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  AUTHORS   Crameri,A., Whitehorn,E.A., Tate,E. and Stemmer,W.P.
  TITLE     Improved green fluorescent protein by molecular evolution using
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  JOURNAL   Nat. Biotechnol. 14 (3), 315-319 (1996)
  MEDLINE   98294348
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  AUTHORS   Crameri,A. and Kitts,P.A.
  TITLE     pBAD-GFPuv complete sequence
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  AUTHORS   Kitts,P.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-JUN-1996) CLONTECH Laboratories, Inc., 1020 East
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COMMENT     This vector can be obtained from CLONTECH Laboratories, Inc.,
1020
            East Meadow Circle, Palo Alto, CA 94303-4230, USA. To place an
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            International customers, please contact your local distributor.
For
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            This sequence has been compiled from information in the
sequence
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BACTERIAL TRANSFORMATION

Introduction:

Genetic transformation occurs when a cell takes up and expresses a new piece of genetic material. In many bacteria this transformation takes place within the bacterial plasmid DNA eg. *Escherichia coli* and *Agrobacterium tumefaciens*. Plasmids are small circular extra-chromosomal bits of DNA contained within the bacteria cell. The insertion of the gene(s) usually provides the organism with a new trait(s) (eg. pest or antibiotic resistance). For example a gene used for the production of insulin in humans has been cloned into a plasmid and transformed into bacteria. Under the right conditions these transformed bacteria will produce authentic human insulin to treat diabetic patients.
Green Fluorescent Protein (GFP) is expressed by a gene found in the bioluminescent jellyfish *Aequorea victoria*. This causes the jellyfish to fluoresce and glow in the dark. Therefore if this gene is cloned into a plasmid and transformed into bacterial cells, the newly transformed bacterial cells should fluoresce a green colour when placed under UV light.
Expressions of traits are regulated by specific promoters. If ampicillin is added to the media, the gene for ampicillin resistance on the plasmid is switched on allowing the bacteria to grow. The GFP gene is turned on in presence of the sugar arabinose.
In this practical we will supply you with a plasmid containing the genes for expressing GFP and ampicillin resistance and *E. coli* cells that will be transformed with the new plasmid. If the transformation is successful the *E. coli* will grow on media containing ampicillin and fluoresce with UV light.

Laboratory technique

Sterile conditions are an important factor with all facets of molecular biology. An attempt should be made to minimise contamination of the experiment. Contamination can be found on hands, bench tops and equipment etc. It is important that gloves are worn during the practical and equipment used is sterile and not touched by any contaminated surface.

The host organism is *E. coli* K-12 strain, which is a non-pathogenic bacteria.

All used disposable pieces of equipment (i.e. loops, pipette tips, plates and gloves) should be placed in the bins to be autoclaved.

Ultra violet light can cause damage to eyes and skin, so always make sure that a perspex barrier is between you and the UV light.

Experimental Procedure:

1. Label one of the micro tubes with **+DNA** and the other **-DNA**. Both tubes should have your group name.

2. Transfer, using a pipette and sterile tip, 200µL of the transformation solution to each micro tube. (The transformation solution is 50mM $CaCl_2$, pH7.4 which neutralises the repulsive negative charges of the phosphate backbone of the DNA and the phospholipids of the cell membrane allowing the DNA to pass through the cell membrane.)

3. Place the micro tubes on ice.

4. Using a sterile loop pick a single colony of *E. coli* from the culture plate and immerse the loop into the **+DNA** micro tube. Spin the loop once immersed in the transformation solution to disperse the bacteria thoroughly. Replace back on ice. Repeat for the **-DNA** micro tube.

5. Immerse a sterile loop into the plasmid DNA making sure that there is a film of plasmid solution across the loop. Transfer to the **+DNA** micro tube, spinning once more to mix the plasmid and bacteria. Return to ice. **DO NOT** add plasmid DNA to the -DNA micro tube.

6. Incubate the micro tubes on ice for 10 minutes.

7. While you are waiting label the bottom of the four agar plates with your group name as follows;

LB plate	-DNA
LB/amp	-DNA
LB/amp	+DNA
LB/amp/ara	+DNA

8. After 10 minutes on ice, transfer the foam rack containing both micro tubes into the water bath at 42°C for exactly 50 seconds. Remove micro tubes and replace on ice for 2 minutes.
(The heat shock increases the permeability of the cell membrane to increase the efficiency that the plasmid enters the. The time of heat shock is crucial for optimum transformation.)

