Practical immunology

UNIT 8

European Initiative for Biotechnology Education

Contributors to this Unit

Lisbet Marcussen (Unit Co-ordinator)
Birgit Sandermann, Elisabeth Strömberg,
Eckhard R. Lucius, Ute Steffens, Christine Labahn-Lucius.

The European Initiative for Biotechnology Education (EIBE) seeks to promote skills, enhance understanding and facilitate informed public debate through improved biotechnology education in schools and colleges throughout the European Union (EU).

EIBE Contacts

AUSTRIA

Rainhart Berner, Höhere Bundeslehr- und Versuchsanstalt für Chemische Industrie Wien, Abteillung für Biochemie, Biotechnologie und Gentechnik, Rosensteingasse 79, A-1170 WIEN.

BELGIUM

Vic Damen / Marleen van Strydonck, R&D Groep VEO, Afdeling Didaktiek en Kritiek, Universiteit van Antwerpen, Universiteitsplein 1, B-2610 WILRIJK.

DENMARK

Dorte Hammelev, Biotechnology Education Group, Foreningen af Danske Biologer, Sønderengen 20, DK-2860 SØBORG.
Lisbet Marcussen, Biotechnology Education Group, Foreningen af Danske Biologer, Lindevej 21, DK-5800 NYBORG.

EIRE

Catherine Adley / Cecily Leonard, University of Limerick, Plassey, LIMERICK.

FRANCE

Gérard Coutouly, LEGPT Jean Rostand, 18 Boulevard de la Victorie, F-67084 STRASBOURG Cedex.
Laurence Simonneaux, Ecole Nationale de Formation Agronomique, Toulouse-Auzeville, Boîte Postale 87, F-31326 CASTANET TOLOSAN Cedex.

GERMANY

Horst Bayrhuber / Eckhard R. Lucius / Regina Rojek / Ute Harms / Angela Kroß, Institut für die Pädagogik der Naturwissenschaften, Universität Kiel, Olshausenstraße 62, D-24098 KIEL 1.
Ognian Serafimov, UNESCO-INCS, c/o Jörg-Zürn-Gewerbeschule, Rauensteinstraße 17, D-88662 ÜBERLINGEN.
Eberhard Todt, Fachbereich Psychologie, Universität Gießen, Otto-Behaghel-Straße 10, D-35394 GIEßEN.

ITALY

Antonio Bargellesi-Severi / Stefania Uccelli / Alessandra Corda Mannino, Centro di Biotechnologie Avanzate, Largo Rosanna Benzi 10 , I-16132 GENOVA.

LUXEMBOURG

John Watson, Ecole Européenne de Luxembourg, Département de Biologie, 23 Boulevard Konrad Adenauer, L-1115 LUXEMBOURG.

THE NETHERLANDS

David Bennett, Cambridge Biomedical Consultants, Schuystraat 12, NL-2517 XE DEN HAAG.
Fred Brinkman, Hogeschool Holland, Afdeling VP&I, Postbus 261, NL-1110 AG DIEMEN.
Guido Matthée, Hogeschool van Arnhem en Nijmegen, Technische Faculteit, HLO, Heijendaalseweg 45, NL-6524 SE NIJMEGEN.
Liesbeth van de Grint / Jan Frings, Hogeschool van Utrecht, Educatie Centrum voor Biotechnologie, FEO, Afdeling Exacte Vakken, Biologie, Postbus 14007, NL-3508 SB UTRECHT.

SPAIN

Maria Saez Brezmes / Angela Gomez Niño, Facultad de Educación, Universidad de Valladolid, Geologo Hernández Pacheco 1, ES-47014 VALLADOLID.

SWEDEN

Margareta Johanssen, Föreningen Gensyn, PO Box 37, S-26800 SVALÖV.
Elisabeth Strömberg, Östrabo Gymnasiet, PO Box 276, Kaempegatan 36, S-45181 UDDEVALLA.

THE UNITED KINGDOM

Wilbert Garvin, Northern Ireland Centre for School Biosciences, NIESU, School of Education, The Queen's University of Belfast, BELFAST, BT7 1NN.
John Grainger / John Schollar / Caroline Shearer, National Centre for Biotechnology Education, The University of Reading, PO Box 228, Whiteknights, READING, RG6 6AJ.
Jill Turner, Department of Science and Technology Studies, University College London, Gower Street, LONDON, WC1 6BT.
Paul Wymer, The Wellcome Centre for Medical Science, The Wellcome Trust, 210 Euston Road, LONDON, NW1 2BE.

EIBE Co-ordinator

Horst Bayrhuber, Institut für die Pädagogik der Naturwissenschaften an der Universität Kiel, Olshausenstraße 62, D-24098 KIEL 1, Germany. Telephone: + 49 (0) 431 880 3137 (EIBE Secretary: Regina Rojek). Facsimile: + 49 (0) 431 880 3132.

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European Initiative for Biotechnology Education

MATERIALS

Contents

World Wide Web

Few areas are developing as rapidly as biotechnology. So that they can be revised and kept up-to-date then distributed at minimum cost, the EIBE Units are published electronically.

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With this software, you can view or print the EIBE Units. In addition, you will be able to 'navigate' around and search the documents with ease.

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Development

The *TriChem* ELISA kit used in the first activity in this Unit was developed by and can be bought from:

TriChem
Bernhard Olsensvej 23
DK-2830 Virum, DENMARK
Telephone : + 45 (0) 45 85 82 83

The copyright of the kit is owned by *TriChem*. This kit was remodelled for class-use by "The Danish Immunology Group", published in *Immunologiske Småforsøg*, Nucleus Forlag ApS (1994).
ISBN: 87 87661 83 7.

