Investigations with *ELISA*

using the SAPS *ELISA* kit for *Botrytis*

Student Guide

Science &Plants for Schools

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Contents

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Note on units

The standard unit used by scientists for measuring a very small volume of solution is the microlitre (µl). For consistency, and to avoid confusing users of this kit, we have therefore used microlitres (µl) and millilitres (ml) in this publication.
1000 µl = 1 ml (= 1 cm^3)
1000 ml = 1 litre (= 1 dm^3)

Safety

Students, teachers and technicians in schools and colleges using the kit are advised to read the Safety Notice on page 5 and to ensure that the employer's risk assessment has been carried out before attempting any practical work. Further safety guidance is given in the *Technical Guide*.

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INTRODUCTION

This booklet is part of the SAPS *ELISA* kit for *Botrytis*. The kit will allow you to become familiar with the practical procedures associated with *ELISA* techniques, and then to use these in further investigations or projects that you can carry out yourself. This *Student Guide* provides a detailed practical protocol for using the materials and equipment in the kit. It also gives you relevant background information to help you understand the theoretical aspects of the topic and some suggestions for further reading. The *Technical Guide* is a separate booklet, intended for teachers and technicians. It provides full details for preparing for the practical sessions and further information about resources relevant to the techniques described.

The ***E***nzyme-***L***inked ***I***mmuno***S***orbent ***A***ssay (*ELISA*) is an immunological technique commonly used in medicine and in scientific research. It uses monoclonal or polyclonal antibodies to detect specific molecules in, for example, extracts from plant or animal tissues. *ELISA*s can also be used to make quantitative estimates of the particular molecule detected. The technique is extremely sensitive and can be made highly specific, so it can be used to detect very small quantities of specific organisms or molecules.

Immunoassays utilise the exquisite specificity and sensitivity of mammalian immune systems. When, for example, a foreign organism, protein, glycoprotein, lipopolysaccharide or complex carbohydrate, is injected into an animal, its immune system responds by making antibodies (Ab). These antibodies specifically recognise and bind to particular sites on the foreign molecule, which is known as the antigen (Ag).

The *ELISA* system depends on the discovery that proteins and carbohydrates, including antibodies and many antigens, bind to polystyrene or clear plastic. This allows a series of reactions to take place, in sequence, on an immobilised surface. When the reactions are positive, a ladder like series of molecules builds up. The formation of such a series can be detected by using an enzyme conjugated to the final antibody. When the enzyme is incubated with an appropriate (colourless) substrate, it gives a coloured product. The intensity of the colour produced is proportional to the concentration of the target antigen present in the original sample, thus allowing quantitative estimates to be made. This sequence of events is summarised in Fig. 1.

Some everyday applications will let you see how and where *ELISA* techniques are used as a diagnostic tool – an aid in detective work – and why it is useful to have some understanding of the processes involved. *ELISA* techniques are, for example, used to detect minute quantities of trace components in food – this can be important for people with a food allergy, say to peanuts. *ELISA* techniques are used extensively to test for the presence of microbes causing infectious diseases – in humans, for example, for HIV, rubella and herpes. In plants *ELISA*s can detect fungal diseases (such as *Botrytis* as used in this kit) or be used to

