense 900  $\mu\text{L}$  of the diluent to all the labeled sterile tubes.
- Step 3. Use a micropipette to transfer 100  $\mu\text{L}$  of the test solution to the first tube and mix. This is the first ten-fold dilution.
- Step 4. Use a micropipette with new sterile tip to carry out a second ten-fold dilution.
- Step 5. Continue the series of ten-fold dilutions until the last tube.



**Figure 27: Ten-fold serial dilutions**

## Appendix 6

### Calculation of arithmetic and geometric means

There are several methods for measuring the central tendency of a set of numbers.

One method is to calculate the **arithmetic mean**. To do this, add up all the values and divide the sum by the number of values. For example, if there are a set of “n” numbers, add the numbers together for example:  $a + b + c + d$  and so on. Then divide the sum by “n”.

One problem with the arithmetic mean is that its value will be influenced disproportionately by a single extreme value.

Another method is to calculate the **geometric mean**. To do this, multiply the values together and then, if there were “n” numbers, take the “n<sup>th</sup>” root. Single extreme values then have less influence.

This method is particularly useful when results are recorded in logarithmic notation. To multiply you only have to add the log indices. To approximate the geometric mean, you take the arithmetic mean of the log indices.

#### Worked Example

You have recorded the following set of values in a serological test. To calculate the arithmetic mean, you must transform these to real numbers.

$$\begin{aligned}2^3 &= 8 \\2^4 &= 16 \\2^4 &= 16 \\2^6 &= 64\end{aligned}$$

$$\text{Calculation of arithmetic mean} = \frac{8 + 16 + 16 + 64}{4} = \frac{104}{4} = 26$$

$$\text{Calculation of the geometric mean} = \sqrt[4]{(8 \times 16 \times 16 \times 64)} = \sqrt[4]{(131072)} = 19$$

Calculation of the geometric mean using the log indices =

$$\text{Sum of the indices} = \frac{3 + 4 + 4 + 6}{4} = \frac{17}{4} = 4.25$$

$$\text{The geometric mean is then} = 2^{4.25} = 19.7$$

## Appendix 7

### Dilutions

There are situations when practicing the methods in this manual when you will be required to prepare a final volume of a diluted solution from a concentrated stock solution. This will require calculating the volume of the stock solution and the volume of diluent to prepare the diluted solution. These volumes can be calculated from the ratio of the concentration of the stock solution and the concentration of the diluted solution.

A useful formula for calculating dilution volumes is  $C_1V_1 = C_2V_2$

$C_1$  = Concentration of stock solution

$V_1$  = Volume of stock solution

$C_2$  = Concentration of diluted solution

$V_2$  = Volume of diluted solution

Make sure you recognize the units involved.

For  $C_1$  and  $C_2$  the units of concentration (amount/volume) must be the same.

For  $V_1$  and  $V_2$  the units of volume must be the same.

This formula can be applied to all situations requiring dilution of a stock solution.

#### Worked example

This example is taken from a recent I-2 Newcastle disease vaccine trial carried out at the John Francis Virology Laboratory.

Aliquots of I-2 ND vaccine stock were stored at  $-70^{\circ}\text{C}$ . One aliquot had been removed and titrated and had an Infectivity Titre of  $10^{9.3}$  EID<sub>50</sub> per mL.

On the day of vaccination, a second aliquot of the vaccine stock was removed and thawed to prepare diluted vaccine. Calculate the volume of vaccine stock and diluent required to prepare 5 mL of diluted vaccine to give  $10^6$  EID<sub>50</sub> in a volume of 30  $\mu\text{L}$  (0.03 mL) to be delivered by eye-drop.

$$C_1 = 10^{9.3} \text{ EID}_{50} / \text{mL}$$

$$V_1 = ?$$

$$C_2 = 10^6 \text{ EID}_{50} / 0.03 \text{ mL}$$

$$V_2 = 5 \text{ mL}$$

$$C_1V_1 = C_2V_2 \quad V_1 = (C_2V_2) \div C_1$$

$$V_1 = (10^6 \text{ EID}_{50} / 0.03 \text{ mL} \times 5 \text{ mL}) \div 10^{9.3} \text{ EID}_{50} / \text{mL}$$
$$= 0.084 \text{ mL} = 84 \mu\text{L}$$

To prepare the diluted vaccine, measure 5 mL of diluent. Use a micropipette to remove 84  $\mu\text{L}$  of diluent and discard. Add 84  $\mu\text{L}$  of undiluted vaccine to the diluent. Mix well.