Lisbet Marcussen
Educational Biotechnology Group
Nyborg Gymnasium
Skolebakken 13
DK-5800 Nyborg
e-mail: lisbetma@post2.tele.dk

The *STEFFENS* ELISA kit used in the third activity was developed and can be bought from: STEFFENS BIOTECHNISCHE ANALYSEN GmbH
Baumgartenstr. 5
D-79285 Ebringen (FRG)
This kit was remodelled for class use by Eckhard R. Lucius, Ute Steffens and Christine Labahn-Lucius at The Institut für die Pädagogik der Naturwissenschaften (see EIBE Secretariat address).

Contributors

- Lisbet Marcussen (Unit Co-ordinator)
  Nyborg Gymnasium og HF, Nyborg, Denmark.
- Birgit Sandermann Justesen
  Bjerringbro Gymnasium, Bjerringbro, Denmark.
- Elisabeth Strömberg
  Östrabo Gymnasiet, Uddevalla, Sweden.
- Eckhard R. Lucius, Ute Steffens, and Christine Labahn-Lucius
  IPN, The University of Kiel, Germany

Design, illustration and typesetting:
Caroline Shearer, NCBE,
The University of Reading, Whiteknights, Reading, RG6 6AJ. The United Kingdom.

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About this Unit

These materials have been devised by practising teachers and educationalists from several European countries, brought together with financial support and encouragement from DG XII of the European Commission, under the auspices of EIBE, the *European Initiative for Biotechnology Education*.

The EIBE materials have been extensively tested in workshops involving teachers and students from across Europe.

The views expressed in this Unit and the activities suggested herein are those of the authors and not of the European Commission.

Particular attention should be paid to the general safety guidelines given inthe introduction to this Unit, and to the specific safety guidelines throughout the text.

⚠ Safety

In all of the EIBE Units, we have tried to check that all recognized hazards have been identified and that suitable precautions are suggested.

Where possible, the proposed procedures are in accordance with commonly-adopted general risk assessments. If a special risk assessment may be necessary, this has been indicated.

However, users should be aware that errors and omissions can be made, and that different employers and educational authorities adopt different standards. Therefore, before doing any activity, users should *always* carry out their own risk assessment. In particular, any local rules issued by employers or educational authorities MUST be obeyed, whatever is suggested in the EIBE Unit.

Unless the context dictates otherwise, it is assumed that:

- practical work is carried out in a properly equipped and maintained science laboratory;
- any mains-operated equipment is properly maintained;
- care is taken with normal laboratory operations such as heating substances;
- good laboratory practice is observed when chemicals or living organisms are used;
- eye protection is worn whenever there is any recognised risk to the eyes;
- pupils and/or students are taught safe techniques for activities such as handling chemicals and microorganisms.

Trichem ELISA kit for classroom use

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Introduction

ELISA (Enzyme Linked Immuno Sorbent Assay) is a sensitive immunoassay that uses an enzyme linked to an antibody or antigen as a marker for the detection of a specific protein, especially an antigen or antibody.

The ELISA test method is simple and easy to use for quick determination of, for example:

- determination of drug use;
- testing for different infectious diseases, e.g. HIV, Lyme disease;
- detecting genes from different mutants (for instance, those introduced by genetic engineering);
- medical genetics.

This Unit aims to give the students as well as the teachers an opportunity to do their own ELISA investigation. Having gained a better understanding of this frequently-used and important modern method, they should be be better-qualified to participate in the debate about its application.

For further information about this activity please contact The Danish Immunology Group or TriChem (see adresses at the start of this Unit).

The following investigation uses the ELISA test principle to analyse blood samples from pigs to determine whether the animals have been exposed to certain bacteria.

By producing a toxin the bacterium *Pasteurella multocida* causes different deformities in the bones of the snout of the infected pigs. This leads to bad biting function, sneezing and a number of different attacks on the respiratory organs. The attack is often followed by an attack by *Bordetella bronchiseptica*, worsening the condition. The result is slow growth and economic losses for the farmer. Therefore it is important to stop the infection as early as possible. The ELISA test method can be used for detecting the toxin as well as for detecting the bacterium *Bordetella bronchiseptica*.

In this experiment antigens from the bacterium *Bordetella bronchiseptica* (*Bb*) are used. On the first day the bacterial antigens are added to the wells of the ELISA-plates (coating). The next day the plates are ready for an analysis of the blood samples from the pigs. If a pig is infected with *Bb* the blood test will show a positive reaction with the antigens in the wells and a colour reaction will result.

Guidelines for the teacher

Aim

To analyse blood samples from pigs using the ELISA method.

Organisation

Day one — about 15 minutes
Day two — about 2 x 45 minutes.

Safety

5-aminosalicylic acid is not toxic and it is used in gram doses for treating human diseases, but related compounds are known to be mutagenic. Therefore it is necessary to handle 5-aminosalicylic acid with care - use rubber gloves.

Procedure

Day 1

Preparing the reagents

PBS

Dissolve the contents of the bottle marked "PBS" in 2.5 litres of deionized water. Check the pH — it should be about 7.1 to 7.5. If necessary, adjust with 5M NaOH or 5M HCl.

Leave 30 cm^3 of this solution for making the COATING SOLUTION.

To the rest of the PBS add the contents of the bottle marked "TWEEN 20". Wash the bottle with a little PBS in order to get it all out. Mix thoroughly, and mark the final solution WASHING BUFFER. Cover the container.

Then add the contents of the bottle marked "Bb ANTIGEN" to the 30 cm^3 of PBS. Mix well! This is now the COATING SOLUTION.