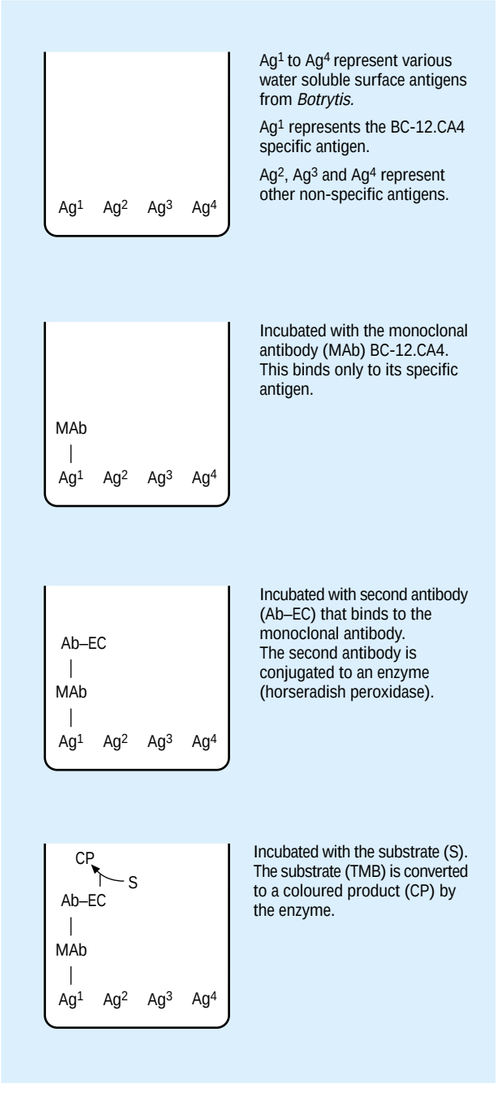


Fig 1 Sequence of events at the surface of the wells at a molecular level. Note how the different antigens are held on the surface, but only the correct (specific) antigen links with the first (monoclonal) antibody. This in turn links with the second antibody (conjugated to an enzyme) which is visualised when the enzyme reacts with the substrate. This illustrates the system used to detect *Botrytis* in the SAPS *ELISA* kit.

detect viral diseases in vegetatively propagated plants, such as potatoes. In humans, *ELISA*s can be used to detect the presence of drugs, or a metabolite derived from them, or determine hormone levels in the blood as a step in the diagnosis of a particular condition a person may be suffering from. The detection of the hormone HCG in urine is the basis of pregnancy test kits that can be bought from chemists. *ELISA*s can also be used to detect genes – or more strictly the proteins they code for – which have been introduced by genetic modification in transgenic organisms.

The SAPS *ELISA* kit uses a monoclonal antibody (BC-12.CA4) to detect the fungus *Botrytis*. This monoclonal antibody can detect any species of *Botrytis*, including *B. cinerea*. This common plant pathogen attacks a wide range of plant material including strawberries, raspberries, grapes, vegetables and flowers. The kit uses a plate-trapped antigen *ELISA* system which has been adapted for use in schools and colleges. In developing this kit, consideration has been given to safety and to the need to obtain meaningful results within a short time scale, say within a 2 hour practical session. Included in the protocol is an investigation which detects *Botrytis* infection in raspberries. Frozen raspberries can be used, which means this investigation could be carried out at any time of year.

The use of a plant pathogen in the kit avoids most of the restrictions associated with work with human pathogens. In teaching, the principles explored give the necessary support for students to gain an understanding of topics relating to monoclonal antibodies in human health and disease as well as its application to an understanding of plant diseases. The kit should help to illustrate the important uses of monoclonal antibodies in diagnostics in plant disease, medicine and veterinary medicine.

The SAPS *ELISA* kit is appropriate for use with post-16 students, including those on AS and A level courses, Scottish Higher Still courses and equivalent vocational courses. It could also be useful to illustrate diagnostic immunological techniques for students in further and higher education.

Before using the kit or carrying out any practical work, students, teachers and technicians in schools and colleges are advised to read the Safety Notice on page 5 and to ensure that the employer's risk assessment has been carried out. Further safety guidance is given in the *Technical Guide*.

Fig 2 Emptying the microwells as used in the *ELISA* technique (*copyright Erica Larkcom*)

STUDENT WORKSHEET

Using *ELISA* – and detecting *Botrytis* infection in raspberries

This worksheet takes you through the practical procedures of the *ELISA* technique and shows how you can use it to detect *Botrytis* infection in raspberries.

You are provided with the items listed. Check through the list and read through the whole worksheet before starting to do any practical work.

Look at the diagrams to make sure you are familiar with all the equipment. You should also read the safety notice and carry out a risk assessment with your teacher before attempting any practical work. Then use the flow chart and the numbered points to take you through the steps of the practical work.

There is sufficient material in the kit for 5 groups of students. For the raspberry investigation, some of these groups are provided with a sample of raspberries that has been inoculated with *Botrytis cinerea*, 2 to 4 days before the experiment. The other raspberry sample has not been inoculated, but is of similar age and has been kept under similar conditions to the inoculated raspberries. The other groups of students are provided with this uninoculated sample. Both samples use frozen raspberries that were defrosted 2 days before the experiment.