9. After 2 minutes on ice remove micro tubes and place on the bench top. Add 200µL of the LB broth solution to both micro tubes. Incubate for 10 minutes at room temperature.
(This 10 minute incubation while allow the transformed cells to grow and express the ampicillin resistance protein, beta-lactamase.)

10. Tap the base of the closed micro tubes with your finger to mix the bacterial suspension. Add 100µL of the suspension from both micro tubes to the appropriate plates as follows;

100µL	**-DNA**	LB plate
100µL	**-DNA**	LB/amp plate
100µL	**+DNA**	LB/amp plate
100µL	**+DNA**	LB/amp/ara plate

11. Using a sterile loop for each plate, spread the suspension evenly over the surface of the media.

12. Wrap each plate with parafilm, taping them in a stack upside down. Incubate in the 25°C room for one week. Check colony formation at the beginning of next weeks practical, for growth and fluorescence.
(Colony formation can be achieved over night by using a 37°C incubation temperature.)

Question 1. We have given you a plasmid with the gene that encodes the GFP already in it. The gene for GFP was not originally present in the plasmid. Briefly explain what processes you think we used to get the GFP gene into the plasmid.

Question 2. Describe how you could use two LB/agar plates, some *E. coli* and some ampicillin to determine how E. coli cells are affected by ampicillin.

Question 3. Explain the reasoning behind plating the -DNA transformation onto LB and LB/amp plates.

Question 4. Why do you think the bugs are incubated for 10 minutes after the L-broth has been added at step nine ?

Question 5. Assuming everything has gone according to plan in this prac, what do you expect to find on each of your four plates next week ? When answering this, make sure you give the reasons why you these results.

Lab Requirements

Group of 20 students

10 X LB plate with E. coli

20 X LB plate 10g LB per 500mlDI
8g Agar

40 X LB/Amp plate 10g LB
0.0375g Amp per 500mL DI
8g Agar

20 X LB/Amp/Ara plate 10g LB
0.0375g Amp
2.5g L+Arabanose per 500mL
8g Agar

60 X Petri dishes
100 X Micro tubes
20 X Micro tube racks
20 X Marker pen fine
20 X pipette P200 & tips
20 X disposable sterile loops packs of 25
20 X 1mL transformation solution (50mM $CaCl_2$ MM 147.02)
20 X 1mL LB broth
20 X timer
20 X paintbrushes
20 X floaties

Each Bench

4 X Esky of ice
4 X Parafilm
4 X scissors

Class

Plasmid DNA
1 X Waterbath 42°C
UV transilluminator
25°C or 37°C incubator
Microwave
6 X autoclave bags
waste beakers

Bacterial Practical

Bacteria belong to the Prokaryotes group, and are the oldest in evolutionary terms. They are the most abundant and smallest cellular organisms in the world. Some bacteria have such a rapid cell division they can double their population in short periods of time eg. *E. coli* at 20 minutes under optimum conditions. The main differences between bacteria and other cellular organisms are; the lack of a nuclear membrane and the composition of their cell wall. The classification of bacteria has mainly been based on their size, shape and cell wall. They come in rod, filamentous, spherical, etc. The chemical assay of their cell wall sorts them into Gram -ve or Gram +ve.

As a group we will discuss the growth habits and safety concerns in dealing with bacterial cultures.

Also the possibilities of painting with bacteria using the agar plates as the 'canvas'.

Things to consider are:

- Bacteria can be found almost everywhere in life.
- Many bacteria can cause health and safety issues.
- The source of the bacteria being it from body fluids or the environment.
- The speed in which bacteria multiply.
- Use of antibiotics for the selection of bacterial strains.
- The disposal of the bacterial cultures after use.

Lab requirements.

100 X Petri dishes
LB broth
Nutrient broth
Agar
Microwave
Nutrient broth
500ml schott X 2
Cotton buds autoclaved
Microscopes compound X 3
Microscopes dissect X 3
Streptomycin
Slides X 10
coverslips X 10
loops
parafilm X 4
scissors X 4
razor blades

Fungal Practical

Fungi, once thought of as plants, are now classified as their own kingdom. Most fungi are multicellular, however the yeasts are unicellular. Yeasts have a growth habit similar to that of bacteria, forming colonies on an agar plate. Filamentous fungi produce masses of strands known as mycelium. When this mycelium is tightly packed it can produce complex spore-producing structures such as mushrooms (Basidiomycetes) and puffballs (Ascomycetes). Fungi can reproduce sexually with + and - mating strains. They also reproduce asexually with the production of spores and budding

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Demonstration:

Under the compound and dissecting microscopes are various plates and slides showing some of the features of fungus. Observe the growth habits, colours and structures that are produced by fungi.