The microtitre plates are made of plastic and treated with radiation in order to increase the binding-capacity and to ensure sterility.

The antigen is a killed (boiled) extract of the bacterium *Bordetella bronchisepta(Bb)*.

Day 2

Preparing the sera

Serum 1:
Negative control from a pig which has not been infected with *Bb*.

Sera 3 to 10:
Unknown sera from pigs. Four of these are negative — the other four are more or less positive.

Serum 12:
Positive control.
All the positive sera come from healthy pigs which have been vaccinated with killed *Bb*.

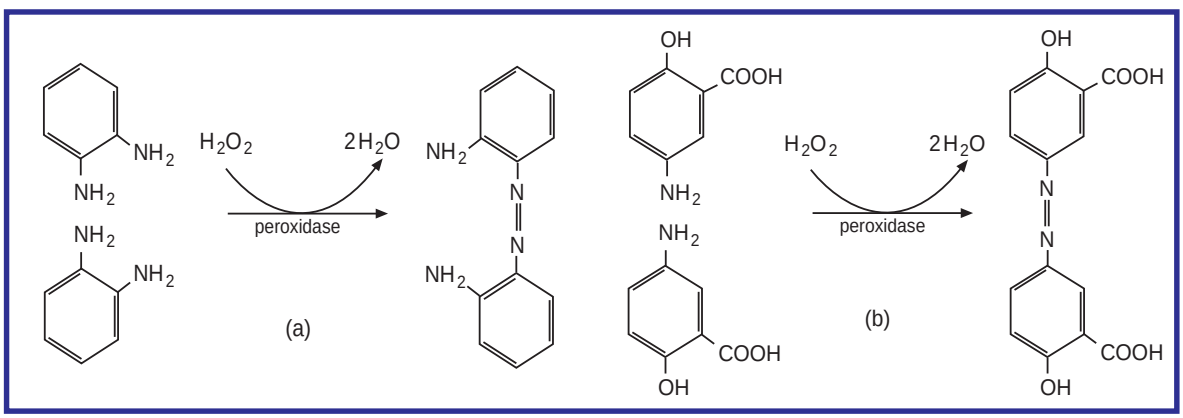
Preparing the conjugation solution

The conjugation solution must be freshly prepared as follows:
Add 25 cm^3 of washing buffer to the bottle marked BSA and mix the solution until it is clear, then add contents of the bottle marked "conjugate". Wash the bottle with BSA washing buffer in order to get everything out. Mix well.
The conjugate is the rabbit anti-pig IgG conjugated to the enzyme peroxidase. (This enzyme is extracted from horse radish.)

BSA
(Bovine Serum Albumin, from cattle)
BSA is used to avoid non-specific adsorption of antibodies or conjugate to the surface of the wells. BSA is used in such a high concentration that it will absorb to unoccupied sites on the surface.

Substrate solution

Important -this solution must be freshly-made!
Dissolve 5-aminosalicylic acid from the bottle marked "Substrate" in contents of the bottle marked "Substrate buffer H_2O_2". Mix until dissolved. Use gloves.



Above: a) oxidation of o -phenylendiamine b) oxidation of 5-amino-salicylic acid

Teacher's note of the results

Answers to the questions

1. Negative sera are 4, 5, 8 and 9.
   Positive sera are (in decreasing order of reaction): 6, 7, 10 and 3.
   Serum no. 1 is a negative control taken from a pig not infected with *Bb*.
   Serum No. 12 is a positive control.
3. Measles viruses are used as antigens
   In the conjugate an antibody to human IgG is used.
4. Even killed pathogenic material can be dangerous to humans. It is difficult to provide human serum that is 100% free from human pathogens such as hepatitis or HIV.

Note:
A positive titre does not necessarily mean that the animal is infected. It could have recovered after having been infected.

Equipment and materials

Please note, that when the kit is bought some of the reagents should be kept in a freezer.

Micropipettes up to 100 µl
Beaker 100 cm^3
Measuring cylinders 20 cm^3, 100 cm^3
Magnetic stirrer
3 litre glass flask
2.5 litres deionized water
5M HCl
5M NaOH
Rubber gloves (extra)
Marking pens (permanent ink)
1 bottle of antigen (boiled extract from the bacterium *Bordetella bronchiseptica*) *
1 box containing 10 vials of pig sera *
1 bottle of substrate — 5-aminosalicylic acid (30 mg) *
1 bottle of conjugate — rabbit anti-pig IgG conjugated to the enzyme peroxidase *
1 vial of substrate buffer with H_2O_2 *
Microtitre plates with lids *
1 bottle of salts for making PBS (phosphate buffer)*
1 bottle (2.5 cm^3) of Tween 20 (synthetic detergent)*
Plastic pipettes *
Rubber gloves *
1 bottle of BSA (Bovine Serum Albumin) *
Plastic spoons *
Waste bags *

* included in the kit.

Student Guide

Procedure

The first day:

Coating the wells with antigen:

1. Add 100 µl antigen solution to the wells marked A, B and C (a total of 36 wells).
2. Cover the wells and allow the plates to stand at room temperature until the next day. If you cannot continue the experiment the following day, then place the plates in the refrigerator.

Second day:

Removing excess antigen:

1. Remove contents of the wells (a quick shake over the sink)
2. Shake the plate in order to dry the wells

Washing:

3. Fill the coated wells with washing buffer. Wait for one minute!
4. Empty completely (shake dry if necessary)
5. Repeat steps 3 and 4 twice.