SAFETY NOTICE

- All work must be supervised
- Observe good laboratory practice
- Wipe up spills. Wash skin if splashed.
- If you are asthmatic, wear a face mask because fungal spores may be released into the air during the experiment
- If you have sensitive skin, wear gloves throughout the procedure

Take care not to contaminate reagents – use a clean pipette or Pastette for each solution. It may be useful to label these to prevent cross contamination.

You must take particular care with the TMB (tetramethylbenzidine) liquid substrate solution which contains TMB (harmful), methanol (toxic) and dimethyl sulphoxide (irritant). You should therefore avoid contact with skin and eyes.

You may dispose of very small volumes of the TMB liquid by washing it down the sink with excess water.

Materials required per group

▶ supplied from the kit

4 small disposable plastic tubes – labelled as follows
- PBS (phosphate buffered saline), contains 5 ml of PBS
- *Botrytis* filtrate (surface washings)
- fruit (such as raspberry)
- fruit filtrate (fruit extract)

1 large disposable plastic tube
- contains 30 ml PBST (phosphate buffered saline with *Tween* – see note 1)

2 x 1 ml pipettes
5 Pastettes (deliver 20 µl* droplets)
4 microwells (4 in a strip, to be designated 1, 2, 3, 4) – described also as 'wells'
2 microcups – labelled as follows
- MAb (monoclonal antibody), contains 0.5 ml MAb
- Ab-EC (second antibody-enzyme conjugate), contains 0.5 ml Ab-EC

2 muslin squares
colour chart

▶ supplied by the school / college

1 pair of forceps
1 glass rod
paper towel
disposable gloves
marker pen
raspberries (see below)
1 container for waste (e.g. 500 ml beaker)

Materials to be shared

cultures of the fungus *Botrytis cinerea* (in tubes on agar slopes)
3 raspberries, uninoculated
3 raspberries, inoculated with *Botrytis*
1 bottle of TMB (tetramethyl benzidine) – substrate for the enzyme which is linked to the second antibody (Ab-EC). (See note 2 and safety notice)

Notes

1 *Tween* is a detergent. Its presence reduces the likelihood of non-specific binding of antibodies and antigens to each other and to the plastic surface of the microwells.
2 The enzyme in Ab-EC converts colourless TMB to a blue colour.

* There are 1000 µl in one millilitre (1 ml = 1 cm^3). See Note on units on page 2.

Practical procedures

You will set up your tests in the 4 microwells, designated 1, 2, 3, 4, as follows.

1 = PBS only (acts as control)
2 = antigen from the known *Botrytis* culture
3 = sample from uninoculated raspberry
4 = sample from inoculated raspberry

Each 'group' is provided with *either* an inoculated *or* an uninoculated sample.

Washing the antigen from the surface of the fungus

1 ● Use a 1 ml (1 cm^3*) pipette to transfer 2 ml of PBS into the tube containing the culture of the fungus.
● Use the tip of your plastic pipette to rub the surface of the culture gently.

2 ● Use the same pipette to draw off approximately 1 ml of the fungal wash.
(This now contains the fungal antigen.)
● Filter this solution through muslin into the tube labelled '*Botrytis* filtrate' (surface washings). You may find it helpful to moisten the muslin with water and use an elastic band to hold it in the tube.
DISCARD USED PIPETTES.

Preparing the fruit samples for diagnosis of *Botrytis*

3 ● Use clean forceps to transfer a small sample of the fruit, e.g. half a raspberry, into the tube labelled 'fruit'.

4 ● Use a 1 ml pipette to transfer 1 ml of PBS into the tube.

5 ● Use a glass rod to gently break up the fruit to form a pulp.

6 ● Filter the pulp through moist muslin into the tube labelled 'fruit filtrate' (fruit extract).
● Label this tube 'uninoculated' or 'inoculated' as appropriate.

Coating the wells

7 ● Label the microwells (wells) 1, 2, 3 and 4 with a marker pen.

8 ● Use a clean pastette to transfer 4 drops (80 µl) of PBS into well 1.
● Use the same pastette to transfer 4 drops of liquid from the tube labelled *Botrytis* filtrate (step 2), into well 2.