## Appendix 8

### Notes on centrifugation

#### Sedimentation

Particles in a suspension will settle in the bottom of a vessel over time. This is due to gravity, which is the effect of the earth's gravitational field. This force is expressed as ***g***. Centrifugation increases the rate of sedimentation by spinning the samples and creating a centrifugal force that acts on the particles.

#### Revolutions per minute (RPM)

The speed of rotation in a centrifuge is expressed as revolutions per minute.

#### Radius of rotation (*r*)

The distance from the centre of rotation to the position in the centrifuge tube of the material being spun is the radius of rotation measured in centimeters. In examples of centrifugation used in this manual, this is the distance from the centre of the centrifuge to the bottom of the tube.

#### Relative centrifugal field (RCF)

Relative centrifugal force is the force acting on samples during centrifugation. It is expressed as multiples of the earth's gravitational field (***g***).

For example 500 ***g*** = 500 RCF

#### Relationship between RCF, RPM and *r*

RCF, RPM and *r* are linked by the equation for calculating RCF.

**$RCF = 11.2 \times r \text{ (RPM/1000)}^2$**  or  **$RCF = 1.12 \times 10^{-5} \text{ (RPM)}^2$** . This equation can be rearranged to calculate RPM from a given RCF.

$$RPM = 1000 \sqrt{(RCF/11.2 \times r)}$$

#### Summary

In this manual, instructions for centrifugation are given as spinning at a given RCF (***g***) for a certain length of time. This value can then be used to calculate the correct RPM for the centrifuge available. A Table shows RCF values rounded to the nearest 10 for a typical bench top centrifuge with a radius of 18.6 cm.

**Table 9: Relationships between RCF (*g*) and RPM for centrifuge with radius (*r*) = 18.6 cm**

RCF <i>g</i>	RPM
500	1 500
850	2 000

## **Appendix 9**

### **Notes on the anatomy of the embryonated egg**

#### **Shell and shell membrane**

The membrane is closely attached to the shell. Together they function as an exchange system and gaseous and liquid molecules pass in both directions. This is why eggs must be incubated in humid conditions. Eggs incubated in low humidity will lose moisture and eventually the embryo will die.

#### **Air sac**

Eggs have a rounded and a pointed end. The air sac is the space at the rounded end and has a function in respiration and pressure adjustments.

#### **Chorioallantoic membrane and allantoic cavity**

The membrane is attached to the embryo and functions to remove soluble, insoluble and gaseous waste products. As the embryo develops, the sac increases in size. The sac contains allantoic fluid into which Newcastle disease virus is shed after inoculation of the allantoic cavity. The fluid is harvested for vaccine production.

Some viruses are propagated by inoculation of the chorioallantoic membrane. This involves placing inoculum on the membrane.

#### **Yolk sac**

This is also attached to the embryo and contains the nutrient-rich yolk. As the embryo develops, the yolk sac decreases in size to approximately 1 cm diameter 3 days before the embryo hatches.