Adding the sera:

6. Shake the thawed solutions of the different sera.
7. Add 100 µl of serum 1 to the wells marked A, B and C in column 1 (3 wells).
   CHANGE PIPETTE TIP !
8. Add 100 µl of serum 3 to the wells marked A, B and C in column 3.
   Row 2 is left untouched
   CHANGE PIPETTE TIP!
9. Continue the same way by filling the wells in column 4, 5, 6, 7, 8, 9, 10 and 12. Columns 2 and 11 are not used.
   REMEMBER TO CHANGE PIPETTE TIPS EVERY TIME YOU CHANGE SERUM.
10. Incubate the plates for 15 minutes at room temperature.

Removing excess serum:

11. Empty all the wells at the same time. Shake dry if necessary.
12. Wash, empty and dry as described in steps 3 and 4 three times

Adding conjugate:

13. Add 100 µl of conjugate to all the wells marked A, B and C.
14. Incubate the plates for 15 minutes at room temperature.
15. Wash, empty and dry as described in step 12.

Adding substrate:

16. Add 100 µl of the substrate solution to each of the wells.
17. Wait for the colour to develop. Note the results in a table by using a scale from 0 to 5 to express the colour intensity. 0 is negative and 5 is the most positive. You may also describe the colours.

Removal of waste:

Place pipettes and plates in a plastic bag and throw away. The remaining solutions can be poured into the sink.

Results and evaluation

1. Which of the unknown sera were positive ?
   Arrange the sera in order of an increasingly positive result.

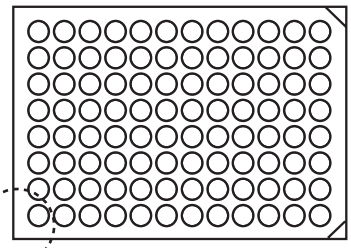
2. What kind of possible errors can be detected in this analysis?

3. If you wish to do a similar test to detect the antibodies of the human measles virus, which of the reagents would have to be changed?

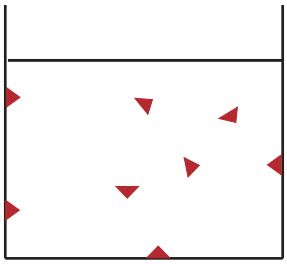
4. Why do you think that we are not using a measles test in this investigation?

ELISA testing proteins

Enzyme-Linked ImmunoSorbent Assay

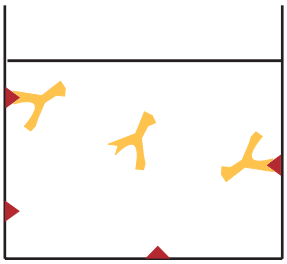


After each reagent has been added, the plate is incubated, then the wells are washed out to remove unreacted materials.



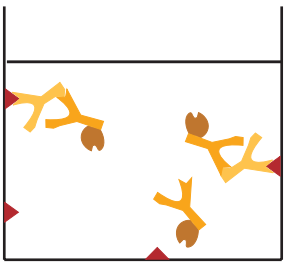
1. Protein (antigen) added that binds to the well.

WASH to remove unbound molecules.



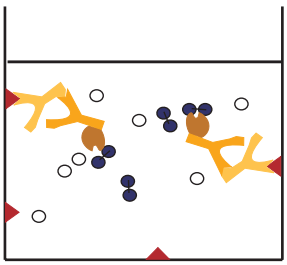
2. Sample added. Antibodies bind to the bound antigen.

WASH to remove unbound molecules.



3. Enzyme linked antibody conjugate added. Conjugate binds to the bound antibodies.

WASH to remove unbound molecules.



4. Colourless enzyme substrate added, in the presence of the the bound enzyme a colour develops.

How to detect egg in different food by double immuno-diffusion

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Eggs are used in many different food products such as hamburgers, pastas and sometimes in ice cream. Many people are allergic to eggs even in very small quantities. It is possible to detect even small traces of egg white or yolk by using antibodies against egg albumin. The method used is called double immunodiffusion.

When antigen and antibody react together near their equivalence point they often form cross-linked precipitates. If the reaction occurs in a supporting medium, such as an agar gel, the reactants form precipitation arcs or lines, which can be used to identify antigens and antibodies in complex mixtures.

The precipitates are formed because antibodies and antigens have more than one binding site so large cross linked structures are formed. The further away from their equivalence point the fewer precipitates.

The method of double immuno-diffusion was developed by the Swede Örjan Ouchterlony about 30 years ago. His method is called “double”, referring to the fact that in this procedure antigen and antibody are allowed to migrate towards each other in a gel, and an arc or line of precipitation is formed where the two reactants meet.

This precipitation reaction is highly specific and sensitive, and is used today by people working with diagnoses, protein detection or comparing antigens and antibodies.

Guidelines for the teacher

Aim

To detect egg-albumin in different foods.

Organisation

Day one: 60 minutes plus overnight until the lines have formed (see teacher’s note)
20 minutes for analysing the result. (If you wish to stain too it will take another 2 hours and 45 minutes).

Safety

No particular precautions are necessary.

Notes

Punch pattern

It isnecessary to punch a regular pattern in the gel. Therefore it can be a good idea to start making the first punch pattern near the edge, leaving space for a second trial. Use a straw or a plastic pipette.
The precipitation lines will form quicker if the distance between the wells is reduced. A distance of 5 mm will show a result after 24 hours and a distance of 10 mm will give results after 48 hours.

After the first day:

If you use microscope slides and it is very warm or dry in the laboratory, the micro-scope slides must be kept humid, e.g. in a Petri dish with a wet paper towel or filter paper and plastic foil until the next day. If it is necessary to keep the slides for many days, it is recommended to keep them in a plastic bag (moist) with a little preservative to avoid mould. Avoid damaging the gel.