9 ● Sharing the fruit filtrate with a neighbouring group – transfer (from step 6)
– 4 drops of liquid from the uninoculated fruit filtrate into well 3
– 4 drops of liquid from the inoculated fruit filtrate into well 4
Use a fresh pastette for each sample.
DISCARD USED PASTETTES AND OTHER APPARATUS INTO THE WASTE CONTAINER PROVIDED.
Leave for at least 10 minutes

10 ● Empty all 4 wells by inverting them above the waste container or sink.
● Remove the last drops by tapping the wells upside down on a pad of paper towel.
● Wash all wells thoroughly (3 times) with PBST.
For each washing use a 1 ml pipette to fill the wells with PBST. Empty each well, then fill again with PBST. Repeat 3 times, removing all buffer each time.
Wells may be tapped upside down on a pad of the paper towel to remove remaining droplets of liquid.
(Do not worry if there are bubbles at the bottom of the wells – these disappear when the next reagent is added.)
After the last wash, it is important to make sure that no liquid remains in the wells.
Keep the 1 ml pipette for dispensing PBST in steps 12 and 14 below.

Adding the monoclonal antibody (MAb) to the wells

11 ● Use a clean Pastette to add 4 drops of the MAb (BC-12.CA4) to each well.
Leave for at least 10 minutes

12 ● Empty all 4 wells by inverting them above the waste container or sink.
Remove the last drops by tapping the wells upside down on a pad of paper towel.
● Wash each well 3 times with PBST (*see step 10*).
After the last wash, it is important to make sure that no liquid remains in the wells.
DISCARD USED PASTETTES.

Adding the antibody-enzyme conjugate (Ab-EC) to the wells

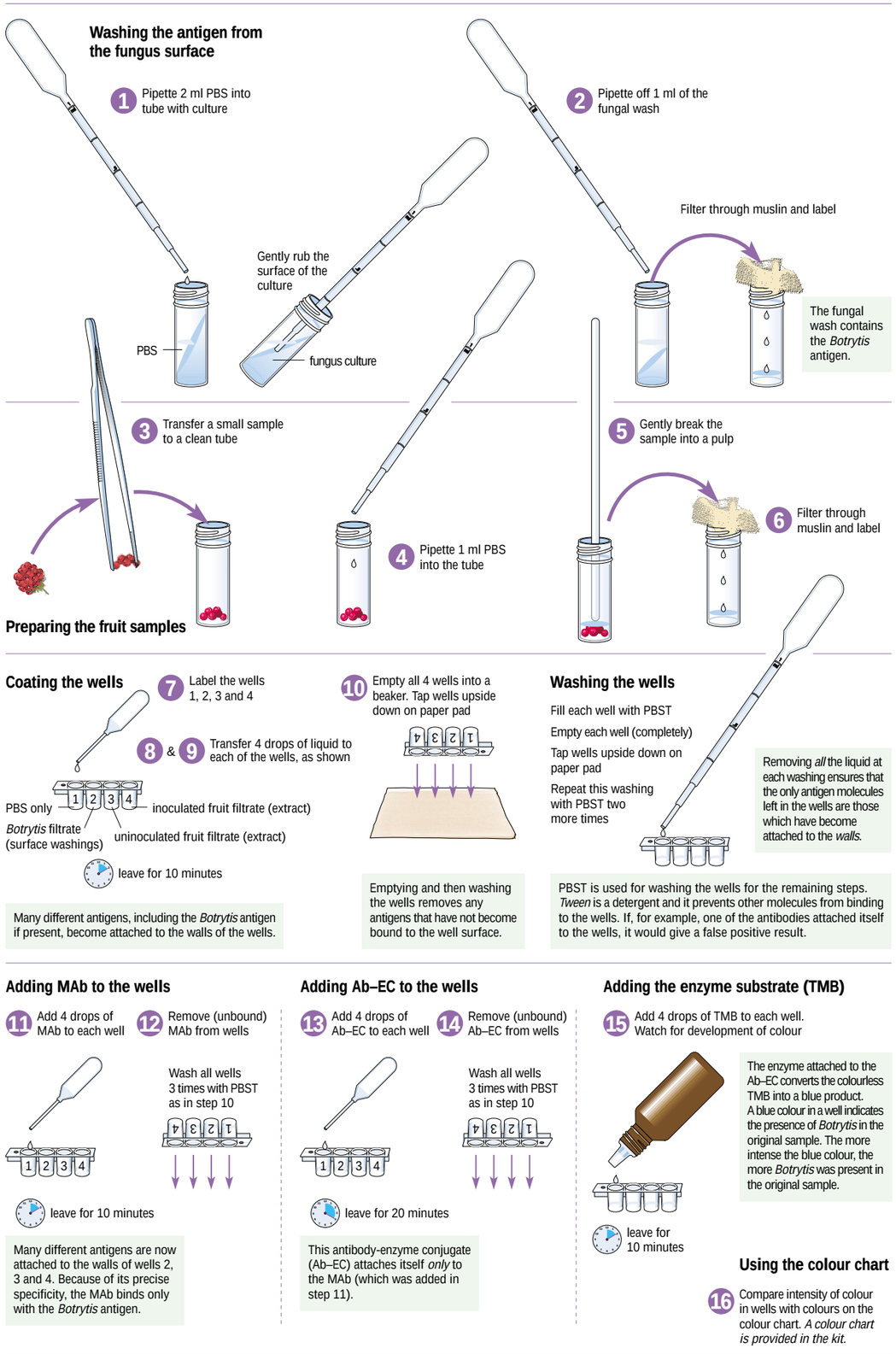
13 ● Use a clean Pastette to add 4 drops of the Ab-EC to each well.
Leave for at least 20 minutes