Making a slide.

Using a dissection needle, remove a thin slice of the fungal culture and place on to a new microscope slide. Chop up into smaller pieces and add a drop of the biological dyes. Gently push on a cover slip and blot off any excess dye with a tissue or filter paper. Observe the slide under a compound microscope.

An alternative method of producing a slide is by using sticky tape. Open the culture and press gently a piece of sticky tape onto the fungal mycelium. Remove and stick onto a new glass slide. Observe under a compound microscope.

Dyeing using extracted fungal pigments. *

Provided is a solution of dye extracted from puffball fungi. Using this dye and fabrics supplied, ranging from silk to cotton create a new design.

*** Donna Franklin, SymbioticA Resident during her Masters of Creative Art (Textiles Major) is the source for much of the material and knowledge associated with this section.**

Lab requirements.

100 X Petri dishes
Puff balls
Range of fabrics
Aluminum sulphate
Pots
Silk
Gas cooker
Detergent
Vinegar
White wool raw
demo posters
Donna fashion
Dissecting needles
Aniline blue
Gongo red
Fusarium
Aureobasidium
Yellow culture
Orange culture
parafilm
scissors
razor blades
demo slides
sticky tape
PHOTOS OF DONNAS FUNGI AND MICRO DETAILS

Plant Tissue Culture

Plant tissue culture has many valuable uses in agricultural research. It is used for the micro propagation of selected cultivars in fruit crops, floriculture and the nursery industry. Plant tissue culture material can also be used for genetic engineering i.e. cloning.

Tissue culture involves growing cells, tissues or plantlets on a nutrient medium (which has usually been solidified with agar), in a sterile environment such as glass jar (*in vitro means* "in glass") under favourable conditions of temperature and light.

The nutrient medium consists of minerals, vitamins and sugar supplemented with growth regulators that control the development of the plants.

Many Western Australian native plants produce seeds that are difficult to germinate artificially. Therefore the use of tissue culture in the form of an embryo rescue may increase the chances of germinating the seed successfully. This is done by excising the seed embryo to overcome any chemical and/or physical dormancy. Once removed the embryo can be grown on a tissue culture media.

You should wear a lab coat and gloves. (There is no eating, drinking, etc. in a lab!)

PRACTICAL

Part 1 Embryo Rescue

- Spray the bench and under the culture hood with 70% ethanol and wipe dry with the tissue paper.
- Light the Bunsen burner or ethanol burner outside the hood.
- Place a sterile Petri dish into the hood.
- Tear open the sterile instruments in the laminar flow and attach the blade to the scalpel handle. (<u>If you fill uncomfortable please ask for help</u>). Lay the instruments on an open Petri dish.
- Dip the instruments in ethanol and flame**, holding the scalpel <u>horizontally</u>.**
- Allow the tools to cool.
- With sterile forceps and working under the hood, excise the embryo from the seed, using a Petri dish as a cutting board.
- In the hood, open a new vial containing nutrient media. Place the embryo on the filter paper.

- Replace the lid tightly, label and date the new tissue culture tube.

Part 2 Lateral bud micro propagation.

- Setting up the culture hoods as described in part 1.
- Remove, under the hood, a section of cultured plant material and place it on a newly opened petri dish.
- Cut a single node piece of stem, removing the leaf.
- Making sure the node is the right way up, push the base into a new tube of media.
- Replace the lid tightly, label and date the new tissue culture tube.

Lab requirements

6 X Culture cabinets (Botany)
3 X spray bottles of 70% ethanol
2 X boxes of tissues
6 X Bunsen burner on gas or ethanol burners
100% ethanol
200 X Petri dishes
Petunia cultures
African Violet cultures
Pea seeds
10 X pre autoclaved tools (scalpel and forceps)
Box scalpel blades
Glass jars for 100% ethanol
5 X M&S nutrient mix pacs
sprinkle of BAP
4 X parafilm
4 X scissors
100 X pre autoclaved 250ml vials
100 X pre autoclaved 30ml vials with filter paper
20ml 1M NaOH
Litmus paper
2 X 2L beaker
Portable balance
Weigh boats
10 X Marker pens
10 X plastic spoons
100g Sucrose
50g Agar
Microwave
Gloves S M L
Hand soap
FIRE EXTINGUISHER

Mammalian Tissue Culture

Tissue Culture is the technique for growing cells from multicellular organisms in a liquid medium for research.