The slides will be ready after one night but you can keep them for up to one week in the fridge.

Reference solution
Since it can be difficult to prepare the reference solution, it might be a good idea to whip some of the egg white first, and then make the reference solution by using the fluid part of the egg white.

Egg-albumin - the antibody
Freeze-dried antibody is diluted in TRIS-buffer according to the instuctions with the antibody. This solution can be aliquoted into eppendorf tubes and stored in the freezer.

Waste
Everything can be handled as normal waste.

Staining
Staining is not necessary, but it might help to get a clearer result. Amido black and Coomassie Brilliant Blue solution may be used for staining.

Equipment and materials

Microscope slides or small (diameter 5 cm) Petri dishes
A straw or plastic pipette with a diameter of 2.5 mm for punching wells in the gel
Micropipettes: 0–10 cm^3
Humid chamber for the microscope slides e.g. a plastic box with a wet paper towel or you can use small plastic Petri dishes (of 5 cm diameter)
Homogenizer / Liquidiser
Hair drier*
Filter papers
Weight — about 1 kg*
Box for staining the gel*
TRIS-buffer 0.01M, pH 8.0
Agarose solution 1% in TRIS-buffer
Reference solution: 0.01% egg white diluted in deionized water
Rabbit-antiserum against egg albumin (*Pharmacia* AS-23)
Acetic acid
Methanol
Amido black solution (0.1 g amido black dissolved in 100 cm^3 mixture made up of acetic acid, methanol and deionized water in the proportions 10:70:20)*
Sodium chloride solution — 0.9%
Destaining solution containing acetic acid 10%, methanol 70% and deionized water 20%*
* only needed if you wish to stain.

Student Guide

Procedure

Preparing the samples

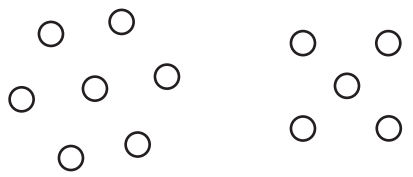
1. Homogenize 5 g of the sample in 5 cm^3 of water as well as possible.
2. Centrifuge the mixed sample 15 minutes at 6 000 rpm (or 10 minutes at 9 500 rpm).
3. Filter the supernatant through a piece of cloth into a clean test tube

Casting the gel

4. Dissolve the correct amount of agarose in TRIS-buffer (1%). Stir.
   For 1 Petri dish (5 cm) you will need 3-5 cm^3 and for one microscope slide you will need 3.5 cm^3.
   TAKE CARE! — IT BOILS EASILY!
5. Let it cool to 60–80 °C.
6. Place the Petri dishes (or microscope slides) on a horizontal table close to the edge. Remove the lids.
7. Pour the hot gel into the dishes or on the glass — a layer of about 2–3 mm is enough. Cover with the lids.
8. Let the gel set — this will take about 5–10 minutes.

Punching the wells

9. Use a straw or a pipette with a diameter of about 2.5 mm to punch wells in the gel. Be careful and make them with vertical sides.
   Remove the gel plug by sucking with the pipette or by using a needle.
   Make one of the following well patterns.



Adding antibody

N.B. Before filling the wells mark each well on the bottom of the Petri dish or the microscope slide.

10. Fill the centre well with the antibody (anti-egg albumin) — usually 5–10 µl will be enough.
    DO NOT OVERFILL.

Adding antigens

11. Fill every second outer well with the reference solution.
    DO NOT OVERFILL.
12. Fill the other outer wells with your test-samples. Note which sample is in the different wells.
    DO NOT OVERFILL.

    Let the diffusion take place by leaving the Petri dishes / slides overnight in a humid chamber at room temperature or in the refrigerator.

13. Later you can study the white precipitation lines / arcs where there has been a positive reaction. It might be easier to see the lines if you hold the slides / dishes against a dark background.

Staining (optional)

14. Remove non-precipitated proteins by soaking the gel in 0.9% NaCl solution for 60 minutes at room temperature.
15. Remove the salt solution, and fill the box with deionized water.

    Leave it for 60 minutes at room temperature.

16. Remove the water.
    Press the gel under a layer of 10 filter papers and a weight of about 1 kg for about 15 minutes.
17. Dry the gel by using a hairdryer.
18. Cover the gel with staining solution and leave for 10 minutes.
19. Remove the staining solution.
    Destain with the destaining solution for 10 minutes.
    It might be necessary to repeat the destaining process.

Results

The detection limit for egg white is 2.5 mg in 100 g. If there are enough chicken proteins in the sample you will notice cross-reactions. Also, yolk contains enough egg white proteins to make a detection possible.

STEFFENS ELISA Kit for classroom use

Introduction

In this kit, a polyclonal antibody to the Pelargonium flower break virus (PFBV) is immobilised on a special comb enabling the test to be carried out both simply and economically within a school timetable.
The kit makes it possible to prove, both sensitively and safely, a PFBV infection in 2 x 10 samples. Positive and negative controls are provided on the comb.
The sensitivity of the test was established by extracting infected plant material (*Chenopodium quinoa*) in sample buffer and diluting it. The virus could still be established in a 1 : 1,000 - dilution of a 10% extract (> 100 mOD over the healthy control).
The reagents of the test kit will remain stable until the expiry date quoted on the label when stored at 4 °C.

References

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Hollings, M. and Stone, O.M. (1974) *Description of plant viruses*. CMI / AAB, 130, 4.
Nellen, U. (1992) The ELISA test: a universal procedure for the identification of antigens on the basis of biotechnologically produced monoclonal antibodies - information and school-experiment. *Biotechnology Education* (3) 3, 107–112.