14 ● Empty all 4 wells by inverting them above the waste container or sink.
Remove the last drops by tapping the wells upside down on a pad of paper towel.
● Wash each well 3 times with PBST (*see step 10*).
After the last wash it is important to make sure that no liquid remains in the wells.
DISCARD USED PASTETTES.

Adding the enzyme substrate (TMB)
***– see SAFETY NOTICE* on page 5**

15 ● Add 4 drops of TMB liquid substrate solution (from the dropper bottle) to each well.
● Wait for the colour to develop.
The colour should be visible within 5 minutes but may take up to 30 minutes to develop fully.

* 1 ml = 1 cm^3. See Note on units on page 2.



Using the colour chart

16 ● After 20 minutes, compare the intensity of colour in the wells with the colours on the colour chart provided to obtain a quantitative estimate of the fungus. The numbers 2 to 128 represent increasing concentration of *Botrytis* antigen.

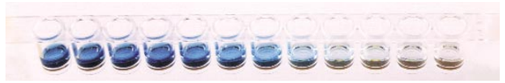


Fig 3 A dilution series prepared to show the range of colours obtained with known concentrations of *Botrytis* (*copyright Oxford University, Department of Plant Sciences*)

Suggestions for further work or other investigations with *ELISA*

When you have become familiar with the basic practical procedures in the *ELISA* protocol, you can extend the method to devise and carry out other investigations using the *ELISA* techniques. Some further suggestions for investigations are given below, and there are more in the *Technical Guide*.

1 Dilution series

You could set up a dilution series, using the surface washings from the fungal culture. This would enable you to show that the intensity of colour obtained in the final step is proportional to the starter concentration of the antigen solution used to coat the wells. You could then check your colours against those in the colour chart supplied.

2 Investigating a range of infected fruit and vegetables for *Botrytis* infection

This could give you ideas for project work. For these investigations, it is recommended that the incubation with the antigen is carried out overnight. But first you should check that the uninfected host tissue does not contain high levels of peroxidase. This is necessary because peroxidases naturally present in the tissues would themselves give a positive colour reaction with the enzyme substrate, TMB. For details of how to do this test, see the *Technical Guide*.

3 Investigating the effect of temperature on the rate of infection by *Botrytis*

Inoculate a suitable fruit and place samples, each in a sealed container, at different temperatures (e.g. in a refrigerator, at room temperature, above a heater or in an incubator). After a suitable time interval, use the *ELISA* kit to monitor the intensity and rate of infection that has developed.

4 Investigating the effect of humidity on the rate of infection by *Botrytis*

Inoculate a suitable fruit and place samples in containers with different humidities. Humidity can, for example, be varied by using silica gel in one container and water in another. After about a week, use the *ELISA* kit to monitor the intensity and rate of infection that has developed.