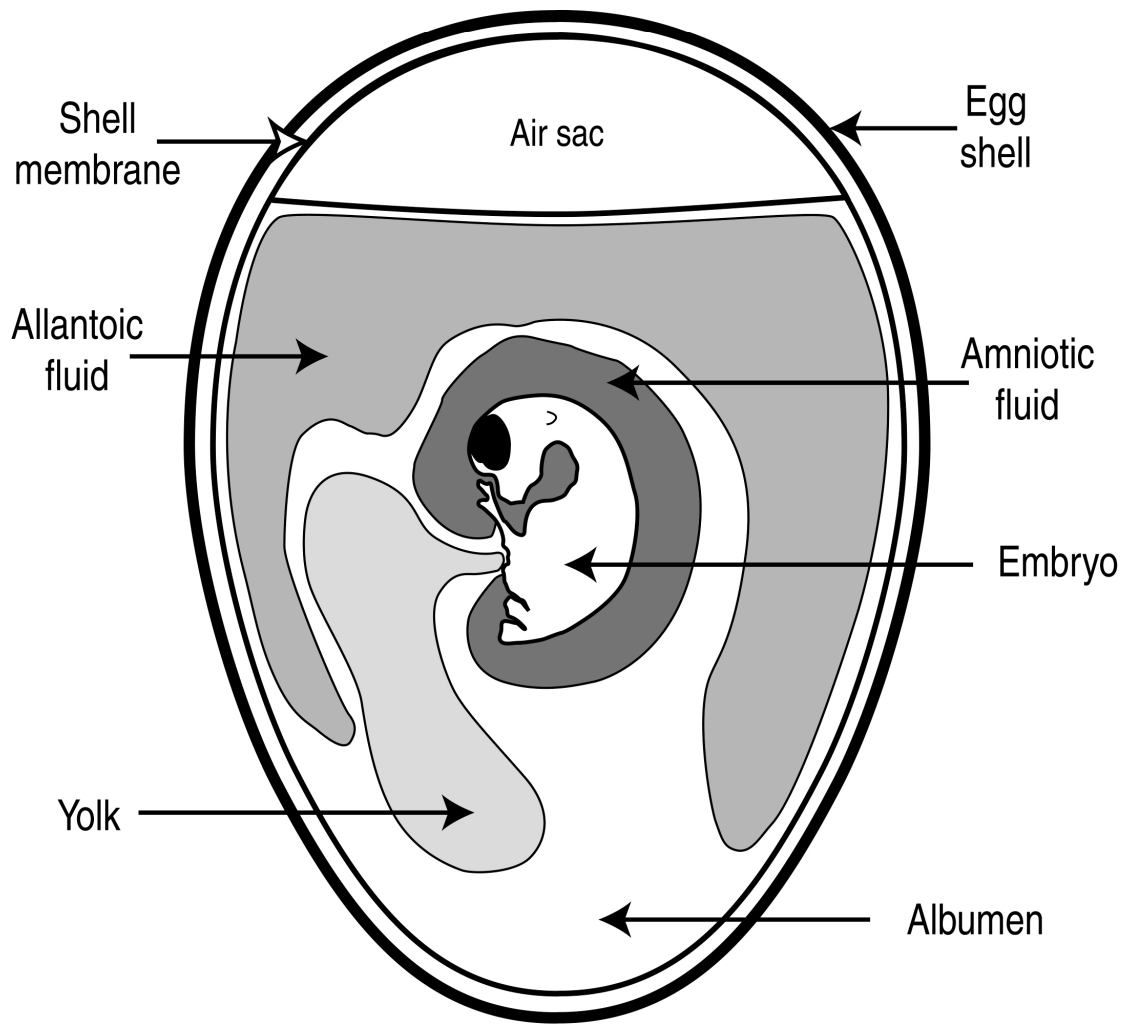
#### **Amniotic Sac**

This sac surrounds the embryo. It is filled with liquid and serves to protect the embryo against physical damage as well as functioning as an area of exchange of molecules. As the embryo develops, the membrane stretches and the amniotic sac is barely visible in the fully developed embryo.

#### **Albumen**

The egg white, which consists mainly of protein.

To examine the structure of embryonated eggs at various stages of development, remove the entire contents of the egg into a Petri dish.



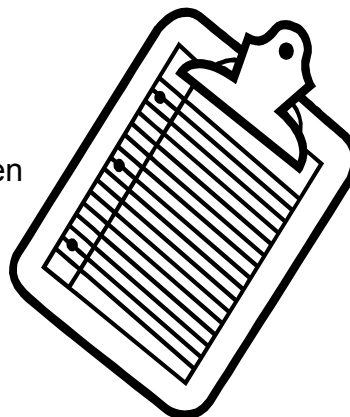
**Figure 28: The anatomy of a ten-day old embryonated egg**



## Appendix 10

### Calculation and recording sheets

Two calculation and recording sheets have been prepared and included in **Appendix 10**. They can then be photocopied or used as templates for handwritten sheets or day book entries. Examples of completed recording sheets are also included



#### Reed Muench calculation sheet

This sheet has been designed to assist with the calculation of the 50 percent endpoint using the Reed Muench method. An example of a completed sheet has also been included. See **Section 12** for details about using the Reed Muench method for calculating the infectivity titre.

#### Microwell plate recording sheet

A recording sheet has also been prepared to assist with recording results of the HA and HI test carried out in microwell plates. Two examples of completed sheets are included.

**Example 1:** This completed recording sheet shows the results of the first part of the HI test, the titration of the Newcastle disease virus antigen, followed by testing of antigen diluted to give 4 HA units per 25  $\mu$ L volume.

**Example 2:** This completed recording sheet shows the results of 8 serum samples tested for Newcastle disease virus antibodies using the HI test.