Guidelines for the teacher

Materials

For each test you will need:

Directly from the *STEFFENS* ELISA test kit for 2 x 12 analyses (Order No. 04093P00):
In an airtight plastic bag together with drying agents but individually packed:
A special comb with 12 prongs, on which the PFVB antibody is immobilised
3 strips of 12 reaction wells
10 extraction bags (plastic bags with cotton gauze)
50 cm^3 ready-to-use sample buffer (yellow)
12 disposable pipettes (for 10 samples, diluted conjugate and substrate)
1 graduated disposable pipette (for the sample buffer)
1 non-graduated disposable pipette with a fine tip (for the concentrated conjugate)
1 minitube with 0.05 cm^3 concentrated conjugate (colourless)
1 small screw-top bottle with 1.6 cm^3 conjugate buffer (blue).
1 small screw-top bottle with 1.6 cm^3 ready to use substrate solution (colourless)
1 pipette stand (part of the packing)

From the kit, but previously prepared:
(All the reagents should be brought to room temperature before starting the test.)
The three strips of wells should be placed in the stand formed from the cardboard of the inner cover of the box (cf. hints and tips).
The concentrated conjugate should be transferred as completely as possible to the blue conjugate buffer, using the fine-tipped disposable pipette. Take care that drops do

not stick to the lid. The contents should be mixed thoroughly.

In addition, you will need:
Up to 10 Pelargonium plants from different locations
Pair of scissors
Homogenizer (e.g. the top of a screwdriver or another solid object like a pestle)
Cold tap water
Microtiter photometer (650 nm), if available (results can be evaluated visually)
Waste container

Organisation

The following is a suggested teaching plan.
Day 1
- Introduce the topic
- Antigen-antibody-reactions
- Virus illnesses
- Homework - gather plant material (complete Pelargonium plants or freshly-picked leaves)

Day 2
- Homogenize the 10 samples in the extraction bags (group work for students).
- Prepare the wells and solutions and load the samples
- Pupils or teacher carry out the individual reaction steps.
- Evaluate the results.

Day 3
- Discuss the results.
- What factors favour virus infection?
- Aims of plant breeding

Hints and tips

Setting up the kit: The ELISA kit has been designed for 2 sets of 12 analyses, with all the parts supplied in duplicate. Half of the components can therefore be left in the box and stored in a cool place (4 °C) for later use.

Pipettes: The pipette tips should not be touched.

Homogenizer: A smooth object, e.g.. the handle of a screwdriver or a pestle can be used in place of a homogenizer.

Special comb: The comb for the antigen-antibody test can be found together with the three strips of wells in a separate plastic bag which also contains desiccant. A polyclonal antibody is immobilised on the prongs of the comb, which specifically recognises the *Pelargonium Flower Break Virus* (PFBV). Prong 11 (green) of the comb has been additionally covered with a negative control, prong 12 (red) with a positive control. The comb should only be held by its green handle. Avoid touching the prongs.

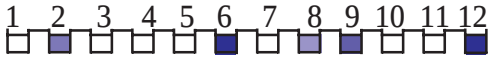
Preparation of the wells: The cover of the interior insertion of the box should be opened up and half of the extraction bags and pipettes taken out. The cover of the inner compartment should be unfolded so that it forms a rack to hold the 3 strips of wells. When removed from the plastic bag the three strips can be placed in the stands. The series and orientation is unimportant. The comb is best left in the bag so that its prongs are not touched unnecessarily. The drying agent is no longer needed and can be discarded.

Conjugate: The concentrated conjugate must be transferred as completely as possible to the blue conjugate buffer using the disposable pipette with the fine tip, watch out for drops of the concentrated conjugate remaining on the cover. Dispose of the pipette after use. Conjugate and buffer should be mixed thoroughly.

Waste: After the test has been evaluated, wash the comb and wells with running water before disposal. Do not try to reuse the strips, even after thorough washing, since they can give incorrect positive results.

Teacher's note of the results

The following schematic illustration of the third row of wells shows a typical result:



Well 11 (negative control - corresponds to the green point on the comb), no colour reaction should occur.

Well 12 (positive control - corresponds to the red dot on the comb), an intense blue colour should be formed .
Compare these with the results of the samples (Wells 1 to 10). The colour tones vary between light and dark blue according to the intensity of infection of the plant material used.

Safety

Chemicals

Sample buffer composition: TRIS / HCl buffer, polyvinylpyrrolidone, sodium chloride, Tween 20, sodium azide, dye - E102.

Conjugate composition: phosphate buffer, horse radish peroxidase, bovine serum albumin, Tween 20, Bronidox L (5-bromo-5-nitro-1,3-dioxane), dye E 131.

Substrate composition: TMB (Tetramethylbenzidine), buffer, H_2O_2.

N.B. The reagents contain Na-Azide and Bronidox L as stabilizers. These are toxic when swallowed!

Disposal
No special problems

Guarantee and liability
EIBE can give no guarantees and can accept no responsibility in respect of materials or chemical agents of the kit.
STEFFENS Biotechnische Analysen GmbH guarantees that the delivered products have been tested thoroughly in order to ensure that the kit fulfils the specifications and corresponds to the description given. Further guarantees are not given.
STEFFENS Biotechnische Analysen GmbH can accept no responsibility for damage which arises due to an improper storage and application of the product.

Table of Results

Probe No.	Details (sample name, origin)	Remarks (test notes)	Result
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11	negative control		colourless
12	positive control		blue

Student Guide

Introduction

Immunobiology is one of the most important research fields of applied biology today. It dates from the start of this century when Paul Ehrlich (Noble Prize 1908) discovered the role of the antibodies in resistance to infectious diseases.