Tissue culture is often a generic term that refers to both organ culture and cell culture and the terms are often used interchangeably. Cell cultures are derived from either primary tissue explants or cell suspensions. Primary cell cultures typically will have a finite life span in culture whereas continuous cell lines are, by definition, abnormal and are often transformed cell lines.

A. Growth pattern. Cells will initially go through a quiescent or lag phase that depends on the cell type, the seeding density, the media components, and previous handling. The cells will then go into exponential growth where they have the highest metabolic activity. The cells will then enter into stationary phase where the number of cells is constant, this is characteristic of a confluent population (where all growth surfaces are covered).

B. Harvesting. Cells are harvested when the cells have reached a population density which suppresses growth. Ideally, cells are harvested when they are in a semi-confluent state and are still in log phase. Cells that are not passaged and are allowed to grow to a confluent state can sometime lag for a long period of time and some may never recover. It is also essential to keep your cells as happy as possible to maximize the efficiency of transformation. Most cells are passaged

Proteolytic enzymes - Trypsin, collagenase, or pronase, usually in combination with EDTA, causes cells to detach from the growth surface. This method is fast and reliable but can damage the cell surface by digesting exposed cell surface proteins. The proteolysis reaction can be quickly terminated by the addition of complete medium containing serum

C. Media and growth requirements

1. Physiological parameters
A. temperature - 37C for cells from homeotherm
B. pH - 7.2-7.5 and osmolality of medium must be maintained
C. humidity is required
D. gas phase - bicarbonate conc. and CO_2 tension in equilibrium
E. visible light - can have an adverse effect on cells; light induced production of toxic compounds can occur in some media; cells should be cultured in the dark and exposed to room light as little as possible;

2. Medium requirements:

A. Nutrient media (DMEM)
B. serum - contains a large number of growth promoting activities such as buffering toxic nutrients by binding them, neutralizes trypsin and other proteases, has undefined effects on the interaction between cells and substrate, and contains peptide hormones or hormone-like growth factors that promote healthy growth.
C. antibiotics - although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants.

3. Feeding - 2-3 times/week.

SAFETY CONSIDERATIONS

Assume all cultures are hazardous since they may harbor latent viruses or other organisms that are uncharacterized. The following safety precautions should also be observed:
pipetting: use pipette aids to prevent ingestion and keep aerosols down to a minimum
no eating, drinking, or smoking
wash hands after handling cultures and before leaving the lab
decontaminate work surfaces with disinfectant (before and after)
autoclave all waste
use biological safety cabinet (laminar flow hood) when working with hazardous organisms. The cabinet protects worker by preventing airborne cells and viruses released during experimental activity from escaping the cabinet; there is an air barrier at the front opening and exhaust air is filtered with a HEPA filter make sure cabinet is not overloaded and leave exhaust grills in the front and the back clear (helps to maintain a uniform airflow)
use aseptic technique
dispose of all liquid waste after each experiment and treat with bleach

Tissue Culture of Mammalian Cells

PASSAGING CELLS

-Cells should be kept at 37°C in 75cm^2 flasks (up to 1x10^7 cells) in 5% CO_2 atmosphere (incubator).
-Remove medium from flask of confluent cells (60-70% for myoblasts).
-Wash 2x with 10ml of PBS and remove. (Medium contains trypsin inhibitor - FCS)
-Add 1-2ml of trypsin/EDTA and rock gently to cover all cells.
-Incubate cells at 37°C for 2-3 minutes.
-Check for cells lifting off the flask. Tilt and knock the side of the flask until the cells purge and turn the film of trypsin/EDTA cloudy. Check for maximum cell "lift off" under the microscope.
-Add about 5ml of medium plus serum and gently resuspend cells to break up cell clumps.
-Ideally a cell count should be made of this suspension so that you know how much to add to a new flask that will seed a new flask and reach confluency in about 3-4 days. You may alter this volume so that the number of cells varies to give you a confluent flask in a day or a week, although it is recommended to seed at least 1x10^5 cells for healthy growth.
-Add to a final volume of 15 ml of medium and update passage number and date on the side of the flask.
-Incubate at 37°C.
N.B. Sterilize hood and all possible apparatus before and after use with alcohol. Wear gloves.

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