BACKGROUND INFORMATION

Plant diseases caused by *Botrytis cinerea*

Botrytis cinerea is a pathogenic fungus of plants. It infects a wide range of plant species and plant tissues. It is responsible for significant economic losses in the horticultural industry. The fungus may infect fruits, vegetables and flowers in the field, in the glasshouse, and during transport and in storage. *B. cinerea* is common on tomato stems, strawberries, grapes and cherries. *Botrytis* infection of roses produces clear spots on the petals and sporulating growth on the flower heads. It may also infect stored produce such as cabbages, onions, carrots, apples and pears.

Fig 4 Ripe fruits of red raspberry showing grey mould, typical of infection by *Botrytis cinerea* (*copyright Scottish Crop Research Institute*)

B. cinerea is able to invade living plants, kill the tissues and then continue to live off the dying and dead plant material. It thus has both parasitic and saprotrophic stages in its life cycle. This mode of life is typical of necrotrophic fungi and it means that spores can be produced for a large part of the year. The main infective agents are the air-borne spores (conidia) which can remain viable for long periods. Infection usually takes place during flowering or harvesting, when spores landing on the surface of fruits and flowers may germinate, penetrate the cuticle, and then pass into a dormant phase. Infections may not immediately be evident, but can remain latent or dormant until the fruits ripen or the flowers develop fully. This often occurs during transit to the market place or once items have been purchased and have been taken home. The result in both cases is the deterioration of the product and spoilage.

Fig 5 Harvested fruits of red raspberry affected by *Botrytis cinerea* (*copyright Scottish Crop Research Institute*)

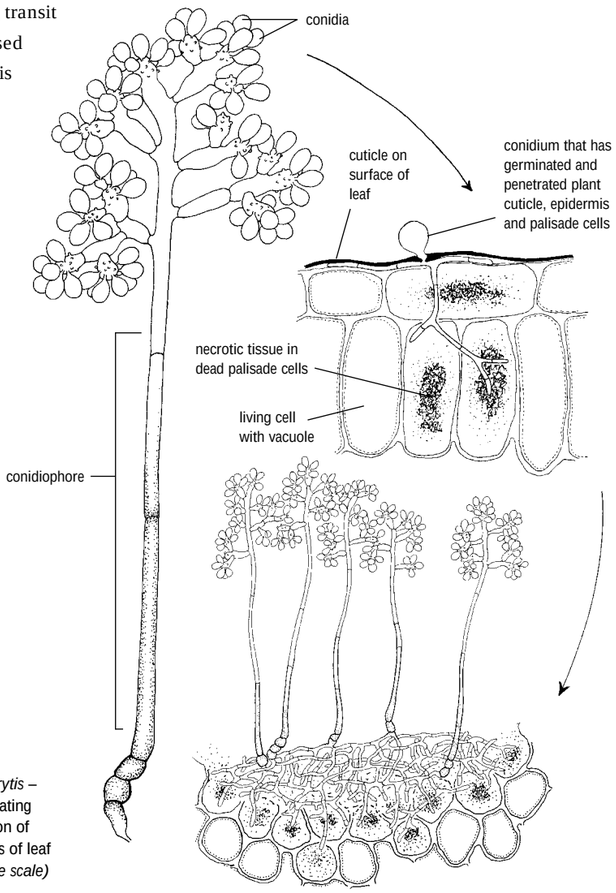


Fig 6 Infection of leaf by *Botrytis* – penetration of leaf by germinating conidium, followed by invasion of fungal hyphae through tissues of leaf *(parts of drawing not to same scale)*

Antibodies and the production of monoclonal antibodies (MAbs)

Antibodies are secreted by B-lymphocytes (a type of white blood cell), circulating in the blood of birds and mammals. They are present in the cell-free component, known as the plasma or antiserum. Antibodies are present also in other body fluids, such as saliva, tears, bronchial secretions, prostate and vaginal secretions and colostrum.

Antibodies are generated as a natural immune response to infection from microorganisms and to foreign molecules or particles (referred to as **antigens**). This response forms the basis of many medical immunisation programmes, in which a low level of a living or inactivated organism is injected into a mammalian host. The immune system is thus triggered to produce antibodies against the organism's antigens.