Immune resistance in vertebrates depends on the formation of a specific antigen-antibody complex. An antigen is any substance capable of stimulating the production of an antibody. Antigens can be any foreign organic compound, such as polysaccharides, proteins, peptides or nucleic acids.

Certain endogenous globular proteins, called immunoglobulins, act as 'antibodies'. These 'recognise' certain surface structures of the antigen on a molecular level and combine to form an insoluble complex.

This feature of antibodies is used in the process of immunological diagnosis. Minute traces of antigens (10^{-8} g cm^{-3} per sample) can be identified. Formerly, antibodies were extracted from animals which had been injected with particular antigens, however this gave a very poor yield of antibody. This has been improved with the development of the 'hybridoma-process', which is now used to produce 'monoclonal' antibodies. Biotechnology techniques are used to produce hybrid cells from a combination of animal antibody forming lymphocytes and tumour cells, these have the combined characteristics of being able to constantly multiply and to produce 'monoclonal' antibodies.

Antibodies used in a diagnostic test need to be labelled in order to make the antigen-antibody complex visible. Antibodies are frequently combined with an enzyme for this purpose. The enzyme can then react with a substrate to transform it to a coloured product. The colour reaction indicates a positive sample.

The ELISA test is based on this principle: Enzyme Linked Immuno Sorbent Assay. A wide variety of antigens can be examined with this method, including those which are of viral origin.

Viral illnesses can not only affect animals and people, but also plants. The spread of infectious material by the intensive propagation methods used in agriculture and in nurseries has meant that the ELISA technique is gaining increasing importance in this field.

This test detects a virus infection of geraniums. Infection by the *Pelargonium flower break virus* (PFBV) causes the blossoms to change their colours undesirably. This is a common infection of geraniums so there should be no problem in finding positive samples to test. The principle of the test is based on the so-called 'sandwich technique': An immobilised antibody, binds to the antigen if it is present in the sample, a second antibody then binds to the immobilised antigen to form an antibody - antigen - antibody complex. The second antibody is marked with an enzyme, which transforms the colourless substrate into a coloured product.

In the *STEFFENS* ELISA kit the first antibody to PFBV is immobilised on the prongs of a special comb.

1st reaction

PFBV antigens from the samples are bound to the immobilised antibodies on the comb. The antigen-antibody complex is formed on the prongs of the comb.

2nd reaction

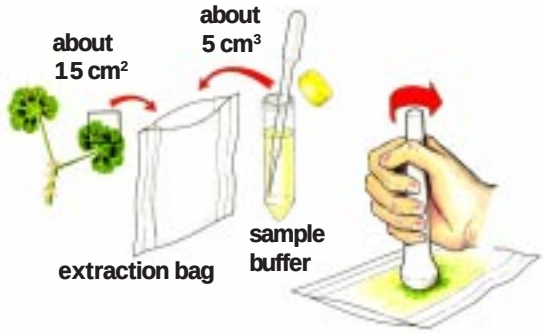
A second PFBV antibody, which is marked with horse radish peroxidase (enzyme conjugate) is bound to the antigen-antibody complex on the prongs of the comb.

3rd reaction

The antigen-antibody complex which is marked with the enzyme transforms the substrate tetramethylbenzidine to a blue-coloured product. PFBV infected probes show this blue colouring, non-infected probes remain colourless.

Procedure

1. Preparing the samples (10 min.)

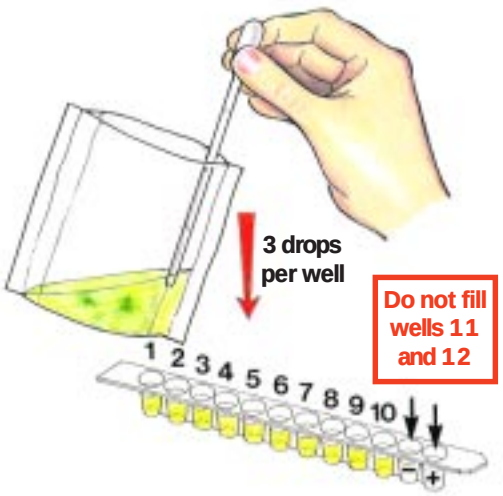


1.1 Place a piece of leaf about 15 cm^2 (about 0.5 g) in the middle of an extraction bag between the cotton gauze.

1.2 Add about 5 cm^3 probe buffer (yellow) with the graduated pipette. Avoid any contamination of the probe buffer with plant extract.

1.3 Place the extraction bag on a smooth solid surface and crush the leaf tissue by moving the homogenisor over the bag with moderate pressure using circular movements, until a fine suspension forms. The release of chlorophyll (green colour) is a good indication of the degree of extraction.

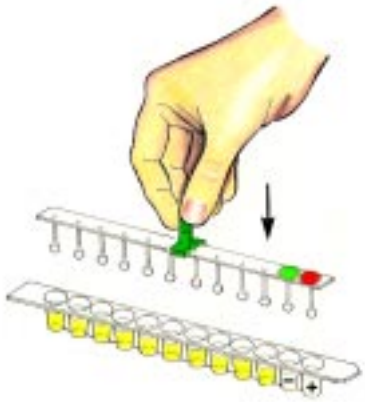
2. Dispensing the samples (5 min.)



2.1 Number the samples 1-10 and transfer 3 drops of each sample into the wells of the first reaction strip, working from left to right. Use a clean pipette for each sample.