See **Section 11** Serology for more details of the HI assay.

**Note:** A summary of the results on the loose recording sheets should be entered in the daybook.

For example, record in the day book:

- Results of HA test
- Calculated infectivity titre in EID<sub>50</sub> /mL.
- Dilution used in the HI test
- HI titres of all the samples tested.

The sheets should then be stored in a file and available for future reference.

Name: .....  
 Date: .....  
 Experiment:.....

### Calculation of EID<sub>50</sub> /mL of virus suspension using Reed and Muench mathematical technique

Dilution of Inoculum Include range of dilutions from 100% infected (all HA +ve) to 0% infected (all HA -ve)	Number of eggs infected (HA +ve)	Number of eggs not infected (HA -ve)	Accumulated numbers			Percentage infected
			Infected (A)	Not infected (B)	Total (A+B)	A/(A+B) x 100
	↑	↓				
	↑	↓				
	↑	↓				
	↑	↓				
	↑	↓				

**Determine the dilution of inoculum producing 50 percent infection of eggs.**

If necessary, use the Reed Muench formula to calculate the index:

$$\text{Index} = \frac{\% \text{ infected at dilution immediately above } 50\% - 50\%}{\% \text{ infected at dilution immediately above } 50\% - \% \text{ infected at dilution immediately below } 50\%} =$$

Apply the index calculated using this formula to the dilution that produced the infection rate immediately above 50 percent =

This dilution of the virus suspension contained one EID<sub>50</sub> unit of virus in 0.1mL.

1mL of the virus suspension will contain ten times the reciprocal of the calculated dilution.

Therefore Infectivity Titre of virus suspension/mL = .....

### Example of completed sheet

Name: John Smith

calculation of EID<sub>50</sub> /mL of virus suspension using

Date: 6/6/01

Reed and Muench mathematical technique

Experiment: Titration of I-2 wet vaccine

Dilution of Inoculum Include range of dilutions from 100% infected (all HA +ve) to 0% infected (all HA -ve)	Number of eggs infected (HA +ve)	Number of eggs not infected (HA -ve)	Accumulated numbers			Percentage infected
			Infected (A)	Not infected (B)	Total (A+B)	A/(A+B) x 100
10 <sup>-6</sup>	5    ↑	0    ↓	11	0	11	11/11 = 100%
*10 <sup>-7</sup>	4    ↑	1    ↓	6	1	7	6/7 = 86%
10 <sup>-8</sup>	1    ↑	4    ↓	2	5	7	2/7 = 29%
10 <sup>-9</sup>	1    ↑	4    ↓	1	9	10	1/10 = 10%
10 <sup>-10</sup>	0    ↑	5    ↓	0	14	14	0/14 = 0%

**Determine the dilution of inoculum producing 50 percent infection of eggs.**

If necessary, use the Reed Muench formula to calculate the index:

$$\text{Index} = \frac{(\% \text{ infected at dilution immediately above } 50\%) - 50\%}{(\% \text{ infected at dilution immediately above } 50\%) - (\% \text{ infected at dilution immediately below } 50\%)} = \frac{86 - 50}{86 - 29} = \frac{36}{57} = 0.6$$

Apply the index calculated using this formula to the dilution that produced the infection rate immediately above 50 percent  
 $= 10^{-7.6}$

(Mark dilution with \*)

This dilution of the virus suspension contained one EID<sub>50</sub> unit of virus in 0.1 mL.

1mL of the virus suspension will contain ten times the reciprocal of the calculated dilution.

Therefore Infectivity Titre of virus suspension in EID<sub>50</sub>/mL =  $10 \times 10^{7.6} = 10^{8.6}$  EID<sub>50</sub>/mL

## Example 1: Completed sheet used in HA test

### MICROWELL PLATE RECORDING SHEET FOR HI/HA TESTS

Plate No.....1..... Virus.....Newcastle disease  
 Name .....Jane Smith.....  
 RBC .....1%.....  
 Date:.....30.5.01.....  
 Samples  
 Titration of I-2 wet vaccine stored at 4°C

Notes  
 RBC added at 1200  
 D = dead egg, not tested  
**X** = Haemagglutination  
 • = No haemagglutination