Antibodies are large globular proteins known as **immunoglobulins** (**Ig**). There are five main classes of antibodies, designated IgA, IgD, IgE, IgG and IgM. Of these, IgG antibodies are the most common and are involved in recognising proteins whereas IgM antibodies, which are the largest, are important in recognising carbohydrates.

All antibodies have the same basic molecular structure but differ considerably in size and properties. The basic unit consists of two long or 'heavy' chains (H) and two short or 'light' chains (L). These chains are held together by disulphide bonds. The diagram (Fig 7) shows how these chains give the antibody a Y-shaped structure. The stem of the 'Y' is known as the **Fc** portion and the amino acid sequence of the **Fc** portion is constant for a particular species of animal. Thus the Fc portion of antibodies raised in mice differs from the Fc portion of antibodies raised in rats or in horses. At the end of each arm of the Y, the antibody unit has two **binding sites**. These binding sites are made up from amino acids at the ends of both the heavy and the light chains. This is known as the 'variable region' because rearrangement of the amino acid sequences in this region can result in different binding sites (potentially up to 10^{10} different sites). It is the variable region which provides the specificity for the antibody to bind to different antigens.

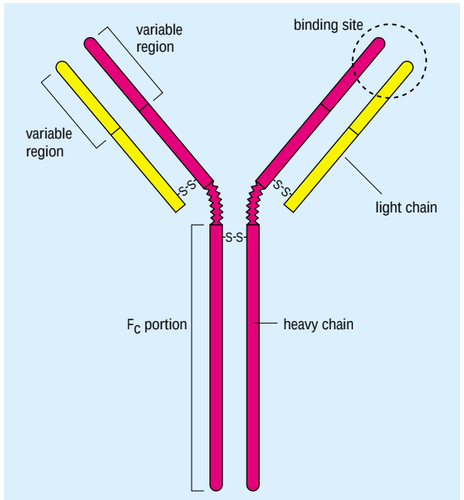


Fig 7 Structure of an antibody molecule

When an animal is injected with a purified antigen, a number of different antibodies are produced. These antibodies recognise different binding sites (**epitopes**) on the same molecule. Some binding sites may be found on other similar or unrelated molecules and in this case the antiserum containing the antibodies is said to be *cross-reactive*. If, however, the binding sites of the antibody recognise only those of the antigen, the antiserum containing the antibodies is then said to be *specific*. It is this type of antiserum that is the most useful for diagnostic purposes. Viruses, for example, because of their molecular simplicity, generally induce a highly specific response. More complex organisms, such as fungi, induce a number of antibodies that are cross-reactive and recognise both related and unrelated species of fungi and host molecules.

If antisera (containing antibodies) are to be useful for diagnostic purposes, they must be taxonomically specific. To obtain such antisera, it is necessary first to identify and purify the taxonomically specific molecules and then use these as the immunogens to raise antisera. Alternatively, we can immunise with a mixture of antigens and raise taxonomically specific 'monoclonal antibodies'.

The term **monoclonal antibody** (**MAb**) is used to describe antibodies, all with the same binding specificity, that are produced from identical cells. Production of monoclonal antibodies is now possible using **hybridoma technology**. The technique involves the fusion of B-lymphocytes from spleens of rats or mice with **myeloma cells**. Myeloma cells are abnormal because they are tumour cells and, unlike lymphocytes, they grow and divide continuously. The fused cells are called **hybridomas**. Like the myeloma cells, the hybridomas can also grow and divide continuously, and they are able to secrete antibodies.

The hybridoma technique was developed in 1975 by Georges Köhler and Céser Milstein for which they were awarded a Nobel Prize in 1984. It utilises the fact that any one B-lymphocyte (antibody-producing cell) secretes antibodies of one type only, all with the same binding specificity. This is the same antibody as that secreted by the original parent lymphocyte. These cells, grown as clones from single hybridoma cells, can then be grown in culture to provide an indefinite supply of the required specific antibody.

The procedure for production of monoclonal antibodies by hybridoma technology is summarised in the flow chart (Fig 8).