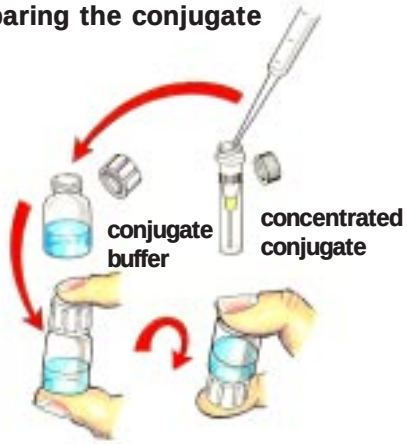
2.2 Leave wells 11 and 12 on the extreme right empty, they remain empty for the first reaction.

3. First incubation (10 min.)



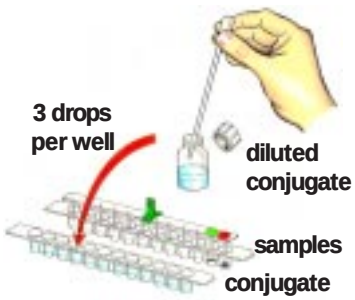
3.1 Place the comb in the first reaction strip so that the controls (which are immobilised on the comb - the green and red dots) match the empty wells 11 and 12. This starts the first reaction which needs 10 min. incubation time. (Use this time to prepare the conjugate).

4. Preparing the conjugate



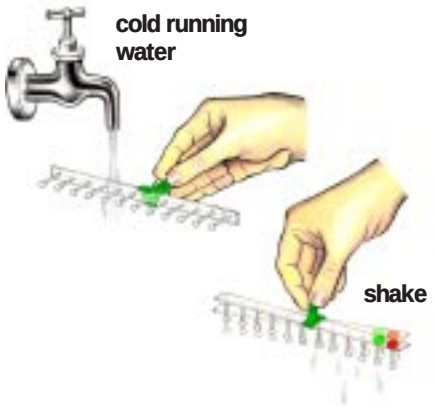
4.1 Using the pipette with the fine tip, transfer the concentrated conjugate into the blue conjugate buffer as entirely as possible. Take care not to leave any drops on the cover. Mix thoroughly.

5. Dispensing the conjugate



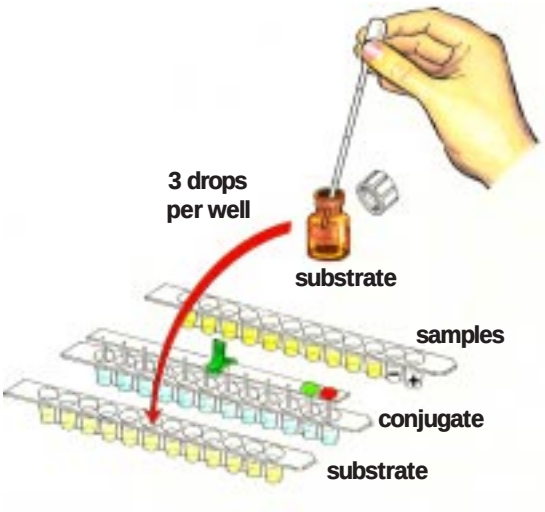
5.1 Using a new pipette, put 3 drops of the diluted conjugate into every well (including 11 and 12) of the second row. Avoid contaminating the third (empty) strip of wells.

6. Washing the comb (30 sec.)



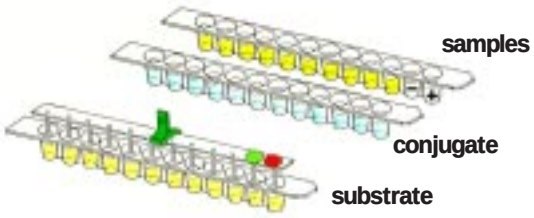
6.1 After 10 minutes incubation, remove the comb from the first row.
6.2 Wash the prongs under cold, gently running water for 15 to 30 sec. Do not hold the comb vertically under the running water to prevent cross contamination. Thorough washing is necessary to avoid background colouring.
6.3 Remove any water remaining on the comb with a few quick shakes.

7. Incubating with conjugate (10 min.) and dispensing the substrate



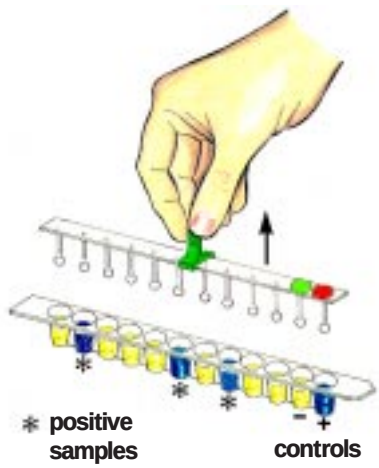
7.1 Place the comb in the second row, again with the controls (red and green dots) on the right side.
7.2 Incubate for 10 min.
7.3 Using a new pipette place 3 drops of the substrate solution into every well of the third strip (including 11 and 12).
N.B. The substrate is sensitive to light. Avoid working in direct sunlight.

8. Incubating with substrate (11 min.)



8.1 After incubation, wash the comb again thoroughly under cold running water, as described in step 6.
8.2 Place the comb in the third row of wells (containing the substrate) with the dots on the right-hand side. Incubate for 10 min. Avoid direct sunlight.

9. Evaluation



9.1 The test is best evaluated immediately after removing the comb at the end of the last incubation. Either by looking and recording the colour development visually or with the help of a microtiter photometer at 650 nm.
9.2 The controls are intended to check the test performance. The test has worked perfectly if the positive control shows an intensive blue colour, while the negative controls remain colourless.
Each sample with a colour reaction stronger than the negative control is infected.
If the sample has less colour or is the same as the negative control, it is probably not infected. Infection cannot be completely excluded however, as the virus concentration of the sample may be less than the sensitivity of the test.