Sample A 5.4.01																	Sample B 3.5.01				
		1	2	3	4	5	6	7	8	9	10	11	12								
A 10 <sup>-10</sup>	A	•	•	•	•	•			•	•	•	•	•	A	B 10 <sup>-10</sup>						
A 10 <sup>-9</sup>	B	•	•	<b>X</b>	•	•			•	•	•	•	•	B	B 10 <sup>-9</sup>						
A 10 <sup>-8</sup>	C	<b>X</b>	•	•	•	•			•	<b>X</b>	<b>X</b>	•	•	C	B 10 <sup>-8</sup>						
A 10 <sup>-7</sup>	D	•	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>			<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>D</b>	D	B 10 <sup>-7</sup>						
A 10 <sup>-6</sup>	E	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>			<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	E	B 10 <sup>-6</sup>						
A 10 <sup>-5</sup>	F	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>			<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	<b>X</b>	F	B 10 <sup>-5</sup>						
RBC control	G	•	•											G							
	H													H							
		1	2	3	4	5	6	7	8	9	10	11	12								

**Note:** One plate has been used for testing allantoic fluid from two sets of eggs.  
 Set A inoculated with titrated sample from 5.4.01 and Set B inoculated with titrated sample from 3.5.01.

## Example 2: Completed sheet used in HI test

### MICROWELL PLATE RECORDING SHEET FOR HI/HA TESTS

Plate No.....1.....

Name .....John Smith

.....

Date:....30.5.01.....

Virus.....Newcastle disease

RBC .....1%.....

Samples

Titration of antigen for HI test

Testing of 4HA units of antigen

HI test of standard positive and negative serum using 4HA units of Ag

Notes

Ag = antigen

Rows A and B RBC added at 1000

Ag diluted 1 in 64 with PBS

Rows E to H, Ag added 1130

Rows C to H , RBC added 1200

**X** = Haemagglutination

• = No Haemagglutination

		1	2	3	4	5	6	7	8	9	10	11	12		
ND Ag	A	X	X	X	X	X	X	X	X	•	•	•	•	A	HA titre = $2^8 = 256$ HA units
ND Ag	B	X	X	X	X	X	X	X	X	•	•	•	•	B	HA titre = $2^8 = 256$ HA units
1/64 dilution of Ag	C	X	X	•	•	•	•	•	•	•	•	•	•	C	HA titre = $2^2 = 4$ HA units
1/64 dilution of Ag	D	X	X	•	•	•	•	•	•	•	•	•	•	D	HA titre = $2^2 = 4$ HA units
Positive serum	E	•	•	•	•	•	•	X	X	X	X	X	•	E	HI titre = $2^6$
Positive serum	F	•	•	•	•	•	•	X	X	X	X	X	•	F	HI titre = $2^6$
Negative serum	G	X	X	X	X	X	X	X	X	X	X	X	•	G	HI titre = $2^0$
Negative serum	H	X	X	X	X	X	X	X	X	X	X	X	•	H	HI titre = $2^0$
		1	2	3	4	5	6	7	8	9	10	11	12		

### Example 3: Completed sheet used in HI test

#### MICROWELL PLATE RECORDING SHEET FOR HI/HA TESTS

Plate No.....1..... Virus.....Newcastle disease.....

Name ....Jane Smith..... RBC.....1%.....

.....  
 Samples..... Dose Response

Date: .....11.5.01..... Trial Serum samples collected 30.1.01.....

Notes.  
 Antigen added 1300  
 RBC added 1330  
 .....  
**X** = Haemagglutination (HA)  
**•** = Haemagglutination inhibition (HI)

Sample numbers		1	2	3	4	5	6	7	8	9	10	11	12	HI titres log base 2	
20	A	•	•	•	•	X	X	X	X	X	X	X	•	A	4
25	B	•	•	X	X	X	X	X	X	X	X	X	•	B	2
1	C	•	X	X	X	X	X	X	X	X	X	X	•	C	1
27	D	•	•	X	X	X	X	X	X	X	X	X	•	D	2
36	E	•	•	•	X	X	X	X	X	X	X	X	•	E	3
42	F	•	•	•	X	X	X	X	X	X	X	X	•	F	3
6	G	X	X	X	X	X	X	X	X	X	X	X	•	G	0
55	H	•	•	•	•	•	X	X	X	X	X	X	•	H	5
		1	2	3	4	5	6	7	8	9	10	11	12		

## MICROWELL PLATE RECORDING SHEET FOR HI/HA TESTS

Plate No..... Virus..... Notes .....  
 Name ..... RBC.....  
 ..... Samples.....  
 Date:.....  
 .....  
 .....

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