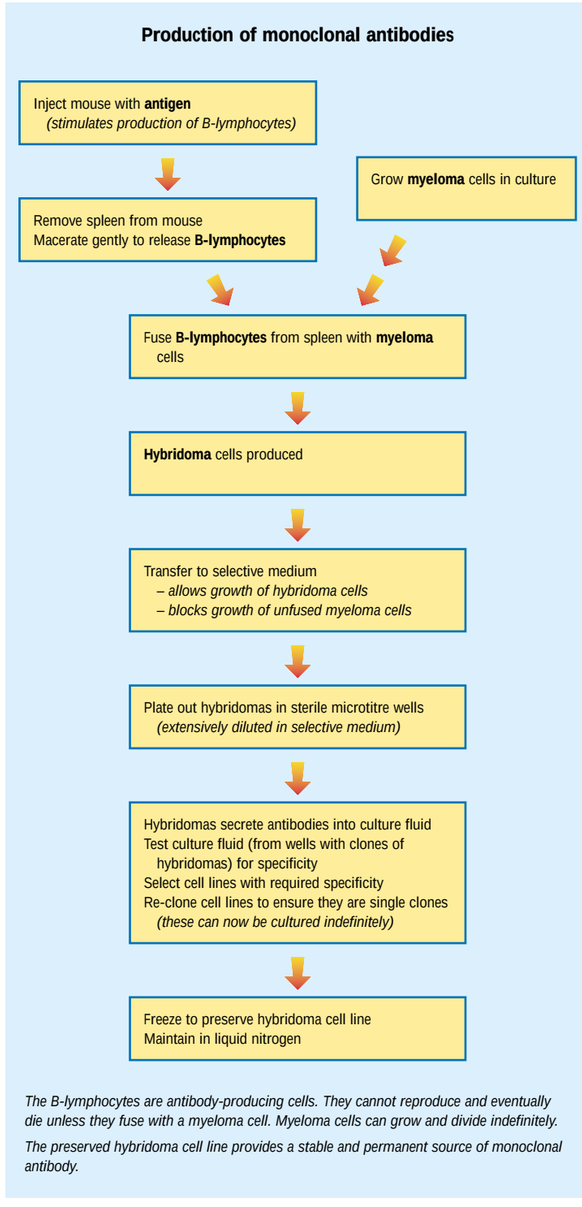


Fig 8 Stages in production of monoclonal antibodies

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SAPS website at www.saps.org.uk

Glossary

Antibody
A protein molecule which is secreted by antibody producing cells (B-lymphocytes) in response to the presence of specific substances considered to be foreign to mammalian systems. Their production is a specific event in the immune response.

Antigen
Generally a large molecule, either protein, glycoprotein or complex carbohydrate, that is foreign to the mammalian immune system and is recognised by and binds to its respective antibody. In some cases, this may be the same as the immunogen.

Antiserum
The cell free component of blood that contains the antibodies, often referred to as polyclonal antiserum.

Colostrum
The first milk secreted by a female mammal after giving birth.

Conidia
Asexual airborne spores produced by many fungi. These spores are not enclosed in a spore-forming organ, but arise from specialised hyphal cells (conidiophores).

ELISA
Enzyme-Linked Immunosorbent Assay

Epitope
Region of a (macro)molecule that is recognised by the specific binding site of an antibody.

Hybridoma cells
Cells which have resulted from the fusion of antibody-producing cells (lymphocytes), from the spleen of an immunised rat or mouse, with myeloma cells (cancer cells from the lymphatic system). They divide continuously secreting antibodies (monoclonal antibodies) with only one level of specificity. Selected hybridoma cell lines may be cloned and the cells preserved by freezing in vials in a special fluid and storing in liquid nitrogen. Cultures of the cells can provide unlimited quantities of monoclonal antibodies.

Immunoassay
The qualitative and/or quantitative measurement or detection of a molecule or organism using its reaction with a specific antibody (or antibodies).

Immunogen
The stimulatory agent, generally the same as the antigen, which triggers an immune response.

Immunoglobulins
Globular proteins present in the cell free component of the blood which recognise and bind to specific molecules, foreign to the body, known as antigens.

Monoclonal antibody
A specific antibody produced by a clone of selected hybridoma cells.

Necrotroph
A fungus which can invade living plant tissue, and continue to grow and survive on dead plant tissues.

Polyclonal antibodies
Antibodies which are present in the cell free component of blood known as the antiserum which can recognise a number of epitopes (binding sites) either on the same molecule or organism or on a range of organisms and molecules. They are derived from more than one parental B-lymphocyte.

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